Aus dem Max von Pettenkofer-Institut für Hygiene und Medizinische
Mikrobiologie der Ludwig-Maximilians-Universität München

Lehrstuhl: Bakteriologie
Komm. Vorstand: Prof. Dr. Rainer Haas

Biochemical Analysis Of Phosphorylation Signalling Through The *Legionella pneumophila* Quorum Sensing System

Dissertation

zum Erwerb des Doktorgrades der Naturwissenschaften
am Max von Pettenkofer-Institut
der Medizinischen Fakultät
der Ludwig-Maximilians-Universität München

vorgelegt von
Ursula Schell
aus Veszprém

2014

Gedruckt mit Genehmigung der Medizinischen Fakultät
der Ludwig-Maximilians-Universität München

Betreuer: Prof. Dr. Hubert Hilbi
Zweitgutachter: Prof. Dr. Reinhard Geiger

Dekan: Prof. Dr. med. Dr. h.c. Maximilian Reiser, FACR, FRCR

Tag der mündlichen Prüfung: 25.06.2015

STATUTORY DECLARATION

I declare that I have written this thesis with the title: “Biochemical Analysis Of Phosphorylation Signalling Through The *Legionella pneumophila* Quorum Sensing System“, independently and that I have not used other sources than declared. I declare as well that I have not submitted a dissertation (neither the entire dissertation nor parts) to another examination board.

_____ _____

Place, Date Ursula Schell

EIDESSTATTLICHE ERKLÄRUNG

Ich versichere hiermit eidesstattlich, dass ich meine Dissertation mit dem Thema: „Biochemical Analysis Of Phosphorylation Signalling Through The *Legionella pneumophila* Quorum Sensing System“ selbstständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe. Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

_____ _____

Ort, Datum Ursula Schell

CONTENTS

NOMENCLATURE

Gene products of *lqs* genes are numbered such that the first methionine of the wild-type protein is designated "1" in the amino acid sequence. Positions are shown as numbers after the particular amino acids. Amino acid substitutions are termed as follows: The native amino acid is designated in one-letter code, followed by the respective amino acid position in the protein. The amino acid introduced by (site-directed) mutagenesis is terminally added in one-letter code (Example: $LqsR_{D108N}$). Unless otherwise noted, nucleotide positions indicate the distance from the transcriptional start site (+1).

ABBREVIATIONS

AI	autoinducer
Amp^R	ampicillin resistance
APS	ammonium persulfate
bp, kb	base pairs, kilo base pairs
BSA	bovine serum albumine
ß-MeOH	beta-mercaptoethanol
°C	degree Celsius
Da, kDa	Dalton, kilo Dalton
DNA	deoxyribonucleic acid
DNAse	desoxyribonucleinase
dNTP	desoxyribonucleotidetriphosphate
DTT	dithiothreitol
Fig.	figure
GFP	green fluorescent protein
gen. DNA	genomic DNA
His	histidine
HK	histidine kinase
IgG	immunoglobulin G
IPTG	isopropyl β-D-1-thiogalactopyranoside
Kan^R	kanamycin resistance
LAI-1	*Legionella* autoinducer- 1
LSP	low speed pellet
mAU	milli arbitrary units
mM, M	millimolar, molar
ms, s, min, h	millisecond, second, minute, hour
ng, µg, mg	nanogramm, microgramm, milligramm
nm, cm	nanometre, centimetre
Ni^{2+}-NTA-agarose	nickel-charged resin (Ni^{2+}-nitrilotriacetic-acid)
OD_x	optical density at x nm
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TBS	Tris-buffered saline

PCR	polymerase chain reaction
PI	propidiumiodide
PMSF	phenylmethansulfonylfluorid
QS	quorum sensing
RIU	relative intensity unit
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecylsulfate
SN	supernatant
Tab.	table
TAE	Tris-acetate-EDTA
TCA	trichloroacetic acid
TEMED	N,N,N',N'–tetramethylethylendiamine
Tet^R	tetracycline resistance
Tris	tris(hydroxymethyl)aminomethane
TG	Tris-glycerol
V	Volt
WT	wild-type

LIST OF PUBLICATIONS

Publication used in this thesis:

- Schell U., Kessler A., Hilbi H., **Phosphorylation signalling through the *Legionella* quorum sensing histidine kinases LqsS and LqsT converges on the response regulator LqsR,** Mol. Micro., 2014 Jun;92(5):1039-55

Additional publications:

- Anetzberger C., Schell U., Jung K., **Single cell analysis of *Vibrio harveyi* uncovers functional heterogeneity in response to quorum sensing signals.** BMC Microbiol. 2012 Sep 18;12:209.
- Anetzberger C., Reiger M., Fekete A., Schell U., Stambrau N., Plener L., Kopka J., Schmitt-Kopplin P., Hilbi H., Jung K., **Autoinducers act as biological timers in *Vibrio harveyi*.** PLoS One., 2012; 7(10):e48310.
- Kessler A., Schell U., Sahr T., Tiaden A., Harrison C., Buchrieser C., Hilbi H., **The *Legionella pneumophila* orphan sensor kinase LqsT regulates competence and pathogen-host interactions as a component of the LAI-1 circuit.**, Environ. Microbiol., 2013 Feb;15(2):646-62.

SUMMARY

Quorum sensing represents an aspect of intercellular communication, which bacteria use to coordinate their behaviour based on the population density. To achieve this, small chemical signalling molecules termed autoinducers are produced, secreted and sensed. Quorum sensing systems are generally two-component systems (TCS) and employ the transfer of phosphoryl groups for signal transduction. TCS consist of two conserved components, a sensor kinase and a response regulator protein. The sensor kinase, which is regulated by external environmental stimuli (such as autoinducer molecules), is autophosphorylated at a conserved histidine residue, creating a high-energy phosphoryl group that is subsequently transferred to an aspartate residue in the response regulator. This protein frequently is a transcription factor, which upon phosphorylation undergoes dimerization, thereby eventually leading to alterations in gene expression. This cell density-dependent regulation of gene expression enables the bacterial population to adapt to changing environmental conditions.

Legionella pneumophila is a Gram-negative bacterium and the causative agent of Pontiac fever or Legionnaires´ disease. The infection occurs aerogen via inhalation of aerosols from contaminated hot water pipes, air conditioning systems or cooling towers. Besides environmental endemic amoebae, *L. pneumophila* also infects human alveolar macrophages.

For cell-cell communication, *L. pneumophila* employs the autoinducer LAI-1 (3-hydroxypentadecane-4-one), which is produced and detected by the Lqs (*Legionella* quorum sensing) system. The system is encoded by the *lqs* cluster, consisting of the autoinducer synthase LqsA, the putative sensor kinase LqsS and the response regulator LqsR, as well as an orphan LqsS homologue termed LqsT. Lqs-regulated processes include uptake and intracellular growth of *L. pneumophila* in phagocytes, production of extracellular filaments, natural competence for DNA acquisition and expression of a genomic “fitness island“.

In this thesis the individual components of the Lqs system were characterised biochemically, and the signal transduction cascade was reconstructed *in vitro*. Phosphorylation experiments using inverted membrane vesicles and [γ-^{32}P]-ATP showed that LqsS and LqsT are autophosphorylated at a conserved histidine residue (H200 or H204) located in their cytoplasmic histidine kinase domain. Immuno-precipitation revealed that LqsS and, albeit less efficiently, LqsT are bound by LqsR. Autophosphorylation of either sensor kinase was prevented by LqsR, dependent on the conserved aspartate residue (D108) in its receiver domain. LqsR catalysed the dephosphorylation of phospho-LqsS or phospho-LqsT, and

efficient dephosphorylation required D108. Additionally, *in vitro* phosphorylation of LqsR at D108 with acetyl phosphate caused dimerization of the response regulator. Moreover, LqsS and LqsT heterologously produced by *E. coli* localized to the membrane fraction. However upon heterologous production, only LqsT was autophosphorylated by ATP at the conserved histidine, suggesting that LqsS requires an *L. pneumophila* co-factor for phosphorylation.

The influence of LAI-1 on the signal transduction cascade was also analysed. These studies revealed that LAI-1 inhibited the autophosphorylation of both sensor kinases, LqsS and LqsT, whereas it had no influence on the phosphotransfer reaction.

In summary, these results indicate that the signal transduction based on the *L. pneumophila* sensor kinases LqsS and LqsT converges via phosphotransfer on the response regulator LqsR, and that the autoinducer LAI-1 negatively regulates this system.

ZUSAMMENFASSUNG

Quorum Sensing ist ein interzellulärer Kommunikationsprozess, den Bakterien verwenden um ihr Verhalten auf Grund der Populationsdichte zu koordinieren. Dafür werden kleine chemische Signalmoleküle, Autoinduktor genannt, produziert, sekretiert und detektiert. Im Allgemeinen handelt es sich bei Quorum Sensing Systemen um Zweikomponentensysteme (TCS), die einen Phosphotransfer für die Signalweiterleitung verwenden. TCS bestehen aus zwei konservierten Komponenten, einer Sensorkinase und einem Antwortregulator. Die Histidinkinase wird über externe Umweltreize (Autoinduktoren) reguliert und am konservierten Histidinrest autophosphoryliert. Dabei wird eine „energiereiche" Phosphorylgruppe kovalent gebunden und anschließend auf den Aspartatrest des Antwortregulators übertragen. Dieser dient seinerseits häufig als Transkriptionsfaktor, wobei die Phosphorylierung Dimerisierung, und dadurch letztendlich eine Veränderung der Genexpression induziert. Diese Zelldichte-abhängige Regulation der Genexpression ermöglicht der Bakterienpopulation eine hohe Anpassungsfähigkeit an sich verändernde Umweltbedingungen.

Legionella pneumophila ist ein Gram-negatives Bakterium und als Auslöser des Pontiac Fiebers oder der Legionärskrankheit bekannt. Die Infektion erfolgt aerogen über Inhalation von Aerosolen aus kontaminierten Warmwasserleitungen, Klimaanlagen oder Kühltürmen. Neben in der Umwelt natürlich vorkommenden Amöben dienen dem Pathogen auch menschlichen Alveolarmakrophagen als Wirt.

Die Zell-Zell Kommunikation erfolgt bei *L. pneumophila* über den Autoinduktor LAI-1 (3-Hydroxypentadecan-4-on), welcher durch das Lqs (*Legionella* quorum sensing) System produziert und detektiert wird. Das System wird codiert durch das *lqs* Cluster, bestehend aus der Autoinduktorsynthase LqsA, der mutmaßlichen Sensorkinase LqsS, dem Antwortregulator LqsR sowie einem unabhängigen LqsS-Homolog, LqsT genannt. Zu den Lqs-regulierten Prozessen gehören Aufnahme und intrazelluläres Wachstum der Bakterien in Phagozyten, Produktion von extrazellulären Filamenten, natürliche Kompetenz für die DNA-Aquisition und die Expression einer genomischen „Fitness-Insel".

Im Rahmen dieser Arbeit sollte eine biochemische Charakterisierung der einzelnen Komponenten des Lqs Systems vorgenommen und die Signaltransduktionskaskade *in vitro* rekonstruiert werden. Phosphorylierungsexperimente mit invertierten Membranvesikeln und [γ-^{32}P]-ATP zeigten, dass LqsS und LqsT am konservierten Histidinrest (H200 oder H204) in der zytoplasmatischen Histidin-Kinasedomäne autophoshoryliert werden. Die Immuno-

päzipitation machte deutlich, dass sowohl LqsS als auch- mit einer geringeren Effizienz- LqsT durch LqsR gebunden werden. Die Autophosphorylierung beider Sensorkinasen wurde durch LqsR verhindert. Dies war abhängig vom konservierten Aspartatrest (D108) in der Empfänger-Domäne. LqsR katalysierte die Dephosphorylierung von Phospho-LqsS oder Phospho-LqsT in Abhängigkeit von D108. Des Weiteren bewirkte die *in vitro* Phosphorylierung von LqsR an D108 durch Acetylphosphat eine Dimerisierung des Antwortregulators. In *E. coli* heterolog produzierte LqsS- und LqsT-Kinasen konnten ebenfalls in der Membranfraktion lokalisiert werden. Jedoch wurde hier nur LqsT durch ATP autophosphoryliert, und zwar am konservierten Histidin. Dies legt die Annahme nahe, dass LqsS einen weiteren *L. pneumophila* Kofaktor für die korrekte Faltung und/oder Autophosphorylierung benötigt.

Weiterhin wurde auch der Einfluss von LAI-1 auf die Signaltransduktionskaskade des Lqs-Systems untersucht. Diese Studien zeigten, dass der Autoinduktor die Autophosphorylierung beider Sensorkinasen, LqsS und LqsT, inhibierte, wohingegen er keinen Einfluss auf den Phosphotransfer hatte.

Diese Resultate veranschaulichen, dass die Signaltransduktion von *L. pneumophila* ausgehend von den Sensorkinasen LqsS und LqsT über Phosphotransfer auf dem Antwortregulator LqsR konvergiert und der Autoinduktor LAI-1 dieses System negativ reguliert.

1 INTRODUCTION

1.1 Bacterial communication

1.1.1 Quorum sensing

Scientific research on bacterial cell-cell communication started in the early 1970s and began with the marine bacterium *Vibrio fischeri,* which is a Gram-negative, symbiotic bacterium regulating bioluminescence production via quorum sensing (Engebrecht *et al.*, 1983, Nealson & Hastings, 1979). At this point, it was believed that the ability to communicate through chemical signals was a characteristic trait of eukaryotes. The discoveries from Engebrecht, Hastings and Nealson established the basis for subsequent scientific investigations of bacterial communication. This special type of communication was termed quorum sensing (QS) and is based on population density-dependent production, detection and response to small diffusible molecules termed autoinducers (AI). This process allows the bacteria to gauge changes in cell density and adjust gene expression accordingly (Fig. 1). The AI concentration in the environment increases simultaneously with a growing bacterial population. Subsequently, detection of the minimal stimulatory (threshold) concentration of the AI leads to an alteration of the bacterial gene expression. Quorum sensing controls the expression of those genes that are beneficial for groups of bacteria acting in synchrony (Bassler *et al.*, 1994, Fuqua & Greenberg, 2002).

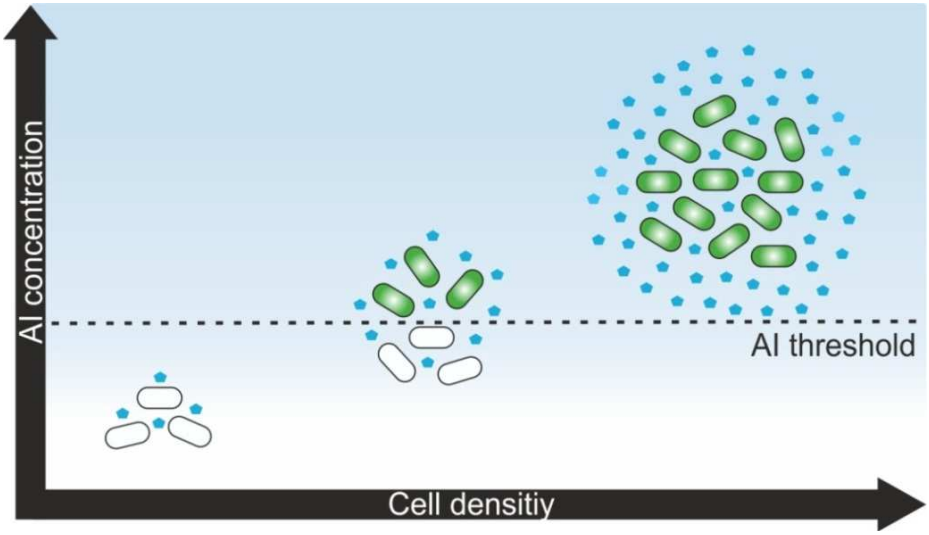

Fig. 1: Scheme of a simple quorum sensing system. Bacterial cells (white/green) produce and secrete small signaling molecules, termed autoinducers (AI, shown by blue pentagons). With increasing cell density the AI concentration in the environment reaches a certain threshold and induces changes in the common behavior of the population. Model adapted from previous publication (Keller & Surette, 2006).

The term “quorum” is Latin and described in the Roman Empire the smallest number of members that was needed for legal decisions in the Senate. Among the diverse bacterial processes regulated by quorum sensing are symbiosis, virulence, motility, biofilm formation, bioluminescence, sporulation, competence and the expression of antibiotics (Miller & Bassler, 2001). Quorum sensing systems are generally two-component systems (TCSs) and can be divided into two classes: LuxIR-type quorum sensing systems in Gram-negative bacteria and oligopeptide-type quorum sensing circuits in Gram-positive bacteria.

The simplest and elementary form of a quorum sensing circuit is the LuxIR system, existing in in over 25 species of Gram-negative bacteria (Miller & Bassler, 2001). These quorum sensing systems contain homologues of the two *Vibrio fischeri* regulatory proteins named LuxI and LuxR (Fig. 2A). The LuxI-type autoinducer synthase produces a species-specific acyl-homoserine lactone (HSL), which works as an autoinducer and diffuses through the cytoplasmic membrane. With increasing cell density the extracellular as well as the intracellular concentration increases. Upon reaching the threshold concentration (~1-10 µg/ml) the AI is detectable by the transcription activator LuxR-like protein, which regulates in the case of *V. fischeri* the expression of the *luxICDABE* operon (Miller & Bassler, 2001). This operon encodes the enzymes responsible for light production of this symbiotic bacterium (Engebrecht & Silverman, 1984). Besides *V. fischeri* among other bacteria also *Agrobacterium tumefaciens* and *Pseudomonas aeruginosa* use this type of quorum sensing system (Passador *et al.*, 1993, Piper *et al.*, 1999).

Gram-positive bacteria evolved a different type of quorum sensing system using oligopeptides instead of HSL as signalling molecules. These systems transport the AIs via specific ATP-binding cassette (ABC) transporters. With increasing cell density the secreted peptide autoinducer accumulates in the bacterial environment and interacts with the membrane bound sensor kinase. This binding triggers a phosphorylation signalling cascade converging on the response regulator, eventually changing the transcription of specific target genes (Fig. 2B). This type of quorum sensing system is composed of typical elements of two-component systems. Among other processes, competence of *Bacillus subtilis* and *Staphylococcus pneumoniae* and virulence of *Staphylococcus aureus* are regulated this way (de Kievit & Iglewski, 2000, Tortosa & Dubnau, 1999).

Bacteria like *Vibrio cholerae* or *Vibrio harveyi* process information on cell density over a QS circuit which represents a hybrid system of the canonical Gram-negative and the Gram-positive bacterial QS system (Bassler *et al.*, 1993, Bassler, 1999).

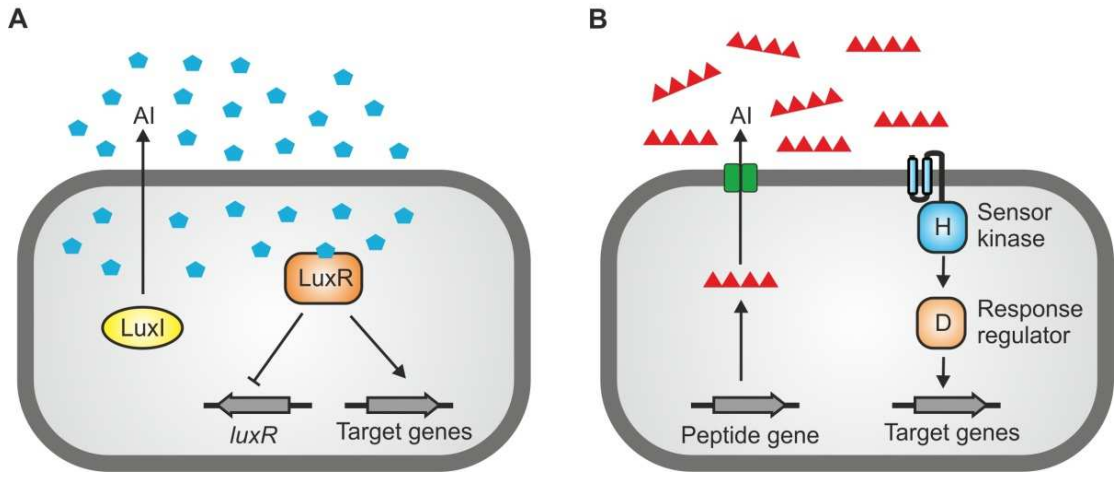

Fig. 2: General models for LuxIR- and peptide-mediated quorum sensing systems. A. In typical Gram-negative LuxIR circuits, the LuxI-type protein catalyzes the synthesis of an N-(3-oxohexanoyl)-homoserine lactone (HSL) autoinducer (blue pentagons). At the threshold concentration of the autoinducer, the response regulator protein LuxR binds the HSL and promotes the expression of target genes and simultaneously represses the own transcription. **B.** Gram-positive bacteria produce oligopeptide autoinducers (red triangles). The peptide signal is secreted by an ABC-transporter (green squares). The AI is detected via a two-component signal transduction system, leading to the phosphorylation of a response regulator protein, which regulates the transcription of target genes. Model adapted from previous publication (Miller & Bassler, 2001).

1.1.2 Bacterial two-component systems

Two-component systems link the stimulus- and response-mechanism in bacteria and are one of the most effective signal transduction systems. TCSs play an essential role in bacterial behaviour such as adaptation to environmental changes, induction of pathogenesis and regulation of cell-cell communication (quorum sensing). TCSs utilize phosphorylation signalling which is an evolutionarily conserved mechanism and is established in all domains of life (Manning *et al.*, 2002). In eukaryotes, the general phosphorylation sites of kinases are either serine or threonine residues, or less commonly, tyrosines (Blom *et al.*, 1999), while in bacteria histidine kinases are more common (Robinson, Buckler *et al.* 2000, Stock, Robinson *et al.* 2000).

Numerous TCSs are encoded in bacterial genomes correlating with the genome size and the complexity of the organism´s lifestyle (Ulrich & Zhulin, 2007). Thereby the numbers range from tens (e.g. *Bacillus subtilis*, *Escherichia coli*) to over hundred (e.g. cyanobacteria, *Myxococcus xanthus*). However, some bacteria do exist that do not encode TCS, such as *Mycoplasma* spp.(Gao *et al.*, 2007). The basic module of TCSs are two conserved proteins;

the sensor histidine kinase (HK) and the response regulator (RR) with specific conserved domains, utilizing a central mechanism of phosphotransfer to create a signalling pathway (Stock *et al.,* 2000, Robinson *et al.*, 2000). In addition to conserved domains, HKs and RRs contain variable domains adapted to the specific system in which they function. HKs are usually transmembrane proteins that function as sensors, with variable domains involved in sensing diverse physical and chemical stimuli. RRs are typically multi-domain proteins with variable effector domains controlling the output response (Robinson *et al.,* 2000).

The TCS signal transduction pathway includes three chemical reactions (Fig. 3). Initially the high energy γ-phosphoryl group from ATP, which is used as a substrate, is transferred to the conserved histidine (His) core of the HK, resulting in autophosphorylation. His phosphorylation occurs on the imidazole nitrogen, producing a high energy phosphoamidate bond. The equilibrium of this autophosphorylation reaction favours the unphosphorylated HK protein, since at typical intracellular ATP/ADP ratios only a small percentage of the HK appears in a phosphorylated state (Stock, Robinson *et al.* 2000).

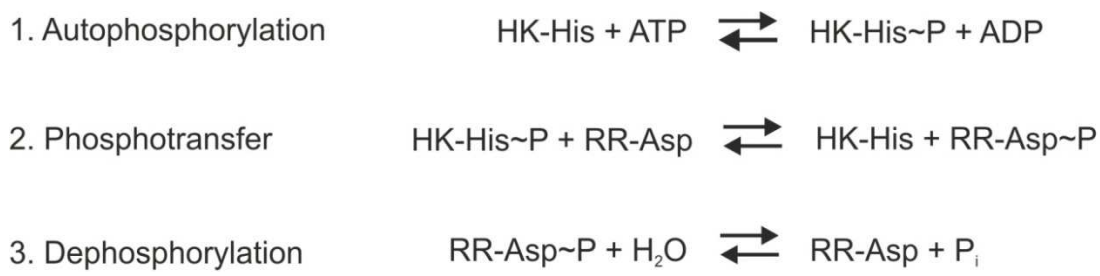

Fig. 3: Chemistry of the basic two-component signal transduction pathway. The basic two-component signal transduction pathway comprises of tree reactions: 1. Autophosphorylation, 2. Phosphotransfer, 3. Dephosphorylation. Model adapted from previous publication (Stock *et al.*, 2000)

In a second reaction the RR catalyses the transfer of this phosphoryl group from the phospho-His residue to a conserved aspartate (Asp) within its own regulatory domain creating a high energy carbonic acid phosphoric acid mixed anhydride. This activates the RR and generates a specific response (Stock *et al.,* 2000). To terminate signalling the phosphoryl group from the RR phospho-aspartate residue is dephosphorylated. All three reactions are catalysed through divalent metal ions, such as Mg^{2+} (Stock *et al.*, 2000).

There are two basic pathway architectures of TCSs. The simplest form is the phosphotransfer pathway involving a HK and RR pair performing one phosphotransfer step/event (Fig. 4A and B). In more complex systems multiple His-containing and Asp-containing domains in various proteins are involved, generating a phosphorelay (Fig. 4C).

1.1.2.1 Structure of histidine kinases

In the TCS pathway the HK represents the essential link between the bacterial environment and the cellular response. HKs function as receptors for the extracellular stimuli and regulate the information flow through the signalling pathway. HKs are transmembrane proteins composed of a diverse N-terminal periplasmic sensory domain linked to a C-terminal cytoplasmic kinase core, consisting of the dimerization and histidine-phosphotransfer (DHP-) domain and the catalytic ATP binding (CA-) domain (Fig 4) (Robinson *et al.,* 2000).

The N-terminal sensing domain of the HK is designed for detection of environmental stimuli. These sensing domains share little sequence similarity, as the HKs contain a wide variety of extracellular, intracellular and/or transmembrane sensor domains designed for specific ligand/stimulus interactions (Stock *et al.,* 2000). In transmembrane HKs, the sensor domains are connected via one or more transmembrane helices to the cytoplasmic kinase core. Sometimes the transmembrane helices are followed by a linker domain, the functions of which are poorly understood, but it is assumed that they act as structural relay for signal transduction (Zhu & Inouye, 2003). Also Per-ARNT-Sim (PAS) domains have been found in HKs. These cytosolic sensing modules detect a wide variety of chemical and physical stimuli (Moglich *et al.,* 2009). PAS domains are small (~100 amino acids) and typically located adjacent to the last transmembrane region of the sensing domain and N-terminal to the kinase domain (Stock *et al.,* 2000).

HKs sense extracellular stimuli and integrate them into a phosphorylation signal by catalysing the ATP-dependent phosphorylation of the conserved His residue. This autophosphorylation reaction is a bimolecular reaction in which one HK monomer phosphorylates a second monomer at the conserved His residue within the DHP-domain (Surette *et al.,* 1996). The characteristic feature of the HK family is the kinase core which is defined by five signature sequences containing conserved amino acids (H-, N-, G1-, F- and G3 boxes). The N, G1, F and G2 boxes form the ATP binding pocket, and the H box contains the conserved His residue that is the site of phosphorylation (Parkinson & Kofoid, 1992) (Fig. 4A and C). According to their domain organisation HKs can be grouped into two major classes (Bilwes *et al.,* 1999). In Class I HKs, the conserved His is located within the dimerization (DHP-) domain, contiguous to the nucleotide binding domain. In contrast, in Class II HKs, the H-box containing region is distant from the CA domain, separated by specific domain insertions (Dutta *et al.,* 1999).

The HK family is extremely diverse ranging from orthodox HKs with only one DHP-domain, e.g. LqsS in *L. pneumophila*, to hybrid kinases with multiple phospho-acceptor and phospho-

donor sites, e.g. CqsS in *V. cholerae* (Tiaden & Hilbi, 2012a) (see below). The structural architecture of the HKs results form the combination of sensing, catalytic and auxiliary domains and reflects the adaption to specific requirements of the signalling system (Stock *et al.,* 2000).

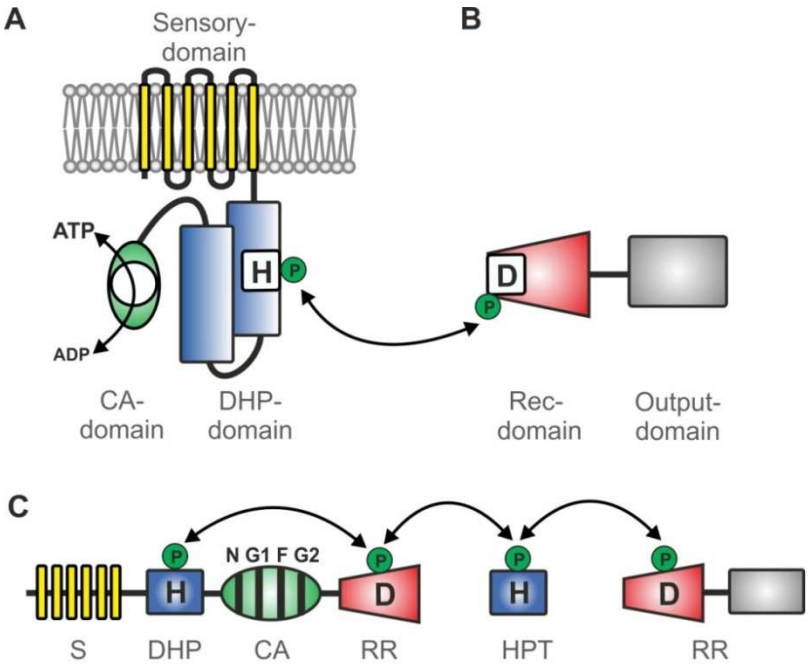

Fig. 4: Modular domains of a two-component signal transduction pathway. The basic two-component system requires a histidine kinase (HK) and a response regulator protein (RR) to generate phosphotransfer pathway (**A, B**). **A.** HK comprise three conserved components: the transmembrane sensory domain (yellow), the dimerization and histidine phosphotransfer (DHP-) domain (blue) and the catalytic ATP binding (CA-) domain (green). **B.** RR contains a receiver (Rec-) domain (red) and an output domain (grey). The phosphotransfer occurs between phospho-histidine (H) of the HK and the phospho-aspartate (**D**) in the downstream RR. **C.** To produce a multicomponent phosphorelay the basic elements can be combined in many different ways. This exemplary pathway uses a Class I hybrid sensor kinase containing both a HK and a regulatory part (receiver domain); additionally the phosphoryl group is transferred to a downstream phosphotransfer (HPT) protein, at last terminating on the RR.

1.1.2.2 Architecture of response regulators

The RR represents the second part of the TCS and is the final regulatory element of the pathway, functioning as a phosphorylation-activated switch. Most RRs show a two-domain architecture with a conserved N-terminal regulatory domain linked to a variable C-terminal effector domain (Stock *et al.,* 2000) (Fig. 4B). As the regulatory or receiver (Rec-) domain is quite similar in all RRs, the chemotaxis RR protein CheY from *E. coli* is generally used as

representative model (Stock *et al.*, 1990). The Rec-domain is a conserved α/β helical domain consisting five five-stranded parallel β-sheets surrounded by five amphipathic helices. Several highly conserved residues in the receiver domain of RRs have important roles in signal transduction and catalysis of phosphotransfer and dephosphorylation (Gao *et al.*, 2007). The phosphorylation site contains a cluster of conserved acidic residues including the aspartic acid at the C-terminal end of β3. Two additional acidic residues in the β1-α1 loop-position bind a divalent metal ion, commonly Mg^{2+}, which is required to catalyse both phospho-transfer and -hydrolysis (Gao *et al.*, 2007).

RR are activated by catalysing the phosphotransfer form the His residue of the HK to the conserved Asp in the own regulatory domain. Thereby HKs are not the sole phospho-donors for RR, as high energy compounds like acetyl phosphate, carbamoyl phosphate, imidazole phosphate, and phosphoramidate can also fulfil that function (Lukat *et al.*, 1992). In addition to phosphotransfer activity, RRs regulate the lifetime of their activated state via dephosphorylation. The half life of RR ranges from seconds to hours, corresponding to the specific adaptive response (Lukat *et al.*, 1992, Stock *et al.*, 1991, Haldimann *et al.*, 1997).

Phosphorylation of the RR promotes a conformational change altering the molecular structure, and the response is achieved through a distinct set of inter- and intramolecular interactions of the output domain (Stock *et al.*, 2000). Thereby RRs exhibit a high structural and functional diversity optimized for various effector functions. The majority of RRs are transcription factors (~65 %), others have C-terminal domains with enzymatic activity (~11 %), and some completely lack the effector domain (~14 %). Only few show RNA (~1 %) or protein binding (~2 %) or exhibit other activities (~7 %) (Gao *et al.*, 2007).

It is a general assumption that regulatory domains of RR exist in equilibrium between two conformational states, the active and the inactive form, and that phosphorylation of the Rec-domain initiates a shift in equilibrium towards the active form (Stock *et al.*, 2000). In most cases, the phosphorylated regulatory domain plays an active role. Phosphorylation can promote either oligomerisation (Porter *et al.*, 1993) or dimerization (Toro-Roman *et al.*, 2005) or the combination of both processes (Anand *et al.*, 1998). In contrast, activation might also implicate a relief of inhibition, as some RRs can be activated by deleting the N-terminal regulatory domain (Huala *et al.*, 1992).

1.1.2.3 TCS specificity

Although most bacteria encode dozens of TCSs there is relatively little or no crosstalk, indicating that the individual signalling pathways are highly specific. In general there are

three key mechanisms defining the specificity of two-component pathways at the level of phosphotransfer: molecular recognition, phosphatase activity, and substrate competition (Podgornaia & Laub, 2013).

The molecular recognition is delineated by pairs of residues located in one part of the HK and/or RR. These residues are amino acids that co-evolve. These pairs are either located in the same protein, where they form intramolecular contacts which maintain the structural integrity of the protein conformation, or they are located in opposite proteins, and have coevolved to preserve the interaction of a related kinase and regulator pair (Podgornaia & Laub, 2013). As the correct RR interacts with its specific HK, the access of an incorrect substrate/RR is prevented and only the correct cognate RR is phosphorylated (Skerker *et al.*, 2008).

Secondly, phosphatase activity determines the level of phosphorylated RR and is correlated to the signal input. The level of phosphorylated RRs can potentially be controlled either through formation or hydrolysis of the phosphoryl residue (Stock *et al.*, 2000). One possibility is the direct coupling between the HK stimulus and the RR response, such that the growing signal input increases the ratio of autophosphorylation of the HKs and therefore provides a greater number of phosphoryl groups being transferred to the RR. Alternatively, HKs can posses a phosphatase activity, leading to HK-mediated RR dephosphorylation, or RRs can also exhibit intrinsic autophosphatase activity. The phosphatase activity of the HK minimizes undesired cross-talk by dephosphorylating its specific RR when it is accidently phosphorylated by another kinase or a small molecule phospho-donor (background phosphorylation) (Podgornaia & Laub, 2013).

Finally, TCS specificity is enhanced by the competition between response regulators for phosphorylated kinases. Generally, the relative cellular concentration of the RR exceeds that of the associated HK, enhancing the probability of the RR for binding to a cognate kinase by effectively outcompeting nonrelated regulators (Groban *et al.*, 2009).

1.2 The pathogen *Legionella pneumophila*

Legionella spp. can cause in humans mainly two types of “Legionellosis”: Legionnaires’ disease and Pontiac Fever. Legionnaires’ disease is a severe pneumopathy, whereas Pontiac fever is a milder form where patients suffer from flu-like symptoms, but no pneumonia (Kaufmann *et al.*, 1981). Legionnaires’ disease was named after an epidemic outbreak of pneumonia at the American Legion convention in Philadelphia in 1976 (Fraser *et al.*, 1977,

McDade *et al.*, 1977). The infection occurs aerogen both through inhalation of bacteria-harbouring aerosols and by micro aspiration of contaminated water. Cause of infection are water sources such as hot water storage tanks, showers, whirlpools, air-conditioning units, plumbing systems and cooling towers (Stout & Yu, 1997) (Fig. 5). People at risk of this opportunistic pathogen are mainly immuno-suppressed individuals, such as elderly, smokers as well as inpatient and ambulatory patients in hospitals (Carratala & Garcia-Vidal, 2010, Hilbi *et al.*, 2010). However, transmission by interpersonal contact has never been reported (Fraser *et al.*, 1977, Steinert *et al.*, 2002, Molofsky & Swanson, 2004). The most suitable clinical tests for *Legionella* are urine antigen tests, cultures of sputum, serology or identification by PCR (Phin *et al.*, 2014). Treatment of Legionellosis involves an antibiotic therapy with macrolides or fluoroquinolones (Hilbi *et al.*, 2010). Nevertheless the average case fatality rate is over 5 % in Europe and in the USA (WHO, 1997).

The clinically most relevant species of this pathogen are *Legionella pneumophila* and *Legionella longbeachae*. Both cause the same diseases but are adapted to different environmental habitats (Hilbi *et al.*, 2011a). In contrast to *L. pneumophila, L. longbeachae* colonizes not only aquatic habitats, but is mostly associated with soil and plants. Therefore, Legionnaires' disease due to *L. longbeachae* is thought to have a different route of transmission, which is not fully identified yet. All confirmed cases were associated with potting soil mixes and composts and not with contaminated water systems (Steele *et al.*, 1990, Lindsay *et al.*, 2012). In Europe, *L. pneumophila* is responsible for 90 % or more of the Legionnaires' disease cases and the most common causative agent for the remainder is *L. longbeachae*. In Australia and New Zealand, reported cases of *L. longbeachae* infection occur as often as cases of *L. pneumophila* infection (Amodeo *et al.*, 2010).

To date there are over 50 described species of *Legionellae* representing 73 serogroups in the family *Legionellaceae.* The members of the genus *Legionella* continue to increase (Fields *et al.*, 2002) *L. pneumophila* is the best studied species and is described as Gram-negative, mono-flagellated, obligate aerobic bacterium with a phenotypic appearance, which can range between rod-shaped and filamentous up to 20 µm long, depending on the cultivation medium and growth phase (Piao *et al.*, 2006, Benson & Fields, 1998).

1.2.1 Life cycle

L. pneumophila is a ubiquitous waterborne parasite, colonizing a wide range of natural or man-made water sources all around the world (Rowbotham, 1980). The bacteria can both survive and replicate within intracellular and extracellular niches and have adopted a biphasic

life cycle consisting of a replicative and a transmissive (virulent) phase (Molofsky & Swanson, 2004) (Fig. 5).

Like most bacteria, *L. pneumophila* colonizes in the environment complex aquatic biofilms of prokaryotic and eukaryotic organisms, including *Pseudomonas aeruginosa* or *Klebsiella pneumoniae* (Hilbi *et al.*, 2011a, Mampel *et al.*, 2006) (Fig. 5). The bacteria naturally replicate in free-living protozoa, including amoebae (*Dictyostelium discoideum, Acanthamoeba, Hartmanella, Naegleria* or *Vahlkampfia* spp.), ciliates (*Tetrahymena* spp.) or nematodes (*Caenorhabditis elegans*) (Solomon *et al.*, 2000, Steinert & Heuner, 2005, Fields, 1996, Brassinga *et al.*, 2010) (Fig. 5). Several of these species were adapted as laboratory model systems for intracellular replication, including *Acanthamoeba castellanii, Hartmanella vermiformis* or *Dictyostelium discoideum* (Fields, 1996, Hägele *et al.*, 1998, Solomon & Isberg, 2000). Of note, growth of *Legionella* spp. in the absence of hosts has been reported only in laboratory media (Fields, 1996, Hagele *et al.*, 2000).

Signals triggering the switch from the replicative to the transmissive life cycle phase are limited nutrients (amino acids) and the simultaneous increase of the intracellular alarmone guanosine 3´,5´-bispyrophosphate (ppGpp) (Hammer & Swanson, 1999, Molofsky & Swanson, 2004). ppGpp levels control a complex regulatory network for transmissive traits comprising the sigma factors RpoS (δ^{38}), RpoN (δ^{54}), FliA (δ^{28}) and the LetA/S two-component system (Bachman & Swanson, 2001, Hales & Shuman, 1999, Heuner *et al.*, 1997, Hammer & Swanson, 1999).

In the transmissive growth phase *L. pneumophila* represses multiplication and instead starts to express virulence traits such as the flagellar apparatus, the type IV pilus machinery, Icm/Dot-dependent and -independent virulence factors and regulatory proteins like the QS response regulator LqsR (Tiaden *et al.*, 2007, Molofsky & Swanson, 2004) (Section 1.3.2).

When nutrient supply is exhausted, *L. pneumophila* exits the cell and searches for new hosts. Therefore, at the end of the replication period, the pathogen not only increases its resistance to extracellular stress but also exhibits pore-forming activity to escape the host (Alli *et al.*, 2000, Hales & Shuman, 1999, Molofsky & Swanson, 2003).

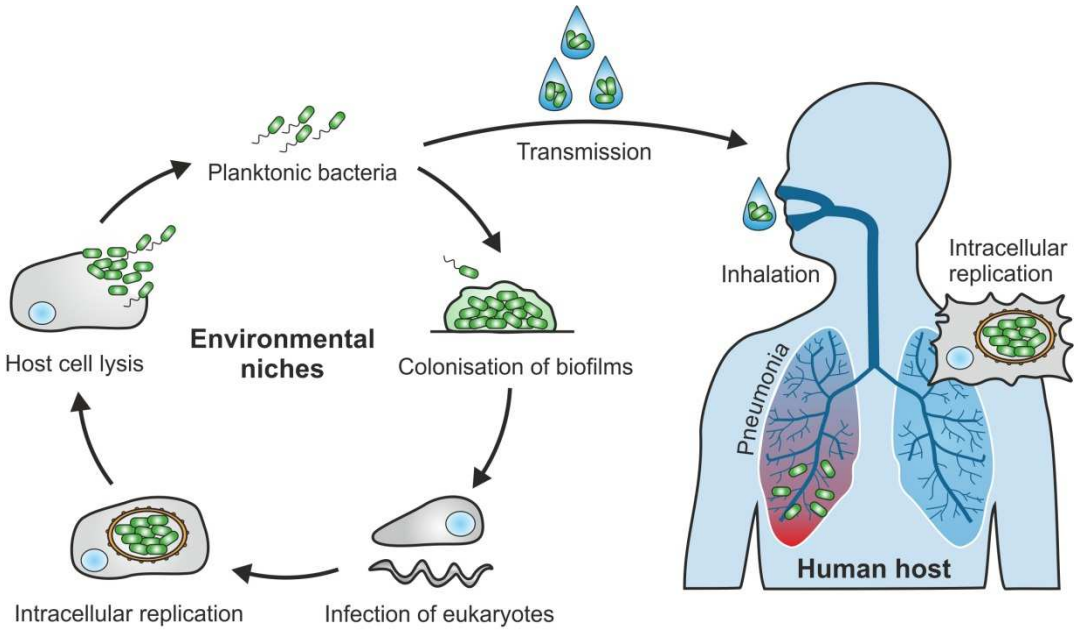

Fig. 5: Schematic life cycle of *L. pneumophila*. The life cycle of *L. pneumophila* consists of a replicative and a transmissive phase. In environmental niches *L. pneumophila* infects and replicates within amoebae and other hosts (ciliates, nematodes), colonizes surfaces and grows in biofilms, and persist in nematodes. Upon reaching the stationary growth phase, *L. pneumophila* is released from its environmental niches and, via inhalation of contaminated aerosols, infects human lung macrophages. Macrophage-resistance is a prerequisite to cause Legionnaires' disease in humans. Model adapted from previous publication (Hilbi *et al.*, 2011a).

The virulence and transmissive traits enable the pathogen to evade its protozoan host, survive as a planktonic cell and re-establish a replicative niche within a new host, where the cycle repeats. In humans *L. pneumophila* can infect and replicate within alveolar macrophages and cause a severe pneumonia. As *L. pneumophila* cannot be transmitted via interpersonal contact (e.g. droplet infection), as known for other pulmonary disease pathogens, such as *Streptococcus pneumonia* or *Haemophilus influenzae*, humans represent a dead end in the life cycle of this bacterium (Jin *et al.*, 2007, Hilbi *et al.*, 2011b, Tikhomirova & Kidd, 2013).

1.2.2 Intracellular replication

L. pneumophila is a facultative intracellular pathogen and therefore, gains competitive advantage in comparison to other microorganisms that can replicate only either inside or outside of host cells. Intracellular replication provides two main advantages: the evasion of environmental predators and initialisation of a niche to evade humoral and cellular immune response of the infected host (Isberg *et al.*, 2009). The pathogen´s life cycle in amoebae and

macrophages is remarkably similar, enforcing the assumption that the virulence strategy of *L. pneumophila* was evolutionary selected in aquatic habitats, i.e. the adaptation of the bacteria to protozoa is a precondition for their adaptation to macrophages (Fields *et al.*, 2002, Molofsky & Swanson, 2004). In order to ensure intracellular replication in host cells *L. pneumophila* utilises diverse mechanisms and translocated factors. Attachment and uptake are the first steps of infection (Fig. 6), during which *L. pneumophila* expresses several adhesins to provide specific binding to host cells, such as RtxA, PilEL, EnhC and Hsp60 which mediate attachment to *A. castellanii* (Garduno *et al.*, 1998). Furthermore, *L. pneumophila* expresses major outer membrane proteins (MOMPs) ensuring the binding to macrophages via host cell complement receptors (Bellinger-Kawahara & Horwitz, 1990). After host cell attachment the bacteria are either taken up via macropinocytosis or by phagocytosis (Hilbi *et al.*, 2001). In both cases the uptake is depending on both actin and actin-binding coronin (Lu & Clarke, 2005).

Shortly after uptake, the bacteria evade lysosome fusion and remodel the phagosome into a replication-permissive vacuole, the *Legionella*-containing vacuole (LCV). To this end the LCV recruits a series of components of host cell organelles to the vacuolar membrane, including early and late endosomes, mitochondria, followed by ribosome-covered membranes derived from the endoplasmic reticulum (ER) (Isberg *et al.*, 2009) (Fig. 6). In order to generate an intracellular replication-permissive niche, *L. pneumophila* manipulates the host cell and establishes a stable LCV by translocating about 300 different effector proteins via the Icm/Dot type IV secretion system (T4SS) into the host cell (Hubber & Roy, 2010, Zhu *et al.*, 2011). These effectors manipulate many host pathways and inhibit the phagosome maturation, vacuole acidification and bacterial degradation (Hubber & Roy, 2010). Some of these effector proteins share sequence homologies with eukaryotic proteins or harbour functional eukaryotic domains, which were likely acquired by trans-kingdom horizontal gene transfer (Hilbi *et al.*, 2011a, Molofsky & Swanson, 2004). Within the LCV, *L. pneumophila* starts to multiply by switching to the replicative phase of its life-cycle (Section 1.2.1), during which genes involved in amino acid biosynthetic pathways as well as amino acid and iron uptake transport systems are upregulated (Faucher *et al.*, 2011). Furthermore, genes involved in glycerol catabolism are also upregulated, suggesting that glycerol is used as a carbon source for intracellular growth. As intracellular replication is coming to an end, *L. pneumophila* shifts into the infectious, transmissive phase of the life cycle, which is characterised by the upregulation of invasion and virulence genes, such as the flagellar machinery and substrates of the Icm/Dot T4SS (Hoffmann *et al.*, 2014). For

termination of the growth cycle, *L. pneumophila* has to exit the host cell and search for new prey. To achieve this, several processes have been described, such as lysing host cells by secreted proteins, induction of apoptosis or also non-lytic release (Müller *et al.*, 1996, Molmeret *et al.*, 2007, Chen *et al.*, 2004). At the end of this process the pathogen can begin a new infection cycle (Molofsky & Swanson, 2004).

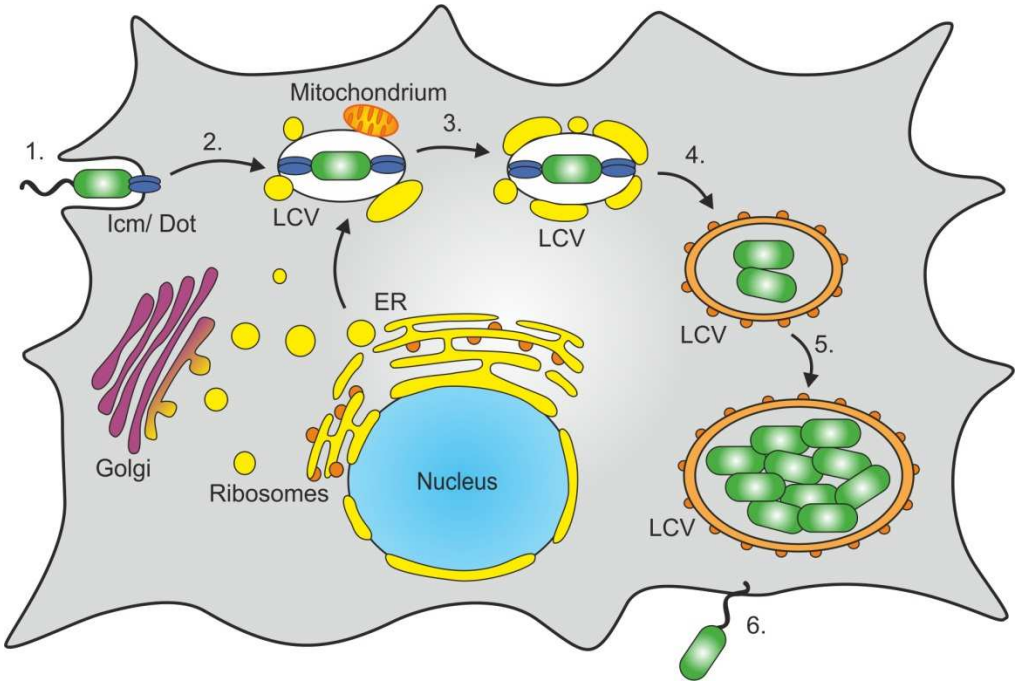

Fig. 6: Formation of the intracellular replication vacuole of *L. pneumophila*. The infection cycle can be devided into six main stages. 1. Pathogen uptake through the Icm/Dot T4SS, 2. Vesicles derived from the endoplasmatic reticulum (ER) and mitochondria appear close to the LCV, 3. Vesicles that surround the LCV attach and expand onto the surface, 4. Membranes that surround the LCV become similar to rough ER in appearance and get coated with ribosomes, 5. Intracellular replication within the LCV, 6. Bacterial exit. Model adapted from previous publication (Isberg *et al.*, 2009).

1.3 Quorum sensing system of *L. pneumophila* and *V. cholerae*

1.3.1 The *L. pneumophila lqs* gene cluster

At present, cell-cell communication via quorum sensing is seen as the most important signal transduction system in bacteria (Bassler & Losick, 2006). While there is a huge variety of different chemical signals, only three classes are present in Gram-negative bacteria: acylated homoserine lactones, α-hydroxyketones and furanosyl borate diester (Ng & Bassler, 2009, Miller & Bassler, 2001). An α-hydroxyketone (AHK) signalling circuit is both utilized by

L. pneumophila and *Vibrio cholerae* for their communication. *V. cholerae* is, like *L. pneumophila,* an opportunistic pathogen and is responsible for outbreaks of the severe disease, cholera (Nelson *et al.*, 2009). Bioinformatic analysis of the genomes revealed that *L. pneumophila* and *V. cholerae* possess homologous QS gene clusters, known as *lqs* (Tiaden *et al.*, 2007) and *cqs* (Miller *et al.*, 2002). *Legionella* quorum sensing (*lqs*) and cholerae quorum sensing (*cqs*) genes encode cognate pairs of an AI synthase and a sensor histidine kinase (LqsA/LqsS and CqsA/CqsS), which produce and sense the corresponding AHK molecules: *Legionella* autoinducer-1 (LAI-1: 3-hydroxypentadecane-4-one) and *Cholerae* autoinducer-1 (CAI-1: 3-hydroxytridecane-4-one) (Spirig *et al.*, 2008a, Higgins *et al.*, 2007) (Fig. 7). Additionally, the *lqs* gene cluster encodes the putative response regulator LqsR and a homologue of the *Escherichia coli* HdeD (HNS-dependent expression D), the function of which is still unknown (Tiaden *et al.*, 2007, Tiaden *et al.*, 2010c). Furthermore, *L. pneumophila* produces an additional orphan sensor kinase, termed LqsT, which is not encoded in the *lqs* cluster but is located near the effector genes *sdeD, sdcA* and *sidC* (Kessler *et al.*, 2013). This HK bears 31 % similarity to LqsS (Kessler *et al.*, 2013).

The two AI synthases LqsA and CqsA share 45 % identity and are functional pyridoxal-5'-phosphate (PLP)-dependent aminotransferase enzymes (Spirig *et al.*, 2008b, Kelly *et al.*, 2009). Accordingly, both AI synthases contain conserved lysine residues ($CqsA_{K236}$ and $LqsA_{K258}$) that covalently bind PLP.

The biosynthesis of the AI molecules LAI-1 and CAI-1 can be divided into two reaction steps an AI-synthase dependent reaction followed by the AI-synthase independent reaction. For the biosynthesis of *V. cholerae* CAI-1, (S)-adenosylmethionine (SAM) or (S)-3-aminobutyrate (SAB) is used together with decanoyl-CoA or octanoyl-CoA as substrate. Presumably, SAM (or SAB) is coupled with dodecanoyl-CoA for the synthesis of LAI-1 in *L. pneumophila*. These aminotransferase reactions are catalysed by CqsA and, likely, LqsA. Through this process several partially unstable chemical intermediates are produced such as Am-CAI-1, Am-C8-CAI-1, Ea-CAI-1, Ea-C8-CAI-1 and, analogously, Am-LAI-1, Ea-LAI-1. These compounds are converted into CAI-1 and LAI-1 by spontaneous hydrolysis and dehydrogenase reactions, or by so far unknown mechanisms (Tiaden & Hilbi, 2012b, Kelly *et al.*, 2009).

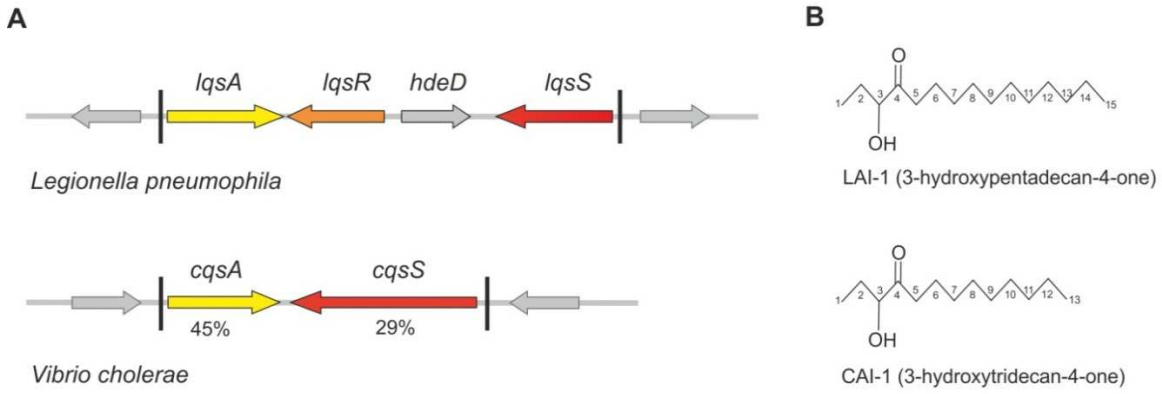

Fig. 7: Genetic organisation of *L. pneumophila* and *V. cholerae* QS gene cluster and the corresponding autoinducers. A. The *lqs* and *cqs* loci harbour the AI synthases (*lqsA, cqsA*), cognate sensor kinases (*lqsS, cqsS*) and the *Legionella* response regulator *lqsR*. **B.** AI molecules synthesized by LqsA and CqsA are LAI-1 and CAI-1. The percent identity with the corresponding *L. pneumophila* proteins are indicated. Model adapted from previous publication (Tiaden & Hilbi, 2012b).

The potential LAI-1 sensor LqsS and the CAI-1 receptor CqsS and belong to the class of six-transmembrane-helix sensor histidine kinases and share 29 % identity (Fig. 7). LqsS and CqsS are located in the inner bacterial membrane and couple the detection of the AI molecules via an N-terminal receptor domain to the C-terminal signal transduction part of the protein (Tiaden *et al.*, 2010a). As CqsS has an additional receiver domain with a conserved aspartate residue, it belongs to the class of hybrid sensor kinases. For signal recognition by CqsS the first three transmembrane helices were found to harbour motifs that are essential for ligand binding and signal transduction (Ng *et al.*, 2011, Ng *et al.*, 2010). These motifs are also conserved in LqsS, supporting the notion that *V. cholerae* CqsS and *L. pneumophila* LqsS share a common ligand-binding domain (Tiaden & Hilbi, 2012b).

In both QS systems the HKs are expected to catalyse the autophosphorylation by ATP at the conserved histidine residues ($LqsS_{H200}$, $CqsS_{H194}$) (Wei *et al.*, 2012, Tiaden & Hilbi, 2012a) (Fig. 8). In the *L. pneumophila* system the phosphoryl group is subsequently likely transferred directly from the sensor kinase to D108 of the response regulator LqsR. By contrast, in *V. cholerae* the phosphoryl group is shuttled from H194 to D618, which lies within the hybrid sensor kinase, and during the following phosphorelay the phosphate is transported via the orphan phosphotransfer protein LuxU (H58) to the receiver domain of the response regulator LuxO (D47) (Freeman & Bassler, 1999a, Freeman & Bassler, 1999b).

Besides CAI-1, *V. cholerae* senses a second chemical signal, known as AI-2 (autoinducer 2). AI-2 is a furanosyl borate diester and is produced and recognized through the LuxS/LuxPQ

system. In *V. cholerae* both QS systems operate in parallel by integrating the AI signals in a joint phosphorylation cascade (Miller *et al.*, 2002) Nevertheless, the CqsS system represents the major QS system in *V. cholerae* (Wei *et al.*, 2012).

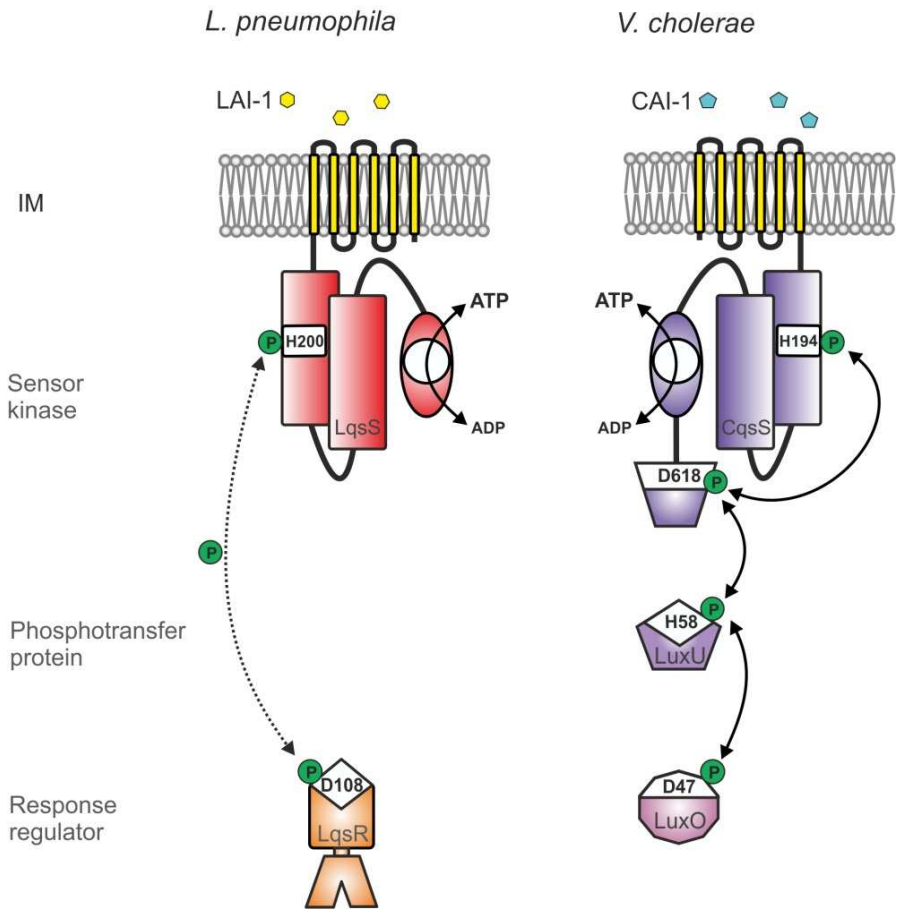

Fig. 8: Signal transduction cascade of *L. pneumophila* LqsS and *V. cholerae* CqsS sensor kinases. LqsS (red) is an orthodox HK, while CqsS (purple) is a hybrid histidine kinase coupled to a phosphorelay system. The autoinducer signal is presumably detected in both sensor kinases by six trans-membrane helices (yellow) representing the sensory domain. The HK catalyse the autophosphorylation of the conserved histidine residues (LqsS: H200, CqsS: H194) by ATP. In the *L. pneumophila* system the phosphoryl group is subsequently likely transferred from the sensor kinase directly to D108 of the response regulator LqsR. By contrast, in *V. cholerae* the phosphoryl group is shuttled from H194 to D618 which lies within the hybrid sensor kinase. During the following phosphorelay the phosphate is transported via the orphan phosphotransfer protein LuxU (H58) to the receiver domain of the response regulator LuxO (D47). LqsS and CqsS are presumably bifunctional kinases/phosphatases (IM = inner membrane).

1.3.2 The *lqs* signalling circuit

In *L. pneumophila* the *lqs* signalling circuit is linked to the stationary growth phase regulatory network. Stationary phase in *L. pneumophila* is induced by a response to the

production of the second messenger ppGpp by the synthases RelA and SpoT. In concert with ppGpp, the alternative sigma factors RpoS (δ^{38}), RpoN (δ^{54}) and FliA (δ^{28}) as well as the LetAS TCS control bacterial transmission (Bachman & Swanson, 2001, Hales & Shuman, 1999, Heuner *et al.*, 1997, Hammer & Swanson, 1999).

The RR LqsR plays a decisive role in the *L. pneumophila* QS system, as it is a prototypic member of a novel family of RRs and (i) controls the transition form the replicative to the transmissive phase of the life cycle, (ii) promotes interaction between the bacterium and phagocytes and (iii) regulates the expression of transmissive/virulence traits (Tiaden *et al.*, 2007, Kessler *et al.*, 2013). Production of LqsR is dependent on the alternative sigma factor RpoS as well as the response regulator LetA and is further regulated at a post-transcriptional level by the sRNAs *rsmYZ* and the small RNA-binding protein CsrA (Sahr *et al.*, 2009, Tiaden *et al.*, 2007) (Fig. 9).

The TCS LetA/LetS, which is homologous to the *V. cholerae* VarA/VarS system, is required for induction of the transmissive phase of the *L. pneumophila* life cycle. LetA directly upregulates the expression of the small non-coding sRNAs (*rsmY* and *rsmZ*) which bind and inhibit the global regulatory protein CsrA, thus relieving the repression of transmissive traits (Molofsky & Swanson, 2003, Rasis & Segal, 2009). CsrA is a conserved small RNA-binding protein, an essential activator of intracellular replication and a global repressor of transmission traits. Furthermore, LetA and RpoS regulate the expression of the RNA-binding protein Hfq, so that the amount of *csrA* mRNA increases and CsrA can promote entry into the replicative growth phase (McNealy *et al.*, 2005).

1.3.3 The *cqs* signalling pathway

In *V. cholerae* the *cqs* system regulates gene expression in a density dependent manner. At low cell density, when the concentration of CAI-1 is below the threshold level, CqsS acts as a kinase and autophosphorylation occurs. The phosphoryl group is subsequently transferred to the phosphotransfer protein LuxU. Phosphorylated LuxU in turn, transfers the phosphoryl group to the response regulator LuxO which induces the expression of small quorum regulatory RNAs (*qrr1-qrr4*) (Lenz *et al.*, 2004, Freeman & Bassler, 1999a, Freeman & Bassler, 1999b) (Fig. 8 and 9). The expression of the sRNAs further requires the small nucleoid protein Fis, which directly binds to the promoters of the sRNAs (Lenz & Bassler, 2007). Mediated by the RNA chaperone Hfq, the *qrr1-qrr4* sRNAs destabilize the *hapR* mRNA and thus prevent production of the QS master regulator. HapR is inactive at low cell densities and active at high cell densities (Ng & Bassler, 2009), and can act both as

transcriptional repressor (e.g. virulence and biofilm formation) and activator (e.g. protease and competence). At high cell density, the HK CqsS binds CAI-1 and switches from a kinase to a phosphatase (Wei *et al.,* 2012). This event reverses the phosphate flow through the circuit which dephosphorylates LuxU and LuxO. Transcription of the *qrr1-qrr4* sRNAs is not induced and so HapR is produced. Also the TCS system VarAS provides further regulatory input to QS-dependent gene regulation. The response regulator VarA and the sensor kinase VarS are homologous to *L. pneumophila* LetAS and promote the expression of three sRNAs (*csrBCD*) (Tiaden *et al.*, 2010a). These sRNAs bind to and inhibit the activity of the global regulatory protein CsrA which in turn regulates the expression of the *qrr* sRNAs and of the master regulator *hapR* (Ng & Bassler, 2009).

1.3.4 AHK regulated processes

For both *L. pneumophila* and *V. cholerae,* survival and interaction in natural habitats- through the ability to communicate both within and between species- is a critical feature. The *lqs* system regulates many essential processes in *L. pneumophila* including pathogen-host cell interactions, production of virulence factors (Tiaden *et al.,* 2008, Tiaden *et al.,* 2007), formation of extracellular filaments (Tiaden *et al.,* 2010c), expression of a genomic fitness island as well as natural competence (Kessler *et al.,* 2013).

Analysis of *L. pneumophila* mutants demonstrated that strains lacking *lqsT* or both sensor kinases (*lqsS* and *lqsT*) show increased salt resistance, greatly enhanced natural competence for DNA acquisition and impaired uptake by phagocytes compared to wild-type bacteria (Kessler *et al.,* 2013). LqsS and LqsT, are differentially expressed in the post-expontential growth phase, and transcriptome studies revealed that the corresponding mutant strains show reciprocal gene regulation for genes encoding translocated effector proteins and a genomic fitness island (Kessler *et al.,* 2013). The genomic fitness island is a 133 kb region in the *L. pneumophila* genome, flanked by putative DNA-mobilizing genes and encoding multiple metal ion efflux pumps (Tiaden *et al.*, 2010b).

Furthermore, strains lacking *lqsS* (Tiaden *et al.,* 2010a), *lqsT* (Kessler *et al.,* 2013), *lqsR* (Tiaden *et al.,* 2007), or the entire *lqs* cluster (*lqsA–lqsR–hdeD–lqsS*) (Tiaden *et al.,* 2008) are severely impaired for host cell uptake and intracellular replication. The Δ*lqsS* and Δ*lqsR* strains produce extracellular filaments, and thus, sediment more slowly than wild-type bacteria (Tiaden *et al.,* 2010a). Finally, *L. pneumophila* lacking *lqsA* is only slightly impaired for pathogen-phagocyte interactions (Tiaden *et al.,* 2010a), but outcompeted by wild-type bacteria upon co-infection of *A. castellanii* (Kessler *et al.,* 2013).

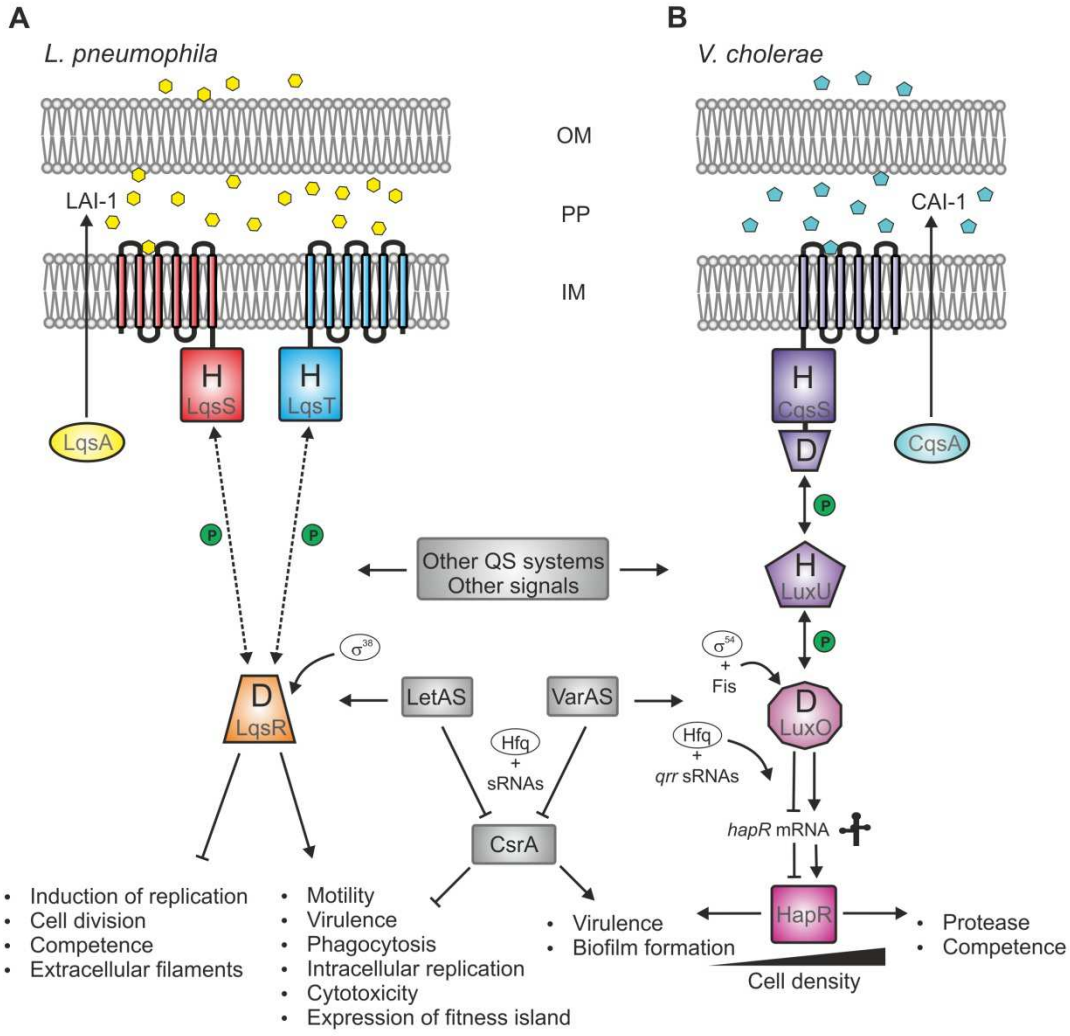

Fig. 9: The AHK signalling circuits of *L. pneumophila* and *V. cholerae*. A. In *L. pneumophila*, the LAI-1 signalling circuit is linked to the stationary growth phase regulatory network and consists of the autoinducer synthase LqsA (producing LAI-1), the sensor kinases LqsS and LqsT and the response regulator LqsR. The stationary sigma factor RpoS (δ^{38}) and the TCS LetAS system regulate the expression of transmission traits (motility, virulence). LetA directly upregulates the expression of the sRNAs, which, together with the RNA chaperone Hfq, titrate the global repressor of transmissive traits CsrA. **B.** In *V. cholerae*, CAI-1 signalling is linked to convergent density-dependent regulatory circuits and comprises the autoinducer synthase CqsA (producing CAI-1) and the sensor kinase CqsS. At low cell density, the response regulator LuxO is phosphorylated by the phosphotransferase LuxU. Together with the sigma factor RpoN (δ^{54}) and the small nucleoid protein Fis, phospho-LuxO induces the expression of the sRNAs *qrr1-qrr4*. The sRNAs, together with the RNA chaperone Hfq, destabilize the *hapR* mRNA and thereby prevent production of the master regulator HapR. At high bacterial density, LuxO is dephosphorylated and inactive, the *qrr1–qrr4* sRNAs are not induced, and HapR is produced. The two-component system VarAS is homologous to *L. pneumophila* LetAS and promotes the expression of the sRNAs *csrBCD*, which inhibit the activity of the global regulatory protein CsrA and regulate the expression of HapR. OM = outer membrane, PP = periplasm, IM = inner membrane. Model adapted from previous publication (Tiaden *et al.*, 2010a).

1.4 Aims of the thesis

The Lqs quorum sensing system of *L. pneumophila* regulates various traits including virulence, formation of extracellular filaments, natural competence for DNA acquisition and expression of a genomic fitness island. However at this stage the Lqs system has only been characterised by genetic and phenotypic means (Kessler *et al.*, 2013).

The aim of this thesis was the detailed biochemical characterisation of the *L. pneumophila* quorum sensing system by analysing the single components and their interactions *in vitro*. Via phosphorylation assays it was to be shown that LqsS and LqsT are membrane localized sensor kinases and have autophosphorylation activity at a conserved histidine residue (H200 or H204) located in their cytoplasmic histidine kinase domain. Additionally, LqsS and LqsT heterologously produced by *E. coli* localized to the membrane fraction as well. However, only LqsT was autophosphorylated by ATP at the conserved histidine, suggesting that LqsS requires an *L. pneumophila* co-factor for correct folding and/or phosphorylation.

The response regulator LqsR was so far also only analysed via genetic and phenotypic analysis (Kessler *et al.*, 2013), but the *in vitro* activity of this protein remained unknown. LqsR was to be heterologously produced and purified from *E.coli* in order to obtain biochemically active protein. Through various approaches the impact of LqsR on the signalling cascade was investigated. Direct interaction of the sensor kinases LqsS and LqsT with the response regulator LqsR was shown by immuno-precipitation. Moreover the influence of LqsR on the autophosphorylation of either/each sensor kinase was to be analysed. In addition, *in vitro* phosphorylation-mediated conformational change of LqsR was analysed. Finally, the influence of the putative kinase ligand on of the signal transduction cascade was to be validated.

2 MATERIALS AND METHODS

2.1 Materials and laboratory equipment

Tab. 1: Chemicals & consumables

Material	Manufacturer
ACES	AppliChem (Darmstadt)
Activated charcoal powder	Fluka (Buchs)
Agar	BD Biosciences (Franklin Lakes)
Agarose	Biozym (Hessisch Oldendorf)
Ampicillin	Roth (Karlsruhe)
Anti-His antibody	Qiagen (Hilden)
Anti-M45 antibody	Genovac AG (Freiburg)
Anti-LqsR antibody	Neosystem (Strasbourg)
[γ ^{32}P]-ATP	Perkin Elmer (Rodgau)
Bacto yeast extract	BD Biosciences (Franklin Lakes)
Bovine serum albumin (BSA)	AppliChem (Darmstadt)
Chloramphenicol	Sigma (Deisenhofen)
DNA standard (2-Log-DNA Ladder)	New England Biolabs (Frankfurt)
DNase	Roche (Basel)
DNeasy tissue kit	Qiagen (Hilden)
dNTPs	Invitrogen (Karlsruhe)
ECL detection kit	GE Healthcare (Chalfont St Giles)
FeN_3O_9 x 9 H_2O	Sigma (St. Louis)
Gene pulser cuvette	Bio-Rad (München)
Glutathione sepharose 4B	GE Healthcare (Chalfont St Giles)
Synthetic LAI-1	D. Trauner, C. Hedberg, J. Schulz
L-cysteine	Sigma (St. Louis)
Milk powder	Roth (Karlsruhe)
Ni^{2+}-NTA-agarose	Qiagen (Hilden)
Nonidet-P40 (99 %)	AppliChem (Darmstadt)
Nucleo spin-gel & PCR clean up	Macherey-Nagel (Düren)
Nucleo spin-plasmid	Macherey-Nagel (Düren)

Phusion DNA polymerase	Finnzymes (Espoot)
PMSF	Sigma-Aldrich (München)
Restriction enzymes	New England Biolabs (Frankfurt)
Page ruler prestained protein ladder	Thermo (Waltham)
T4 DNA ligase	New England Biolabs (Frankfurt)
TEMED	Biomol Feinchemikalien (Hamburg)

All materials that do not appear in the table above were obtained in purity grade *pro analysis* from the companies AppliChem (Darmstadt), Bayer (Leverkusen), Biomol (Hamburg), BioRad (München), Biozym Diagnostics GmbH (Hess. Oldendorf), Fluka (Neu-Ulm), Gibco/BRL (Eggenstein), ICN Biomedicals Inc. (Aurora, Ohio), E. Merck (Darmstadt), Roche Diagnostics (Mannheim), Roth (Karlsruhe) or Serva (Heidelberg), respectively.

Tab. 2: Laboratory equipment

Laboratory equipment	**Manufacturer**
ÄKTA explorer	GE Healthcare (Frankfurt a. Main)
Autoclave Varioklav classic	H&P (Oberschleißheim)
Benchtop centrifuge 5417R	Eppendorf (Hamburg)
Electrophoresis chamber Mini-Protean 3	Bio-Rad (München)
Electrophoresis chamber Mini-Subcell GT	Bio-Rad (München)
Electrophoresis chamber Subcell GT	Bio-Rad (München)
Electroporation device GenePulser XCell	Bio-Rad (München)
French press SIM AMINCO	Spectronic (New York)
Gel imaging system ChemiDoc MP System	Bio-Rad (München)
Gel imaging system GelDoc EQ	Bio-Rad (München)
Hot plate magnetic stirrer RCT basic	IKA (Staufen)
Ice maker AF30	Scotsman (Vernon Hills)
Incubation cabinet Certomat BS-1	Sartorius (Goettingen)
Incubation cabinet Oribital shaker	Thermo (Waltham)
Incubator Heraeus BR6000	Thermo (Waltham)
Incubator Heraeus Function Line	Thermo (Waltham)
Medical Film processor FPM-100A Fuiji-Film	EU (Düsseldorf)
Mixer Vortex-Genie 2	IKA (Staufen)
pH-meter Level 1	inoLab (Weilheim)

Power supply PAC100	Bio-Rad (München)
Precision balance BP61-S	Sartorius (Goettingen)
Precision balance PG2002-S	Mettler-Toledo (Greifensee)
Protein transfer device MAXI-Semi-Dry-Blotter	Roth (Karlsruhe)
Rocking platform shaker Mini MR-1	Biosan (Riga)
Spectrophotometer Helios Epsilon	Thermo (Waltham)
Spectrophotometer NanoDrop ND-1000	PeqLab (Erlangen)
T3 Thermocycler	Biometra (Göttingen)
Ultra centrifuge Optima TL	Beckman Coulter (Krefeld)
UV Transilluminator	Bio-Rad (München)

2.2 Strains, plasmids and oligonucleotides

Tab. 3: Bacterial strains

Strain	Genotype	Reference
E. coli		
BL21(DE3)	*fhuA2 [lon] ompT gal (λ DE3) [dcm]⊿hsdS λ DE3 = λ sBamHIo ⊿EcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 ⊿nin5*	Novagen
TKR2000	*OkdpFABCDE thi rha lacZ nagA trkA405 trkD1 atp706*	(Studier *et al.*, 1990)
TOP10	*recA1 endA1 gyrA96 hsdR17 supE44 relA1 (lacproAB)/F' [traD36proAB*lacIq lacZ M15]*	Invitrogen
L. 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)
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., 2010c)
NT03 (Δ*lqsR*)	JR32 *lqsR*::Km	(Tiaden et al., 2010c)
NT05 (Δ*lqsS*)	JR32 *lqsS*::Km	(Tiaden et al., 2010c)

Tab. 4: Plasmids

Plasmid	Characterisation	Sequence
pCR-2	pGEX-4T-1-GST-*sidC*	(Weber *et al.*, 2006)
pCR-33	pMMB207C-RBS-M45	(Weber et al., 2006)
pKK-LuxN-6His	pKK223-3-*luxN*-His_6	(Timmen *et al.*, 2006)
pQE-LuxU-6His	pQE30-*luxU*-His_6	(Timmen et al., 2006)
pNT-35	pGEX-6P-1-GST-*lqsR*	(Schell *et al.*, 2014)
pRB-4	pET-28a(+)-His-$lqsR_{D108N}$	(Schell et al., 2014)
pUC19	*oriR* (pMB1), *lacI*, *lacZ´*, Amp^R	Lab collection
pTS-23	pET-28a(+)-His-*lqsR*	(Tiaden et al., 2007)
pUS-1	pMMB207-C-RBS-M45-*lqsS*	(Schell et al., 2014)
pUS-2	pMMB207-C-RBS-M45-*lqsT*	(Schell et al., 2014)
pUS-5	pMMB207-C-RBS-M45-$lqsS_{H200Q}$	(Schell et al., 2014)
pUS-6	pMMB207-C-RBS-M45-$lqsT_{H204Q}$	(Schell et al., 2014)

Tab. 5: Oligonucleotides

Oligonucleotide	Sequence 5' - 3'	Comments
oAT-LqsR-fo	ATATGGATCCATGCAACATTTCTCAATACC	5´ *lqsR* (fo), *Bam*HI
oAT-LqsR-re	ATATCTCGAGCCTTAATTCAGAAACATTTCC	3´ *lqsR* (re), *Xho*I
$oLqsR_{D108A}$-fo	CAGTAGTCGTTGTTGCGTATGCAATGCCGGGTATGGATGG	5' $LqsR_{D108A}$ (fo)
$oLqsR_{D108A}$-re	CCATCCATACCCGGCATTGCATACGCAACAACGACTACTG	3' $LqsR_{D108A}$ (re)
$oLqsR_{D108N}$-fo	CAGTAGTCGTTGTTAATTATGCAATGCCGGGTATGGATGGATTAGAG	5' $LqsR_{D108N}$ (fo)
$oLqsR_{D108N}$-re	CTCTAATCCATCCATACCCGGCATTGCATAATTAACAACGACTACTG	3' $LqsR_{D108N}$ (re)
$oLqsS_{H200Q}$-fo	ATGATTGCCCAAGAATTGCGTTCACCATTG	5' $LqsS_{H200Q}$ (fo)
$oLqsS_{H200Q}$-re	ACGCAATTCTTGGGCAATCATGCCTGC	3' $LqsS_{H200Q}$ (re)
$oLqsT_{H204Q}$-fo	AGTATCGCTCAAGATTTAAGAACGCCG	5' $LqsT_{H204Q}$ (fo)
$oLqsT_{H204Q}$-re	TCTTAAATCTTGAGCGATACTTCCAGC	3' $LqsT_{H204Q}$ (re)
oUS-LqsS-fo	TAAGGAGGATCCATGTCACAACTAAAAAAAATAGTG	5´ *lqsS* (fo), *Bam*HI

oUS-LqsS-re	AGAAAAGTCGACTTAAACCGAGCCTGGAAAACTCAG	3' *lqsS* (re), *Sal*I
oUS-LqsT-fo	AATTAAGGATCCATGCAAAGGTTAAAAAATATA	5' *lqsT* (fo), *Bam*HI
oUS-LqsT-re	GTTAAAGTCGACTTAATCAATTTTGGGGAATTT	3' *lqsT* (re), *Sal*I

2.3 Cultivation procedures

2.3.1 Cultivation of *Escherichia coli*

E. coli strains shown in Table 3 were cultivated aerobically at 37 °C in Luria-Bertani (LB)-medium (1 % NaCl (w/v); 1 % tryptone (w/v); 0.5 % (w/v) yeast extract) or KML-medium (1 % (w/v) tryptone; 1 % (w/v) KCl; 0.5 % (w/v) yeast extract). Agar plates were produced by addition of 1.5 % agar (w/v). Antibiotics were added in concentrations of 30 µg/ml (ampicillin, chloramphenicol, kanamycin). Bacterial growth was controlled by measuring the optical density in the spectrophotometer (Thermo, Waltham) at 600 nm.

2.3.2 Cultivation of *Legionella pneumophila*

L. pneumophila strains shown in Table 3 were grown at 37 °C on CYE agar plates (1 % ACES (w/v), 1 % Bacto yeast extract (w/v), Activated charcoal puriss p.a.; powder 0.2 %, 1.5 % agar, 0.04 % L-cysteine (w/v), 0.025 % FeN_3O_9 x 9 H_2O) (Feeley *et al.*, 1979). ACES and the yeast extract were dissolved in H_2O and the pH adjusted to 6.9 with 10 M KOH. After addition of the activated charcoal, autoclaving and cooling to 50 °C the filter-sterilised cysteine- and iron-solutions were added. If necessary chloramphenicol was supplemented to a final concentration of 5 µg/ml. After 3 days of cultivation on CYE plates, bacteria were transferred in 3 ml AYE liquid medium (1 % ACES (w/v), 1 % Bacto yeast extract (w/v), 0.04 % L-cysteine(w/v), 0.025 % FeN_3O_9 x 9 H_2O) (Horwitz, 1983) with a starting OD_{600} of 0.1 and grown aerobically at 37 °C as long as necessary.

2.3.3 Permanent storage

Freshly cultivated cultures with an OD of 2.5 were mixed 1:1 with sterile glycerol (v/w 50 %) and snap frozen in liquid nitrogen. Strains were stored as stocks at -80 °C.

2.4 Molecular biology and genetic methods

2.4.1 Isolation of genomic and plasmid DNA

Genomic and plasmid DNA were isolated from 3 ml overnight cultures according to the protocol of the manufacturer using kits from Qiagen (Hilden) or Macherey-Nagel (Düren), eluted in 30-50 µl elution buffer or nuclease free water and stored at -20 °C. DNA concentrations were determined by Nano Drop 1000 Spectrophotometer (Peqlab, Erlangen).

2.4.2 Modification of DNA

DNA manipulations were performed according to standard protocols (Sambrook *et al.*, 1989). *In vitro* modification of DNA, such as restriction and ligation were performed according to requirements of manufacturers. To inhibit religation, linearised vectors were treated with alkaline phosphatase (CIP) (NEB, Frankfurt).

2.4.3 Polymerase chain reaction (PCR)

DNA fragments were amplified *in vitro* by PCR (Mullis & Faloona, 1987). Reactions were carried out in a T3 Thermocycler (Biometra, Göttingen) using Phusion DNA polymerase (Finnzymes, Espoot) and the listed oligonucleotides (Table 5).

The PCR protocol consisted of an initial denaturation step at 94 °C for 4 min, followed by 30 cycles of annealing at 55 °C for 30 sec, elongation at 72 °C for 90 sec and a denaturation step at 94 °C for 1 min. The reaction was terminated by continuous cooling at 4 °C.

2.4.4 Electrophoretic analysis of DNA fragments

Analytical and preparative analysis of DNA fragments was obtained by agarose gel electrophoresis. Agarose gels were prepared containing 0.8 % (w/v) agarose solved in TAE-Buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA) and 0.2 µg/ml ethidium bromide.

DNA samples were mixed with 10x sample-buffer (50 % (v/v) glycerol, 0.1 M EDTA, 1 % (w/v) SDS, 0.1 % (w/v) Bromphenol blue). Gels were run at 100 V for 30 min in an Electrophoresis chamber Mini-Subcell GT (Bio-Rad, München). DNA fragments were detected on UV-Transilluminator (Bio-Rad, München) at 304 nm and sizes were determined using 2-Log DNA Ladder (NEB, Frankfurt).

DNA fragments were extracted via Nucleo Spin-Gel & PCR clean up Kit (Macherey-Nagel, Düren) from agarose gels and the concentration was determined by Nano Drop-Spectrophotometer (Peqlab, Erlangen).

2.4.5 DNA sequence analysis

Sequencing of double stranded DNA was performed according to chain-terminating method of Sanger *et al*., 1977 by GATC-Biotech (Konstanz). Sequences were analysed with CLC-DNA Worchbench 6 (Qiagen, Hilden).

2.4.6 Construction of plasmids

DNA manipulations were performed according to standard protocols, and plasmids were isolated using commercially available kits (Macherey-Nagel). The genes encoding *lqsS* and *lqsT* were amplified from *L. pneumophila* genomic DNA using the oligonucleotides listed in Table 5, restriction digested with *BamHI/SalI* and cloned into the vector pCR-33 (pMMB207C-RBS-M45), yielding pUS-1 and pUS-2. The expression vectors encoding $LqsS_{H200Q}$ (pUS-5) or $LqsT_{H204Q}$ (pUS-6) were constructed from pUS-1 and pUS-2 using the Quickchange site-directed mutagenesis kit according to the manufacturer`s recommendation (Stratagene) and the oligos listed in Table 5. The vector encoding GST-LqsR (pNT-35) was constructed by PCR using the oligos oAT-LqsR-fo/-re (Table 5) and *L. pneumophila* genomic DNA as a template, restriction digested with *BamHI/XhoI* and cloned into pGEX-6P-1. The expression vectors encoding His-$LqsR_{D108A}$ (pRB-3) or His-$LqsR_{D108N}$ (pRB-4) were constructed using the Quick change kit and the oligos listed in Table 5. To this end, *lqsR* was released from pTS-23 (His-LqsR) by digestion with BamHI/NdeI, cloned into pUC19 (used as a template 1 for site directed mutagenesis), and finally cloned back into pET-28a(+). All PCR-generated DNA fragments were verified by sequencing.

2.4.7 Competent cells and transformation

2.4.7.1 Chemically competent cells and transformation

Transformation of *E. coli* cells with plasmid DNA was performed with a modified RbCl-method of Promega. 1 ml of an *E. coli* overnight culture (TOP10, BL21) was added to 100 ml LB medium. At an OD_{600} of 0.5 the bacteria were centrifuged at 4 °C and 4500 × g

for 5 min and resuspended in 50 ml ice-cold TFB1 solution (30 mM potassium acetate, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 100 mM RbCl, 15 % glycerol, pH adjusted with acetic acid to 5.8). After a subsequent centrifugation, the cells were resuspended in 1 ml ice-cold TFB2 (100 mM MOPS pH 6.5, 75 mM $CaCl_2$, 10 mM RbCl, 15 % glycerol, pH adjusted with potassium hydroxide to 6.5) and incubated on ice for 15-60 min. Aliquots of 50 µl were snap-frozen and stored at -80 °C.

For transformation 50-100 ng of plasmid was added to chemically competent *E. coli* on ice and incubated for 30 min. The bacteria were heat-shocked for 1 min at 42 °C and put back on ice for 2 min. After addition of 450 µl LB medium and incubation at 37 °C for 1 h, the bacteria were plated onto selective LB agar.

2.4.7.2 Electrocompetent cells and electroporation

1 ml of a pre-stationary phase *L. pneumophila* culture was added to 30 ml AYE. At an OD_{600} between 0.3 and 0.5 the bacteria were cooled and washed 3 times with sterile, ice-cold 10 % glycerol (10 ml, 2.5 ml, 160 µl). Aliquots of 25 µl were snap frozen and stored at -80 °C. 100 ng of plasmid was added to electrocompetent *L. pneumophila* on ice. After transfer into a cuvette with a 2 mm electrode gap (gene pulser cuvette) and electroporation (2.5 kV, 200 Ω, 25 µF), 450 µl AYE were added. The bacteria were incubated for 5 h at 37 °C on a turning wheel and plated onto selective CYE agar.

2.5 Biochemical and analytical methods

2.5.1 Preparation of inverted membrane vesicles

L. pneumophila JR32 and Δ*lqsT* or *E. coli* TOP10 harbouring plasmids encoding wild-type or mutant LqsS or LqsT were grown in AYE or LB medium with Cm at 37 °C. Overnight bacterial cultures were diluted to an OD_{600} of 0.1 in fresh medium with Cm, incubated in Erlenmeyer flasks until an OD_{600} of 0.5 and induced by addition of 1 mM IPTG for 8 h. Cells were harvested by centrifugation (20 min at 7000 × g; 4 °C), resuspended and homogenized 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, 100 µM DCCD) and disrupted at 10000 psi by a French press. Afterwards, the suspension was centrifuged (10 min at 7000 × g, 4 °C), followed by an ultracentrifugation step (1 h at 87000 × g, 4 °C) to separate soluble proteins from membranes and insoluble proteins. LqsS- and LqsT-containing membrane vesicles were washed in low ionic buffer (1 mM Tris/HCl, pH 8.0, 3 mM EDTA) and resuspended in TG buffer (50 mM

Tris/HCl, pH 8.0, 10 % (v/v) glycerol). The protein amount was estimated quantitatively after a modified method of Peterson (Peterson, 1977).

2.5.2 Purification of LqsR

His-LqsR or GST-LqsR was purified from the cytosolic fraction of *E. coli* BL21(DE3)/ pTS-23 or pNT-35, respectively. Cells were grown aerobically in LB medium at 37 °C and induced with IPTG (1 mM) during exponential growth for 4 h. Cells were harvested and lysed as described above. His-LqsR was purified by affinity chromatography using nickel-nitrilotriacetic acid Ni^{2+}-NTA-agarose (Qiagen) equilibrated with equilibration buffer (buffer-E) (10 mM imidazole, 10 % 1 (v/v) glycerol, 50 mM Tris/HCl, pH 8.0, 10 mM β-mercaptoethanol). His-tagged proteins were eluted with buffer E containing 250 mM imidazole. During the entire purification process the temperature was maintained at 4 °C to minimize proteolysis. Purified proteins were dialyzed against elution buffer lacking imidazole. GST-LqsR was purified using glutathione-sepharose beads according to the manufacturer`s recommendation (GE Healthcare). Briefly, the soluble fraction containing recombinant fusion protein was applied to a glutathione-sepharose column for 1.5 h at 4 °C, washed three times with TBS buffer (50 mM Tris pH 8, 150 mM NaCl) and eluted with elution buffer (50 mM Tris, pH 8.0, 10 mM reduced glutathione). Proteins were examined by SDS-PAGE followed by Coomassie Brilliant Blue staining and found to be ~95 % pure. Protein concentrations were determined using the Nano Drop 1000 Spectrophotometer (Thermo Scientific).

2.5.3 *In vitro* phosphorylation

2.5.3.1 Kinase activity of sensor kinases

Phosphorylation assays were performed with inverted membrane vesicles containing 25 µg M45-LqsS or M45-LqsT (approximately 2 µM) or the corresponding histidine mutant proteins. Reactions were carried out in phosphorylation buffer (50 mM Tris pH 8.0, 500 mM KCl, 5 mM $MgCl_2$, and 10 % (v/v) glycerol, 2 mM DTT). Experiments were initiated by addition of 100 µM ATP and 2 µCi [γ-^{32}P]-ATP from a 3000 Ci/mmol stock solution (Perkin Elmer). The samples were incubated at 25 °C, terminated with SDS-PAGE loading buffer, and the reaction products were separated by SDS-PAGE. Gels were dried at 75 °C on filter paper under vacuum, exposed to a phospho-screen overnight, and analysed using a scanner and ImageJ software. Further analysis was carried out by using normalized, background-

subtracted intensity values, defined as RIU (relative intensity units). The autophosphorylation of the *V. cholerae* sensor kinase LuxN by [γ-^{32}P]-ATP and phospho-transfer to LuxU was assayed as described (Timmen *et al.*, 2006).

2.5.3.2 Dephosphorylation

Where indicated, 2 µg (approximately 10 µM) of His-LqsR, His-LqsR$_{D108N}$ or heat-denatured (15 min, 95 °C) His-LqsR were used. Reactions were carried out in phosphorylation buffer (50 mM Tris pH 8.0, 500 mM KCl, 5 mM $MgCl_2$, 10 % (v/v) glycerol, 2 mM DTT). Experiments were initiated by addition of 100 µM ATP and 2 µCi [γ-^{32}P]-ATP from a 3000 Ci/mmol stock solution (Perkin Elmer). The samples were incubated at 25 °C, terminated with SDS-PAGE loading buffer, and the reaction products were separated by SDS-PAGE. Gels were dried at 75 °C on filter paper under vacuum, exposed to a phospho-screen overnight, and analysed using a scanner and ImageJ software. Further analysis was carried out by using normalized, background-subtracted intensity values, defined as RIU. The autophosphorylation of the *V. cholerae* sensor kinase LuxN by [γ-^{32}P]-ATP and phospho-transfer to LuxU was assayed as described (Timmen *et al.*, 2006).

2.5.3.3 *In vitro*-phosphorylation with [γ-^{32}P]-acetyl phosphate

[^{32}P]-acetyl phosphate (AcP) was synthesized according to a modified protocol of (Quon *et al.*, 1996), using 0.3 U *E. coli* acetate kinase (Sigma) and 10 µCi of [γ-^{32}P]-ATP (6000 Ci/mmol, Perkin Elmer) in 7.5 µl AKP buffer (50 mM Tris/HCl pH 7.5, 600 mM potassium acetate, 10 mM $MgCl_2$, 1 mM DTT) incubated for 20 min at room temperature. [^{32}P]-acetyl phosphate was separated from acetate kinase by ultrafiltration (10K device, Amicon).

For radio-phosphorylation of LqsR, 10 mM [^{32}P]-acetyl phosphate was added to 2 µg of purified His-LqsR or His-LqsR$_{D108N}$ in phosphorylation buffer (25 mM Tris pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM DTT). Excessive [^{32}P]-acetyl phosphate was removed by passage over a PD SpinTrap G-25 column (GE Healthcare). The samples were incubated at 30 °C, terminated with loading buffer, and the reaction products were separated by SDS-PAGE. Gels were dried at 75°C on filter paper under vacuum or blotted on a nitrocellulose membrane, exposed to a phospho-screen overnight and analysed using a FujiFilm FLA3000 scanner.

2.5.3.4 Phosphotransfer

Phosphotransfer assay was performed with inverted membrane vesicles containing 50 µg M45-LqsT (approximately 4 µM) or the corresponding histidine mutant protein. In assays containing His-LqsR or His-$LqsR_{D108N}$, 4 µg (approximately 20 µM) of these proteins were used. Reactions were carried out in phosphorylation buffer (50 mM Tris pH 8.0, 500 mM KCl, 5 mM, 10 $MgCl_2$, 10 % (v/v) glycerol, 2 mM DTT). Experiments were initiated by addition of 50 mM ATP or 100 mM acetyl phosphate (Sigma). The samples were incubated for 20 min at 25 °C and centrifuged for 1 h at 55000 × g. The extent of dimerization of His-LqsR or His-$LqsR_{D108N}$ was estimated by analytical gel filtration chromatography as described in section 2.5.3.5. 0.3 ml fractions from 13.3-14.8 ml (LqsR dimer) and 14.8 ml-16.6 ml (LqsR monomer) were analysed by Western blot using a rabbit anti-LqsR antibody (1:100; Tiaden *et al.*, 2007).

2.5.3.5 Gel filtration chromatography

In order to evaluate phosphorylation-dependent dimerisation of His-LqsR or His-$LqsR_{D108N}$, the proteins were incubated with 100 mM acetyl phosphate (Sigma) in phosphorylation buffer (25 mM Tris pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM DTT) at 25 °C. The extent of dimerisation of His-LqsR or His-$LqsR_{D108N}$ was estimated by analytical gel filtration chromatography using an analytical Superdex 200 10/300 GL column (GE Healthcare) at a flow rate of 0.2 ml/min. Standard proteins used were the gel filtration calibration kit LMW (GE Healthcare), comprising conalbumin (75 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa), and aprotinin (6.5 kDa). 2 µg of the standards, LqsR or $LqsR_{D108N}$ were loaded onto the column.

2.5.4 Pull-down experiments

2.5.4.1 Binding of LqsR to LqsS and LqsT

To investigate binding of LqsR to LqsS or LqsT, 2 µg purified GST-LqsR was incubated on a rotation wheel (1.5 h, 4 °C) with 25 µg M45-LqsS or M45-LqsT membrane vesicles and 10 µl washed glutathione-sepharose beads in a total volume of 200 µl binding buffer (50 mM Tris pH 8, 200 mM KCl, 1 mM DTT, 1 mM EDTA, 0.5 % NP-40, 10 mM $MgCl_2$). Beads were washed 4 times with 1 ml binding buffer, resuspended in 20 µl SDS loading buffer (350 mM Tris/HCl pH 6.8, 10 % SDS, 30 % (v/v) glycerol, 0.6 M DTT, 0.1 mg/ml Bromophenol Blue), boiled and centrifuged. The supernatant was analysed by Western blot using a mouse anti-M45 antibody (1:1000; Genovoc AG). As controls, beads only and beads

with GST-SidC (pCR-87) were used (Weber *et al.*, 2006, Ragaz *et al.*, 2008). 8 µl of membrane vesicle suspension of LqsS or LqsT (1:50 dilution) were used as input controls.

2.5.4.2 Binding of phosphorylated LqsR to LqsS and LqsT

To analyse binding of LqsR and phospho-LqsR to LqsS or LqsT, 4 µg purified His-LqsR was incubated with 100 mM acetyl phosphate (Sigma) in phosphorylation buffer (25 mM Tris/HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM DTT) and separated by gel filtration chromatography using an ÄKTA Purifier (GE Healthcare) equipped with a Superdex 200 10/300 GL column (GE Healthcare). The column was equilibrated with chromatography buffer (25 mM Tris/HCl pH 7.5, 50 mM NaCl). Fractions corresponding to monomeric or dimeric LqsR were collected and adjusted to the same protein concentration. The samples were then incubated on a wheel (1 h, 4 °C) with 25 µg M45-LqsS or M45-LqsT membrane vesicles and 10 µl washed Ni-NTA-beads in a total volume of 200 µl binding buffer (50 mM Tris pH 8, 10 mM DTT, 10 mM $MgCl_2$). Beads were washed four times with 1 ml binding buffer, resuspended in 20 µl SDS loading buffer, boiled and centrifuged. The supernatant was analysed by Western blot using either a rabbit anti-LqsR antibody (1:100; Tiaden *et al.*, 2007) or a mouse anti-M45 antibody (1:1000; Genovac AG). Beads alone and beads with His-LuxU were used as controls. 10 µl of membrane vesicle suspension of LqsS or LqsT (1:50 dilution) were used as input controls.

3 RESULTS

At the onset of this thesis nearly all knowledge about the *L. pneumophila* QS system was based upon genetic analysis or investigation of phenotypic properties, and the biochemical characterisation of the signalling cascade was not addressed.

Bioinformatic analysis of the *L. pneumophila* genome revealed a gene cluster homologous to the *cqsAS* QS system in *V. cholerae* (Tiaden *et al.,* 2007, Miller *et al.,* 2002). Based on sequence homology and functional analysis of QS genes, a first model of the signal transduction cascade was constructed and some of its functions have been revealed (Section 1.3.4; Figure 9A) (Tiaden *et al.,* 2010a, Kessler *et al.,* 2013). LqsA catalyses the production of the autoinducer LAI-1, which is likely recognized by the sensor kinases LqsS and LqsT. Both sensor kinases presumably transmit the signal to the putative response regulator LqsR, which in turn controls the transition form replicative to transmissive life cycle stages, promotes interaction between the bacterium and phagocytes and regulates expression of transmissive/virulence traits (Tiaden *et al.,* 2007, Kessler *et al.,* 2013)

The Lqs system provides an excellent subject for biochemical studies, as the main components LqsS, LqsT, LqsR, LqsA and the corresponding ligand LAI-1 have already been identified (Tiaden *et al.,* 2010a, Kessler *et al.,* 2013). Furthermore, the hydrophobic presumably aliphatic tail renders LAI-1 a membrane-permeable molecule, which allows the study of receptor-ligand interactions in inverted membrane vesicles. The biochemical studies provided in this thesis characterise the individual components of the Lqs system and significantly add to the reconstruction of the complex QS signal transduction cascade in *L. pneumophila in vitro*. These results validate our model based on genetic data, thus giving new insights on the regulation of signal detection and transduction. The main points addressed here are (i) sensor kinase autophosphorylation, (ii) signal transduction to the RR LqsR, (iii) response of LqsR upon phosphorylation and (iv) influence of the autoinducer LAI-1 on sensor kinase phosphorylation and signalling.

3.1 Expression and purification of Lqs proteins in *L. pneumophila* and *E. coli*

The primary aim of these experiments was the production of inverted membrane vesicles (MV) containing the Lqs HKs, LqsS and LqsT, or the corresponding His-mutants $LqsS_{H200Q}$

and $LqsT_{H204Q}$. These HK-containing MVs were in turn used to analyse autophosphorylation by [γ-^{32}P]-ATP and in a second step, the phosphotransfer to the RR LqsR.

To achieve this, the HK were overproduced in different expression systems, *L. pneumophila* or *E. coli*, in the later case to exclude the necessity of further cofactors for their autophosphorylation.

3.1.1 Expression of the sensor kinases LqsS and LqsT and preparation of inverted membrane vesicles

The preparation of inverted membrane vesicles was already established for the *V. harveyi* QS sensor kinases LuxN/LuxQ (Timmen *et al.*, 2006, Jung *et al.*, 2007), and we successfully adapted this method for the LqsS and LqsT sensor kinases from the *L. pneumophila* Lqs system. The gene sequences of LqsS and LqsT were analysed with various bioinformatics programs (ProtParam; Protein model portal) of the ExPASy server in order to predict the size, localisation and molecular function in the bacterial cell (SIB, 2014). LqsS and LqsT have a molecular mass of 48.5 kDa and 48.6 kDa, respectively and were predicted to show a phosphorelay sensor kinase activity and to localize in the bacterial membrane. To anchor to the cytoplasmic membrane both LqsS and LqsT harbour six hydrophobic α-helices (Tiaden & Hilbi, 2012b).

Upon overproduction both N-terminally M45-tagged sensor kinases and their corresponding His-mutants were produced in roughly equal amounts in *L. pneumophila* JR32 or *E. coli* TOP10 upon treatment with IPTG. The HKs localised to the LSP (low speed pellet) and in major parts to the membrane fraction. The proteins could be detected only in traces in the cytosolic fraction (Fig. 10). The localisation of the sensor kinases in the LSP is probably due to their presence in intact cells and protein aggregates. Furthermore, the amount of LqsS/$LqsS_{H200Q}$ and LqsT/$LqsT_{H204Q}$ incorporated into the MV was similar.

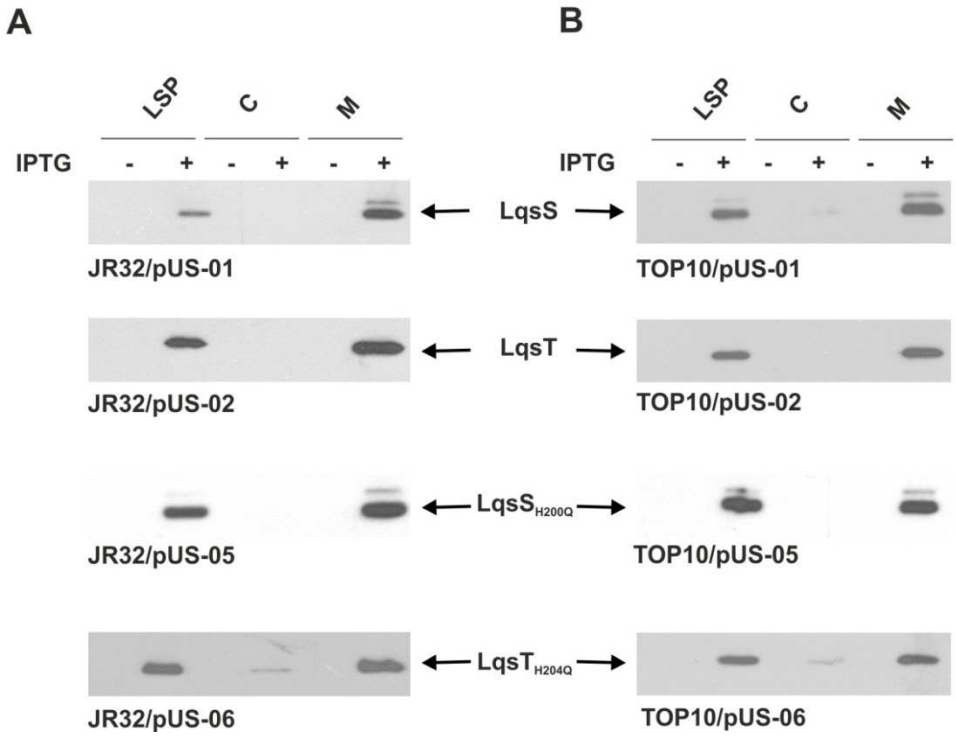

Fig. 10: Production and membrane localisation of LqsS and LqsT in *L. pneumophila* and *E. coli*. **A.** *L. pneumophila* JR32 or **B.** *E. coli* TOP10 producing M45-LqsS (pUS-1), M45-LqsT (pUS- 2), M45-LqsS$_{H200Q}$ (pUS-5) or M45-LqsT$_{H204Q}$ (pUS-6) upon induction with 1 mM IPTG (37 °C, 8 h). The cells were lysed by French press, and fractionated into low speed pellet (LSP), cytoplasm (C) and membrane (M). The M45 fusion proteins were visualized by Western blot using an anti-M45 antibody.

3.1.2 Heterologous expression and purification of the response regulator LqsR

The gene sequence of LqsR was analysed by various programs (ProtParam; Protein model portal) of the ExPASy server and predicted to encode a protein of molecular mass of 41.4 kDa and to be a soluble cytosolic protein (SIB, 2014). Either of two C-terminal tags, His or GST, were added to the response regulator gene during the cloning process. Upon overproduction in *E. coli* TOP10, His-tagged LqsR as well as the Aspartate mutant LqsR$_{D108N}$ (both 42.4 kDa) were restricted to the cytosolic fraction, where it could be isolated from via Ni^{2+}-NTA-agarose to a purity of 95 %. GST-LqsR (65.1 kDa) was purified by glutathione-sepharose-agarose to a purity of ≥95 % (Fig 11). Smaller bands appearing in the eluate fractions presumably represent LqsR degradation products.

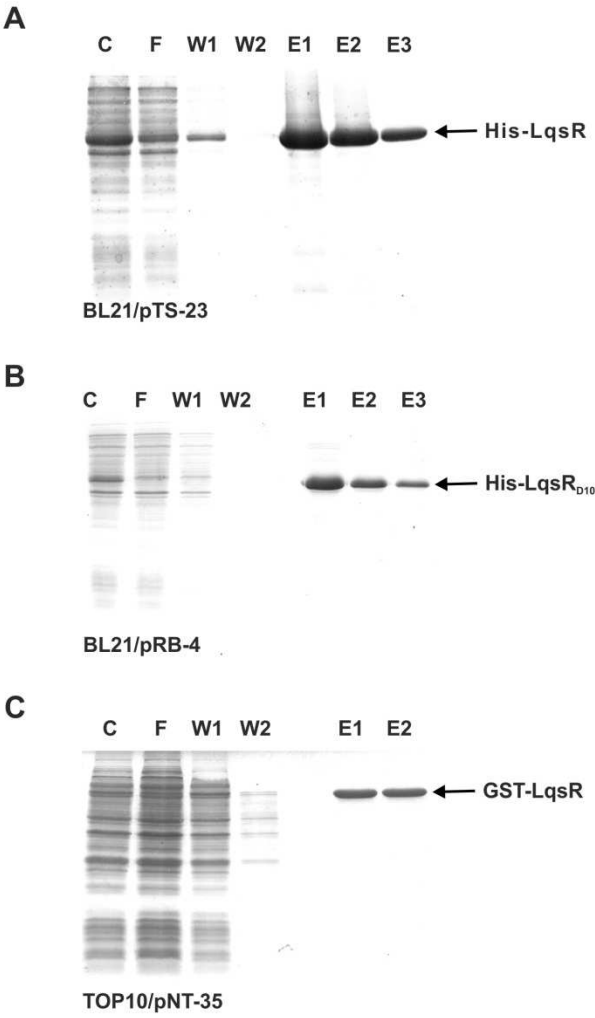

Fig. 11: Production and purification of LqsR and LqsR$_{D108N}$ in *E. coli*. A. *E. coli* BL21 producing His-LqsR (pTS-23), **B.** His-LqsR$_{D108N}$ (pRB-4), or **C.** *E. coli* TOP10 GST-LqsR (pNT-35) upon induction with 1 mM IPTG (37 °C, 4 h). The cells were lysed by French press, and the lysate was fractionated into low speed pellet and cytoplasm (C). The tagged proteins were purified from the cytoplasm via Ni^{2+}-NTA-agarose beads **A., B.** or via gluthatione-sepharose beads **C.** The proteins in the fractions were separated by 15 % SDS-Page and visualized by Coomassie Brilliant Blue staining. C = cytoplasm, F = flow-through, W = wash, E = eluate

3.1.3 Heterologous expression and purification of Lux proteins in *E. coli*

In order to establish the correct procedure for phosphorylation in the Lqs system we used a previous published system of the *V. harveyi* LuxN/LuxU TCS as a positive control. To this end, the *V. harveyi* Lux proteins were overproduced and purified according to Timmen *et al.*, 2006. A minor portion of His-LuxN (97.1 kDa) localized to the low speed and cytosolic fraction and the majority was found, as expected, in the membrane fraction (Fig 12A). Further bands appearing on the Western blot were due to unspecific binding of the His antibody. His-tagged LuxU (13.9 kDa) was restricted to the cytosolic fraction, where it could be isolated from by Ni^{2+}-NTA-agarose to a purity of 80-90 % (Fig 12B).

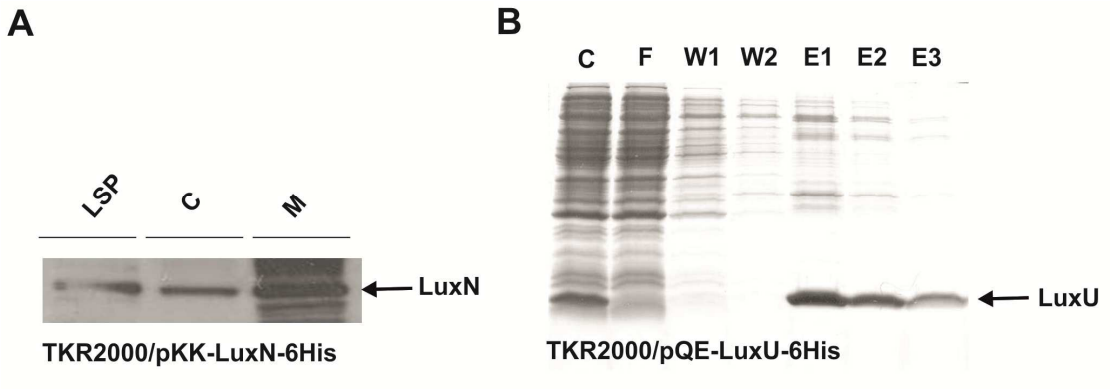

Fig. 12: Production and membrane localization of LuxN and purification of LuxU in *E. coli*. **A.** *E. coli* TKR2000 cells producing LuxN (pKK-LuxN-6His) were harvested at late exponential growth phase. The cells were lysed by French press, and the lysate was fractionated into low speed pellet (LSP), cytoplasm (C) and membrane (M). The His-fusion protein was visualized by Western blot using an anti-His antibody. **B.** LuxU (pQE-LuxU-6His) was overproduced and purified from *E. coli* TKR2000 (IPTG induction for 3 h). The His-tagged protein was purified from the cytosolic fraction by affinity chromatography using Ni^{2+}-NTA-agarose beads. The fractions were separated by 15 % SDS-Page and visualized by Coomassie Brilliant Blue staining. C = cytoplasm, F = flow-through, W = wash, E = eluate

3.2 Biochemical and functional analysis of the *L. pneumophila* Lqs system

3.2.1 Establishment of LqsS and LqsT autophosphorylation

To investigate autophosphorylation of the sensor kinases *in vitro*, we over-expressed M45-LqsS or M45-LqsT in *L. pneumophila*, prepared inverted membrane vesicles and examined [γ^{32}P]-ATP-induced phosphorylation. Inverted membrane vesicles provide access of the phospho-donor compound to the cytoplasmic section of the HKs, and therefore, allow phosphorylation at the conserved histidine of the cytoplasmic kinase domain (Timmen *et al.*, 2006). Data were analysed by quantifying intensities of phospho-protein bands, defined as relative intensity units (RIU). We optimized autophosphorylation of MV harbouring M45-LqsS or M45-LqsT by preparing and incubating them with [γ-^{32}P]-ATP under different conditions. Different types of salts, NaCl and KCl, were tested at different concentrations in mM range. Furthermore, we performed the reactions at altered pHs, 7 and 8 (Fig. 13). Optimal for autophosphorylation of both HKs was a combination of 500 mM KCl at a pH of 8. The usage of NaCl (500 mM), minor KCl concentrations (100 mM) and reduction of the pH (pH 7) resulted in reduced autophosphorylation. Moreover, both LqsS and LqsT were

phosphorylated to a different extent. LqsT exhibited a higher phosphorylation than LqsS under all conditions.

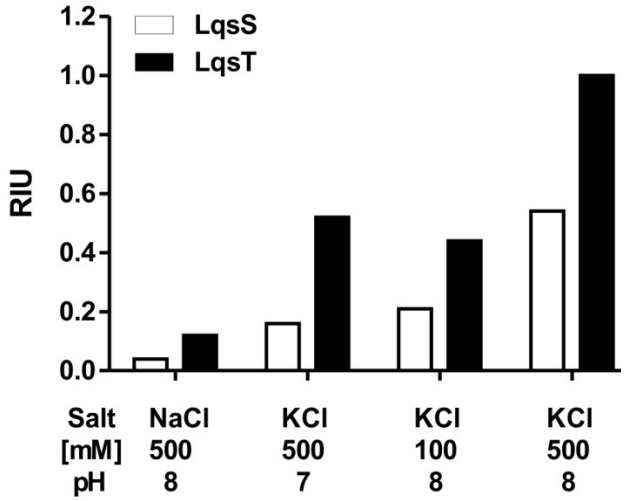

Fig. 13: Optimization of auto-phosphorylation of LqsS and LqsT in *L. pneumophila* membrane vesicles. Inverted membrane vesicles of *L. pneumophila* JR32 producing M45-LqsS (pUS-1) or M45-LqsT (pUS-2), were prepared and incubated with [γ-^{32}P]-ATP under different conditions (diverse salt types, salt concentrations and pHs). Samples were taken for SDS-PAGE/autoradiography after 10 min. Data represent one typical experiment, and band intensities were normalized to LqsT (RIU = relative intensity units).

3.2.2 Autophosphorylation kinetics of LqsS and LqsT

We assessed the phosphorylation kinetics for LqsS or LqsT by incubating either HK with [γ^{32}P]-ATP and taking samples after 0 min, 1 min, 2.5 min, 5 min, 10 min and 20 min. Incubation of the MVs in the presence of [γ-^{32}P]-ATP led to rapid and stable autophosphorylation of both sensor kinases (Fig. 14A). LqsS phosphorylation occurred with an initial rate 0.11 RIU min^{-1}, while LqsT was labelled at a rate of 0.29 RIU min^{-1}. The maximal phosphorylation level was reached after 10 min. Moreover, the amount of phospho-LqsS and phospho-LqsT remained constant, indicating that these HK possessed no phosphatase activity. Both LqsS and LqsT were phosphorylated to a different extent; LqsT exhibited a two-fold higher phosphorylation than LqsS after 10 or 20 min. Western blotting proved that this difference could not be attributed to higher protein production of LqsT (Fig. 14A and B).

To test the hypothesis that the conserved histidines H200 or H204 are the phosphorylation sites in the DHp domain of LqsS or LqsT, we exchanged the amino acid for a glutamine residue by site-directed mutagenesis to yield the mutant proteins LqsS$_{H200Q}$ or LqsT$_{H204Q}$. Autoradiographs indicated that, compared to wild-type LqsS and LqsT, LqsS$_{H200Q}$ and LqsT$_{H204Q}$ were not autophosphorylated, even though the same protein amount was used (Fig. 14B).

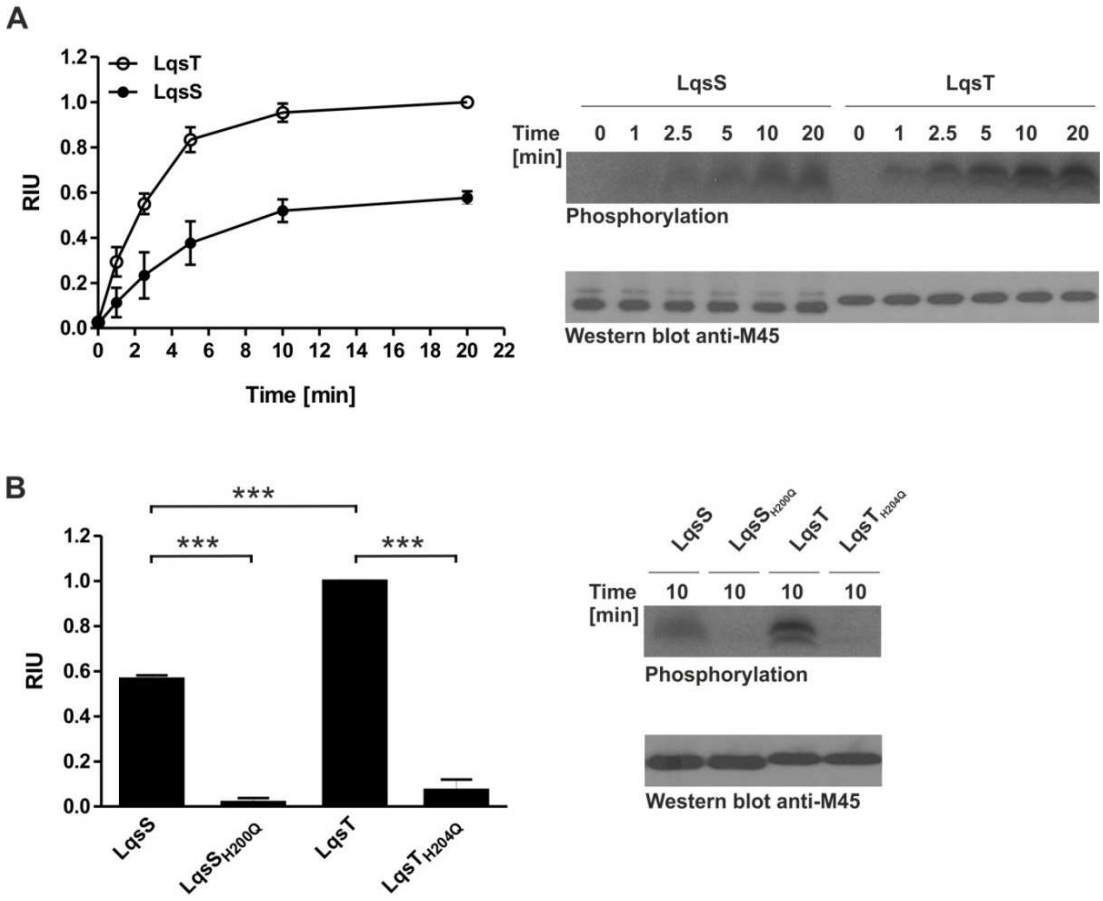

Fig. 14 : Autophosphorylation kinetics of LqsS and LqsT in *L. pneumophila* membrane vesicles. Inverted membrane vesicles *of L. pneumophila* JR32 producing **A.** M45-LqsS (pUS-1) or M45-LqsT (pUS-2), or **B.** M45-LqsS$_{H200Q}$ (pUS-5) or M45-LqsT$_{H204Q}$ (pUS-6) were prepared and incubated with [γ-^{32}P]-ATP. Samples were taken for SDS-PAGE/autoradiography **A.** at given time points or **B.** after 10 min. Data represent means and standard deviation of three independent experiments, and band intensities were normalized to LqsT (RIU, relative intensity units). Data points (> 2.5 min) are significantly different (**p<0.05, ***p<0.001; unpaired Student´s *t*-test). Representative autoradiographs and Western blots are shown.

3.2.3 LqsT does not contribute to LqsS autophosphorylation

In order to test whether LqsT contributes to LqsS autophosphorylation we overproduced M45-LqsS in the *L. pneumophila* Δ*lqsT* strain. In the Δ*lqsT* strain M45-LqsS localised to the membrane fraction and in similar amounts compared to the wild-type strain JR32 (Fig. 10A and 15A). LqsS-harbouring MVs from wild-type JR32 and the Δ*lqsT* strain both showed autophosphorylation to the same extent after 10 min incubation with [γ-^{32}P]-ATP. Therefore, we can exclude the possibility that LqsS is cross-phosphorylated by LqsT (Fig. 15B).

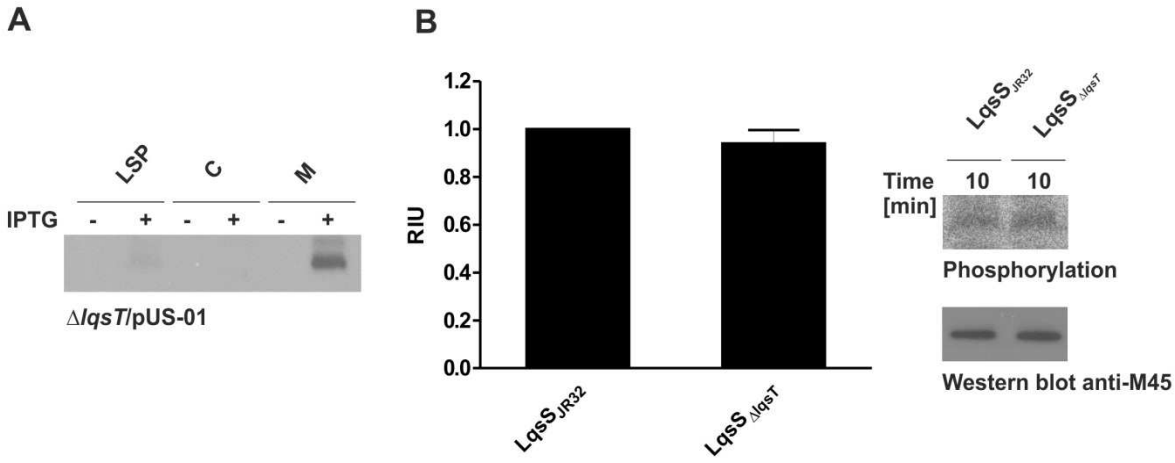

Fig. 15: Production and autophosphorylation of LqsS in *L. pneumophila* Δ*lqsT*. **A.** *L. pneumophila* Δ*lqsT* producing M45-LqsS (pUS-1) upon induction with 1 mM IPTG (37°C, 8 h). The cells were lysed by French press, and the lysate was fractionated into low speed pellet (LSP), cytoplasm (C) and membrane (M). The M45 fusion proteins were visualized by Western blot using an anti-M45 antibody (+, with IPTG; -, without IPTG). **B.** Inverted membrane vesicles of *L. pneumophila* wild-type (JR32) or Δ*lqsT* (AK01) producing M45-LqsS (pUS-1) were prepared and incubated with [γ-^{32}P]-ATP. Samples were taken for SDS-PAGE/autoradiography after 10 min. Data represent means and standard deviation of three independent experiments, and band intensities were normalized to LqsS overproduced in the wild-type JR32 strain (RIU = relative intensity units).

3.2.4 Interaction of LqsS and LqsT with the response regulator LqsR

In TCSs an interaction between the His-domain of the HK and the Asp-domain in the receiver domain of the RR takes place upon signal transduction or phosphotransfer *(*Stock *et al.,* 2000). To determine binding between LqsS and LqsT and the putative RR LqsR we performed pull-down assays between membrane vesicles overexpressing either sensor kinase and purified GST-tagged LqsR (Fig. 16). GST-LqsR was incubated with membrane vesicles harbouring a M45-tagged sensor kinase together with glutathione-sepharose beads, washed several times, and an anti-M45 Western blot was performed to detect the amount of LqsS or LqsT bound by LqsR. Under these conditions both LqsS and LqsT interacted with LqsR. However, an approximately two-fold higher amount of LqsS bound LqsR compared to LqsT. SidC (Substrate of Icm/Dot transporter) or glutathione-sepharose beads alone were used as controls for unspecific binding. SidC is translocated by *L. pneumophila* into the host cell via Icm/Dot T4SS and does not interact with the bacterial membrane-bound sensor kinases. Upon incubation of membrane vesicles harbouring either M45-tagged LqsS or LqsT with purified GST-tagged SidC or glutathione-sepharose beads alone, significantly less LqsR

bound to the sensor kinases. Furthermore, LqsT showed more unspecific binding to SidC or the glutathione-sepharose beads compared to LqsS. Taken together, both LqsS and LqsT interacted with LqsR and approximately two-fold more LqsS bound to LqsR, suggesting that LqsS binds LqsR with higher affinity than LqsT (Fig. 16).

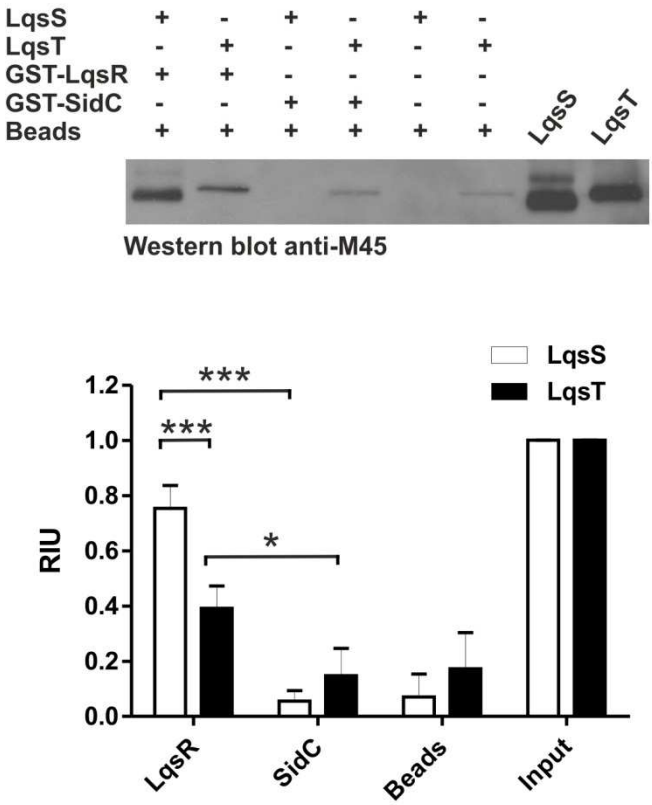

Fig. 16: Interaction of the sensor kinases LqsS and LqsT with the response regulator LqsR. Membrane vesicles of *L. pneumophila* producing M45-LqsS (pUS-1) or M45-LqsT (pUS-2) were prepared and incubated with purified GST-LqsR or, as a control, GST-SidC, followed by the addition of glutathione-sepharose beads. Eluates from washed beads were analysed by Western blot using an anti-M45 antibody. White or black bars show the quantification for LqsS or LqsT respectively. Data represent means and standard deviation of three independent experiments, and band intensities were normalized to input controls of LqsS and LqsT (*p<0.05; ***p<0.001; unpaired Student's *t*-test).

3.2.5 Inhibition of autophosphorylation of LqsS or LqsT by LqsR

To further characterise the signal transduction in *L. pneumophila in vitro*, we investigated the influence of the response regulator LqsR on the autophosphorylation of the sensor kinases. To this end we examined the [γ^{32}P]-ATP-dependent autophosphorylation of LqsS and LqsT in the presence of LqsR or the $LqsR_{D108N}$ mutant protein, where the conserved aspartate residue in the receiver domain is exchanged against asparagine. MVs containing M45-tagged sensor kinase were mixed with purified His-LqsR, His-$LqsR_{D108N}$ or heat-denatured LqsR (LqsR h.d.) and compared to autophosphorylation of the HKs only. These experiments revealed that autophosphorylation of both sensor kinases LqsS and LqsT was completely inhibited by native LqsR (Fig. 17A and B). The inhibition was dependent on the conserved aspartate residue (D108) in the Rec-domain of the RR, as $LqsR_{D108N}$ inhibited

autophosphorylation with significantly lower efficiency. In contrast, addition of heat-denatured LqsR to the membrane vesicles had no effect on the autophosphorylation kinetics or -levels of either LqsS or LqsT, compared to untreated samples (Fig. 17).

Subsequently we studied the dose-dependency of sensor kinase autophosphorylation inhibition by LqsR or $LqsR_{D108N}$. To this end, MVs harbouring either M45-LqsS (Fig. 17C) or M45-LqsT (Fig. 17D) were treated with increasing amounts of purified His-LqsR or His-$LqsR_{D108N}$, and autophosphorylation was quantified by RIU after 10 min. Whereas the autophosphorylation of LqsS was very efficiently blocked by wild-type LqsR (half maximal inhibition < 0.1 μg), LqsR was approximately eight–fold less efficient at inhibiting LqsT autophosphorylation (half maximum inhibition ~0.8 μg). Moreover, compared to native LqsR, the autophosphorylation of LqsS (half maximum inhibition ~0.8 μg) or LqsT (half maximum inhibition ~2 μg) was inhibited by $LqsR_{D108N}$ with a significantly lower efficiency. The effect on autophosphorylation inhibition of the conserved aspartate residue D108 of LqsR was more pronounced for LqsS (~8-fold) than for LqsT (~2.5-fold). In summary, the autophosphorylation of LqsS or, albeit less efficiently, LqsT, was inhibited completely and in a dose-dependent manner by native LqsR and with significantly lower efficiency by $LqsR_{D108N}$.

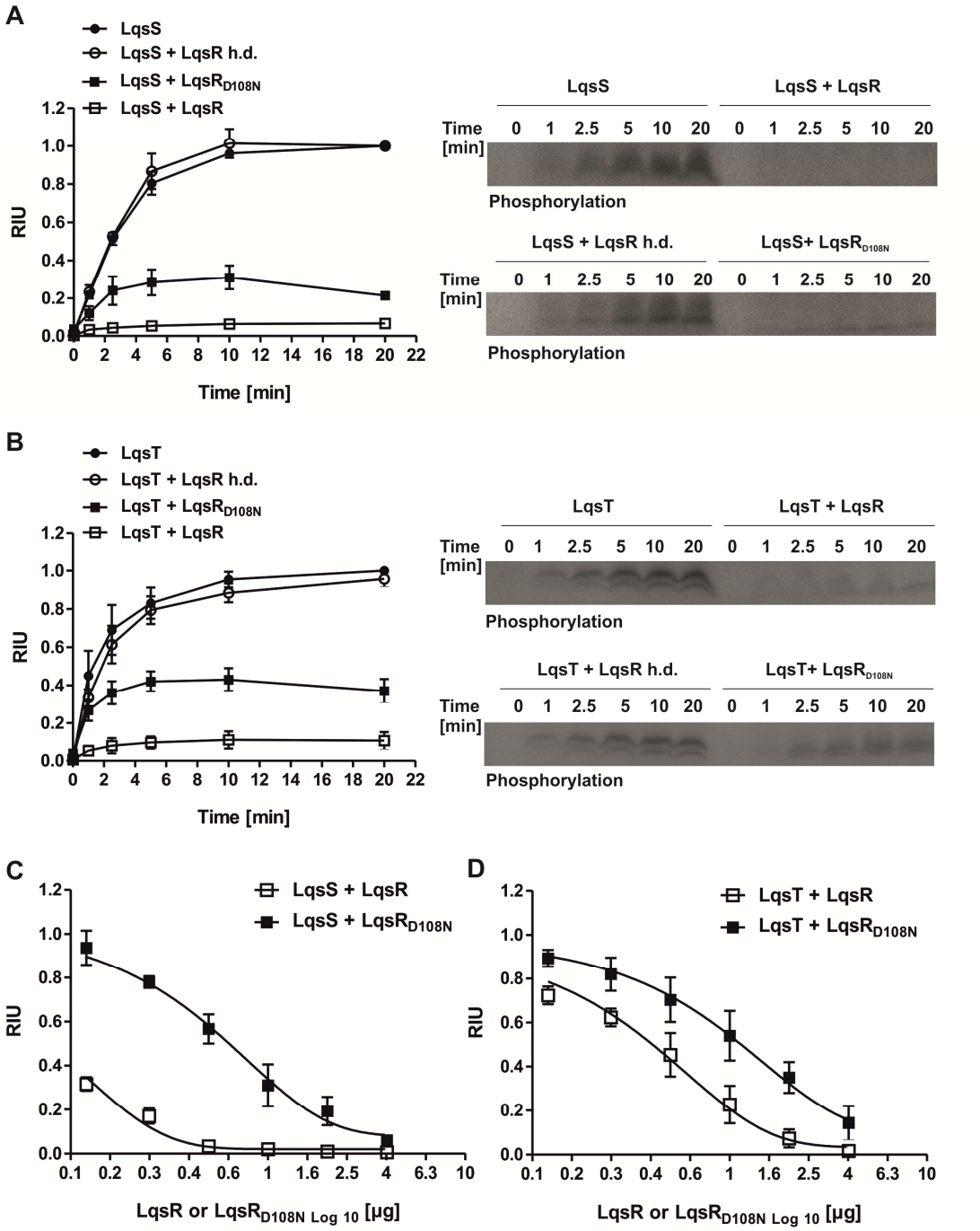

Fig. 17: Inhibition of autophosphorylation of LqsS or LqsT by LqsR. Membrane vesicles of *L. pneumophila* producing **A.** M45-LqsS (pUS-1)or **B.** M45-LqsT (pUS-2) were prepared and incubated with [γ-^{32}P]-ATP in absence or presence of **A., B.** 2 µg or **C., D.** the amount indicated of purified His-LqsR, His-LqsR$_{D108N}$ or heat-denatured His-LqsR (h.d.). Samples were taken for SDS-PAGE/autoradiography **A., B.** at given time points or **C., D.** after 10 min. Data represent means and standard deviation of three independent experiments, and band intensities were normalized to sensor kinase autophosphorylation in absence of LqsR. Data points (> 2.5 min, except LqsR h.d.) are significantly different (*p<0.05; unpaired Student's *t*-test) (RIU = relative intensity units). Representative autoradiographs and Western blots are shown.

3.2.6 Dephosphorylation of phosphorylated sensor kinases by LqsR

LqsR might impede autophosphorylation of LqsS or LqsT by catalysing the dephosphorylation of phospho-sensor kinases or by preventing the phosphorylation reaction. To discriminate among these two mechanisms, the sensor kinases LqsS or LqsT were first phosphorylated, followed by addition of native LqsR, $LqsR_{D108N}$ or heat-denatured LqsR. Both HKs, phospho-LqsS (Fig. 18A) or phospho-LqsT (Fig. 18B) were found to be dephosphorylated instantly upon addition of native LqsR, and only 20 % of LqsS or 40 % of LqsT remained phosphorylated.

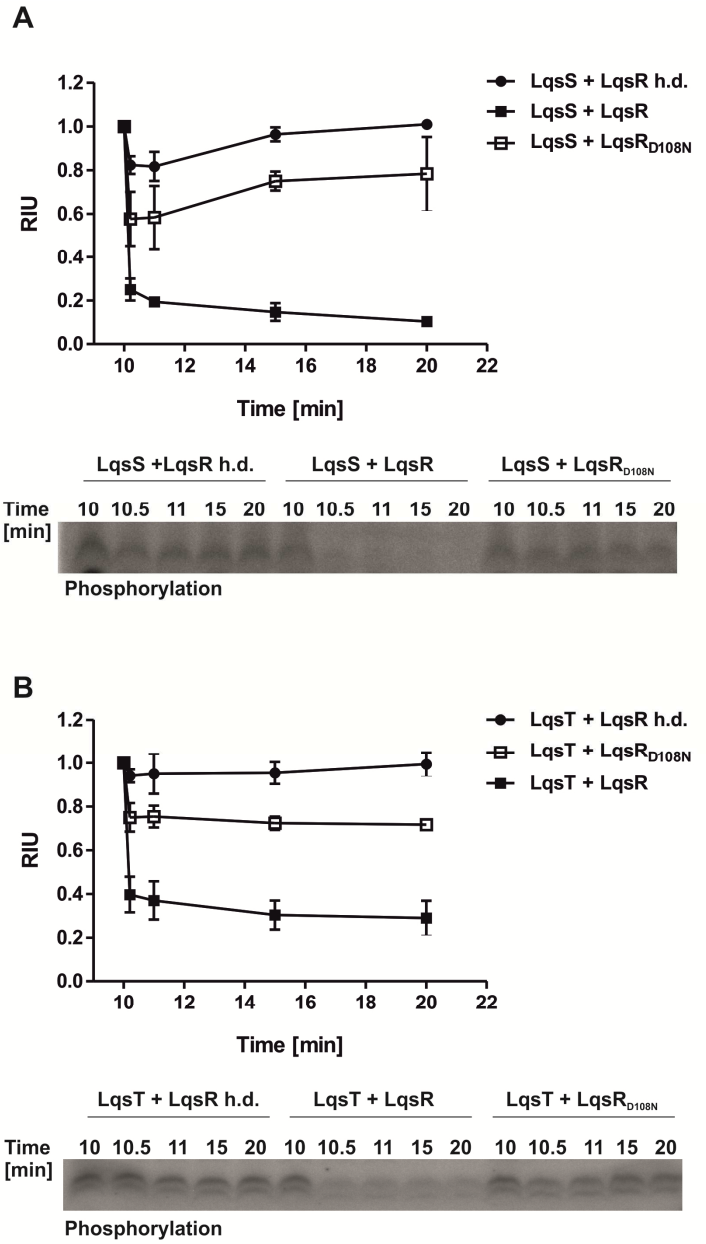

Fig. 18: Dephosphorylation of phospho-LqsS or phospho-LqsT by LqsR. Membrane vesicles of *L. pneumophila* producing **A.** M45-LqsS (pUS-1) or **B.** M45-LqsT (pUS-2) were prepared and incubated with [γ-^{32}P]-ATP for 10 min, followed by incubation with 2 µg purified His-LqsR, His-$LqsR_{D108N}$ or heat-denatured His-LqsR. Samples were taken for SDS-PAGE/ autoradiography at the time points indicated. Data points are significantly different (*p<0.05; unpaired Student's *t*-test) (RIU = relative intensity units).

Upon addition of $LqsR_{D108N}$ a significant proportion of the phospho-sensor kinases was also immediately dephosphorylated. However, in contrast to native LqsR, approximately 60-70 % of LqsS and 80 % of LqsT remained phosphorylated. Heat-denatured LqsR did not affect the extent of phosphorylation of the phospho-sensor kinases. In summary, these results suggest that LqsR catalyses the dephosphorylation of phospho-LqsS or phospho-LqsT rather than interfering with the phosphorylation of the sensor kinases, and this process is dependent on the conserved aspartate in the receiver domain.

3.2.7 Phosphorylation and dimerisation of LqsR

Conformational changes and dimerisation or oligomerisation of response regulators can be induced upon phosphorylation by high energy molecules like acetyl phosphate (AcP) (Lukat *et al.*, 1992). Phosphorylation of LqsR was detected via SDS-PAGE while dimerization was demonstrated by an increase in the molecular mass observed via analytic gel-filtration.

To test whether the conserved aspartate D108 in the LqsR receiver domain is phosphorylated, we incubated purified His-LqsR or His-$LqsR_{D108N}$ with 10 mM [^{32}P]-AcP for up to 6 h (Fig. 19A). The maximal phospho-LqsR amount was reached after 1 h of AcP treatment (1 RIU) and subsequently decreased with a half-life of approximately 6 h. At the same time, $LqsR_{D108N}$ was barely phosphorylated (0.2 RIU). In contrast, incubation of purified His-LqsR with radiolabelled [γ-^{32}P]-ATP did not result in detectable phosphorylation of the protein (Fig. 22A). Thus, LqsR can be phosphorylated *in vitro* at D108 by AcP but not by ATP.

To assess whether LqsR oligomerises upon phosphorylation of D108, purified His-LqsR or, as a negative control, His-$LqsR_{D108N}$ was incubated with 100 mM AcP for up to 6 h, and oligomerisation was determined by analytical gel filtration chromatography. Indeed, treatment of LqsR with AcP led to a shift of the main protein peak eluting from the column from an apparent molecular mass of 40 kDa (15.2 ml elution volume) to 80 kDa (13.8 ml elution volume). The sizes correspond to monomeric and dimeric His-LqsR respectively (Fig. 19B). The LqsR dimerisation was already detectable as a peak ‘shoulder’ of dimeric protein after 10 min, the monomeric and dimeric forms were present in approximately equal amounts at 2 h, and dimeric LqsR predominated at 6 h. To confirm the identity of LqsR, protein fractions eluting at the volume of dimeric phospho-LqsR were collected and analysed by SDS-PAGE, which revealed a band with an apparent molecular weight of 40 kDa (Fig. 19C). In contrast, the aspartate mutant $LqsR_{D108N}$ exhibited no dimerisation over the entire 6 h period of AcP treatment. These results support the link between dimerisation and

phosphorylation of LqsR and, furthermore, identify the conserved aspartate residue (D108) as the functionally phosphorylation site.

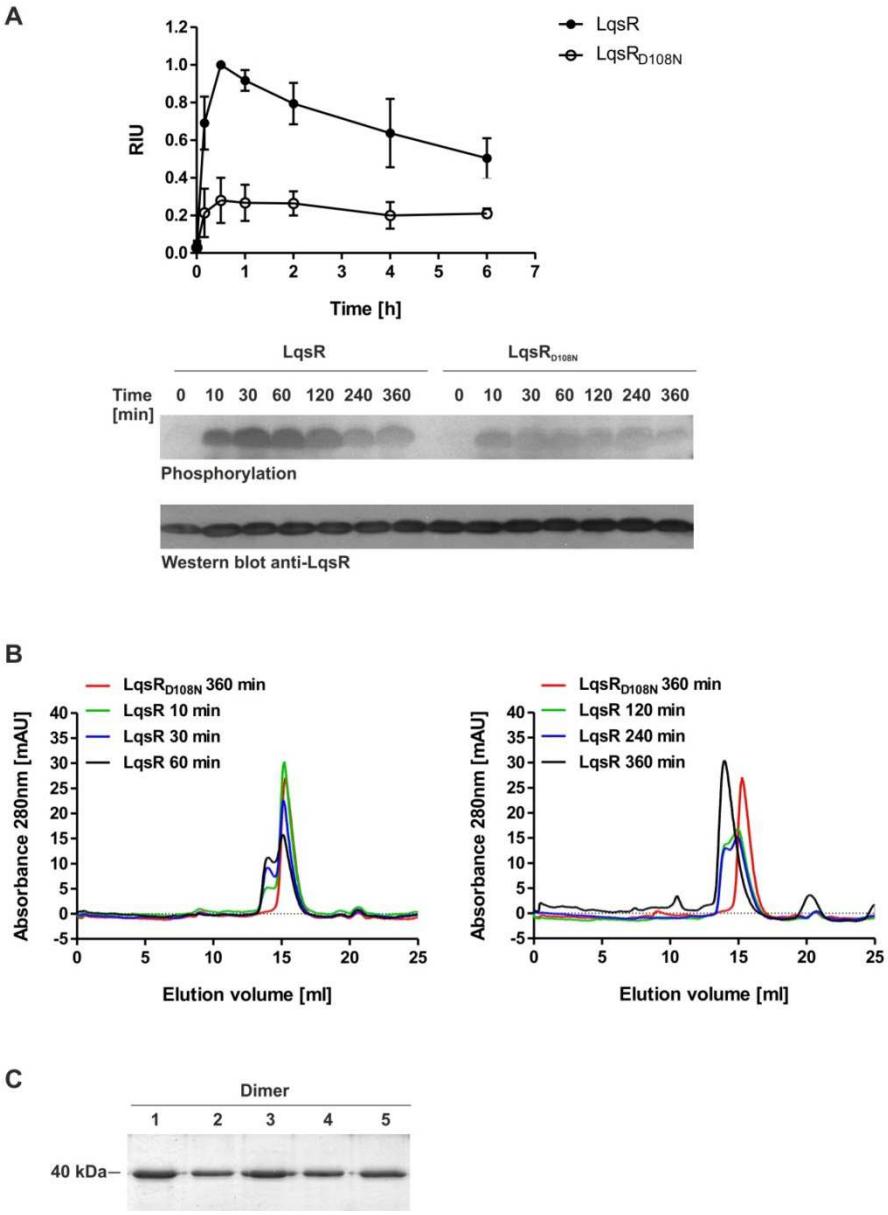

Fig. 19: Phosphorylation and dimerization of LqsR. A. Purified His-LqsR or His-LqsR$_{D108N}$ was treated with 10 mM [^{32}P]-acetyl phosphate (AcP), and phosphorylation was analysed by SDS-PAGE/ autoradiography at the time points indicated. Data represent means and standard deviation of three independent experiments. Data points are significantly different ($p < 0.05$; unpaired Student's *t*-test). Representative autoradiographs and Western blots are shown. **B.** Purified His-LqsR or His-LqsR$_{D108N}$ was treated with 100 mM AcP, and dimerisation was analysed by analytical gel filtration chromatography. LqsR or phospho-LqsR eluted at approximately 40 kDa (15.2 ml) or 80 kDa (13.8 ml), corresponding to the monomeric or dimeric form, respectively. **C.** Protein fractions (1-5) eluting at the volume of dimeric phospho-LqsR were collected and analysed by SDS-PAGE. A single band appearing at 40 kDa corresponds to LqsR.

To test the stability of phospho-LqsR over a longer period of time we incubated purified His-LqsR with either H_2O or 100 mM AcP for up to 6 h and analysed the oligomerisation by gel filtration chromatography. Subsequently the reaction mix was stored at 4°C, and samples were taken every 12h. Results proved that LqsR remained in a stable phosphorylated state at lower temperatures (4°C), even 24h after phosphorylation (Fig. 20).

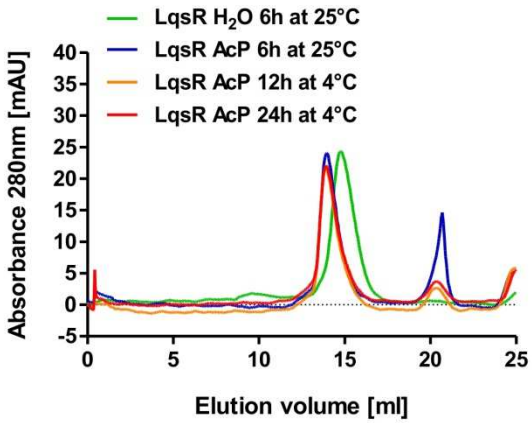

Fig. 20: Phosphorylation and dimerization of LqsR. Purified His-LqsR was treated either with H_2O or with 100 mM AcP, and dimerization was analysed by analytical gel filtration chromatography. Samples were taken after 6h of incubation, the reaction was then transferred to 4°C and samples were taken 12h and 24h after temperature shift. LqsR or phospho-LqsR eluted at about 40 kDa (15.2 ml) or 80 kDa (13.8 ml), corresponding to the monomeric or dimeric form, respectively.

3.2.8 Interaction of phospho-LqsR with sensor kinases

Furthermore, we also compared the binding of monomeric LqsR and dimeric phospho-LqsR to the sensor kinases LqsS and LqsT. First we determined if phosphorylation influenced the binding of His-LqsR to the Ni^{2+}-NTA-beads. To this end, AcP-treated purified His-LqsR was separated by gel filtration chromatography, and pooled fractions corresponding to monomeric or dimeric (phosphorylated) LqsR were incubated with Ni^{2+}-NTA-beads, washed several times and eluted by heat-denaturation (Fig. 21A). The Western blot indicates that the same amount of LqsR and phospho-LqsR was bound to the Ni^{2+}-NTA-beads. In addition, monomeric or dimeric His-LqsR or His-LuxU were also analysed by SDS-PAGE to confirm identical amounts in each sample (Fig. 21B).

Subsequently, membrane vesicles containing LqsS or LqsT were incubated with pooled fractions corresponding to monomeric or dimeric (phosphorylated) LqsR and with Ni^{2+}-NTA-beads. The same amounts of monomeric or dimeric LqsR bound to LqsS or LqsT,

indicating that phosphorylation of the response regulator did not modulate its binding to the sensor kinases. Purified *V. cholerae* His-LuxU was used as a control, which did not bind to either sensor kinase (Fig. 21C).

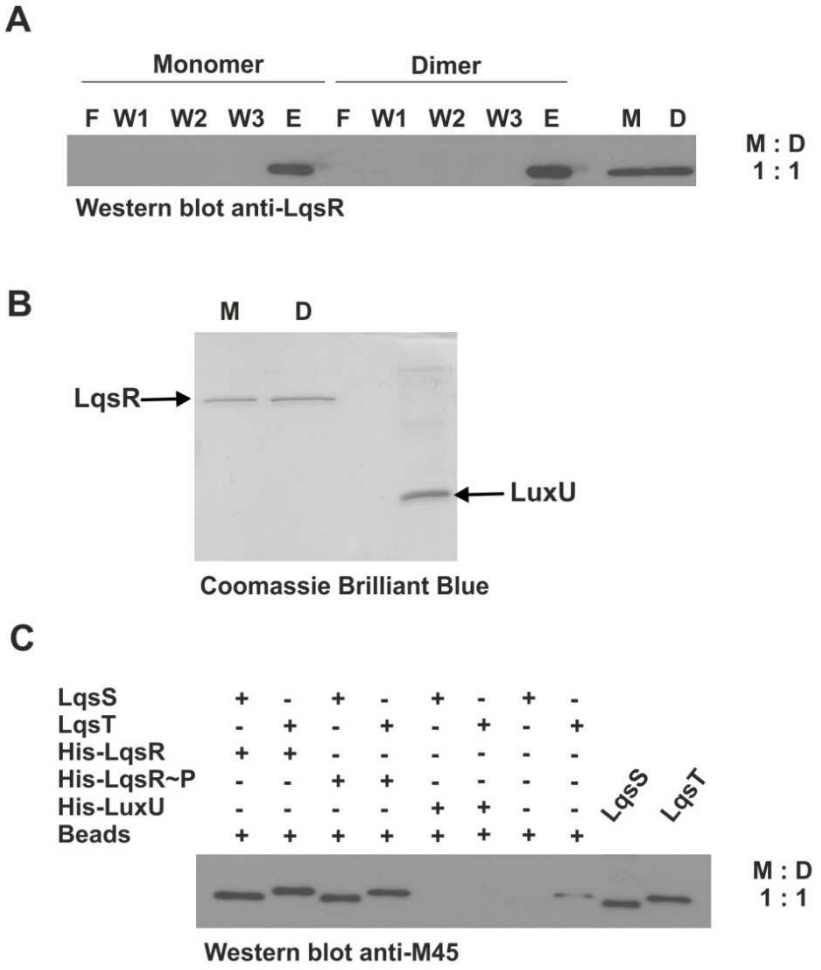

Fig. 21: Interaction of the LqsR monomer and dimer with the sensor kinases LqsS and LqsT. A. 4 µg purified His-LqsR were incubated with 100 mM AcP in phosphorylation buffer and separated by gel filtration chromatography. Fractions corresponding to monomeric or dimeric LqsR were collected, adjusted to the same protein concentration and incubated with 25 µg M45-LqsS or M45-LqsT in *L. pneumophila* MVs and 10 µl washed Ni-NTA beads in a total volume of 200 µl binding buffer. The flow-through (F), washing steps (W1-3) and the eluate (E) of the Ni^{2+}-NTA-beads, as well as LqsR monomer and dimer samples, were analysed by Western blot using a polyclonal anti-LqsR antibody. **B.** The amounts of monomeric or dimeric His-LqsR or His-LuxU were also analysed by SDS-PAGE stained with Coomassie Brillant Blue (M = monomer, D = dimer). **C.** Washed Ni^{2+}-NTA-beads were resuspended in 20 µl SDS loading buffer, boiled and centrifuged, and the supernatant was analysed by Western blot using a mouse anti-M45 antibody. Beads only, beads with His-LuxU or 10 µl of membrane vesicle suspension of M45-LqsS or M45-LqsT (1:50 dilution) were used as controls. Representative Western blots of three independent experiments are shown **A., C.**

Repeated attempts to demonstrate phospho-transfer activity from phospho-LqsS or phospho-LqsT to LqsR, possibly yielding phospho-LqsR were unsuccessful (Fig 22A). By contrast, control assays using the *V. cholerae* sensor kinase LuxN and the phospho-relay protein LuxU confirmed that the sensor kinase is autophosphorylated by [γ-^{32}P]-ATP, and the phosphate residue of phospho-LuxN was readily transferred to LuxU, yielding phospho-LuxU (Fig. 22B)

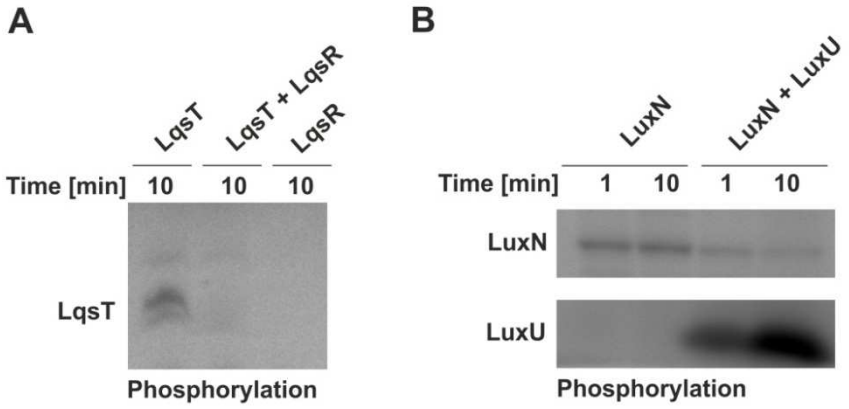

Fig. 22 Autophosphorylation and phosphotransfer. A. Membrane vesicles of *L. pneumophila* JR32 producing the sensor kinase M45-LqsT and the purified response regulator His-LqsR were either alone or as a mixture incubated with [γ-^{32}P]-ATP for 10min. **B.** The *V. harveyi* sensor kinase LuxN and the phospho-relay protein LuxU were used as positive controls for autophosphorylation and phosphotransfer, respectively. 25 µg inverted membrane vesicles of *E. coli* TKR2000 producing His-LuxN (pKK-LuxN-6His) were prepared and incubated with [γ-^{32}P]-ATP in absence or presence of 0.5 µg of His-LuxU (pQE-LuxU-6His). Samples were taken for SDS-PAGE/autoradiography after 1 or 10 min. Representative autoradiographs are shown.

3.2.9 Dimerisation of LqsR promoted by phospho-LqsT

For further analysis of the interaction of the sensor kinases and LqsR, we determined whether a phosphorylated kinase would promote the phosphorylation-dependent dimerisation of the response regulator. To this end, membrane vesicles containing M45-tagged LqsT or $LqsT_{H204Q}$ were mixed with purified His-LqsR or His-$LqsR_{D108N}$, and 50 mM ATP was subsequently added to start the reaction. After 20 min of incubation the samples were centrifuged for 1 h at 55000 x g, and dimerisation of the response regulator present in the supernatant was detected by analytical gel filtration (Fig. 23) and Western blot (Fig. 24). As a positive control, purified His-LqsR was treated with 100 mM AcP. The gel filtration chromatogram indicated a small peak corresponding to dimeric LqsR along with mainly monomeric LqsR. Fractions of the dimeric and monomeric LqsR were collected and samples were analysed by Western blot.

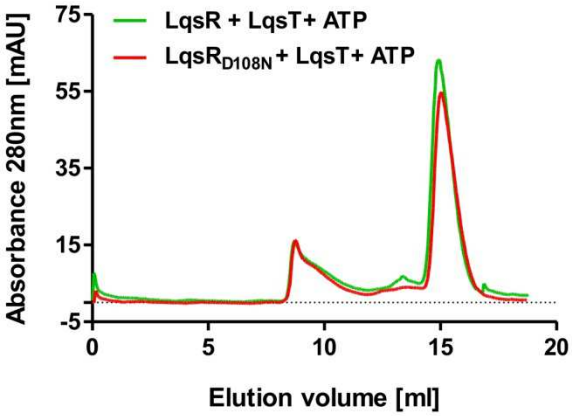

Fig. 23: Phosphorylation and dimerization of LqsR. Purified His-LqsR or LqsR$_{D108N}$ was mixed with LqsT containing MVs and treated with 50 mM ATP for 20 min. Dimeric LqsR present in the supernatant was then determined by analytical gel filtration chromatography. LqsR or phospho-LqsR eluted at about 40 kDa (15.2 ml) or 80 kDa (13.8 ml), corresponding to the monomeric or dimeric form respectively.

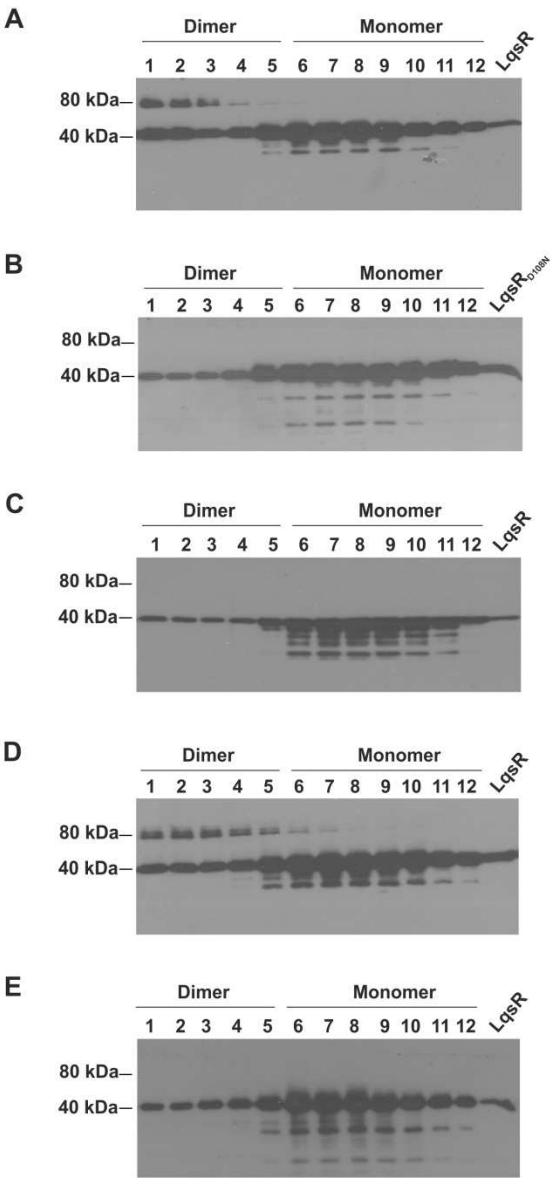

Fig. 24 Phospho-LqsT promotes dimerization of LqsR. A., C. Membrane vesicles of *L. pneumophila* JR32 producing M45-LqsT (pUS-2) or **C.** M45-LqsT$_{H204Q}$ (pUS-6) were prepared and incubated for 20 min with purified His-LqsR and 50 mM ATP, **B.** M45-LqsT (pUS-2) with His-LqsR$_{D108N}$ and 50 mM ATP, or **D.** purified His-LqsR with 100 mM AcP, **E.** purified His-LqsR with 50 mM ATP. After centrifugation, dimerization of LqsR in the supernatant was analysed by analytical gel filtration chromatography, and fractions corresponding to monomeric (40 kDa) or dimeric (80 kDa) LqsR were analysed by Western blot. Representative Western blots of duplicate experiments are shown.

Interestingly, compared to negative controls, a more prominent 40 kDa anti-LqsR-reactive band, as well as an 80 kDa band was visible under these conditions (Fig. 24A). If the assay was performed with membrane vesicles containing either M45-tagged LqsT and His-$LqsR_{D108N}$ (Fig. 24B), or M45-tagged $LqsT_{H204Q}$ and LqsR (Fig. 24C), the column fractions corresponding to dimeric LqsR contained approximately 10-fold less 40 kDa LqsR, and no 80 kDa LqsR band was detected. Finally, upon treatment of purified His-LqsR with AcP and separation by gel filtration under the same conditions, the 40 kDa anti-LqsR-reactive band, as well as the 80 kDa band, were visible in Western blots (Fig. 24D) whereas upon treatment of purified His-LqsR with ATP and separation by gel filtration under the same conditions only a 40 kDa anti-LqsR-reactive band was detectable (Fig. 24E). Taken together, these results indicate that autophosphorylation of LqsT in presence of LqsR leads to the phosphorylation-dependent formation of LqsR dimers.

3.2.10 Autophosphorylation kinetics of LqsS and LqsT in *E. coli*

In order to reconstruct the *L. pneumophila* QS signalling cascade in *E. coli* we cloned and overexpressed LqsS or LqsT and subsequently prepared inverted membrane vesicles. Treatment of these vesicles with [γ-^{32}P]-ATP revealed stable autophosphorylation only in the case of LqsT, while LqsS as well as $LqsS_{H200Q}$ or $LqsT_{H204Q}$ remained undetectable by radiography (Fig 25A and B). The phosphorylation kinetics and final level in *E. coli* was similar to that obtained in *L. pneumophila*, reaching a maximum after 10 min and with no change following this point (Fig 25). This suggests that autophosphorylation of LqsS requires a further factor or factors. Further verification of the protein input by Western blot proved that the same protein amount was used (Fig. 25B). Upon production in *E. coli*, similar amounts of M45-LqsS or M45-LqsT accumulated in the membrane fraction (Fig. 10, Fig. 25A).

Next, we analysed the inhibition of autophosphorylation of M45-LqsT by purified His-LqsR. Similar to *L. pneumophila*-derived membrane vesicles, the autophosphorylation of LqsT produced in *E. coli* was inhibited completely by native LqsR. Furthermore, $LqsR_{D108N}$ inhibited the process with significantly lower efficiency, and heat denatured LqsR had no effect on the kinetics or extent of phosphorylation of LqsT (Fig. 25C). Finally, the dephosphorylation of phospho-LqsT was most efficiently catalysed by LqsR, followed by $LqsR_{D108N}$, and heat-denatured LqsR had almost no effect (Fig. 25D). In summary, LqsT heterologously produced in *E. coli* localizes to membrane vesicles, is autophosphorylated by

ATP with similar kinetics and to a similar extent as in *L. pneumophila*, and LqsR inhibits autophosphorylation by catalysing dephosphorylation of phospho-LqsT.

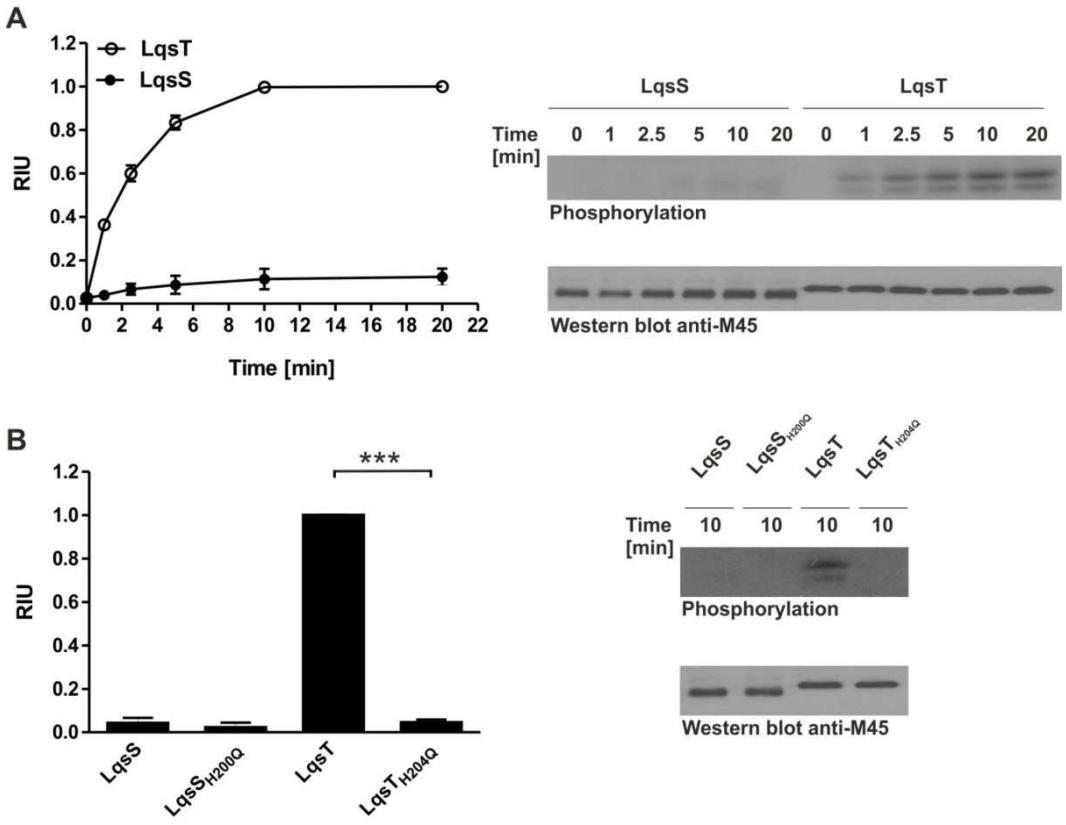

Fig. 25: Autophosphorylation kinetics of LqsS and LqsT in *E. coli* membrane vesicles. Membrane vesicles of *E. coli* TOP10 producing M45-LqsS (pUS-1), M45-LqsT (pUS-2) **A.**, or M45-LqsS$_{H200Q}$ (pUS-5) or M45-LqsT$_{H204Q}$ (pUS-6) **B.**, were prepared and incubated with [γ-^{32}P]-ATP. Samples were taken for SDS-PAGE/autoradiography **A.** at given time points or **B.** after 10 min. Data represent means and standard deviation of three independent experiments, and band intensities were normalized to autophosphorylation of LqsT (***p<0.001, unpaired Student's *t*-test). Representative autoradiographs and Western blots are shown (RIU = relative intensity units).

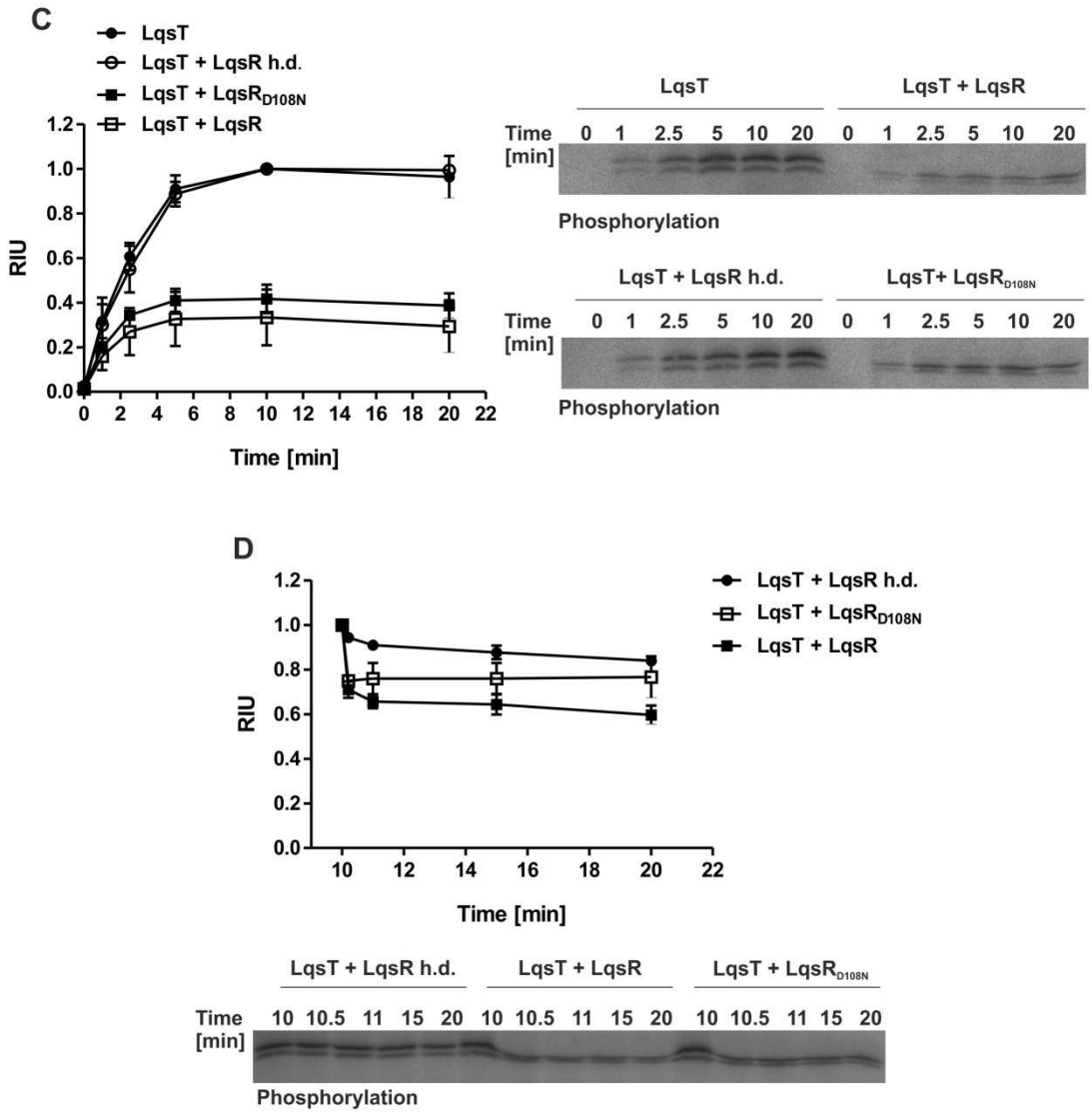

Fig. 25: Autophosphorylation kinetics of LqsS and LqsT in *E. coli* membrane vesicles. **C.** and **D.** Membrane vesicles of *E. coli* producing M45-LqsT (pUS-2) were prepared and either incubated with [γ-^{32}P]-ATP in absence or presence of **C.** 2 µg purified His-LqsR, His-LqsR$_{D108N}$ or heat-denatured His-LqsR, or **D.** incubated with [γ-^{32}P]-ATP for 10 min, followed by incubation with 2 µg purified His-LqsR, His-LqsRD108N or heat-denatured His-LqsR. Samples were taken for SDS-PAGE/ autoradiography at given time points. Data represent means and standard deviation of 3 independent experiments, and band intensities were normalized to LqsS or LqsT phosphorylation prior to addition of LqsR. Data points (**A, C; D:** > 2.5 min) are significantly different (*p<0.05; unpaired Student´s *t*-test). Representative autoradiographs are shown.

3.3 Influence of LAI-1 on the Lqs system

In previous experiments we assessed the kinase activity of the sensor kinases LqsS and LqsT, demonstrated the interaction of LqsS, LqsT and the response regulator LqsR and verified the dimerisation of LqsR upon phosphorylation. These experiments document the reconstruction of the Lqs signal transduction pathway *in vitro*. To expand on these results we examined the impact of the autoinducer LAI-1 and cognate AI compounds (CAI-1, (*S*)- or (*R*)-amino-LAI-1/ R-amino-CAI-1), on the QS system by exploring the reaction of the sensor kinases to the stimuli. Furthermore, we determined the ligand specificity of LqsS and LqsT and quantified the influence of LAI-1 on the dephosphorylation of and phosphotransfer from the sensor kinases.

3.3.1 Inhibition of sensor kinase autophosphorylation by LAI-1

In order to explore the effect of the autoinducer LAI-1 on autophosphorylation of LqsS or LqsT *in vitro*, we overproduced the M45-tagged sensor kinases in *L. pneumophila*, prepared inverted membrane vesicles using a French press and determined autophosphorylation upon addition of [γ-^{32}P]-ATP. The samples were treated with either LAI-1 or DMSO as a control, and autophosphorylation was quantified by densitometry within 20 min reaction time. Under these conditions, 500 µM LAI-1 reduced the phosphorylation rate and the phosphorylation level of M45-LqsS (Fig. 26A) or M45-LqsT (Fig. 26B) to a similar extent (approximately 30 %). In the range between 100 µM and 1 mM the phosphorylation inhibition by LAI-1 was dose-dependent, reaching 60 % inhibition at maximum (Fig. 26C). These results indicate that LAI-1 has either an inhibiting effect on the kinase activities of LqsS and LqsT or promotes intrinsic autophosphatase activities.

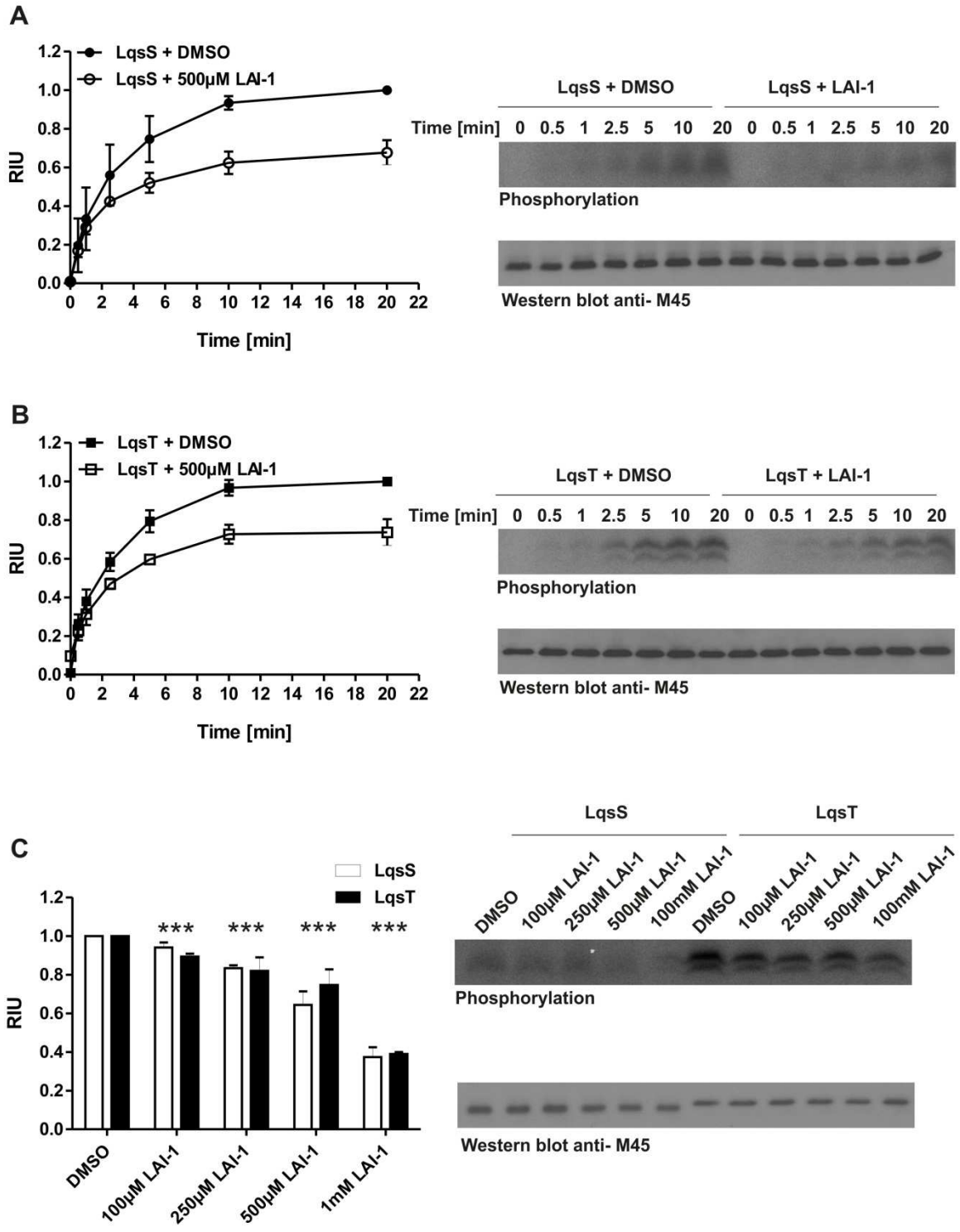

Fig. 26: LAI-1 inhibits autophosphorylation of the sensor kinases LqsS and LqsT. Membrane vesicles of *L. pneumophila* JR32 producing A. M45-LqsS (pUS-01) or B. M45-LqsT (pUS-02) were prepared and incubated with [γ-^{32}P]-ATP in absence or presence of A., B. 500 µM LAI-1 or C. the concentration of LAI-1 indicated. Samples were taken for SDS-PAGE/ autoradiography **A., B.** at given time points or **C.** after 10 min. Data represent means and standard deviation of three independent experiments (***p<0.001; unpaired Student's *t*-test). Representative autoradiographs and Western blots are shown (RIU = relative intensity units).

3.3.2 Ligand specificity of LqsS and LqsT

We tested the ligand specificity of LqsS and LqsT by using inverted membrane vesicles containing M45-LqsS or M45-LqsT. The samples were treated with 500 µM of (*S*)- or (*R*)-LAI-1, (*S*)- or (*R*)-amino-LAI-1, or DMSO as a control, and autophosphorylation with [γ-^{32}P]-ATP was quantified by densitometry (RIU) after 10 min. Under these conditions, (*S*)- or (*R*)-LAI-1 decreased the phosphorylation level of LqsS and LqsT by about 20-30 % (Fig. 27A and B). In contrast, (*S*)- or (*R*)-amino-LAI-1 (Am-LAI-1) did not affect the phosphorylation of LqsS, but decreased the phosphorylation level of LqsT by 40 % ((*S*)-Am-LAI-1) and even by 50 % ((*R*)-Am-LAI-1) (Fig. 27A and B).

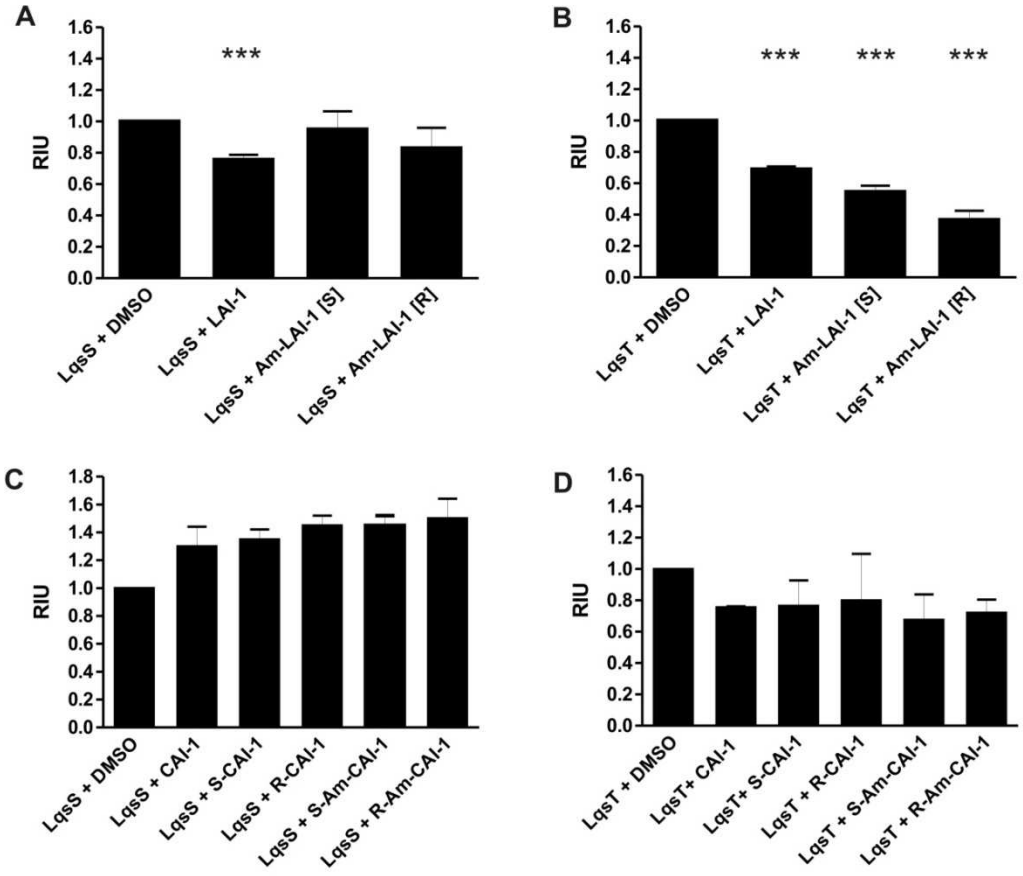

Fig. 27: Ligand specificity of LqsS and LqsT. Membrane vesicles of *L. pneumophila* producing **A.** M45-LqsS (pUS-01) or **B.** M45-LqsT (pUS-02) were prepared and incubated with [γ-^{32}P]-ATP in absence or presence of 500 µM of the autoinducers indicated. Samples were taken for SDS-PAGE/ autoradiography after 10 min. Data represent means and standard deviations of three **A.** (***p<0.001; unpaired Student's *t*-test) or two **B.** independent experiments. Representative autoradiographs and Western blots are shown (RIU = relative intensity units).

Furthermore, we tested the effects on LqsS or LqsT phosphorylation of the *V. cholerae* autoinducer molecules CAI-1 and (*S*)- or (*R*)-amino-CAI-1 by auto-phosphorylation with [γ-^{32}P]-ATP after 10 min. The racemic mixture of (*S*)- and (*R*)-CAI-1 and (*S*)- or (*R*)-Am-CAI-1 showed opposite effects on LqsS and LqsT: while it promoted the autophosphorylation of LqsS, it decreased the phosphorylation level of LqsT.

Taken together, these results suggest that the tested LAI-1-related autoinducers decrease the phosphorylation of LqsT and, to a lesser extent, LqsS, whereas CAI-1-derived autoinducers have an antagonistic effect on LqsS.

3.3.3 Effect of LAI-1 on the stability of phosphorylated LqsS and LqsT

Next, we tested the effect of LAI-1 on the stability of phospho-LqsS or phospho-LqsT. To this end, membrane vesicles of *L. pneumophila* producing M45-LqsS or M45-LqsT were prepared and incubated with [γ-^{32}P]-ATP for 10 min, followed by incubation with 500 µM LAI-1 or DMSO as a control. Samples were taken for SDS-PAGE/autoradiography at given time points within a 20 min reaction. Under these conditions LAI-1 neither affected the phosphorylation level of LqsS (Fig. 28A) nor of LqsT (Fig. 28B). Thus, LAI-1 does not appear to stimulate the intrinsic autophosphatase activities of the kinases.

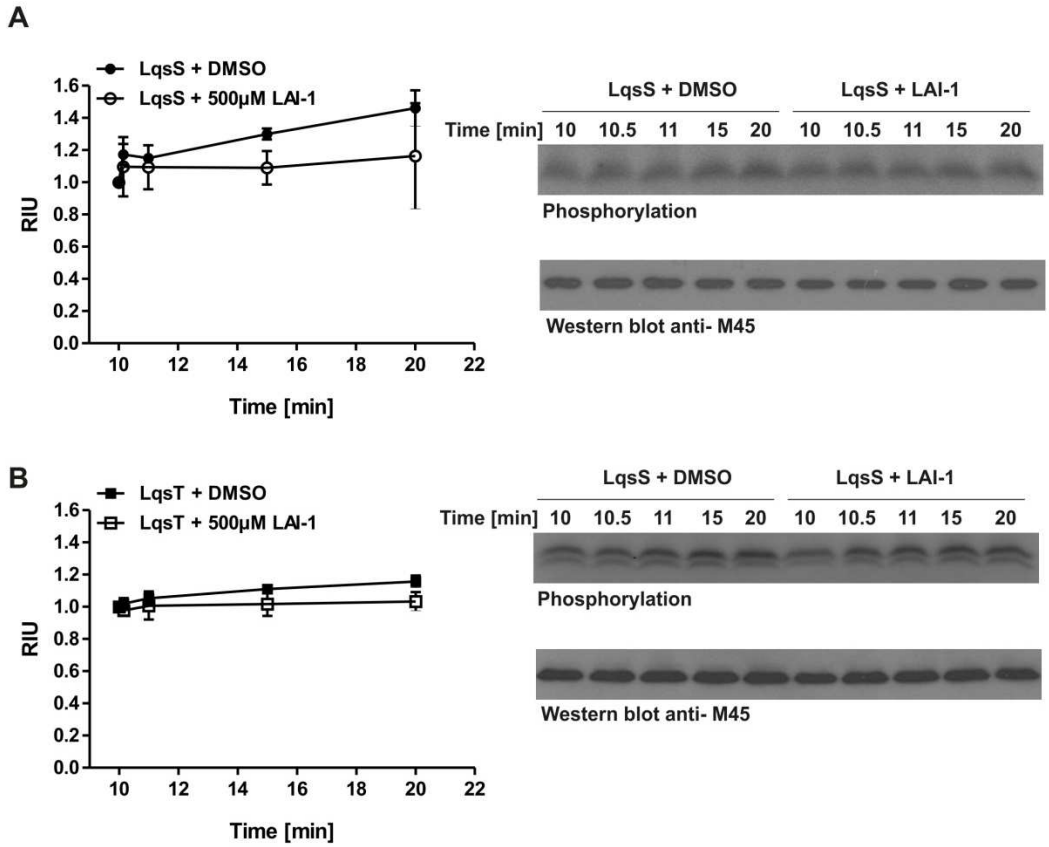

Fig. 28: Effect of LAI-1 on the stability of phospho-LqsS and phospho-LqsT. Membrane vesicles of *L. pneumophila* producing **A.** M45-LqsS (pUS-01) or **B.** M45-LqsT (pUS-02) were prepared and incubated with [γ-^{32}P]-ATP for 10 min, followed by incubation with DMSO or 500µM LAI-1. Samples were taken for SDS-PAGE/autoradiography at given time points. Data represent means and standard deviation of three independent experiments. Representative autoradiographs are shown (RIU = relative intensity units).

3.3.4 Role of LAI-1 for phosphotransfer processes

In addition to the inhibition of the autophosphorylation of the sensor kinases, LAI-1 binding could also affect downstream phosphotransfer processes by preventing LqsS/LqsT and LqsR interaction. Purified His-LqsR, but not heat-denatured His-LqsR or the His-LqsR$_{D108N}$ mutant protein, efficiently dephosphorylates phospho-LqsS or phospho-LqsT in *L. pneumophila* membrane vesicles (Section 3.2.6). To test whether LAI-1 effects the dephosphorylation of phospho-LqsS or phospho-LqsT, inverted membrane vesicles containing a M45-tagged sensor kinase were prepared and incubated with [γ-^{32}P]-ATP for 10 min, followed by incubation with purified His-LqsR or heat-denatured His-LqsR in presence or absence of 500 µM LAI-1. Under these conditions LqsR, but not the heat-inactivated response regulator, rapidly catalysed the dephosphorylation of approximately 80 % phospho-LqsS (Fig. 29A) or

50 % phospho-LqsT (Fig. 29B), respectively. Yet, LAI-1 had no effect on either the dephosphorylation of the phospho-kinases by LqsR, nor the lack of dephosphorylation by heat-inactivated LqsR. Therefore, LAI-1 does not affect the dephosphorylation by or phosphotransfer onto LqsR.

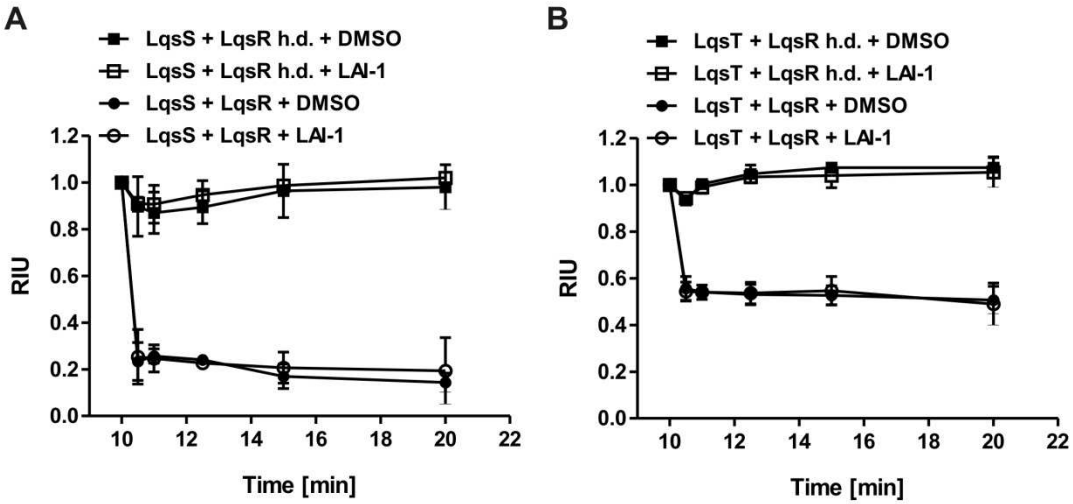

Fig. 29: Effect of LAI-1 on dephosphorylation of phospho-LqsS and phospho-LqsT by LqsR. Membrane vesicles of *L. pneumophila* producing **A.** M45-LqsS (pUS-01) or **B.** M45-LqsT (pUS-02) were prepared and incubated with [γ-^{32}P]-ATP for 10 min, followed by incubation with purified His-LqsR, heat-denatured His-LqsR or His-LqsR$_{D108N}$ in presence or absence of 500 µM LAI-1. Samples were taken for SDS-PAGE/ autoradiography at given time points. Data represent means and standard deviation of three independent experiments. Representative autoradiographs are shown (RIU = relative intensity units).

4 DISCUSSION

The Lqs system plays a crucial role for adaptation to environmental changes. Previous studies identified and characterised the *L. pneumophila* sensor kinases LqsS and LqsT, which as components of the QS circuit, regulate a wide range of features (Tiaden *et al.*, 2010b, Kessler *et al.*, 2013). However, detailed information about the interactions of individual components and the control of signal transduction in the Lqs system remained unknown. Therefore, biochemical and structural studies were required for reconstruction of the Lqs QS system *in vitro*.

4.1 Biochemical characterisation of the Lqs system

In this thesis we biochemically analysed the sensor kinases LqsS and LqsT and proved that they undergo autophosphorylation at the conserved histidine residues and interact both with the putative response regulator LqsR. In a process dependent on the conserved aspartate in the receiver domain, LqsR inhibited autophosphorylation of the sensor kinases and catalysed the dephosphorylation of phospho-LqsS and phospho-LqsT. Furthermore, LqsR was phosphorylated by [γ-^{32}P]-acetyl phosphate and dimerised upon phosphorylation.

Both sensor kinases where rapidly autophosphorylated to a different extent; LqsT exhibited a two-fold higher phosphorylation level than LqsS. Autophosphorylation of both sensor kinases were dependent on the conserved histidine residue H200 and H204 (Fig. 14). LqsS-harbouring MVs from wild-type *L. pneumophila* and the Δ*lqsT* strain showed both autophosphorylation to the same extent (Fig. 15). Therefore, we could exclude possible cross autophosphorylation of the two sensor kinases and its contributions to the individual autophosphorylation level of the single sensor kinases. A second band of an unidentified phospho-protein was observed upon autophosphorylation of LqsT-containing membrane vesicles from *L. pneumophila* (Fig. 14) or *E. coli* (Fig. 25). This additional phospho-protein band might represent a C-terminal phospho-LqsT fragment, lacking the N-terminal M45-tag, or LqsT might phosphorylate another unidentified protein which is conserved in both *L. pneumophila* and *E. coli*.

Differential phosphorylation levels of the sensor kinases may represent an intrinsic aspect of the regulatory mechanism of the Lqs system. The *lqsS* and *lqsT* genes are reciprocally expressed in the post-exponential growth phase of *L. pneumophila*, and transcriptome analysis of Δ*lqsS* or Δ*lqsT* mutant strains revealed a reciprocal regulation of up to 90 % of

the genes. Reciprocally regulated genes include components of the Icm/Dot T4SS and a number of Icm/Dot substrates. This supports the notion that the sensor kinases have partially antagonistic regulatory functions in the post exponential growth phase (Kessler *et al.*, 2013). Further characterisation of mutant strains lacking both sensor kinases (Δ*lqsS* and Δ*lqsT*) revealed the lack of phenotype reversion by overexpression of *lqsA*, confirming that *L. pneumophila* does not produce other/additional LAI-1 sensitive histidine kinases than LqsS and LqsT (Kessler *et al.*, 2013). The presence of two homologous sensor histidine kinases with antagonistic roles represents a new characteristic of quorum sensing circuits (Fig. 30).

LqsS and LqsT lack the C-terminal aspartate-containing phospho-receiver domain, which is present in the *Vibrio* spp. hybrid histidine sensor kinase CqsS (Ng *et al.*, 2010, Tiaden *et al.*, 2010a, Tiaden & Hilbi, 2012b). Therefore, phospho-groups are likely transferred from the histidine residue of LqsS and LqsT onto the receiver domain of a phospho-acceptor protein. A possible interaction partner is the putative response regulator LqsR which is encoded in the *lqs* cluster. This matches our observations that, LqsR binds to LqsS as well as to LqsT (Fig. 16) and, dependent on the conserved aspartate in the receiver domain, catalyses dephosphorylation of the sensor kinases (Fig. 17).

The interaction of HKs with the cognate RR is the basic principle for phosphotransfer in TCSs (Stock *et al.*, 2000). The RR LqsR binds both sensor kinases, yet to a different extent (Fig. 16). QS genes encoding a sensor kinase and the cognate response regulator are usually located in close vicinity of each other in the bacterial genome. The apparently higher binding strength suggests an evolutionally conserved functional correlation between LqsS and LqsR as they exist together within the *lqs* gene cluster, while LqsT is located as an “orphan” in the genome (Tiaden & Hilbi, 2012a, Lerat & Moran, 2004). Moreover, the participation of an additional accessory *L. pneumophila* protein that is required for the binding of LqsS or LqsT to LqsR seems rather unlikely. Due to the fact that the sensor kinases need to be overproduced to be detected in the pull-down assay, this putative factor would have to be active at sub-stoichiometric amounts. Besides, since LqsR precipitated both sensor kinases, the factor would either have to interact with both sensor kinases or two distinct factors would have to be bound by LqsR. Finally, LqsR inhibited the autophosphorylation of LqsT by catalysing the dephosphorylation of phospho-LqsT also in membrane vesicles derived from *E. coli* (Fig. 25). Thus, if binding/dephosphorylation activity would require additional factors, they would have to be conserved in *E. coli* as well.

By using the high-energy molecule acetyl phosphate we proved the phosphorylation capability of purified LqsR (Fig. 19A), its dependence on the conserved aspartate residue D108 (Fig. 17, Fig. 18 and Fig. 19B), estimated the half-life of phospho-LqsR and also demonstrated the influence of phosphorylation on oligomerisation of the RR (Fig. 19). Though, we failed in repeated attempts to demonstrate a direct phospho-transfer from the phosphorylated sensor kinases to the RR. It is possible that the phospho-transfer activity of LqsS and LqsT is very slow and/or the kinases exhibit a high intrinsic phospho-LqsR phosphatase activity, as demonstrated for other sensor histidine kinases (Stock *et al.,* 2000). Furthermore, also other proteins present in the MV membrane might act as auxiliary phosphatases and thereby shorten the half-life of phospho-LqsR to an extent that phosphotransfer from the histidine sensor kinases to LqsR cannot be observed anymore. Interestingly, the dose-dependent inhibition of sensor kinase autophosphorylation (Fig. 17) and catalysis of dephosphorylation (Fig. 18) required to a large extent the conserved aspartate residue (D108) in the receiver domain of the RR. It is therefore likely that the underlying mechanism of LqsR activity might be phospho-transfer to D108 rather than phosphatase/hydrolase activity of the phosphorylated HKs. Binding of LqsR to the phosphorylated sensor kinases might also promote conformational changes of the HK and thereby promote phosphate hydrolysis, as the $LqsR_{D108N}$ mutant protein that cannot accept the phosphate group still showed residual dephosphorylation activity (Fig. 18 and Fig. 25).

Upon phosphorylation by acetyl phosphate homogenously monomeric LqsR quantitatively dimerised (Fig. 19). In addition, phosphorylation-dependent dimerization of LqsR was also promoted by phospho-LqsT, providing a direct functional link between the sensor kinase and the response regulator (Fig. 23 and Fig. 24). Dimeric and monomeric LqsR were separated by analytical gel filtration (Fig. 23). Interestingly, compared to negative controls, Western blot analysis of column fractions corresponding to the LqsR dimer not only revealed more LqsR of an apparent molecular weight of 40 kDa, but also an additional band at 80 kDa (Fig. 24). This band was observed upon incubation of LqsR with LqsT/ATP or with acetyl phosphate, but neither upon incubation of $LqsR_{D108N}$ with LqsT/ATP nor of LqsR with $LqsT_{H204Q}$/ATP. The 80 kDa band may represent dimeric LqsR generated by phosphotransfer form phosphorylated LqsT to the aspartate residue of the RR. Remarkably, the dimer formation seems to be so stable that it is preserved even under the reducing SDS-PAGE conditions.

Phosphorylation-induced conformational changes, e.g. dimerisation or oligomerisation, are a characteristic feature of response regulators in TCS. Yet, the output domains of RRs exhibit a

high structural and functional diversity optimized for various effector functions such as DNA-binding, expression of enzymatic functions (Galperin, 2010, Gao & Stock, 2010, Gao *et al.*, 2007) (Section 1.1.2.2). Whereas the output domain of LqsR does not show any similarity to known domains, the observed dimerization of the protein is in agreement with the notion that it is a response regulator, and thus, LqsR represents a prototypic member of a novel family of response regulators.

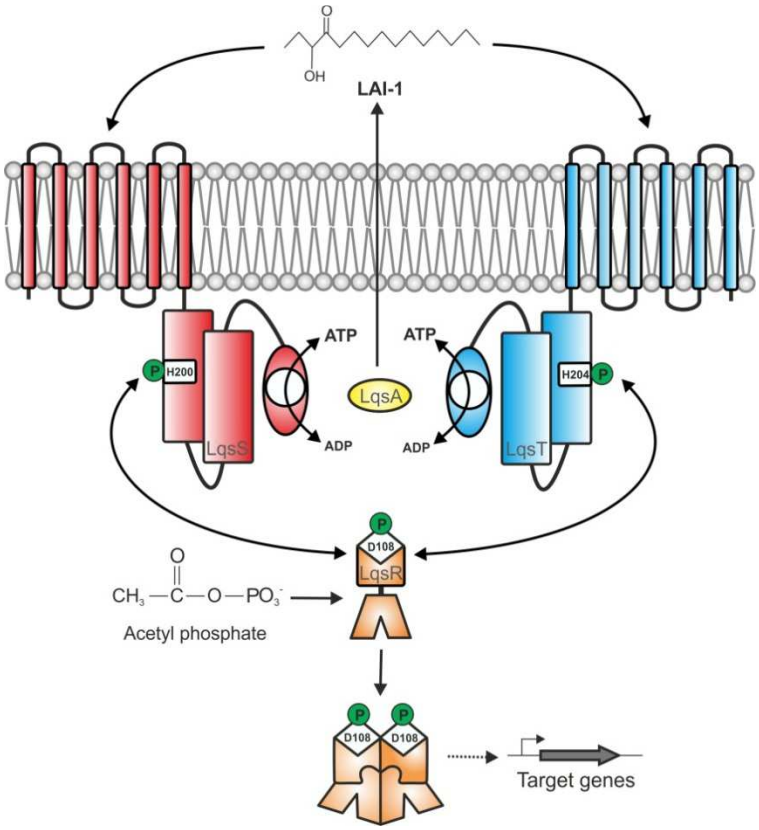

Fig. 30: The *L. pneumophila* Lqs system and the LAI-1 signalling circuit. The *L. pneumophila* Lqs (*Legionella* quorum sensing) system consists of the autoinducer synthase LqsA producing the α-hydroxyketone signalling molecule LAI-1 (*Legionella* autoinducer-1), the homologous sensor kinases LqsS and LqsT, and the prototypic response regulator LqsR. Phosphorylation converges on LqsR, resulting in dimerization of phospho-LqsR.

Environmental bacteria, including *Legionella*, *Polaromonas*, *Burkholderia*, *Nitrococcus* and *Vibrio* spp., contain quorum sensing systems homologous to Lqs/Cqs (Tiaden *et al.*, 2010a, Hornung *et al.*, 2013) (Section 4.2). The *lqs* cluster system is the only one which additionally encodes an HdeD homologue of unknown function (Tiaden *et al.*, 2008). The sensor kinase adjacent to *hdeD*, *lqsS*, is not autophosphorylated upon ectopic expression in *E. coli*, in contrast to the orphan gene product LqsT (Fig. 25). Perhaps, LqsS requires an additional

L. pneumophila factor such as HdeD to become autophosphorylated. However, our preliminary attempts to show an effect of HdeD on autophosphorylation of LqsS were not successful.

In summary, we identified LqsS and LqsT as sensor histidine kinases, which both interact with and are dephosphorylated by LqsR, a putative response regulator that dimerises upon phosphorylation. Our investigations form the basis for further biochemical characterisation of the unique Lqs system.

4.2 LqsR structure analysis

Homologues of the *lqsR* gene, present in the *L. pneumophila lqs* cluster, can be found in the genomes of four further genera of environmental bacteria, *Nitrococcus*, *Burkholderia*, *Polaromonas* and *Ralstonia* (Tiaden *et al.*, 2007) (Fig. 31). The clustering of the autoinducer synthase, sensor kinase and response regulator is conserved in bacterial species that harbour an LqsR homologue, suggesting an evolutionarily conserved functional correlation between the *lqsR*, *lqsA* and *lqsS* genes (Tiaden *et al.*, 2010a, Tiaden *et al.*, 2010b).

LqsR harbours a signal receiver domain in the N-terminal part (amino acid 77-157) which is homologous to the RR CheY from *E. coli* (Fig. 31). Receiver domains of RRs are conformationally dynamic, and phosphorylation shifts the equilibrium from an inactive to a primary active conformation. In case of LqsR, phosphorylation during signal transduction at the conserved aspartate residue, D108, promotes the formation of a homodimer, which is probably the active form of the RR. Phosphorylation of the RR does usually not substantially change the secondary structure, rather the secondary structure elements are slightly repositioned, causing backbone deviations of a few angstroms. However, these changes dramatically affect the molecular surface, altering both topological and electrostatic features (Stock *et al.*, 2000). The phosphorylation-induced conformational changes provide a molecular surface in the regulatory domain of RR proteins for multiple protein-protein interactions with HKs, auxiliary phosphatases or components of the transcriptional machinery (Stock & Guhaniyogi, 2006). The LqsR receiver domain contains a putative intermolecular recognition site (5 amino acids; MP[X]MDG) for the recognition of corresponding unknown interaction partners (Fig. 31). LqsR contains a further conserved residue, K157, which is homologous to one of three residues (KPV) in the α4-β5-α5 interdomain surface of CheY. These conserved amino acids commonly represent the locus of the largest differences between inactive and active conformations, providing the surface that

mediates dimerisation of receiver domains in the active state of CheY, and other RRs such as DrrB, DrrD, and MtrA (Barbieri *et al.*, 2010). Thus, this region could indicate a possible dimerisation site in LqsR.

The C-terminal part of LqsR does not show any similarity to known domains, like the DNA-binding helix-turn-helix motif, and thus, LqsR represents a prototypic member of a novel family of response regulators.

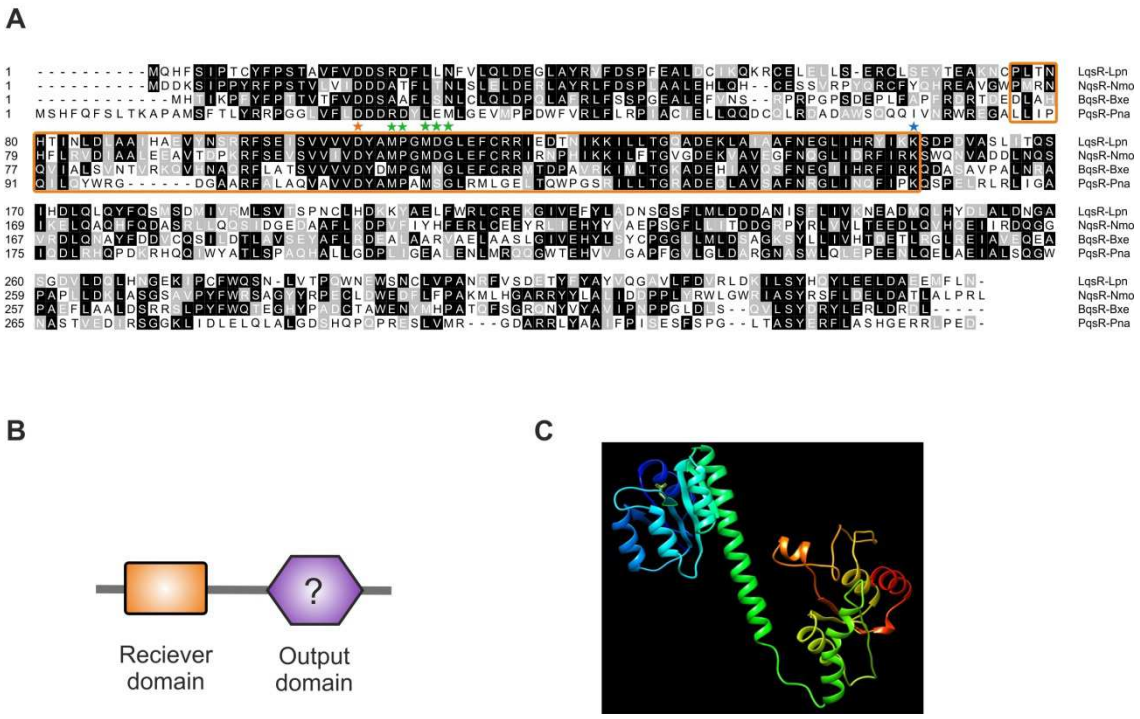

Fig. 31: Structural analysis of the *L. pneumophila* response regulator LqsR. A. Alignment of LqsR homologues. LqsR from *Legionella pneumophila* (LqsR-Lpn), *Nitrococcus mobilis* (NqsR-Nmo), *Burkholderia xenovorans* (BqsR-Bxe) and *Polaromonas naphtalenivorans* (PqsR-Pna) were aligned using the CLUSTALW algorithm. Identical amino acids are shaded in black. The conserved aspartic acid residue, D108, in LqsR (orange star), the intermolecular recognition site (green stars) and the dimerization interface (blue star) are marked within the response regulator receiver domain identified by the NCBI conserved domain database. **B.** Structural organisation of LqsR. **C.** Putative 3D structure design of LqsR using UCSF Chimera program.

4.3 Influence of LAI-1 on QS signalling

In *L. pneumophila*, QS signalling relies on two sensor kinases, LqsS and LqsT, which sense the autoinducer LAI-1 and whose signal transduction pathway converges on the RR LqsR (Section 4.1). We were able to reconstruct the Lqs phosphorylation pathway *in vitro* using

inverted membrane vesicles containing LqsS and LqsT derived from *L. pneumophila,* together with the purified downstream RR LqsR (Section 4.1).

As the structure of LAI-1 is known, LAI-1 and a set of ligands including Amino-LAI-1 as well as CAI-1 and Amino-CAI-1 were produced as either racemic mixtures or as enantiopure compounds (Dr. D. Trauner, Dr. C. Hedberg, Dr. J. Schulz).

Thus, we discovered that LAI-1 binding regulates to a similar extent the autophosphorylation of the sensor kinases LqsS and LqsT at H200 and H204 respectively (Fig. 26A and B), and that this inhibition was dose dependent (Fig. 26C). Furthermore, LAI-1 has an inhibiting effect on the kinase activities of LqsS and LqsT, since autophosphatase activity and LqsR-dependent dephosphorylation were not affected by ligand binding (Fig. 28 and Fig. 29).

Autophosphorylation of HKs in our experiments were never completely impaired by LAI-1 at any time or concentration (Fig. 26), in agreement with the assumption that the AI does not act as a switch for the on/off state of LqsS and LqsT, but rather as a fine-tuning compound for the initial autophosphorylation and subsequent phospho-LqsR concentration in the signal transduction cascade. During the transition form low to high cell density the altered autophosphorylation activity affects phospho-LqsR generation, in turn regulating the transcription of target genes and QS-related processes (Tiaden *et al.,* 2007, Kessler *et al.,* 2013) (Fig. 32). Thus, rapid phosphorylation of LqsR is expected to occur when LAI-1 disappears (Fig. 32). This AI inhibitory mechanism of the senor kinases is consistent with previous results of the QS system in *Vibrio* spp., where CAI-1 regulates the CqsS receptor (Wei *et al.,* 2012, Henke & Bassler, 2004). It further suggests that the ligand binding induces a conformational change in the sensor kinases which alters the interaction between the CA and DHP domains (Stock *et al.,* 2000, Neiditch *et al.,* 2006, Borkovich & Simon, 1990) (Section 1.1.2.1). Thus, the presence of LAI-1 impairs new rounds of autophosphorylation of LqsS and LqsT.

Our experiments revealed a constant, but slow autophosphatase activity of phospho-LqsR which showed a half-life of 6 hours (Fig. 19). This dephosphorylation ensures the reduction and thereby inactivation of phospho-LqsR. In general, half-lives of RRs correlate with their function in cellular processes, e.g. the chemotaxis regulator CheY in *E. coli* has half-life of only 10 seconds, as bacteria have to change their orientation quite rapidly (Lukat *et al.,* 1992, Stock *et al.,* 1991), whereas RRs that control long-term processes such as antibiotic resistance show a much higher phospho-stability e.g. VanR in *E. coli* has an estimated half-life up to 13 hours (Haldimann *et al.,* 1997). LqsR triggers the transition from the replicative to the transmissive growth phase of *L. pneumophila* (Section 1.2.1), thus for this scheduled

process a very short-lived phospho-RR would be obstructive, whereas a persistent phospho-RR is permissive.

The model we postulate for the Lqs system is based on our biochemical studies and quantitative real-time (qRT)-PCR data (Kessler *et al.*, 2013). It compromises three states corresponding to three different growth phases (Fig. 32). In the early exponential growth phase (OD_{600} 0.5-2) *lqsR* expression starts time-delayed before *lqsS*, whereas the *lqsT* expression stays constant. Under these conditions mainly LqsT is present and may phosphorylate LqsR to a low level. Thus, LqsR is mainly inactive and replicative traits of *L. pneumophila* are promoted (Fig. 32).

At the beginning of the post exponential growth phase (OD_{600} 2-3) AI-synthase production and consequently also the LAI-1 concentration is low. As the inhibition of the sensor kinases is dose-dependent (Fig. 26C), both sensor kinases may be autophosphorylated to a relatively high level and transfer phospho-groups onto LqsR. In addition, the expression of *lqsS* and *lqsT* are negatively correlated. As LqsS concentration is high and the sensor kinase binds LqsR with a higher affinity than LqsT (Fig. 16) it is likely that more phospho-groups are transmitted to LqsR by the sensor kinase LqsS (Fig. 32). Although *lqsT* expression is six-fold lower than *lqsS*, LqsT is stronger autophosphorylated than LqsS (Fig. 14) and therefore may also contribute to LqsR phosphorylation. In the late post exponential growth phase (OD_{600} 3-4) LqsS and LqsR levels decrease, but with the long half-life of phospho-LqsR, the amount of active LqsR is maximised and stays constant, inducing the expression of *L. pneumophila* virulence traits (Fig. 32). In the stationary growth phase the LAI-1 level maximises, which triggers the inhibition of new rounds of sensor kinase autophosphorylation (Fig. 26), leading to progressive inactivation of the RR due to its own phosphatase activity (Fig. 32).

To see an effect on the QS system *in vitro*, relatively high amounts of LAI-1 (500 µM) were needed (Fig. 26-29), while in *in vivo* experiments low, physiologically relevant, concentrations between 10-50 µM LAI-1 showed an effect on *L. pneumophila* (Aline Kessler, unpublished data). This might be due to the accessibility of the sensor kinases, as in our *in vitro* system the sensory domain lies inside the inverted membrane vesicles. Although LAI-1 is a small molecule it is quite hydrophobic, which may lead to only a fraction transiting the membrane to reach the sensory domains. As other mechanical treatments of the MVs together with LAI-1 (freezing and thawing, sonication) did not increase the inhibitory effect on the autophosphorylation of the sensor kinases, it may also be the lack of sensitivity due to an artificial system. Since other publications, such as Wei *et al.*, 2012 utilized the

same ligand concentration (500 µM CAI-1) for the *V. cholerae* Cqs QS system *in vitro*, it seems to be less an AI-specific feature than a common experimental problem.

We further analysed the ligand receptor specificities of the LqsS and LqsT sensor kinases in detail (Fig. 27). The results suggested that the tested LAI-1-related autoinducers decreased the phosphorylation of LqsT and, to a lesser extent, LqsS, whereas CAI-1-derived autoinducers have an antagonistic effect on LqsS. Indeed, the differences between LqsS and LqsT seem to reflect their function as, at least partly, antagonistic sensors (Kessler *et al.*, 2013). The *lqsT* and *lqsS* genes are reciprocally expressed in the post-exponential growth phase of *L. pneumophila*, and 90 % of the genes that are up-regulated in absence of *lqsS* are down-regulated in *L. pneumophila* lacking *lqsT*. The existence of two homologous sensor histidine kinases with antagonistic roles represents a novel feature of quorum sensing circuits. As phosphorylation signalling through these antagonistic sensor kinases converges on LqsR, the kinases probably bind and interact differently with the response regulator, signal to common as well as diverse response regulators, and respond contrarily to agonists and antagonists.

A detailed description of a complex regulatory system like the QS system of *L. pneumophila* requires information of every individual component, including its spatial and temporal organization. Each component in the system represents an individual protein module, any one of which can exist in several different states. Interactions between the components occur continuously; interference at any point can change the average state of a given component over time. Our biochemical studies provide molecular details for the reconstruction of the Lqs system *in vitro*. Among QS systems the organization of Lqs system seems to be unique as it includes two homologous sensor kinases, LqsS and LqsT (Kessler *et al.*, 2013). In *L. pneumophila*, phosphorylation signalling through these histidine kinases converges on LqsR. Analogously, in *Vibrio* spp. two or even three different autoinducer systems based on a acyl-homoserine lactone, the furanosyl borate diester AI-2, and an α-hydroxyketone signal, converge on LuxO to regulate virulence and other traits (Miller *et al.*, 2002, Henke & Bassler, 2004, Ng & Bassler, 2009, Tiaden *et al.*, 2010a). As *L. pneumophila* apparently does not to produce the commonly known acyl-homoserine lactones (AHL) or the furanosyl-borate-diester (AI-2), and only uses the AHK signalling molecule LAI-1, a unique and more complex regulatory mechanism might be required to integrate distinct α-hydroxyketone signals, to accommodate different signalling thresholds, and/or to promote antagonistic responses.

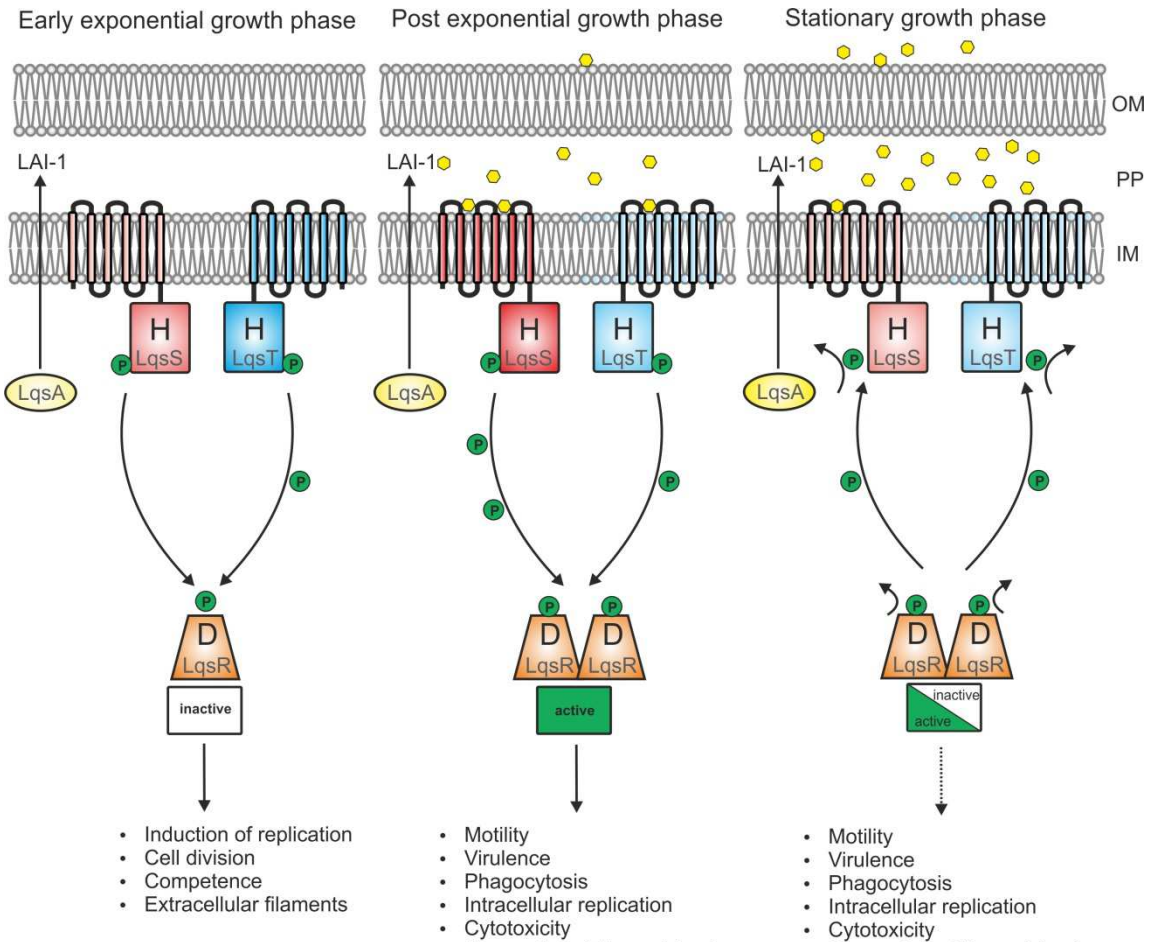

Fig. 32: *L. pneumophila* Lqs QS system at early, post exponential and stationary growth phase. At low cell density, the response regulator LqsR is mainly inactive promoting *L. pneumophila* replicative traits and competence. At post exponential growth phase both sensor kinases LqsS and LqsT, phosphorylate LqsR, causing dimerisation and activation of the RR. This triggers expression of *L. pneumophila* virulence traits. At stationary growth phase the phospho-LuxR concentration slowly decreases as autophosphorylation of the sensor kinase reduces upon interaction with LAI-1 and due to LqsR phosphatase activity, OM = outer membrane, PP = periplasm, IM = inner membrane.

4.4 Lqs cluster distribution

The *Legionella* quorum sensing (*lqs*) cluster was identified via a bioinformatic analysis of the *L. pneumophila* genome, screening for homologues of the *Vibrio cholerae* quorum sensing system *cqsAS* (Tiaden *et al.*, 2007). It is present in all *L. pneumophila* strains sequenced to date: Philadelphia (*lpg2731* to *lpg2734*), Paris (*lpp2787* to *lpp2790*), Lens (*lpl2656* to *lpl2659*), and Corby (*lpc0402-lpc0401-lpc0399-lpc0396*) (Tiaden *et al.*, 2008). The Lqs cluster comprises four genes (*lqsA-lqsR-hdeD-lqsS*), of which three are involved in the

composition of the signal transduction pathway while the function of HdeD in the Lqs system or elsewhere remains still unknown (Tiaden *et al.*, 2007). Each gene in the Lqs cluster is expressed under control of its own promoter. The clustering and orientation of the *lqsA-lqsR-lqsS* genes are conserved among different bacterial species, and therefore indicate a functional relationship of the individual genes (Tiaden *et al.*, 2007) (Fig. 33).

The *lqs* cluster of *L. pneumophila* Philadelphia is flanked by several genes encoding components of the bacterial inner membrane respiratory chain. The 5´ upstream region harbours *dsbH* (*lpg2729*), encoding a putative disulfide oxidoreductase and *cycB* (*lpg2730*), encoding cytochrome c5. The 3´ downstream region contains an operon that includes the four ORFs *lpg2735* to *lpg2738*, encoding the heme biosynthesis proteins porphobilinogen deaminase (*hemC*), uroporphyrinogen III synthetase (*hemD*), uroporphyrinogen III methylase (*hemX*), and protoporphyrinogen IX and coproporphyrinogen III oxidase (*hemY*) (Tiaden *et al.*, 2008).

In contrast to *L. pneumophila* with its single Lqs system, *V. cholerae* possesses two QS systems, *cqsAS* and *luxPQS*. Accordingly, *V. cholerae* produces two autoinducers CAI-1 and AI-2. CAI-1 is made by all *Vibrios* and thus is used for intra-genera communication. By contrast, AI-2 is made and detected by a vast array of bacterial species, and thus, AI-2 is used for inter-genera communication (Bassler & Losick, 2006). As LAI-1 is only produced by *L. pneumophila* it seems likely that LAI-1 is used for intra-species communication. The differential production of AIs might reflect the evolutionary adaption to distinct lifestyles of *Legionella* and *Vibrio*. While *Legionella* spp. can colonize extracellular niches such as multi-species biofilms, their preferential environmental niche is, as an intracellular pathogen, the amoebal fauna of biofilms (Hilbi *et al.*, 2011b). Consequently, intracellular *L. pneumophila* might avoid the competitors which would normally be encountered in nutrition-rich extracellular ecological niches, and accordingly, may have lost or never acquired QS systems that support broad inter-species communication (Tiaden & Hilbi, 2012b). This is in agreement with the hypothesis that inter-species communication systems are not essential for intracellular bacteria, as no species other than *L. pneumophila* possess the *lqs* system (Spirig *et al.*, 2008a, Cazalet *et al.*, 2010, Tiaden *et al.*, 2010a).

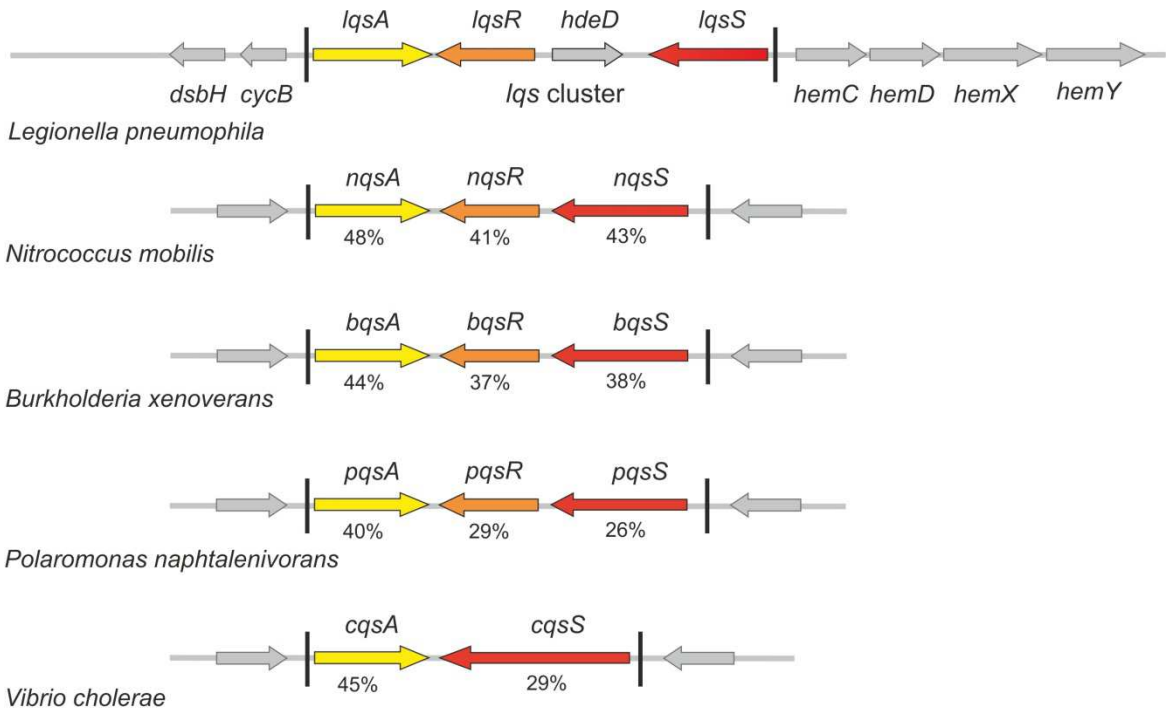

Fig. 33 : Alignment of homologous quorum sensing gene clusters. QS gene clusters form *L. pneumophila* (*lqs, Legionella* quorum sensing), *N. mobilis, B. xenoverans, P. naphtalenivorans* and *V. cholerae* are shown. The percent identity with the corresponding *L. pneumophila* proteins is indicated. Model adapted from previous publication (Tiaden *et al.*, 2007).

The *lqs* genes are located in a genomic region of *L. pneumophila* that seems to represent a hot spot for recombination, as the corresponding site in *L. longbeachae* harbours a homologue of the putative effector gene *legG2* (*llo0327, lpg0267*). This gene is flanked by transposase elements and localizes upstream of a homologue of *E. coli hdeD* (Tiaden & Hilbi, 2012b). *L. pneumophila* likely acquired the *lqsA-lqsR-lqsS* cluster by horizontal gene transfer from other environmental bacteria, such as *Nitrococcus*, *Burkholderia* or *Polaromonas* spp., which also harbour gene cluster homologues of *lqsA* and *lqsS* (Tiaden & Hilbi, 2012b, Tiaden *et al.*, 2007) (Fig. 33). Interestingly *L. pneumophila* is the only bacterium where an additional gene, *hdeD*, is present in the Lqs cluster, where it interrupts the otherwise convergently transcribed *lqsS* and *lqsR* genes (Tiaden *et al.*, 2007) (Fig. 33). The *lqsS* and *lqsR* genes might originate from a larger *cqsS*-like ancestor gene, which was split by the resident *hdeD* locus by recombinatory events that integrated a *cqs*-like cassette into the *L. pneumophila* genome (Tiaden & Hilbi, 2012b). The prevalence of putative AI synthases and sensors suggests that intra- and inter-species AHK-based cell-cell communication is wide-spread among bacteria.

4.5 Quorum sensing inhibition

Quorum sensing is a well documented, wide-spread mechanism for gene regulation and coordination of bacterial behaviour on population level, used by bacterial pathogens for regulation of invasion, defence and spread. Due to the ongoing evolution of antibiotic-resistant pathogens, the interest in alternative therapeutic strategies has increased immensely over the last years. Identification of autoinducers that regulate QS-dependent processes, and also chemical compounds and enzymes that promote quorum sensing inhibition (QSI), could be used to manipulate bacterial processes, especially pathogenic traits that endanger human health and productivity of animal breeding and agriculture (Fig. 34). As all QS systems have been found to share three processes; namely signal production, signal accumulation and signal detection, QSIs can target at least one of these (LaSarre & Federle, 2013). Blocking cellular communication by QSI serves as an effective tactic to disrupt cooperative actions of bacterial populations.

One of the most-studied QSI strategies is the degradation of autoinducer molecules through enzymatic inactivation. Quorum quenching enzymes are especially known to target the class of acylhomoserinelactones (AHL), inactivating them by enzymatic cleavage. The three known classes of AHL cleaving enzymes are lactonases, acylases and oxidoreductases (Dong *et al.*, 2001).

In addition to enzymatic inactivation, signal sequestration by antibodies has been investigated recently. Here, monoclonal antibodies targeting the *Pseudomonas aeruginosa* AHL 3oxo-C_{12}-HSL inhibited QS signalling *in vitro*, suggesting a therapeutic potential for this approach (Kaufmann *et al.*, 2006). Antibody-complex formation of QS molecules might be of therapeutic benefit by targeting two routes, i.e. by inhibiting activation of QS cascades and the production of virulence factors, and/or by preventing AHL-induced host cell cytotoxicity (Kaufmann *et al.*, 2008).

Furthermore, the use of AI antagonists can inhibit signal detection and thus the induction of QS-regulated processes such as biofilm formation and virulence traits (Lyon *et al.*, 2002). The underlying principle involves the production of compounds whose structures diverge from the natural AI, but still bind to the corresponding sensory receptors of pathogens. This generates non productive signal-receptor complexes and thus blocks the downstream QS pathway. For AIs such as AHL of *P. aeruginosa*, molecules that show no obvious similarity to the AI were identified by *in silico* analysis, which bind to the same protein pocket as the AHL (Muh *et al.*, 2006).

An alternative and less studied approach is the inhibition of AI synthesis. Suppression of AI production could theoretically be achieved either through suppression of the substrate biosynthesis pathway, or via inactivation of a synthase enzyme (LaSarre & Federle, 2013). In the Gram-negative pathogenic bacterium *Burkholderia glumae* the AHL signalling molecule is produced by the AI synthase TofI. Here a strong inhibitor, J8-C8, was identified which competitively occupyied the binding site for the acyl chain of the TofI cognate substrate, acylated acyl-carrier protein (Chung *et al.*, 2011).

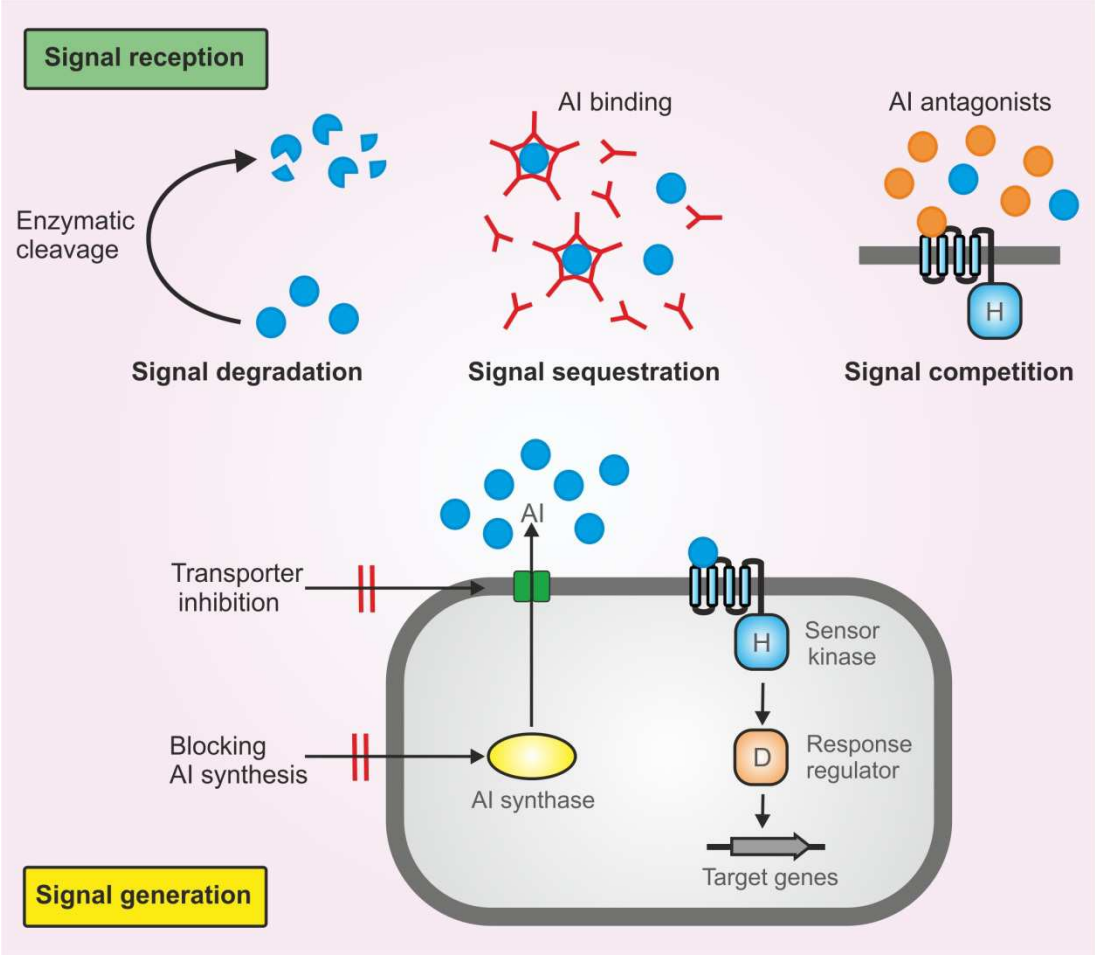

Fig. 34: Overview of quorum sensing inhibition strategies. Signal transduction in QS systems depends on autoinducer molecules (AI) and can be disturbed by either blocking the signal generation or inhibiting the signal reception. The AI production can be directly blocked by inhibition of the AI synthesis pathway. Furthermore the release of AIs, which can not diffuse through the bacterial membrane, can be inhibited by inactivation of the corresponding transporter. QS-active bacteria are susceptible for quorum quenching mechanisms like enzymatic inactivation of autoinducers (AI), signal sequestration through antibodies and inhibition of bacterial signal detection by AI antagonists. Model adapted from previous publication (Whitehead *et al.*, 2001).

Since the initial discovery of quorum sensing over 40 years ago, the fundamental understanding of QS systems has increased significantly. New QS systems and signal molecules have been identified, additional information about the mechanistic mode of operation of QS systems has emerged, and the potential of quorum sensing inhibitors has been discovered. Prophylactic antibiotic use in humans, as well as the general misuse of antibiotics in livestock, have certainly contributed to increasing bacterial resistance rates (Bartlett *et al.*, 2013), and the pharmaceutical and biotechnology companies mostly lack novel antibiotic compounds (Bush, 2012). However, as antibiotic resistance development is unavoidable, it might be the time for consideration of new antimicrobial strategies directed at the neutralization of virulence mechanisms rather that directly targeting the viability of pathogens (Zhu & Kaufmann, 2013). Quorum quenching strategies might become an effective alternative either as a single therapeutic approach or in combination with antibiotics for infection prevention and contagion treatment.

LITERATURE

Alli, O. A., L. Y. Gao, L. L. Pedersen, S. Zink, M. Radulic, M. Doric & Y. Abu Kwaik, (2000) Temporal pore formation-mediated egress from macrophages and alveolar epithelial cells by *Legionella pneumophila*. *Infection and immunity* **68**: 6431-6440.

Amodeo, M. R., D. R. Murdoch & A. D. Pithie, (2010) Legionnaires' disease caused by *Legionella longbeachae* and *Legionella pneumophila*: comparison of clinical features, host-related risk factors, and outcomes. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* **16**: 1405-1407.

Anand, G. S., P. N. Goudreau & A. M. Stock, (1998) Activation of methylesterase CheB: evidence of a dual role for the regulatory domain. *Biochemistry* **37**: 14038-14047.

Bachman, M. A. & M. S. Swanson, (2001) RpoS co-operates with other factors to induce *Legionella pneumophila* virulence in the stationary phase. *Molecular microbiology* **40**: 1201-1214.

Barbieri, C. M., T. R. Mack, V. L. Robinson, M. T. Miller & A. M. Stock, (2010) Regulation of response regulator autophosphorylation through interdomain contacts. *The Journal of biological chemistry* **285**: 32325-32335.

Bartlett, J. G., D. N. Gilbert & B. Spellberg, (2013) Seven ways to preserve the miracle of antibiotics. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **56**: 1445-1450.

Bassler, B. L., (1999) How bacteria talk to each other: regulation of gene expression by quorum sensing. *Current opinion in microbiology* **2**: 582-587.

Bassler, B. L. & R. Losick, (2006) Bacterially speaking. *Cell* **125**: 237-246.

Bassler, B. L., M. Wright, R. E. Showalter & M. R. Silverman, (1993) Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. *Molecular microbiology* **9**: 773-786.

Bassler, B. L., M. Wright & M. R. Silverman, (1994) Sequence and function of LuxO, a negative regulator of luminescence in *Vibrio harveyi*. *Molecular microbiology* **12**: 403-412.

Bellinger-Kawahara, C. & M. A. Horwitz, (1990) Complement component C3 fixes selectively to the major outer membrane protein (MOMP) of *Legionella pneumophila* and mediates phagocytosis of liposome-MOMP complexes by human monocytes. *J Exp Med* **172**: 1201-1210.

Benson, R. F. & B. S. Fields, (1998) Classification of the genus *Legionella*. *Seminars in respiratory infections* **13**: 90-99.

Bilwes, A. M., L. A. Alex, B. R. Crane & M. I. Simon, (1999) Structure of CheA, a signal-transducing histidine kinase. *Cell* **96**: 131-141.

Blom, N., S. Gammeltoft & S. Brunak, (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *Journal of molecular biology* **294**: 1351-1362.

Borkovich, K. A. & M. I. Simon, (1990) The dynamics of protein phosphorylation in bacterial chemotaxis. *Cell* **63**: 1339-1348.

Brassinga, A. K., J. M. Kinchen, M. E. Cupp, S. R. Day, P. S. Hoffman & C. D. Sifri, (2010) *Caenorhabditis* is a metazoan host for *Legionella*. *Cellular microbiology* **12**: 343-361.

Bush, K., (2012) Improving known classes of antibiotics: an optimistic approach for the future. *Current opinion in pharmacology* **12**: 527-534.

Carratala, J. & C. Garcia-Vidal, (2010) An update on *Legionella. Current opinion in infectious diseases* **23**: 152-157.

Cazalet, C., L. Gomez-Valero, C. Rusniok, M. Lomma, D. Dervins-Ravault, H. J. Newton, F. M. Sansom, S. Jarraud, N. Zidane, L. Ma, C. Bouchier, J. Etienne, E. L. Hartland & C. Buchrieser, (2010) Analysis of the *Legionella longbeachae* genome and transcriptome uncovers unique strategies to cause Legionnaires' disease. *PLoS genetics* **6**: e1000851.

Chen, J., K. S. de Felipe, M. Clarke, H. Lu, O. R. Anderson, G. Segal & H. A. Shuman, (2004) *Legionella* effectors that promote nonlytic release from protozoa. *Science* **303**: 1358-1361.

Chung, J., E. Goo, S. Yu, O. Choi, J. Lee, J. Kim, H. Kim, J. Igarashi, H. Suga, J. S. Moon, I. Hwang & S. Rhee, (2011) Small-molecule inhibitor binding to an N-acyl-homoserine lactone synthase. *Proceedings of the National Academy of Sciences of the United States of America* **108**: 12089-12094.

de Kievit, T. R. & B. H. Iglewski, (2000) Bacterial quorum sensing in pathogenic relationships. *Infection and immunity* **68**: 4839-4849.

Dong, Y. H., L. H. Wang, J. L. Xu, H. B. Zhang, X. F. Zhang & L. H. Zhang, (2001) Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. *Nature* **411**: 813-817.

Dutta, R., L. Qin & M. Inouye, (1999) Histidine kinases: diversity of domain organization. *Molecular microbiology* **34**: 633-640.

Engebrecht, J., K. Nealson & M. Silverman, (1983) Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. *Cell* **32**: 773-781.

Engebrecht, J. & M. Silverman, (1984) Identification of genes and gene products necessary for bacterial bioluminescence. *Proceedings of the National Academy of Sciences of the United States of America* **81**: 4154-4158.

Faucher, S. P., C. A. Mueller & H. A. Shuman, (2011) *Legionella pneumophila* transcriptome during intracellular multiplication in human macrophages. *Front Microbiol* **2**: 60.

Feeley, J. C., R. J. Gibson, G. W. Gorman, N. C. Langford, J. K. Rasheed, D. C. Mackel & W. B. Baine, (1979) Charcoal-yeast extract agar: primary isolation medium for *Legionella pneumophila*. *J Clin Microbiol* **10**: 437-441.

Fields, B. S., (1996) The molecular ecology of *Legionellae*. *Trends in microbiology* **4**: 286-290.

Fields, B. S., R. F. Benson & R. E. Besser, (2002) *Legionella* and Legionnaires' disease: 25 years of investigation. *Clinical microbiology reviews* **15**: 506-526.

Fraser, D. W., T. R. Tsai, W. Orenstein, W. E. Parkin, H. J. Beecham, R. G. Sharrar, J. Harris, G. F. Mallison, S. M. Martin, J. E. McDade, C. C. Shepard & P. S. Brachman, (1977) Legionnaires' disease: description of an epidemic of pneumonia. *The New England journal of medicine* **297**: 1189-1197.

Freeman, J. A. & B. L. Bassler, (1999a) A genetic analysis of the function of LuxO, a two-component response regulator involved in quorum sensing in *Vibrio harveyi*. *Molecular microbiology* **31**: 665-677.

Freeman, J. A. & B. L. Bassler, (1999b) Sequence and function of LuxU: a two-component phosphorelay protein that regulates quorum sensing in *Vibrio harveyi*. *Journal of bacteriology* **181**: 899-906.

Fuqua, C. & E. P. Greenberg, (2002) Listening in on bacteria: acyl-homoserine lactone signalling. *Nat Rev Mol Cell Biol* **3**: 685-695.

Galperin, M. Y., (2010) Diversity of structure and function of response regulator output domains. *Current opinion in microbiology* **13**: 150-159.

Gao, R., T. R. Mack & A. M. Stock, (2007) Bacterial response regulators: versatile regulatory strategies from common domains. *Trends in biochemical sciences* **32**: 225-234.

Gao, R. & A. M. Stock, (2010) Molecular strategies for phosphorylation-mediated regulation of response regulator activity. *Current opinion in microbiology* **13**: 160-167.

Garduno, R. A., E. Garduno & P. S. Hoffman, (1998) Surface-associated hsp60 chaperonin of *Legionella pneumophila* mediates invasion in a HeLa cell model. *Infection and immunity* **66**: 4602-4610.

Groban, E. S., E. J. Clarke, H. M. Salis, S. M. Miller & C. A. Voigt, (2009) Kinetic buffering of cross talk between bacterial two-component sensors. *Journal of molecular biology* **390**: 380-393.

Hägele, S., J. Hacker & B. C. Brand, (1998) *Legionella pneumophila* kills human phagocytes but not protozoan host cells by inducing apoptotic cell death. *FEMS microbiology letters* **169**: 51-58.

Hagele, S., R. Kohler, H. Merkert, M. Schleicher, J. Hacker & M. Steinert, (2000) *Dictyostelium discoideum*: a new host model system for intracellular pathogens of the genus *Legionella*. *Cellular microbiology* **2**: 165-171.

Haldimann, A., S. L. Fisher, L. L. Daniels, C. T. Walsh & B. L. Wanner, (1997) Transcriptional regulation of the *Enterococcus faecium* BM4147 vancomycin resistance gene cluster by the VanS-VanR two-component regulatory system in *Escherichia coli* K-12. *Journal of bacteriology* **179**: 5903-5913.

Hales, L. M. & H. A. Shuman, (1999) The *Legionella pneumophila rpoS* gene is required for growth within *Acanthamoeba castellanii*. *Journal of bacteriology* **181**: 4879-4889.

Hammer, B. K. & M. S. Swanson, (1999) Co-ordination of *Legionella pneumophila* virulence with entry into stationary phase by ppGpp. *Molecular microbiology* **33**: 721-731.

Henke, J. M. & B. L. Bassler, (2004) Three parallel quorum-sensing systems regulate gene expression in *Vibrio harveyi*. *Journal of bacteriology* **186**: 6902-6914.

Heuner, K., J. Hacker & B. C. Brand, (1997) The alternative sigma factor sigma28 of *Legionella pneumophila* restores flagellation and motility to an *Escherichia coli fliA* mutant. *Journal of bacteriology* **179**: 17-23.

Higgins, D. A., M. E. Pomianek, C. M. Kraml, R. K. Taylor, M. F. Semmelhack & B. L. Bassler, (2007) The major *Vibrio cholerae* autoinducer and its role in virulence factor production. *Nature* **450**: 883-886.

Hilbi, H., C. Hoffmann & C. F. Harrison, (2011a) *Legionella* spp. outdoors: colonization, communication and persistence. *Environ Microbiol Rep* **3**: 286-296.

Hilbi, H., C. Hoffmann & C. F. Harrison, (2011b) *Legionella* spp. outdoors: colonization, communication and persistence. *Environ Microbiol Rep* **3**: 286–296.

Hilbi, H., S. Jarraud, E. Hartland & C. Buchrieser, (2010) Update on Legionnaires' disease: pathogenesis, epidemiology, detection and control. *Molecular microbiology* **76**: 1-11.

Hilbi, H., G. Segal & H. A. Shuman, (2001) Icm/Dot-dependent upregulation of phagocytosis by *Legionella pneumophila*. *Molecular microbiology* **42**: 603-617.

Hoffmann, C., C. F. Harrison & H. Hilbi, (2014) The natural alternative: protozoa as cellular models for *Legionella* infection. *Cellular microbiology* **16**: 15-26.

Hornung, C., A. Poehlein, F. S. Haack, M. Schmidt, K. Dierking, A. Pohlen, H. Schulenburg, M. Blokesch, L. Plener, K. Jung, A. Bonge, I. Krohn-Molt, C. Utpatel, G. Timmermann, E. Spieck, A. Pommerening-Roser, E. Bode, H. B. Bode, R. Daniel, C. Schmeisser & W. R. Streit, (2013) The *Janthinobacterium* sp. HH01 genome encodes a homologue of the *V. cholerae* CqsA and *L. pneumophila* LqsA autoinducer synthases. *PLoS One* **8**: e55045.

Horwitz, M. A., (1983) The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. *J Exp Med* **158**: 2108-2126.

Huala, E., J. Stigter & F. M. Ausubel, (1992) The central domain of *Rhizobium leguminosarum* DctD functions independently to activate transcription. *Journal of bacteriology* **174**: 1428-1431.

Hubber, A. & C. R. Roy, (2010) Modulation of host cell function by *Legionella pneumophila* type IV effectors. *Annu Rev Cell Dev Biol* **26**: 261-283.

Isberg, R. R., T. J. O'Connor & M. Heidtman, (2009) The *Legionella pneumophila* replication vacuole: making a cosy niche inside host cells. *Nature reviews. Microbiology* **7**: 13-24.

Jin, Z., S. Romero-Steiner, G. M. Carlone, J. B. Robbins & R. Schneerson, (2007) *Haemophilus influenzae* type a infection and its prevention. *Infection and immunity* **75**: 2650-2654.

Jung, K., T. Odenbach & M. Timmen, (2007) The quorum-sensing hybrid histidine kinase LuxN of *Vibrio harveyi* contains a periplasmically located N terminus. *Journal of bacteriology* **189**: 2945-2948.

Kaufmann, A. F., J. E. McDade, C. M. Patton, J. V. Bennett, P. Skaliy, J. C. Feeley, D. C. Anderson, M. E. Potter, V. F. Newhouse, M. B. Gregg & P. S. Brachman, (1981) Pontiac fever: isolation of the etiologic agent (*Legionella pneumophilia*) and demonstration of its mode of transmission. *American journal of epidemiology* **114**: 337-347.

Kaufmann, G. F., J. Park, J. M. Mee, R. J. Ulevitch & K. D. Janda, (2008) The quorum quenching antibody RS2-1G9 protects macrophages from the cytotoxic effects of the *Pseudomonas aeruginosa* quorum sensing signalling molecule N-3-oxo-dodecanoyl-homoserine lactone. *Molecular immunology* **45**: 2710-2714.

Kaufmann, G. F., R. Sartorio, S. H. Lee, J. M. Mee, L. J. Altobell, 3rd, D. P. Kujawa, E. Jeffries, B. Clapham, M. M. Meijler & K. D. Janda, (2006) Antibody interference with N-acyl homoserine lactone-mediated bacterial quorum sensing. *Journal of the American Chemical Society* **128**: 2802-2803.

Keller, L. & M. G. Surette, (2006) Communication in bacteria: an ecological and evolutionary perspective. *Nature reviews. Microbiology* **4**: 249-258.

Kelly, R. C., M. E. Bolitho, D. A. Higgins, W. Lu, W. L. Ng, P. D. Jeffrey, J. D. Rabinowitz, M. F. Semmelhack, F. M. Hughson & B. L. Bassler, (2009) The *Vibrio cholerae* quorum-sensing autoinducer CAI-1: analysis of the biosynthetic enzyme CqsA. *Nat Chem Biol* **5**: 891-895.

Kessler, A., U. Schell, T. Sahr, A. Tiaden, C. Harrison, C. Buchrieser & H. Hilbi, (2013) The *Legionella pneumophila* orphan sensor kinase LqsT regulates competence and pathogen-host interactions as a component of the LAI-1 circuit. *Environ Microbiol* **15**: 646-662.

LaSarre, B. & M. J. Federle, (2013) Exploiting quorum sensing to confuse bacterial pathogens. *Microbiology and molecular biology reviews : MMBR* **77**: 73-111.

Lenz, D. H. & B. L. Bassler, (2007) The small nucleoid protein Fis is involved in *Vibrio cholerae* quorum sensing. *Molecular microbiology* **63**: 859-871.

Lenz, D. H., K. C. Mok, B. N. Lilley, R. V. Kulkarni, N. S. Wingreen & B. L. Bassler, (2004) The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*. *Cell* **118**: 69-82.

Lerat, E. & N. A. Moran, (2004) The evolutionary history of quorum-sensing systems in bacteria. *Molecular biology and evolution* **21**: 903-913.

Lindsay, D. S., A. W. Brown, D. J. Brown, S. J. Pravinkumar, E. Anderson & G. F. Edwards, (2012) *Legionella longbeachae* serogroup 1 infections linked to potting compost. *Journal of medical microbiology* **61**: 218-222.

Lu, H. & M. Clarke, (2005) Dynamic properties of *Legionella*-containing phagosomes in *Dictyostelium amoebae*. *Cellular microbiology* **7**: 995-1007.

Lukat, G. S., W. R. McCleary, A. M. Stock & J. B. Stock, (1992) Phosphorylation of bacterial response regulator proteins by low molecular weight phospho-donors. *Proceedings of the National Academy of Sciences of the United States of America* **89**: 718-722.

Lyon, G. J., J. S. Wright, A. Christopoulos, R. P. Novick & T. W. Muir, (2002) Reversible and specific extracellular antagonism of receptor-histidine kinase signaling. *The Journal of biological chemistry* **277**: 6247-6253.

Mampel, J., T. Spirig, S. S. Weber, J. A. Haagensen, S. Molin & H. Hilbi, (2006) Planktonic replication is essential for biofilm formation by *Legionella pneumophila* in a complex medium under static and dynamic flow conditions. *Applied and environmental microbiology* **72**: 2885-2895.

Manning, G., G. D. Plowman, T. Hunter & S. Sudarsanam, (2002) Evolution of protein kinase signaling from yeast to man. *Trends in biochemical sciences* **27**: 514-520.

McDade, J. E., C. C. Shepard, D. W. Fraser, T. R. Tsai, M. A. Redus & W. R. Dowdle, (1977) Legionnaires' disease: isolation of a bacterium and demonstration of its role in other respiratory disease. *The New England journal of medicine* **297**: 1197-1203.

McNealy, T. L., V. Forsbach-Birk, C. Shi & R. Marre, (2005) The Hfq homolog in *Legionella pneumophila* demonstrates regulation by LetA and RpoS and interacts with the global regulator CsrA. *Journal of bacteriology* **187**: 1527-1532.

Miller, M. B. & B. L. Bassler, (2001) Quorum sensing in bacteria. *Annual review of microbiology* **55**: 165-199.

Miller, M. B., K. Skorupski, D. H. Lenz, R. K. Taylor & B. L. Bassler, (2002) Parallel quorum sensing systems converge to regulate virulence in *Vibrio cholerae*. *Cell* **110**: 303-314.

Moglich, A., R. A. Ayers & K. Moffat, (2009) Structure and signaling mechanism of Per-ARNT-Sim domains. *Structure* **17**: 1282-1294.

Molmeret, M., M. Santic, R. Asare, R. A. Carabeo & Y. Abu Kwaik, (2007) Rapid escape of the dot/icm mutants of *Legionella pneumophila* into the cytosol of mammalian and protozoan cells. *Infection and immunity* **75**: 3290-3304.

Molofsky, A. B. & M. S. Swanson, (2003) *Legionella pneumophila* CsrA is a pivotal repressor of transmission traits and activator of replication. *Molecular microbiology* **50**: 445-461.

Molofsky, A. B. & M. S. Swanson, (2004) Differentiate to thrive: lessons from the *Legionella pneumophila* life cycle. *Molecular microbiology* **53**: 29-40.

Muh, U., M. Schuster, R. Heim, A. Singh, E. R. Olson & E. P. Greenberg, (2006) Novel *Pseudomonas aeruginosa* quorum-sensing inhibitors identified in an ultra-high-throughput screen. *Antimicrob Agents Chemother* **50**: 3674-3679.

Müller, A., J. Hacker & B. C. Brand, (1996) Evidence for apoptosis of human macrophage-like HL-60 cells by *Legionella pneumophila* infection. *Infection and immunity* **64**: 4900-4906.

Mullis, K. B. & F. A. Faloona, (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods in enzymology* **155**: 335-350.

Nealson, K. H. & J. W. Hastings, (1979) Bacterial bioluminescence: its control and ecological significance. *Microbiological reviews* **43**: 496-518.

Neiditch, M. B., M. J. Federle, A. J. Pompeani, R. C. Kelly, D. L. Swem, P. D. Jeffrey, B. L. Bassler & F. M. Hughson, (2006) Ligand-induced asymmetry in histidine sensor kinase complex regulates quorum sensing. *Cell* **126**: 1095-1108.

Nelson, E. J., J. B. Harris, J. G. Morris, Jr., S. B. Calderwood & A. Camilli, (2009) Cholera transmission: the host, pathogen and bacteriophage dynamic. *Nature reviews. Microbiology* **7**: 693-702.

Ng, W. L. & B. L. Bassler, (2009) Bacterial quorum-sensing network architectures. *Annual review of genetics* **43**: 197-222.

Ng, W. L., L. J. Perez, Y. Wei, C. Kraml, M. F. Semmelhack & B. L. Bassler, (2011) Signal production and detection specificity in *Vibrio* CqsA/CqsS quorum-sensing systems. *Molecular microbiology* **79**: 1407-1417.

Ng, W. L., Y. Wei, L. J. Perez, J. Cong, T. Long, M. Koch, M. F. Semmelhack, N. S. Wingreen & B. L. Bassler, (2010) Probing bacterial transmembrane histidine kinase receptor-ligand interactions with natural and synthetic molecules. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 5575-5580.

Parkinson, J. S. & E. C. Kofoid, (1992) Communication modules in bacterial signaling proteins. *Annual review of genetics* **26**: 71-112.

Passador, L., J. M. Cook, M. J. Gambello, L. Rust & B. H. Iglewski, (1993) Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science* **260**: 1127-1130.

Peterson, G. L., (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Analytical biochemistry* **83**: 346-356.

Phin, N., F. Parry-Ford, T. Harrison, H. R. Stagg, N. Zhang, K. Kumar, O. Lortholary, A. Zumla & I. Abubakar, (2014) Epidemiology and clinical management of Legionnaires' disease. *The Lancet infectious diseases*.

Piao, Z., C. C. Sze, O. Barysheva, K. Iida & S. Yoshida, (2006) Temperature-regulated formation of mycelial mat-like biofilms by *Legionella pneumophila*. *Applied and environmental microbiology* **72**: 1613-1622.

Piper, K. R., S. Beck Von Bodman, I. Hwang & S. K. Farrand, (1999) Hierarchical gene regulatory systems arising from fortuitous gene associations: controlling quorum sensing by the opine regulon in *Agrobacterium*. *Molecular microbiology* **32**: 1077-1089.

Podgornaia, A. I. & M. T. Laub, (2013) Determinants of specificity in two-component signal transduction. *Current opinion in microbiology* **16**: 156-162.

Porter, S. C., A. K. North, A. B. Wedel & S. Kustu, (1993) Oligomerization of NTRC at the glnA enhancer is required for transcriptional activation. *Genes & development* **7**: 2258-2273.

Quon, K. C., G. T. Marczynski & L. Shapiro, (1996) Cell cycle control by an essential bacterial two-component signal transduction protein. *Cell* **84**: 83-93.

Ragaz, C., H. Pietsch, S. Urwyler, A. Tiaden, S. S. Weber & H. Hilbi, (2008) The *Legionella pneumophila* phosphatidylinositol-4 phosphate-binding type IV substrate SidC recruits endoplasmic reticulum vesicles to a replication-permissive vacuole. *Cellular microbiology* **10**: 2416-2433.

Rasis, M. & G. Segal, (2009) The LetA-RsmYZ-CsrA regulatory cascade, together with RpoS and PmrA, post-transcriptionally regulates stationary phase activation of *Legionella pneumophila* Icm/Dot effectors. *Molecular microbiology* **72**: 995-1010.

Robinson, V. L., D. R. Buckler & A. M. Stock, (2000) A tale of two components: a novel kinase and a regulatory switch. *Nature structural biology* **7**: 626-633.

Rowbotham, T. J., (1980) Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. *J Clin Pathol* **33**: 1179-1183.

Sadosky, A. B., L. A. Wiater & H. A. Shuman, (1993) Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. *Infection and immunity* **61**: 5361-5373.

Sahr, T., H. Bruggemann, M. Jules, M. Lomma, C. Albert-Weissenberger, C. Cazalet & C. Buchrieser, (2009) Two small ncRNAs jointly govern virulence and transmission in *Legionella pneumophila*. *Molecular microbiology* **72**: 741-762.

Sambrook, J., E. F. Fritsch & T. Maniatis, (1989) *Molecular Cloning, a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

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

SIB, (2014) ExPASy - Bioinformatics Resource Portal. *www.expasy.org*.

Skerker, J. M., B. S. Perchuk, A. Siryaporn, E. A. Lubin, O. Ashenberg, M. Goulian & M. T. Laub, (2008) Rewiring the specificity of two-component signal transduction systems. *Cell* **133**: 1043-1054.

Solomon, J. M. & R. R. Isberg, (2000) Growth of *Legionella pneumophila* in *Dictyostelium discoideum*: a novel system for genetic analysis of host-pathogen interactions. *Trends in microbiology* **8**: 478-480.

Solomon, J. M., A. Rupper, J. A. Cardelli & R. R. Isberg, (2000) Intracellular growth of *Legionella pneumophila* in *Dictyostelium discoideum*, a system for genetic analysis of host-pathogen interactions. *Infection and immunity* **68**: 2939-2947.

Spirig, T., A. Tiaden, P. Kiefer, C. Buchrieser, J. A. Vorholt & H. Hilbi, (2008a) The *Legionella* autoinducer synthase LqsA produces an α-hydroxyketone signaling molecule. *The Journal of biological chemistry* **283**: 18113-18123.

Spirig, T., A. Tiaden, P. Kiefer, C. Buchrieser, J. A. Vorholt & H. Hilbi, (2008b) The *Legionella* autoinducer synthase LqsA produces an alpha-hydroxyketone signaling molecule. *The Journal of biological chemistry* **283**: 18113-18123.

Steele, T. W., J. Lanser & N. Sangster, (1990) Isolation of *Legionella longbeachae* serogroup 1 from potting mixes. *Applied and environmental microbiology* **56**: 49-53.

Steinert, M., U. Hentschel & J. Hacker, (2002) *Legionella pneumophila*: an aquatic microbe goes astray. *FEMS Microbiol Rev* **26**: 149-162.

Steinert, M. & K. Heuner, (2005) *Dictyostelium* as host model for pathogenesis. *Cellular microbiology* **7**: 307-314.

Stock, A. M. & J. Guhaniyogi, (2006) A new perspective on response regulator activation. *Journal of bacteriology* **188**: 7328-7330.

Stock, A. M., V. L. Robinson & P. N. Goudreau, (2000) Two-component signal transduction. *Annual review of biochemistry* **69**: 183-215.

Stock, J. B., G. S. Lukat & A. M. Stock, (1991) Bacterial chemotaxis and the molecular logic of intracellular signal transduction networks. *Annual review of biophysics and biophysical chemistry* **20**: 109-136.

Stock, J. B., A. M. Stock & J. M. Mottonen, (1990) Signal transduction in bacteria. *Nature* **344**: 395-400.

Stout, J. E. & V. L. Yu, (1997) Legionellosis. *The New England journal of medicine* **337**: 682-687.

Studier, F. W., A. H. Rosenberg, J. J. Dunn & J. W. Dubendorff, (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods in enzymology* **185**: 60-89.

Surette, M. G., M. Levit, Y. Liu, G. Lukat, E. G. Ninfa, A. Ninfa & J. B. Stock, (1996) Dimerisation is required for the activity of the protein histidine kinase CheA that mediates signal transduction in bacterial chemotaxis. *The Journal of biological chemistry* **271**: 939-945.

Tiaden, A. & H. Hilbi, (2012a) alpha-Hydroxyketone synthesis and sensing by *Legionella* and Vibrio. *Sensors (Basel)* **12**: 2899-2919.

Tiaden, A. & H. Hilbi, (2012b) α-Hydroxyketone synthesis and sensing by *Legionella* and *Vibrio*. *Sensors* **12**: 2899-2919.

Tiaden, A., T. Spirig, P. Carranza, H. Bruggemann, K. Riedel, L. Eberl, C. Buchrieser & H. Hilbi, (2008) Synergistic contribution of the *Legionella pneumophila lqs* genes to pathogen-host interactions. *Journal of bacteriology* **190**: 7532-7547.

Tiaden, A., T. Spirig & H. Hilbi, (2010a) Bacterial gene regulation by α-hydroxyketone signaling. *Trends in microbiology* **18**: 288-297.

Tiaden, A., T. Spirig, T. Sahr, M. A. Walti, K. Boucke, C. Buchrieser & H. Hilbi, (2010b) The autoinducer synthase LqsA and putative sensor kinase LqsS regulate phagocyte interactions, extracellular filaments and a genomic island of *Legionella pneumophila*. *Environ Microbiol* **12**: 1243-1259.

Tiaden, A., T. Spirig, T. Sahr, M. A. Walti, K. Boucke, C. Buchrieser & H. Hilbi, (2010c) The autoinducer synthase LqsA and putative sensor kinase LqsS regulate phagocyte interactions, extracellular filaments and a genomic island of *Legionella pneumophila.* *Environ Microbiol* **12**: 1243-1259.

Tiaden, A., T. Spirig, S. S. Weber, H. Bruggemann, R. Bosshard, C. Buchrieser & H. Hilbi, (2007) The *Legionella pneumophila* response regulator LqsR promotes host cell interactions as an element of the virulence regulatory network controlled by RpoS and LetA. *Cellular microbiology* **9**: 2903-2920.

Tikhomirova, A. & S. P. Kidd, (2013) *Haemophilus influenzae* and *Streptococcus pneumoniae*: living together in a biofilm. *Pathogens and disease* **69**: 114-126.

Timmen, M., B. L. Bassler & K. Jung, (2006) AI-1 influences the kinase activity but not the phosphatase activity of LuxN of *Vibrio harveyi*. *The Journal of biological chemistry* **281**: 24398-24404.

Toro-Roman, A., T. Wu & A. M. Stock, (2005) A common dimerization interface in bacterial response regulators KdpE and TorR. *Protein science : a publication of the Protein Society* **14**: 3077-3088.

Tortosa, P. & D. Dubnau, (1999) Competence for transformation: a matter of taste. *Current opinion in microbiology* **2**: 588-592.

Ulrich, L. E. & I. B. Zhulin, (2007) MiST: a microbial signal transduction database. *Nucleic acids research* **35**: D386-390.

Weber, S. S., C. Ragaz, K. Reus, Y. Nyfeler & H. Hilbi, (2006) *Legionella pneumophila* exploits PI(4)P to anchor secreted effector proteins to the replicative vacuole. *PLoS pathogens* **2**: e46.

Wei, Y., W. L. Ng, J. Cong & B. L. Bassler, (2012) Ligand and antagonist driven regulation of the *Vibrio cholerae* quorum-sensing receptor CqsS. *Molecular microbiology* **83**: 1095-1108.

Whitehead, N. A., M. Welch & G. P. Salmond, (2001) Silencing the majority. *Nature biotechnology* **19**: 735-736.

WHO, (1997) *The World Health Health Report*.

Zhu, J. & G. F. Kaufmann, (2013) Quo vadis quorum quenching? *Current opinion in pharmacology* **13**: 688-698.

Zhu, W., S. Banga, Y. Tan, C. Zheng, R. Stephenson, J. Gately & Z. Q. Luo, (2011) Comprehensive identification of protein substrates of the Dot/Icm type IV transporter of *Legionella pneumophila*. *PloS one* **6**: e17638.

Zhu, Y. & M. Inouye, (2003) Analysis of the role of the EnvZ linker region in signal transduction using a chimeric Tar/EnvZ receptor protein, Tez1. *The Journal of biological chemistry* **278**: 22812-22819.

DANKSAGUNG

An allererster Stelle gilt mein Dank meinem Doktorvater Hubert Hilbi. Ich danke dir für diese großartige Chance eine Doktorarbeit auf diesem tollen Projekt bestreiten zu dürfen. Vielen Dank für deine unermüdliche Motivation, tausende deiner Ideen („have you ever tried…?“), zeitlich unbegrenzte Diskussionen und immer ein offenes Ohr.

Weiterhin danke ich meiner Quorum Sensing Partnerin Aline Kessler, die zu jeder Schwierigkeit im Projekt einen Lösungsansatz wusste und mir immer mit Rat und Tat zur Seite stand. Go Team Quorum!

Ich danke ferner allen Mitgliedern aus der Arbeitsgruppe Hilbi die mir nicht nur tolle Kollegen, sondern auch gute Freunde waren. Dazu gehören: Chris „knows everything“ Harrison, Ivo „Käsefreund & Süßigkeiten-Asket“ Finsel und sein Gefährte Christian „Lasagne“ Manske, Gudrun „Organisatorin“ Pfaffinger, Ina „The Transporter“ Haneburger, Maria „Gehe gern Bergsteigen“ Wagner, Stephanie „Sport“ Dolinsky und Stephen „The Beaver“ Weber.

Mein besonderer Dank gilt Sylvia Simon und Christine Nagel. Beide wissen immer über alles und jeden bescheid und sind ein unerschöpflicher Quell neuer Ideen. Sie gehören zu den aufrichtigsten, freundlichsten und mutigsten Menschen die ich kennenlernen durfte. Vielen Dank für eure Freundschaft und die schöne gemeinsame Zeit.

Weiterhin danke ich meinen Freunden Verena, Ute, Dani & Flo, Susi & Christoph, Britta & Stephan, Julia & Bernd, Nici, Daniel, Christian. Vielen Dank für eure Unterstützung, all die tollen Partys, Grillfeiern und Geburtstage.

Zuletzt geht mein Dank an meine Familie. Meine Eltern, die mich großgezogen und mir mein Studium ermöglicht haben. Meine Schwester die mich immer mit ihrer entspannten Art auf den Boden der Tatsachen zurückholt. Meinem Mann Uli, der immer an meiner Seite steht und immer an mich glaubt. Vielen Dank für eure Liebe und Treue.