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Role of the sensor kinase LqsT and the transcription factor SinR in α-hydroxyketone-mediated signalling in *Legionella pneumophila*

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Contents

List of Abbreviations

%	Percent
°C	Degrees Celsius
µg	Microgram
µl	Microlitre
ACES	*N*-(2-acetamido)-2-aminoethanesulfonic acid
ad	Adjusted to
AHK	α-hydroxyketone
AHL	Acyl-homoserine lactone
Ap	Ampicillin
AYE	ACES yeast extract
bp	Base pairs
CAI-1	Cholera autoinducer 1
CFU	Colony forming unit
Cm	Chloramphenicol
Cm^R	Resistance to chloramphenicol
ddH_2O	Double distilled water
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dpi	Days post infection
EDTA	Ethylenediaminetetraacetate
EHEC	Enterohaemorrhagic *Escherichia coli*
EMSA	Electrophoretic moboility shift assay
EtOH	Ethanol
ER	Endoplasmatic reticulum
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
fo	Forward primer
g	Gravity
GAP	GTPase-activating protein
GDF	GDI displacement factor
GDI	GDP dissociation inhibitor
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
h	Hour
HEPES	2-(4-(2-hydroxyethyl) piperazin-1-yl) ethanesulfonic acid
HGT	Horizontal gene transfer
His_6-tag	Affinity tag composed of 6 histidine residues
Icm	Intracellular multiplication
IMAC	Immobilised ion metal affinity chromatography
IPTG	Isopropyl-β-D-thiogalactopyranoside

kb	Kilobase
kDa	Kilo-Dalton
Km	Kanamycin
Km^R	Resistance to kanamycin
L	Litre
LAI-1	*Legionella* autoinducer 1
LB	Luria-Bertani medium
LCV	*Legionella*-containing vacuole
Lpn	*Legionella pneumophila*
Lqs	*Legionella* quorum sensing
M	Molar, Mol per litre or: DNA fragment size marker
MC	Multimeric complex
MeOH	Methanol
MGE	Mobile genetic element
mif	Mature infective form
min	Minute
MOI	Multiplicity of infection
MOMP	Major outer membrane protein
ml	Millilitre
mM	Millimolar
MME	Monomethyl ether
mol	Unit or amount of substance
MOPS	3-(N-Morpholino)propane
mRNA	Messenger RNA
OD_{600}	Optical density at a wave length of 600 nm
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pH	Negative common logarithm of the concentration of hydrogen ions
PI	Phosphoinositide
PMA	Phorbol 12-myristate 13-acetate
Poly(dI-dC)	Poly(deoxyinosinic-deoxycytidylic) acid
PPAR	peroxisome proliferator activated receptor
PYG	Peptone yeast extract glucose
qRT	quantitative real-time
QS	Quorum sensing
RNA	Ribonucleic acid
RFU	Relative fluorescence unit
RPMI	Roswell Park Memorial Institute
RU	Relative units

SEC	Size-exclusion chromatography
SDS	Sodium dodecyl sulfate
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SorC	Sörensen phosphate buffer
STPK	Serine/threonine protein kinase
T4SS	Type IV secretion system
TCS	Two-component system
TEMED	N,N,N',N'-Tetramethylethylendiamine
Tris	2-Amino-2-(hydroxymethyl)-1, 3-propanediol
tRNA	Transfer RNA
re	Reverse primer
U	Unit
V	Volt
v/v	Volume per volume
Vch	*Vibrio cholerae*
w/v	Weight per volume
wt	Wild-type

List of Publications

Schell, U., Kessler, A., Hilbi, H. (2014). Phosphorylation signalling through the *Legionella* quorum sensing histidine kinases LqsS and LqsT converges on the response regulator LqsR. *Molecular Microbiology* **92:**1039-1055.

Kessler A., Schell U., Sahr T., Tiaden A., Harrison C., Buchrieser C., Hilbi H. (2013). The *Legionella pneumophila* orphan sensor kinase LqsT regulates competence and pathogen-host interactions as a component of the LAI-1 circuit. *Environmental Microbiology* **15:**646-62.

Tiaden A., N., Kessler A., Hilbi H. (2013). Analysis of *Legionella* infection by flow cytometry. *Methods in Molecular Biology*. **954:**233-49.

Summary

Legionella pneumophila is an amoeba-resistant, opportunistic pathogen which performs cell–cell communication through the α-hydroxyketone (AHK) signalling molecule 3-hydroxypentadecane-4-one (LAI-1, *Legionella* autoinducer-1). AHK signalling is mediated by the *lqs* (*Legionella* quorum sensing) gene cluster encoding the LAI-1 autoinducer synthase LqsA, the cognate sensor kinase LqsS and the prototypic response regulator LqsR of unknown output function.

Here we show that the Lqs system includes an 'orphan' homologue of LqsS termed LqsT. Compared with wild-type *L. pneumophila*, strains lacking *lqsT* or both *lqsS* and *lqsT* show increased salt resistance, greatly enhanced natural competence for DNA acquisition and impaired uptake by phagocytes. Sensitive novel single round growth assays and competition experiments using *Acanthamoeba castellanii* revealed that Δ*lqsT* and Δ*lqsS*-Δ*lqsT*, as well as Δ*lqsA* and other *lqs* mutant strains are impaired for intracellular growth and cannot compete against wild-type bacteria upon co-infection. In contrast to the Δ*lqsS* strain, Δ*lqsT* does not produce extracellular filaments. The phenotypes of the Δ*lqsS*-Δ*lqsT* strain are partially complemented by reintroducing either *lqsT* or *lqsS*, but are not reversed by overexpression of *lqsA*, suggesting that LqsT and LqsS are the sole LAI-1-responsive sensor kinases in *L. pneumophila*. In agreement with the different phenotypes of the Δ*lqsT* and Δ*lqsS* strains, *lqsT* and *lqsS* are differentially expressed in the post-exponential growth phase. Transcriptome studies indicated that 90% of the genes, which are downregulated in absence of *lqsT* are upregulated in absence of *lqsS.* Reciprocally regulated genes encode either translocated effector proteins implicated in virulence or components of a 133 kb genomic 'fitness island', which also harbours a gene that resembles SinR-family transcription factors encompassing a conserved HTH-motif. The characterisation of a corresponding deletion mutant demonstrated that *sinR* in *L. pneumophila* promotes pathogen-host cell interactions (such as efficient phagocytosis and intracellular replication) and biofilm formation, while repressing natural transformation. *sinR* expression is negatively controlled during stationary growth by *lqsS* and to a smaller extent by *sinR*. In addition to this autoregulation, SinR was shown to induce expression of *lqsR* and *lqsA* in *L. pneumophila*. Indeed, *in vitro* EMSA studies revealed that SinR directly interacts with the promoter of *sinR* and, albeit less efficiently, with the *lqsA* upstream region. Finally, we produced highly diffracting protein crystals of the novel response regulator LqsR. The determination of the high resolution X-ray structure from these crystals might provide insight into its function in *L. pneumophila*.

Together, these data reveal a unique organisation of the *L. pneumophila* Lqs system comprising two partially antagonistic LAI-1-responsive sensor kinases, LqsT and LqsS, which converge on the reponse regulator LqsR and regulate distinct pools of genes implicated in pathogen–host cell interactions, competence, expression of a genomic island or production of extracellular filaments. As a novel component, the transcription factor SinR expands the AHK signalling circuit and crosstalks with the stationary phase regulatory network controlling pathogen-phagocyte interactions and other features of *L. pneumophila*.

Zusammenfassung

Legionella pneumophila ist ein amöbenresistentes opportunistisches Pathogen, welches Zell-Zell-Kommunikation durch das α-Hydroxyketon (AHK) Signalmolekül 3-Hydroxypentadekan-4-on (LAI-1, *Legionella* Autoinducer-1) ausübt. Die AHK Signaltransduktion wird durch das *lqs* (*Legionella* quorum sensing) Gencluster vermittelt, welches für die Autoinducersynthase LqsA, die dazugehörige Sensorkinase LqsS, sowie den prototypischen Responsregulator LqsR kodiert.

Diese Arbeit beschreibt ein genomisch isoliertes Homolog der Sensorkinase LqsS, genannt LqsT. Verglichen mit Wildtypbakterien wiesen *L. pneumophila*-Stämme mit *lqsT*-Deletion oder *lqsS*-*lqsT*-Doppelmutation eine erhöhte Salzresistenz, gesteigerte natürliche Kompetenz für die DNA-Aufnahme, sowie verminderte Aufnahmeeffizienz in Phagozyten auf. Sensitive, neuartige Wachstums- und Kompetitionsexperimente mit *Acanthamoeba castellanii* zeigten für die Δ*lqsT* und der Δ*lqsS*-Δ*lqsT* Doppelmutante, sowie die Δ*lqsA* und andere *lqs* Mutantenstämme, reduziertes intrazelluläres Wachstum und einen Kompetitionsnachteil gegen Wildtypbakterien bei Koinfektion. Im Gegensatz zu Δ*lqsS* produzierte Δ*lqsT* keine extrazellulären Filamente. Die Phänotypen der Δ*lqsS*-Δ*lqsT* Mutante wurden teilweise komplementiert entweder durch *lqsT* oder *lqsS*, nicht aber durch Überproduktion von *lqsA.* Dies führte zur Annahme, dass LqsT und LqsS die einzigen beiden LAI-1-reaktiven Sensorkinasen in *L. pneumophila* sind. Übereinstimmend mit den verschiedenen Phänotypen der Δ*lqsT* und Δ*lqsS* Stämme wurden *lqsT* und *lqsS* in der post-exponentiellen Wachstumsphase unterschiedlich exprimiert. Transkriptionsstudien indizierten, dass 90% der in Abwesenheit von *lqsT* reprimierten Gene in der *lqsS*-Mutante induziert sind. Reziprok regulierte Gene kodieren für translozierte Virulenz-fördernde Effektorproteine, oder Komponenten einer 133 kb grossen genomischen „Fitnessinsel“, welche einen SinR-artigen Transkriptionsfaktor mit HTH-Motiv enthält. Die Charakterisierung einer *sinR*-Deletionsmutante demonstrierte, dass *sinR* einerseits die Biofilmproduktion und Pathogen-Wirtszelleninteraktionen, wie die Aufnahmeeffizienz und die intrazelluläre Replikation förderte, andererseits die natürliche Transformation inhibierte. Die Expression von *sinR* wurde in der stationären Phase durch *lqsS* und in geringerem Masse durch *sinR* selbst reprimiert. Neben dieser Autoregulation induzierte SinR die Expression von *lqsR und lqsA* in *L. pneumophila.* Eine direkte Interaktion von SinR mit der Promoterregion von *sinR und lqsA* wurde *in vitro* in EMSA-Experimenten bestätigt. Darüber hinaus könnte die hochauflösende Struktur der hier präsentierten hochdiffraktierenden Proteinkristalle des neuartigen Responsregulators LqsR Aufschluss liefern über seine bisher unbekannte Funktion in *L. pneumophila*.

Zusammenfassend beschreibt diese Doktorarbeit die einzigartige Organisation des *L. pneumophila* Lqs Systems mit den zwei partiell antagonistischen LAI-1-reaktiven Sensorkinasen LqsS und LqsT, welche auf den Responseregulator LqsR konvergieren. Die beiden Sensorkinasen regulieren unterschiedliche Genpools mit Auswirkung auf Pathogen-Wirtszellinteraktionen, Kompetenz, Expression einer genomischen Fitnessinsel, sowie auf die Produktion von extrazellulären Filamenten. Als neuartiges Signalelement erweitert der pleiotrope Transkriptionsfaktor SinR die AHK Signalkaskade und kommuniziert mit dem Regulationsnetzwerk

der stationären Wachstumsphase, welches Pathogen-Wirtszellineraktionen und andere Eigenschaften von *L. pneumophila* kontrolliert

1. Introduction

1.1 Adaptation of *Legionella pneumophila* to host cells and the environment

Legionella pneumophila was first identified in 1976 as a Gram-negative opportunistic pathogen belonging to the gamma-subgroup of proteobacteria (Fraser *et al.*, 1977). The water-borne bacteria ubiquitously persist in aquatic habitats, either as individual planktonic cells or attached to biotic/abiotic surfaces as part of multispecies biofilm communities. Moreover, *L. pneumophila* is a facultative intracellular bacterium that infects and replicates within a wide range of hosts. Mostly found engulfed by their natural hosts, free-living amoebae such as *Acanthamoeba* and *Hartmanella* spp*.,* the pathogen also colonises the social soil amoeba and model organism *Dictyostelium discoideum* (Steinert and Heuner, 2005, Solomon *et al*., 2000, Fields *et al.,* 1996). However, since *L. pneumophila* is also associated with human infection, and thus, the co-evolution of *L. pneumophila* with phagocytic unicellular protozoa most likely preadapted this pathogen for the contact with a new host: functionally related human phagocytes (Steinert *et al.*, 2002, Horwitz and Silverstein, 1980). The infection and growth of *Legionella* in human alveolar macrophages gives rise to Legionnaires' disease, a severe pneumonia charachterised by multisystem defect (Diederen *et al.*, 2008) or a milder, flu-like ailment termed Pontiac-fever. Legionellosis occurs through the inhalation of contaminated aerosols (Atlas *et al.*, 1999), and its epidemiology is strongly associated with a variety of technical water systems such as showers, cooling towers, or air conditioning systems. In addition to sporadic epidemic outbreaks, nosocomial pneumonia due to *L. pneumophila* is a major issue for public health services around the world. Among the more than 50 species of the genus *Legionella*, strain *L. pneumophila* serogroup 1 accounts for the majority of cases of human Legionnaires' disease (84% worldwide, 95% in Europe). Relevant risk factors include age, sex, smoking, immunosuppression as well as underlying diseases like diabetes, cancer or AIDS (Marston *et al.*, 1994).

Four genomes of different strains of *L. pneumophila* have been sequenced and published: *L. pneumophila* strain Philadelphia (Chien *et al.*, 2004), *L. pneumophila* strains Paris and Lens (Cazalet *et al.*, 2004) and *L. pneumophila* strain Corby (Steinert *et al.*, 2007). The genome size of 3.3 Mb of *L. pneumophila* is relatively large when compared to many other intracellular pathogens such as *Rickettsia*, *Bartonella* or *Chlamydia* spp. (Fuxelius *et al.*, 2007, Saenz *et al.*, 2007, Thomson *et al.*, 2008), and corresponds to a higher number of genes. The genome size reflects the capacity of *Legionella* to adapt to different environmental conditions and hosts. The close association of the pathogen with aquatic protozoa most likely generated a pool of virulence traits, which were acquired by trans-kingdom horizontal gene transfer (HGT) and allow *Legionella* to interact with their host cells. Indeed, all sequenced *Legionella* strains encode a high number of eukaryotic-like proteins that may functionally mimic host cell proteins and could modulate different stages of the intracellular life cycle (Gomez-Valero *et al*., 2011, Cazalet *et al.*, 2004).

L. pneumophila cycles between an intracellular, replicative form and an infectious, cytotoxic state, which promotes transmission to a new host. Cellular differentiation of *L. pneumophila* during this biphasic life cycle is governed by a complex regulatory system that provides a link between the

growth phase and the expression of virulence traits (Swanson *et al.*, 1998). Major regulators involved in the adaptation to extracellular and intracellular environments of the bacteria include specific sigma factors, two-component systems and small regulatory RNAs (Molofsky *et al.*, 2004).

For the development of a successful pathogen–host interaction, a complex regulatory system modulates a pool of virulence traits. Through these systems, *L. pneumophila* manipulates host cell processes required to enter, survive, replicate and evade amoebae or macrophages. Essential contributors to *L. pneumophila* pathogenicity are specialised secretion systems, designated type I–V (Russell *et al.*, 2014, Abdallah *et al*., 2007, Thanassi and Hultgren, 2000). The Lsp type II secretion system (T2SS) is involved in growth within amoeba and macrophages, and secretes a number of hydrolytic enzymes including proteases, aminopeptidases and phospholipases (Rossier *et al.,* 2008, Rossier and Cianciotto, 2001).

The Icm/Dot (intracellular multiplication/defective organelle trafficking type IV secretion system (T4SS) encoded by 25 different genes is the key virulence factor of *L. pneumophila*. The translocation of a large arsenal of approximately 300 effector proteins (encoded by 10% of the bacterial genome) into host cells allows the manipulation of conserved cellular processes such as signal transduction, post-translational modifications and vesicle trafficking pathways. Recent studies elucidated the functions of T4SS-translocated effectors that subvert small GTPases (Sherwood and Roy, 2013, Rothmeier *et al.*, 2013, Itzen and Goody, 2011, Urwyler *et al.*, 2009) phosphoinositide lipids (Haneburger and Hilbi, 2013, Weber *et al.,* 2006), the retrograde vesicle trafficking pathway (Finsel *et al*., 2013) as well as ubiquitinylation and apoptosis factors (Roy, 2014, Rolando and Buchrieser, 2012). Ultimately, *L. pneumophila* phagosome trafficking is redirected, leading to its conversion into an ER-derived organelle permissive for intracellular bacterial replication (Rolando and Buchrieser, 2012, Shin and Roy, 2008). The pathogens organelle is termed the *Legionella*-containing vacuole (LCV) (Zhu *et al.,* 2011, Isberg *et al.,* 2009, Shohdy *et al.*, 2005). LCVs communicate with the endocytic, secretory and retrograde vesicle trafficking pathways and eventually fuse with the endoplasmic reticulum (Hilbi and Haas, 2012). The molecular mechanisms of several *L. pneumophila* effectors acting along the sequential pathogen-host interactions have been elucidated (Section 1.2) and will be further highlighted in their regulatory context (Section 1.3).

1.2 Interactions of phagocytic host cells with *L. pneumophila*

During the first step of the infectious cycle (Figure 1), *L. pneumophila* attaches to and enters its host cell by the use of factors including RtxA, LadC, EnhC, the long pilus $PilE_L$ and the major outer membrane protein (MOMP). Furthermore, the flagellar sigma factor FliA, heat shock protein Hsp60 and LpnE contribute to host cell entry (Newton *et al*., 2007, Molofsky *et al*., 2005, Cirillo *et al*., 2000).

Although the uptake of the pathogen and the subsequent phagosome formation occurs mainly by host-driven actin-dependent phagocytosis, the Icm/Dot T4SS was shown to be a key factor for the enhancement of endocytic events in host cells (Hilbi *et al.,* 2001, Khelef *et al*., 2001). Among the few described Icm/Dot effectors acting very early in infection, two (LaiA and SdeA) have been suggested to play a role in adherence and uptake by macrophages (Chang *et al*, 2005). VipA was discovered as actin nucleator that directly polymerises microfilaments associated with early endosomes, supportive for its role in organelle trafficking (Franco *et al*., 2012, Shohdy *et al*., 2005).

Immediately after phagocytosis of *L. pneumophila* by a host cell the bacterium defines its own intracellular compartment, evading the endocytic pathway by preventing phagosome-lysosome fusion. *L. pneumophila* targets and subverts cellular trafficking processes mainly via the wide range of Icm/Dot-translocated effector proteins (Derré and Isberg 2004, Horwitz and Maxfield, 1984, Horwitz, 1983). The establishment of a modified organelle that permits intracellular replication of the pathogenic bacteria is mediated by the host secretory pathway (Tilney *et al*., 2001). Moreover, initial remodeling of the LCV membrane includes the recruitment of mitochondria and the interaction with early secretory vesicles derived from the smooth endoplasmiatic reticulum (ER) (Tilney *et al*., 2001) as shown by colocalisation with KDEL-GFP as a marker of ER/Golgi (Kagan and Roy, 2002).

Vacuolar ATPases and hydrolases are a prerequisite for the creation of an acidic phagolysosomal microenvironment for the degradation of bacteria (Kornfeld and Mellman, 1989). The translocated effector protein SidK has been identified to target the host V-ATPase subunit VatA, thus inhibiting ATP hydrolysis, proton translocation and subsequently vacuole acidification (Xu *et al*., 2010). Besides the effector-dependent evasion of lysosomal fusion, lipopolysaccharide (LPS) molecules on the *L. pneumophila* surface were shown to delay fusion of late endosomal vacuoles with lysosomes (Fernandez-Moreira *et al*., 2006).

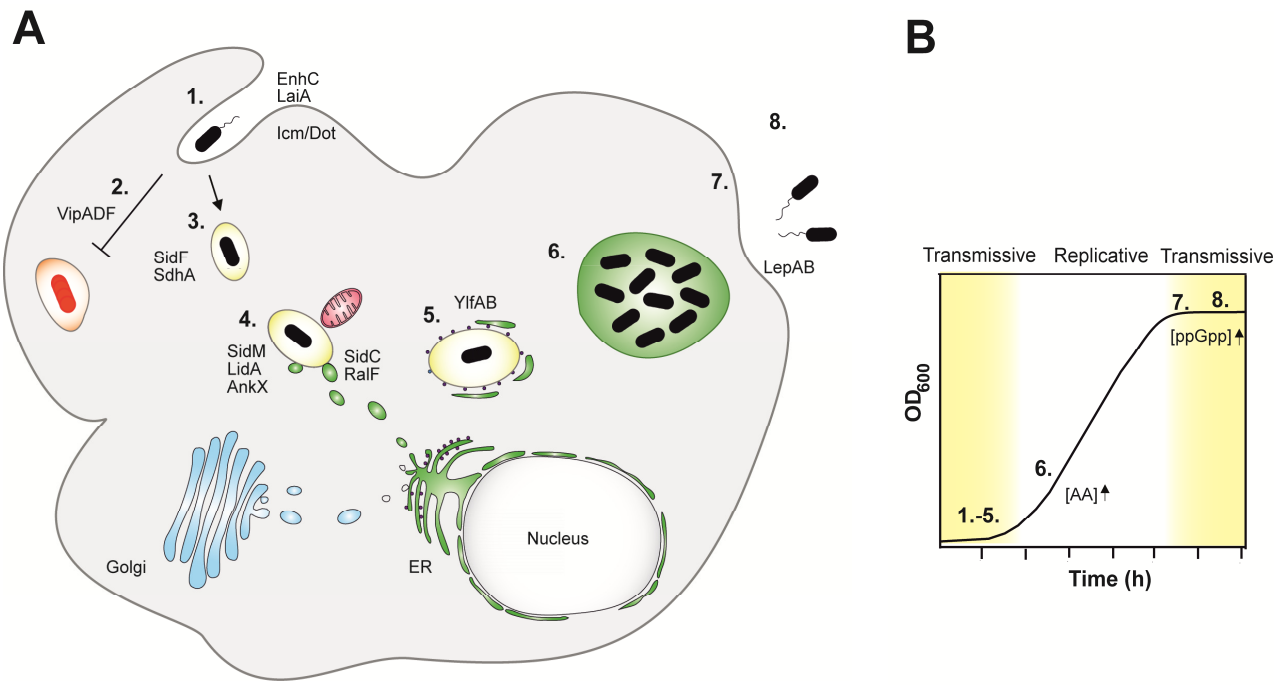

Figure 1: **The growth-phase dependent process of *L. pneumophila* infection.** (**A**) (**1**) Adhesion and entry of *L. pneumophila* into the host cell. (**2**) Evasion of endocytic pathway mediated by VipA, VipD and VipF (**3**). Establishment of the *Legionella* containing vacuole (LCV). (**4**) Recruitment and fusion of ER-derived vesicles with the LCV, e.g. by the translocated effectors LidA or SidM. (**5**) Second step of LCV maturation: Fusion of the modified LCV with ER membranes, delivery of luminal ER proteins to LCV. (**6**). Bacterial replication within rough-ER-like vacuole. (**7**) Switch to transmissive phase and LepA-/LepB-dependent bacterial egress. (**8**) Transmission to environmental niches or infection of new host cells. Major Icm/Dot-translocated effectors involved in the different steps are indicated in black (adapted from Brüggemann *et al*., 2006b) and discussed in Section 1.2. (**B**) Growth-phase dependent regulation of transmissive (virulence) traits throughout the infectious cycle of *L. pneumophila*. Numbers along the growth curve (optical density, OD_{600}) correspond to the different steps of infection depicted in (A). Proteosomal degradation of proteins leads to elevated cellular levels of amino acids (AA) that might promote bacterial replication. Similarly to the spent host cell, nutrient limitation in broth triggers the entry into the stationary growth phase of *L. pneumophila* via the accumulation of the alarmone (p)ppGpp (guanosine 3′,5′-bispyrophosphate), thereby inducing transmissive traits.

Another level of spatial and temporal regulation of LCV formation is associated with phosphoinositide (PI) metabolism, as *L. pneumophila* exploits the signalling properties of PI lipids during the establishment of the replicative vacuole. PI molecules act by anchoring host regulatory proteins. This involves the recruitment and activation of multiple small GTPases that modulate endosomal trafficking and remodel the actin cytoskeleton (Thi and Reiner, 2012). Similarly, the pathogen perturbs the PI lipid profile during the establishment of the replicative vacuole to anchor Icm/Dot substrates to the LCV (Hilbi *et al*., 2011). Recently, Hsu *et al*. reported that the *L. pneumophila* translocated effector SidF functions as a phosphoinositide 3-phosphatase that specifically hydrolyses PI(3,4,5)P3 *in vitro* to possibly generate a PI(4)*P*-enriched LCV (Hsu *et al.*, 2012). Accordingly, the LCV-resident PI(4)*P* was found to bind and recruit the effectors LidA, SidM, SidC and its paralogue SdcA (Del Campo *et al.*, 2014, Schoebel *et al*., 2010, Brombacher *et al.*, 2009, Weber *et al.,* 2006). Furthermore, the resemblance of the LCV to a cis-Golgi network was hypothesised to favour the acquisition of ER vesicles (Weber *et al.*, 2006).

Important host factors that are recruited to the LCV include the small GTPase families ARF and Rab. They associate with ER-derived vesicles and Golgi membranes (Donaldson and Jackson, 2011) as well as with host-tethering proteins produced by *L. pneumophila* (Machner and Isberg, 2006). Small GTPases act as molecular switches which are inactive in their cytosolic GDP-bound form, but when binding GTP, associate with membranes and engage with proteins that modulate membrane structure and function. The coordinated function of the small GTPases Arf1, Sar1 and Rab1 directs the transport and fusion of ER-derived vesicles to the LCV (Nagai and Roy, 2003).

The activation of Rab proteins on a target vesicle is tightly controlled on several levels of regulation and requires (i) the exchange of GDP for GTP by a specific guanine nucleotide exchange factor (GEF) (Pfeffer *et al*., 2001), (ii) the displacement of GDP-dissociation inhibitors (GDIs) by GDI-displacement-factors (GDF) and (iii) Rab inactivation by GTPase-activating proteins (GAPs) that promote hydrolysis of GTP to GDP (Bernards *et al.*, 2003). The first biochemically characterised Icm/Dot-secreted effector protein RalF was identified as an Arf1 GEF (Nagai *et al*., 2005). The bifunctional Rab1 regulator SidM raised particular interest (Ingmundson *et al*., 2007; Machner and Isberg, 2007). One region in SidM is required for Rab1 recruitment to membranes and functions as a GDF, whereas the second region stimulates Rab1 activation by operating as a GEF (Arasaki *et al*., 2012, Ingmundson *et al*., 2007, Machner and Isberg, 2007). Moreover, the interactions of Rab1 with the effectors LidA (Itzen *et al.,* 2011, Derré and Isberg, 2005) or LepB were reported to promote the recruitment of early ER-derived vesicles to the LCV. The effector AnkX was shown to interact with the transport of both endocytic vesicles and secretory vesicles by phosphocholination and hence induce activation of Rab1 on the LCV (Mukherjee *et al*., 2011). A recent study presented the small GTPase-targeting effector LegG1 as a promoter of intracellular growth. The accumulation of LegG1 on the LCV results in the activation of Ran GTPase and the subsequent stabilisation of microtubules, supporting LCV motility (Rothmeier *et al.*, 2013).

Successful budding and docking of ER-derived vesicles is followed by the LCV fusion with rough ER membranes. This second phase of LCV remodelling occurring several hours after infection is characterised by the acquisition of the ER-specific proteins calnexin and glucose-6-phosphatase and the presence of ribosomes on the LCV surface. The GTPase Sar1 contributes to the initial tethering of membranes (Robinson and Roy, 2006). Subsequent interactions of the LCV with the rough ER enables the delivery of resident ER proteins into the LCV lumen. Moreover, active Rab1 promotes the pairing of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) on the target (t-SNARE) membrane (e.g. Sec22b) with a vesicular v-SNARE (e.g. syntaxin) on the LCV membrane, leading to the fusion of ER-vesicles with the LCV membrane. *L. pneumophila* has been suggested to promote fusion of ER-derived vesicles with the LCV via the translocation of t-SNARE-mimicking effector proteins, such as YlfA/B, or the incorporation of a t-SNARE complex present at the LCV or plasma membrane during uptake (Paumet *et al.*, 2009).

The removal of effectors from the LCV after completion of their function was suggested to support successful progression of infection. Polyubiquitination of proteins marks them for proteasomal degradation in a process called ER-associated degradation (ERAD) (Price *et al*., 2009). The U-box-

containing effector LubX mimicks host E3 ubiquitin ligase-activity and thereby mediates SidH removal from the LCV (Kubori *et al.*, 2008). The LCV is decorated with polyubiquitinated proteins, which are targeted for proteasomal degradation leading to elevated cellular levels of amino acids that might power bacterial replication (Price *et al*., 2011).

Once LCV establishment is complete, *L. pneumophila* switches to a replicative phase and starts to proliferate. Transcriptome analyses revealed a profound shift between gene expression profiles of replicative or transmissive bacteria during intracellular growth in *A. castellanii* or macrophages (Faucher *et al.*, 2011, Brüggemann *et al.*, 2006b). A further role in virulence was assigned to a number of regulatory non-coding RNAs due to their altered expression shown during the biphasic life cycle of the opportunistic pathogen (Sahr *et al*., 2012, Weissenmayer *et al.*, 2011).

Following multiple rounds of intracellular replication, nutrient consumption and the accumulation of the alarmone guanosine 3′,5′-bispyrophosphate (ppGpp) trigger the stringent response in *L. pneumophila* and the subsequent transmissive gene expression pattern. Host cell lysis is possibly mediated by a cytolysin/ egress pore (Molmeret *et al.*, 2002) or by the effector proteins LepA and LepB which have been implicated in the active, nonlytic egress from protozoa (Chen *et al.*, 2004b). Subsequent egress from the host cell and infection of neighbouring cells involves the upregulation of flagellar or Icm/Dot-encoding genes. Released bacteria may then reinfect new host cells, disseminate or persist in the environment as planktonic cells, or colonise biofilms.

1.3 Regulation of *L. pneumophila* virulence

In order to establish a replication niche inside their host cells, pathogenic bacteria monitor and adapt to the environmental conditions by the use of sensory and signal transduction systems combined with regulatory cascades to manipulate intracellular processes (Beier and Gross, 2006; Mekalanos, 1992, Miller *et al*., 1989).

Along the biphasic life cycle, *L. pneumophila* exhibits physiologically and morphologically different states that can be mimicked *in vitro*. *L. pneumophila* alternates between distinct phenotypic forms described as intracellular vacuolar pathogens, extracellular planktonic form, filaments associated with biofilms, persistant bacteria, or mature infective form (mif) (Piao *et al*., 2006, Hiltz *et al*., 2004). The differentiation of transmissive *Legionella* into the replicative form is tightly coupled to its metabolic state (Edwards *et al*., 2010, Dalebroux *et al*., 2009, Hammer and Swanson, 1999).: The transcriptome of replicative bacteria revealed the elevated transcription of genes involved in cell division and metabolic processes, e.g. aerobic metabolism, amino acid catabolism as well as the Entner-Doudoroff glycolytic pathway (Brüggemann *et al*., 2006b). Within the LCV, the bacteria efficiently replicate and repress transmissive traits (virulence, motility, stress resistance): Nutrient deficiencies trigger the induction of transmissive features once reaching the late post-exponential phase in broth or in phagocytic host cells (Faucher *et al*., 2011, Brüggemann *et al*., 2006b, Byrne and Swanson, 1998). Late in its life cycle, *L. pneumophila* transforms to a motile, sodium-sensitive and osmosis-resistant state in order to promote the transmission to and manipulation of a new host cell.

1.3.1 Two-component systems control *L. pneumophila* virulence

L. pneumophila constantly senses endogenous, host-derived and environmental stimuli to ensure a periodic transition between environmental and host-associated niches (Molofsky and Swanson, 2004). One of the predominant ways by which *L. pneumophila* regulates the transition between niche-specific gene expression, is defined by two-component systems (TCS). In relation to the large genome size, genome analyses identified a low overall number of TCS (13 histidine kinases, 14 response regulators) in strain Paris (Cazalet *et al*., 2004). The TCS are typically composed of a membrane-located sensor with histidine kinase activity and a cytoplasmic transcriptional regulator. Metabolic stimuli detected by these systems are transformed into a cellular signal by autophosphorylation of the sensor proteins at a conserved histidine residue. The phosphoryl group is then transferred to an aspartic acid residue in the receiver domain of the response regulator, which frequently leads to its dimerisation and hence activation of the highly diverse output domains.

The regulatory network controlling *L. pneumophila* virulence encompasses four TCS (Nevo *et al*., 2014) (Figure 2): (i) the LetAS-RsmYZ-CsrA regulatory cascade including the LetAS TCS, the two small RNAs (sRNAs) RsmY and RsmZ as well as the post-transcriptional carbon storage regulator A (CsrA) regulating the Arf1 GEF gene *ralF*, actin-associated *vipA* and the paralogous effectors *ylfA* and *ylfB*, (ii) PmrAB (Rasis and Segal, 2009, Al-Khodor *et al*., 2009, Zusman *et al.,* 2007) which is under control of RpoS (Hovel-Miner *et al*., 2009) and upregulates expression of the effectors *lepB*,

sdhA and *sidF*, (iii) the CpxRA TCS which additionally controls *sidM* and *icm/dot* apparatus genes and iv) the *Legionella* quorum sensing circuit consisting of *lqsRS* (cognate sensor kinase and response regulator) together with the autoinducer synthase *lqsA* (further discussed in Section 1.3.2). In concert with additional sensor systems such as small RNAs (Sahr *et al.*, 2009), the network encompassing these TCS coordinates various processes during the *L pneumophila* life cycle, including pathogen-host interactions, expression of virulence factors (Nevo *et al.*, 2014), establishment of the LCV (Gomez-Valero *et al*., 2011, Isberg *et al.*, 2009, Franco *et al.*, 2009), biofilm and filament formation and the regulation of a genomic 'fitness island' (Tiaden *et al.,* 2010b, Tiaden *et al*., 2007). The *icm/dot* and effector genes under direct control of the four TCS are discussed in Section 2.2 and illustrated in Figure 2 (Altman and Segal, 2008).

Whether in extracellular or intracellular environments, differentiation of transmissive *L. pneumophila* to the replicative form is coupled to its metabolic state (Edwards *et al.,* 2010, Dalebroux *et al.*, 2009, Hammer and Swanson, 1999). Together with alternative sigma factors, the aforementioned TCS orchestrate the biphasic life cycle of *L pneumophila* in a growth-phase dependent manner (Figure 2): During the exponential phase, the RNA-binding global regulator CsrA binds to target mRNAs and repress the translation of transmissive (virulence, motility) genes, thus promoting the replicative (metabolism) transcriptional programme. The reversible switch from replicative to transmissive phase is triggered initially through the production of the 'alarmone' ppGpp by the synthetase RelA and the bifunctional synthetase/hydrolase SpoT (Dalebroux *et al*., 2009, Zusman *et al.*, 2002, Hammer and Swanson, 1999) or at the end of the infection cycle by reduction of amino acid and/or fatty acid biosynthesis. The accumulation of (p)ppGpp upon nutrient starvation was shown to coordinate the entry into the stationary phase and is sufficient to promote the induction of the transmission regulon (Hammer and Swanson, 1999). ppGpp may act as a direct transcriptional activator of *rpoS* and *letA* in starved bacteria by binding to the *rpoS* promoter, (Lange *et al*., 1995). Consequently, high concentrations of ppGpp lead to the upregulation of RpoN and flagellar (FliA) sigma factors, and the subsequent exhibition of virulence traits (Bachman and Swanson, 2001).

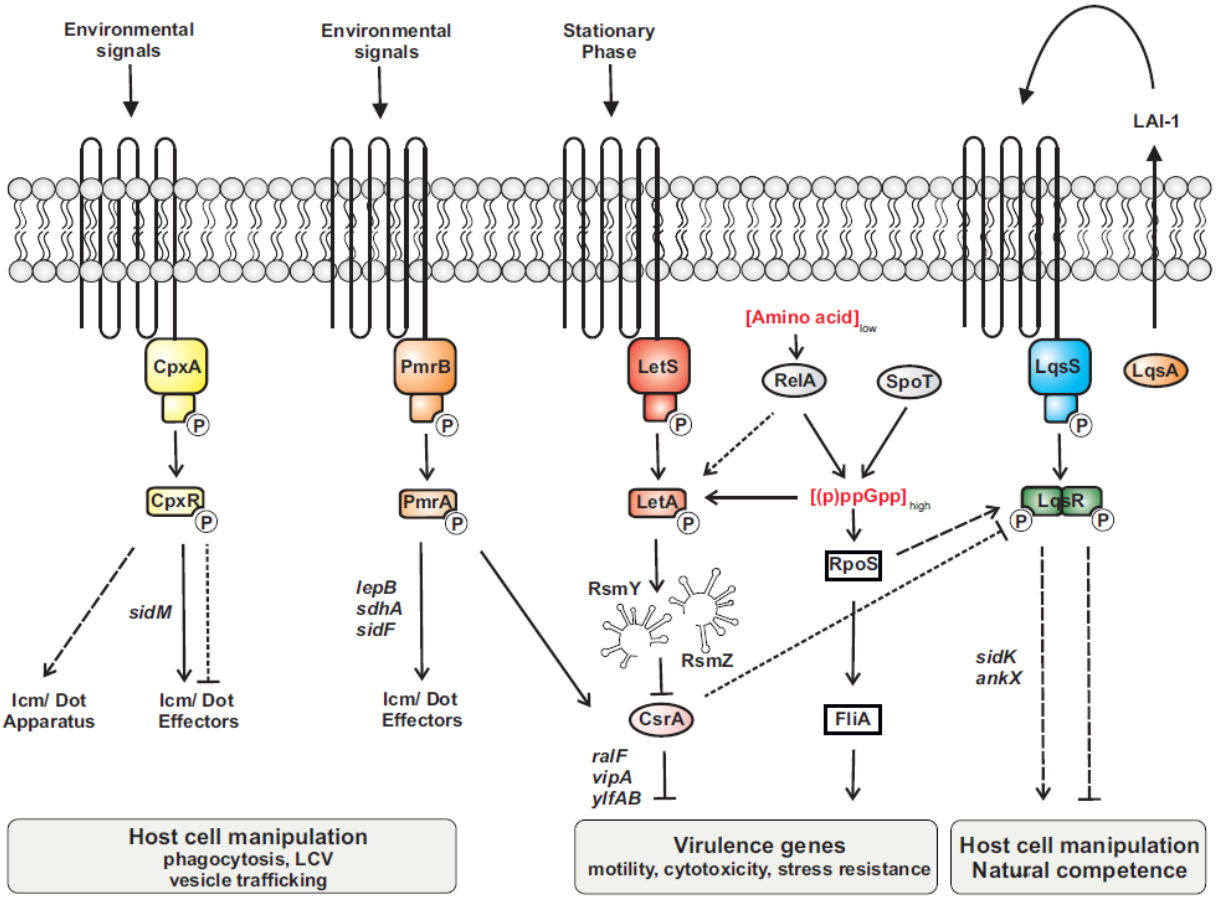

Figure 2: Model of the TCS controlling the transition from the replicative to the virulent phase of *L. pneumophila*. The TCSs CpxRA, PmrAB, LetAS and LqsRS and the regulatory cascade LetAS-RsmYZ-CsrA are schematically illustrated. In addition, regulated effector proteins whose function is known or predicted to play a role in this process are depicted. During the replicative phase, nutrients are abundant and the post-transcriptional carbon storage regulator A (CsrA) represses transmission traits and promotes replication. Upon nutrient depletion at the onset of stationary phase, RelA stimulates the production of the alarmone ppGpp. Other signals may stimulate the enzyme SpoT to contribute to the accumulation of ppGpp, which in turn induces the LetAS TCS and the alternative sigma factor RpoS. Together with FliA, LetA and RpoS induce transmission traits. Active LetA induces the production of small regulatory RNAs RsmY and RsmZ to sequester CsrA and relieve post-transcriptional repression. LAI-1 (*Legionella* autoinducer-1) is produced by LqsA and likely binds to LqsS that phosphorylates and activates the cognate response regulator LqsR, whose production is dependent on RpoS as well as on RsmYZ and CsrA. Additionally, the response regulators CpxR and PmrA positively regulate Icm/Dot components and substrates, respectively. Solid and dashed lines represent direct and indirect regulation, respectively. Model adapted from (Tiaden and Hilbi, 2012, Jules and Buchrieser, 2007, Molofsky and Swanson, 2004).

Upon entry into the stationary phase, the sensor kinase LetS is activated by a so far unknown signal and phosphorylates LetA, its cognate response regulator. LetA directly upregulates the expression of the two small non-coding RNAs RsmY and RsmZ, which bind and sequester multiple CsrA molecules from their target mRNAs and thereby releave the repression of transmissive traits by CsrA. Moreover, RpoS excerts further levels of regulation by controlling (i) PmrAB which itself contributes to the *csrA* gene regulation, (ii) non-coding RNAs RsmY and RsmZ upon activation of LetAS, and (iii) production of the response regulator LqsR which is co-regulated post-

transcriptionally by the sRNAs RsmYZ and by CsrA. Moreover, CpxA activation might be linked to *L. pneumophila* adherence to host cells, as CpxR-activated effectors were shown to translocate into host cells early during infection.

After internalisation into a host cell, *L. pneumophila* establishes the LCV by TCS-dependent expression of virulence genes and the subsequent translocation of different sets of effectors in a timely controlled fashion (Section 1.2). Indeed, the interplay between two regulators was shown to result in two distinct groups of effectors: One group is expressed and activated by the PmrAB TCS during the exponential growth phase and the second group of effectors is de-repressed by the LetAS TCS during stationary phase. During these stepwise events, the bacteria are converted to a replicative, avirulent, non-motile form. When nutrients are depleted, the bacteria enter the transmissive phase and express virulence proteins, resulting in lysis of the host cells and the initiation of a new infection round (for reviews see Shin and Roy, 2008 and Steinert *et al*., 2007).

Finally, *L. pneumophila* contains an α-hydroxyketone (AHK) signalling circuit consisting of the autoinducer synthase LqsA, the putative cognate sensor kinase LqsS, and the response regulator LqsR (Figure 2, Kessler *et al*., 2013, Tiaden *et al*., 2010b). AHK signalling is linked to the stationary growth phase regulatory network of *L. pneumophila* via LqsR. Production of LqsR requires RpoS and also depends to a smaller extent on the response regulator LetA (Tiaden *et al.,* 2007). Furthermore, the small RNAs RsmY and RsmZ and CsrA act as posttranscriptional regulators of LqsR production (Sahr *et al*., 2009).

1.3.2 Gene regulation by α-hydroxyketone mediated signalling

In addition to exogenous signals, *L. pneumophila* also synthesises, releases and detects small, membrane-diffusible signalling molecules to promote intra- and interspecies cell–cell communication (Spirig *et al*., 2008). Upon reaching a certain threshold concentration, autoinducer molecules trigger signalling pathways and gene regulation to functionally coordinate a bacterial population in a process termed quorum sensing. Quorum sensing represents a means of bacterial cell density-dependent gene regulation in order to control processes, such as virulence, biofilm formation, competence or bioluminescence (Camilli and Bassler, 2006, Fuqua and Greenberg, 2002).

Among the various chemical classes of autoinducers known thus far, only the AHK LAI-1 (*Legionella* autoinducer-1, 3-hydroxypentadecane-4-one) has been identified by tandem mass spectrometry as a signalling molecule in *L. pneumophila* (Spirig *et al*., 2008). LAI-1 and the closely related *Vibrio cholera*e CAI-1 (cholera autoinducer-1, 3-hydroxytridecane-4-one) are the first biologically relevant 3-hydroxy-4-keto compounds belonging to the class of AHKs (Higgins *et al*., 2007).

LAI-1 and CAI-1 are produced and detected by the *lqs* (*Legionella* quorum sensing) and *cqs* (*cholera* quorum sensing) genes, respectively. The *lqs* genes encode the cognate pair of an autoinducer synthase LqsA and the putative sensor histidine kinase LqsS. LqsA and LqsS are homologues of CqsA and CqsS, sharing 45% and 29% identity, respectively (Tiaden and Hilbi, 2012).

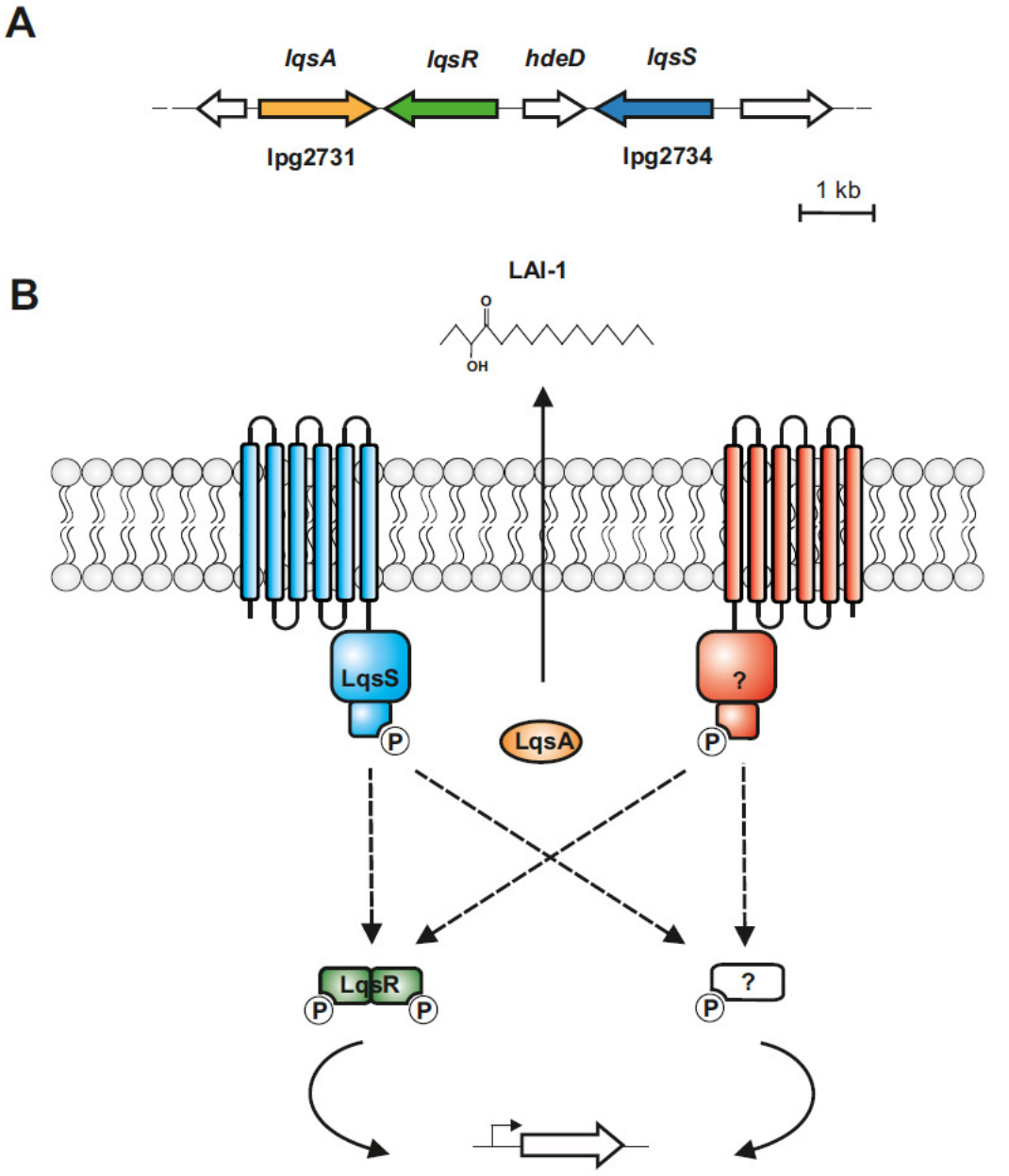

Figure 3: The *L. pneumophila lqs* gene cluster encodes components of the LAI-1 signalling circuit. (**A**) Genomic organisation of the *L. pneumophila lqs* (*Legionella* quorum sensing) gene cluster (*lpg2731–2734*). The *lqs* cluster comprises the autoinducer synthase *lqsA*, the response regulator *lqsR*, a homologue of *E. coli hdeD* (HNS-dependent expression D) and the cognate sensor kinase *lqsS*. (**B**) Scheme of the *L. pneumophila* LAI-1 (*Legionella* autoinducer-1) signalling circuit. The α-hydroxyketone (AHK) signalling molecule 3-hydroxypentadecane-4-one LAI-1 is produced by the autoinducer synthase LqsA and presumably detected by the sensor kinase LqsS and perhaps other sensors which transmit the signal through the response regulator LqsR and/or other response regulators. Dashed arrows indicate hypothetical or indirect interactions (published in Kessler *et al*. (2013).

Functional similarity was reported for the pyridoxal-5'-phosphate (PLP)-dependent aminotransferases LqsA and CqsA, as *lqsA* partially complemented a *cqsA* mutation in *V. cholerae* (Spirig *et al*., 2008). The sensor kinases which belong to the class of six-transmembrane helices couple the detection of AHK signalling molecules via an N-terminal receptor domain to a C-terminal signal transduction module. In the case of the hybrid sensor kinase CqsS, this signal module in addition contains a receiver domain with an aspartate residue. In *Vibrio spp*., the cognate CqsA/CqsS system promotes regulation of virulence and biofilm formation in a cell density-dependent manner (Henke and Bassler, 2004, Miller *et al*., 2002), as well as natural competence (Antonova and Hammer, 2011, Suckow *et al.*, 2011).

Furthermore, the *lqs* gene cluster additionally encodes the putative response regulator LqsR and a homologue of *Escherichia coli* HdeD (HNS-dependent expression D) linked to acid resistance in *E. coli*. The four genes *lqsA*, *lqsS*, *lqsR* and *hdeD* are divergently transcribed and expressed from individual promoters (Sahr *et al*., 2012). Moreover, the *lqs* gene cluster is present in the genomes of all *L. pneumophila* strains sequenced to date: Philadelphia (*lpg2731-2734*), Paris (*lpp2787-2790*), Alcoy (*lpa_03985, lpa_03987*, *lpa_03988, lpa_03991*), Corby (LPC_0396, LPC_0398, LPC_0401, LPC_0402), as well as Lens (*lpl2656*-2659). The clustering of the *lqsR* gene with *lqsA* and *lqsS* is conserved in bacterial species possessing an LqsR homologue, e.g. *Burkholderia* and *Janthinobacterium spp*. (Hornung *et al*., 2013), implying an evolutionarily conserved functional relation between LqsA/LqsS and LqsR.

As the prototypic member of a novel response regulator family, LqsR contributes to the stationary-phase regulatory network, mediated by the alternative sigma factor RpoS and to a lesser extent by LetA (Tiaden *et al*., 2007, discussed in Section 1.3.1). LqsR harbours a canonical, CheY-like N-terminal receiver domain (amino acids 80-160) including the conserved aspartate residue (D108). The C-terminal fragment of LqsR is of unknown function and does not show homology to any known signal output domains.

Previous functional analysis of *L. pneumophila* lacking either single genes or the entire *lqs* cluster revealed that the Lqs system controls various processes, including pathogen-host cell interactions, production of virulence factors, formation of extracellular filaments and regulation of a genomic island (Tiaden *et al.*, 2010b, 2008, 2007). The Lqs components liekly act in the stationary phase, as, compared to wild-type bacteria, mutant strains lacking *lqsS* or *lqsR* exhibited differential transcriptional profiles exclusively during stationary growth. Interestingly, the phenotypes of the Δ*lqsS* mutant strain were reversed by overexpression of *lqsA*, suggesting the existence of another LAI-1 responsive sensor in *L. pneumophila.*

1.4 Elements of genome plasticity in *L. pneumophila*

In addition to the core genome encoding essential metabolic functions, bacterial genomes also harbour a range of accessory genes likely acquired by horizontal gene transfer (HGT) (Juhas *et al*., 2009). HGT between bacterial strains and species is a key mechanism of genome evolution, contributing to the diversification and adaptation of microorganisms and significantly affecting genome plasticity (Treangen and Rocha, 2011, Skippington and Ragan, 2011, Dagan *et al*., 2008, Gogarten and Townsend, 2005, Ochman *et al*., 2000). A successful HGT event can be split in three successive steps. The first step is DNA transfer from one cell to another, involving free DNA (by transformation), encapsidated DNA (by transduction or lysogenisation), or cell-to-cell contact (by conjugation). Secondly, DNA is acquired by the daughter cells during division either by replication of the incoming DNA as a plasmid or by integration/transposition into a replicon. The third step is marked by the evolutionary success of the strain. It may be correlated to advantageous functions encoded by the transferred genes that result in better adaptation of the recipient cell to the environment or colonisation of novel niches.

HGT mainly corresponds to the acquisition of a mobile genetic element (MGE; Frost *et al.*, 2005). In particular, this process involves a DNA fragment that moves from cell to cell (intercellular mobility) or within a genome (intracellular mobility) and that carries some or all sequences and genes involved in its mobility (Toussaint and Merlin, 2002). The traditional classes of MGEs, represented by conjugative plasmids, transposons and integrated prophages, frequently carry additional adaptation genes that contribute to the success of their transfer). Beyond the traditional classes of MGE, other types have been reported to rely on a combination of transfer and maintenance mechanisms (Guerillot *et al*., 2013, Wozniak and Waldor, 2010, Beaber *et al*., 2002).

Genomic islands (GEIs) define a superfamily of horizontally acquired DNA elements that play a key role in bacterial evolution and adaptation, the dissemination of antibiotic resistance and virulence genes, and formation of novel catabolic pathways, ultimately enhancing the fitness of their host (Figure 4, Juhas *et al.*, 2009, Gaillard *et al*., 2006, Dobrindt *et al*., 2004, Hacker and Kaper, 2000). Generally, GEIs comprise a large spectrum of variable genetic organisation and functionality, and thus, only share conserved analogous core and structural features required for their maintenance, rather than being phylogenically related (Vernikos and Parkhill, 2008). Most GEIs are characterised by (i) large syntenic DNA blocks of 10-200 kb harbouring relatively novel genes that are present in only few strains, (ii) ununsual G+C percentage or codon usage, and (iii) their frequent insertion at tRNA genes. Moreover, GEIs are often flanked by direct repeats (DR) which arise during site-specific integration into the target site (Schmidt and Hensel, 2004).

Other systems involved in the mobilisation of GEIs include plasmid conjugation machineries, integrons, insertion sequence (IS) elements, transposons, phages or integrases (Buchrieser *et al.*, 1998). Integrons are characterised by the formation of large arrays of gene cassettes by homologous recombination, associated with transposons or conjugative plasmids, mediating the spread of HGEs (Dobrindt *et al*., 2004). All of these elements contribute to the dynamic character of

bacterial chromosomes by allowing excision of GEIs and their transfer to other recipients. Accessory genes offering a selective advantage for host bacteria might especially contribute to their genomic plasticity and complex evolution. Thus, depending on the life-style of their particular host species, GEIs may be further specified according to their gene content, encoding pathogenicity, symbiosis, metabolic, resistance or fitness traits (Schmidt and Hensel, 2004, Dobrindt *et al.,* 2004).

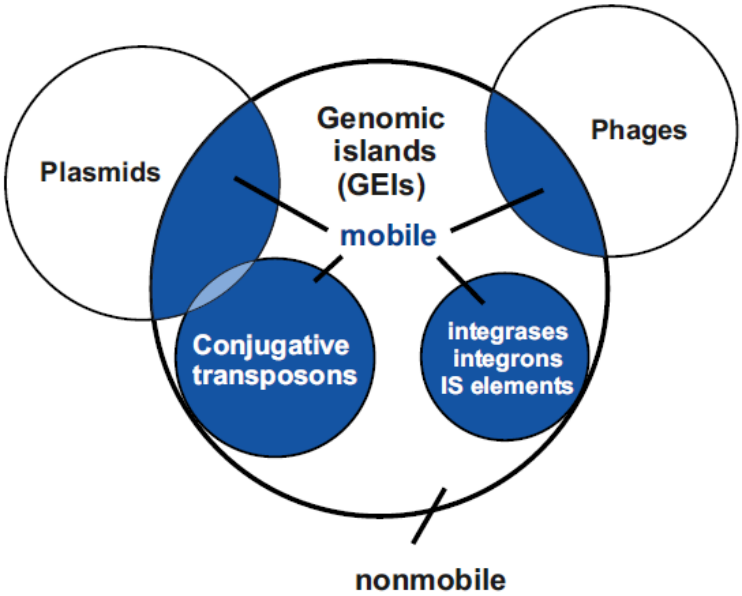

Figure 4: Typical components of genomic islands. Genomic islands (GEIs) include further classes of mobile elements, such as conjugative transposons, integrated plasmids, nonreplicative but excisable elements (integrases, integrons, insertion sequences (IS), and cryptic or damaged phages. Blue areas indicate self-mobile GEIs. Modified after Juhas *et al*., 2009.

A high plasticity was observed among the genome sequences of *L. pneumophila* (D'Auria *et al*., 2010, Schroeder *et al*., 2010 Glöckner *et al*., 2008, Cazalet *et al*., 2004, Chien *et al*., 2004), *L. longbeacheae* (Cazalet *et al.,* 2010, Kozak *et al*., 2010) and the draft genome of *L. drancourtii* (Moliner *et al*., 2009), represented by multiple chromosomal islands containing a G+C content different from the 38% of the core genome.

The marked cellular and genetic plasticity is an intrinsic propertiy of *L. pneumophila*, which allows the bacteria to colonise different ecological niches or hosts. Most likely, the long-term convergent co-evolution of the pathogen with various prokaryotes or eukaryotic hosts has favoured inter-kingdom horizontal gene transfer (Moliner *et al*., 2010, Franco *et al*., 2009, Cazalet *et al*., 2008). During the different stages of the intracellular life cycle, *L. pneumophila* diverts host cell functions by an abundance of eukaryote-like proteins containing domains implicated in protein-protein interactions (Cazalet *et al*., 2004). Of great interest are the F-box and U-box-containing proteins which may functionally mimic and interfere with the host cell's ubiquitin machinery (Brüggemann *et al*., 2006a). Additionally, the bacterial eukaryote-like serine/threonine protein kinases (STPKs) may engage host signal transduction pathways.

Numerous features of genetic mobility found in *L. pneumophila* may facilitate the transfer and uptake of DNA, e.g. *L. pneumophila* harbours an alternative T4SS implicated in conjugation of

plasmid DNA (Segal *et al.*, 1999; 1998; Vogel *et al.,* 1998) and has been described as naturally competent (Sexton and Vogel, 2004, Stone and Kwaik, 1999). Natural transformation by competence is a major mechanism of HGT in bacteria. Competence is defined as the genetically programmed physiological state that allows the active import of DNA from the environment and the subsequent genotypic and phenotypic transformation (Charpentier *et al*., 2011). The conditions that promote competence development are multiple and often elusive

A putative genomic island of *L. pneumophila* Philadelphia-1 is a 133 kb region harbouring 125 open reading frames (*lpg0973–lpg1096*). This region has a higher G+C content than the core genome, is located adjacent to the $tRNA^{Thr}$ gene *lpg0972* and flanked by presumed DNA-mobilising elements such as integrases, transposases and phage-like genes. Interestingly, 67 out of these 125 genes are upregulated 1.5–8.5-fold in the absence of the putative sensor kinase *lqsS* (Tiaden *et al.*, 2010b). The 133 kb gene cluster shows high genomic plasticity and is further divided into two regions. Region I (*lpg0973–lpg1003*, 26 kb) harbours many conserved, yet unknown genes, which possibly encode pili components. Region II (*lpg1006–lpg1096*, 107 kb) encodes the subunits of a FoF1 ATP synthase and various putative metal ion resistance transport proteins. The region from *lpg1008* to *lpg1035* was previously identified as a 40 kb efflux pump genomic island, which is induced in *L. pneumophila* upon (but is not required for) infection of macrophages (Rankin *et al.*, 2002). Moreover, in strain Philadelphia-1 region II, a SinR family transcription regulator is located between *lpg1055* and *lpg1056* (1'153'652–1'153'389 bp, previously not annotated). The corresponding gene was also identified in the genome of strain Paris (*lpp2326*). In summary, the 133 kb genomic region fulfils the criteria of a canonical genomic fitness island (Dobrindt *et al.*, 2004) and might play a role during pathogen–phagocyte interactions.

Another genomic island present in four of six *L. pneumophila* strains (Schroeder *et al*., 2010, Cazalet *et al*., 2004, Chien *et al.,* 2004) and in two *L. longbeacheae* strains (Cazalet *et al*., 2010, Kozak *et al.,* 2010) is defined by the *lvh* region, which encodes a T4SS (Segal *et al*., 1999). The *lvh* region shows a higher G+C content than the core genome, is inserted at tRNA sites and flanked by mobility genes. Moreover, this genomic mobile element can be excised from the chromosome and exist as a multicopy plasmid in the lag phase of bacterial growth (Doleans-Jordheim *et al.*, 2006). Finally, these genomic islands have been postulated to be mobile and transferred by horizontal gene transfer, thus contributing to the genetic diversity of the species *L. pneumophila* (Cazalet *et al.*, 2008).

1.5 Aims of the thesis

L. pneumophila harbours a plethora of regulatory elements to adapt to and switch between the aquatic environment and the intracellular milieu of phagocytic cells during a biphasic life cycle. This complex regulatory network involves AHK-mediated signal transduction to modulate essential traits including virulence, biofilm formation and environmental fitness. The signalling molecule LAI-1 is synthesised by the autoinducer synthase LqsA and is presumably detected by the sensor kinase LqsS. Strikingly, the virulence and sedimentation phenotypes of *L. pneumophila* lacking *lqsS* are reverted upon overexpression of *lqsA*, suggesting that LAI-1 is also recognised by alternative sensors. AHK signalling is transduced via the prototypic response regulator LqsR that harbours an N-terminal receiver domain and a C-terminal part, which shares no homology with any known response regulator output domains.

The 133 kb putative genomic fitness island upregulated in *L. pneumophila* lacking *lqsS* encodes a putative response regulator homologous to SinR, the master regulator of biofilm formation in *Bacillus subtilis* (Colledge *et al.*, 2011). In strain Philadelphia-1, this regulator has not been annotated nor investigated so far.

The objective of this thesis was to analyse AHK-mediated signal transduction and gene regulation in *L. pneumophila* by the homologous sensor kinases LqsS and LqsT using genetic, biochemical and cellular microbial approaches. Moreover, the role of the *L. pneumophila sinR* homologue in the AHK-signalling network was to be investigated in terms of pathogen-host cell interactions, gene regulation and natural competence. Finally, crystallisation and determination of the high resolution structure of the response regulator LqsR was initiated to provide insight into its molecular function. This study should contribute to a biochemical analysis of the elements of the Lqs system, the signal transduction cascade and the cross-talk among its constituents.

2. Materials and methods

2.1 Materials

2.1.1 Laboratory equipment

Item	Model	Manufacturer
Autoclave	STERIMAQUET	MAQUET (Rastatt)
Autoclave	Varioklav classic	H+P (Oberschleissheim)
Benchtop centrifuge	5417R	Eppendorf (Hamburg)
Centrifuge	5810	Eppendorf (Hamburg
Centrifuge	Sorvall RC5C Plus	Dupont (Wilmington)
CO_2 incubator	Heraeus HeraCell 240	Thermo (Waltham)
Concentrator	Amicon filter (MWCO 30000)	Millipore (Zug)
Colony counter	Countermat flash	IUL (Barcelona)
Cryo stream	Cryostream Plus	Oxford Cryosystems (Oxford)
Crystallisation 96-well plate	96-well Crystal Quick	Greiner (Frickenhausen)
Crystal score imaging system	RockImager	Formulatrix (Waltham)
Crystallisation robot	Mosquito	TTP (Dürnten)
Crystallisation kit	Clear Strategy I	Qiagen (Hombrechtikon)
Crystallisation kit	Clear Strategy I	Qiagen (Hombrechtikon)
Crystallisation kit	JCSG	Molecular Dimensions
Crystallisation kit	Morpheus	Molecular Dimensions
Crystallisation kit	Nextal PEG Suite	Qiagen (Hombrechtikon)
Crystallisation kit	PEGs	Qiagen (Hombrechtikon)
Crystallisation kit	PACT	Qiagen (Hombrechtikon)
Culture microscope	Primo Vert	Zeiss (Oberkochen)
Diaphragm vacuum pump	MZ 2C	Vacuubrand (Wertheim)
Electrophoresis chamber	Mini-Protean 3	Bio-Rad (Munich)
Electrophoresis chamber	Mini-Subcell GT	Bio-Rad (Munich)
Electrophoresis chamber	Subcell GT	Bio-Rad (Munich)
Electroporation device	GenePulser XCell	Bio-Rad (Munich)
FACS system	FACS Canto	BD BioSciences (Heidelberg)
Gel filtration column	HILoad™ 16/60 Superdex 200	GE Healthcare (Glattbrugg)
Gel imaging system	ChemiDoc MP System	Bio-Rad (Munich)
Gel imaging system	GelDoc EQ	Bio-Rad (Munich)
Hot plate magnetic stirrer	RCT basic	IKA (Staufen)
Incubation cabinet	Certomat BS-1	Sartorius (Goettingen)
Incubation cabinet	Oribital shaker Forma	Thermo (Waltham)
Incubator	Heraeus BR6000	Thermo (Waltham)
Incubator	Heraeus Function Line	Thermo (Waltham)
Incubator	IPP500	Memmert (Schwabach)
Mixer	Vortex-Genie 2	IKA (Staufen)
pH-meter	Level 1	inoLab (Weilheim)

Item	Model	Manufacturer
Pipettes	Pipetman	Gilson (Middleton)
Power supply	PAC100	Bio-Rad (Munich)
Precision balance	PG2002-S	Mettler-Toledo (Greifensee)
Precision balance	BP61-S	Sartorius (Goettingen)
Protein purification System	Äkta™ 10 purifier	GE Healthcare
Protein transfer device	MAXI- Semi-Dry-Blotter	Roth (Karlsruhe)
Rocking platform shaker	Duomax 1030	Heidolph (Schwabach)
Rolling mixer	RM5-35s 1732	Fröbel (Lindau)
Spectrophotometer	Helios Epsilon	Thermo (Waltham)
Spectrophotometer	NanoDrop ND-1000	PeqLab (Erlangen)
SEC column	Superdex 200 16/60	GE Healthcare (Glattbrugg)
IMAC column	HisTrap HP	GE Healthcare (Glattbrugg)
Superspeed centrifuge	Sorvall RC-5B	DuPont (Wilmington)
Suspension mixer	CMV	Fröbel (Lindau)
Thermal cycler	T3	Biometra (Goettingen)
Thermal mixer	Thermomixer comfort	Eppendorf (Hamburg)
UV-transilluminator		Bachofer (Reutlingen)
Water bath	Wasserbad 1005	GFL (Burgwedel)

2.1.2 Chemicals and consumables

Item	Supplier
ACES	AppliChem (Drmstadt)
Acrylamid/ bisacrylamid	Serva (Heidelberg)
Activated charcoal powder	Fluka (Buchs)
Anti-histidine antibody	Qiagen (Germantown)
Agar	BD Biosciences (Franklin Lakes)
Agarose	Biozym (Oldendorf)
β-Mercaptoethanol	Sigma Aldrich (Buchs)
Bacteriological peptone	BD Biosciences (Franklin Lakes)
Bacto proteose peptone	BD Biosciences (Franklin Lakes)
Bacto yeast extract	BD Biosciences (Franklin Lakes)
DNA ladder (1 kb plus)	Life Technologies (Grand Island)
DNA purification kit	Machery Nagel (Dueren)
DNase I	Roche (Basel)
DreamTaq PCR master mix	Thermo (Waltham)
FCS	Life Technologies (Grand Island)
FeN_3O_9 x 9 H_2O	Sigma (Saint Louis)
Gene pulser cuvette	Bio-Rad (Munich)
D(+)-glucose monohydrate	Fluka (Buchs)
KH_2PO_4	Fluka (Buchs)
LB agar	Life Technologies (Grand Island)
LB broth base	Life Technologies (Grand Island)
L-cysteine	Sigma (St. Louis)
L-glutamine	Life Technologies (Grand Island)
Na_2HPO_4	Fluka (Buchs)
Phusion polymerase	Thermo (Waltham)
Plastic cell containers	TPP (Trasadingen)
PMA	Fisher Scientific (Reinach)
Protease inhibitor (cOmplete, mini)	Roche (Basel)
Protein ladder (PageRuler prestained 10-170K)	Thermo (Waltham)
SDS	Serva (Heidelberg)
Sterile syringe filters 0.22 µm pore size	Millipore Merck (Darmstadt)
Restriction enzymes	Thermo (Waltham)
RPMI 1640	Life Technologies (Grand Island)
Sodium citrate x 2 H_2O	Fluka (Buchs)
T4 DNA ligase	New England Biolabs (Ipswich)

Chemicals not listed were obtained from Roth (Karlsruhe) or Sigma-Aldrich (Buchs).

2.1.3 Strains and plasmids

Strain	Relevant pheno-/genotype	Reference
Escherichia coli		
BL21NiCo(DE3)		Invitrogen
TOP10		Novagen
Legionella pneumophila		
AK01 (Δ*lqsT*)	JR32 *lqsT*::Km	Kessler *et al.* (2013)
AK02 (Δ*lqsS*-Δ*lqsT*)	JR32 *lqsS*::Km *lqsT*::Gm	Kessler *et al.* (2013)
AK03 (Δ*sinR)*	JR32 *sinR*::Km	This work
GS3011 (Δ*icmT*)	JR32 *icmT*3011::KmΩ	Segal and Shuman (1998)
JR32	*L. pneumophila* serogroup 1 Philadelphia-1 salt-sensitive isolate of AM511	Sadosky *et al.* (1993)
NT02 (Δ*lqsA*)	JR32 *lqsA*::Km	Tiaden *et al.* (2010b)
NT03 (Δ*lqsR*)	JR32 *lqsR*::Km	Tiaden *et al*. (2007)
NT04 (Δ*hdeD*)	JR32 *hdeD*::Km	Tiaden *et al.* (2008)
NT05 (Δ*lqsS*)	JR32 *lqsS*::Km	Tiaden *et al.* (2010b)
Acanthamoeba castellanii		ATCC 30234
Dictyostelium discoideum	Ax3	Zhou *et al.* (1995)
HL-60	Human monocytes	ATCC CCL-240
RAW 264.7	Murine macrophage cell line	ATCC TIB-71

Plasmid	Description	Reference
pAK-2	pMMB207C, *gfp* (constitutive), *lqsT* (P_{lqsT})	Kessler *et al.* (2013)
pAK-6	pMMB207C-RBS-*lqsT*	Kessler *et al.* (2013)
pAK-15	pGEM-T-Easy, *lqs* flanking region, Cm	Kessler *et al.* (2013)
pAK-18	pMMB207C, *gfp* (constitutive), *sinR* (P_{sinR})	This work
pAK-21	pET28(+)-*sinR*	This work
pBSL141	*oriR* (pBM1), Ap, MCS::Gm	Alexeyev *et al*. (1995)
pCM-5	pMMB207-*lqsA-gfp* (ASV)	Christian Manske
pCM-6	pMMB207-*lqsR-gfp* (ASV)	Christian Manske
pGEM-T-Easy	Cloning of PCR products, Ap	Promega
pLAW344	*oriT* (RK2), *oriR* (ColE1), *sacB*, Cm, Ap	Wiater *et al*. (1994)
pMMB207C-RBS-*lcsC*	*Legionella* expression vector for LcsC (with ribosome binding site, RBS)	Weber *et al.* (2006)
pNT-1	pUCBM20, *lqs* genomic region	Tiaden *et al.* (2007)
pNT-28	pMMB207C, *gfp* (constitutive)	Tiaden *et al.* (2007)
pNT-31	pMMB207C, *gfp* (constitutive), *lqsS* (P_{lqsS})	Tiaden *et al*. (2010b)
pNT-36	pMMB207C, *gfp* (constitutive), *lqsA* (P_{lqsA})	Tiaden *et al*. (2010b)
pNT-46	pLAW344, *lqsT*::Km	Kessler *et al.* (2013)
pNT-47	pLAW344, *lqsT*::Gm	Kessler *et al.* (2013)
pRB-3	pET28(+)-*lqsR*-D109A	Schell *et al.* (2014)
pRB-4	pET28(+)-*lqsR*-D109N	Schell *et al.* (2014)
pTS-1	pMMB207C, *lqs* region	Spirig *et al*. (2008)
pTS-2	pMMB207C-RBS-*lqsA*	Spirig *et al*. (2008)
pTS-3	pMMB207C-RBS-*lqsS*	Tiaden *et al*. (2010b)
pTS-10	pMMB207C-RBS	Tiaden *et al*. (2007)
pTS-23	pET28(+)-*lqsR*	Schell *et al.* (2014)
pUC4K	*oriR* (pBR322), Ap, MCS::Km	Amersham
pUS-11	pMMB207-*sinR-gfp* (ASV)	Ursula Schell
pXDC42	GFP reporter construct (promoterless *gfp*)	Charpentier *et al*. (2011)
pXDC91	GFP reporter construct (P_{comEA}–*gfp*)	Charpentier *et al*. (2011)

2.1.4 Oligonucleotides

Oligonucleotide	Sequence 5'-3'	Description
oC-LqsA-fo	GGCGATCTGCAGTTCTTTTCCCTTGTGTGCA	5' flanking seq. of *lqsA* (fo)
oC-LqsA-re	GCACAAGGATCCTGGTTGTAGTCCAACAGC	5' flanking seq. of *lqs*A (re)
oC-LqsS-fo	GGCGGTCAACAAGAGGCCATCCGGAAGTT	3' flanking seq. of *lqsS* (fo)
oC-LqsS-re	GGCGTACTGCAGGCAAAGAAATTGGGGGT	3' flanking seq. of *lqsS* (re)
oH1-LqsT-fo	TACAAGAACACAAAACGCCAG	5' flanking seq. of *lqsT* (fo)
oH1-LqsT-re	TATAGACGGGATCCTTAACCTTTGCATGATTCC	5' flanking seq. of *lqsT* (re)
oH2-LqsT-fo	TATAGACGGGATCCCCCCAAAATTGATTAATTCC	3' flanking seq. of *lqsT* (fo)
oH2-LqsT-re	ATGCCTGAAGAGACGAGCAC	3' flanking seq. of *lqsT* (re)
oCR-fo	GGGTTACTGCAGACTGGATCTCAACAGCG	5' flanking seq. of Cm^R (fo)
oCR-re	GCAACGCTGCAGAGACAATAACTGCC	3' flanking seq. of Cm^R (re)
o-LqsT-fo	CCTGTCCATATGCAAAGGTTAAAAAATA	5' *lqsT* (fo)
oP-LqsT-fo	TATAGACGGGATCCATGGTATTGTATTATGATGC	5' P_{lqsT} +*lqsT* (fo)
o-LqsT-re	TATAGACGGGATCCAGGAATTAATCAATTTTGGG	3' *lqsT* (re)
oH1-SinR-fo	AAAAAATCTAGAGCGTGCTGATTGGTCC	5' flanking seq. of *sinR* (fo)
oH1-SinR-re	GCGCGCGGATCCTTGTTTTTTCATTC	5' flanking seq. of *sinR* (re)
oH2-SinR-fo	ATAACTGGATCCTCAGCCTCATAAACC	3' flanking seq. of *sinR* (fo)
oH2-SinR-re	CGTTTATCTAGACGCAGTGCCAGTCATGAC	3' flanking seq. of *sinR* (fo)
o-SinR-fo	AAAAAAACGCGTGTGCTGATTGGTCC	5' P_{sinR}+*sinR* (fo)
oP-sinR-fo	CCTGTCCATATGAAAAAACAAACCG	5' *sinR* (fo)
o-SinR-re	AAAAAAGGATCCTTATGAGGCTGAG	3' *sinR* (re)
oP-FrgA-fo	GCGCGCGAGCTCGGAAGTTGATGGGTATC	5' P_{frgA} (fo)
oP-FrgA-re	CGCGCGTCTAGATATTATCTCCTGAAGG	3' P_{frgA} (re)
oP-LqsA-EMSA-fo	AATCCCCTGCTCCCCAAAATAG	5' P_{lqsA} (fo)
oP-LqsA-EMSA-re	CGCTGGATCCGTGTTAATTACCCTTAAAC	3' P_{lqsA} (re)
oP-LqsR-EMSA-fo	CGGATTTCTTAGCTAAGATAAGGTACG	5' P_{lqsR}(fo)
oP-LqsR-EMSA-re	GCAAAACGTTCCAAAGTTATATCCGCG	3' P_{lqsR} (re)
oP-RpoS-EMSA-fo	GATGCGACTCAACTTTACTTGG	5' P_{rpoS} (fo)
oP-RpoS-EMSA-re	TAGGCAAACGGATAGTTCTC	3' P_{rpoS} (re)

[a] lower case: artificially introduced mucleotides

[b] underlined: restriction sites

2.1.5 Media and buffers

2.1.5.1 *Legionella pneumophila*

Charcoal yeast extract (CYE) agar plates (Feeley *et al.*, 1979)

Component	Per litre	Supplier
ACES	10 g	AppliChem
Bacto yeast extract	10 g	BD Biosciences
Activated charcoal powder p.a. puriss	2 g	Fluka
Agar	15 g	BD Biosciences
L-cysteine	0.4 g in 10 ml H_2O	Sigma
FeN_3O_9 x 9 H_2O	0.25 g in 10 ml H_2O	Sigma

Antibiotic	Final concentration	Supplier
Chloramphenicol	5 mg/l	Roth
Gentamicin	10 mg/l	Roth
Kanamycin	50 mg/l	Roth

For the preparation of CYE (Charcoal yeast extract) agar plates ACES and Bacto yeast extract were dissolved in H_2O and the pH was adjusted to 6.9 using 10 M KOH. Following addition of activated charcoal and agar, the solution was autoclaved and cooled to 50°C. Filter sterilised L-cysteine and iron solutions were pipetted aseptically to the mixture. If necessary antibiotics were supplemented at the concentrations indicated. Plates were stored at 4°C.

ACES yeast extract (AYE) medium (Horwitz *et al.,* 1984)

Component	Per litre	Supplier
ACES	10 g	AppliChem
Bacto yeast extract	10 g	BD Biosciences
L-cysteine	0.4 g in 10 ml H_2O	Sigma
FeN_3O_9 x 9 H_2O	0.25 g in 10 ml H_2O	Sigma

Antibiotic	Final concentration	Supplier
Chloramphenicol	5 mg/l	Roth
Gentamicin	10 mg/l	Roth
Kanamycin	30 mg/l	Roth

AYE medium was generated by dissolving ACES and Bacto yeast extract in 900 ml of H_2O and adjusting the pH to 6.9 using 10 M KOH. Cysteine and iron were separately dissolved in 10 ml

H_2O each and slowly added while stirring. The solution was filtered using a glass fiber filter 6-8 times and subsequently filter-sterilised. The medium was stored at 4°C.

2.1.5.2 *Escherichia coli*

Luria-Bertani (LB) agar

Component	Per litre	Supplier
LB agar	32 g	Life Technologies

Antibiotic	Final concentration	Supplier
Chloramphenicol	30 mg/l	Roth
Kanamycin	30 mg/l	Roth

LB agar was dissolved in H_2O. The solution was autoclaved and cooled to 50°C. If required, antibiotics were added at the concentrations indicated. Plates were stored at 4°C.

Luria-Bertani (LB) medium

Component	Per litre	Supplier
Luria-Bertani broth base	20 g	AppliChem

LB broth base was dissolved in H_2O. The solution was autoclaved and cooled to 50°C. If required, antibiotics were added at the concentrations indicated. Plates were stored at 4°C.

ZYM-5052 autoinduction medium (Studier, 2005)

Component	Final concentration	Supplier
N-Z amine AS	1%	AppliChem
Yeast Extract	5%	BD Biosciences
1000x Trace metals		
$FeCl_3$	10 µM	Sigma-Aldrich
$CaCl_2$	4 µM	Sigma-Aldrich
$MnCl_2$	2 µM	Sigma-Aldrich
$ZnSO_4$	2 M	Sigma-Aldrich
$CoCl_2$	1 µM	Sigma-Aldrich
$CuCl_2$	1 µM	Sigma-Aldrich
$NiCl_2$	1 µM	Sigma-Aldrich
Na_2MoO_4	1 µM	Sigma-Aldrich
Na_2SeO_3	1 µM	Sigma-Aldrich
H_3BO_3	1 µM	Sigma-Aldrich

50x5052 mixture		
Glycerol	54 mM	Sigma-Aldrich
Glucose	2.8 mM	Sigma-Aldrich
α-Lactose	5.6 mM	Sigma-Aldrich
50xM mixture		
Na_2HPO_4	25 mM	Sigma-Aldrich
KH_2PO_4	25 mM	Sigma-Aldrich
NH_4Cl	50 mM	Sigma-Aldrich
Na_2SO_4	5 mM	Sigma-Aldrich
$MgSO_4$	2 mM	Sigma-Aldrich

The ZYM-5052 medium was prepared according to the recipe described by Studier (2005). Briefly, N-Z-amine AS and yeast extract were dissolved in 1 L H_2O in a 2 l Erlenmeyer flask, autoclaved and cooled. A stock solution of 0.1 M $FeCl_3$ was dissolved in a 100-fold dilution of 0.12 M HCl. This solution was combined with autoclaved stock solutions of the other metals to make a 1000x trace metal mixture. 50x5052 and 50xM were prepared, filter-sterilised and added to a final concentration as indicated. Kanamycin was added to a concentration of 30 µg/µl.

TFB1

Component	Per litre	Supplier
Potassium acetate	2.82 g (30 mM)	Roth
KCl	7.46 g (100 mM)	Roth
$CaCl_2$ x 2 H_2O	1.48 g (10mM)	Roth
MnCl	6.3 g (50 mM)	Roth
Glycerol	150 ml (15%)	Roth

The pH was adjusted to 5.8 with 0.2 M acetic acid prior to filter-sterilisation. 40 ml aliquots were stored at -20°C.

TFB2

Component	Per 100 ml	Supplier
MOPS	0.42 g (10 mM)	Roth
KCl	7.46 g (100 mM)	Roth
$CaCl_2$ x 2 H_2O	1.11 g (75 mM)	Roth
KCl	0.074 g (10 mM)	Roth
Glycerol	15 m (15%)	Roth

The pH was adjusted to 6.5 with KOH prior to filter-sterilisation. 4 ml aliquots were stored at 20°C.

2.1.5.3 *Dictyotelium discoideum*

HL5 medium (Watts and Ashworth, 1970)

Component	Per litre	Supplier
D(+)-glucose monohydrate	11 g	Fluka
BBL yeast extract	5 g	BD Biosciences
Bacto proteose peptone	5 g	BD Biosciences
Bacteriological peptone	5 g	Oxoid
Na_2HPO_4	0.355 g (2.5 mM)	Fluka
KH_2PO_4	0.34 g (2.5 mM)	Fluka

The pH was adjusted to 6.5 with 1 M KOH or 1 M HCl and the medium autoclaved and stored at 4°C.

MB medium (Solomon *et al.,* 2000)

Component	Per litre	Supplier
Yeast Extract	7 g	Oxoid
Bacto proteose peptone	14 g	BD BioSciences
MES buffer	4.26 g (20 mM)	AppliChem

MB medium was prepared immediately before use. The pH was adjusted to 6.9 using 1 M KOH, autoclaved and stored at 4°C.

SorC (Malchow *et al*., 1972)

Component	Per litre	Supplier
$NaHPO_4$	0.28 g (2 mM)	Fluka
KH_2PO_4	2.04 g (15 mM)	Fluka
$CaCl_2$ x 2 H_2O	7.35 mg (50 mg)	Roth

The pH was adjusted to 6.0 using KOH, autoclaved and stored at 4°C.

2.1.5.4 *Acanthamoeba castellanii*

PYG (Peptone yeast extract glucose) medium (Moffat and Tompkins, 1992)

Component	Per litre	Supplier
Bacto proteose peptone	20 g	BD BioSciences
BBL yeast extract	1 g	BD BioSciences
$MgSO_4$ x 7 H_2O	10 ml (4 mM)	Roth
Sodium citrate x 2 H_2O	3.4 ml (1 M)	Roth
$Fe(NH_4)_2$ x 7 H_2O	10 ml (0.25 M)	Roth
Na_2HPO_4	10 ml (0.25 M)	Roth
KH_2PO_4	2.5 mg (2.5 mM)	Fluka
D(+)-glucose monohydrate	19.8 g	Fluka

The pH was adjusted to 6.5 with 1 M KOH or 1 M HCl. After addition of 50 ml 2 M glucose the medium was filter-sterilised using a glass-fiber filter 5 times and stored at 4°C.

Lo Flo medium

Component	Per litre	Supplier
LoFlo base	20 g	ForMedium (Norfolk)

Ac buffer (Moffat and Tompkins, 1992)

Component	Per litre	Supplier
$MgSO_4$ x 7 H_2O	985.9 mg (4 mM)	Oxoid
$CaCl_2$ x 2 H_2O	44 mg (0.4 mM)	BD BioSciences
Sodium citrate x 2 H_2O	999 mg (3.4 mM)	AppliChem
Na_2HPO_4 x 7 H_2O	86 mg (0.05 mM)	
KH_2PO_4	2.5 mg (2.5 mM)	
NH_4Cl	2.6 mg (0.05 mM)	
$FeSO_4$	7.55 mg (0.05 mM)	

The pH was adjusted pH to 6.5 using 1 M HCl, autoclaved and stored at 4°C.

Phosphate buffered saline (PBS) 10x

Component	Final concentration	Supplier
NaCl	80 g	Roth
KCl	2 g	Roth
Na_2HPO_4	14.2 g	Fluka
KH_2PO_4	2.4 g	Fluka

The pH was adjusted to 7.4 with 1 M NaOH or 1 M HCl, autoclaved and stored at room temperature.

2.2 Methods

2.2.1 *Legionella pneumophila*

2.2.1.1 Cultivation of *L. pneumophila*

The bacterial strains used are listed in Section 2.1.3. *L. pneumophila* was grown on CYE agar plates (Feeley *et al*., 1979), or in AYE broth, supplemented with chloramphenicol (Cm, 5 µg/ml), gentamicin (Gm, 10 µg/ml) or kanamycin (Km, 50 µg/ml), if necessary.
L. pneumophila grown for 3 days on CYE plates were used to inoculate 3 ml of AYE liquid medium in a 15-ml polystyrene test tube at an OD_{600} of 0.1 for subsequent incubation on a turning wheel at 37°C.

2.2.1.2 *L. pneumophila* glycerol stocks

Late-exponential *L. pneumophila* liquid cultures (Section 2.2.1.1) were mixed with 50% glycerol in a 1:2 ratio and frozen in liquid nitrogen in cryo tubes for storage purpose at -80°C.

2.2.1.3 Electrocompetent *L. pneumophila*

Exponentially grown overnight culture of *L. pneumophila* (Section 2.2.1.2) was diluted 1:30 and used to inoculate 30 ml of AYE. At an OD_{600} between 0.3 and 0.6, the bacteria were cooled on ice and washed 3 times with sterile, ice-cold 10% glycerol (10 ml, 2.5 ml, 160 µl). 40-µl aliquots were frozen in liquid nitrogen and stored at -80°C.

2.2.1.4 Transformation of *L. pneumophila* by electroporation

40 µl electrocompetent *L. pneumophila* (Section 2.2.1.3) were mixed with a minimum of 150 ng of plasmid DNA on ice. Bacteria were transferred into a 2 mm electrode-gap electroporation cuvette and subjected to electroporation (2.5 kV, 200 Ohm, 25 µF, 5 ms). After addition of 450 µl AYE, bacteria were incubated at 37°C at 800 rpm for 5 h and plated onto selective CYE agar.

2.2.2 *Escherichia coli*

2.2.2.1 Cultivation of *E. coli*

E. coli strains were cultured in LB medium, supplemented with Cm (30 µg/ml) or Km (30 µg/ml), if required. Protein overproduction in *E. coli* was performed in ZY50-52 medium (Section 2.1.5.2) as described elsewhere (Studier, 2005).

2.2.2.2 Preparation of chemocompetent *E. coli*

A late-exponential *E. coli* overnight culture (Section 2.2.2.1) was used to inoculate 100 ml of LB medium at 37°C at 180 rpm. At an OD_{600} of 0.5, bacteria were cooled on ice and washed with 40 ml of ice-cold TFB1 (Section 2.1.5.2) and subsequently with 4 ml of ice-cold TFB2. Bacterial aliquots of 40 µl were flash-frozen in liquid nitrogen and stored at -80°C.

2.2.2.3 Transformation of *E. coli* by heat shock

40 µl of chemocompetent *E. coli* (Section 2.2.2.2) were incubated with 100 ng of plasmid DNA on ice for 30 min. After performing a heat shock at 42°C for 45 s, bacteria were mixed with 450 µl of LB, incubated for 90 min at 800 rpm at 37°C and plated on selective LB agar.

2.2.2.4 Glycerol stocks of *E. coli*

Overnight *E. coli* liquid cultures (Section 2.2.2.1) grown in LB medium were mixed with 50% glycerol in a 1:2 ratio and frozen in liquid nitrogen in cryo tubes for storage purpose at -80°C.

2.2.3 Mammalian and protozoan cell lines

2.2.3.1 Cultivation of mammalian cell lines

The murine macrophage cell line RAW264.7 and human HL-60 cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C in RPMI (Roswell Park Memorial Institute) 1640 medium (Life Technologies) supplemented with 10% (v/v) fetal calf serum and 2 mM L-glutamine (50 µg/ml). HL-60 cells were differentiated into macrophages with phorbol 12-myristate 13-acetate (PMA, 100 ng/ml, 48 h) as described by Hilbi *et al*. (2001).

2.2.3.2 Storage of mammalian cell lines

Cells were cultivated to 80% confluence in a 75 cm^2 culture flask, spun down and resuspended in 3-4 ml freezing medium (70% RPMI 1640, 20% FCS, 10% DMSO). Cryo tubes were filled with 1 ml-aliqouts of the prepared suspension and were transferred to a freezing box (containing isopropanol and precooled at 4°C for 1 h), frozen at -80°C overnight and stored in liquid N_2.

2.2.3.3 Cultivation of protozoan cell lines

Acanthamoeba castellanii (ATCC 30234) was grown in PYG medium (Section 2.1.5.4) at 30°C (Segal and Shuman, 1999, Moffat and Tompkins, 1992). *Dictyostelium discoideum* wild-type strain Ax3 was grown in HL5 medium (Section 2.1.5.3) at 23°C as described (Weber *et al*., 2006).

2.2.3.4 Storage of protozoan cell lines

9 ml of *D. disoideum* or *A. castellanii* cultures were grown to 80% confluence in a 75 cm^2 culture flask (Section 2.2.3.3), and resuspended in 3-4 ml freezing medium (80% HL-5 or PYG, 10% FCS, 10% DMSO). Cryo tubes were filled with 1 ml-aliqouts of the prepared suspension and were transferred to a freezing box (containing isopropanol and precooled at 4°C for 1 h), frozen at -80°C overnight and stored in liquid N_2.

2.2.4 Construction of vectors for expression, allelic exchange and competence

Cloning and DNA manipulations were performed according to standard protocols (Promega, NEB, Fermentas, Roche). DNA fragments were amplified using primers listed in Section 2.1.4 and plasmids were isolated using commercially available kits from Qiagen or Macherey-Nagel. All constructs were verified by DNA sequencing (GATC, Germany).

To construct the allelic exchange vectors pNT-46 (*lqsT*, Km^R) or pNT-47 (*lqsT*, Gm^R), approximately 0.6 kb each of the 5′ and 3′ flanking sequences of *lqsT* (TH1, TH2) were amplified by PCR using the primer pairs oH1-LqsT-fo/-re and oH2-LqsT-fo/-re respectively. The PCR products and a Km resistance cassette from pUC4K or a Gm resistance cassette from pBSL141 were digested with *Bam*HI and ligated into pGEM-T-Easy in a four-way ligation reaction. The resulting clones were analysed by restriction digestion and sequenced. Using *Not*I the TH1-Km^R-TH2 or TH1-Gm^R-TH2 fragments were then cloned into the pLAW344 suicide vector, yielding plasmid pNT-46 or pNT-47 respectively.

The deletion plasmid for pAK-16 was constructed as follows: Using primer pairs oH1-SinR-fo/re and oH2-SinR-fo/re, 0.7 kb of the 5’ and 3’ flanking sequences of *sinR* (SH1, SH2) were amplified by PCR and together with a Km resistance cassette cloned into pLAW344 using the restriction sites *Xba*I and *Bam*HI.

The expression vectors pAK-2 (*lqsT* under control of P_{lqsT}, constitutive *gfp* expression) and pAK-6 (*lqsT* under control of P_{tac}) were constructed by PCR amplification using chromosomal DNA of *L. pneumophila* JR32 as template and the primer pairs oP-LqsT-fo/-re (including the 5′ UTR of *lqsT*), or oLqsT-fo/oP-LqsT-re respectively (Section 2.1.4). The PCR products were ligated into pGEM-T-Easy, liberated by digestion with either *Bam*HI or *Nde*I/*Bam*HI and cloned into plasmid pNT-28 (*Bam*HI within the additional MCS) or into pMMB207C-RBS-*lcsC* (*Nde*I/*Bam*HI). All PCR products were sequenced (GATC, Munich).

The complementation plasmid pAK-18 was generated by amplifying *sinR* and its promoter by PCR, digestion with *Xba*I and *Bam*HI and cloning into pNT-28.

GFP-transcriptional fusions of P_{frgA} and P_{sinR} were constructed using primer pairs oP-FrgA-fo/re and oP-SinR-fo/re and cloned into the *Sac*I and *Xba*I sites of pCM-4, yielding pAK-17 and pUS-11, respectively.

For the construction of the vector encoding His-SinR (pAK-21), a PCR fragment amplified with the primer pair oSinR-fo/re was cloned into pET28a(+) using the restriction sites *Nde*I and *Bam*HI.

For competence assays, the vector pAK-15 harbouring a Cm resistance cassette was generated as follows: flanking sequences of *lqsA* and *lqsS* (0.5 kb each) were amplified by PCR using the primer pair oC-LqsA-fo/-re and oC-LqsS-fo/-re, and pNT-1 as a template. The Cm resistance cassette fragment (1.1 kb) was amplified by PCR using the primer pair oCR-fo/-re and pTS-1 as a template. The PCR products were digested with *Pst*I and ligated into the pGEM-T-Easy vector in a four-way ligation reaction. The resulting clones were analysed by restriction digestion and sequenced for correct inserts.

2.2.5 Construction of chromosomal *lqsT*, *lqsS-lqsT* and *sinR* deletion strains

Allelic exchange by double homologous recombination using counter-selection on sucrose was performed essentially as described (Tiaden *et al*., 2007; Wiater *et al*., 1994). To construct a chromosomal *lqsT* or *sinR* deletion strain, *L. pneumophila* JR32 was transformed by electroporation with pNT-46 or pAK-16, respectively. Co-integration of the plasmids was assayed by selection on CYE/Km (5-7 d, 30°C). Several clones thus obtained were re-streaked on CYE/Km, grown overnight in 96-well plates containing AYE medium at 180 rpm and streaked on CYE/Km containing 2% sucrose (Suc). After an incubation period of 3-5 d at 37°C, single colonies were spotted on CYE/Cm, CYE/Km/2% Suc and CYE/Km plates to screen for Cm^S, Km^R, Suc^R colonies. Double-cross-over events and thus deletion mutants were confirmed by PCR screening and sequencing.

For construction of the double mutant strain *lqsS-lqsT*, pNT-47 was transformed into Δ*lqsS*. Subsequent selection steps were performed on CYE/Gm. Candidate deletion mutant clones were screened by PCR and confirmed by sequencing.

2.2.6 Production and purification of LqsR

The LqsR expression vectors encoding His-$LqsR_{D108A}$ (pRB-3) or His-$LqsR_{D108N}$ (pRB-4) were generated from His-LqsR (pTS-23) (Tiaden *et al*., 2007) by site-directed mutagenesis as described in Schell *et al*. (2014). The constructs were confirmed by sequencing and transformed into *E. coli* BL21(DE3) cells (Robichon *et al.,* 2011). For large-scale protein production, bacterial cells grown overnight at 37°C in LB were used to inoculate main cultures of ZYM-5052 autoinduction medium at a ratio of 1:500. Bacterial cells were grown at 30°C for 24 h, harvested (4'000 x g, 4°C, 20 min) and resuspended in lysis buffer (50 mM Tris-HCl pH 7.5, 10 mM β-mercaptoethanol, 10% glycerol, 500 mM NaCl) containing lysozyme and Roche Complete protease inhibitor cocktail (Roche). After cell disruption by sonication, the lysate was centrifuged (24'000 x g, 4°C, 1 h) and filtrated (0.45 µm pore size filter units). Target proteins in the obtained supernatant were purified by immobilised metal ion affinity chromatography (IMAC) using an Äkta purifier system equipped with a HisTrap HP affinity column (GE Healthcare) and buffer B (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 10 mM β-MeOH, 500 mM Imidazole, 10% glycerol). The fractions containing LqsR protein were pooled and concentrated to 5 ml using a Centriprep device (Microcon Ultrafree concentrator with a 30 kDa cut-off membrane (Millipore). Gel filtration of the samples was performed using a Superdex 200 16/60 size-exclusion chromatography (SEC) column (GE Healthcare) in SEC buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM β-MeOH). Protein purity and integrity was assessed by SDS-PAGE according to Lämmli (1970). Protein concentrations were determined using the NanoDrop 1000 apparatus (Thermo Scientific).

The protein-containing fractions were collected, concentrated to ~50 mg/ml for crystallisation, and frozen in aliquots for storage at -80°C. All samples LqsR wild type, $LqsR_{D108A}$ and $LqsR_{D108N}$ were treated likewise.

2.2.7 Crystallisation of LqsR

All recombinant protein samples His-LqsR wild type, His-LqsR$_{D108A}$ and His-LqsR$_{D108N}$ were screened initially using approximately 480 crystallisation conditions chosen from various commercially available kits: Clear Strategy I, Clear Strategy II, JCSG, Morpheus (Molecular Dimensions), PACT and PEGs (Qiagen). Sitting drop vapour-diffusion crystallisation experiments and automated seeding were set up at 20°C using a Mosquito crystallisation robot (TTP). In initial crystallisation screens, 0.3 µl reservoir solution was added to 0.3 µl protein solution in 96-well CrystalQuick Plates (Greiner), the reservoir wells contained 90 µl of the screen solution. To test if removal of the His tag has an effect on the crystallisation of LqsR, Thrombin cleaved LqsR$_{D108A}$ was tested using the screens PEGs Suite and PACT.

Crystal optimisation was performed with His-LqsR wild type, His-LqsR$_{D108A}$ and His-LqsR$_{D108N}$. applying the previously desbribed microseeding technique (Marsh and D'Arcy, 2007, Ireton and Stoddard, 2004, Bergfors, 2003). The seed preparations were made using the 'seed-bead' kit from Hampton Research, as described by Luft and DeTitta (1999). Briefly, crystals obtained from initial screen conditions were placed in 50 µl of their respective reservoir solution and mechanically homogenised on a vortex apparatus for 3 min at full speed. These seeds were stored as 50 µl aliquots and frozen at -80°C. Dilutions of the seed stocks (between 10- and 1000-fold) were generated with the respective reservoir solutions and stored likewise. Similarily, crystals obtained for LqsR wild type and LqsR$_{D108A}$ in the following PACT screen conditions were used to produce seed bead stocks: (0.2 M sodium acetate, 0.1 M bis Tris propane pH 7.5, 20% PEG 3350) and (0.2 M lithium chloride, 0.1 M HEPES pH 7.0, 20% PEG 6000), respectively. Images of all drops were collected after a period of 2 days using a Crystal Score imaging system (RockImager). Crystals were soaked briefly in cryoprotectant that consisted of 85/15% (v/v) reservoir solution with glycerol prior to data collection in an N_2 cold stream. Data were collected at 100 K at the beamlines X06SA and X06DA at the Swiss Light Source (SLS, Paul Scherrer Institute, Villigen, Switzerland) and were processed and merged with the XDS program (Kabsch, 2010).

2.2.8 Production and purification of SinR

His-SinR was purified from the cytosolic fraction of BL21(DE3)/pAK-21. Cells were grown aerobically in LB medium at 30°C and induced with 0.1 mM IPTG during exponential growth for 10 h. Bacterial cells were harvested by centrifugation (20 min at 6000 x g; 4°C), resuspended and homogenised in lysis buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol (v/v), 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, 30 ng/ml DNase I) and disrupted at 10000 psi by French press. After centrifugation (10 min at 6000 x g, 4°C), the supernatant containing His-SinR was subjected to purification by affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA)-agarose (Qiagen) equilibrated with buffer E (10 mM imidazole, 10% (v/v) glycerol, 50 mM Tris-HCl pH 8.0, 10 mM β-MeOH). His-tagged proteins were eluted using buffer E containing 250 mM imidazole. His-SinR was examined by SDS-PAGE and subsequent Coomassie Brilliant Blue staining. Protein concentrations were determined using the NanoDrop 1000 apparatus.

2.2.9 Electrophoretic mobility shift assays (EMSA)

For electrophoretic mobility shift assay (EMSA), amplicons spanning the promoter regions of *sinR*, *lqsA*, or *lqsR* (P_{sinR}, P_{lqsA}, or P_{lqsR}) were generated by PCR amplification using primer sets oSinR-fo/re, oLqsA-EMSA-fo/re, oLqsR-EMSA-fo/re, or oRpoS-EMSA-fo/re respectively. *sinR* and *lqsA* promoter fragments were restriction digested with *Eco*RI, yielding DNA probes A, B and E, F, respectively (see Section 3.3.1.2, Figures 21 and 22). DNA probes (65 pmol) were incubated with various concentrations of His_6-SinR (0, 10, 20, 40, 60, 80, 100, 120, 140 µM) in a 20-µl reaction mixture containing 50 mM Tris-HCl, pH 8.0; 750 mM KCl; 2.5 mM EDTA, 62.5% (v/v) glycerol; 0.5% Triton-X 100, 1 mM dithiothreitol (DTT). Each reaction was supplied with 1 µg of the copolymer poly(deoxyinosinic-deoxycytidylic) acid (poly(dI-dC)) to prevent unspecific binding of protein to the promoter regions of interest. After 10 min of incubation at room temperature, the binding reaction mixtures were resolved by electrophoresis for approximately 1 h at 180 V on a 6% nondenaturing polyacrylamide gel in 1×TBE buffer. Visualisation and digital image capture of the EtBr-stained bands were performed using a Bio-Rad imager.

2.2.10 Host cell interaction experiments

2.2.10.1 Phagocytosis

Phagocytosis of GFP-labelled *L. pneumophila* by *A. castellanii*, *D. discoideum* or RAW 264.7 macrophages was assessed by FACS (fluorescence-activated cell sorting) as described (Tiaden *et al*., 2007, Weber *et al*., 2006). Briefly, cells were seeded onto a 24-well plate (2.5×10^5 *A. castellanii*/ml, 5×10^5 *D. discoideum*/ml, or 2 x 10^5 RAW macrophages/ml) and were allowed to adhere for 1–2 h. *L. pneumophila* AYE overnight cultures grown for 21 h were diluted in the corresponding culture media and used for infection of the amoebae and macrophages at an MOI of 50. The infection was synchronised by centrifugation (10 min, 880 × g), and the infected cells were incubated at 30°C (*A. castellanii*), 25°C (*D. discoideum*) or 37°C (RAW 264.7 cells). At 40 min post infection the infected cells were washed three times with Ac buffer (*A. castellanii*), SorC buffer (*D. discoideum*), or PBS (RAW 264.7 cells). Cells were detached by vigorously pipetting in culture supernatant, and GFP fluorescence was measured using a FACS Canto flow cytometer (BD BioSciences) and a scatter gate adjusted for *A. castellanii, D. discoideum* or RAW 264.7 cells. The viability of *L. pneumophila* (CFU) and expression of GFP (typically 80-90%) was routinely controlled.

2.2.10.2 Intracellular replication

For an iIntracellular replication assay *A. castellanii, D. discoideum* or RAW 264.7 macrophages were washed once, suspended in Ac buffer (*A. castellanii*), MB medium (*D. discoideum*) or PBS (RAW 264.7 macrophages), seeded onto a 96-well plate (2.5×10^5 *A. castellanii*/ml, 5×10^5 *D. discoideum*/ml or 2×10^5 RAW macrophages/ml) and allowed to adhere. *L. pneumophila* was grown for 21 h in AYE broth, diluted in Ac buffer (*A. castellanii*) or MB medium (*D. discoideum*) or RPMI 1640 (RAW 264.7 cells) and used to infect the amoebae at an MOI of 0.1. The infection was synchronised by centrifugation, and the infected amoebae were incubated at 30°C (*A.*

castellanii), 25°C (*D. discoideum*) or 37°C (RAW macrophages). At days 1-5 post infection, samples were taken by resuspending the infected amoebae in culture supernatant.

Single round intracellular growth of GFP-producing *L. pneumophila* was assayed in *A. castellanii* amoebae or RAW macrophages, which were transferred to fresh medium (PYG or RPMI 1640, respectively) 2 days before the experiment. One day before the experiment, the cells were resuspended and seeded into a black 96-well clear bottom plate (Perkin-Elmer) at a density of 2×10^4 (amoebae) or 8×10^4 (RAW 264.7 macrophages) cells per well and allowed to adhere overnight. *L. pneumophila* transformed with pNT-28 (constitutively producing GFP) were grown overnight in AYE/Cm to an OD_{600} of 3.0 ($\sim 2 \times 10^9$ bacteria/ml) and diluted to 8×10^6 bacteria/ml in LoFlo low fluorescence medium (Formedium). The cells were infected at an MOI of 20 with 100 µl of diluted *L. pneumophila* suspension by centrifugation at 450 × g for 10 min, and then incubated at 30°C for 48 h or several days. GFP fluorescence was quantified at multiple time points using a plate reader (FLUOstar Optima, BMG Labtech). To correlate fluorescence readings with bacterial viability, the cells were lysed at set time points using 0.8% saponin (amoebae) or ddH_2O (macrophages), dilutions were plated on CYE plates, and CFU were recorded.

2.2.10.3 Co-infection experiments

For co-infection competition assays the protocol of Herrmann *et al*. was modified (Herrmann *et al*., 2011). *A. castellanii* amoebae (5×10^4 per well, 96-well plate) in Ac buffer were infected at an MOI of 0.01 each with wild-type *L. pneumophila* and the Km-resistant mutant strain to be tested. The infected amoebae were grown for 21 days at 37°C. Every third day the supernatant and amoebae lysed with 0.8% saponin were diluted 1:1000, fresh amoebae were infected (50 µl homogenate per 200 µl amoebae culture volume), and aliquots were plated on CYE agar plates containing Km or not to determine CFU.

2.2.10.4 Gentamicin protection assay

Phagocytosis of *L. pneumophila* by HL-60 macrophages was analysed by a gentamicin protection assay as described (Hilbi *et al*., 2001). Briefly, HL-60 macrophages differentiated with PMA (100 ng/ml) were infected at an MOI of 100 with *L. pneumophila* strains grown for 21 h in AYE broth, washed and resuspended in RGN medium (RPMI 1640, 2 mM L-glutamine, 10% normal human AB serum). After centrifugation, the infected macrophages were incubated for 20 min at 37ºC, washed with PBS, and the medium was replaced by RGN medium containing gentamicin (0.1 mg/ml). After another 40 min, the medium was aspirated, the infected macrophages were lysed by addition of 100 µl H_2O, and 20 µl of the lysate were plated onto CYE agar plates.

2.2.11 Salt sensitivity test

The stress resistance of *L. pneumophila* was analysed using 0.5 ml of cultures in exponential (OD_{600}=2.0) or stationary (OD_{600}=3.8) growth phase. The bacteria were harvested and resuspended in 0.5 ml PBS (control) or the medium containing either 10 mM H_2O_2 (10 min), 0.1 M citric acid pH 3 (10 min) or 5 M NaCl (30 min). The bacteria were collected by centrifugation, resuspended in 0.5 ml of PBS and plated in appropriate dilutions on CYE plates.

2.2.12 Biofilm formation and sedimentation

Biofilm formation of *L. pneumophila* under static conditions was assayed in 96-well polystyrene microtitre plate by crystal violet incorporation after 5 days as described (Mampel *et al*., 2006). Briefly, stationary phase *L. pneumophila* overnight cultures were adjusted to OD_{600} of 0.2, and 200 µl were transferred to a 96-well microtitre plate. Following incubation at 37°C for 3 days, the biomass of biofilm was quantified by crystal violet staining assay. Bacteria were stained with 200 µl of a 0.2% crystal violet solution in each well for 15 min, washed 3 times with 340 µl sterile distilled water and solubilised in 200 µl of 95% ethanol at room temperature for 15 min. Absorbance was measured at OD_{595} with a microplate reader. Each experiment was repeated three times with nine technical replicates for each condition and control.

To assay sedimentation, the bacteria were grown for 3–4 days on CYE agar plates, resuspended in 1–2 ml AYE medium at an OD_{600} of 3.5 and let sediment for 6–12 h at room temperature (Tiaden *et al*., 2010).

2.2.13 Natural competence

Analysis of natural competence was performed according to Charpentier *et al.* (2011), with the following modifications: *L. pneumophila* wild-type and *lqs* mutant strains were grown in 5 ml of AYE broth to post-exponential growth phase (OD_{600} 2.6–3.0) or to the OD_{600} indicated. The cultures were centrifuged, and after removing 2 ml of AYE, resuspended in the remaining 3 ml of the medium. The bacteria were then incubated without agitation at 30°C for 24 h with 300 ng of linear DNA (purified PCR product). The PCR fragments consisted of either a Gm resistance cassette inserted into the 5′ and 3′ flanking regions of *lqsT* (amplified with the primers oH1-LqsT fo/oH2-LqsT-re using pNT-47 as template), or a Cm resistance cassette plus flanking regions of the *lqs* cluster (amplified with the primers oC-LqsA-fo/oC-LqsS-re using pAK-15 as template). Subsequently, the strains were plated in appropriate dilutions on CYE agar plates supplemented with 5 µg/ml Cm or 10 µg/ml Gm, and CFU were determined after 3 days. For the Δ*lqsS*-Δ*lqsT* strain (Km^R, Gm^R) only linear DNA conferring Cm resistance was used.

2.2.14 GFP reporter experiments

Overnight liquid cultures of *L. pneumophila* strains harbouring transcriptional *gfp* fusion reporter constructs were grown to an OD_{600} of 1.5-2.0 and diluted to an initial OD_{600} of 0.1 in AYE/Cm (200 µl/well), and the 96-well plate was incubated at 37°C at 150 rpm. Growth and expression of the green fluorescent protein (GFP) gene fusions were monitored by measuring the absorbance at 600 nm (A_{600}) and fluorescence (RFU, relative fluorescence units; excitation, 485 nm; emission, 520 nm) using a microtitre plate reader (FluoStar Optima, BMG Labtech). Values are expressed as relative fluorescence units (RFU) per OD_{600} and represent means and standard deviations of sextuplicates.

3. Results

3.1 The *Legionella pneumophila* sensor kinase LqsT, a homologoue of LqsS

Bioinformatic analysis of the *L. pneumophila* strain Philadelphia-1 genome revealed a gene (*lpg2506*), which was homologous to the *L.pneumophila lqsS* and *V. cholerae cqsS* sensor histidine kinases (Miller *et al.*, 2002) and therefore termed *lqsT*. The putative histidine kinase LqsT

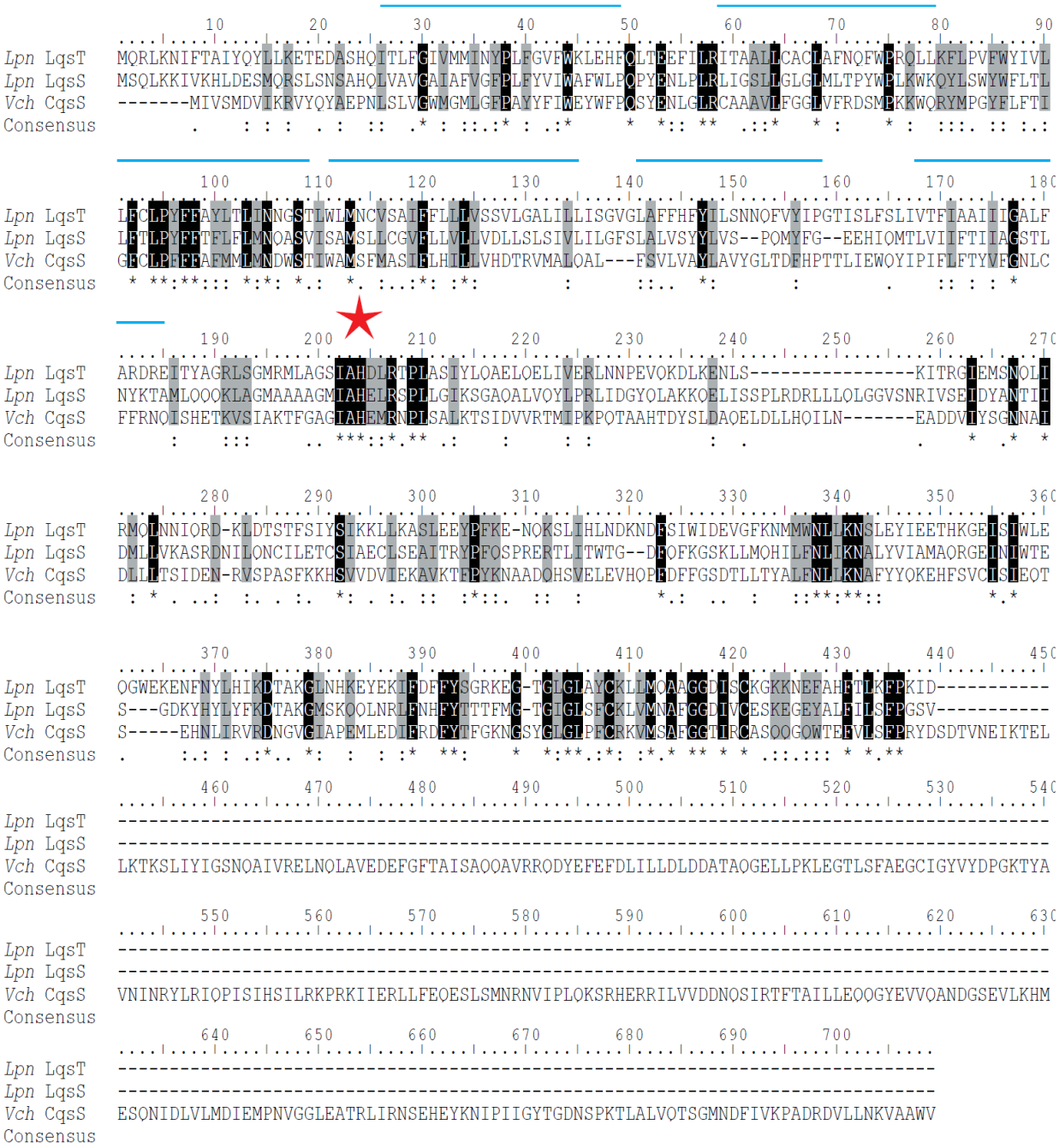

Figure 4: Sequence comparison of Lqs/Cqs family sensor histidine kinases. The sequences of the Lqs/Cqs family sensor histidine kinases LqsT *(lpg2506*) and LqsS (*lpg2733*) of *Legionella pneumophila* Philadelphia-1 and CqsS of *Vibrio cholerae* C6706str El Tor (Accession Q9KM66) were aligned using the ClustalW algorithm and Bioedit. The degree of residue shading was determined by using BioEdit at setting 'Blosum62'. Black and grey shading indicate 100% and 80% residue similarity, respectively. The putative six transmembrane helices are marked with a blue line, the conserved histidine residue ($LqsT_{H204}$, $LqsS_{H200}$ and $CqsS_{H195}$) and putative phosphorylation site is represented by a red star.

shares 31% sequence identity with *L. pneumophila* LqsS including six transmembrane helices adjacent to the periplasmic signal recognition domain. Analogously to LqsS, LqsT also lacks the C-terminal receiver domain of the *V. cholerae* hybrid sensor kinase CqsS (Figure 4).

In contrast to *cqsS* and *lqsS*, the 'orphan' gene *lpg2506* is located in a distance to the *lqs* cluster in the genome, in the vicinity of three genes encoding Icm/Dot substrates (Figure 5): *sdeD* (*lpg2509*), *sdcA* (*lpg2510*) and *sidC* (*lpg2511*) (Luo and Isberg, 2004, Weber *et al.*, 2006, Ragaz *et al.*, 2008, respectively). Moreover, the *lpg2506* gene is expressed from its own promoter (Sahr *et al.,* 2012) and does not bear any resemblance to other genes apart from *lqsS*

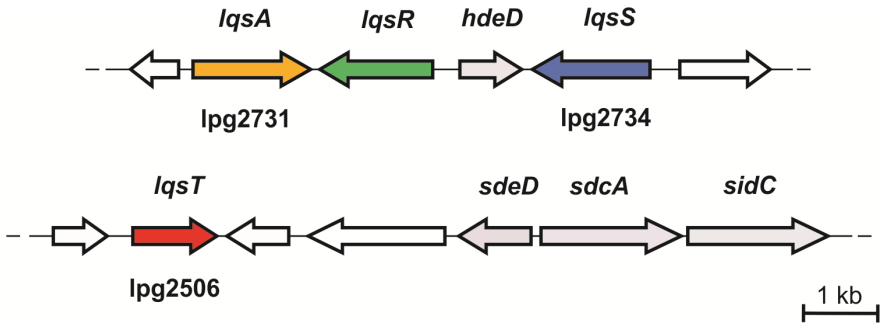

Figure 5: The *lqs* system in *L. pneumophila*. Genomic organisation of the *L. pneumophila lqs* gene cluster (*lpg2731–2734*) and *lqsT* (*lpg2506*). The *lqs* cluster comprises the autoinducer synthase *lqsA*, the response regulator *lqsR*, a homologue of *E. coli hdeD* (HNS-dependent expression D) and the cognate sensor kinase *lqsS*. The 'orphan' sensor kinase *lqsT* localises in the vicinity of the effector genes *sdeD*, *sdcA* and *sidC*.

To characterise the *lqsT* gene on the genetic level, defined chromosomal deletion mutants of *L. pneumophila* Philadelphia-1 lacking *lqsT* (Δ*lqsT*; AK01), or *lqsS* and *lqsT* (Δ*lqsS*-Δ*lqsT*; AK02) were constructed by allelic exchange. The strains Δ*lqsT* or Δ*lqsS*-Δ*lqsT* replicate at the same rate as wild-type bacteria in AYE broth (Figure 6).

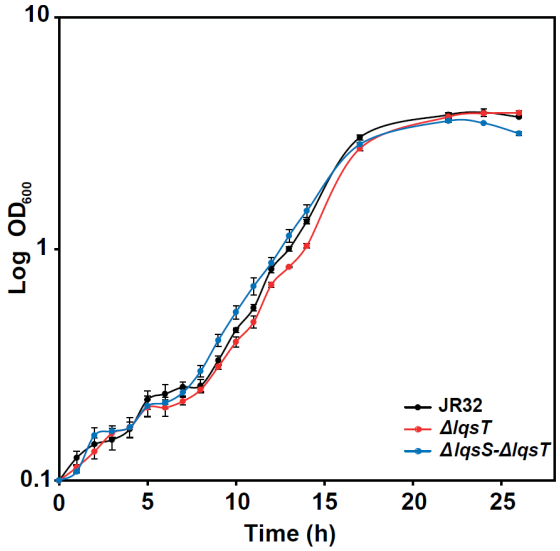

Figure 6: Growth of the *L. pneumophila* Δ*lqsT* and Δ*lqsS*-Δ*lqsT* mutant strains in AYE broth. *L. pneumophila* wild type, Δ*lqsT* or Δ*lqsS*-Δ*lqsT* mutant strains were grown in AYE broth at 37°C and the optical density (OD_{600}) was monitored over time. Data represent means and standard deviations of triplicates and are representative of at least three independent experiments.

Moreover, the Δ*lqsT* mutant strain was identical to wild-type bacteria regarding morphology, growth on agar plates at different pH values (pH 6.5–7.5) and at lower temperature (25°C), persistence (14 days in water) or biofilm formation (Kessler *et al.*, 2013).

3.1.1 Salt sensitivity and sedimentation of *L. pneumophila* lacking *lqsT* or *lqsS* and *lqsT*

The transition from the exponential to the post-exponential growth phase confers profound phenotypic changes to *L. pneumophila*. In particular, the expression of various virulence attributes is linked to specific growth conditions. Wild-type *L. pneumophila* are sensitive to sodium chloride, a trait that closely correlates with virulence, such that avirulent strains are more salt-resistant (Hales and Shuman, 1999, Byrne and Swanson, 1998). To compare the salt sensitivity of Δ*lqsT* or Δ*lqsS*-Δ*lqsT* to wild-type or *lqs* mutant bacteria, the strains were grown to stationary phase and spotted in serial dilutions on CYE agar plates in the presence or absence of 100 mM NaCl. In the presence of salt, plating efficiency of the Δ*lqsT* or Δ*lqsS*-Δ*lqsT* strains was 1000-fold higher compared with wild-type bacteria, akin to the Δ*lqsR* and Δ*lqsS* mutant strains (Figure 7). Expression of *lqsT* on a plasmid under control of its native promoter complemented the phenotype both in the Δ*lqsT* and in the Δ*lqsS*-Δ*lqsT* background. Similarly, salt sensitivity was restored by introducing *lqsS* on a plasmid under the control of the endogenous promoter either in the Δ*lqsS* or in the Δ*lqsS*-Δ*lqsT* background. On the other hand, exponentially growing *lqs* mutant strains did not display any differences in salt sensitivity (Kessler *et al.*, 2013).

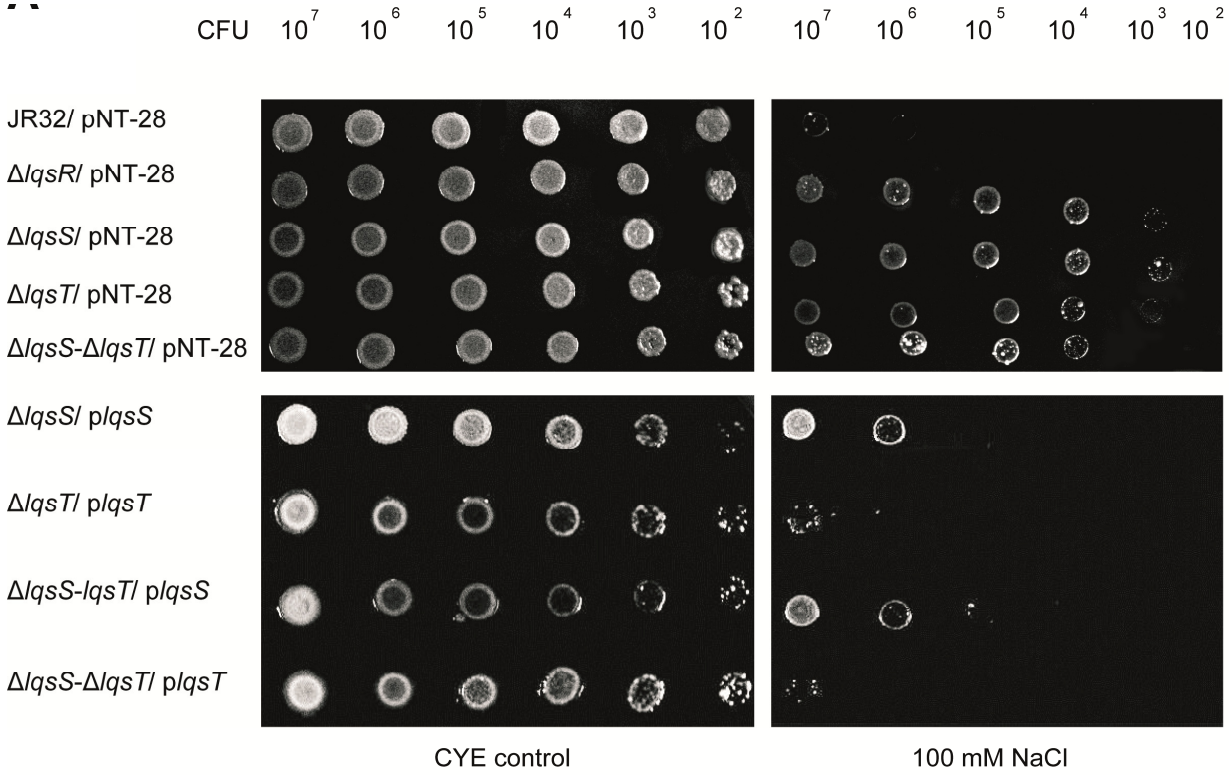

Figure 7: Salt sensitivity of *L. pneumophila* Δ*lqsT* or Δ*lqsS*-Δ*lqsT* mutant strains. *L. pneumophila* wild-type, Δ*lqsR, ΔlqsS, ΔlqsT or* Δ*lqsS*-Δ*lqsT* mutant strains harbouring a vector control (pNT-28) or a plasmid expressing *lqsS* (p*lqsS,* pNT-31) or *lqsT* (p*lqsT;* pAK-2) under control of their native promoters were grown to stationary growth phase in AYE broth at 37°C and spotted in decreasing concentrations onto CYE agar plates either with or without 100 mM NaCl (control). Similar results were obtained in 3 independent experiments. Published in Kessler *et al.*, 2013.

Together, these results indicate that the absence of *lqsT* decreases the bacterial salt sensitivity during stationary phase, and *lqsT* and *lqsS* may functionally substitute each other.

Wild-type *L. pneumophila* grown for 3–4 days on CYE agar plates and suspended in AYE medium were observed to settle to the bottom of polystyrene test tubes and form a loose cell pellet after 12-18 hours. By contrast, strains lacking *lqsR* or *lqsS* exhibited impaired sedimentation behaviour due to the formation of extracellular filaments (Tiaden *et al*., 2010b). To test whether *lqsT* played a role in the production of extracellular filaments, sedimentation of the Δ*lqsT* or Δ*lqsS*-Δ*lqsT* strains was compared to wild-type bacteria and the Δ*lqsR* and Δ*lqsS* strains (Figure 8). Consistent with previous results, the Δ*lqsR* and Δ*lqsS* mutant strains remained partially resuspended with limited sedimentation. By contrast, Δ*lqsT* formed a wild-type-like cell pellet, indicating that the formation of extracellular filaments implicated in sedimentation did not depend on *lqsT*. Bacterial cell pellets of the Δ*lqsS* and Δ*lqsS*-Δ*lqsT* strains were of similar size, indicating that the absence of *lqsS* promotes the production of extracellular filaments in a dominant manner.

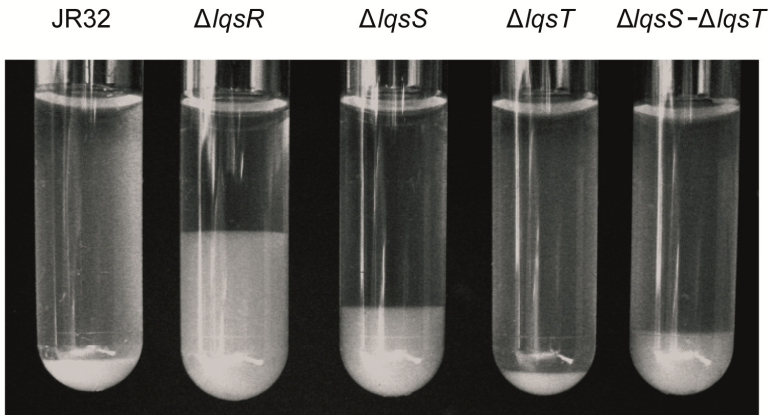

Figure 8: Sedimentation behavior of *L. pneumophila* lacking *lqsT* or *lqsS* and *lqsT*. *L. pneumophila* wild type*,* Δ*lqsR,* Δ*lqsS,* Δ*lqsT or* Δ*lqsS*-Δ*lqsT* were grown for 3 days on CYE agar plates, resuspended in 1 ml of AYE broth at an OD_{600} of 3.5 and analysed for sedimentation at room temperature for 12 h. Similar results were obtained in at least three independent experiments. Published in Kessler *et al.*, 2013.

3.1.2 The role of *lqs* genes in the regulation of natural competence

Natural competence for transformation is a mode of horizontal gene transfer used by bacteria to acquire DNA from their environment. This process is mediated by a set of proteins dedicated to uptake and subsequent homologous recombination and integration of transforming DNA (Seitz *et al.*, 2014): Transport of exogenous DNA through the outer membrane involves type IV pili. The passage of DNA through the cytoplasmic membrane in *Bacillus subtilis* was shown to be mediated

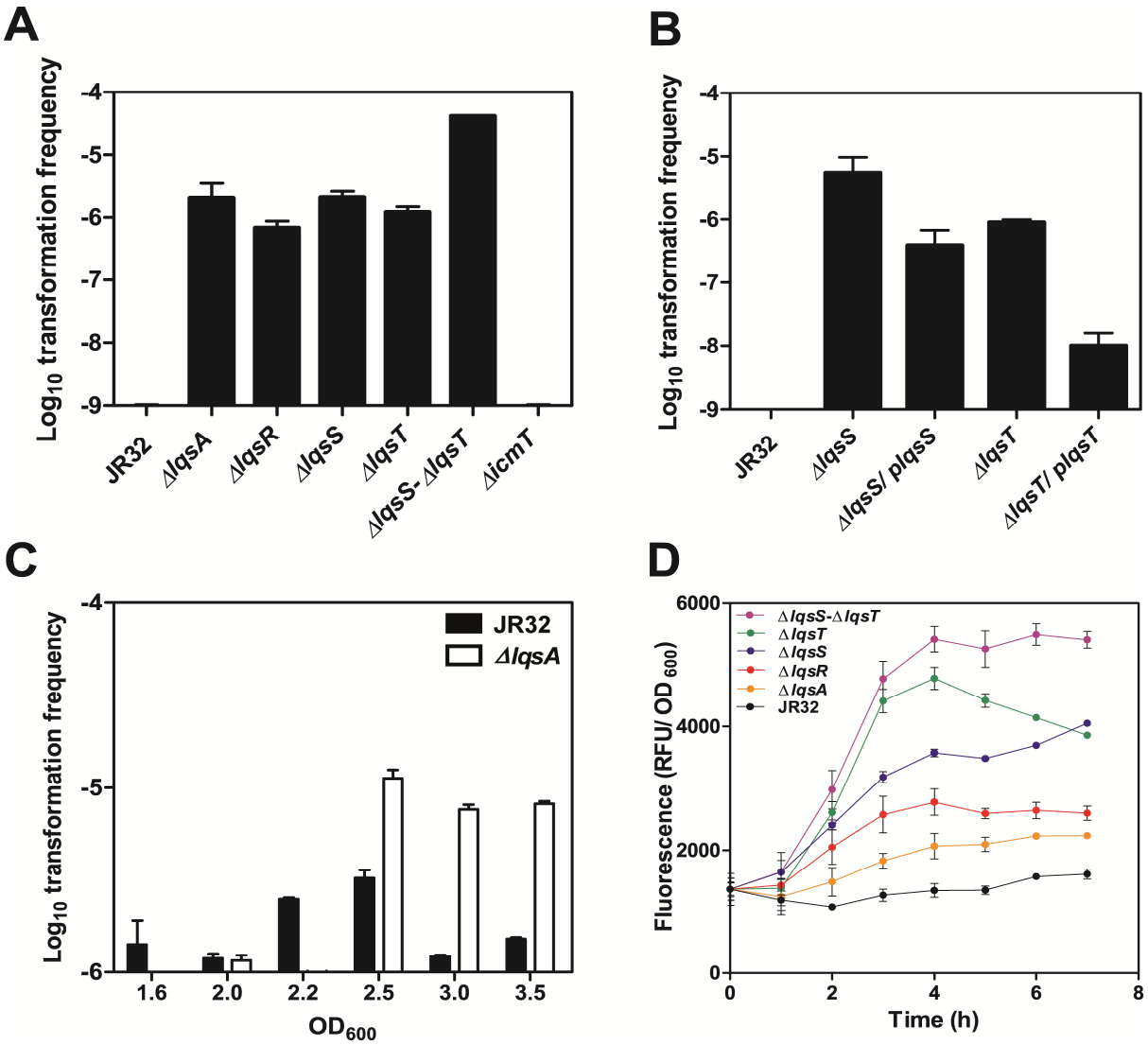

Figure 9: *Lqs* genes regulate natural competence for transformation in *L. pneumophila*. *L. pneumophila* wild-type, Δ*lqsA,* Δ*lqsR,* Δ*lqsS,* Δ*lqsT,* Δ*lqsS*-Δ*lqsT* or Δ*icmT* strains were grown in AYE broth (A, B, D) to post-exponential growth phase (OD_{600} 2.6–3.0) or (C) to the OD_{600} indicated and incubated at 30°C for 24 h with linear DNA encoding (A, B) a Cm or (C) a Gm resistance cassette flanked by fragments homologous to the chromosomal up- and downstream regions of either the *lqs* cluster or *lqsT*. Transformation efficiency was quantified by determining CFU on selective agar plates. (D) P_{comEA}-dependent production of GFP relative to cell density (relative fluorescence units, RFU/OD_{600} was monitored over time in *L. pneumophila* wild-type, Δ*lqsA*, Δ*lqsR*, Δ*lqsS*, Δ*lqsT* or Δ*lqsS*-Δ*lqsT* strains harbouring a transcriptional P_{comEA}–*gfp* fusion reporter construct. The data shown are means and standard deviations of duplicates (A–C) or triplicates (D) and representative of at least two independent experiments. *P*-value (A–C) < 0.05 (unpaired Student's *t*-test) for an OD_{600} > 2.5. Published in Kessler *et al.*, 2013.

by the membrane-anchored dsDNA binding protein ComEA (Johnsborg *et al*., 2007, Chen *et al*., 2004a). To investigate any potential link between natural transformation and small-molecule signalling in *L. pneumophila*, the *lqs* mutant strains were tested for the ability to take up exogenous DNA. For this purpose, wild-type and *lqs* deletion bacteria were grown to late exponential growth phase and incubated with linear DNA. This DNA fragment comprised a Cm resistance cassette flanked by fragments homologous to the chromosomal up- and downstream regions of the *lqs* cluster. Selection for Cm resistance yielded only low numbers of wild-type bacteria, arguing for a generally low transformation frequency of this strain under the conditions applied. In stark contrast, four orders of magnitude more colonies appeared for strains lacking *lqsA*, *lqsR*, *lqsS* or *lqsT* (Figure 9A). Moreover, approximately 50 times more colonies of the Δ*lqsS*-Δ*lqsT* strain were obtained, compared with Δ*lqsS* or Δ*lqsT*. Natural competence of an *icmT* mutant strain lacking a functional Icm/Dot system was as low as the wild-type strain, suggesting that the T4SS, which also functions as a conjugation apparatus (Segal and Shuman, 1998), was not involved in the uptake of free DNA. The increased competence of the Δ*lqsS* or Δ*lqsT* strains was partially reverted by providing either *lqsS* or *lqsT* under the control of their native promoters, indicating that the genes are able to restore the phenotype (Figure 9B). Finally, in absence of the linear DNA fragment wild-type *L. pneumophila* or Δ*lqsS*-Δ*lqsT* failed to develop Cm-resistant transformants (Kessler *et al.*, 2013).

Given that quorum sensing-controlled features are commonly regulated by cell density, natural competence of *L. pneumophila* was assessed at various stages throughout the growth curve. To this end, Δ*lqsA* and wild-type bacterial cultures of varying optical densities were exposed to linear DNA fragments comprising a Gm resistance cassette flanked by fragments homologous to the chromosomal up- and downstream regions of *lqsT*. Transformation frequency of the strains was quantified by selection for and counting of Gm resistant colony-forming units. Natural competence of wild-type bacteria was low within the OD_{600} range of 1.5–3.5. In contrast, the transformation frequency of the Δ*lqsA* mutant strain was significantly elevated at an OD_{600} above 2.5. This implied that competence of *L. pneumophila* is indeed regulated by the bacterial density and in particular by the growth phase, yet presumably repressed by LAI-1 signalling at high cell densities (Figure 9C). Similarly, increased competence of a strain lacking *lqsR* was observed only for cultures with an OD_{600} larger than 2.5 (Kessler *et al.*, 2013).

The gene encoding for the small DNA-binding periplasmic protein ComEA has been described as the most differentially expressed gene of those required for natural transformation of *L. pneumophila* (Charpentier *et al.*, 2008). As a direct indicator of competence, production of GTP under the control of the P_{comEA} promoter was therefore analysed in the *lqs* mutant strains (Figure 9D). Late-exponential overnight cultures of bacterial strains harbouring a transcriptional P_{comEA}–*gfp* fusion were diluted to an initial OD_{600} of 0.1 in a microtitre plate and incubated at 600 rpm at 37°C. Compared with the wild-type strain, P_{comEA}-induced *gfp*-expression was significantly upregulated in bacteria laking *lqsA, lqsR, lqsS* or *lqsT* and even more pronounced in Δ*lqsS*-Δ*lqsT.* This observation was in line with the findings obtained for the uptake and integration of linear DNA by homologous recombination (Figure 9A). After 4 h growth the normalised P_{comEA}-dependent

fluorescence intensity was enhanced approximately 1.5 × (Δ*lqsA*), 2.0 × (Δ*lqsR*), 2.7 × (Δ*lqsS*), 3.6 × (Δ*lqsT*) or 4.0 × (Δ*lqsS*-Δ*lqsT*) compared with wild-type bacteria. Strains harbouring the corresponding promoterless vector control did not produce detectable GFP (Kessler *et al.,* 2013). In summary, these results indicate that the *lqs* genes negatively regulate natural transformation of *L. pneumophila* at high cell densities, where *lqsS* and *lqsT* are assumed to control competence in a synergistic manner.

3.1.3 Uptake and intracellular replication of *L. pneumophila* lacking *lqsT* or both *lqsS* and *lqsT*

Previous reports demonstrated a decreased efficiency in phagocytic uptake for *L. pneumophila* strains lacking a functional Icm/Dot T4SS (Weber *et al*., 2006, Hilbi *et al.,* 2001), *lqsR* (Tiaden *et al.,* 2007), *lqsS* (Tiaden *et al*., 2010b) or the entire *lqs* cluster (Tiaden *et al*., 2008).

To test the involvement of *lqsT* in phagocytic uptake of *L. pneumophila*, amoebae or macrophages were infected with various GFP producing *lqs* deletion mutants, and uptake of the bacteria was quantified by flow cytometry. Compared with the wild type, fewer Δ*lqsT* mutant bacteria were taken up by *A. castellanii* (Figure 10A) or *D.discoideum* amoebae (50% or 25%, respectively), and approximately 10 times fewer Δ*lqsS*-Δ*lqsT* mutant bacteria were phagocytosed by *A. castellanii*. Thus, the absence of *lqsT* had a smaller impact on uptake by phagocytes than the deletion of *lqsS*, and bacteria lacking both sensor kinases exhibited a synergistic phenotype. These data were corroborated in a gentamicin protection assay, where the uptake by human HL-60 macrophage-like cells of *L. pneumophila* lacking either *lqsT, lqsS or lqsR* was also found to be impaired (Figure 10B).

A putative role of *lqsT* in intracellular replication was assessed by infecting *A. castellanii* with *L. pneumophila* strains constitutively producing GFP. The replication efficiency of bacteria lacking *lqsT* as indicated by fluorescence intensity was comparable to the wild-type or *lqsA* mutant strain (Figure 10C). The growth defect observed for *the* Δ*lqsS*-Δ*lqsT* deletion strain was more profound than for Δ*lqsS*, but the double mutant strain still replicated more efficiently than Δ*lqsR* bacteria. Furthermore, multiple round intracellular replication assays were performed with *A. castellanii* (Kessler *et al*., 2013) or murine RAW 264.7 macrophages (Figure 10D) infected at a low MOI (0.1). In this test the Δ*lqsT,* Δ*lqsS,* Δ*lqsS*-Δl*qsT* or other *lqs* mutant strains grew similarly to wild-type bacteria. In summary, host cell uptake and replication experiments revealed that *lqsT* regulates the interactions of *L. pneumophila* with phagocytes, yet *lqsT* appears to play a minor role compared with *lqsS.*

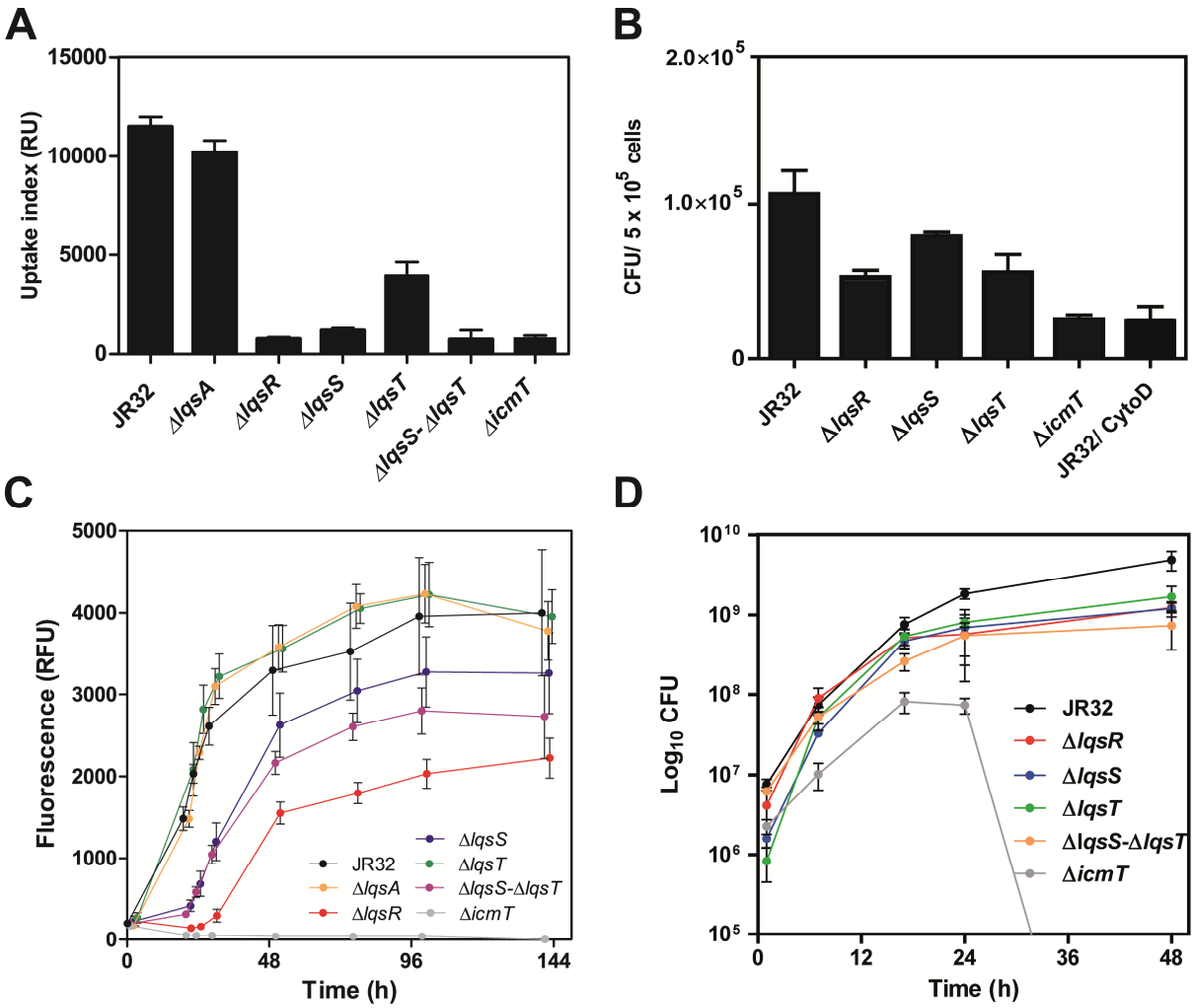

Figure 10. *Lqs* genes promote phagocytosis of *L. pneumophila* and intracellular replication in host cells. (**A**) *A. castellanii* amoebae were infected (MOI 50) with *L. pneumophila* wild-type, Δ*lqsA*, Δ*lqsR,* Δ*lqsS,* Δ*lqsT,* Δ*lqsS*-Δ*lqsT* or Δ*icmT* mutant strains harbouring a vector constitutively producing GFP (pNT-28). Uptake was quantified by flow cytometry defining an uptake index as the product of the number of cells above the gate threshold and the fluorescence intensity of the cells (relative units, RU). (**B**) Human HL-60 cells differentiated with PMA into macrophages were infected (MOI 50) with the aforementioned strains with or without the actin polymerisation inhibitor cytochalasin D (CytoD), and the uptake efficiency was determined by a gentamicin protection assay. (**C**) *A. castellanii* amoebae were infected (MOI 20) in a 96-well plate with the above strains harbouring pNT-28, and the production of GFP was monitored over time by using a microtitre plate fluorescence reader (relative fluorescence units, RFU). (**D**) Murine RAW 264.7 macrophages were infected (MOI 0.1) with the aforementioned *L. pneumophila* strains, the infected cells were lysed with H_2O, and bacterial growth was quantified by CFU counting.The data shown are means and standard deviations of triplicates and representative of three independent experiments. P-value (A) < 0.001 (unpaired Student's *t*-test) for all mutant strains except Δ*lqsA*. Published in Kessler *et al.*, 2013.

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3.1.4 Competition of *L. pneumophila* wild-type and *lqs* mutant strains in *A. castellanii*

The Δ*lqsT* mutant strain was only slightly impaired in intracellular replication compared to wild-type bacteria. In order to pinpoint the role of *lqsT* in the interactions between *L. pneumophila* and its hosts, a co-infection experiment was established. To this end, *A. castellanii* was co-infected with wild-type *L. pneumophila* and different *lqs* mutant strains at a 1:1 ratio (MOI 0.01 each) and grown at 37°C for 21 days. At the time points indicated, CFU of wild-type and mutant *L. pneumophila* were quantified by plating appropriate dilutions on CYE as well as on CYE/Km agar plates. Under the conditions used, the Δ*lqsA,* Δ*lqsR,* Δ*lqsS,* Δ*lqsT,* Δ*lqsS*-Δ*lqsT* mutant strains failed to compete against wild-type bacteria and were eradicated within 15–21 days (Figure 11). Compared with the Δ*lqsT* or Δ*lqsS*-Δ*lqsT* mutants, the strains lacking *lqsA*, *lqsR* or *lqsS* persisted more robustly for up to 15 days before being outcompeted by wild-type bacteria. Finally, an *L. pneumophila* strain lacking *hdeD*, a gene of unknown function located in the *lqs* cluster (Tiaden *et al*., 2008) (Section 1.3.2, Figure 3), persisted throughout the course of the experiment, indicating that the Km resistance cassette used to construct the mutants did not *per se* impair the strains. Together, these results demonstrate a profound intracellular competitive disadvantage for strains lacking individual *lqs* genes.

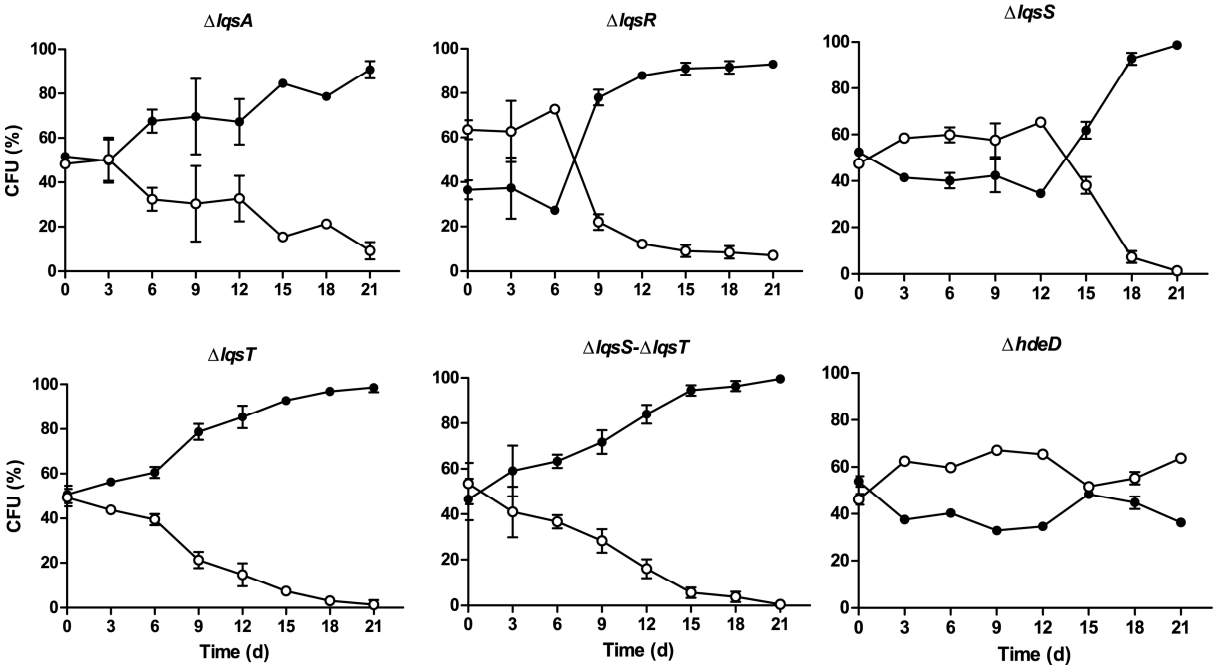

Figure 11: *Lqs* deletion strains are outcompeted by wild-type *L. pneumophila* upon co-infection of *A. castellanii*. *A.castellanii* amoebae in Ac buffer were co-infected (1:1 ratio, MOI 0.01 each) in 96-well plates with wild-type *L. pneumophila* (filled circles) and the Δ*lqsA,* Δ*lqsR,* Δ*lqsS,* Δ*lqsT,* Δ*lqsS*-Δ*lqsT* or Δ*hdeD* mutant strains (open circles), and grown at 37°C for 21 days. Every third day the supernatant and lysed amoebae were diluted 1:1000, fresh *A. castellanii* amoebae were infected (50 µl homogenate per 200 µl of amoebae culture volume), and aliquots were plated on CYE agar plates in presence or absence of Km for CFU quantification. The data shown are means and standard deviations of triplicates and representative of three independent experiments. Published in Kessler *et al.*, 2013.

3.1.5 Overexpression of *lqsA* does not reverse phenotypes of Δ*lqsS*-Δ*lqsT*

A mutant strain defective for a LAI-1-responsive sensor kinase is likely insensitive to LAI-1 signalling. Therefore, overproduction of the LAI-1-producing autoinducer synthase LqsA, should have no effect on *L. pneumophila* strains lacking LAI-1-responsive sensors. Unexpectedly however, overexpression of *lqsA* in the Δ*lqsS* strain reversed phenotypes of the mutant, suggesting that *L. pneumophila* produces another LAI-1-responsive sensor (Tiaden *et al.,* 2010b). To investigate on a genetic level whether LqsT is a LAI-1-responsive sensor, *lqsA* was expressed under the control of its own promoter in *lqs* mutants, and uptake and salt sensitivity were compared to bacteria lacking both sensor kinases (strain Δ*lqsS*-Δ*lqsT)*. Additional experiments were performed to analyse potential reversion of the phenotypes by supplying the sensor kinases on a plasmid.

Overexpression of *lqsA* partially restored the enhanced salt resistance of the Δ*lqsS* and Δ*lqsT* mutant strains but did not reach wild-type levels in the Δ*lqsS*-Δ*lqsT* or Δ*lqsR* strains (Figure 12A). When provided on a plasmid, *lqsA* expression was able to restore the uptake defect of the single sensor kinase mutants Δ*lqsS* or Δ*lqsT*. In contrast, uptake efficiency of the Δ*lqsS*-Δ*lqsT* or Δ*lqsR* strains was not restored to wild-type level upon expression of *lqsA* (Figure 12B). Previous reports demonstrated that *lqsA* also enhanced the uptake efficiency of wild type or Δ*lqsA*, but not of Δ*icmT*, confirming that a functional Icm/Dot T4SS is required for efficient uptake (Tiaden *et al*., 2010b). Finally, these observations indicate that LqsS and LqsT are likely the only two LAI-1-responsive sensor kinases produced by *L. pneumophila*.

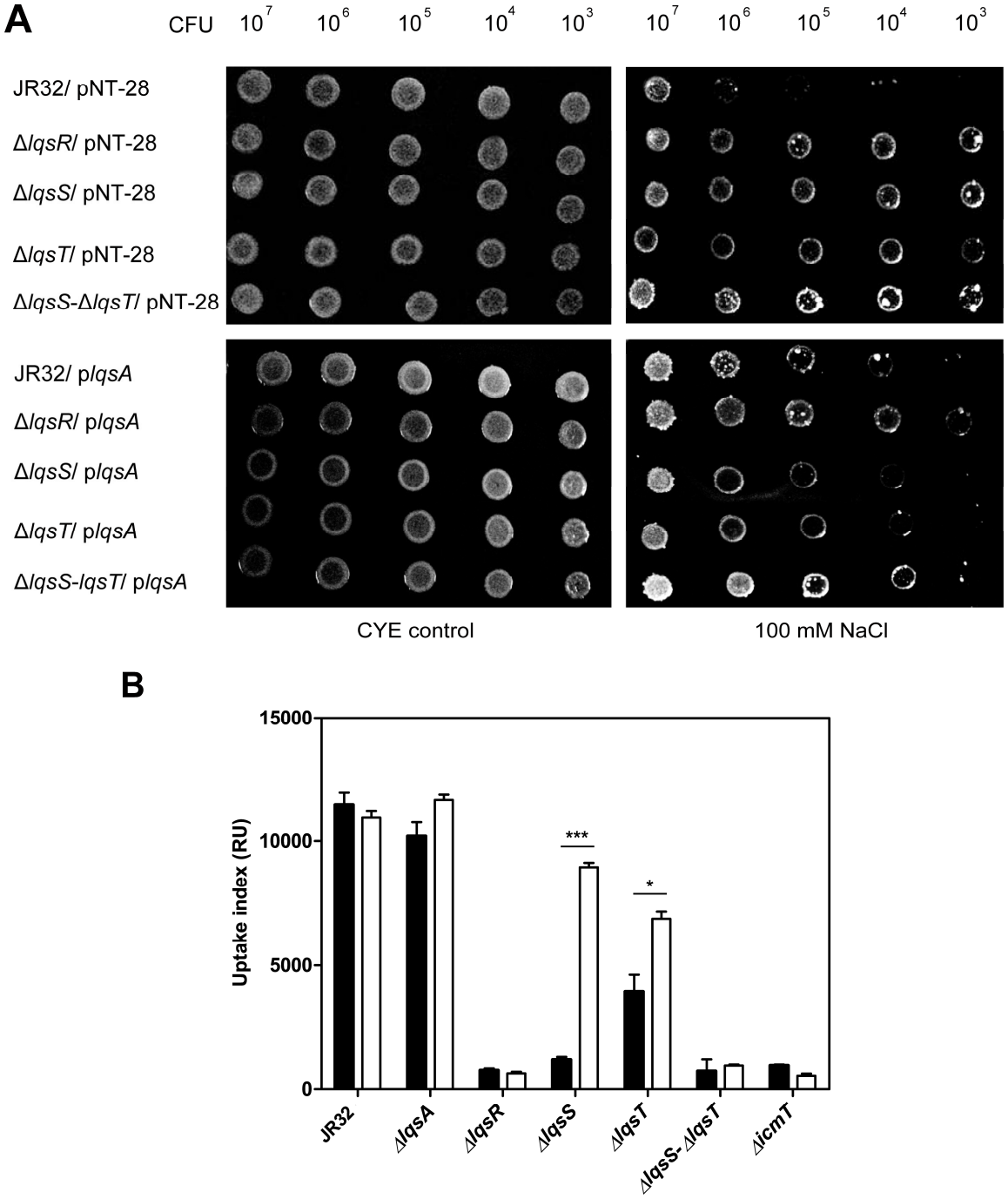

Figure 12*: lqsA* does not revert phenotypes of *L. pneumophila* Δ*lqsS*-Δ*lqsT*. (**A**) *L. pneumophila* wild-type, Δ*lqsR, ΔlqsS, ΔlqsT or* Δ*lqsS*-Δ*lqsT* harbouring a vector control (pNT-28, black bars) or a plasmid expressing *lqsA* (pNT-36, white bars) were spotted in decreasing concentrations onto CYE agar plates supplemented with or without 100 mM NaCl and incubated for 3 days. (**B**) *D. discoideum* amoebae were infected (MOI 50) with *L. pneumophila* wild-type, Δ*lqsA, ΔlqsR, ΔlqsS, ΔlqsT, Δlqs*S-Δ*lqsT or* Δ*icmT* mutant strains harbouring a vector constitutively producing GFP (pNT-28), or GFP and LqsA under control of its own promoter (pNT-36). The percentage of infected amoebae was quantified by flow cytometry (uptake index: relative units, RU; *P < 0.05; ***P < 0.001, unpaired Student's *t*-test). The data shown are means and standard deviations of triplicates (A) and representative of three independent experiments.Published in Kessler *et al*.. 2013.

3.1.6 Regulation of *L. pneumophila* gene expression by *lqsT* and *lqsS*

To characterise the impact of the *lqs* genes in the different growth phases of *L. pneumophila*, the expression of *lqsA*, *lqsR*, *lqsS* and *lqsT* genes was investigated by quantitative real-time (qRT)-PCR during the course of bacterial growth in AYE medium (Kessler *et al.,* 2013). In the post-exponential growth phase, *lqsA* showed a 2-fold and *lqsR* and *lqsS* a six-fold higher expression compared to the exponential growth phase. *lqsS* and *lqsT* were most differentially regulated in the late post-exponential growth phase (OD_{600} 3.5–4.0). To follow up on this finding, the impact of the deletion of *lqsS* and *lqsT* on genome-wide transcription during the late post-exponential growth phase was analysed by using DNA microarray (experiments performed by Tobias Sahr, published

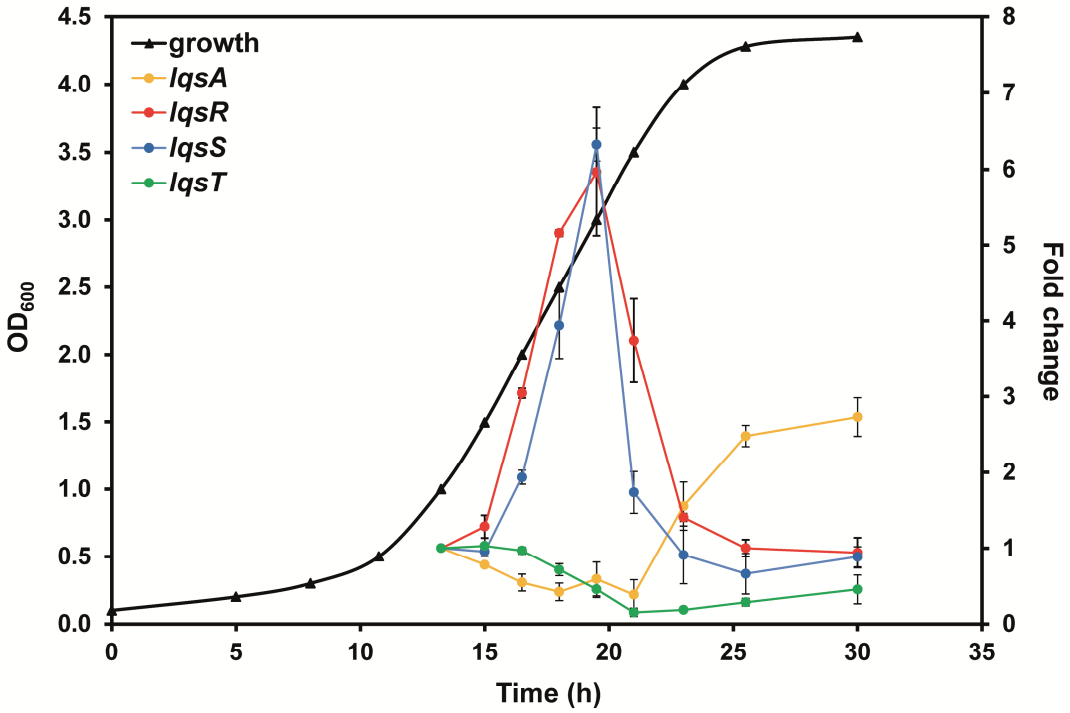

Figure 13: *lqsS* and *lqsT* are differentially regulated in the late post-exponential growth phase. qRT-PCR data showing relative expression of *lqsA, lqsR, lqsS* and *lqsT* along the *L. pneumophila* growth curve in AYE medium. Data were normalised to the expression level at an OD_{600} of 0.5. Experiment was performed by Tobias Sahr. Published in Kessler *et al.*, 2013.

in Kessler *et al.*, 2013). Direct or indirect targets of the *L. pneumophila* sensor kinase LqsT were identified in transcriptomic studies: In the post-exponential growth phase (OD_{600} 4.0), no genes were upregulated in an *lqsT* mutant strain compared to the wild-type strain JR32. On the other hand, microarray data revealed downregulation of 105 genes by a factor of at least 1.5-fold in a strain lacking *lqsT* (Kessler *et al.*, 2013). In addition to constituents of the region I (22 genes) or region II (19 genes) of the 133 kb genomic fitness island (Tiaden *et al.*, 2010b), *lvrA* and *lvrB* of the *Legionella vir* region were found to be repressed in absence of *lqsT*. Interestingly, a number of genes downregulated in absence of *lqsT* encode components of the Icm/Dot T4SS (IcmML, DotBC) or 13 different Icm/Dot substrates (Kessler *et al.*, 2013). Furthermore, downregulation in the *lqsT* deletion mutant was observed for the genes encoding the following factors: macrophage

infectivity potentiator (Mip), chitinase, flagellum components (FlgG, FlgH), 19 kDa peptidoglycan-associated lipoprotein (Pal), major outer membrane proteins, oxidative stress factors, cold shock proteins (CspA, CspC, CspD), cell division factors (MraW, MraZ, FtsL, MinD), DNA-binding proteins and transcription factors (Fis, HU-β, GyrA, RpoS), metabolic enzymes (GlnA, LysAC, LpxC), or a putative non-coding RNA (ncRNA18).

While none of the genes repressed in absence of *lqsT* was also downregulated in *L. pneumophila* lacking *lqsS*, 95 of the 105 genes downregulated in absence of *lqsT* (90%) showed enhanced expression levels in absence of *lqsS* (Figure 14). Interestingly, several genes under reciprocal regulation in absence of *lqsT* or *lqsS* encode Icm/Dot substrates. Taken together, LqsS and LqsT regulate a vast number of genes in an inverse manner, suggesting at least a partial antagonistic function of the two sensor kinases.

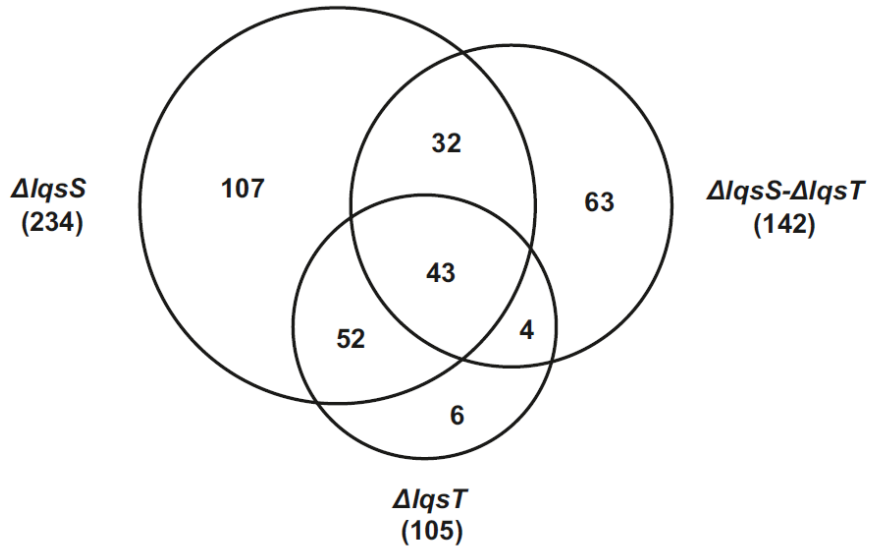

Figure 14: Venn diagram of *lqsT*- and *lqsS*-regulated genes. Genome-wide comparative transcription analysis in stationary growth phase of genes downregulated in absence of *lqsT*, or upregulated in absence of either *lqsS* alone or *lqsS* and *lqsT*. Pubished in Kessler *et al.,* 2013.

Of note, in absence of both *lqsS* and *lqsT*, a total of 142 genes were upregulated at least 1.5-fold compared to wild-type *L. pneumophila.* However, only one gene of unknown function (*lpg0893*) was repressed under these conditions (late post-exponential phase, OD_{600} 4.0). Enhanced expression in Δ*lqsS*-Δ*lqsT* was observed for *lvrA*, as well as constituents of region I (five genes) and region II (one gene) of the 133 kb genomic fitness island alongside various Icm/Dot components plus 26 Icm/Dot substrate genes. Moreover, deletion of both *lqsS* and *lqsT* revealed induced expression of genes encoding enhanced entry proteins (EnhB, EnhC), major outer membrane proteins, phagosomal transporter (PhtA), Mip, Pal, chitinase, pyoverdine synthesis protein (PvcA), type IV pilin (PilA), flagellum components (FlgH), oxidative stress factors, global stress protein (GspA), cold shock proteins (CspA, CspD), cell division factors (FtsZ, MinD, MinE), DNA-binding proteins and transcription factors (Fis, HU-β, GyrA, RpoD), or a putative non-coding RNA (ncRNA18). A gene of unknown function (*lpg2395*) was upregulated more than 10-fold in absence of both *lqsS and* Δ*lqsT* strain (Kessler *et al.*, 2013) or even more than 11-fold in a Δ*lqsR* strain (Tiaden *et al.,* 2007).

A comparison of genes upregulated in absence of *lqsS* and *lqsT* with genes regulated in strains lacking the individual sensor kinase genes revealed that 75 of the 142 genes upregulated in absence of *lqsS* and *lqsT* (53%) were also upregulated in *L. pneumophila* lacking only *lqsS*, while 47 of the 105 genes downregulated in an *lqsT*-deficient strain (45%) were upregulated in an *lqsS*-*lqsT* double deletion strain (Figure 14). Finally, a direct comparison of the transcriptome of *L. pneumophila* lacking both *lqsS* and *lqsT* with a strain lacking only *lqsT* revealed that in absence of both sensor kinases 209 genes were upregulated and 96 genes were downregulated at least 1.5 times (Kessler *et al*., 2013). Many genes differentially regulated encode components required for protein production, bioenergetics, metabolism, virulence, motility, cell division and regulation. The differential regulation of genes in absence of *lqsT* or both *lqsS* and *lqsT* was confirmed by qRT-PCR for the *mip* (*lpg0791*) and chitinase (*lpg1116*) genes, as well as for genes of region I of the genomic fitness island (*lpg0976*, *lpg0978*, *lpg0980*, *lpg0987*, *lpg0992*). Among others, the iron-regulator-encoding gene *frgA* was shown to be 8-fold upegulated in the absence of *lqsS* and *lqsT* compared to *L. pneumophila* wild-type. To substantiate this finding, a P_{frgA}-*gfp* reporter construct was transformed into the *lqs* mutant strains and the expression patterns were compared to wild-type *L. pneumophila* (Figure 15).

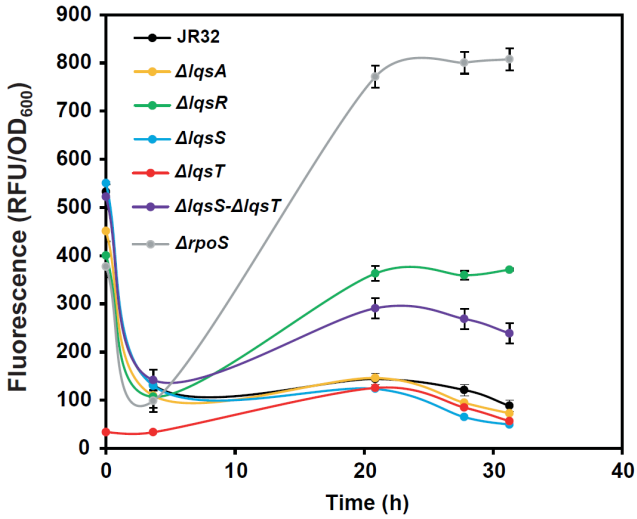

Figure 15: Expression of *frgA in L. pneumophila* is regulated by *rpoS*, *lqsR* and simultaneously by *lqsS* and *lqsT*. P_{frgA}-dependent production of GFP relative to cell density (relative fluorescence units, RFU/OD_{600} was monitored over time in *L. pneumophila* wild-type, Δ*lqsA*, Δ*lqsR*, Δ*lqsS*, Δ*lqsT*, Δ*lqsS*-Δ*lqsT or* Δ*rpoS* strains harbouring a transcriptional P_{frgA}–*gfp* fusion reporter construct. The data shown are means and standard deviations of quadruplicates and representative of at least three independent experiments.

Taken together, this indicates that the *lqsS* and *lqsT* genes are reciprocally regulated in the post-exponential growth phase of *L. pneumophila*, and transcriptome studies performed under these conditions revealed that the regulatory pattern of an *lqsS*-deficient strain is also reciprocally correlated to Δ*lqsT*. Furthermore, the transcriptome pattern of a strain lacking both *lqsS* and *lqsT* resembles a strain lacking *lqsS.* These results indicate that in the post-exponential growth phase LqsS and LqsT may have at least partially antagonistic regulatory functions, yet LqsS controls additional aspects of *L. pneumophila* physiology and virulence.

3.2 Purification and crystallisation of LqsR

3.2.1 Production and purification of His-LqsR in *E. coli*

For the large scale production of His-LqsR WT, His-LqsR$_{D108A}$ and His-LqsR$_{D108N}$, the corresponding plasmids pTS-23, pRB-3 and pRB-4 were freshly transformed into *E. coli* BL21(DE3). The recombinant His-tagged LqsR proteins were successfully produced and purified to electrophoretic homogeneity by Ni^{2+}-chelating affinity and size-exclusion chromatography using an Äkta Purifier System (GE Healthcare). The purified His-LqsR WT, His-LqsR$_{D108A}$ and His-LqsR$_{D108N}$ proteins represented by the peak fractions of the resulting chromatograms were analysed by SDS–PAGE and found to be 95% pure as indicated by the major band at 41.4 kDA (Figure 16). From hereon, LqsR and its derivatives will be used synonymously for the His-tagged proteins throughout the thesis.

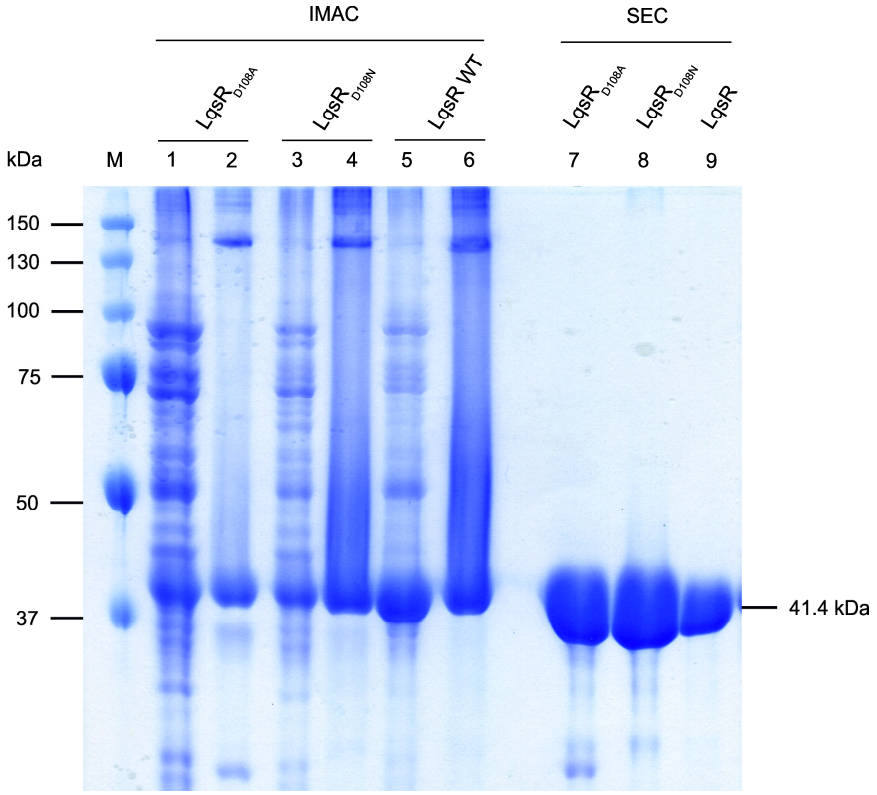

Figure 16: SDS–PAGE analysis of His-LqsR, His-LqsR$_{D108A}$ and His-LqsR$_{D108N}$. Lanes 1, 3, and 5 depict peak fractions of wash fraction eluted from the Histrap HP column. Lanes 2, 4, and 6 represent peak fractions of protein eluted from Histrap FF column (IMAC). Lanes 7-9 indicate peak fractions of protein eluted from the HiLoad Superdex 200 pg column (SEC). 20 µl of sample were boiled with 5x loading buffer and loaded on each lane. M, molecular weight marker (kDa); IMAC, immobilised metal ion chromatography; SEC: size-exclusion chromatography.

3.2.2 Crystallisation of His-LqsR

Preliminary screening of crystallisation conditions for LqsR, $LqsR_{D108A}$ and $LqsR_{D108N}$ was carried out by sitting-drop vapour-diffusion and several commercially available screening kits; approximately 500 individual conditions were tested. After 5-7 d incubation, $LqsR_{D108A}$ and $LqsR_{D108N}$ crystals of different shapes were obtained in various solution conditions. Promising microcrystals of $LqsR_{D108N}$ were obtained after 3-5 d in 0.1 M sodium HEPES pH 7.5, 25% (w/v) polyethylene glycol monomethyl ethanolamine (MME) 2000.

According to preliminary X-ray diffraction analysis of the crystals, optimisation of the initial conditions was required to increase the size of the crystals and to improve the crystal quality. Microseeding has become a well established strategy during the optimisation of crystallisation conditions (Bergfors, 2003). Ireton and Stoddard (2004) and Marsh and D'Arcy (2007) refer to this technique as ‘microseed matrix screening', a method where poorly diffracting crystals were used to seed into similar, but non-identical conditions, resulting in a markedly improved crystal form. We therefore employed a microseeding technique for the crystallisation of the $LqsR_{D108N}$ protein.

$LqsR_{D108N}$ crystallised best in the Nextal PEG Suite condition containing 20% polyethylene glycol (PEG) 3350 and 0.2 M lithium chloride. From these crystals a seed-bead stock was generated. Similarily, crystals obtained for LqsR and $LqsR_{D108A}$ in the following PACT screen conditions were used to produce seed bead stocks. LqsR: 0.2 M sodium acetate, 0.1 M bis-Tris propane pH 7.5, 20% PEG 3350 and $LqsR_{D108A}$: 0.2 M lithium chloride, 0.1 M HEPES pH 7.0, 20% PEG 6000.

The created microseeds were used in subsequent microseeding experiments in order to achieve optimised crystal morphology. In the microseeding plates, conditions containing 25% PEG 2000, 0.1 M sodium HEPES pH 7.5 produced the most promising crystals. Interestingly, $LqsR_{D108N}$ protein crystals could be obtained after matrix seeding under conditions that significantly differed from those which provided the initial crystal seed stocks. For example, $LqsR_{D108N}$ seeds were isolated from 20% PEG 3350, 0.2 M lithium chloride, but subsequently generated crystals in drops containing 25% PEG 2000, 0.1 M sodium HEPES pH 7.5. The new crystals obtained by using the seeding technique consistently diffracted to 3.6 Å resolution. Crystals were soaked briefly in cryoprotectant that consisted of 85%/15% (v/v) reservoir solution with glycerol prior to data collection in an N_2 cold stream. Data were collected at 100 K at the beamlines X06SA and X06DA at the Swiss Light Source (SLS, Paul Scherrer Institute, Villigen, Switzerland) and were processed and merged with the XDS program (Kabsch, 2010).

In efforts to improve the diffraction resolution of the $LqsR_{D108N}$ crystals, additional optimisation parameters (pH range 7.2-7.8, PEG 2000 MME range 5%-30%) were tested using the microseeding technique. $LqsR_{D108N}$ was crystallised in 24-well plates by hanging drop vapor diffusion in a setup of drops consisting of 2 µl protein solution + 2 µl reservoir solution + 0.5 µl of concentrated seed bead stock.

The optimised conditions obtained from microseeding crystallisation experiments consisted of PEG 2000 MME in the concentration range 20–30% (w/v) and 0.1 M sodium HEPES in the pH range 7.4-7.6. Applying these conditions in microseeding experiments using the concentrated seed stock solution, His-LqsR$_{D108N}$ crystals with representative dimensions of 0.1 × 0.1 × 0.1 mm and 0.2–0.3 × 0.05 × 0.03 mm and several different crystal morphologies were grown at 20°C in 3–4 d in 24-well plates by hanging-drop vapour diffusion. In this assay, the best protein crystals resulted from the His-LqsR$_{D108N}$ derivative in solution conditions of 22.5% PEG 2000 MME, pH 7.6 and 0.1 M sodium HEPES. These crystals diffracted to 2.4 Å resolution (Figure 17A). Diffraction data collection is to 90% complete and initial attempts to solve the structure using molecular replacement are currently in progress.

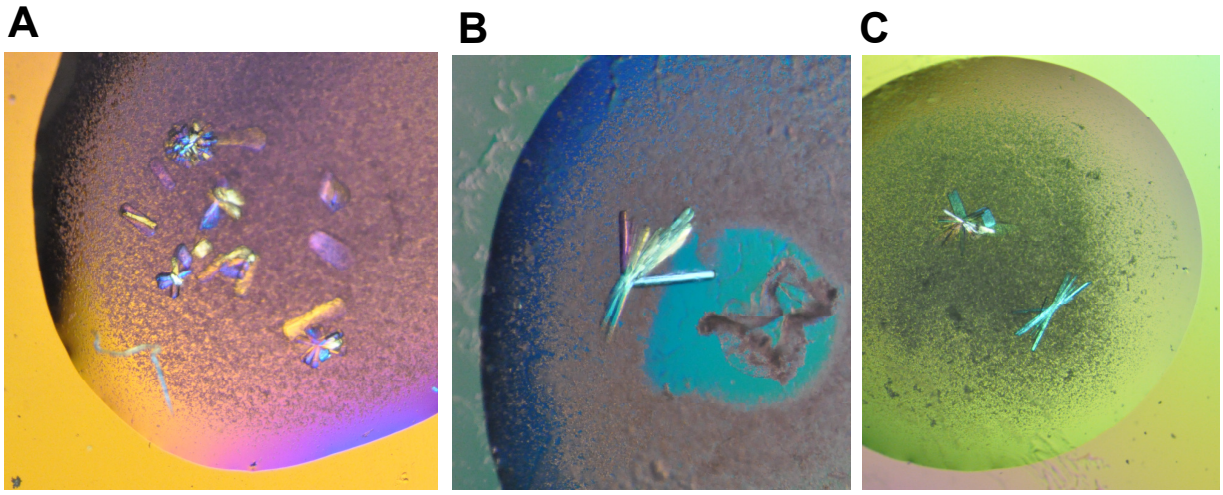

Figure 17: Images of His-LqsR$_{D108N}$ crystals used for diffraction analysis. Crystals formed in crystallisation drops consisting of (**A**) 20% PEG 2000 MME, pH 7.6 and 0.1 M sodium HEPES or (**B**, **C**) 22.5% PEG 2000 MME, pH 7.6 and 0.1 M sodium HEPES.

3.3 Identification and characterisation of the putative transcription regulator SinR

The *sinR* gene of *Bacillus subtilis* encodes a transcriptional regulator that is known to be involved in the biosynthesis of exopolysaccharides in biofilms (Kearns *et al*., 2005). *L. pneumophila* possesses a gene encoding a SinR family transcription regulator that genomically maps between *lpg1055* and *lpg1056* (1'153'652–1'153'389 bp) in Region I of the putative 133 kb genomic fitness island (Figure 18). While not yet annotated in *L. pneumophila*, a 100% identical gene was identified in the genome of strain Paris (*lpp2326*), Lens (*lpl1052*) and Alcoy (*lpa_1638*). The putative *sinR* gene is located in the vicinity of genes encoding an ATP synthase F1 beta chain *atpD1* (*lpg1054*), a guanylate cyclase (*lpg1056*) and a sensory GGDEF family protein (*lpg1057*). The *L. pneumophila* transcriptional regulator SinR is a putative member of the DNA binding xenobiotic response element (XRE) family, containing a HTH (helix-turn-helix) motif at its N-terminus.

The present study focuses on the role of the *L. pneumophila* SinR homologue in physiological processes and pathogen-host cell interactions.

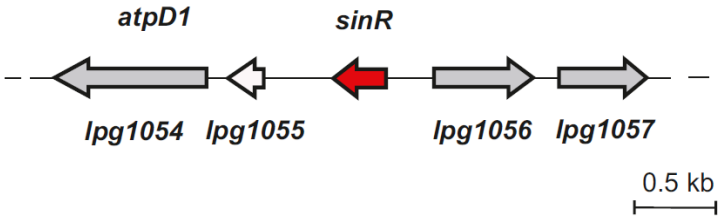

Figure 18: Genomic region of *sinR* in *L. pneumophila*. *sinR* (1'153'652–1'153'389 bp) is located proximally to the genes encoding the beta chain of an ATP synthase F1 *(atpD1, lpg1054*), a guanylate cyclase (*lpg1056*) and a sensory GGDEF family protein (*lpg1057*). Grey and white shading represents functionally annotated and unknown loci, respectively.

3.3.1 The effects of *sinR* on *lqsA*, *lqsR* and *sinR* expression

3.3.1.1 *sinR* is located in a putative genomic fitness island regulated by *lqsS*

In previous transcriptome studies the expression of the putative *sinR*-like transcription regulator was upregulated 8.5-fold in the absence of *lqsS* compared to *L. pneumophila* wild type grown to stationary phase. Notably, qRT-PCR revealed a sevenfold higher upregulation for the *sinR* locus in the absence of *lqsS* (Tiaden *et al.*, 2010). To analyse the correlation between the *lqs* system and *sinR* regarding gene regulation, expression of *sinR* was monitored in the different *lqs* mutant strains and Δ*sinR* using a transcriptional P_{sinR}-*gfp* fusion construct. Compared to wild-type bacteria *sinR* was significantly upregulated in the late exponential to early stationary growth phase in the Δ*lqsS* strain, validating the microarray data (Tiaden *et al.*, 2010). Moreover, *sinR* expression in the other *lqs* deletion strains did not differ from wild-type bacteria and was only slightly induced in a *sinR*-defective strain under standard growth conditions (Figure 19A). Interestingly, the *sinR* mutant exhibited elevated *sinR* expression levels only very late during stationary growth (Figure 19B). This finding implies that the expression of *sinR* is temporally controlled in the late growth phases of *L. pneumophila*, i.e. by *lqsS* and by *sinR* in a repressive manner. Thus, in addition to the strong repressive role of *lqsS* on *sinR* expression, the autoregulation of *sinR* expression is likely governed by a negative feedback loop.

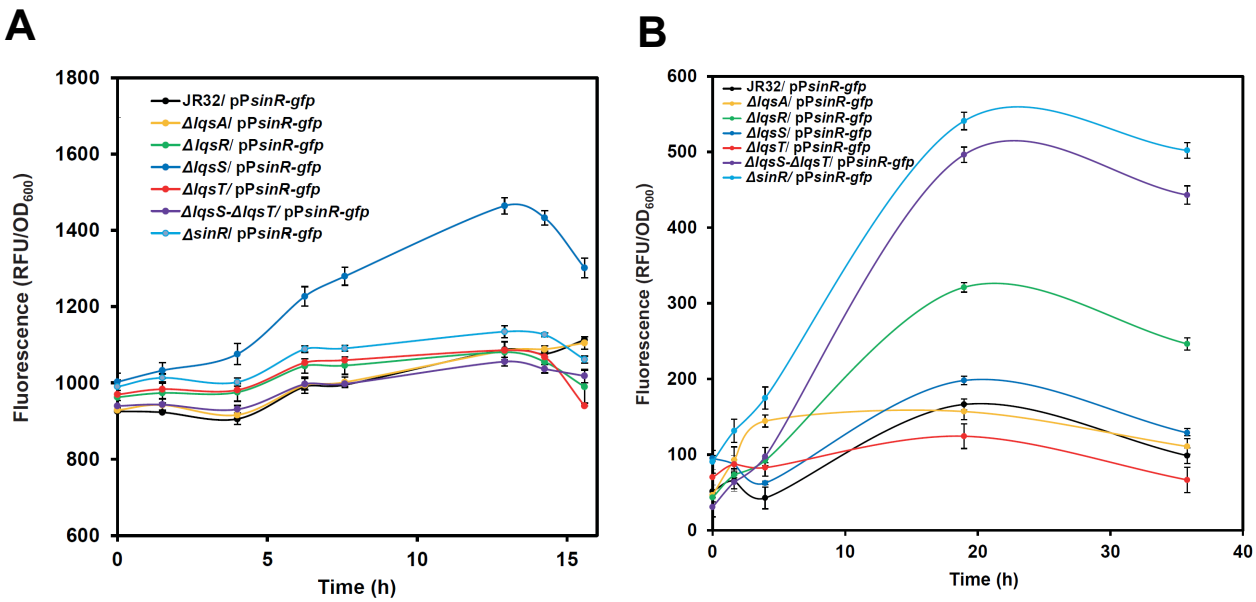

Figure 19: Expression of *sinR in L. pneumophila* is regulated by *lqsS* and *sinR*. P_{sinR}-dependent production of GFP normalised to bacterial cell density (relative fluorescence units, RFU) was monitored over time in *L. pneumophila* wild-type, Δ*lqsA*, Δ*lqsR*, Δ*lqsS*, Δ*lqsT*, Δ*lqsS*-Δ*lqsT or* Δ*sinR* strains harbouring a transcriptional P_{sinR}–*gfp* fusion construct. Strains were set up at (**A**) a start OD_{600} of 0.5 or (**B**) an OD_{600} of 0.8. The data shown are means and standard deviations of quadruplicates and representative of at least three independent experiments.

3.3.1.2 *sinR directly* regulates the expression of *lqsA and sinR*

In order to analyse putative target genes regulated by *sinR in vivo*, expression patterns of *lqsA*, *lqsR* and *sinR* was investigated in the *lqs* and *sinR* mutant strains via GFP production. Compared to the wild-type strain, expression levels of *lqsA* were reduced in Δ*lqsR (*by 50%*),* in Δ*lqsS*-Δ*lqsT*

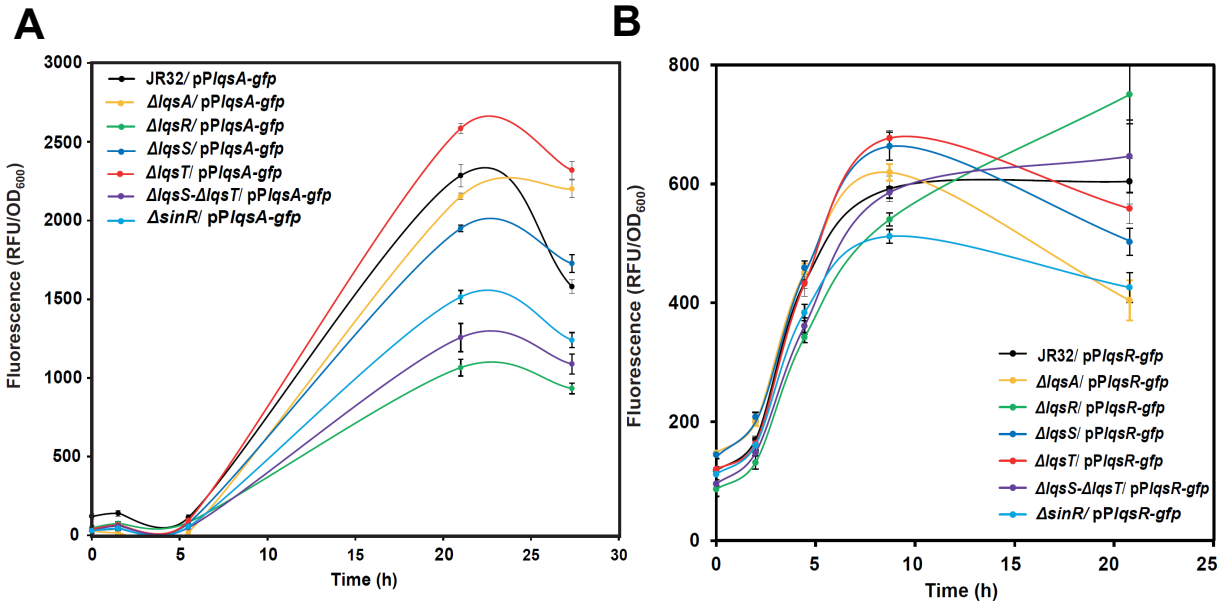

Figure 20: Expression of *lqsA in L. pneumophila* is regulated by *sinR, lqsR, lqsS* and *lqsT*. (**A**) P_{lqsA}- or (**B**) P_{lqsR}-dependent production of GFP normalised to cell density (relative fluorescence units, RFU) was monitored over time in *L. pneumophila* wild-type, Δ*lqsA*, Δ*lqsR*, Δ*lqsS*, Δ*lqsT*, Δ*lqsS*-Δ*lqsT or* Δ*sinR* strains harbouring a transcriptional P_{lqsA}–*gfp or* P_{lqsR}–*gfp* fusion reporter construct. The data shown are means and standard deviations of quadruplicates and representative of at least three independent experiments.

(45%), and in Δ*sinR* (42%) (Figure 20A). In contrast, *lqsR* expression only revealed differential regulation in the Δ*sinR* background compared to wild-type *L. pneumophila* (Figure 20B).

Based on the results obtained in the expression analysis, SinR was expected to directly interact with the *sinR* promoter and perhaps also to bind to the upstream regions of the *lqsA* and *lqsR* loci. To test this possibility, we purified SinR from *E. coli* cells and assessed binding of SinR (10-100 µM) to the promoter regions of *sinR, lqsA and lqsR* in gel electrophoretic mobility shift experiments. SinR specifically bound to both its own promoter, and albeit less efficiently, to the promoter sequence of *lqsA*. By contrast, SinR did not bind to the *lqsR* or *rpoS* promoter, and LqsR did not interact with the promoter region of *sinR* or *lqsR*, either.

Figure 21 demonstrates that the mobility of the *sinR* promoter fragment was gradually decreased and revealed multiple bands with increasing concentrations of SinR. At a minimal SinR concentration of 10 µM, efficient band shifting occurred, without any free DNA probe remaining at higher concentrations of SinR. Several defined protein-DNA complexes of differing stoichiometry

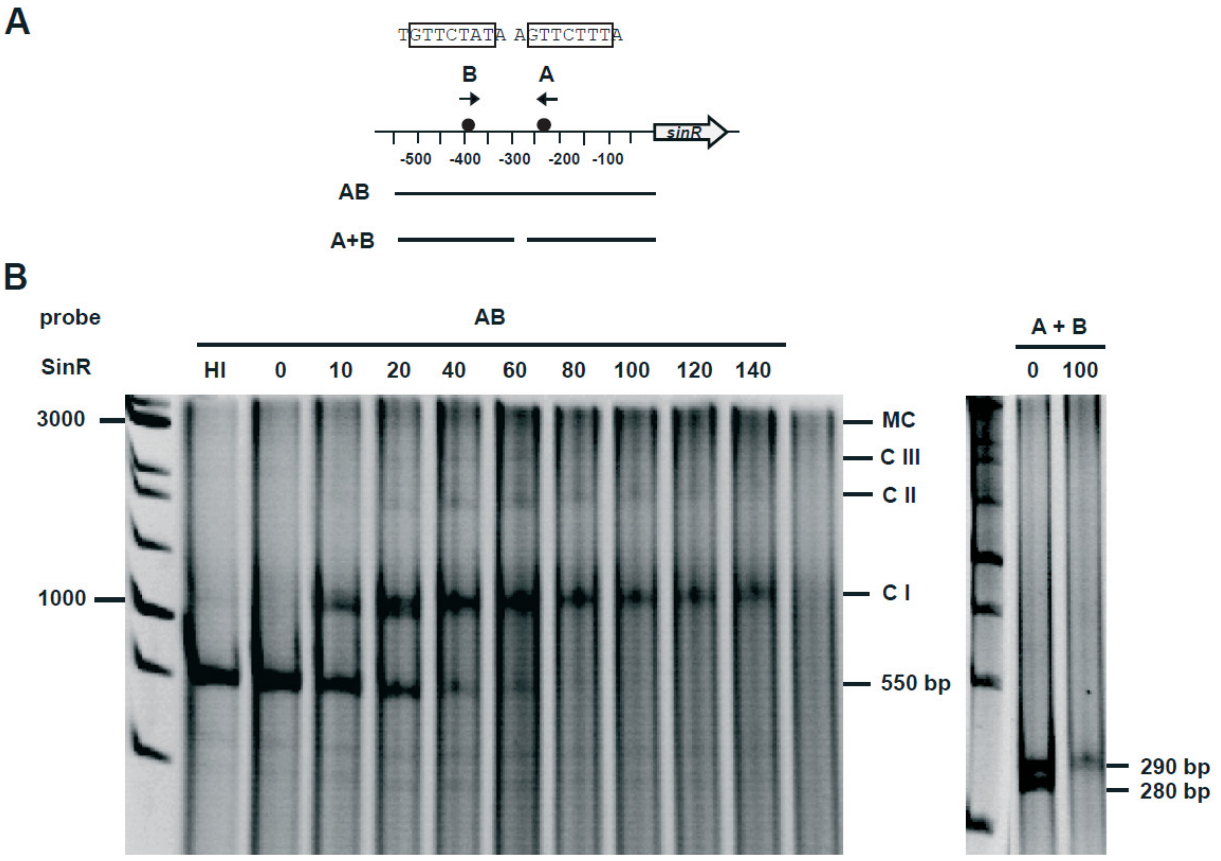

Figure 21: Interaction of *sinR* promoter DNA with the SinR regulator. (**A**) Map of the intergenic regions upsteam of *L. pneumophila sinR*, with SinR binding motifs A and B located at -227and -393 bp relative to the start of the *sinR* ORF, respectively. Arrows indicate the orientation of the motif and the DNA fragments used for EMSA experiments are depicted below. (**B**) EMSA were performed using purified SinR incubated at the concentrations indicated with 65 pmol of amplified *sinR* promoter fragment AB or with restriction digested DNA fragment (A+B). Control reactions were done with heat-inactivated SinR (HI) or did not contain DNA (blank). M, DNA marker; CI, CII and CIII, protein-DNA complexes; MC, multimeric complex. The reaction products were analysed on a 6% native polyacrylamide gel and visualised by ethidum bromide staining. Data shown were reproduced at least three times.

were resolved, resulting in a 'ladder' of bands in which each stoichiometric step is represented: Upon interaction with the *sinR* promoter His-SinR efficiently formed three distinct complexes (CI, CII and CIII), likely with 1, 2 and 3 SinR dimers bound per DNA molecule, respectively, as well as a multimeric complex (MC) (Figure 21).

The upstream region of *sinR* in *L. pneumophila* harbours sequences which were shown to mediate SinR binding in *B. subtilis* (Kearns *et al.*, 2005): A near-perfect inverted repeat containing the consensus sequences GTTCTAT and AAAGAAC is located at -393 and -227 bp relative to the start of the ORF (Figure 21). Separation of the two putative consensus SinR binding sites by restriction digestion of the *sinR* promoter sequence resulted in a less efficient band shift upon incubation with SinR (Figure 21, probe A+B). Specifically, since each individual site is nonpalindromic and cannot contact SinR alone, this implies that each motif is equivalent to a half-site with a spacer of 159 bp. This result reveals the requirement of both conserved SinR binding sites to be present on the same molecule of DNA to guarantee specific DNA-protein interaction. These data are in agreement with the results obtained from transcriptional profiling (Section 3.3.1.2, Figure 19), suggesting that the SinR regulator repressed *sinR* transcription via binding these sites, and confirms that expression of *sinR* is directly autoregulated.

In contrast, SinR bound weakly to the promoter region of *lqsA* and only at high concentrations of protein used (120 µM), where free DNA probe could still be observed (Figure 22). Interestingly, the *lqsA* promoter region contains only one single putative SinR binding site (GTTCTTT) which might mediate the interaction with the SinR regulator less stringently than the binding of SinR to its own promoter (Figure 22A). Indeed, interaction of SinR with the *lqsA* promoter generated a band shift that was barely visible, not displaying any distinct protein-DNA complex formation (Figure 22B).

Similarly to the *sinR* promoter, the *lqsA* upstream region was restriction digested to yield two fragments, one of them containing the putative SinR binding site (Figure 22A). No difference in band shift efficiency was observed among *lqsA* promoter fragments regardless of the presence (Figure 22, lanes 1-6) or absence (Figure 22, lanes 7-17) of the SinR binding site. This finding suggests that binding of SinR to the *lqsA* promoter does not exclusively depend on the presence of the SinR binding motif on the DNA fragment provided. Additional, thus far unidentified binding elements are postulated to be involved in the interaction between the *lqsA* upstream region and SinR. In accordance with the decreased expression profile of *lqsA* observed in a Δ*sinR* strain, SinR is assumed to act as a direct activator of *lqsA* transcription.

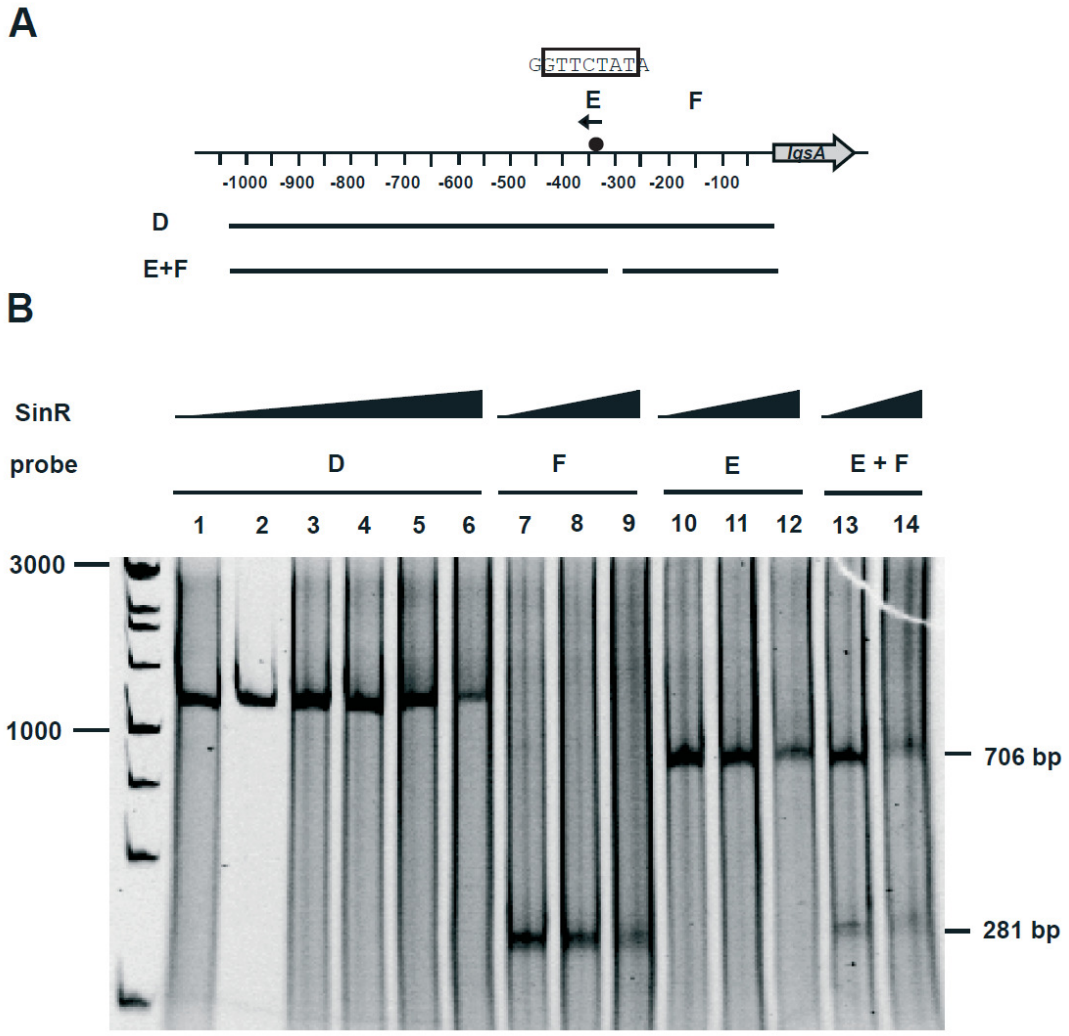

Figure 22: Interaction of SinR with the *lqsA* promoter region. (**A**) Map of the intergenic region upsteam of *L. pneumophila lqsA* harbouring the SinR binding motif E located at -374 bp relative to the start of the *sinR* ORF. Arrows indicate the orientation of the motifs and the DNA fragments used for EMSA experiments are depicted below. (**B**) Titration of *lqsA* promoter DNA with SinR regulator by EMSA was performed using increasing concentrations of purified SinR incubated with 65 pmol of amplified *lqsA* promoter region fragment D (lanes 2-6; 0, 20, 60, 100, 120 µM), F (lanes 7-9; 0, 60, 120 µM), E (lanes 10-12; 0, 60, 120 µM) and combined E+F (lanes 13 and 14; 0, 120 µM). Heat-inactivated SinR was used as control (Lane 1). The reaction products were analysed on a 6% native polyacrylamide gel and visualised by ethidum bromide staining. Data shown were reproduced at least three times.

No interaction of SinR with the promoter region of *lqsR* was observed as no band shift was apparent in the conditions tested (Figure 23). In control reactions, LqsR did not bind to the promoters of *lqsR* nor *sinR*, either. These findings together with *in vivo* expression profiles suggest that sinR regulates lqsR in an indirect manner, perhaps via the master regulator of stationary phase gene regulation, the sigma factor RpoS. We tested this hypothesis by using a probe corresponding to the *rpoS* promoter region in EMSA analysis. However, SinR clearly did not bind to rpoS promoter sequence (Figure 23), suggesting the presence of intermittent factor(s) along the sinR-lqsR regulatory cascade.

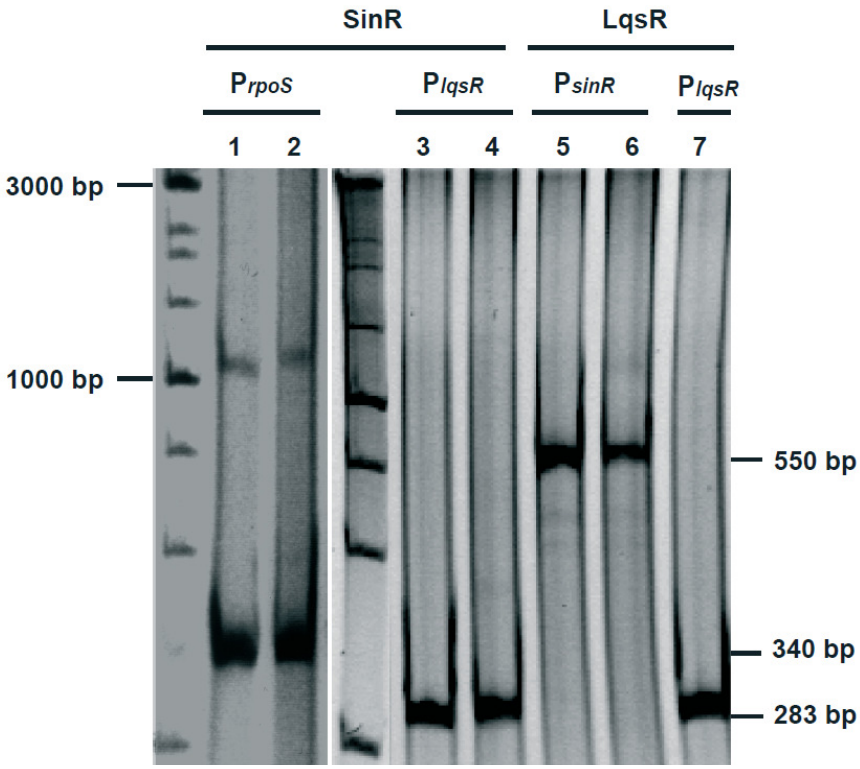

Figure 23: Mobility shift analysis of SinR binding to promoters of *lqsR and rpoS*. EMSA were performed using purified SinR incubated with promoter region fragments (65 pmol) of *rpoS* (P_{rpoS}, lanes 1, 2) or *lqsR* (P_{lqsR}, lanes 3, 4). Purified LqsR was incubated with P_{sinR} (lanes 5 and 6) or P_{lqsR} (lane 7). Samples of lanes 1, 3 and 5 contained 20 µM of heat-inactivated protein and lanes 2, 4, 6, and 7 contained 100 µM of native protein. Data shown were reproduced at least three times.

Collectively, these results indicate that SinR acts as a repressor of *sinR* transcription and as an activator of *lqsA* by directly interacting with their promoter regions *in vitro*. On the other hand, the regulatory effect of *sinR* on *lqsR* expression observed in *gfp* reporter studies presumably is mediated in an indirect fashion, independent of *rpoS*.

3.3.2 *sinR* controls biofilm formation of *L. pneumophila*

To corroborate the hypothesis that SinR-mediated regulation might be of relevance during late phases of stationary growth, an *L. pneumophila* strain lacking *sinR* was tested for the ability to form biofilms, a trait linked to the stationary phase of the bacteria. For this purpose, Δ*sinR* was compared to wild-type and Δ*rpoS* bacteria in a crystal violet staining assay (Figure 24A). To quantify biofilm formation, late stationary phase AYE liquid cultures were diluted in a microtitre plate, and incubated for 5 d at 30°C and the adherent cells were fixed, resuspended in a crystal violet solution, and transferred to a separate plate before their absorbance (OD_{595}) was measured using a microtitre plate reader.

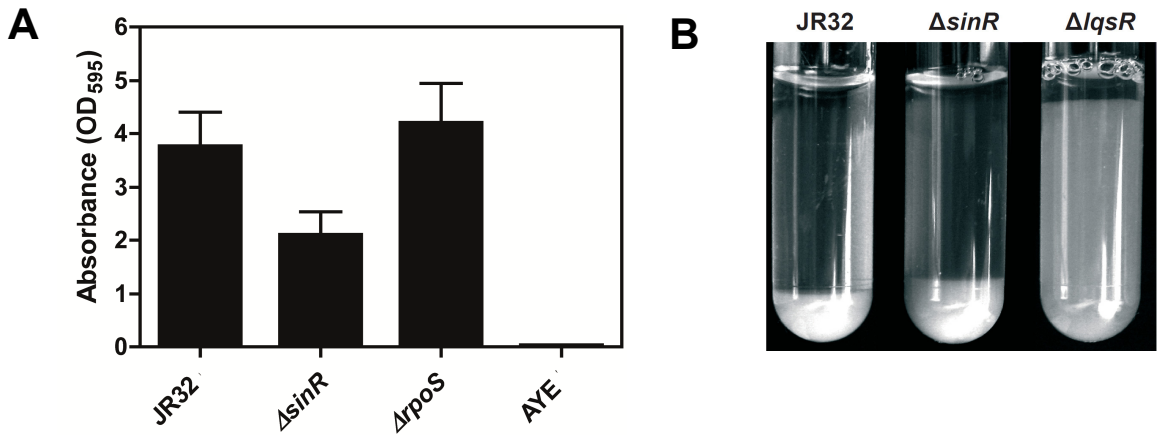

Figure 24: Analysis of biofilm formation and sedimentation of *L. pneumophila ΔsinR*. (**A**) Crystal violet staining of 5 days old biofilms of *L. pneumophila* wild type, Δ*sinR,* Δ*rpoS* and of non-inoculated wells (AYE) of a microtitre plate. Data shown are means and standard deviations representative of three independent experiments. (**B**) *L. pneumophila* wild type, Δ*sinR* and Δ*lqsR* were grown for 3 days on CYE agar plates, resuspended in 1 ml of AYE broth at an OD_{600} of 3.5 and analysed for sedimentation after 12 h at room temperature. P-value (A) < 0.001 (unpaired Student's *t*-test). Data shown are representative for three individual experiments.

The *sinR* mutant exhibited significantly impaired biofilm formation under the conditions tested, as it produced 50% less biomass within 5 days. This finding proposes that *sinR* promotes biofilm formation in *L. pneumophila* (Figure 24A). Moreover, a *sinR*-defective strain showed wild-type-like sedimentation behavior, suggesting that *sinR* does not affect the production of extracellular filaments (Figue 24B). In contrast, a strain lacking the sigma factor *rpoS*, required for expression of transmissive traits of *L. pneumophila*, was not impaired in biofilm formation, consistent with previous reports (Mampel *et al*., 2006).

3.3.3 Effects of *sinR* on uptake and intracellular replication

The Icm/Dot T4SS and LqsR promote the efficient uptake *of L. pneumophila* by amoebae and macrophages (Tiaden *et al*., 2007, 2008, Weber *et al*., 2006, Hilbi *et al.,* 2001). To test whether SinR plays a role in efficient uptake of *L. pneumophila* by phagocytes, *D. discoideum* or *A. castellanii* were infected with a *sinR* mutant producing GFP, uptake was quantified by flow cytometry and then plotted as uptake index. Compared with wild-type *L. pneumophila* ca. 60% fewer amoebae were infected with the Δ*sinR* strain (Figure 25). The phenotype could be barely complemented by providing sinR under control of its own promoter on a plasmid. This might be due to a non-physiological gene dose of *sinR*, similar to previous observations obtained for *lqsR* (Tiaden *et al*., 2007).

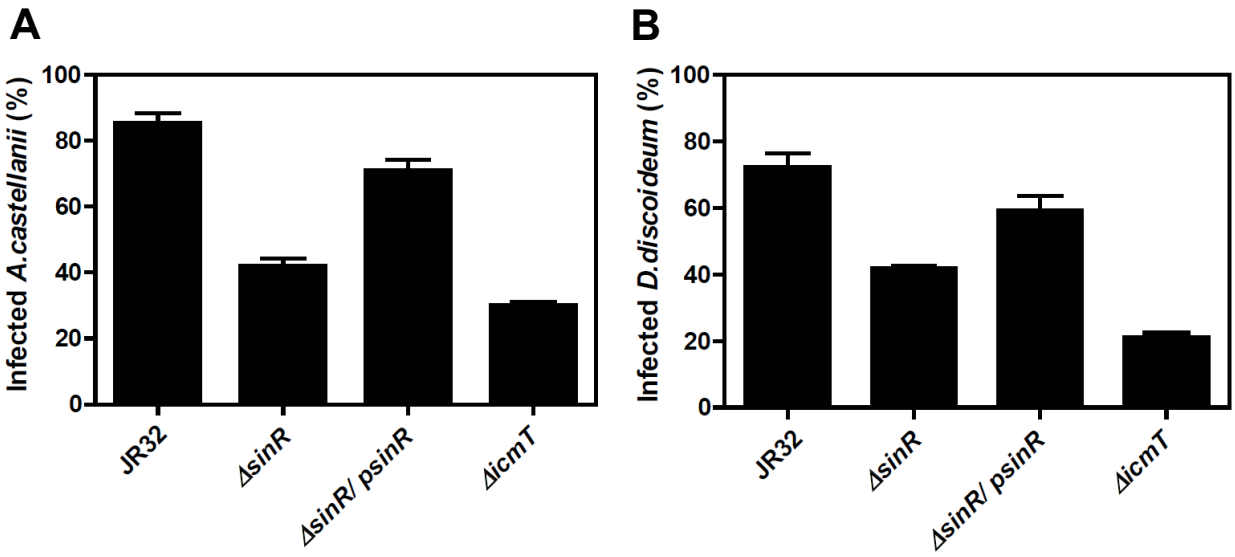

Figure 25: *sinR* promotes the efficient uptake of *L. pneumophila* by phagocytic host cells. (**A**) *A. castellanii* amoebae or (**B**) *D. discoideum* were infected at an MOI of 50 with wild type, Δ*sinR* or Δ*icmT* mutant *L. pneumophila* harbouring a vector constitutively expressing GFP (pNT-28) or *sinR* expressed from its endogenous promoter (P_{sinR}; pAK-18). The percentage of infected host cells was quantified by flow cytometry. P-value (A, B) < 0.001 (unpaired Student's *t*-test). Data shown are means and standard deviations representative of at least three independent experiments.

To investigate the impact of *sinR* in intracellular replication, single round replication assays were performed, where *A. castellanii* were infected with *L. pneumophila* strains constitutively producing GFP (MOI 20). A *sinR*-deficient strain was severely impaired for intracellular replication compared with wild-type bacteria. This replication defect could be partially restored by introducing *sinR* under its native promoter (Figure 26A). Moreover, compared with *L. pneumophila* wild type, a *sinR* mutant exhibited impaired intracellular replication in RAW macrophages (Figure 26B).

Upon co-infection of *A. castellanii*, intracellular growth of Δ*sinR* and *L. pneumophila* wild type was monitored over 21 days and quantified by CFU determination. The *sinR*-deficient bacteria were impaired for intracellular growth compared to the wild-type strain (Figure 26C). These data strongly imply a role for *sinR* in the regulation of pathogen–host interactions.

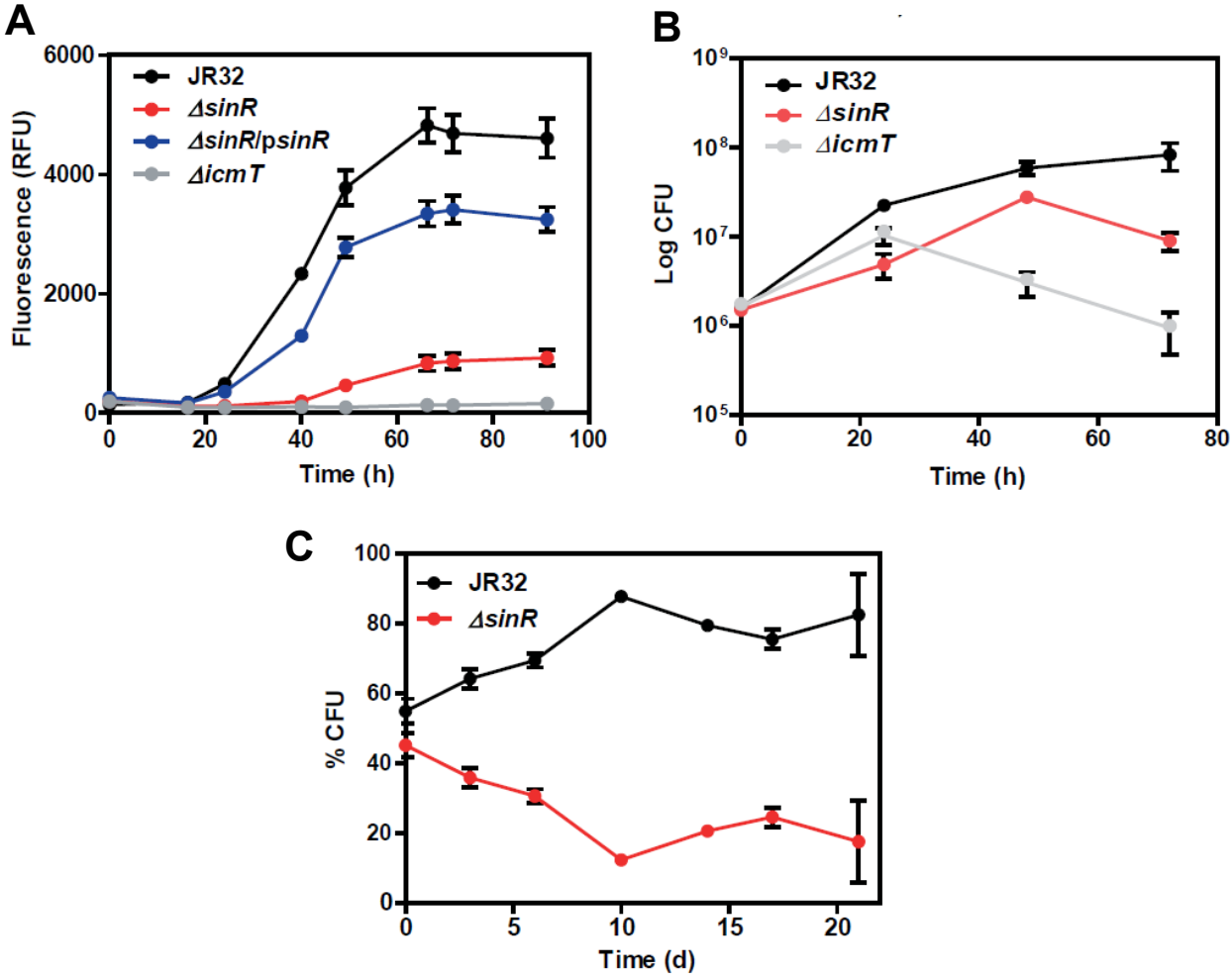

Figure 26: SinR is involved in intracellular growth and competition of *L. pneumophila* in host cells. (**A**) Single-round replication was analysed by infecting *A. castellanii* with *L. pneumophila* strains constitutively expressing *gfp* (pNT-28) or *sinR* under its native promoter (pAK-18) at an MOI of 20. Intracellular growth was measured over time using a microtitre plate fluorescence reader. (**C**) Co-infection experiments of *A. castellanii* with *L. pneumophila* wild type and Δ*sinR* at an MOI of 0.01 each. Intracellular growth was monitored over 21 days by CFU counting. (**B**) Intracellular growth of *L. pneumophila* wild type, Δ*sinR* and Δ*icmT* deletion mutants in RAW 264.7 cells (MOI 0.1) was quantified over time by determining CFU. Data are based on means and standard deviations representative of at least three independent experiments.

3.3.4 *sinR* controls natural competence in *L. pneumophila*

Next, a putative role of *sinR* in the regulation of competence induction and natural transformation of *L. pneumophila* was assessed. In addition to the direct analysis of DNA uptake and integration efficiency resulting from homologous recombination, the expression of the competence gene *comEA* was used as an indicator for competence induction. As shown in Figure 27, a strain lacking *sinR* displayed a four orders of magnitude higher transformation frequency, as well as upregulated expression of *comEA,* compared to *L. pneumophila* wild type. Enhanced competence (termed hypercompetence) resulting from the deletion of *sinR* implies a role for *sinR* acting as a repressor in the complex pathway controlling natural transformation and the uptake of exogenous DNA. Whether this regulation is of the direct or indirect type requires further investigation.

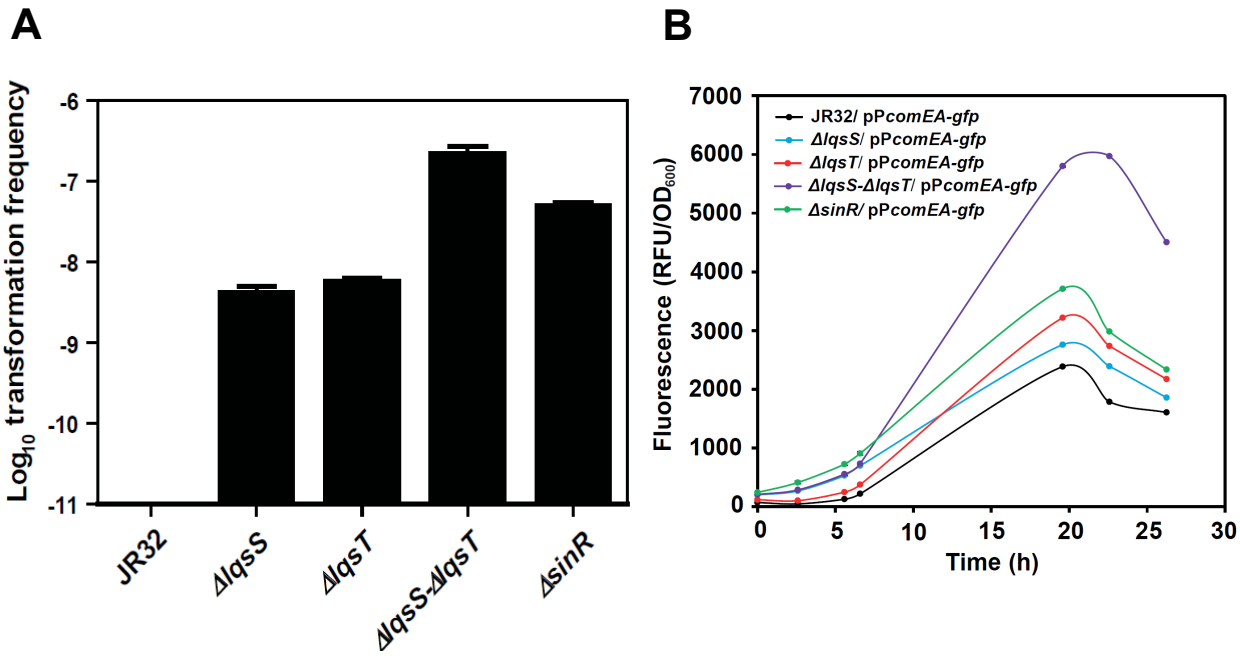

Figure 27: *sinR* controls natural competence of *L. pneumophila*. (**A**) *L. pneumophila* wild type and mutant strains were grown to post-exponential growth phase (OD_{600} 2.6–3.0) and incubated at 30°C for 24 h with linear DNA encoding a Cm resistance cassette flanked by fragments homologous to the chromosomal up- and downstream regions of *lqsT*. Transformation efficiency was quantified by determining CFU on selective agar plates. (**B**) Expression of *comEA* was analysed by monitoring P_{comEA}-dependent production of GFP normalised to cell density (relative fluorescence units, RFU) over time in *L. pneumophila* wild-type and mutant strains. The data shown are means and standard deviations of quadruplicates and representative of at least three independent experiments.

3.4 Summary of results

In conclusion, the *lqs* system of *L. pneumophila* was demonstrated to govern a plethora of virulence and morphology traits, ranging from intracellular replication and natural competence to the formation of extracellular filaments. Speficically, the sensor kinases LqsS and LqsT may have at least partially antagonistic regulatory functions, with LqsS controlling separate aspects of *L. pneumophila* physiology and virulence, such as a 133 kb genomic fitness island. As a member thereof, *sinR* was identified and characterised to play a role in bacterial pathogenicity, biofilm formation and competence, exerting regulatory functions in addition to and interconnected with the AHK signalling circuit.

4. Discussion

Gene regulation by small signalling molecules is utilised as a means of cell-cell communication by many bacteria to coordinate population behaviour (Bassler and Losick, 2006, Camilli and Bassler, 2006, Fuqua and Greenberg, 2002). A novel class of signalling molecules, the α-hydroxyketones (AHK), were discovered in the facultative human pathogens *L. pneumophila* and *V. cholerae*. Analogously to the *V. cholerae* CqsAS system, the *lqs* gene cluster encodes the LAI-1 autoinducer synthase LqsA, the cognate sensor kinase LqsS and the response regulator LqsR. This study documents the identification and characterisation of an 'orphan' homologue of LqsS, termed LqsT, which contributes to various functions of the Lqs system, such as pathogen-host cell interactions, bacterial virulence, salt resistance, natural competence and formation of extracellular filaments. Moreover, AHK signalling and in particular *lqsS* regulates a 133-kb genomic fitness island, which harbours a SinR-like transcription factor that was shown to coregulate virulence, natural competence and biofilm formation of *L. pneumophila.*

4.1 Autoinducer regulatory circuits in *L. pneumophila* and *V. cholerae*

L. pneumophila and *V. cholera*e are Gram-negative aquatic microorganisms and opportunistic human pathogens. They respond to environmental conditions and adjust their gene expression programs either according to the growth phase or population density, respectively. A biphasic life style allows *L. pneumophila* to cycle between a replicative state with upregulation of metabolic pathways and a transmissive state (virulence, motility and stress resistance traits) (Brüggemann *et al*., 2006b).

Quorum sensing circuits, in concert with additional sensor systems enable to coordinate various processes during their life cycle (Figure 28), including pathogen-host interactions and production of virulence factors (Tiaden *et al*., 2007, 2008, Zhu *et al*., 2002, Miller *et al.*, 2002), biofilm and filament formation (Waters *et a*l., 2008, Zhu *et al*., 2003, Hammer *et al*., 2003), regulation of a genomic fitness island (Tiaden *et a*l., 2008) and natural competence (Kessler *et al*., 2013, Section 3.1.2, Figure 9).

AHK signalling participates in the stationary phase regulatory network in *L. pneumophila*, and so the Lqs system promotes gene expression in the stationary growth phase. The *L. pneumophila* and *V. cholerae* AHK signalling circuits are comprised of the *lqs* or cholera QS (*cqs*) gene clusters, which produce and detect the signalling molecules LAI-1 and CAI-1 ((S)-3-hydroxytridecan-4-one), respectively (Figure 28). In *L. pneumophila*, the response regulator LqsR links AHK signalling to the stationary growth phase regulatory network.

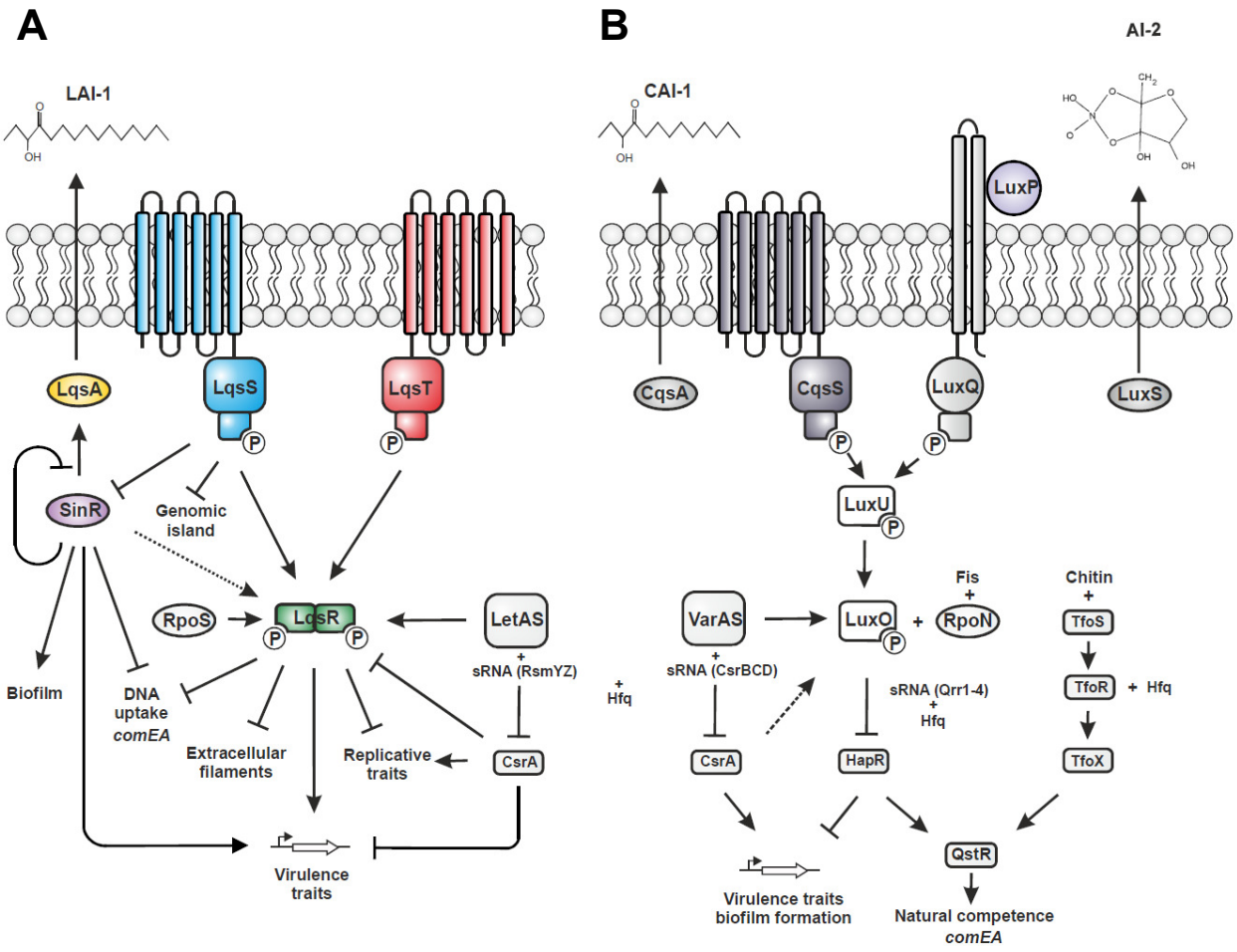

Figure 28: **Model of the regulatory network controlling the transition from the replicative to the virulent phase**. (**A**) In *L. pneumophila*, the AHK signalling circuit is linked to the stationary growth phase regulatory network and comprises the autoinducer synthase LqsA (producing LAI-1), the putative cognate sensor kinase LqsS and the response regulator LqsR. Phosphorylation signalling through LqsS and its orphan homologie LqsT converges on LqsR. Alongside LqsR, the expression of transmission traits (virulence, motility) is controlled by the stationary sigma factor RpoS (sigma38) and the two-component system LetAS, a homologue of *V. cholerae* VarAS. Active LetA induces the production of LqsR and the small regulatory RNAs RsmYZ to sequester the RNA-binding repressor CsrA and relieve post-transcriptional repression, thereby promoting replication. The transcription regulator SinR is interlinked with the AHK signalling circuit via its control of virulence, biofilm formation and natural competence. Furthermore, SinR directly regulates *lqsA* transcription. (**B**) In *V. cholerae*, AHK signalling is executed by the autoinducer synthase CqsA (producing CAI-1) and the sensor kinase CqsS. The TCS VarAS inhibits the production of the global repressor CsrA via the sRNAs *csrBCD*. Together with these systems LuxPQS (AI-2 sensing) converges on LuxO. At low cell density, the response regulator LuxO is phosphorylated by the phosphotransferase LuxU. Together with the sigma factor RpoN and the small nucleoid protein Fis, phosphorylated LuxO induces the expression of the sRNAs *qrr1-qrr4*, which destabilise the *hapR* mRNA and prevent production of the master regulator HapR and thus repress virulence traits. High bacterial density leads to LuxO dephosphorylation and inactivation. As the production of Qrr1-Qrr4 sRNA is repressed, HapR is generated, resulting in the production of QstR and the development of natural competence, which is in parallel induced via the chitin-TfoX pathway. Model adapted from (Tiaden *et al*., 2010a, Jules and Buchrieser, 2007, Molofsky and Swanson, 2004).

The production of LqsR depends on RpoS and LetA, requiring the sRNAs RsmYZ in addition to the RNA-binding protein CsrA at a post-transcriptional level. The essential activator of intracellular replication CsrA was shown to bind nascent mRNA, where it promotes Rho-dependent transcription attenuation or inhibits translation initiation of transmissive genes (Figueroa-Bossi *et al*., 2014). Additionally to the alternative sigma factors RpoS, RpoN and FliA, the two-component system LetAS, homologous to *V. cholerae* VarAS is involved in the induction of transmissive traits. LetA directly promotes the expression of *rsmYZ*, which bind and sequester CsrA together with the RNA chaperone Hfq. This leads to the released repression of transmissive traits and thus entry into the replicative growth phase. Hypothetically, the AHK-mediated response regulator LqsR can directly act as transcriptional regulator of virulence traits, interacting with the sRNAs *rsmYZ* or the replicative phase regulator CsrA to de-repress the transmissive regulon (Forsbach-Birk *et al*., 2004, Molofsky and Swanson, 2003, Fettes *et al*., 2001). Thus, similar to the *V. cholerae* VarAS two-component system converging with the CqsAS quorum sensing system (Lenz *et al*., 2007), the *L. pneumophila* LetAS two-component system may crosstalk with the *lqs* system in the stationary growth phase.

In parallel to AHK signalling, *V. cholerae* employs two additional regulatory units to repress virulence and biofilm formation at high bacterial density. The linkage of several modular two-component systems (TCS) in QS circuits might offer an advantage for the integration of multiple signals and the fine tuning of an adaptive response. The CqsAS and LuxSPQ systems in *V. cholerae* are structurally distinct from the archetypical QS systems found in *Vibrio fisheri* (LuxRI) or *Pseudomonas aeruginosa (*LasRI and RhlRI), where the LuxI-type autoinducer synthase produces acyl homoserine lactone-based autoinducers (AHL), which directly bind to cytosolic LuxR-type transcriptional regulators (Camilli and Bassler, 2006, Fuqua and Greenberg, 2002).

The two quorum sensing systems CqsAS and LuxSPQ in *V. cholerae* converge on the shared phosphorelay protein LuxU, which serves as a specific cross-communication module between the AHK and AI-2 signalling circuits: LuxS synthesises the furanosyl borate ester compound AI-2, which is detected by the sensor histidine kinase LuxQ in conjunction with the periplasmic AI-2 binding protein LuxP (Milton *et al*., 2006, Bassler *et al*., 2004). At low cell density, the response regulator LuxO is phosphorylated by the histidine phosphotransferase LuxU and, together with RpoN, phosphorylated LuxO activates the expression of four small non-coding sRNAs, the ‘quorum regulatory RNAs’ Qrr1-Qrr4. The small nucleoid protein Fis directly binds and stimulates the Qrr1-4 RNA promoters. Furthermore, these sRNAs destabilise the *haprR* mRNA in an Hfq-dependent fashion, inhibiting the production of the master regulator HapR. Upon reaching high cell densities, dephosphorylated and inactive LuxO prevents the induction of Qrr1-4 sRNAs and the production of HapR. Analogously to the CAI-1- and AI-2-based circuits, the VarAS TCS, homologous to *L. pneumophila* LetAS, implies a third component of QS-dependent gene regulation affected by the upregulation of the sRNAs *csrBCD*, which inhibit CsrA activity. CsrA in turn regulates *luxO* and the expression of Qrr sRNAs and *hapR*. HapR inhibits virulence, but induces natural competence via controlling the expression of the transcriptional regulator *qstR* (Lo Scrudato, 2013*)*. Overall, LAI-1 promotes virulence of *L. pneumophila*, while CAI-1 represses these traits in *V. cholerae*.

4.2 The orphan sensor histidine kinase LqsT contributes to AHK signalling in *L. pneumophila*

Within the wide scope of intra- and interspecies cell-cell communication, a remarkable spectrum of small signalling molecules is utilised by bacteria to regulate gene expression, including the commonly used acyl-homoserine lactones (AHLs) or the furanosyl borate AI-2 (Winans, 2011, Bassler and Losick, 2006, Camilli and Bassler, 2006, Fuqua and Greenberg, 2002). However, *L. pneumophila* does not employ the aforementioned compounds, instead producing the AHK signalling molecule LAI-1 (Spirig *et al*., 2008). The LAI-1 autoinducer synthase LqsA and the putative cognate sensor kinase LqsS are encoded in the *lqs* gene cluster (*lqsA-lqsR-hdeD-lqsS*). Based on previous reports where phenotypes of an *L. pneumophila* strain lacking *lqsS* were reversed by overexpression of *lqsA*, an alternative sensor kinase (or kinases) was hypothesised to respond to the AHK signalling molecule LAI-1 (Tiaden *et al*., 2010b). This work is focused on the characterisation of the orphan LqsS homologue termed LqsT located distally to the *lqs* cluster. While phenotypes of the mutant strains lacking single sensor kinase (Δ*lqsS*, Δ*lqsT*) (such as salt resistance or impaired host cell uptake) can be reversed by overexpression of *lqsA*, the corresponding phenotypes of a double deletion strain (Δ*lqsS*-Δ*lqsT*) are not (Section 3.1.5 and Table 12). These results suggest that under the conditions tested *L. pneumophila* does not produce additional LAI-1 sensors, and that LqsS and LqsT are the sole sensor kinases responsive to LqsA-generated LAI-1.

Table 12: Summary of Δ*lqsA*, Δ*lqsS* or Δ*lqsR* mutant strain phenotypes.

Phenotype	JR32	Δ*lqsA*	Δ*lqsR*	Δ*lqsS*	Δ*lqsT*	Δ*lqsS*-Δ*lqsT*
Sodium resistance	+	+	++	+++	+++	++++
Sedimentation	++++	++++	+	++	++++	+
Natural competence	+	+++	+++	+++	+++	++++
Uptake by host cells	++++	++++	++	+++	++++	++
Intracellular replication	++++	++++	++	+++	++++	++
Effect of *lqsA* on						
Salt sensitivity	No	No	No	Yes	Yes	No
Uptake by host cells	Yes	Yes	No	Yes	Yes	No

A Δ*lqsT* strain was only slightly impaired in pathogen–host interactions (Section 3.1.3, Figure 9), similarly to a strain lacking the autoinducer synthase *lqsA*,). Perhaps, under the conditions used, the expression of *lqsA* is tightly regulated, and the corresponding protein is produced in minor amounts or in an inactive state. In agreement with this notion, the signalling molecule produced by LqsA, 3-hydroxypentadecan-4-one (LAI-1), was detected only upon overexpression of *lqsA* in *L. pneumophila* (or *E. coli*) (Spirig *et al*., 2008), and in low concentrations (pmol-range) in wild-type *L. pneumophila* (Michael Witting, unpublished data). The *V. cholerae* sensor kinase CqsS recognises

3-hydroxytridecan-4-one (CAI-1) produced by CqsA (Higgins *et al*., 2007) or by LqsA as a side product (Spirig *et al*., 2008). *L. pneumophila* LqsA was reported to generate either LAI-1 (C12 acyl tail), or to a smaller extent CAI-1 (C10 acyl tail) and derivatives with C11 or C13 acyl tails, reflecting a relaxed product specificity upon heterologous production of LqsA in *E. coli*. Thus, in *L. pneumophila*, additional sensor kinases may preferentially recognise LqsA-produced α-hydroxyketones with a carbon chain length different from the main product LAI-1. Alternatively, or in addition, different sensor kinases might have different affinities to the same α-hydroxyketone molecule, thus modulating signal transduction on a further level. However, the *in vivo* biosynthesis pathway of the physiologically relevant LAI-1 derivatives in *L. pneumophila* requires further investigation.

As shown for *L. pneumophila* Δ*lqsA*, a *V. cholerae* Δ*cqsA* mutant was not significantly impaired for virulence, as determined by the production of virulence factors and by colonisation studies in a mouse model (Hammer and Bassler, 2003, Zhu and Mekalanos, 2003, Miller *et al.,* 2002, Zhu *et al*., 2002). Functional redundancy of the three QS systems in *V. cholerae* might account for the absence of a virulence phenotype of the *cqsA and cqsS* mutant strains (Ng *et al.,* 2011, Higgins *et al*., 2007). Analogously, *L. pneumophila* might possess additional autoinducer/sensor kinase systems functioning in parallel to the Lqs signalling pathway. These systems might mask phenotypes of a Δ*lqsA* mutant, respond to products of LqsA and signal through a set of common downstream components including LqsR to jointly regulate target genes.

Interestingly, the presence of the two LAI-1-responsive sensor kinases LqsS and LqsT converging on one response regulator in the Lqs system implies a unique organisation (Section 1.3.2, Figure 3), which has not been reported for other AHK-responsive circuits such as the Cqs system of *Vibrio* spp. In *V. cholerae* and *V. harveyi* two or three different autoinducer systems based on AHL, AI-2 and AHK signals converge further downstream to regulate virulence and other traits (Henke and Bassler, 2004, Miller *et al*., 2002). In contrast, as *L. pneumophila* does not produce AHL and AI-2, the complexity of AHK signalling allows (i) integration of distinct AHK signals, (ii) accommodation of different signalling thresholds and/or (iii) transmission of antagonistic responses.

The increased salt resistance of the Δ*lqsS*-Δ*lqsT* sensor kinase double mutant strain is reversed by providing either plasmid-encoded *lqsT* or *lqsS* (Section 3.1.1, Figure 7). This finding suggests that LqsS and LqsT are in part functionally redundant and might sense the same or similar AHK signalling molecules. Both LqsS and LqsT lack a C-terminal phospho-receiver domain as well as a a conserved aspartate present in the *Vibrio* spp. hybrid sensor kinase CqsS (Tiaden and Hilbi, 2012, Tiaden *et al*., 2010a). Therefore, phosphorylated LqsS and LqsT are postulated to transfer the phosphate residue onto the receiver domain of a distinct protein, most likely to the putative response regulator LqsR encoded in the *lqs* cluster. Indeed, pull-down assays revealed direct interactions between the response regulator and the cognate sensor histidine kinases: LqsS and LqsT are bound by LqsR or phospho-LqsR. Furthermore, LqsS and LqsT undergo autophosphorylation by [γ-^{32}P]-ATP at a conserved histidine residue (H_{200} or H_{204}) located in their

cytoplasmic histidine kinase domain. Through a process dependent on the conserved aspartate (D_{108}) in the receiver domain, the response regulator prevented autophosphorylation of both sensor kinases by catalysing the dephosphorylation of phospho-LqsS or phospho-LqsT. Moreover, LqsR dimerised upon phosphorylation at D_{108} following the addition of either acetyl-phosphate or phospho-LqsT (Schell *et al.*, 2014).

In agreement with the findings from the aforementioned *in vitro* studies, these data indicate that phosphorylation signalling through the *Legionella* quorum sensing histidine kinases LqsS and LqsT indeed converges on the response regulator LqsR. The kinases might (i) bind and interact differently with the response regulator, (ii) signal to common as well as distinct response regulators, and/or (iii) respond contrarily to agonists and antagonists (Schell *et al*., 2014). The complex crosstalk among the two sensor kinases is expected to involve both common and distinct response regulators. Corroborating this hypothesis, the complexity of LAI-1-mediated signalling in *L. pneumophila* is reflected by some of the phenotypes of the strains lacking *lqsS* or *lqsT,* being (i) qualitatively similar but quantitatively different (Section 3.1, Figures 7A, 9D, 10A, 11), (ii) inverse for Δ*lqsS* and Δ*lqsT* (Kessler *et al.,* 2013), or (iii) specific for Δ*lqsS* (Figures 7B and 10C) (Kessler *et al*., 2013). Ultimately, the determination of the high resolution structure of the obtained LqsR crystals might provide insight into the structure-function relationship in the context of signal perception and response relay.

4.3 *lqsT* and *lqsS* reciprocally regulate gene expression in *L. pneumophila*

The more pleiotropic phenotypes of the Δ*lqsS* mutant compared with Δ*lqsT* are also reflected in the distinct transcriptomes of the mutant strains compared with wild-type *L. pneumophila* (Tiaden *et al*., 2010b). In absence of *lqsS* a total of 234 genes are upregulated, many of which involve protein production, metabolism and bioenergetics, and are not regulated by *lqsT*. The reciprocal regulons of LqsS and LqsT parallel their antagonistic function in *L. pneumophila* virulence, with differential expression of *lqsT* and *lqsS* occurring in the post-exponential growth phase (Kessler *et al*., 2013).

The differential regulation of more than 100 genes in absence of *lqsT* (Kessler *et al*., 2013) might explain the impaired virulence of *L. pneumophila* lacking the sensor kinase, i.e. components and substrates of the Icm/Dot T4SS or other virulence and transmission factors are downregulated (e.g. Mip, chitinase, flagellum components). Furthermore, downregulation of protection and replication factors (cold shock proteins, major outer membrane proteins, oxidative stress factors, cell division components) might impede survival in the bactericidal intracellular environment. Other phenotypes exhibited by the Δ*lqsT* mutant, e.g. the enhanced natural competence, do not directly correspond to the transcriptome pattern. Most likely, this phenotypic set is controlled more indirectly and results from a complex regulatory network including pleiotropic DNA-binding proteins and transcription factors such as Fis, HU-β, DNA gyrase (GyrA) and the stationary sigma factor RpoS (sigma38) (Kessler *et al.*, 2013). Moreover, the second messenger cyclic di-GMP (c-di-GMP) might also play a role in the regulation of virulence processes. C-di-GMP is produced from GTP by diguanylate cyclases, degraded by phosphodiesterases and acts through various effector proteins or riboswitches (Hengge *et al.*, 2009, Schirmer *et al*., 2009). The catalytic site of diguanylate

cyclases contains a GGDEF motif. Three c-di-GMP-metabolising enzymes of the *L. pneumophila* Lens strain displaying diguanylate cyclase (Lpl0780), phosphodiesterase (Lpl1118), and bifunctional diguanylate cyclase/phosphodiesterase (Lpl0922) activities were implicated in the survival and intracellular replication of *L. pneumophila.* The corresponding deletion mutants were partially defective for the escape of the *Legionella*-containing vacuole (LCV) from the host degradative endocytic pathway, resulting in decreased survival rate (Allombert *et al*., 2014).

The *lqsT* and *lqsS* genes are reciprocally expressed in the post-exponential growth phase, and 90% of the genes downregulated in absence of *lqsT* are upregulated in *L. pneumophila* lacking *lqsS* (Section 3.1.6, Figures 13 and 14; Kessler *et al*., 2013). Genes inversely regulated by *lqsT* and *lqsS* include components of the Icm/Dot T4SS and a number of Icm/Dot substrates (Kessler *et al*., 2013). Thus, LqsT and LqsS are assumed to represent antagonistic sensors. The presence of two homologous sensor kinases with antagonistic roles is a novel feature of quorum sensing circuits.

In summary, LAI-1 signalling in *L. pneumophila* is characterised by a unique organization involving two AHK-responsive sensor kinases, LqsS and LqsT, and a complex cross-talk among the two signal transduction pathways. However, while both sensor kinases apparently respond to signal(s) produced by LqsA, the physiological signal(s) are currently unknown. Since AHK-triggered sensor kinases can respond to both agonists and antagonists (Wei *et al*., 2012, Bolitho *et al*., 2011), LqsT and LqsS might signal in parallel or even synergistically *in vivo*. This hypothesis is supported by the more severe phenotypes of *L. pneumophila* lacking both *lqsT* and *lqsS*, compared to the single-deletion mutant strains.

Accordingly, the transcriptome of an *L. pneumophila* straing lacking the entire *lqs* cluster was reflected by even stronger phenotypes in virulence (e.g. T4SS-translocated effectors), motility (flagella components) and growth (*fis* and *hfq* genes) in the stationary phase (Tiaden *et al*., 2008). Notably, transcriptome analysis of a Δ*lqs* strain in the stationary growth phase revealed the downregulation of crucial Icm/Dot-secreted effector proteins including SidC, SidD, SidH, SidG, SdcA, four SidE paralogues, RalF, SidM/DrrA and LidA. In addition, a set of Enh paralogues implicated in efficient phagocytosis (Cirillo *et al.,* 2001, 2000), several eukaryotic-like proteins, flagellum genes (*flaA* and *fliA*), and multiple transmissive phase-induced regulatory proteins, including GGDEF regulators was downregulated. While the defects in the flagellum and the absence of Enh might explain the less efficient phagocytosis of the *lqs* mutant, the impaired intracellular replication might correlate with the lack of Icm/Dot-secreted factors. The effector proteins SidC, RalF, SidM or LidA contribute to the early establishment of the LCV by interfering with vesicle trafficking between the ER and Golgi network (Ninio and Roy, 2007). Supported by microarray and *in vivo* experiments, the *lqs* genes act synergistically during the transition from the replicative to the transmissive, virulent phase to establish a productive pathogen-host interaction. This switch is partly governed by the control of expression and activation of the Icm/Dot substrates that are translocated during consecutive phases of the infectious cycle (Section 1.2).

Previous comparative transcriptome analysis revealed a distinct correlation between the gene regulation patterns of Δ*lqsS* and Δ*lqsR* and a wild-type strain overexprexpressing *lqsA* (JR32/pNT-36) (Tiaden *et al*., 2010b). These findings suggest that LqsS and LqsR, as well as LqsA and LqsS share a common signal transduction pathway. Based on the transcriptome data, LqsA and LqsR seem to cooperate less prominently, corresponding to the mild impact of an *lqsA* deletion in contrast to the strong and pleiotropic phenotypes of a strain lacking *lqsR*.

4.3.1 *lqsS* regulates a 133 kb genomic fitness island

While absence of *lqsT* resulted in downregulation of the 133 kb genomic fitness island, deletion of *lqsS* induced the expression of this gene cluster (Tiaden *et al*., 2010b). These islands have recently been classified as integrative and conjugative elements capable of horizontal gene transfer (Wee *et al*., 2013). As an element of genomic plasticity, the entire 133 kb element regulated by *lqsS* contributes to genetic diversity of the species *L. pneumophila*, since in *L. pneumophila* Philadelphia-1 the regions I and II (*lvrA1-helC*) are contiguous, while in *L. pneumophila* Lens the two genes (*lpl1038*, *lpl1044*) are separated by 5 kb (Cazalet *et al*., 2004, Chien *et al*., 2004) and in *L. pneumophila* Paris *lvrA1* (*lpp1076*) is even located 1530 kb away from *helC* (*lpp2373*). The locus includes region I (putative pili components), which is separated by *lvrB1-lvrA1* (*lpg1004-1005*) from region II (multiple metal ion efflux systems). The 133 kb element is present in all sequenced genomes of *L. pneumophila*, and in two-thirds of 217 strains tested by hybridisation (Cazalet *et al*., 2008), implying a crucial role in the bacterial life cycle. Typical mobilising attributes of genomic islands (discussed in Section 1.4) map to the 133 kb region: Flanking sequences of regions I (*lpg0969-lpg1000*) and II (*lpg1006-lpg1069*) contain a tRNA gene (*lpg0972*) located upstream of integrase-encoding loci (*lpg0980*, *lpg1070*), a conjugative coupling factor TraD (*lpg0983*) and a transposase gene *tnpA* (*lpg1071*). Furthermore, an ISL3-family insertion sequence (IS) element maps between *lpg1062* and *lpg1063* (Figure 29).

Interestingly, in the wild-type strain overexpressing *lqsA* all induced genes cluster to region I of the 133 kb genomic island, as compared to a Δ*lqsA* mutant. In summary, the 133 kb region exhibits characteristics of a mobile fitness island, which was incorporated via horizontal gene transfer into the genomes of different *L. pneumophila* strains after their divergence from a common ancestor.

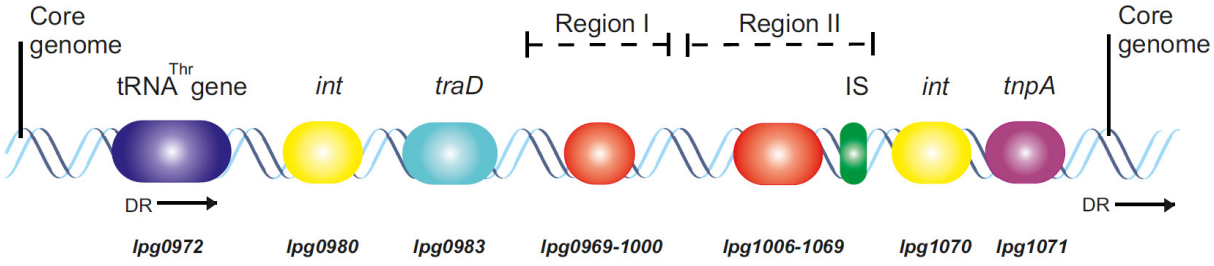

Figure 29: **Genetic organisation of the 133 kb genomic island in *L. pneumophila* Philadelphia-1.** Regions I and II of the genomic fitness island were chromosomally inserted in the vicinity of a tRNA^{Thr} gene and are flanked by genes coding for integrases (*int*; *lpg0980, lpg1070*), conjugation coupling factor *traD* (*lpg0983*) and transposase *tnpA* (*lpg1071*). An insertion sequence (IS) element is located close to the 3' end of region II. Direct repeats (DR) represent sites of insertion and deletion of DNA fragments.

A functional annotation was assigned to a distinct section of region II of the fitness island ranging from *helA* (*lpg1008*) to *pcoA/copA2* (*lpg1035*): This portion was previously described as a 40 kb efflux pump genomic island (Kim *et al.,* 2009, Rankin *et al*., 2002, McClain *et al*., 1996), harbouring bacterial genes that were found to be induced during, but dispensable for, infection of macrophages.

The *V. cholerae* 57.3 kb pathogenicity island VPI-2 is hypothesised to be important in pathogenesis, either directly in cholera virulence or indirectly in the transfer and integration of the island (Jermyn *et al.,* 2005). A VPI-2-resident neuraminidase and a gene cluster involved in the utilisation of specific amino sugars as an alternative nutrient source may contribute to the survival of the bacterium in different ecological niches, and thus provides a significant competitive advantage to pathogenic *V. cholerae* strains (Jermyn *et al.,* 2002). In *L. pneumophila*, reciprocal regulation of the 133 kb fitness island by the two sensor histidine kinases LqsS and LqsT might correlate with the *A. castellanii* competition data, where the Δ*lqsT* strain was more efficiently outcompeted than the Δ*lqsS* strain (Figure 10). The amoeba competition assay is a sensitive, yet complex readout, selecting for intracellular survival and replication of the bacteria, as well as for extracellular persistence and fitness. Thus, the 133 kb genomic fitness island might promote survival and persistence during pathogen–phagocyte interactions under the conditions tested (Kessler *et al.,* 2013).

4.3.2 The transcriptional regulator SinR is part of a 133 kb genomic fitness island

As a part of the *lqsS*-regulated 133 kb genomic fitness island, a SinR-like regulator belonging to the xenobiotic response element (XRE) family of transcription factors harbouring a HTH-binding motif was identified in *L. pneumophila*. P_{sinR}-dependet *gfp* expression was strongly induced in absence of *lqsS* in the stationary growth phase, and only mildly elevated in a Δ*sinR* strain. Yet, upon prolonged incubation, *sinR* displayed upregulated expression at very late stationary growth stages. Conceavably, these growth conditions trigger specific stress or starvation processes that ultimately affect *sinR* expression in addition to the bacterial growth phase. SinR shows similarity to the *B. subtilis* homologue, the master regulator of biofilm formation (Colledge *et al.,* 2011). Biofilms are structured microbial communities of cells attached to an inert or living surface by means of a matrix of self-produced extracellular polymeric substances (Mattick *et al*., 2002). Interestingly, *L. pneumophila* lacking *sinR* was defective for biofilm formation (Figure 24). Of the known *L. pneumophila* sigma factors, only the flagellar sigma factor FliA has been implicated in the regulation of biofilm production to date (Mampel *et al.,* 2006). It will be interesting to determine whether *sinR* promotes biofilm production in *L. pneumophila* like in *B. subtilis*.

Δ*sinR* exhibited no difference in sedimentation behaviour compared to *L. pneumophila* JR32, implying a wild-type-like absence of extracellular filament production. Moreover, *sinR*-deficient bacteria were deficient in uptake and intracellular replication in macrophages, *D. discoideum*, and *A. castellanii* and were outcompeted by wild-type *L. pneumophila* in *A. castellanii*, suggesting a role in pathogen host-cell interactions (Section 3.3.3, Figure 11). In addition to the *lqs* system, *sinR* was identified as novel factor controlling natural competence in *L. pneumophila*, which reduces the

transformation efficiency and represses the transcription of the DNA uptake machinery components *comEA* (Section 3.3.4, Figure 10*)*.

Furthermore, expression analyses showed that *sinR* induced expression of *lqsA and lqsR* in the late stationary phase and repressed transcription of its own gene, suggesting an autoregulatory feedback loop. This regulation is supported by gel band-shift data, where SinR was shown to directly interact with conserved SinR binding motifs located in the promoter regions of *sinR* and *lqsA*. Copies of the 7-bp SinR DNA binding consensus sequence are found in different numbers and arrangements at SinR-regulated promoters, suggesting that it can be bound in a variety of orientations and valencies (Kearns *et al*., 2005, Colledge *et al*., 2011). SinR bound with higher affinity to the full-length *sinR* promoter, which harbours two copies of the 7-bp SinR DNA binding consensus sequence GTTCTTT in inverted orientation. In contrast, binding of SinR to a single motif present in the *lqsA* upstream region appeared to be weaker. These data are in agreement with a recent report showing that SinR of *B. subtilis* is able to bind *in vitro* to the single motif, in addition to the inverted repeat motifs (Ogura *et al*., 2014). This implies that SinR is adapted to binding DNA elements which contain 2-fold rotational symmetry and that these sites are required for SinR interaction with the *sinR* upstream intergenic region on the bacterial chromosome. Differing from common binding geometries adopted by HTH-domains of prokaryotic proteins (Huffman and Brennan, 2002), SinR is assumed to bind to DNA elements located unusually far from the promoter, separated by as much as 159 bp. Due to their nonpalindromic characteristic, high-affinity interaction with SinR required both binding motifs of the *sinR* promoter to be present on the same molecule of DNA (Section 3.3.1.2, Figure 21). Similarly, the *M. tuberculosis* EspR dimer was shown to contact two operator DNA elements linked by a 177 base spacer (Rosenberg *et al.*, 2011). In this model, one EspR monomer binds to one site, while the second one contacts the other site, allowing for cooperative binding of the dimer with concomitant looping of the intervening DNA. Further biochemical studies such as genome-wide ChIP-on-Chip experiments in combination with the high resolution structure determination are required to decipher the mode of SinR binding to its operators.

The possibility of additional factors cooperating with SinR in promoter regions of target genes also needs to be considered. In fact, activation of multiple SinR target promoters in *B. subtilis* was reported to involve formation of a complex with the co-factor SlrR. Upon binding to the *degU* promoter, the SlrR/SinR complex is in turn removed by a phosphorylated response regulator, DegU, in dependence on RNA polymerase (Ogura *et al*., 2014). Moreover, biofilm synthesis requires SlrR/SlrA to activate the *eps* and *ypxM* operons by antagonising SinR (Kobayashi, 2008). SinR is a wide-range DNA-binding protein that is essential for the late-growth phase processes of competence and motility in *B. subtilis* and also acts as a repressor of other loci, e.g., the sporulation gene *spo0A* (Cervin *et al*., 1998, Mandic-Mulec *et al*., 1995). The broad spectrum of functions regulated by SinR in *L. pneumophila* allows speculations about the involvement of further factors in the fine-tuning of SinR target gene expression. This direct transcriptional control might be mediated by the formation of protein-protein complexes that occlude differential operator sequences of regulated promoter regions. Furthermore, transcriptional profiling using a *sinR*-

defective strain by microarray and qRT-PCR analysis will provide detailed insight into the extensive regulon of SinR, including the indirectly regulated genes.

Collectively, the pleiotropic DNA-binding protein SinR was identified as affecting several processes of *L. pneumophila* virulence and physiology at the onset of the stationary growth phase. Part of these phenotypes are co-regulated by the *lqs* system (pathogen-host interactions and natural competence), yet *sinR* also exhibits separate functions (biofilm formation). In addition to inducing *lqsR* expression in an indirect manner, *sinR* is also assumed to be a positive factor in *lqsA* transcription, directly binding to its promoter region. Thus, *sinR* might provide a novel link between the AHK signalling cascade and the interconnected regulatory systems governing adaptation of *L. pneumophila* to the transition from the exponential growth to stationary phase.

4.4 AHK signalling mediates natural competence of *L pneumophila* and *V. cholerae*

L. pneumophila strains lacking *lqs* genes displayed significantly increased natural competence and efficient acquisition of extracellular DNA (Section 3.1.2, Figure 10). Natural competence depends on type IV pili (Stone and Kwaik, 1999) and is significantly enhanced in certain strains upon microaerophilic growth at 37°C or aerobic growth at 30°C (Sexton and Vogel, 2004). Moreover, genotoxic stress caused by UV radiation or compounds such as mitomycin C or antibiotics were shown to promote natural competence for transformation of *L. pneumophila* (Charpentier *et al*., 2011). In support of previous studies (Sexton and Vogel, 2004, Stone and Kwaik, 1999), the competence of *L. pneumophila* wild-type strain Philadelphia-1 was found to be low (Section 3.1.2, Figure 10A). However, the Δ*lqsA, ΔlqsR, ΔlqsS or* Δ*lqsT* strains exhibited a 4-orders of magnitude higher transformation efficiency, with the Δ*lqsS*-Δ*lqsT* deletion mutant having an even stronger phenotype. Substantiating these data, the expression of the *comEA* promoter was upregulated in all *L. pneumophila* strains lacking individual *lqs* genes and most profoundly in the Δ*lqsS*-Δ*lqsT* strain (Section 3.1.2, Figure 10D). Thus, *lqsS* and *lqsT* seem to control competence in a synergistic manner.

L. pneumophila lacking *lqs* genes was induced to take up extracellular DNA only at high bacterial densities ($OD_{600} > 2.5$; Figure 10), akin to *V. cholerae*. In contrast to *L. pneumophila*, however, *V. cholerae* DNA acquisition and *comEA* transcription was abolished in absence of *cqsA* or *cqsS*, and enhanced by AHK signalling in biofilms or by synthetic CAI-1 (Antonova and Hammer, 2011, Suckow *et al.,* 2011). Taken together, AHK signalling reciprocally regulates competence as well as virulence in *L. pneumophila* and *V. cholerae*. While AHK signalling represses competence and promotes virulence in *L. pneumophila*, CAI-1 signalling promotes natural competence and repress virulence as well as biofilm formation in *V. cholerae* (Figure 28; Suckow *et al*., 2011, Henke and Bassler, 2004, Miller *et al.,* 2002). Biofilm formation was not affected by the *lqs* genes in *L. pneumophila* (Tiaden *et al*., 2010b). Interestingly, the SinR-type transcription factor characterised in this study was found to inhibit natural competence, as was observed for the *lqs* components. As *sinR* was not only found to control its own expression, but also to induce *lqsA* transcription, SinR

establishes a novel link between the development of natural competence and AHK signalling. Analogously, several regulatory pathways, i.e. growth on chitin surfaces and quorum sensing, are involved in the development of natural competence in *V. cholerae*. The major regulators of these two cascades, TfoX and HapR, were shown to activate the expression of the common transcriptional regulator QstR, which itself links chitin-induced TfoX activity with quorum sensing (Section 4.1, Figure 28; Lo Scrudato, 2013). In the absence of chitin oligomers, TfoX translation is inhibited. Specifically, the membrane-bound transcriptional regulator TfoS cannot dimerise and activate the transcription of the small RNA TfoR, leading to an inhibitory stem-loop structure in the *tfoX* mRNA (Yamamoto *et al.*, 2011). In the presence of chitin, these oligomers may interact with and dimerise the membrane-bound transcriptional regulator TfoS, which allows the cytoplasmic DNA-binding domains to activate expression of *tfoR*. TfoR then interacts with Hfq and positively regulates *tfoX* translation, resulting in activation of the genes required for competence (Dalia *et al*., 2014). In *L. pneumophila*, detailed mechanisms of DNA uptake, including the involvement of the type IV-like pilus and ComEA remain to be uncovered.

Finally, the *lqs* system together with the transcription factor SinR represents a major negative regulatory element of natural competence of *L. pneumophila* and also functionally links the *lqs* cluster with the orphan *lqsT* gene. The *in vivo* conditions under which *lqs*-repressed natural competence of *L. pneumophila* is induced remain elusive. Of special interest is the identification of a pivotal role of LAI-1 in the horizontal transfer of DNA and the acquisition of fitness-enhancing features.

4.5 Evolutionary and environmental implications of AHK-mediated signalling

The *V. cholerae* CqsAS QS system was identified in several *Vibrio* spp. including *V. harveyi* and *V. parahaemolyticus* and other marine bacteria (Henke *et al*., 2004). Studies using a luminescent *V. cholerae* CAI-1 reporter strain revealed that CAI-1 from *V. harveyi* (or other *Vibrio* spp.) controls gene expression in *V. cholerae* (and *vice versa*). Therefore, cell–cell communication via CAI-1 functions across the species barrier.

The *cqsA* and *cqsS* genes are transcribed in opposite directions in *V. cholerae* and the corresponding homologues in other environmental bacteria retain this orientation. In contrast, the *lqsR* homologues appear to form an operon with the *lqsS/cqsS* genes. The *hdeD* homologue exclusively present in *L. pneumophila* disrupts this putative operon structure, but also introduces novel regulatory elements. Upstream of *hdeD* a ‘lux box’-type operator consensus is present, which binds LuxR-type regulators such as LetA and promotes the expression of several autoinducer synthases and other genes regulated by quorum sensing.

The LAI-1 synthase-encoding gene *lqsA* is present in all *L. pneumophila* genomes sequenced so far (Glöckner *et al.*, 2007, Chien *et al.*, 2004, Cazalet *et al*., 2004) as well as in several other clinical or environmental isolates (Spirig *et al*., 2008). On the other hand, neither *L. longbeachae*, nor *Legionella* spp. other than *L. pneumophila* apparently possess the *lqs* system (Cazalet *et al.,*

2010). The *L. pneumophila lqs* cluster is flanked by a putative hot spot for genomic recombination, as the corresponding region in *L. longbeachae* harbours a homologue of the putative effector gene *legG2* (*llo0327*, *lpg0267*), flanked by transposase elements and localises upstream of an *E. coli hdeD*-like gene. Since *hdeD* interrupts the convergently transcribed *lqsS* and *lqsR,* it is tempting to speculate that these two genes originate from one single, larger *cqsS* ancestor gene. Perhaps, recombinatory events might have introduced a *cqs*-like cluster into the *L. pneumophila* genome. Homologuous gene clusters containing *lqsA, lqsS* and *lqsR* (but not *hdeD*) have been discovered in other environmental bacteria, including *Nitrococcus mobilis*, *Burkholderia xenovorans* and *Polaromonas* spp. (Tiaden *et al.*, 2007), corroborating the notion that *L. pneumophila* might have acquired the *lqsA-lqsR-lqsS* cluster by horizontal gene transfer.

AHK signalling is prevalent among family members of the Legionellaceae (*Legionella* spp.), Vibrionaceae (*Vibrio* spp., *Photobacterium* spp.), Burkholderiaceae (*Burkholderia* spp., *Cupravidus* spp.) and Chlorobiaceae (*Chlorobium* spp., *Prostheochloris* spp.). Given the prevalence of Lqs/Cqs systems among a number of environmental bacterial species of different genera, AHK molecules might also contribute to more widespread crosstalk between different bacterial genera using this class of signalling molecules (Section 4.6).

4.6 Interkingdom signalling

Beyond bacterial cell-cell communication, small signalling molecules are mediators of the prokaryote-host cell interplay in a process termed interkingdom signalling (Pacheco *et al.,* 2009). A number of reports have assigned bacterial autoinducers diverse effects on their host cells during the infection process, including mammalian cell physiology (Williams *et al*., 2004), cellular stress pathways (Clarke *et al.,* 2007), epithelial cell migration (Karlsson *et al.,* 2012) and apoptosis (Tateda *et al.,* 2003). Specifically, N-Acylhomoserine lactone (AHL) produced by *P. aeruginosa* was shown to interact with lymphocytes via a cooperative binding model, implying the existence of an AHL membrane receptor (Davis *et al*., 2010). Jahoor and coworkers described a ligand for oxo-C12-HSL, namely the peroxisome proliferator activated receptors beta-delta (PPAR), which upon activation inhibit the expression of proinflammatory genes (Jahoor *et al*., 2008). Binding of these transcription factors to their target promoters was shown to be antagonised by oxo-C12-HSL *in vitro*. Moreover, in a host cell-dependent fashion, AHL was reported to mediate either pro- or anti-inflammatory host responses by subverting NF-κB-dependent gene expression (Kravchenko *et al.*, 2008). It will be interesting to test effects of the *L. pneumophila* autoinducer molecule LAI-1 on eukaryotic host cells. Overall, it is still controversial whether quorum sensing molecules modulate host signaling pathways or if the eukaryotic host uses the conserved microbial compound structures as molecular alarmone to detect and fight infections.

Moreover, signal transduction systems of some pathogenic bacteria were shown to recognise host adrenergic hormones (catecholamines) to promote virulence. The first piece of evidence of crosstalk between eukaryotic and prokaryotic signaling systems was provided by the two-component system QseBC present in the enteric pathogen EHEC (Enterohameorrhagic *E. coli*) (Clarke *et al*., 2006, Figure 30).

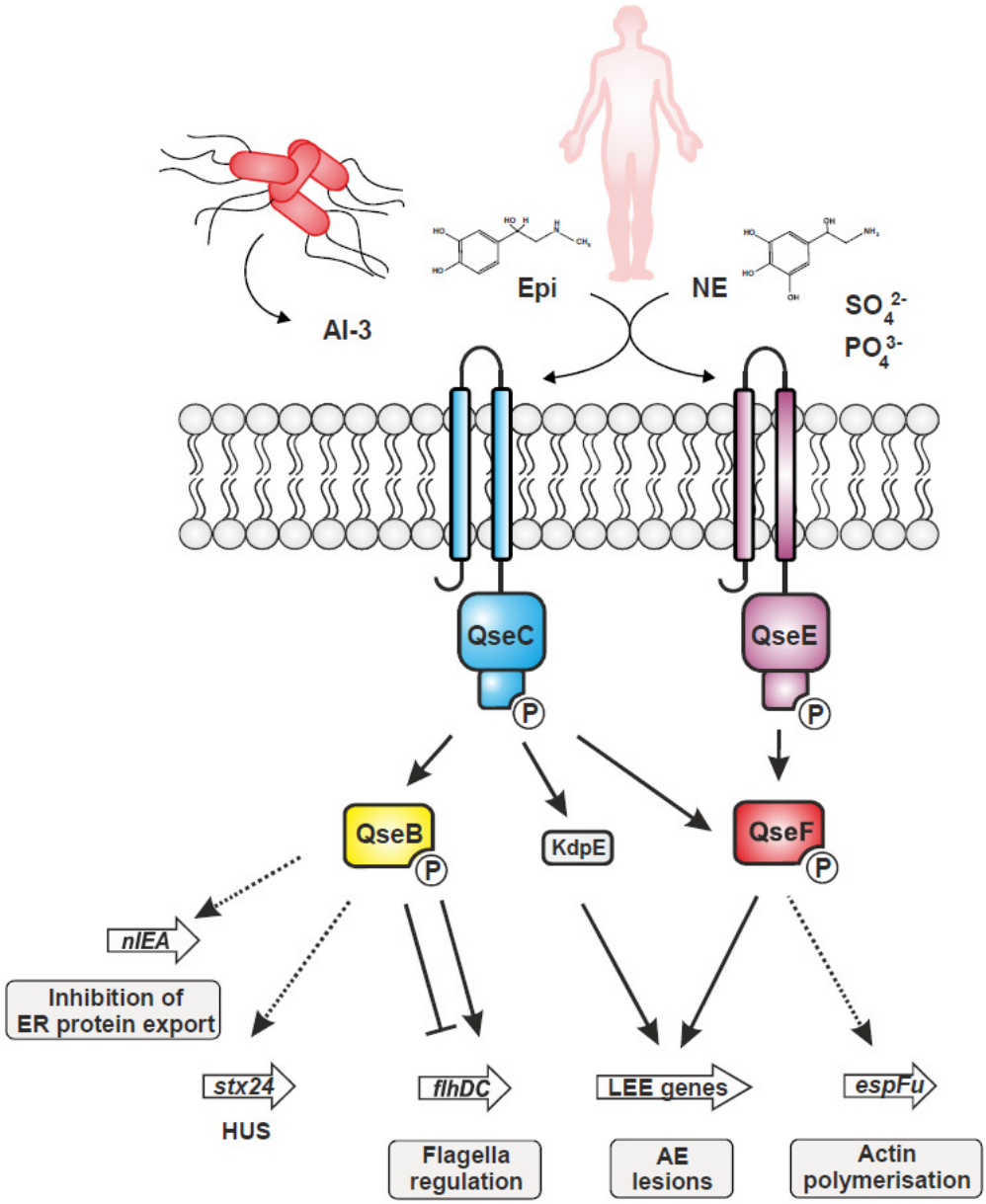

Figure 30: **Model of the QseC and QseE signalling cascades in EHEC**. QseC responds to the human stress hormones epinephrine/norepinephrine and to AI-3 generated by the gut microbial flora. QseC phosphorylates its cognate response regulator QseB, which directly activates transcription of flagellar genes *flhDC* and the virulence gene *nlEA,* and indirectly promotes LEE gene expression. QseF is phosphorylated by both QseC and QseE and indirectly induces expression of Shiga toxin and the virulence factor *espFu*, promoting actin polymerisation. Solid and dashed lines indicate direct positive and indirect regulation, respectively. EHEC. Enterohaemorrhagic *E. coli*; Epi, epinephrine; LEE, locus of enterocyte effacement; NE, norepinephrine; AI-3, autoinducer-3; AE, attaching and effacing, HUS, haemolytic-uremic syndrome. Modified after Hughes *et al*., 2009.

The membrane-embedded sensor kinase QseC not only senses the host hormones epinephrine and norepinephrine, but also binds to AI-3 produced by the microbial gastrointestinal flora. Through the QseC sensor kinase, AI-3 promotes EHEC motility, permitting the bacteria to approach the gut epithelium, where they can be exposed to host-produced epinephrine and norepinephrine (Clarke *et al*., 2006). Upon binding of any of these signalling molecules, QseC autophosphorylates and subsequently phosphorylates a transcription factor, QseB which relays to a complex regulatory cascade, leading to the transcription of key virulence genes. Interestingly, QseB binds to different sites in the target promoters depending on its phosphorylation state. Thus, one single response regulator is able to modulate gene expression by both activating or repressing the same gene. Upon recognition by the QseCB system, adrenergic hormones were revealed to regulate expression of the locus of enterocyte effacement (LEE), Shiga toxin and motility genes (*flhDC*) in EHEC (Njoroge and Sperandio, 2012, Figure 30).

A second two-component system (QseEF), composed of the sensor kinase QseE and the response regulator QseF, was reported to cooperate with QseCB to control virulence in EHEC (Reading *et al*., 2007). Like QseC, QseE also senses the host hormone epinephrine, but in contrast, does not react to the bacterial signal AI-3. *qseEF* transcription is activated by epinephrine via QseC. The QseEF system is not involved in regulation of flagella and motility, but plays an important role in activating genes necessary for AE lesion formation (Reading *et al*., 2007) and also activates production of Shiga toxin (Figure 31).

The complex multitude of effects governed by adrenaline and noradrenaline target both bacteria and the host response: Although the NE stress hormones mostly benefit the bacteria to navigate their infectious pathway, under certain conditions the noradrenergic compounds provide a unique advantage to the host in order to manipulate bacterial pathogens: While the downregulation of the lipopolysaccharide (LPS) modifying enzymes PmrF and PagL causes an increase in sensitivity to polymyxin B, it also concomitanty reduces activation of the TLR-4 recpeptors, thereby alleviating the host inflammatory response to infection (Kawasaki *et al.,* 2004). Homologues of the QseBC signal transduction system were discovered in more than 25 human or plant pathogenic species and mutants of EHEC, in *Salmonella* Typhimurium, and *Francisella tularensis* were attenuated in infected animals (Rasko *et al*., 2008).

5. Conclusions and future perspectives

Bacterial cell-cell communication involving the novel class of AHK signalling molecules is known to regulate different functions involved in microbial pathogenesis. The *lqs* system of *L. pneumophila* was shown to control virulence, biofilm or extracellular filament formation, natural competence and the expression of a genomic fitness island. The transcription factor SinR was found to be functionally linked to the resident AHK signalling pathway, allowing speculations regarding further regulatory elements expanding this complex network. Due to its vast array of controlled traits, quorum sensing is deemed to be an interesting target to modulate pathogenesis. Quorum sensing inhibitors may quench the virulence phenotypes exerted by pathogenic bacteria and complement antibiotic treatment, a highly desirable strategy in response to alarming global reports of continuous emergence of antibiotic-resistant microbes (Naik *et al*., 2013). Of note, agonists or inhibitors of LqsS or LqsT family sensor kinases as well as chemicals targeting LAI-1 or its derivatives pose great potential for therapeutic applications. The antimicrobial function of quorum sensing inhibitors acts at various stages of infection processes ranging from the generation or detection of the signal molecule by its receptor to the activation of the QS regulon.

Future research on AHK signal transduction in *L. pneumophila* might encompass the direct impact of synthetic LAI-1 (i) on mechanistic interactions among the Lqs proteins in biochemical profiles, (ii) on pathogen host-cell processes using cellular approaches, and (iii) on global gene expression studies of *L. pneumophila* or its host in the context of interkingdom signalling. Ultimately, the identification of new chemical communication modules and their crosstalk between different kingdoms will provide insights into pathogenicity and open new avenues for antimicrobial research.

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