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The *Legionella pneumophila kai* operon is implicated in stress response and confers fitness in competitive environments

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Summary

Legionella pneumophila uses aquatic protozoa as replication niche and protection from harsh environments. Although *L. pneumophila* is not known to have a circadian clock, it encodes homologues of the KaiBC proteins of *Cyanobacteria* that regulate circadian gene expression. We show that *L. pneumophila kaiB*, *kaiC* and the downstream gene *lpp1114*, are transcribed as a unit under the control of the stress sigma factor RpoS. KaiC and KaiB of *L. pneumophila* do not interact as evidenced by yeast and bacterial two-hybrid analyses. Fusion of the C-terminal residues of cyanobacterial KaiB to *Legionella* KaiB restores their interaction. In contrast, KaiC of *L. pneumophila* conserved autophosphorylation activity, but KaiB does not trigger the dephosphorylation of KaiC like in *Cyanobacteria*. The crystal structure of *L. pneumophila* KaiB suggests that it is an oxidoreductase-like protein with a typical thioredoxin fold. Indeed, mutant analyses revealed that the *kai* operon-encoded proteins increase fitness of *L. pneumophila* in competitive environments, and confer higher resistance to oxidative and sodium stress. The phylogenetic analysis indicates that *L. pneumophila* KaiBC resemble *Synechosystis* KaiC2B2 and not circadian KaiB1C1. Thus, the *L. pneumophila* Kai proteins do not encode a circadian clock, but enhance stress resistance and adaption to changes in the environments.

Introduction

Legionella pneumophila is an environmental bacterium and a human pathogen. It infects and multiplies within aquatic protozoa like *Acanthamoeba castellanii* and also human alveolar

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macrophages causing a severe pneumonia known as Legionnaires' disease (Fraser *et al*., 1977; McDade *et al*., 1977; Fields *et al*., 2002). *Legionella* is found in natural fresh waters as well as in man-made water systems, which are stressful habitats (Fields *et al*., 2002). Bacteria living under aerobic conditions must deal with the toxic effects of oxygen that can be generated exogenously or endogenously. In natural waters for example, solar radiation can initiate a series of photochemical reactions that produce harmful reactive oxygen species such as hydrogen peroxide and hydroxyl radicals (Scully *et al*., 2003). *Legionella pneumophila* encounters reactive oxygen species in the fresh water environment, as well as in the intracellular environment because of the oxidative burst exerted by the host cells (Newton *et al*., 2010). Therefore the response to oxidative stress and to toxic compounds is a key determinant of survival, spread and virulence of *L. pneumophila*. Indeed, *L. pneumophila* encodes different enzymes to defend itself against these stressors like two catalase-peroxidases: KatA being localized in the periplasm and KatB being localized in the cytoplasm (Bandyopadhyay and Steinman, 2000). These enzymes display strong peroxidase, but weak hydroperoxidase activity. In addition *L. pneumophila* encodes two superoxide dismutases, one Cu/Zn dependent predicted to be located in the periplasm and a Mn/Fe dependent located in the cytoplasm. These are expressed differentially depending on the growth phase (Bruggemann *et al*., 2006). The Cu/Zn superoxide dismutase is expressed in post exponential growth phase and is regulated by the stress sigma factor RpoS. In contrast the Mn/Fe superoxide dismutase is highly expressed during exponential growth (Bruggemann *et al*., 2006). Surprisingly, the typical SoxRS regulator of superoxide dismutase gene expression under conditions of oxidative stress is absent from *L. pneumophila* (Cazalet *et al*., 2004). Additionally, *L. pneumophila* encodes three peroxide-scavenging alkylhydroxide reductase systems that also may be important in protection from reactive oxygen species. AhpC1 (*lpp3037*), AhpC2 (*lpp2299*) and AhpD (*lpp2298*) protect *L. pneumophila* from peroxide damage as chromosomal deletion mutants were two- to eightfold more sensitive to H_2O_2, tert-butyl hydroperoxide, cumene hydroperoxide and paraquat than wild-type *L. pneumophila* (LeBlanc *et al*., 2008). LeBlanc and colleagues (2008) reported that *ahpD* is induced early in exponential phase and repressed postexponentially, concomitant with the induction of *ahpC1*. Our recent expression data obtained by microarray analyses showed that AhpC2D (*lpp2298/lpp2299*) are differently expresse*d in vitro* and *in vivo*. They showed post-exponential phase up-regulation *in vitro*, but were expressed during replicative phase *in vivo* (Bruggemann *et al*., 2006). The gene encoding AhpC1 is predominately transcribed during replicative phase *in vitro* and has been shown to be up-regulated during intracellular growth in macrophages indicating a role in protection from the respiratory burst (Rankin *et al*., 2002). Phylogenetic analysis of the AphC1 peroxidases of *Helicobacter pylori* suggests that thioredoxin and thioredoxin reductase activate the enzyme in a nicotinamide adenosine dinucleotide phosphate-dependent reaction (Baker *et al*., 2001). In contrast, AhpC2 of *L. pneumophila* shares similarities to the complex AhpC/D found in *Mycobacterium tuberculosis* (Bryk *et al*., 2002). The regulation of these different oxidative stress proteins is not understood and may involve many regulators like OxyR, RpoS and perhaps other regulatory factors not yet identified. Thus, to understand how *L. pneumophila* adapts to conditions it faces in natural environments or within the intracellular milieu of protozoa or macrophages, its response to different stressors needs to be deciphered.

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Genome sequence analyses showed that *L. pneumophila* encodes two genes homologous to *kaiC* (*lpp1116*) and *kaiB* (*lpp1115*) of *Cyanobacteria*, but that *L. pneumophila* lacks the *kaiA* gene (Cazalet *et al*., 2004). In *Cyanobacteria*, KaiABC, the circadian core genes, control rhythmic gene expression and regulate different physiological functions like chromosomal compaction and cell division to synchronize them with the Earth rotation (Smith and Williams, 2006; Dong *et al*., 2010). Furthermore, possession of a circadian clock has been shown to enhance the adaptive fitness of *Cyanobacteria* in a wide variety of environmental conditions (Woelfle *et al*., 2004). In prokaryotic organisms, the circadian rhythms have been described only in photosynthetic bacteria (Johnson, 2007), where they were mainly studied in *Cyanobacteria* and lately also in the purple bacteria *Rhodobacter sphaeroides* (Min *et al*., 2005a). Although KaiB and KaiC are found in several prokaryotes belonging to different species (Dvornyk *et al*., 2003; Loza-Correa *et al*., 2010), their implication in circadian clocks has never been shown. Thus the question arises: why are the *kai* genes conserved in *L. pneumophila* and what are their functions? Does *L. pneumophila* encode a circadian clock and/or are these genes involved in improving fitness in the environment like in *Cyanobacteria*? Here we report that the *kai* genes of *L. pneumophila* do not seem to be regulating circadian rhythms, but are implicated in stress response, in particular to oxidative and sodium stress. In line with this function, the crystal structure of KaiB resembles a thioredoxin.

Results

kaiBC genes of *L. pneumophila* are organized in an operon together with a regulatory protein

In the *L. pneumophila* genome, the *kaiBC* (*lpp1115* and *lpp1116*) genes are contiguous as described in *Synechococcus elongatus* and other *Cyanobacteria* and are located next to *lpp1114*, a gene encoding a multidomain protein containing a Per-ARNT-Sim (PAS) sensor domain, a GGDEF and an EAL domain (Fig. 1A). GGDEF and EAL domain-containing proteins determine the cellular concentrations of c-di-GMP by mediating its synthesis and degradation, respectively (Schirmer and Jenal, 2009). The PAS domain is found in many signalling proteins that monitor changes in light, redox potential, oxygen, small ligands and overall energy of a cell (Taylor and Zhulin, 1999). Gene expression data obtained from microarray analyses and quantitative real-time polymerase chain reaction (qRT-PCR) experiments showed that in *L. pneumophila kaiB* and *kaiC* form a transcriptional unit together with *lpp1114* (data not shown). We have previously undertaken a RNAseq analysis of exponentially and post exponentially grown *L. pneumophila* to map all transcriptional start sites (TSS) in the genome (Sahr *et al*., 2012). Using these data, we could map the TSS of the *kai* operon 67nts upstream the translational start with a predicted RpoS promoter sequence upstream the TSS (Fig. 1A). This finding is in accordance with a regulation of this operon by RpoS as indeed seen in a transcriptome analysis of a Δ*rpoS* mutant strain (Table S1). Sequence comparison of *L. pneumophila* KaiC with those of *Cyanobacteria*, revealed that the two Walker motifs [G or A]XXXXGK[T or S] required for the adenosine triphosphate (ATP)/guanosine 5′-triphosphate (GTP)-binding are highly conserved. Furthermore, the potential C-terminal phosphorylation sites Thr405, Ser410 and Ser411 in

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L. pneumophila corresponding to Thr426, Ser431 and Thr432 in *S. elongatus* are present (Fig. 1B).

Legionella pneumophila kai genes are not expressed in a circadian manner

In *S. elongatus*, the expression of the *kaiBC* mRNA shows robust circadian rhythms during continuous light (Tomita *et al*., 2005). Thus, our first approach was to test whether the *kai* genes of *L. pneumophila* growing *in vitro* show circadian activity. We examined the relative mRNA expression levels by real-time PCR of each gene of the operon (*kaiC*, *kaiB* and *lpp1114*) during *in vitro* growth in buffered yeast extract (BYE) medium. Samples were taken at different time points: after 14 h and 16 h corresponding to exponential growth phase at optical density (OD) 2.5 and 3.0, respectively, during post-exponential growth phase (OD 3.8, 20 h) and late post-exponential growth phase (OD 4.2, 24 h). As shown in Fig. 2A the *kaiC*, *kaiB* and *lpp1114* mRNAs are expressed during post exponential growth phase and their expression increases until late exponential growth phase. Thus the *kai* operon is expressed in a typical growth phase-dependent manner as reported for many *L. pneumophila* genes (Bruggemann *et al*., 2006; Sahr *et al*., 2012). RNAseq analyses at exponential and post exponential growth phase confirmed this result (Sahr *et al*., 2012). We then further tested the expression of the *kai* operon during intracellular growth in *A. castellanii*. Amoebae were infected with wild-type *L. pneumophila* and its intracellular replication was monitored at 20°C, an infection temperature that mimics environmental conditions better. Samples were taken each day over 7 days and the relative mRNA expression of the *kai* genes was measured (Fig. 2B). Again, the expression of the *kai* operon genes was correlated with increased intracellular multiplication and no circadian cycles were observed. Thus, the *kai* operon has a growth phase dependent expression controlled by the stress sigma factor RpoS, with the highest levels during late stages of *in vitro* or *in vivo* growth.

Transcriptional profiling of *L. pneumophila* wild type, Δ*kaiC* and Δ*lpp1114* mutant strains

KaiC has been described in *Cyanobacteria* as a regulatory protein. To analyse whether KaiC of *L. pneumophila* has regulatory functions, we constructed two mutants where either the *kaiC* gene (Δ*kaiC : kan*) or the *lpp1114* gene (Δ*lpp1114 : apra*) was replaced by an antibiotic cassette and analysed their transcriptional profiles in different conditions. Repeated efforts to construct a Δ*kaiB* mutant were unsuccessful. *kaiC* is the first gene of the operon. We thus tested the gene expression of *kaiB* and *lpp1114* in the Δ*kaiC* strain to reveal possible downstream effects of the *kaiC* gene deletion. Indeed, qRT-PCR and microarray analyses showed that transcription of *kaiB* and *lpp1114* was abolished in the Δ*kaiC* mutant strain indicating that the entire operon was not transcribed (data not shown). Thus our Δ*kaiC* strain is comparable with a triple mutant Δ*kaiCBlpp1114*.

For genome-wide transcriptional profiling of *in vitro* grown bacteria, RNA was sampled at OD 4.0 after growth of the wild-type and the Δ*kaiC* strains in BYE media at 37°C. This corresponds to the time point of the highest expression of *kaiC* (Fig. 2A). However, even at this time point, only very slight expression changes were observed and the *kai* operon genes themselves were in all experiments only weakly expressed, making the comparison and interpretation of the data difficult. Apart from the *kai* operon genes (*lpp1114-lpp1116*), four genes were identified as significantly down-regulated in the Δ*kaiC* mutant strain as

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compared with the wild type. These genes encode an esterase/lipase (*lpp1856*), a putative coproporphyrinogen oxidase A (*lpp2849*) and two hypothetical proteins (*lpp1823* and *lpp0954*; Table 1). Our analysis did not identify any significantly up-regulated gene in the Δ*kaiC* strain compared with the wild type. As differences in the transcriptional profiles of *L. pneumophila* grown *in vitro* compared with *in vivo* exist (Bruggemann *et al*., 2006), we recovered RNA from *L. pneumophila* wild type and the two mutants after 6 days of infection of *A. castellanii* at 20°C, the time point where the highest level of expression of the *kai* genes was observed by qRT-PCR (Fig. 2B). During *in vivo* growth we identified five genes differentially regulated in both mutant strains [fold change (FC) > 1.5], three of them with significant changes in their expression (Table 2). Interestingly, the genes slightly down-regulated in the Δ*kaiC* strain, were up-regulated 1.5-fold and more in the Δ*lpp1114*. Among the genes down-regulated in the Δ*kaiC* mutant are two that encode major outer membrane proteins (*lpp3032*, *lpp3033*), two hypothetical proteins (*lpp1553* and *lpp2209*) and a small RNA (ncRNA08). This could suggest that KaiC has a negative effect on *lpp1114* expression.

Kai proteins provide fitness to *L. pneumophila* in competitive environments

In order to learn whether the Kai proteins are implicated in intracellular growth of *L. pneumophila*, *Acanthamoeba castellanii*, was infected with the wild-type strain and the Δ*kaiC* and Δ*lpp1114* mutant strains and their intracellularly replication over 72 h at 37°C and over 168 h at 20°C was recorded. No difference in intracellular replication was observed (data not shown). In order to detect more subtle differences, we conducted a competitive growth assay in *A. castellanii* (Kessler *et al*., 2013). The Δ*lpp1114* mutant, as compared with the wild type did not show any difference also in this assay. However, the Δ*kaiC* mutant, that is comparable with a knockout of the entire operon, was clearly less competitive in amoebae.Already after 2 days of infection, only half the number of mutant bacteria, as compared with wild-type bacteria, was recovered, and after 5 days, the mutant was nearly outcompeted (Fig. 3A). When complementing the Δ*kaiC* mutant with the entire operon on a plasmid (pMMB207_*kai*_operon), the phenotype was restored (Fig. 3B). Thus the *kai* operon mutant is likely an example of ‘soft selection’ (Wallace, 1981) where the reduced fitness of one genotype compared with another one is only seen in a competitive environment.

Crystal structure of KaiB demonstrates closer resemblance to thioredoxins than to *Cyanobacteria* KaiB structures

In order to gain further insight into the function of the *L. pneumophila* KaiB and KaiC proteins, we embarked on the structural characterization of these proteins by X-ray crystallography. Diffraction quality crystals were obtained only for the *L. pneumophila* KaiB protein, while the KaiC crystallization was unsuccessful (data not shown). The structure of the KaiB protein was solved to 1.95 Å resolution by the single-wavelength anomalous diffraction technique using selenomethionine-enriched protein samples. The final model contained two full-length (residues 1–90) KaiB molecules in the asymmetric unit with each monomer demonstrating a slightly different conformation between residues 31 and 37 (Fig. 4A). Each KaiB molecule features a two-layer α/β sandwich composed of a four-strand mixed β-sheet surrounded by three α-helices α1, α2′ and α2″ and α5 corresponding to residues 15–27, 50–53 and 80–86 respectively. The secondary structure elements were

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assigned using DSSP (http://swift.cmbi.ru.nl/gv/dssp/; Kabsch and Sander, 1983) and numbered according to corresponding elements in cyanobacterial KaiB proteins (Hitomi *et al*., 2005).

The comparative structural analysis using the Dali server (Holm and Rosenström, 2010; http://ekhidna.biocenter.helsinki.fi/dali_server) demonstrated that KaiB molecule bears strong resemblance to the thioredoxin family protein structures. In particular, KaiB and *Thermus thermophilus* thioredoxin [protein data bank (PDB) 2CVK] structures superimpose with rmsd of 2.3 Å over 84 Cα atoms while sharing only 13% of primary sequence identity (Fig. 4B). In comparison, the closest structural homologue among characterized KaiB proteins – the structure of the KaiB protein from the cyanobacterium *Synechocystis* sp. PCC6803 (PDB 1WWJ) superimposes with KaiB structure with rmsd of 4.3 Å over only 63 Cμ atoms. The secondary structure elements that are conserved between the *L. pneumophila* and *Synechocystis* KaiB molecules primarily include β1 (residues 4–10) and β2 (residues 37–43) strands on the main β-sheet and the α1-helix of the KaiB protein, which spans the N-terminal half (residues 1–46) of this protein (Fig. 4B). By contrast, the C-terminal half of the KaiB molecule shares very little structural similarity with the *Synechocystis* KaiB molecule with only the β4-strand (residues 61–66) matching to corresponding elements in the cyanobacterial protein structure. The β3-strand of the *Synechocystis* KaiB molecule spanning residues 57–60 contributes to the tetramerization interface critical for the circadian rhythm function of this protein (Hitomi *et al*., 2005). The similar region in the KaiB structure corresponds to helices α2′ (residues 45–47) and α2″ (residues 50–53). While this region is involved in the KaiB dimerization interface the orientation of the KaiB monomers in the dimer is significantly different than that of monomers in the *Synechocystis* KaiB tetramer. The α5-helix (residues 80–86) and the β5-strand (residues 71–74) of KaiB match to the corresponding regions in *T. thermophilus* thioredoxin, but not to *Synechocystis* KaiB structure (Fig. 4B).

Furthermore, comparative sequence analysis indicated that KaiB lacks the C-terminal sequence conserved in cyanobacterial KaiB proteins (residues 95–108 in *T. elongatus* KaiB protein; Fig. S1). However, this region is crucial for the function of this protein because the Glu95 to Phe102 deletion in *S. elongatus* PCC7942 KaiB resulted in loss of the circadian rhythm *in vivo* (Iwase *et al*., 2005). The structural comparison of KaiB and cyanobacterial KaiB molecules confirmed that the *L. pneumophila* protein does not contain any structural elements that would correspond to the C-terminal region of the *T. elongatus* KaiB protein. Taken together, the structural analysis of KaiB identifies significant differences between this protein and the structures of cyanobacterial KaiB proteins. The KaiB structure appears lacking the key functionally relevant elements conserved in cyanobacterial KaiB proteins indicating that despite remaining similarities the KaiB protein function has diversified from that of *bona fide* KaiB proteins involved in circadian rhythms. On the other hand the strong structural similarity between KaiB and bacterial thioredoxin proteins points to a possible function of *L. pneumophila* KaiB in the oxidative stress response.

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Legionella pneumophila KaiB and KaiC do not interact

The crystal structure of KaiB suggested that *L. pneumophila* KaiB might not interact with KaiC as the C-terminal residues of KaiB are missing in *L. pneumophila*. To test this hypothesis, we used a yeast two-hybrid approach where KaiB was used as bait and KaiC as target. Both were expressed as fusion protein with hSos and with a myristoylation sequence, respectively, and co-expressed in the *Saccharomyces cervisae* mutant strain cdc25H at 37°C. If KaiB and KaiC interact, the hSos protein would activate the Ras-signalling cascade and allow the strain to grow at 37°C. However, no interaction between KaiB and KaiC of *L. pneumophila* was observed (data not shown). A major disadvantage of assaying protein–protein interactions in a heterologous system is that bacterial-specific post-translational modifications or protein chaperones are not present in the yeast background, which might lead to incomplete or mis-folded proteins. Thus we used a second system, a bacterial two-hybrid approach, the bacterial adenylate cyclase-based two-hybrid (BACTH) system. This method is based on the interaction-mediated reconstitution of the adenylate cyclase activity in *Escherichia coli* lacking endogenous adenylate cyclase (Karimova *et al*., 1998). It uses the catalytic domain of adenylate cyclase (CyaA) from *Bordetella pertussis* that consists of two fragments that are complementary to each other (T25 and T18) and that need to stay in immediate vicinity. The fragments T25 and T18 were fused to KaiC and KaiB respectively. In case the two proteins interact T25 and T18 would be closely enough to synthesize adenosine 3′5′ cyclic monophosphate that the latter binds to the catabolite activator protein and regulates the transcription of genes like the *lac* and *mal* operon. Thus, it is possible to determine if two proteins interact by plating the bacteria on selective media that allow visualizing production of β-galactosidase or maltose fermentation. Again, we did not observe any interaction of *Legionella* KaiB and KaiC when bacteria were plated on McConkey/maltose or LB/X-gal agar (Fig. 5A, panel 1). In order to test the hypothesis that the missing C-terminal residues are responsible for the lack of interaction, we fused the last 13 C-terminal residues (EIGDQAEDDLGLE) present in *T. elongatus* KaiB to the *L. pneumophila* KaiB protein and called it KaiB-T (Fig. S2). Then we used *L. pneumophila* KaiB-T and KaiC fused to T18 and T25, respectively, and repeated the bacterial BACTH two-hybrid assay. Indeed, now interaction between KaiC and KaiB-T occurred (Fig. 5A, panel 2). As positive control, we used KaiC from *S. elongatus* fused to T18 and *L. pneumophila* KaiB-T fused to T25, respectively, which indeed interacted, similarly to the *L. pneumophila* KaiC/KaiB-T interaction (Fig. 5A, panel 3). The quantification of the obtained β-galactosidase levels for each interaction experiment is shown in Fig. 5B. *Legionella* KaiC and KaiB-T showed a similar level of β-galactosidase activity like that of *Cyanobacteria* (Fig. 5B). We then also tested whether Lpp1114 may interact with KaiC by fusing them to T18 and T25 respectively. However, no interaction was detected (data not shown). Thus our results suggest that neither *L. pneumophila* KaiB and KaiC nor KaiC and Lpp1114 interact.

Legionella pneumophila KaiC conserves autokinase activity, but not circadian dephosporylation when KaiB is present

Bacterial proteins containing Walker motifs may display autokinase activity (Doublet *et al*., 1999). *Cyanobacteria* KaiC exhibits autokinase activity *in vitro* (Nishiwaki *et al*., 2000). Furthermore, KaiB of *Cynaobacteria* functions by binding the KaiC protein upon

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phosphorylation of the Ser431 residue (Nishiwaki *et al*., 2007; Rust *et al*., 2007; Qin *et al*., 2010), probably to the CII ring (Akiyama *et al*., 2008). Then a dephosphorylation phase follows and completes one 24 h cycle. Thus KaiB is an attenuator of phosphorylation that happens in a circadian manner (Kitayama *et al*., 2003). In *L. pneumophila* KaiC the Walker motifs and the phosphorylation residues are well conserved (Fig. 1B). To test whether *L. pneumophila* KaiC exhibits autokinase activity, a His6x-KaiC protein of *L. pneumophila* was expressed in *E. coli*, purified with Ni-affinity resin and incubated with [γ-^{32}P] ATP in presence of Mg^{2+}. As a control, we used *S. elongatus* His6x-KaiC. As shown in Fig. 6A, after incubation *L. pneumophila* and *S. elongatus* His6x-KaiC were indeed labelled, revealing that *L. pneumophila* KaiC exhibits autokinase activity. In *Cyanobacteria* the circadian clock can be reconstituted *in vitro* from the KaiA, KaiB, and KaiC proteins in the presence of ATP (Nakajima *et al*., 2005). We therefore tested whether KaiC of *L. pneumophila* can also be dephosphorylated by KaiB *in vitro*, and if the missing C-terminal residues in *L. pneumophila* KaiB play a role. We incubated *L. pneumophila* and *S. elongatus* KaiC with *L. pneumophila* KaiB or KaiB-T and analysed autophosphorylation, as well as dephosphorylation of KaiC. As reported previously, *S. elongatus* KaiC is dephosphorylated in a circadian rhythm; however, only when *L. pneumophila* KaiB-T was added, but not by wild-type *L. pneumophila* KaiB, further indicating that the C-terminal residues are essential and that KaiB of *L. pneumophila* does not interact with KaiC (Fig. 6B). For *L. pneumophila* KaiC instead, no change of the phosphorylation level over time was observed neither in presence of KaiB-T nor KaiB. Thus, our different analyses show that *L. pneumophila* KaiB and KaiC do not interact supporting a different function for these proteins.

Legionella pneumophila Δ*kaiC* and Δ*lpp1114* are more sensitive to oxidative stress than the wild type

Legionella pneumophila lives under aerobic conditions and must deal with the toxic effect of oxygen that is generated in aquatic environments as well as when invading a eukaryotic cell. The structural analysis suggested that KaiB resembles a thioredoxin and the Lpp1114 protein, encoded in the same transcriptional unit contains a PAS domain that potentially senses intracellular redox changes. According to these findings, we hypothesized that the Kai operon may be implicated in regulating the oxidative stress response of *L. pneumophila*. First experiments on bacteria grown on buffered charcoal-yeast extract (BCYE) agar with different concentrations of hydrogen peroxide further substantiated this hypothesis, as the Δ*kaiC* mutant strain was less resistant than the wild-type strain (data not shown). In order to quantify and compare the oxidative stress response of the mutant and the wild-type strain, we first tested the sensitivity of *L. pneumophila* wild type to different concentrations of two oxidative reagents, H_2O_2 and paraquat. *Legionella pneumophila* wild type was highly sensitive to H_2O_2 in the medium as already 30 min of exposure to 1 mM H_2O_2 killed nearly all bacteria. In contrast, the sensitivity to paraquat was less pronounced as only 5 mM and 10 mM paraquat reduced bacterial survival significantly (Fig. 7A). We then selected two conditions for further testing the survival of the wild-type strains over longer exposure: 10 mM paraquat and 1 mM H_2O_2 (Fig. 7B). This showed that *L. pneumophila* is highly sensitive to 1 mM H_2O_2 as already after 10 min of exposure 80% of the bacteria were killed. However, as H_2O_2 does not seem to be stable in the medium, the remaining bacteria recovered and replicated. In contrast, a significant difference between the survival kinetics

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after exposure to 10 mM paraquat was observed with Δ*kaiC* and Δ*lpp1114* mutants showing less resistance to this oxidative stress than the wild-type strain (Fig. 7C, D). When complementing the Δ*kaiC* mutant with the entire operon on a plasmid (pMMB207_*kaiOp*) or Δ*lpp1114* mutant with *lpp1114* (pMMB207_lppp1114) the oxidative stress resistance was similar to that of the wild-type strain (Fig. S3). Hence, our results indicate that the *kai* operon contributes to oxidative stress defense of *L. pneumophila*.

To gain insight in the regulatory cascade implicated in oxidative stress response and to analyse where KaiC may act, we tested the effect of 10 mM paraquat in mutant strains that are thought to either regulate the *kai* operon genes like RpoS, or in genes homologous to other components in the circadian cascade. Apart from the KaiABC operon, CikA, SasA, RpaA, LabA and LdpA are implicated in this regulatory cascade in *Cyanobacteria* (Mackey *et al*., 2011). The best homologue of CikA (Gutu and O'Shea, 2013) in *L. pneumophila* is LetS (37% amino acid identity and e-value of $2e^{-43}$), the sensor kinase of the two component system LetA/LetS implicated in *L. pneumophila* virulence regulation, flagellar motility and cytotoxicity (Hammer *et al*., 2002; Molofsky and Swanson, 2004; Sahr *et al*., 2009). SasA and RpaA, the two-component system important for clock-control gene expression of *S. elongatus* (Taniguchi *et al*., 2007) have their best homologues (37% aa similarity) with StuC and CpxR of *L. pneumophila* respectively. *stuC* encodes a sensor histidine kinase protein and CpxR is a response regulator that can control the expression of different components of the Icm/Dot system (Altman and Segal, 2008). LabA and LdpA of *S. elongatus* are absent from *L. pneumophila* genome. Indeed, Δ*rpoS* and Δ*letA* are impaired for survival by the oxidative stress exerted by paraquat (Fig. S4), suggesting that LetA and RpoS are implicated in the oxidative stress response. In contrast, Δ*stuC* and Δ*stuC/A* are only very slightly affected (Fig. S4). Thus, we conclude that during oxidative stress the *kai* genes are regulated probably directly by RpoS and are influenced by LetA.

KaiC influences the expression of the alkyl-peroxidases *ahpCD* of *L. pneumophila*

To investigate the oxidative stress response of *L. pneumophila* at the transcriptional level, we first analysed the transcriptome of the *L. pneumophila* wild-type strain exposed to 10 mM paraquat as compared with growth in BYE medium without paraquat, using a whole genome microarray described previously (Albert-Weissenberger *et al*., 2010). This approach identified the significant induction of two genes, *lpp2298* (31.2-fold) and *lpp2299* (10.9-fold) encoding alkyl-peroxidases (*ahpCD*; Table 3). We confirmed this result by qRT-PCR where *lpp2299* showed 74-fold up-regulation under oxidative stress conditions (data not shown). Other genes that showed up-regulation in this stress condition were *feoA* (*lpp2712*) with 2.9-fold change, *feoB* (*lpp2711*) with 1.4-fold change and *frgA* (*lpp2846*) with 2.8-fold change. The *feo*-operon is involved in the transport of ferrous iron into bacteria (Cartron *et al*., 2006) and has been described as critical for *L. pneumophila* growth in low-iron conditions, in host cells, and in the mammalian lung. FrgA has been described as an iron-regulated protein of *L. pneumophila* (Cianciotto, 2007; Hickey and Cianciotto, 1997; Robey and Cianciotto, 2002). Furthermore, two genes similar to *pvcA* (*lpp0236*) and *pvcB* (*lpp0237*) showed a fold change of 1.8 and 1.5 respectively. PvcA and PvcB are required for synthesis of pyoverdine chromophore, they serve as signalling molecules controlling gene expression inside the cell and have been reported to be induced in biofilm and regulated by

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iron concentrations available (Stintzi *et al*., 1999; Hindre *et al*., 2008; Table 3). Thus the general transcriptional response of *L. pneumophila* to oxidative stress leads to the induction of two alkyl-peroxidases and iron related genes.

We then compared the transcriptional profile of the Δ*kaiC* and Δ*lpp1114* mutants to *L. pneumophila* wild type after exposure to 10 mM paraquat for 15 min as this was the time point where the mutants showed the highest sensitivity (Fig. 7C,D). We found that *ahpC* and *ahpD* were slightly down-regulated in both mutants (Tables 4 and 5). Furthermore, coproporphyrinogen oxidase A (*lpp2849*), a nicotinamide adenine dinucleotide plus hydrogen (NADH)-ubiquinone oxidoreductase (*lpp0727*) and *fliA*, coding for the sigma factor 28, were slightly down-regulated in the Δ*kaiC* strain (Table 4). qRT-PCR analysis of the alkyl-peroxidase encoding genes *ahpC1* and *ahpC2D* (*lpp3037* and *lpp2298, lpp2299*) during post-exponential growth phase under the effect of 10 mM paraquat confirmed the microarray results (data not shown). Furthermore, the genes encoding FrgA and a NADH-dependent flavin oxidoreductase (*lpp2779*) were down-regulated in the Δ*lpp1114* strain (Table 6). qRT-PCR of the Δ*lpp1114* strain confirmed the reduction of the alkyl-peroxidases AhpCD and also showed a down-regulation of the expression of the *kai* genes (data not shown). Thus, the expression of the alkyl-peroxidase operon and of other genes possibly related to the oxidative stress response of *L. pneumophila* is modulated by KaiC.

Δ*kaiC* and Δ*lpp1114* mutants are more sensitive to sodium stress than the wild type

To further study the implication of the *kai* operon genes in the *L. pneumophila* stress response we analysed the phenotype of the mutant strains under salt stress. It is known that in broth *L. pneumophila* is sodium sensitive only during post exponential growth (Catrenich and Johnson, 1989). It has been shown that sodium sensitivity is also correlated with virulence of *L. pneumophila* as during the intracellular replication period, this bacterium is sodium resistant and lacks flagella (Bachman and Swanson, 2001; Molofsky and Swanson, 2004). Concomitant with macrophage lysis, *L. pneumophila* becomes sodium sensitive and flagellated (Byrne and Swanson, 1998). In order to test whether the Kai proteins are implicated in regulating this distinct phenotype, we grew the wild-type and the mutant strains Δ*kaiC* and Δ*lpp1114* to post exponential growth phase (OD 3.8) and spotted 10 µl of serial dilutions (non diluted to 10^{-5}) of these cultures on BYCE agar plates containing 100 mM NaCl or not. Indeed, both mutants were more sensitive to sodium stress than the wild-type strain, as no growth of the mutant strains could be observed under salt stress above a dilution of 10^{-3}. In contrast the wild-type strain grew on plates containing 100 mM NaCl up to a dilution of 10^{-5} (Fig. 8) indicating that the *kai* operon genes enhance sodium resistance of *L. pneumophila* in post exponential phase.

Evolution of the KaiB and KaiC proteins in *L. pneumophila*

A Basic Local Alignment Search Tool (BLAST) search identified homologs of the *kaiC*, *kaiB* and *lpp114* genes in all eight published *L. pneumophila* genome sequences. However, in *L. pneumophila* Lens, the *kaiC* gene contains a frameshift leading to a truncated gene and KaiC of *L. pneumophila* Alcoy has another start codon leading to a shorter protein, but the two walker motifs are conserved. Furthermore, we examined a collection of 41 *L. pneumophila* isolates belonging to the 15 serogroups (our unpublished data). The *kai* operon

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genes were missing in three of the 41 strains and six isolates had a frameshift in the operon sequence. Analyses of the genome sequences of five *L. longbeachae* strains (Cazalet *et al*., 2010; Kozak *et al*., 2010), one *L. drancourtii* strain (Moliner *et al*., 2010), two *L. dumoffii* strains (Qin *et al*., 2012) and one *L. anisa* strain (unpublished), and the genomes of *L. hackeliae, L. micdadei and L. fallonii* (our unpublished data) revealed that *L. hackeliae* contains the *kai* operon and that *L. fallonii* contains the *kaiB* and *lpp1114* genes but no *kaiC* gene, whereas the entire operon is absent from the remaining five *Legionella* species. This heterogeneous distribution and the mutations present in the operons might point to the fact that this operon is on an evolutionary path to be lost from *Legionella*.

To get further insight into the evolution of the Kai operon proteins of *L. pneumophila* we undertook different phylogenetic analyses. Recently Wiegard and colleagues (2013) published the first functional analyses of paralogous KaiBC genes in *Synechocystis* that encodes three copies of KaiB and KaiC in its genome, showing that the paralogous KaiB2C2 genes do not seem to encode a circadian clock (Wiegard *et al*., 2013). To learn whether the *L. pneumophila* Kai proteins are related to one of the paralogous families or to the genuine Kai clock proteins of *Cyanobacteria*, we undertook a phylogenetic analysis including all paralogues of KaiC and KaiB that may be present in different prokaryotic genomes. We selected the representative Kai protein sequences published by Dvornyk and colleagues (2003) and included the *Legionella* Kai sequences. Interestingly, KaiC of *L. pneumophila* groups with the *Synechocystis* KaiC2 (*sll1595*) protein and not with the KaiC1 sequence that is part of the genuine circadian clock (Fig. 9).

We then undertook phylogenetic analyses of *L. pneumophila* KaiB, that shares 97% amino acid similarity with KaiB of *Cyanobacteria* (Fig. S1). *kaiB* genes are present only in prokaryotic organisms that encode a *kaiC* gene next to it, but not all prokaryotes that contain *kaiC* contain *kaiB* as also observed previously (Dvornyk *et al*., 2003). We constructed the phylogenetic KaiB tree by analysing one representative KaiB protein of each prokaryote that contains it. However, given the short sequence of the KaiB protein and the high conservation of the core residues 8–94 of *S. elongatus* (Iwase *et al*., 2005), no strong evolutionary conclusion can be drawn and only few nodes of the KaiB tree have significant bootstrap support (Fig. S5). Thus we concatenated the KaiB and KaiC sequences to obtain a better resolved and supported tree. As shown in Fig. 10 the *Legionella* KaiCB sequences group with the KaiB2C2 genes (*sll1595, sll1506*) of *Synechocystis* and of other Proteobacteria, like KaiC alone (Fig. 9). In both phylogenetic trees (Fig. 9 and 10) the *Legionella* and *Synechocystis* KaiCB2 proteins cluster. Interestingly, when analysing the neighborhood of KaiCB of each organism it became evident that in four out of six Proteobacteria belonging to the KaiC2B2 cluster, a PAS-domain containing protein is encoded next to the *kai* genes like in *L. pneumophila*. This conserved organization suggests a link between this protein and the Kai proteins and further supports a putative role of this operon in the response to environmental signal(s).

Taken together, our phylogenetic analyses suggest that the *kai* genes of *L. pneumophila* have been acquired by horizontal gene transfer, but probably the origin is not the operon encoding the circadian clock named KaiB1C1 in *Synechocystis*, but the paralogous cluster KaiC2B2 for which no clock function has been demonstrated yet.

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Discussion

Bacteria are facing changing environments to which they need to adapt to survive. To respond they use signal transduction pathways that sense external or internal signals. These are processed to generate an output response that can lead to changes in gene expression, cell movement, cell growth or others. Many organisms can also generate an oscillatory response, in which the output varies over time in a repetitive manner. A striking example of such an oscillatory response is the circadian regulation of genes for oxygenic photosynthesis and nitrogen fixation in some *Cyanobacteria* (Mackey and Golden, 2007). This oscillatory response is regulated in *Cyanobacteria* by the KaiABC genes that are extensively studied in *S. elongatus*. *Legionella pneumophila* resides in aquatic reservoirs within biofilms or protozoa and encounters many different environments when it cycles between hosts. It is not known to possess circadian gene regulation or to have the capabilities of photosynthesis or nitrogen fixation. However it encodes KaiBC proteins in its genome (Gomez-Valero *et al*., 2011).

In this study we show that the three Kai operon genes (*kaiB*, *kaiC* and *lpp1114*) of *L. pneumophila* Paris do not seem to be related to a circadian clock. This finding is in line with our phylogenetic analysis that shows that *L. pneumophila* KaiBC cluster with KaiB2C2 of *Synechocystis* sp., a paralogous copy of KaiC1B1 that does not seem to be involved in circadian rhythms (Wiegard *et al*., 2013; Figs. 9 and 10). Furthermore, KaiB2 and KaiC2 do not interact (Wiegard *et al*., 2013). The close phylogenetic relationship between KaiC2 of *Synechocystis* sp. and *L. pneumophila* KaiC is also seen in the alignment of the three KaiC copies of *Synechocystis* sp. with *L. pneumophila* KaiC. The two main phosphorylation residues in the C-terminal region of *L. pneumophila* KaiC are serine/serine residues like in *Synechocystis* KaiC2, and not serine/threonine (S431 and T432 in *Synechococcus sp.*) like in KaiC1 and in other *Cyanobacteria* (Fig. S6). Thus, KaiBC of *L. pneumophila* seem to be different from the canonical circadian Kai system described for many *Cyanobacteria*.

Our sequence comparison and the structure of KaiB that we solved revealed that the C-terminal amino acids that are critical for the KaiB – KaiC interaction in *Cyanobacteria* are missing in *L. pneumophila* KaiB (Fig. 4). Indeed, the *L. pneumophila* proteins do not interact neither in a yeast-nor a bacterial two-hybrid system (Fig. 5). In contrast, a fusion protein of *L. pneumophila* KaiB with the 13 C-terminal residues (EIGDQAEDDLGLE) of KaiB of the Cyanobacterium *Thermosynechococcus elongatus* that we named KaiB-T interacts with *L. pneumophila* and *S. elongatus* KaiC. This result confirms the prediction obtained from the structure of *L. pneumophila* KaiB and shows that the *L. pneumophila* proteins do not interact and that the missing C-terminal residues are responsible. We thus reasoned that the KaiBC proteins of *L. pneumophila* might not encode a circadian clock, and that *L. pneumophila* does not show circadian gene expression regulated by the Kai proteins. Indeed, monitoring of the *kai* operon gene expression during 5 days of intracellular growth of *L. pneumophila* in *A. castellanii* as well as during *in vitro* growth in BYE medium, revealed no circadian, but growth phase-dependent gene expression with the highest expression in stationary growth phase.

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The question arises what is the function of these proteins in *L. pneumophila*. One hint came from the KaiB structure that we solved. KaiB seems to be the most closely related to thioredoxin. Thus we hypothesized that these proteins in *L. pneumophila* might be involved in oxidative stress response. The knowledge concerning the genes involved in oxidative stress resistance and its regulation in *Legionella* is rather limited. The antioxidative defence genes of *L. pneumophila* described to date, are the Cu/Zn and Fe/Mn dependent superoxide dismutase, the katalase peroxidases *katA* and *katB*, or the alkyl hydroperoxide reductases *ahpC1 and ahpC2D*, whose expression is growth phase dependently regulated (St John and Steinman, 1996; Bandyopadhyay and Steinman, 2000; Bandyopadhyay *et al*., 2003; Bruggemann *et al*., 2006; LeBlanc *et al*., 2006). However, most of the regulators of the oxidative stress response pathways known in other bacteria such as SoxRS, OhrR and PerR are missing in *L. pneumophila*. Only a homologue of OxyR has been described (LeBlanc *et al*., 2008). In *Pseudomonas fluorescens*, a related bacterium found in aquatic environments, it is shown that the oxidative stress resistance pathway is controlled by a complex regulatory cascade, including the two-component system GacS/GacA, the mRNA binding protein RsmA, RpoS and a small RNA (*rgsA*; Heeb *et al*., 2005; González *et al*., 2008). A similar pathway exists in *L. pneumophila*, including sigma factors (RpoN, RpoS, FliA), the LetS/LetA (GacA/GacA) two-component system, the mRNA binding protein CsrA (RsmA) and two small RNAs (*rsmY*, *rsmZ*; Molofsky and Swanson, 2004; Sahr *et al*., 2009; Faucher *et al*., 2011). This pathway controls the entry of bacterial cells into the stationary or transmissive phase, which is thought to promote infection of a new host cell. It is thus tempting to speculate that this regulatory pathway in *L. pneumophila* may also be involved in the oxidative stress response and that KaiCB are part of this cascade.

Indeed, we show that the expression of the *kai* operon genes is under control of the stress sigma factor RpoS and that the Δ*kaiC* and Δ*lpp1114* strains are more sensitive to oxidative stress induced by paraquat than the wild type (Fig. 7). The effect exerted by paraquat in the *kai* mutants is similar or even stronger than that observed in an Δ*rpoS* and Δ*letA* mutant strain (Fig. S5). Additionally, the levels of transcription of the *kai* genes are reduced in the Δ*letA* mutant. LetA as well as RpoS respond to the alarmone ppGpp (Zusman *et al*., 2002; Dalebroux *et al*., 2010) that is required to induce the transmission phenotype in stationary phase (Bachman and Swanson, 2001; Hammer *et al*., 2002). In a Δ*letA* background, *L. pneumophila* is not able to express transmissive traits, including RpoS (Sahr *et al*., 2009). Thus, RpoS regulates directly the sensitivity to oxidative stress of Δ*kaiC* and Δ*lpp1114* and LetAinfluences the stress response indirectly. Furthermore, in response to 10 mM paraquat *L. pneumophila* induces the genes encoding the alkyl hydroperoxide reductases AhpC2D. To our knowledge, this is the first report of an activation of AhpC2D in response to oxidative stress. It was reported that OxyR regulates the expression of the *ahpC*D operon; however, we could not detect any difference in its transcription during oxidative stress due to paraquat. Interestingly, the levels of transcription of *pvcA* and *pvcB*, two proteins regulated by iron concentrations was increased during oxidative stress conditions, similar to what was seen in biofilm, where the alkyl hydroperoxidases were up-regulated together with the PvcA and PvcB (Hindre *et al*., 2008). In contrast, in the Δ*kaiC* mutant strain, the transcriptional levels of the alkyl hydroperoxides was diminished further suggesting that the *kai* genes are implicated in oxidative stress response. In addition to their implication in oxidative stress

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resistance, *kai* operon mutants showed also reduced growth in presence of 100 mM NaCl compared with the wild type. Interestingly, in *Arabidopsis thaliana*, the circadian clock associated protein LIP1, regulates light input to the clock and is required for tolerance to salt stress. However the mechanism is not understood yet (Terecskei *et al*., 2013). Another example of circadian clock proteins linked with salt stress is AqpZ an aquaporine regulated by daily oscillations of intracellular osmolarity in *Synechocystis sp.* (Akai *et al*., 2012).

In *L. pneumophila*, the *kaiBC* genes are in an operon with *lpp1114* encoding a PAS (period circadian protein – aryl hydrocarbon receptor nuclear translocator protein – single-minded protein)-domain containing protein. PAS-domains are found from bacteria to humans, and are described to play a role in sensing changes in the environment. Furthermore, PAS proteins are related to the circadian clock of eukaryotic organisms (McIntosh *et al*., 2010). Thus, it seems likely that Lpp1114 senses via its PAS domain the input signal that activates the Kai proteins. The PAS domain encoded by Lpp1114 shares 70% similarity with the LOV domains of YtvA (a blue light GTP-binding receptor) from *Bacillus subtilis* (Jurk *et al*., 2011) and one from *Listeria monocytogenes* (*lmo0799*; Chan *et al*., 2013). LOV domains are a subset of the larger Per-ARNT-Sim (PAS) domain superfamily (Herrou and Crosson, 2011). The second class, LOV-GGDEF/EAL proteins are predicted to regulate the synthesis and hydrolysis of cyclic-di-GMP and constitute around 20% of bacterial LOV proteins. Lpp1114 might belong to this class. Interestingly, in many organisms, a PAS-domain encoding protein corresponding to this class is located adjacent to KaiC (Fig. 9). Although there is a lack of data on the role of light in the life cycle of *L. pneumophila*, light might function as an environmental signal that stimulates *L. pneumophila* in the extracellular environment to up-regulate pathways that promote infection of a new host. However, further studies are needed to determine if it plays a role in light/redox sensing. Surprisingly, the *kaiC* gene does not affect significantly its capacity to kill *A. castellanii* although the capacity to parasitize protozoa is thought to be the main force driving evolution of *L. pneumophila*. Thus, the acquisition of the *kai* operon genes may not be directly related to protozoan parasitism but may increase fitness of *L. pneumophila* in environments containing reactive oxygen species, e.g. in sunlight water ponds or water systems.

Taken together, our findings indicate that the Kai proteins of *L. pneumophila* do not encode a circadian clock but resemble the KaiB2C2 proteins of *Synechocystis* sp. and are involved in stress response. Phylogenetic analyses suggest that they have been acquired by horizontal gene transfer from *Cyanobacteria*, but it seems likely that they have diverged during evolution.

Material and methods

Bacterial strains and growth conditions

Legionella pneumophila strain Paris and derivates were grown on BCYE agar or in ACES (N-(2-acetamido)-2-aminoethanesulfonic acid)-BYE broth. *Legionella pneumophila* was routinely grown on BCYE agar for 2–3 days at 37°C. For growth in liquid medium, *L. pneumophila* was inoculated at an OD 0.5–0.8 at 37°C under continuous shaking. Bacterial growth was monitored by measuring the optical density (OD) of the culture at a wavelength of 660 nm using a spectrophotometer (Eppendorf France S.A.S., Le Pecq, France). *E. coli*

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strains were grown in Luria-Bertani (LB) broth or LB-agar. When necessary antibiotics were added at the following final concentrations: ampicillin at 100 μg ml^{-1} for *E. coli*; kanamycin at 12.5 μg ml^{-1} for *L. pneumophila* and 50 μg ml^{-1} for *E. coli*; chloramphenicol at 10 μg ml^{-1} for *L. pneumophila*, at 20 μg ml^{-1} for *E. coli*; apramycin at 15 μg ml^{-1} for *L. pneumophila*. Recombinant expression of the KaiC and KaiB proteins was done in *E. coli* BL21 (DE3).

DNA manipulation and construction of mutants

The Δ*kaiC* mutant was obtained by replacing the *kaiC* gene with a kanamycin cassette and the Δ*lpp1114* mutant by replacing *lpp1114* with an apramycin cassette. *Legionella pneumophila* Δ*kaiC* mutant strain was constructed as described previously (Bruggemann *et al*., 2006). To construct the Δ*kaiC* mutant strain, the chromosomal region containing the *kaiC* gene was PCR-amplified using the primers KaiC-F ATCGCATCAACCCCTCCTCATT and KaiC-R TGCCAACAAGCTCTGTATGGGG. The product was cloned in the pGEM-T easy vector (Promega Corporation, Madison, WI, USA) and on the resulting plasmid an inverse PCR was performed using the primers KaiC_inv_BamHI-F CGGGATCCCGCATATC CCTTAATTCC and KaiC_inv_BamHI_R CGGGATCCCG CTCTTGAATCTTGAGTTGAGCACG. The PCR product was digested using BamHI enzyme and ligated with a BamHI-digested kanamycin resistance cassette. The Δ*lpp1114* was constructed by a three-step PCR as previously described (Rolando *et al*., 2013). In the first step, we amplified the apramycin-resistant cassette and the upstream and downstream sequence of the *lpp1114*, flanked by short overlapping regions of the apramycin cassette next to the insertion site. For the upstream sequence we used the primers Lpp1114_mut-for TCGT AAGCTGTCTATCCCAGAG and Lpp1114_inv_rev GCT GATGGAGCTG CACATGAATGCTCCAGAATGGGGAG AAAACC, for the downstream sequence Lpp1114_inv_for GAGCGGATCGGGGATTGTCTTTCCGGATCGATGATG ATAA TGC and Lpp1114_mut-rev AACAAGGGGATA GCAGAACAAG. In a second step, the full-length recombinant DNA was synthesized by combining the three PCR fragments obtained in step one. For complementation experiments, the entire operon, genes *kaiC*, *kaiB* and *lpp1114*, was cloned in the vector pMMB207c. As control *L. pneumophila* Paris wild type and the *kaiC* mutant containing the empty plasmid pMMB207 were used respectively. Mutants and plasmids used in this study are listed in Table 7. *E. coli* DH5α and BL21 (DE3) were used for amplification of recombinant plasmid DNA with backbones: pMMB207, pGEM-T easy vector (Promega), pGEX vector (GE Healthcare en France, Aulnay sous bois, France), pQE30 (QIAGEN Sciences, Germantown, MD, USA), TOPOXL (Invitrogen, Life Technologies SAS, Saint Aubin, France). Genomic and plasmid DNA were prepared, amplified and sequenced according to standard procedures. Nucleotide and protein sequence were analysed using NCBI website (http://www.ncbi.nlm.nih.gov/), ExPAsy (http://www.expasy.ch), T-Coffee (http://www.tcofee.org) and DNA sequence assembly (Gap4).

Intracellular growth in *A. castellanii*

Intracellular growth of *L. pneumophila* in *A. castellanii* was done as previously described (Lomma *et al*., 2010). Briefly, 3-day-old cultures of *A. castellanii* incubated at 20°C in PYG 712 medium (2% bactotryptone, 0.1% yeast extract, 0.1 M glucose, 4 mM $MgSO_4$, 0.4 M

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$CaCl_2$ 0.1% sodium citrate dihydrate, 0.05 mM $Fe(NH_4)_2(SO_4)_2 \times 6H_2O$, 2.5 mM NaH_2PO_3, 2.5 mM K_2HPO_3) were washed using infection buffer (PYG 712 medium without tryptone, glucose and yeast extract) seeded in 25 cm^2 flasks and adjusted to 10^5 cells per ml. *Legionella pneumophila* grown on BYCE agar and diluted in PBS and added to the flask containing *A. castellanii* at multiplicity of infection (MOI) of 0.1 or 100. After an hour of incubation at 37°C or 20°C to allow the invasion of *L. pneumophila*, attached *A. castellanii* were washed carefully twice with infection buffer. Intracellular replication was monitored by taking 300 µl of sample at each time point. To lyse the amoeba, 300 µl the cells were centrifuged (3 min, 14 000 rpm) and vortexed and 100 µl were plated on BYCE agar in serial dilutions.

Competitive assay during infection of *A. castellanii*

For competitive infection assays, *L. pneumophila* strains Paris wild type and the Δ*kaiC* or Δ*lpp1114* mutants or the wild-type strain carrying the empty plasmid and the complemented strains Δ*kaiC*, respectively, were compared. The competition assay was done as previously described (Kessler *et al*., 2013). Briefly, *L. pneumophila* and the respective competitor were both grown to stationary phase on BYCE agar and mixed in equal ratios adjusted to OD 1 in PBS. This mix of bacteria was added to the flask containing *A. castellanii* prepared as described earlier at an MOI of 0.1. After 1 h invasion at 37°C the layer of *A. castellanii* was washed twice with infection buffer. To monitor the total number of bacteria, a 300 µl sample was taken, centrifuged to 14 000 rpm, vortexed and plated on BYCE agar with and without corresponding antibiotic for bacterial selection. After 2 days of incubation at 37°C, a second sample was taken and seeded in BCYE agar plates and BCYE agar plates containing selective antibiotic. Additionally, new 3-day-old cultures of *A. castellanii* in PYG 712 medium were washed twice and adjusted to 10^5 cells per ml and 100 µl of 1×10^{-3} dilution of the bacteria sample was added to the flask starting a new round of infection. Again after 2 days of incubation the same procedure described earlier was performed.

RNA isolation and labelling for array hybridization

For *in vitro*-grown *L. pneumophila*, total RNA for array hybridization and real-time PCR was extracted as described previously (Sahr *et al*., 2012). For total RNA extraction of *L. pneumophila* growing intracellularly in *A. castellanii*, an additional FastPrep step was added to the protocol prior to RNA extraction as described previously (Bruggemann *et al*., 2006). RNA was reverse-transcribed and indirectly labelled with Cy5 and Cy3 (GE Healthcare) by using the cDNA Superscript Indirect Labeling Kit (Invitrogen).

L. pneumophila genome microarray and hybridization experiments

For transcriptional experiments, microarrays for *L. pneumophila* were used as previously described (Bruggemann *et al*., 2006). Array hybridization was performed using 250 pmol of labelled cDNA. At least, two biological replicates were performed for each condition including dye swap. Microarray slides were scanned using GENEPix 4200 AL scanner (Axon Instruments, Molecular Devices, LLC, Sunnyvale, CA, USA) and the laser power was adjusted to balance the two channels. The images were analysed using the GENEPIX PRO 6.0 software. For statistical analysis the R software was used (http://www.r-

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project.org/) and the microarray data was normalized using a loess based method using the BioConductor package marray (http://www.bioconductor.org/packages/bioc/stable/src/contrib/html/marray.html). The gene names in the tables (*lpp*) refer to the strain Paris, available at the LegioList web server (http://genolist.pasteur.fr/LegioList).

Real-time PCR

qRT-PCR was performed to confirm transcriptome results using the total RNA in the same conditions. After DNase treatment, cDNA synthesis was performed with 5 μg RNA and reverse transcriptase (400 U μl^{-1}, Invitrogen) according to the manufacturer's instructions for 2–3 h at 42°C. The experiment was done in 20 μl reaction volume containing 1.2 ng of cDNA, 10 μl SYBR PCR master mix (Applied Biosystems, Life Technologies SAS) and gene-specific primers (300 nM; Table S2). Each sample was done in triplicate and with different cDNA dilutions (non diluted, 1:10, 1:100, 1:1000). The quantity of cDNA for each tested gene was normalized to the constitutively expressed genes, *letA* and *tldD*. The relative gene expression was calculated as the ratio normalized target concentrations ($\Delta\Delta C_t$) using the REST software (http://www.gene-quantification.de/rest.html). Primers were designed using the PRIME3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) leading to amplification products in a size range of 150–250 bp. The amplification was performed using an ABI Prism 7900 HT sequence detection system (PE Applied Biosystems).

Oxidative stress induced by 10 mM paraquat

Legionella pneumophila and its derivates were grown in 50 ml BYE medium until exponential growth (OD 2.5). The cultures were adjusted to OD 1.0 and two aliquots of 5 ml of this culture were taken, centrifuged at 5000 g for 5 min at room temperature and the pellet was resuspend in 5 ml of fresh BYE medium. A final concentration of 10 mM of Paraquat (Sigma-Aldrich, Lyon, France) was added and incubated for 1 h at 37°C under shaking at 250 rpm. Every 10 min a sample of 300 μl was taken and serial dilutions were plated on BYCE to estimate the number of bacterial survival. In parallel, the second aliquot was recovered after 15 min of incubation with 10 mM paraquat, and the RNA was extracted as described earlier, for further transcriptional analysis.

Sodium sensitivity

Sodium sensitivity was tested with *L. pneumophila* grown to post-exponential growth (OD 3.8) in liquid broth. Ten microliters of the culture was pipette in serial dilutions on BCYE agar plates containing or lacking 100 mM NaCl.

Two-hybrid experiments to test protein–protein interaction

We used a yeast two-hybrid approach based on the Cytotrap vector kit (Stratagene, Agilent Technologies, Inc., Life Sciences and Chemical Analysis Group, Santa Clara, CA, USA) according to the manufactures instructions. In this method KaiB was used as bait and KaiC. Briefly, *kaiB* and *kaiC* were cloned in frame into the pSos and pMyr vectors, respectively, and expressed as fusion protein. hSos-*kaiB* was obtained by amplifying *kaiB* using the primer pSos-KaiB-FAGGATCCCCATGGCCATGACTAAG CTAAAGCTG and pSos-

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KaiB-R GTTATTTCTGCTTTGG AAATGGATTAGACGTCGACGCGCGCA. The product was cloned in pSos; pMyr-*kaiC* was obtained by amplifying *kaiC* using the primers pMyr-KaiC-F GCCCGGGC CTCGAGGGTGAATACCAATAAAGCAAAAACGGGA and pMyr-KaiC-R GAGTCCAACAAATTGTTAAGAGAGTAA TCGAGGTCGACTAAT and the product was cloned in pMyr fusing *kaiC* to a myristoylation sequence at the N-terminus. Both fusions were co-expressed in the *Saccharomyces cervisae* mutant strain cdc25H at 37°C.

Secondly we used a bacterial two-hybrid approach based on the BACTH kit (Euromedex, Souffelweyersheim, France). In this method, the T25 and T18 domains were separately fused to the N-termini of KaiC and KaiB/KaiB-T respectively. pKT25-*kaiC* was obtained by amplifying *kaiC* using the primer T25-KaiC-F TCTAGAGGTGAAT ACCAATAAAGCAAAAACG and T25-KaiC-R GAGCT CGGCTCTCTTAACAATT TGTTGGACTC; pUT18-*kaiB* was obtained by amplifying *kaiB* using the primers T18-KaiB-F CTGCAGGATGACTCGAACTAAGCTAAAGC and T18-KaiB-R GAGCTCGGAT CCATTTCCAAAGCAGAAA TAAC. To construct pUT18C-*kaiB-T*, the *kaiB* sequence was used as a template and amplified using the primers T18-KaiB-F and T18-KaiB-T-R GAGCTCGGTTCCAA GCCTAAGTCATCCTC. As control *S. elongatus kaiC* and *kaiB* were cloned in pKT25 and pUT18C respectively. The primers used to amplify *kaiC* were T25-CyKaiC-XbaI-F CTGCAGGATGAGCCCTCGTAAAACCTAC and T25-CyKaiC-KpnI-R GGTACCCGG CTCTCCGGCCCTTT TTCTTG; and for *kaiB* T18-CyKaiB-PstI-F GAGCTCGG GAAGTCGTCGGAATCTTGAAG and T18-CyKaiB-SacI-R TCTAGAGATGACTTCC GCTGAGATGACTAG. All constructs were confirmed by sequencing.

For β-galactosidase activities for the BACTH measurements, cells were grown to OD 4. The culture was diluted 1:10 and the OD at 600 nm was measured. An aliquot of 20 μl of the culture was added to 80 μl of permeabilization solution (Solution A: 0.8 mg ml^{-1} hexadecyltrimethylammonium bromide, 0.4 mg ml^{-1} sodium deoxycholate, 70 mM NaH_2PO_4 (pH 7), 30 mM NaH_2PO_4 20 mM KCl 2 mM $MgSO_4$, and 5.4 β-mercaptoethanol). The permeabilization solution mixture was kept for 30 min at 30°C. Then 600 μl of substrate solution (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mMKCl, 1 mg ml^{-1} o-nitrophenyl-B-D-galactopyranoside and 2.7 μl ml^{-1} β-mercaptoethanol) was added to start the reaction. After 30 min to 1 h at 30°C, the reactions were stopped by adding 1 ml of 1 M Na_2CO_3. Enzyme activity was expressed as the rate of change A420 per h per A600 unit of culture; the enzymatic activity was calculated according to the following formula: Enzymatic activity = 200 × {[(OD420 – OD420 in control tube)/min of incubation] × dilution factor}. The enzymatic activity was given in units per mg considering 1 ml of culture at OD600 = 1 corresponds to 300 μg dry weight bacteria.

Purification of His6-KaiC protein

The *kaiC* genes of *L. pneumophila* and *S. elongatus* were cloned into the expression vector pQE30 using the restriction sites of the primers SalI_KaiC_F TTTGTCGAC TAGTGAATACCAATAAAGCAAAAACG and PstI_KaiC_R TTCTGCAG TTACTCTCTTAACAATTTGTTGG for *L. pneumophila* and Sal_KaiC_F

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TTTGTCGACTAATGAC TTCCGCTGAGATGACTAGCC HindIII_KaiC_R TTTA AGC TTCTAGCTCTCCGGCCCTTTTTC for *S. elongatus*. Both *kaiC* genes were fused to the His6x tag at the N-terminus. For expression of His-KaiC, BL21 (DE3) cells were transformed with pQE30_*kaiC* and cultured at 25°C for 50 h without IPTG (Iwasaki *et al*., 1999). Total protein was extracted and affinity purification of the His6-KaiC protein from cell extracts of each transformant using a nickel-charged resin (Qiagen) was performed. To wash the column buffers with increasing concentration of imidazole were used: W-Buffer (25 mM Tris pH 8, 0.5 M NaCl, 10 mM/30 mM/50 mM/70 mM Imidazole, 5 mM MgCl2). Proteins were eluted with 500 mM Imidazole and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (10%), blotted onto polyvinylidene difluoride membranes and incubated with anti-histidine-antibody (AbCam #ab14923). Primary antibodies were detected with anti-rabbit HRP conjugates IgG (DakoCytomation, Dako France S.A.S., Les Ulis Cedex, France) and immunoblots were revealed with a G : Box system (Syngene Europe office, Cambridge, UK).

KaiB crystallization and structure determination

For structural analysis, *L. pneumophila* KaiB and KaiC genes were cloned into a modified pET15b vector (Novagen, EMD Millipore Corporation, Billerica, MA, USA) in which the tobacco etch virus protease cleavage site replaced the thrombin cleavage site and a double stop codon was introduced downstream the cloning site (Zhang *et al*., 2001). Selenomethionine enriched protein samples was obtained by culturing the *E. coli* BL21(DE3)-RIPL (Agilent Technologies Canada, Inc., Mississauga, Canada) cells containing KaiB or KaiC expressing constructs in M9 SeMET High-Yield growth media (Shanghai Medicilon Inc., Shanghai, China) at 37°C to an optical density (at 600 nm) of approximately 1.2. At this point 0.4 mM IPTG was added to induce KaiC protein expression. After induction, the cells were incubated overnight with shaking at 20°C.

The harvested cells were resuspended in 250 mM NaCl 5% glycerol, 100 mM NH_4 sulphate, 50 mM phosphate–citrate buffer pH 7.5, 0.5 mM tris(2-carboxyethyl) phosphine (TCEP), 0.08% *n*-Dodecyl-β-D-maltopyranoside (DDM) and 5 mM imidazole and lysed by sonication. The lysate was clarified by centrifugation (30 min at 17 000 × g) and the clarified soluble fraction was applied to a metal chelate affinity column charged with Ni^{2+} pre-equilibrated with 300 mM NaCl, 50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) Na pH 7.5, 5% glycerol, 5 mM imidazole. The bound protein was washed with 300 mM NaCl, 50 mM HEPES Na pH 7.5, 5% glycerol, 30 mM imidazole buffer and eluted with 300 mM NaCl, 5% glycerol, 50 mM HEPES pH 7.5, and 250 mM imidazole buffer, Next the polyhistidine tag was cleaved from the fusion protein by treatment with recombinant His-tagged Tobacco Etch Virus (TEV) protease (Tropea *et al*., 2009) followed by dialyses at 4°C in 50 mM HEPES (pH 7.5), 300 mM NaCl, 5% glycerol and 0.5 mM TCEP. The cleaved protein was then resolved from the cleaved His-tag and the His-tagged protease by flowing the mixture through a second Ni^{2+}-chelated affinity column.

Before submission to crystallization trial protein was dialysed in 300 mM NaCl, 10 mM HEPES Na pH 7.5 and 0.5 mM TCEP. Particulate matter was removed from protein samples by passing it through a 0.2 μm Ultrafree-MC centrifugal filter (EMD Millipore Corporation).

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No crystallization was observed for KaiC protein samples. The KaiB protein was crystallized by hanging-drop vapour diffusion by mixing 2 μL of the protein solution (20 mg ml^{-1}) with 2 μL of a precipitant solution, 0.2 M Na Acetate, 0.1 M Bis-Tris pH 6.0, 35% w/v PEG 8 K and 1 mM Zn acetate. Prior to data collection, crystals were transferred into paratone-N and cryo-cooled in a steam of liquid nitrogen.

The crystal structure of *L. pneumophila* KaiB was determined by the single anomalous dispersion method using selenomethionine enriched protein crystals. Initial phases were calculated using Solve (Terwilliger and Berendzen, 1999) from Phenix software suite (Adams *et al*., 2010) and improved by solvent flattening and histogram matching using Resolve (Terwilliger and Berendzen, 1999) prior to model building. The KaiB native crystal diffracted to a 1.95 Å resolution and its structure was solved by molecular replacement using Phaser from the CCP4 suite. The obtained structure model was manually refined using Coot (Emsley and Cowtan, 2004) and Refine from the Phenix suite. The KaiB final model contains 181 residues per asymmetric unit and 150 water molecules; 98.8% and 1.2% residues belonged to the most and additionally favoured regions of the Ramachandran plot respectively. The complete data reduction and the refinement statistics are summarized in Table S3.

Protein phosphorylation assay

The autokinase assay was performed at 30°C as described previously, with some modifications (Nishiwaki *et al*., 2000). In brief, 1 μg of KaiC was incubated for 1 h at 30°C in 10 μl of incubation buffer (20 mM Tris pH 8, 150 mM NaCl, 5 mM MgCl2) containing 5 μCi of [γ-32P]-ATP. Reaction mixtures were subjected to SDS-PAGE (10%) followed by autoradiography.

Phylogenetic analysis of the KaiB and KaiC sequences

Homologues amino acid sequences of the KaiC protein were identified by BlastP search against 1408 completely sequenced genomes of bacteria and archea available at the NCBI database, using the protein sequence of KaiC of *Synechococcus elongatus*. Only hits covering at least 80% of the length of the protein of *S. elongatus* with a minimum e-value of e^{-5} were taking into account. We then selected the representative protein of each species of bacteria and archea that was giving the best hit. Finally, a total of 53 sequences containing KaiC were retrieved belonging each to a different species. In each of those the presence of the KaiB protein sequence was searched by BlastP using the *S. elongatus* KaiB sequence as reference. KaiB was identified in 23 of the 53 organisms. Then the KaiC and KaiB sequence of these 23 organisms were aligned separately and concatenated using the programme Expresso (Taly *et al*., 2011). For the alignments, the best evolutionary model was selected using the program Prottest (Abascal *et al*., 2005) with the Akaike information criterion to select the best-fit model for amino acid substitutions. Then phylogenetic trees for KaiB and Kaic, respectively, were constructed using both a distance method (neighbour-joining) and maximum likelihood with 300 bootstrap replicates. Distance methods were run in MEGA4 (Tamura *et al*., 2007) and the KaiBC tree was obtained by using the program Phyml (Guindon *et al*., 2005). To construct the KaiB tree, we used the 23 sequences identified, aligned them using Expresso, the best evolutionary model was selected using Prottest and

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the phylogenetic tree was constructed using a distance and maximum likelihood using 300 bootstrap replicates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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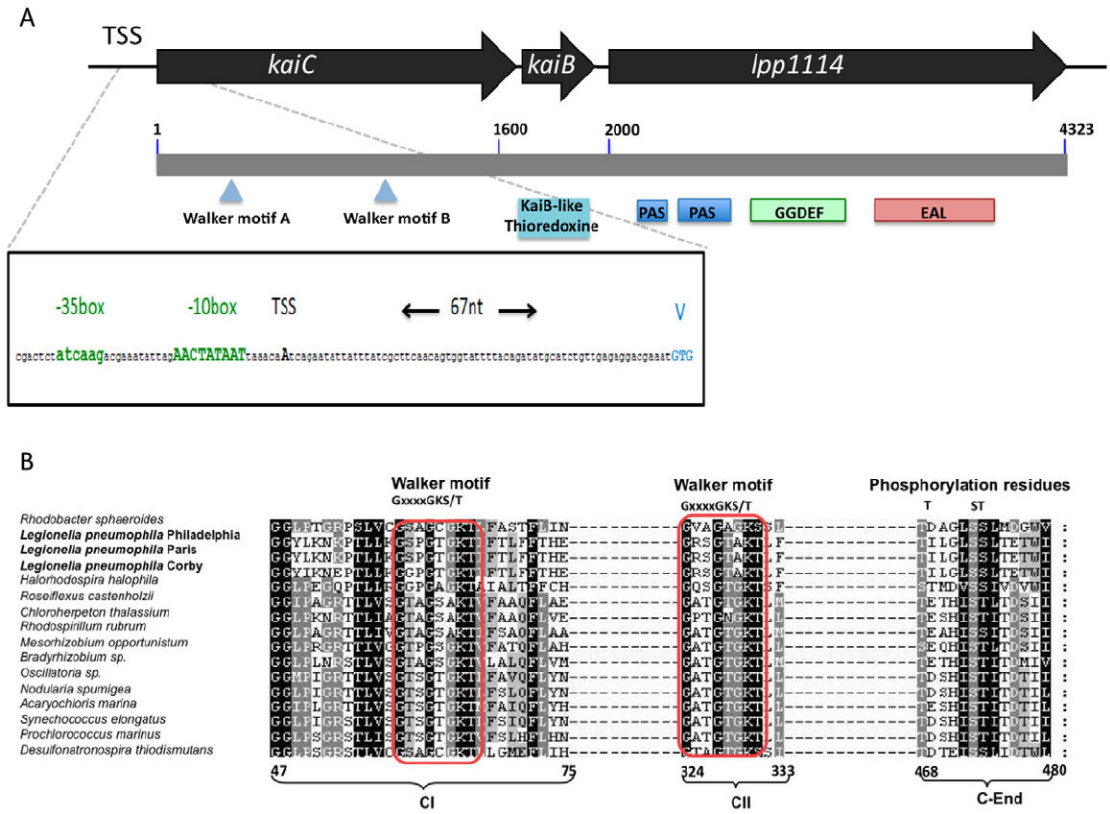

Fig. 1.

Schematic representation of the *kai* operon in *Legionella pneumophila*.

A. Black arrows represent the genes of the *kai* operon: *kaiC (lpp1116), kaiB (lpp1115)* and *lpp1114*. The gray bar indicates the length of the mRNA transcribed starting at the 1 + position of *kaiC*. Below the gray bar, the two Walker motifs found in KaiC are indicated (blue triangles), the domains encoded by each protein are represented as boxes. The transcriptional start site (TSS) is 67 nucleotides upstream the translational start (indicated by V for Valine). Upstream the TSS the −10 and −35 box of a predicted RpoS promoter sequence is shown in green.

B. Alignment of different bacterial KaiC proteins with that of *L. pneumophila*. Red boxes indicate the conserved walker motifs. The numbers below, indicate the amino acid position with respect to the full-length protein sequence of *Synechococcus elongatus*. CI stands for the first KaiC domain, CII stands for the second KaiC domain and C-End indicate the C-terminus of the protein.

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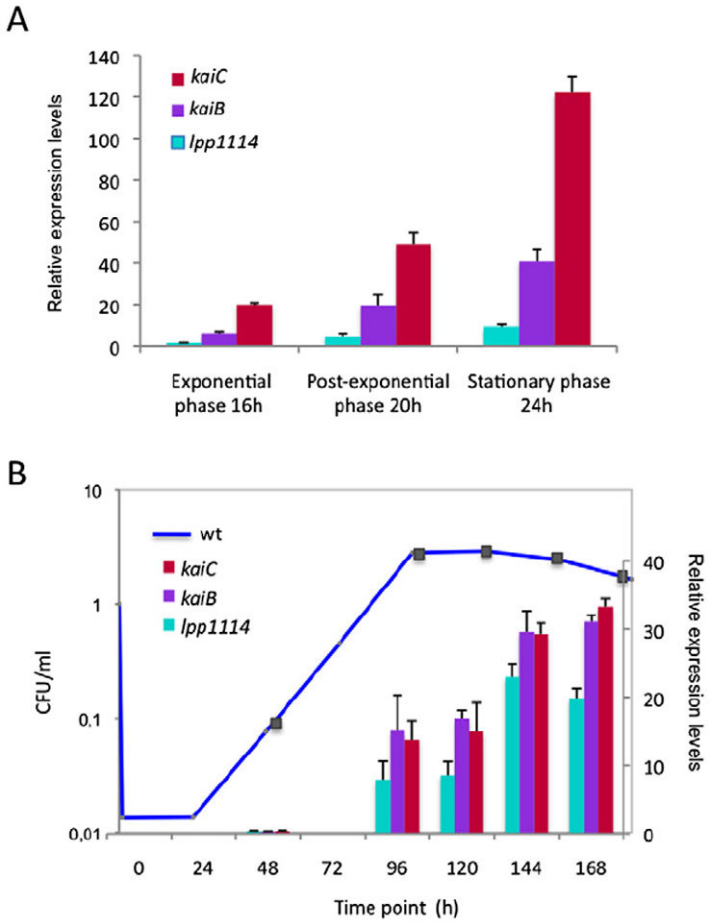

Fig. 2.

The expression of the *kai* operon genes is growth phase dependent *in vitro a*nd *in vivo* in *Acanthamoeba castellanii*.

A. Quantitative real-time polymerase chain reaction of the *kai* operon genes during 24 h *in vitro* growth. The bars represent the relative quantity of mRNA at the sampled time points relative to mRNA levels at exponential phase (OD 2.5). Red bars *kaiC*; purple bars, *kaiB*; cyan *lpp1114*.

B. Relative gene expression of the *kai* operon genes during intracellular growth in *A. castellanii*. Amoebae were infected with *Legionella pneumophila* wild type at MOI = 100 and the infections were followed at 20°C over 7 days (168 h). The squares on the growth curve indicate the time points (hours) of intracellular growth when the mRNA samples were taken. Transcription levels of the genes were calculated with respect to the values obtained at 72 h. The bars represent the mean of two independent experiments ± standard deviation.

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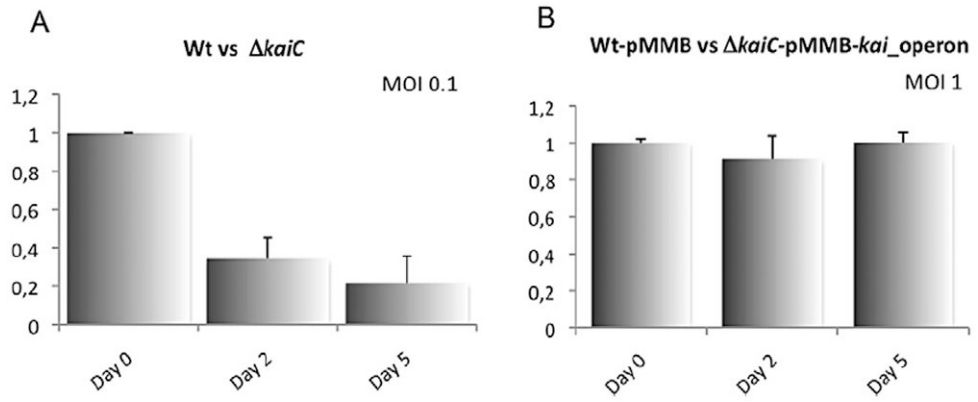

Fig. 3.

The *kai* operon genes confer fitness in competitive intracellular growth in *Acanthamoeba castellanii*.

A. Competitive assay of *Legionella pneumophila* wild type vs Δ*kaiC* after infection of *A. castellanii* with an MOI of 0.1. The graph represents the ratio between wild type and mutant bacteria recovered at day 0, 2 and 5. The Δ*kaiC* mutant is outcompeted by the wild type after two days of infection of *A. castelanii*.

B. Competitive assay of *L. pneumophila* wild type carrying the empty vector pMMB vs Δ*kaiC* carrying the pMMB vector encoding for the entire operon (*kaiOp*). Infection of *A. castellanii* was performed with an MOI of 1. The phenotype is restored when the Δ*kaiC* mutant is complemented with the entire operon *in trans*. The bars represent the mean of at least three independent experiments ± standard deviation.

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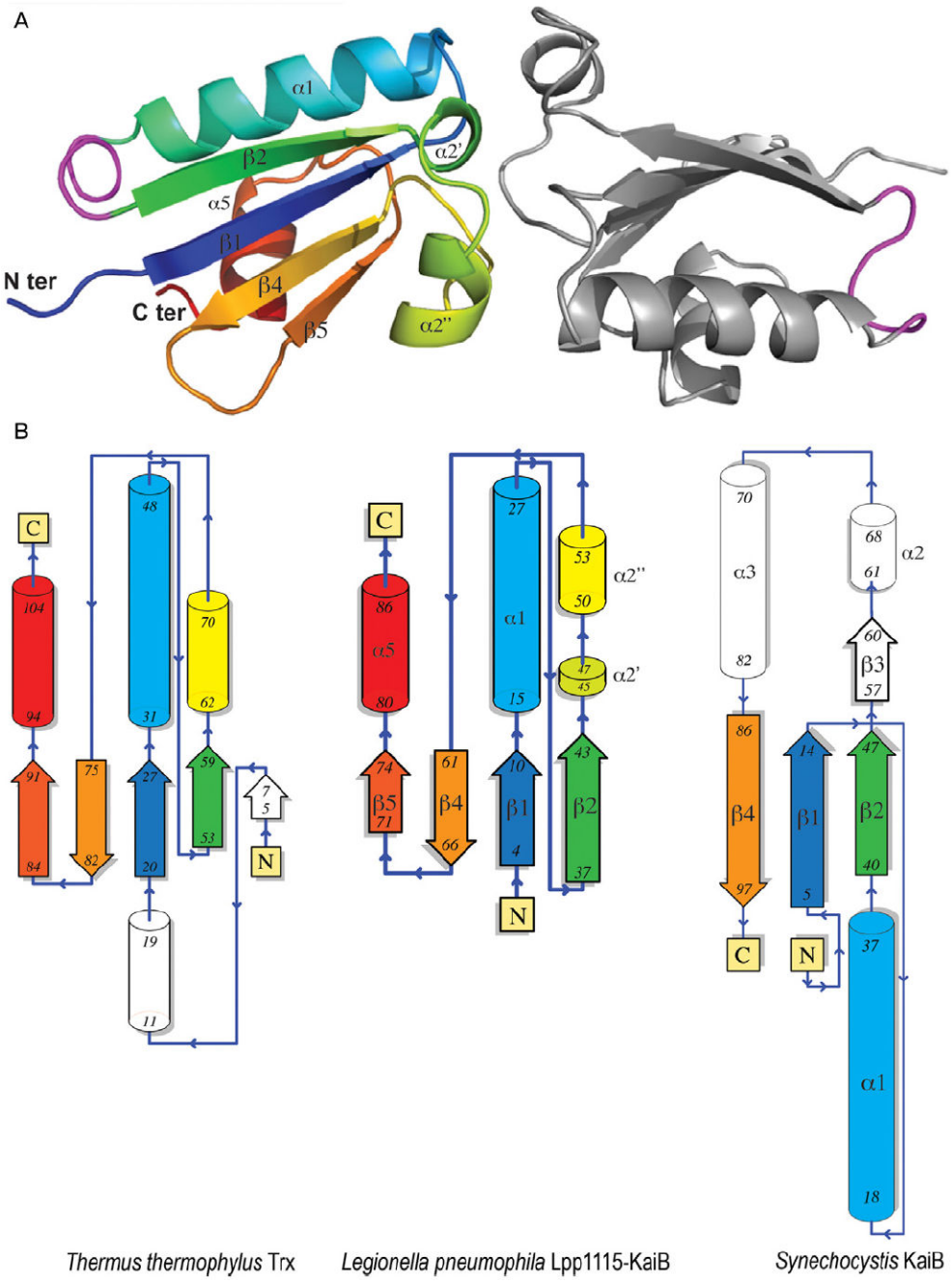

Fig. 4.

Structure of *Legionella pneumophila* KaiB highlights differences between Lpp1115 and other members of the KaiB protein family.

A. The structure of the *L. pneumophila* KaiB protein features two full-length protein (residues 1–90) molecules shown as a ribbon diagram. One of the Lpp1115-KaiB monomers is coloured in *rainbow spectrum* from N-terminus (blue) to C-terminus (red). The secondary structure elements in Lpp1115-KaiB are labelled (α for a helix and β for a strand) in black and numbered according to previously characterized cyanobaterial KaiB proteins. Lpp1115-

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KaiB N- and C-termini are also labelled. The KaiB region between residues 31 and 37 adapting different conformations in each KaiB monomer is coloured in magenta. B. *Legionella pneumophila* KaiB (centre), *Thermus thermophilus* thioredoxin (left) and *Synechocystis* sp. PCC6803 (PDB 1WWJ) *Thermosynechococcus elongatus* BP-1 KaiB (right) secondary structure topologies illustrate the structural similarities between these protein molecules. The secondary structure elements in *L. pneumophila* KaiB are coloured according to rainbow spectrum of the KaiB ribbon diagram presented above. The numbers of first and last residues in each β-strand and α-helix are indicated in black. The secondary structure elements in *T. thermophilus* Thioredoxin (left) and *T. elongatus* BP-1 KaiB (right) are colored according to matching elements in the *Legionella* Lpp1115-KaiB structure. Pymol (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC) and PDBsum software (Laskowski, 2009) were used to generate this figure.

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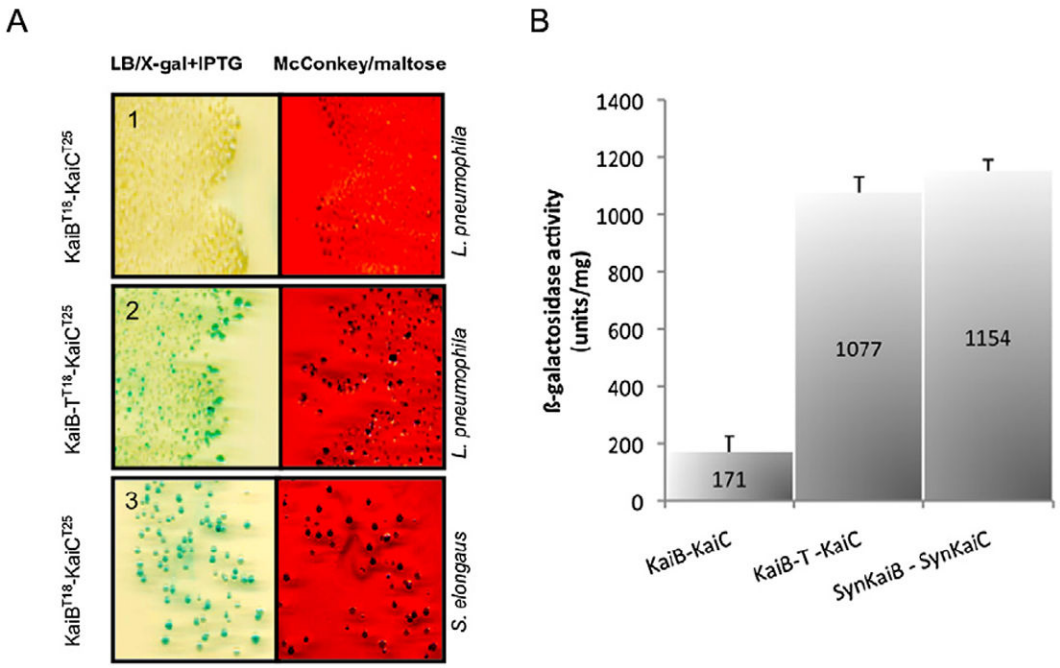

Fig. 5.

Bacterial two hybrid assay suggests that *Legionella pneumophila* KaiC and KaiB do not interact. The interaction test is based on the reconstitution of the adenylate cyclase activity in an *Escherichia coli* strain lacking endogenous adenylate cyclase.

A. KaiC and KaiB from *L. pneumophila* and *Synechococcus elongatus* were fused to T25 and T18-domain of the adenylate cyclase respectively. Interaction assay of KaiC with KaiB of *L. pneumophila* (Panel 1). Interaction assay KaiC with KaiB-T in which the C-terminal residues from *T. elongatus* are fused to *L. pneumophila* KaiB (Panel 2). Interaction assay with, KaiC from *S. elongatus* and KaiB from *L. pneumophila* used as a positive control showed interaction on the indicator media (Panel 3).

B. The β-galactosidase activity was measured for each combination of proteins. KaiB-T and KaiC from *Legionella pneumophila* display similar levels of β -galactosidase suggesting the importance of the C-terminal residues of KaiB for interaction with KaiC. The bars represent the mean values of three independent experiments ± standard deviation.

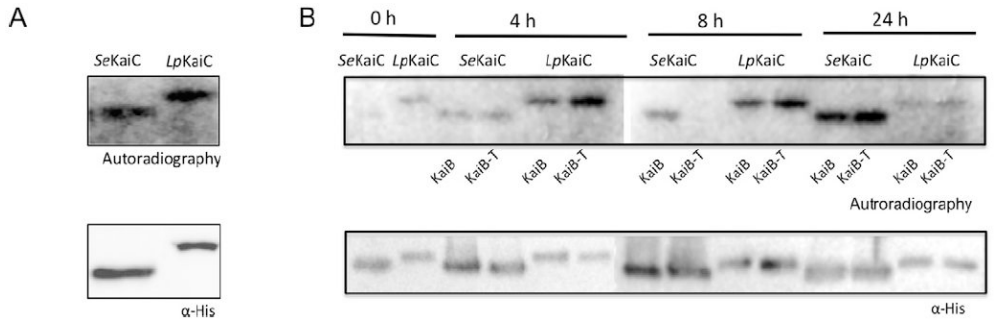

Fig. 6.

Legionella KaiC conserves autokinase activity but is not dephosphorylated by KaiB.

A. Western blot using an α-histidine antibody against the purified *Legionella* 6xHis-*Lp*KaiC (65 kDa) and *S. elongatus* 6xHis-*Se*KaiC (58 kDa) proteins (lower panel). Autophosphorylation of *Se*KaiC and LpKaiC incubated with 5 μCi [γ-^{32}P]-ATP for 1 h at 30°C (upper panel).

B. Kinetics of autophosphorylation and dephosphorylation of *Lp*KaiC and *Se*KaiC in presence of KaiB or KaiB-T of *L. pneumophila*. Recombinant KaiC proteins were incubated with KaiB or KaiB-T, respectively and 5 μCi [γ -^{32}P]-ATP at 30°C for 24 h. Samples were taken at 4 h, 8 h and 24 h of incubation and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis analyses. The phosphorylation state was detected by autoradiography (upper panel) and Western blot of the reaction mixtures with α-His as loading control (lower panel).

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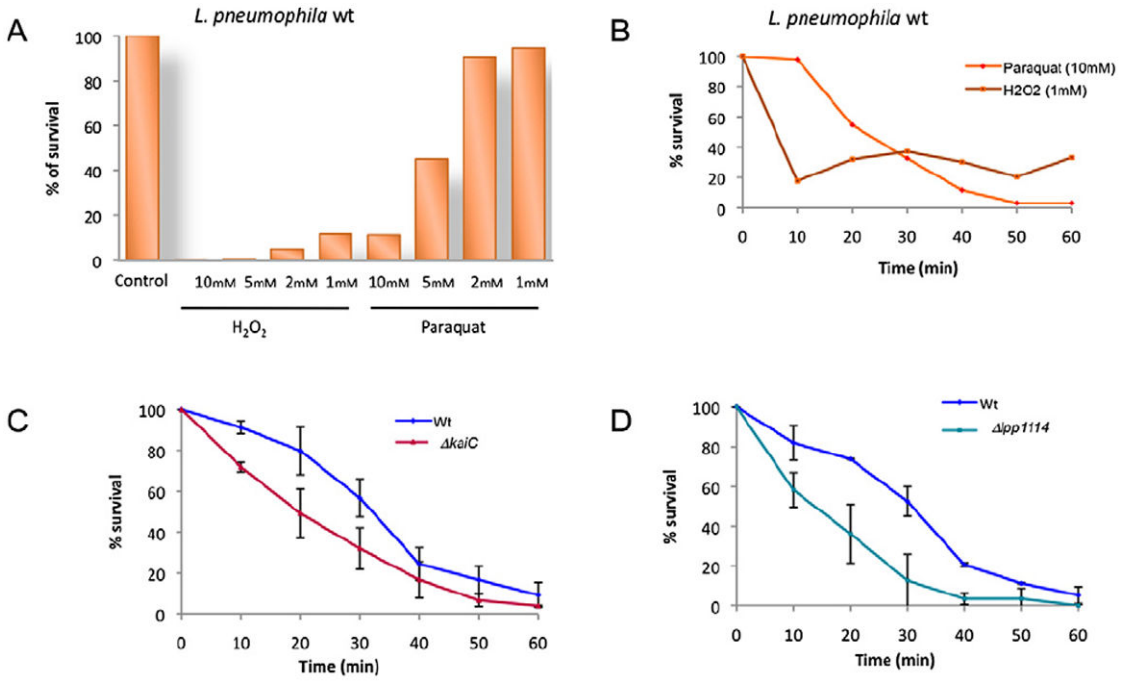

Fig. 7.

Legionella pneumophila ΔkaiC and Δ*lpp1114* mutants are sensitive to oxidative stress exerted by H_2O_2 and paraquat.

A. Percentage of wild-type *L. pneumophila* surviving at different concentrations of H_2O_2 and paraquat after 30 min of exposure.

B. Kinetics of the effect of 10 mM paraquat (purple) and 1 mM H_2O_2 (blue) on the survival of *L. pneumophila* wild type over 60 min C and D. Comparison of the survival kinetics of the wild type and Δ*kaiC* (C) or Δ*lpp1114* mutant strains (D), respectively: blue, wild-type strain; red ΔkaiC*; cyan* Δ*lpp1114*. Each point represents the mean ± standard deviation value of at least two independent experiments.

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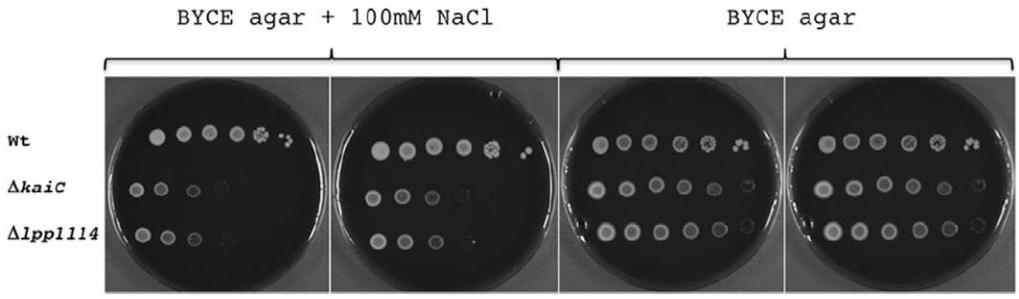

Fig. 8.

The ΔkaiC *and* Δ*lpp1114* mutant strains display a higher sensitivity to 100 mM NaCl than wild-type *Legionella pneumophila. Legionella pneumophila* wild-type and mutant strains were grown to OD 3.8 in buffered yeast extract (BYE) medium and 10 µl of serial dilutions (non diluted to, 10^{-5}) were spotted onto agar containing or not 100 mM NaCl. Plates were incubated for 3 days at 37°C. Representative picture of three independent experiments.

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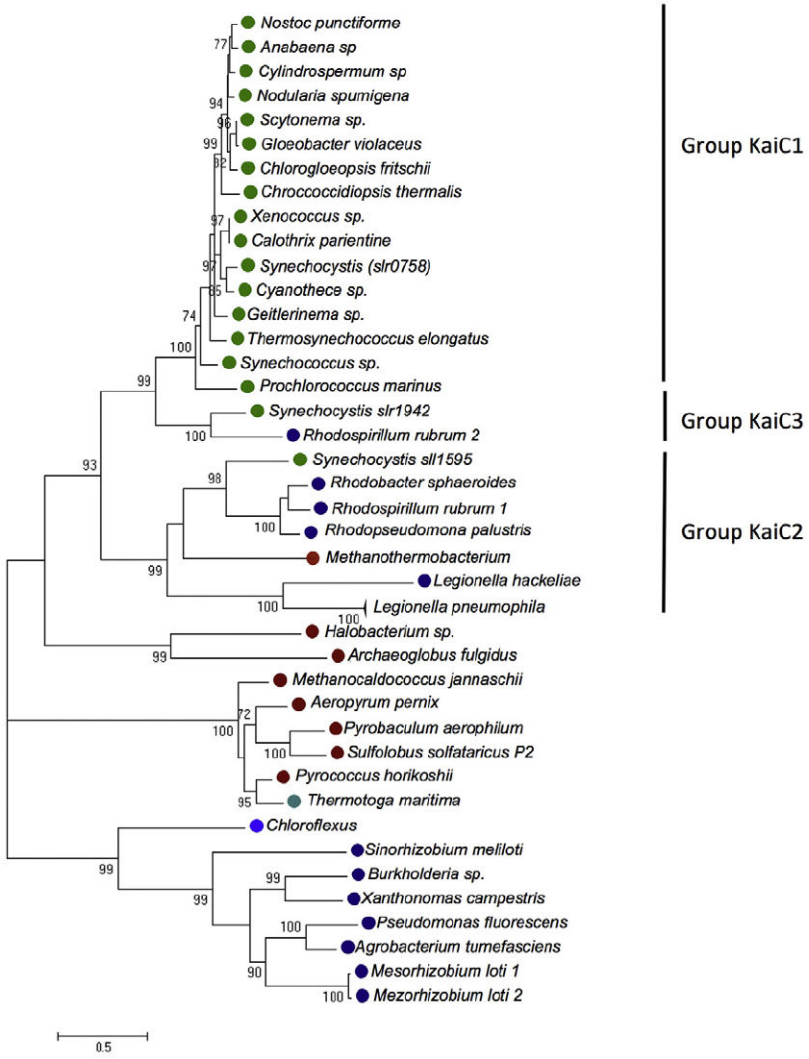

Fig. 9.

Phylogenetic tree of KaiC and its paralogues in selected organism. The tree was constructed using representative KaiC sequences as published previously (Dvornyk *et al*., 2003) and the KaiC sequence of *Legionella pneumophila* was included. KaiC of *L. pneumophila* groups with KaiC2 of *Synechocystis*. Cyanobacteria, green circles; Proteobacteria, dark blue circles; Archaea red circles; Chloroflexus, blue circles; Termotogae, cyan circles.

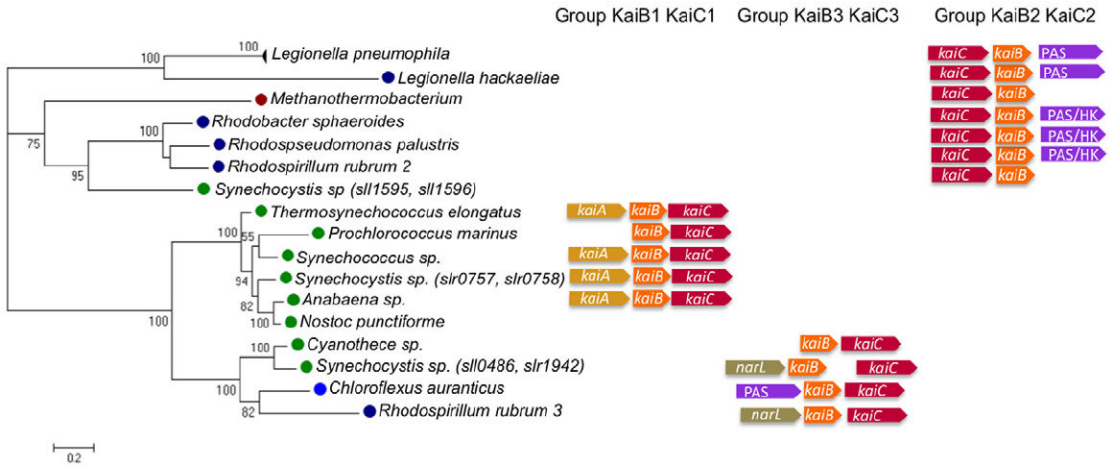

Fig. 10.

Phylogenetic tree of concatenated KaiCB sequences identifies three different groups of KaiBC systems. The tree was constructed using the same representative sequences as published by Dvornyk and colleagues (2003) with the *Legionella* sequences included. The Kai proteins of *Legionella* group with KaiC2B2 from *Synechocystis* (Wiegard *et al*., 2013), Cyanobacteria, green circles; Proteobacteria, dark blue circles; Archaea, red circles; Chloroflexus, blue circles. The right panel shows the chromosomal organization of the three different groups of *kaiB* and *kaiC* as described for the three copies of KaiC and KaiCB of *Synechocystis. kaiB1C1* are the orthologous genes of the circadian genes in *S. elongatus;* kaiB2C2 does not seem to be involved in circadian functions; KaiB3C3 were reported to have a putative involvement in fine-tuning parameters of the circadian clock.

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Table 1

Genes down-regulated in the Δ*kaiC* mutant at OD 4.0 after *in vitro* growth in buffered yeast extract at 37°C.

Gene ID	Product	FC
lpp1115	KaiB – like circadian clock protein	0.158*
lpp1116	KaiC – like circadian clock protein	0.167*
lpp1114	Regulatory protein (GGDEF and EAL domains)	0.237*
lpp1823	Unknown	0.506
lpp0954	Unknown	0.57
lpp1856	Similar to esterase/lipase	0.583
lpp2849	Similar to putative coproporphyrinogen oxidase A	0.604

Significantly differentially regulated genes are marked by an asterisk.

*$P < 0.05$.

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Table 2

Genes with differential expression in the Δ*kaiC* and the Δ*lpp1114* mutant at 144 h after *in vivo* growth in *A. castellanii* at 20°C.

Gene ID	Product	ΔkaiC FC	Δ*lpp1114* FC
lpp3032	Major outer membrane protein	0.482*	2.248*
lpp3033	Major outer membrane protein precursor	0.694	2.035*
lpp2209	Unkown	0.528*	2.165*
lpp1294	Flagelline (*flaA*)	0.672	1.529
ncRNA18	ncRNA18	0.533	1.624
lpp1114	Regulatory protein (GGDEF/EAL domains)	0.593	0.759
lpp1115	KaiB – like circadian clock protein	0.353	1.093
lpp1116	KaiC – like circadian clock protein	0.308	1.09

Significantly differentially regulated genes are marked by an asterisk.

*$P < 0.05$.

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Table 3

Genes up-regulated in *Legionella pneumophila* after exposure to 10 mM paraquat (15 min).

Gene ID	Product	FC
lpp2299	Similar to alkyl hydroperoxide reductase AhpC	31.177**
lpp2298	Similar to alkyl hydroperoxide reductase AhpD	10.945**
lpp2712	Ferrous iron transporter A (FeoA)	2.918
lpp2846	FrgA protein	2.797
lpp2780	Similar to transcriptional regulator- ArsR family	2.796
lpp2711	Ferrous iron transporter B (FeoB)	1.365
lpp0237	Similar to pyoverdine biosynthesis protein PvcB	1.810
lpp0236	Similar to pyoverdine biosynthesis protein PvcA	1.525

Significantly differentially regulated genes are marked by an asterisk.

** $P < 0.001$.

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Table 4

Genes down-regulated in *Legionella pneumophila* Δ*kaiC* compared with the wild type after exposure to 10 mM paraquat (15 min).

Gene ID	Product	FC
lpp2299	Similar to alkyl hydroperoxide reductase AhpC	0.646
lpp2298	Similar to alkyl hydroperoxide reductase AhpD	0.693
lpp2141	Global stress protein GspA	0.71
lpp1115	KaiB – like circadian clock protein	0.715
lpp1116	KaiC – like circadian clock protein	0.745
lpp2461	Unkown	0.75
lpp1942	Unkown	0.756
lpp1746	Sigma factor 28 (*fliA*)	0.758
lpp0727	Similar to nicotinamide adenine dinucleotide plus hydrogen-ubiquinone oxidoreductase	0.764
lpp1208	Similar to protein	0.742
lpp2849	Similar to putative coproporphyrinogen oxidase A	0.768

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Table 5

Genes down-regulated in *Legionella pneumophila* Δ*lpp1114* compared with the wild type after exposure to 10 mM paraquat (15 min).

Gene ID	Product	FC
lpp0623	Unknown	0.482*
lpp0988	Unknown	0.582*
ncRNA20	Non coding small RNA	0.582*
lpp1025	Unkown	0.622
lpp2298	Similar to alkyl hydroperoxide reductase AhpD	0.634
lpp2504	Unknown	0.642
lpp2846	FrgA protein	0.647
lpp1957	Unknown	0.667
lpp0391	Translation elongation factor G (*fusA*)	0.672
lpp0805	Similar to surface antigens (17 kDa)	0.676
lpp1440	Weak similarity to myosin	0.68
lpp2721	RNA polymerase sigma-32 factor (*rpoH*)	0.684
lpp2779	Similar to NADH-dependent flavin oxidoreductase	0.721

Significantly differentially regulated genes are marked by an asterisk.

*$P < 0.05$.

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Table 6

Genes up-regulated in *Legionella pneumophila* Δ*lpp1114* compared with the wild type after exposure to10 mM paraquat (15 min).

Gene ID	Product	FC
plpp0015	Unknown	2.570*
plpp0027	Hypothetical gene	2.623*
plpp0018	Unknown	2.759*
plpp0017	Similar to *Escherichia coli* TraT complement resistance protein	2.910*
plpp0016	Weakly similar to carbon storage regulator CsrA	2.981*
lpp2431	Similar to conserved hypothetical protein	3.002*
lpp2430	Unknown	3.039*
plpp0022	Similar to fimbrial protein precursor (Pilin)	3.321*
lpp2884	Similar to transposase (IS21 family)	3.555*
plpp0024	Weakly similar to conjugative transfer protein TraE	3.566*
lpp2434	Unknown	3.583*
plpp0020	Similar to transposase	3.601*
plpp0023	Weakly similar to conjugative transfer protein TraL	3.623*
lpp2885	Similar to transposase (IS21 family)	3.630*
plpp0021	Similar to plasmid DNA replication protein	3.859*
plpp0023	Unknown	2.570*

Significantly differentially regulated genes are marked by an asterisk.

*$P < 0.05$.

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Table 7

Bacterial strains and plasmids used in this study.

Strain or plasmid	Properties	Reference
Strain		
Escherichia coli		
DH5a	F-φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17 (rk-. mk+) *phoA sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-	Invitrogen
BL21	F-*ompT hsd*SB (rB-mB-) *gal dcm rne131* (DE3)	Invitrogen
Legionella pneumophila		
CIP 107629	*L. pneumophila* strain Paris	Cazalet *etal.*, (2004)
Δ*kaiC*	Paris *lpp1116:*Km	This study
Δ*lpp1114*	Paris *lpp1114:*Apm	This study
Δ*kaiC/*p*kai*Op	Paris *lpp1116:*Km carrying p*lpp1116/lpp1115/lpp1114*	This study
Δ*lpp1114/*p*lpp1114*	Paris *lpp1114:*Ap carrying p*lpp1114*	This study
Plasmids		
pGEM-T Easy	Cloning of PCR products (Amp^r)	Promega
TOPO XL	Cloning of long PCR products (> 3 kb; Km^r)	Invitrogen
pMMB207C	*Legionella* expression vector (Cm^r)	Chen *et al.* (2004)
p*kai*Op	pMMB207C containing the *kai* operon	This study
p*lpp1114*	pMMB207C containing gene *lpp1114*	This study
pQE30	Expression vector N-terminal His6x tag	Qiagen
pKNT25	Expression vector N-terminal T25 (Km^r)	Euromedex
pUT18	Expression vector N-terminal T18 (Ap^r)	Euromedex
pGEX	Expression vector N-terminal GST-tag (Am^r)	GE healthcare

Amp, ampicillin; Ap, apramycin resistance; Cm, chloramphenicol; Km, kanamycin resistance; PCR, polymerase chain reaction.