Adrenergic antagonists restrict replication of Legionella

Christopher F. Harrison, 1 Sébastien Kicka, 2 Agata Kranjc, 3 Ivo Finsel, 1 Gianpaolo Chiriano, 3 Hajer Ouertatani-Sakouhi, 4 Thierry Soldati, 2 Leonardo Scapozza 3 and Hubert Hilbi 1,5

Correspondence
Hubert Hilbi
hilbi@imm.uzh.ch

1 Max von Pettenkofer Institute, Department of Medicine, Ludwig-Maximilians University Munich, 80336 Munich, Germany
2 Department of Biochemistry, University of Geneva, 1211 Geneva, Switzerland
3 School of Pharmaceutical Sciences, Department of Pharmaceutical Biochemistry, University of Geneva and University of Lausanne, 1211 Geneva, Switzerland
4 Faculty of Medicine, University of Geneva, 1211 Geneva, Switzerland
5 Institute of Medical Microbiology, Department of Medicine, University of Zurich, Gloriastrasse 30/32, 8006 Zurich, Switzerland

Legionella pneumophila is a facultative intracellular bacterium, which upon inhalation can cause a potentially fatal pneumonia termed Legionnaires’ disease. The opportunistic pathogen grows in environmental amoebae and mammalian macrophages within a unique membrane-bound compartment, the ‘Legionella-containing vacuole’. Bacteria are exposed to many environmental cues including small signalling molecules from eukaryotic cells. A number of pathogenic bacteria sense and respond to catecholamine hormones, such as adrenalin and noradrenalin, a process mediated via the QseBC two-component system in some bacteria. In this study, we examined the effect of adrenergic compounds on L. pneumophila, and discovered that the adrenergic receptor antagonists benoxathian, naftopidil, propranolol and labetalol, as well as the QseC sensor kinase inhibitor LED209, reduced the growth of L. pneumophila in broth or amoebae, while replication in macrophages was enhanced. Growth restriction was common to members of the genus Legionella and Mycobacterium, and was observed for L. pneumophila in the replicative but not stationary phase of the biphasic life cycle. Deletion of the L. pneumophila qseBC genes indicated that growth inhibition by adrenergics or LED209 is mediated only to a minor extent by this two-component system, implying the presence of other adrenergic sensing systems. This study identifies adrenergic molecules as novel inhibitors of extra- and intracellular growth of Legionella and reveals LED209 as a potential lead compound to combat infections with Legionella or Mycobacterium spp.

INTRODUCTION

Legionella pneumophila is a Gram-negative bacterium, which is found ubiquitously throughout both artificial and natural freshwater sources (Hilbi et al., 2011; Newton et al., 2010). Owing to constant predation from single-celled phagocytes such as amoebae, L. pneumophila has acquired the ability to avoid lysosomal degradation following phagocytosis, instead creating a replication-permissive compartment termed the ‘Legionella-containing vacuole’ (LCV) by means of the Icm/Dot type IV secretion system (T4SS) (Hilbi & Haas, 2012; Hubber & Roy, 2010; Isberg et al., 2009). The processes that allow the infection of freshwater amoebae also enable Legionella spp. to avoid destruction by macrophages of the human immune system (Hilbi et al., 2011; Newton et al., 2010). Owing to this, the bacteria can act as ‘accidental’ human pathogens, infecting lung macrophages and leading to the severe pneumonia known as Legionnaires’ disease. Intra- and inter-species communication through low molecular mass signalling molecules is common among bacteria (Bassler & Losick, 2006; Shank & Kolter, 2009), but is also employed for reciprocal inter-kingdom communication.
between prokaryotes and eukaryotes. Accordingly, pathogenic bacteria can sense and respond to host molecules. Examples include *Pseudomonas fluorescens*, which upon exposure to plant extracts upregulates various genes involved in bio-control through the orphan ‘LuxR solo’ response regulator PsOR (Subramoni et al., 2011). Host-sensing processes are seen not only in plant pathogens, but also in bacteria infecting humans. *Pseudomonas aeruginosa* has been reported to sense host IFN-γ via the OprF outer-membrane protein (Wu et al., 2005), while *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) detects host-produced antimicrobial peptides via the PhoPQ two-component system (TCS) (Bader et al., 2005).

A prominent example of eukaryote to prokaryote communication is adrenergic signalling through the catecholamines adrenalin (epinephrin) and noradrenalin (norepinephrin) (Hughes & Sperandio, 2008). The adrenergic neurotransmitters adrenalin and noradrenalin are pivotal components of eukaryotic stress responses, but also control in an agonistic manner the expression of virulence and motility genes of enterohaemorrhagic *Escherichia coli* (Hughes & Sperandio, 2008). The adrenergic neurotransmission is adrenergic signalling through the catecholamines (Bader et al., 2009). The responses to adrenergic signalling of *E. coli* (Kostakioti et al., 2009), *S. typhimurium* (Bearson & Bearson, 2008; Moreira et al., 2010), *Francisella tularensis* (Rasko et al., 2008), *Aggregatibacter actinomycetemcomitans* (Novak et al., 2010), *Actinobacillus pleuropneumoniae* (Li et al., 2012) and *Edwardsiella tarda* (Wang et al., 2011). Given their broad distribution, small-molecule sensing pathways and in particular the QseBC system are attractive targets for antivirulence compounds (Njoroge & Sperandio, 2009). Indeed, in a high-throughput screen, a novel small molecule blocking QseC was identified, LED209, which reduced the virulence and severity of infection in animal models of EHEC, *S. typhimurium* and *F. tularensis* (Rasko et al., 2008; Hughes & Sperandio, 2008). LED209 is a potent prodrug, which upon cleavage of an aniline group exposes a reactive isothiocyanate and covalently modifies lysine residues in QseC (Curtis et al., 2014).

The QseC sensor kinase discovered in EHEC also seems to be present in *L. pneumophila* (Rasko et al., 2008). Yet, the genes encoding the TCS homologue of QseBC in *Legionella* spp. have been named either pmrAB, qseBC or lrpR, depending on the strain or species involved (Table S1, available in the online Supplementary Material). In *L. pneumophila* strain 130b, the TCS ‘PmrAB’ was implicated in intracellular replication within phagocytes, perhaps as a pH sensor (Al-Khodor et al., 2009), and in *L. pneumophila* strain JR32 the response regulator ‘PmrA’ was found to be a major regulator of Icm/Dot-translocated effector proteins (Zusman et al., 2007). In addition to virulence and motility genes, ‘PmrA’ positively regulates the expression of stress response and metabolic genes, including *csrA* (Al-Khodor et al., 2009; Rasis & Segal, 2009), encoding the RNA-binding global repressor of transmissive traits CsrA (Molofsky & Swanson, 2003; Rasis & Segal, 2009). These results suggest that the ‘PmrAB’ TCS is involved in controlling the switch from the transmissive to the replicative form of *L. pneumophila*. As PmrAB/QseBC homologues exist in *L. pneumophila*, we set out to determine if adrenergic compounds played a role in the process of host-cell infection. In this study we show that some α-adrenergic receptor antagonists, as well as the QseC sensor kinase inhibitor LED209, reduced the extracellular growth of *Legionella* spp. as well as the intracellular growth in amoebae.

**METHODS**

**Bacteria and reagents.** Bacterial strains used in this study are described in Table 1. Constitutive GFP-producing *L. pneumophila* (Tiaden et al., 2007) were grown on charcoal yeast extract (CYE) plates or in ACES yeast extract (AYE) medium (Feeley et al., 1979), with 10 μg/ml chloramphenicol. For infections, the bacteria were grown overnight in AYE at 37 °C to a final OD₆₀₀ of 3.0, at which stage they have...
reached the transmissive, infectious phase. Murine RAW 264.7 macrophages (ATCC TIB-71, lab collection) were maintained in supplemented RPMI 1640 medium, Acanthamoeba castellanii (ATCC 30234) in proteose-yeast extract-glucose (PYG) medium—all cells were passaged twice weekly.

LED209 (Sigma-L8919), adrenergic compounds (http://druginfo.nlm.nih.gov/drugportal/) (Fig. 1) and all other chemicals were from Sigma-Aldrich unless otherwise specified. Stock solutions of the reagents were prepared in DMSO at a concentration of 30–100 mM. The compounds were diluted in the medium used, yielding the final concentrations indicated and a concentration of <0.5 % DMSO, which did not interfere with the assays.

Construction of an *L. pneumophila* qseBC deletion mutant strain. Primers were designed to amplify the flanking regions of the *lpg1291-lpg1292* (qseBC) genes from *L. pneumophila* genomic DNA (Table S2). After amplification, the 1291-flank and 1292-flank DNA fragments were cut with *Xba*I/*Bam*HI. The kanamycin (Kan) resistance cassette was cut from the pUC4K plasmid using *Bam*HI, and ligated with the flanking regions into pGEM-T Easy (Promega) to make pCFH-8 (Table 1). After verification by sequencing, the flank-Kan-flank DNA was transformed into *E. coli* by electroporation with pCFH-9, and stable transformants were selected on CYE-Kan plates (Tiaden et al. 2007). Deletion of the qseBC genes from *L. pneumophila* JR32 (pmrA) was confirmed by PCR and sequencing.

### Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant properties</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. pneumophila</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JR32</td>
<td><em>L. pneumophila</em> Philadelphia-1, serogroup 1, salt-sensitive isolate of AM511</td>
<td>Sadosky et al. (1993)</td>
</tr>
<tr>
<td>Δ<em>icmT</em></td>
<td>JR32 icmT3011::Km (GS3011)</td>
<td>Segal &amp; Shuman (1998)</td>
</tr>
<tr>
<td>Δ<em>lpqR</em></td>
<td>JR32 lpqR::Km</td>
<td>Tiaden et al. (2007)</td>
</tr>
<tr>
<td>Δ<em>rpoS</em></td>
<td>JR32 rpoS::Tn903III-Gm (LM1376)</td>
<td>Hales &amp; Shuman (1999)</td>
</tr>
<tr>
<td>Δ<em>pmaA</em> (qseB)</td>
<td>Gift from lab of G. Segal (Tel Aviv University)</td>
<td>Zusman et al. (2007)</td>
</tr>
<tr>
<td>Δ<em>qseBC</em></td>
<td>JR32 lpg1291-lpg1292::Km</td>
<td>This study</td>
</tr>
<tr>
<td>Lp02</td>
<td><em>L. pneumophila</em> serogroup 1, strain Lp02</td>
<td>O’Connor et al. (2011)</td>
</tr>
<tr>
<td>Apentuple</td>
<td>Lp02 lacking gene clusters 3, 2ab, 6a, 7a, 4a</td>
<td>O’Connor et al. (2011)</td>
</tr>
<tr>
<td><em>L. bozemanii</em></td>
<td>SNRCL no. 1165; Italy, 2002 (water)</td>
<td>Spiris et al. (2008)</td>
</tr>
<tr>
<td><em>L. longbeachae</em></td>
<td>Strain NSW150</td>
<td>Cazalet et al. (2010)</td>
</tr>
<tr>
<td><em>L. micdadei</em></td>
<td>SNRCL no. 634; Bern, 2000 (patient)</td>
<td>Harrison et al. (2013)</td>
</tr>
<tr>
<td><em>K. aerogenes</em></td>
<td>Lab collection (= <em>K. pneumonia</em>)</td>
<td>Harrison et al. (2013)</td>
</tr>
<tr>
<td><em>M. marinum</em></td>
<td>Strain M</td>
<td>Ramakrishnan et al. (2000)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>Strain PAO1</td>
<td>Calciano et al. (1992)</td>
</tr>
<tr>
<td><em>S. flexneri</em></td>
<td>Strain M90T</td>
<td>Sansonetti et al. (1982)</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td>Strain C5</td>
<td>Hermant et al. (1995)</td>
</tr>
<tr>
<td><em>Y. enterocolitica</em></td>
<td>ATCC 23715</td>
<td>ATCC</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCFH-8</td>
<td>pGEM-T-easy, lpg1291-lpg1292 flanking regions, lpg1291-lpg1292::Km, Cm</td>
<td>This work</td>
</tr>
<tr>
<td>pCFH-9</td>
<td>pLAW344, lpg1291-lpg1292::Km</td>
<td>This work</td>
</tr>
<tr>
<td>pGEM-T-easy</td>
<td>Cloning of PCR products, Ap</td>
<td>Promega</td>
</tr>
<tr>
<td>pLAW344</td>
<td>orIR (RK2), orIR (ColE1), sacB, Cm, Ap</td>
<td>Walter et al. (1994)</td>
</tr>
<tr>
<td>pUC4K</td>
<td>orIR (pBR322), Ap, MCS::Km</td>
<td>Amersham</td>
</tr>
</tbody>
</table>

*Ap, Ampicillin resistance; Gm, gentamicin resistance; Km, kanamycin resistance; SNRCL, Swiss National Reference Center for *Legionella*. 

### Extracellular growth assays.

Extracellular replication of *L. pneumophila* in the presence of compounds was determined using previously published protocols (Harrison et al., 2013). Briefly, *L. pneumophila* were taken from CYE plates after 3 days of growth, resuspended in 3 ml AYE at a final OD<sub>600</sub> of 0.02 (matching the bacterial concentration used for intracellular assays; see below) and incubated in test tubes on a rotating wheel overnight at 37 °C. The compounds of interest (usually at 30 μM) or vehicle controls (<0.5 %) were diluted into these cultures. The end concentration of compounds in single point measurements was 30 μM (0.1 % DMSO), and at all concentrations used, DMSO did not interfere with the assays. OD<sub>600</sub> measurements were then taken and the values normalized to the vehicle controls. The growth of *Legionella* spp., *L. pneumophila* mutants, and other bacterial species was assayed in the same manner, albeit with the use of lysogeny broth (LB) or, occasionally, AYE for non-*Legionella* species. Growth of GFP-producing *Mycobacterium marinum* was assayed in BBL Middlebrook 7H9 broth (Difco) supplemented with OAD (BD) by seeding 1 × 10<sup>3</sup> msp : : GFP bacteria into a 96-well plate with or without compounds, and replication was then followed for up to 48 h by monitoring the fluorescence. Time-course growth assays were performed as above, albeit with measurement of fluorescence values three to four times per day for 2 days.

For recovery experiments, *L. pneumophila* cultures were set up in the presence of DMSO (0.1 %), naftopidil (30 μM) or LED209 (30 μM), as described above. Cultures were centrifuged the next morning and bacterial pellets washed in AYE, before resuspension in AYE and further growth for 24 h, at which point the final OD<sub>600</sub> was measured. We opted to use OD as a measure of bacterial growth rather than...
c.f.u. owing to the higher experimental throughput possible. As all cultures were compared against vehicle controls, and the compounds used did not affect the overall optical density (this was verified using controls containing medium/compound alone), it provided us with a simple method to determine relative bacterial growth.

For growth-phase-sensitivity experiments, *L. pneumophila* overnight cultures in replicative or stationary phase, together with bacteria directly resuspended from CYE plates, were diluted to an OD600 of 0.02 and treated with DMSO (0.1 %), naftopidil (30 μM in 0.1 % DMSO) or LED209 (30 μM in 0.1 % DMSO) for 2 h. Cultures were then plated to CYE plates to determine c.f.u.

**Intracellular replication assays.** Intracellular replication of *L. pneumophila* or *M. marinum* in phagocytes (*A. castellanii*, macrophages) was assessed using previously published protocols (Harrison

**Fig. 1.** Adrenergic compounds affect *L. pneumophila* replication. (a) Various adrenergic agonists/antagonists were assayed at a concentration of 30 μM (0.1 % DMSO) for their effect on extra- (black) and intracellular (in *A. castellanii*, blue) replication; and several showed inhibition of bacterial growth. The graph indicates mean and 95 % CI of three separate screens. (b) Replication time-course indicating the dose-dependent inhibition of *L. pneumophila* replication within *A. castellanii* by naftopidil (3.8–60 μM; 0.1 % DMSO), as followed by fluorescence replication assay. The graph indicates representative time-course of infection, each point represents mean ± SD of triplicate wells. AU, arbitrary units. (c–f) Dose–response curves of effect of the adrenergic compounds naftopidil (c), benoxathian (d), propranolol (e) and noradrenalin (f), on *L. pneumophila* replication in medium (black) and within *A. castellanii* (blue). In all samples, the end concentration of DMSO was <0.5 %. Graphs indicate mean ± SD of at least five separate experiments.
et al., 2013; Kicka et al., 2014). In brief, A. castellanii were seeded to 96-well plates at 2 × 10⁴ cells per plate and grown overnight, following which they were infected at an m.o.i. of 20 by GFP-producing L. pneumophila. The compounds of interest (usually at 30–60 μM) or vehicle controls (<0.5 % DMSO) were diluted into the cultures, prior to, concomitant with or after infection. At the concentrations used, DMSO did not interfere with the assays. Infected amoebae were incubated at 30 °C, and the progress of replication was followed by the increase in GFP fluorescence over several days. RAW 264.7 macrophages were cultured in RPMI 1640 containing 5 % FCS (Gibco). Cells were seeded at 8 × 10⁴ cells per well in a 96-well plate, and infection was performed as with A. castellanii, albeit with bacteria and compounds diluted in RPMI 1640. Medium was changed 4 h following infection to ensure removal of non-phagocytosed bacteria.

Data analysis. Data analysis was performed using Microsoft Excel and GraphPad Prism 5. To compare the effect of compound treatment on intracellular replication, fluorescence values were taken from the first time point following entry to stationary phase. The results were then normalized such that medium-only wells (no bacteria) were ‘0’, while vehicle-treated wells were ‘1’ (normal replication). The mean of the replicates (minimum three per plate) was then plotted as dose–response curves, such that each individual point represented the mean of a single experiment. Compound treatments were repeated a minimum of three times to control for the increased variability of bacteria-host-cell interactions. Lines of best fit and associated IC₅₀ values were calculated for each dose–response curve using the Nonlinear fit (log slope) function of Prism 5.

RESULTS

Adrenergic compounds affect L. pneumophila replication

Legionella spp. contain homologues of genes variously referred to as pmrAB or qseBC (Table S1). Homology analysis indicated that the proteins encoded by lpg1291 and lpg1292 in L. pneumophila Philadelphia-1 are 34 or 45 % identical to EHEC QseC and QseB, respectively. Comparison of full-length proteins revealed that the pairwise alignment score for the putative L. pneumophila sensor kinase Lpg1291 (471 aa) and EHEC QseC (449 aa) is significantly higher (30 % overall identity) than the score for the EHEC sensor kinases PmrB (363 aa; 20 % identity) or CpxA (457 aa; 18 % identity). Thus, the L. pneumophila TCS annotated as ‘PmrAB’ is actually more closely related to EHEC QseBC than to EHEC PmrAB. For this reason we refer to lpg1291 and lpg1292 as qseC and qseB throughout this publication, and we sought to test whether L. pneumophila responds to adrenergics.

To determine the role that adrenergic compounds play on L. pneumophila replication, we analysed the effect of a range of compounds known to be associated with effects on the human adrenergic system (http://druginfo.nlm.nih.gov/drugportal/) (Fig. 1). These compounds were assessed for their ability to inhibit replication of L. pneumophila both in medium and intracellularly within the natural host amoeba A. castellanii (Fig. 1a). Both intra- and extracellular replication of L. pneumophila were inhibited by 30 μM α1-adrenoceptor antagonists benoxathain and naftopidil. Inhibition of extra- but not intracellular replication was observed in the presence of propranolol (a β1- and β2-adrenoceptor antagonist) or labetalol (a mixed antagonist of α1- and β1/β2-adrenergic receptors). Further experiments at a range of concentrations showed a dose-dependent inhibition of extra- and intracellular replication by naftopidil (Fig. 1b, c) with IC₅₀ values of 16.9 and 30.3 μM respectively (Tables 2 and S3). Dose-dependent inhibition of replication was also observed from benoxathain (Fig. 1d) and propranolol (Fig. 1e). High concentrations of noradrenaline (a mammalian neurotransmitter and activator of both α and β receptors) also inhibited replication (Fig. 1f). Collectively, these results indicated that compounds that target adrenergic receptors prevent the replication of L. pneumophila.

The response to adrenergic compounds is specific for Legionella and Mycobacterium

As these adrenergic compounds inhibited the growth of L. pneumophila in medium, it seemed logical that they were affecting a bacterial target, rather than one within the host cell. To examine the Legionella species specificity, we screened a range of adrenergic compounds on the species

<table>
<thead>
<tr>
<th>Compound</th>
<th>Parameter</th>
<th>Extracellular</th>
<th>A. castellanii</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naftopidil</td>
<td>IC₅₀ (μM)</td>
<td>16.9 (15.6–18.2)</td>
<td>30.3 (19.9–48.0)</td>
</tr>
<tr>
<td></td>
<td>Hill slope</td>
<td>−3.12 (−3.96–(2.44)</td>
<td>−0.77 (−1.10–(0.44)</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>IC₅₀ (μM)</td>
<td>299 (231–387)</td>
<td>299 (231–387)</td>
</tr>
<tr>
<td></td>
<td>Hill slope</td>
<td>−2.05 (−2.98–1.11)</td>
<td>−2.05 (−2.98–1.11)</td>
</tr>
<tr>
<td>Benoxathain</td>
<td>IC₅₀ (μM)</td>
<td>24.7 (11.6–52.8)</td>
<td>40.8 (5.27–317)</td>
</tr>
<tr>
<td></td>
<td>Hill slope</td>
<td>−1.61 (−3.14–0.07)</td>
<td>−0.93 (−2.30–0.43)</td>
</tr>
<tr>
<td>Propranolol</td>
<td>IC₅₀ (μM)</td>
<td>70.5 (49–101)</td>
<td>231 (15.3–345)</td>
</tr>
<tr>
<td></td>
<td>Hill slope</td>
<td>−2.62 (−4.46–0.88)</td>
<td>−1.43 (−3.21–0.36)</td>
</tr>
<tr>
<td>LED209</td>
<td>IC₅₀ (μM)</td>
<td>1.27 (1.07–1.50)</td>
<td>50.6 (25.8–99.5)</td>
</tr>
<tr>
<td></td>
<td>Hill slope</td>
<td>−1.48 (−1.76–1.21)</td>
<td>−1.94 (−3.03–0.86)</td>
</tr>
</tbody>
</table>

Numbers given in parentheses indicate 95 % CI.
L. pneumophila, L. bozemanii, L. micdadei and L. longbeachae, observing equal growth inhibition in broth for each (Fig. 2a). By contrast, no growth inhibition was observed following compound treatment of other bacterial genera, such as Shigella flexneri, S. typhimurium, Yersinia enterocolitica or Klebsiella aerogenes (Fig. 2a). To address the question whether the adrenergic compounds are inhibitory only in the AYE medium used to grow Legionella spp., we tested the effects of up to 150 μM compounds pindolol, propranolol and labetalol on growth of S. flexneri, S. typhimurium or P. aeruginosa in AYE medium. Yet, also under these conditions the adrenergic compounds did not inhibit the growth of bacteria other than Legionella (Fig. 2b).

In addition, we also tested different adrenergic compounds for their effects on growth of M. marinum in 7H9 medium. Interestingly, benoxathian, naftopidil and propranolol significantly inhibited the growth of M. marinum (Fig. 2c). Thus, the adrenergic compounds affect Legionella as well as Mycobacterium spp., and the inhibitory effect of the compounds is not dependent on the growth medium used.

Finally, no statistically significant difference in growth inhibition by adrenergic compounds was observed between L. pneumophila deletion mutants lacking a component of the Icm/Dot T4SS (ΔicmT), an alternative sigma factor (ΔrpoS), or a response regulator involved in L. pneumophila quorum sensing (ΔlqsR), indicating that these genes do not play a role in the observed effect (Fig. S1). In summary, these results support the hypothesis that the growth-inhibitory effect of adrenergic compounds is specific to members of the genus Legionella and Mycobacterium.

**LED209 restricts replication of Legionella spp. and Mycobacterium marinum**

We then determined whether the response of L. pneumophila to adrenergic compounds was linked to the QseBC pathway. LED209 is a small-molecule inhibitor that has been shown to inhibit the activation of QseBC-mediated virulence pathways (Rasko et al., 2008). Treatment of L. pneumophila with LED209 led to a dose-dependent decrease in both extra- and intracellular replication, with IC50 values of 1.27 and 50.6 μM respectively (Fig. 3a, b, Table 2). Interestingly, the inhibition of extracellular growth by LED209 was over 10-fold more effective than by naftopidil. Moreover, LED209 (but not naftopidil) significantly reduced the intracellular growth of M. marinum in A. castellanii (Fig. 3c), while the compound had no deleterious effect on M. marinum growing in broth (data not shown). On the other hand, LED209 had antibiotic activity against several members of the genus Legionella, but not against other species tested (Fig. 3d). Finally, LED209 inhibited the growth of L. pneumophila deleted for icmT, rpoS or lqsR, or the ‘Δpentuple’ mutant strain (O’Connor et al., 2011), lacking 18.5 % of the genome similar to WT bacteria (Fig. S2).

**LED209 and naftopidil promote growth of L. pneumophila in macrophages**

To this point the intracellular replication of L. pneumophila had been assessed in the natural host A. castellanii; however we also wanted to determine if adrenergic compounds inhibit replication in a model more relevant to human disease. As such, we examined the effect of adrenergic compounds on L. pneumophila replication within RAW 264.7 macrophages (Fig. 4a). Unexpectedly, compounds that inhibited replication in media or in A. castellanii such as...
LED209 or naftopidil led to a dose-dependent increase in replication when used to treat macrophages (Fig. 4b). This was not due to overall differences in the model systems, as antibiotic treatment continued to reduce replication levels (Fig. 4a), and we had previously shown correlation between antibiotic effects in *A. castellanii* and RAW 264.7 macrophages (Harrison et al., 2013). The increased bacterial replication in macrophages correlated with an extended lag phase prior to the onset of growth, as seen in time-course measurements of replication in the presence of different concentrations of LED209 (Fig. 4c) or naftopidil (Fig. 4d). Taking these results together, LED209 and naftopidil inhibit intracellular replication of *L. pneumophila* in broth as well as in *A. castellanii*, while both compounds promote the replication in macrophages. Thus, the molecules do not simply or solely act as inhibitors of bacterial growth. Rather, the compounds might also target the host cells and modulate amoebae and macrophages in a different manner, leading to a decreased or enhanced intracellular replication, respectively.

**Characterization of the efficacy of adrenergic compounds**

To further characterize the effects of LED209 and naftopidil on *L. pneumophila*–phagocyte interactions, we determined their efficacy at various points in the infection process. Host cells were treated with LED209 or naftopidil (i) for 30 min prior to infection, (ii) simultaneously during the infection, or (iii) after a 10 min post-infection pause. We observed that in both *A. castellanii* and RAW 264.7 macrophages the effect of the compounds was heavily dependent on the time of addition (Fig. 5a). Pre-treatment of cultured cells had no effect on replication, whereas treatment starting 10 min after infection had limited effects
on intracellular growth. By contrast, co-treatment with LED209 or naftopidil and L. pneumophila significantly affected the degree of intracellular replication. This suggests that LED209 and naftopidil modulate the entry/uptake of L. pneumophila or the initial phase of LCV formation and thus prevent (A. castellanii) or promote (macrophages) the formation of a replication-permissive compartment.

To establish if LED209 or naftopidil was exerting permanent or temporary effects, L. pneumophila cultures were incubated overnight in the presence of these compounds or, as a control, the bactericidal antibiotic Kan. As no replication occurred, centrifugation of the cultures led to recovery of the original bacterial inoculum, which was washed several times, resuspended in fresh medium, and incubated overnight. As expected, no growth was observed in Kan-treated cultures after the wash-out (Fig. 5b). Treatment with naftopidil led to a temporary halt in replication, as cultures resumed growth following wash-out of the compound. Thus, this compound has a bacteriostatic rather than a bactericidal effect on L. pneumophila. On the other hand, LED209 exerted an intermediate phenotype, as cultures resumed growth but at a slower rate than before the addition of the compound. Taken together, naftopidil is bacteriostatic for L. pneumophila, while LED209 appears to have a bactericidal effect.

L. pneumophila undergoes a two-stage life cycle, alternating between a replicative phase and a stationary, infectious phase, which is characterized by the expression of various virulence factors (Byrne & Swanson, 1998). These two stages of the growth cycle are characterized by differing levels of resistance to antibiotics and salt concentrations.

Fig. 4. LED209 and naftopidil promote growth of L. pneumophila in macrophages. (a) Comparative screen of adrenergic compounds (60 \( \mu \)M) on L. pneumophila replication within RAW 264.7 macrophages, indicating that efficacious adrenergic compounds increase replication, in contrast to antibiotics. The graph indicates mean ± SD of at least three separate experiments. (b) Dose–response curve for LED209 or naftopidil indicates a positive effect on L. pneumophila replication within RAW 264.7 macrophages, as normalized to DMSO. Graph shows mean and 95 % CI from at least three separate experiments. (c, d) Time-course of L. pneumophila replication in RAW 264.7 macrophages at different concentrations of LED209 (c) or naftopidil (d). In all samples (a–d), the end concentration of DMSO was 0.5 %. Graphs (c, d) show mean and 95 % CI of triplicate infections within the same representative experiment (*\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \); Student’s t-test); AU, arbitrary units.
(Byrne & Swanson, 1998; Molofsky & Swanson, 2004), and so could also differ in their response to adrenergic compounds. *L. pneumophila* cultures in exponential growth or stationary stage, or resuspended from plated colonies (effectively also stationary phase), were diluted, and treated with DMSO, LED209 or naftopidil for 2 h, and then viability was determined by c.f.u. plating. We observed that, while both compounds inhibited the viability of replicative *L. pneumophila*, they had no effect on stationary-phase bacteria (Fig. 5c). This was also observed in further experiments, which examined the effect of LED209 on *L. pneumophila* cultures at various OD\textsubscript{600} values (Fig. 5d). In this case, a clear increase in viability occurred after OD\textsubscript{600} 3.0, consistent with the transition into stationary phase.

Initial dose–response experiments with LED209 or naftopidil using *L. pneumophila*-infected macrophages showed that the degree of efficacy appeared to be reduced (Fig. 4), when compared with assays with *A. castellanii*. 

---

**Fig. 5.** Characterization of the efficacy of adrenergic compounds. (a) Time dependency of compound treatment. *A. castellanii* or RAW 264.7 macrophages were treated with 30 μM LED209 (green), 30 μM naftopidil (blue) or 0.1 % DMSO (black) 30 min before, simultaneously, or 10 min after infection with *L. pneumophila*. Graph shows mean and 95 % CI of triplicate measurements within a representative experiment, plotted as GFP fluorescence (AU, arbitrary units) versus time (h). (b) Recovery experiment: *L. pneumophila* was grown for 24 h in the presence of 30 μM LED209, 30 μM naftopidil or 50 μg ml\textsuperscript{-1} Kan, after which no replication was observed. Bacteria were then pelleted, washed and resuspended in fresh AYE, and the OD\textsubscript{600} was measured after a further 24 h. (c) *L. pneumophila* cultures were grown to exponential or stationary growth phase, or resuspended directly from plate, diluted into AYE with 30 μM LED209, 30 μM naftopidil or 0.1 % DMSO, and incubated for 2 h. The total c.f.u. counts were then assayed at the end of this time, normalized to DMSO. LED209 and naftopidil only reduced c.f.u. of cultures in exponential rather than stationary growth phase. (d) Efficacy of 30 μM LED209 in reducing c.f.u. as compared with OD\textsubscript{600} of originating colony indicates a rapid increase in resistance following the shift into stationary phase. Graphs indicate mean±sd (b), or mean and 95 % CI (c, d) of representative triplicates (*P<0.05, ***P<0.001; Student’s t-test).
experiments where intracellular replication in macrophages and *A. castellanii* was assessed in parallel (Fig. 5).

**LED209 but not naftopidil acts partially through QseBC**

Based on the previous results, we hypothesized that LED209 and naftopidil might exert their effects via the QseBC pathway. To test this, we constructed an *L. pneumophila* ΔqseBC deletion mutant and also used a ‘ΔpmrA’ (ΔqseB) mutant strain previously constructed (Zusman *et al.*, 2007). These deletion mutants appeared normal, although we did observe that both strains exhibited a slower growth rate than WT (Fig. S3A). Furthermore, the ΔqseBC strain also showed decreased production of the stationary-phase brown pigment pyomelanin (Fig. S3B).

The response of *L. pneumophila* mutant strains lacking *qseB* or *qseBC* to a range of adrenergic compounds was not significantly different from WT bacteria (Fig. 6a). Next, we compared the sensitivity of the ΔqseB or ΔqseBC mutant strains against LED209. The dose–response curves for LED209 (IC$_{50}$ 1.27 µM, 95 % confidence interval (CI) 1.07–1.50) were shifted in the absence of QseB (IC$_{50}$ 2.1 µM, 95 % CI 1.82–2.59) (Fig. 6b) or QseBC (IC$_{50}$ 3.1 µM, 95 % CI 2.66–3.73) (Fig. 6c), indicating a statistically significant reduction in the sensitivity of the mutant bacteria to this compound, compared with WT *L. pneumophila* (Table S4). Finally, dose–response curves constructed from multiple experiments indicated that there was no difference in the inhibition of *L. pneumophila* WT, ΔqseB or ΔqseBC extracellular growth by naftopidil (with IC$_{50}$ values of 17, 23 and 20 µM, respectively; Fig. 6d). Thus, it seemed that, while LED209 exerted an effect via the QseBC pathway, this compound as well as other adrenergics such as naftopidil also acted on *L. pneumophila* via an alternative pathway.

![Graphs](http://mic.sgmjournals.org)
DISCUSSION

This study shows that growth of *Legionella* species is reduced by a number of adrenergic signalling antagonists, in particular the α1-antagonists benoxathain and naftopidil, or the β1-/-β2-antagonist propranolol. The role of adrenergic compounds in growth of *Legionella* or other intracellular pathogens has not been thoroughly examined. One screen for intracellular inhibitors of *L. pneumophila* did identify carvedilol, a non-selective α1-/β-arenergic antagonist, as an inhibitor of replication in THP-1 macrophage-like cells (Czyż et al., 2014). We did not observe an effect of carvedilol in our *A. castellanii*-based assay, possibly reflecting the different host cells used in these assays and perhaps indicating the importance of host-cell features such as receptor sensitivity, membrane permeability and/or pathogen compartment access of compounds.

In addition to adrenergic compounds, the QseC sensor kinase inhibitor LED209 blocked the extracellular growth of *Legionella* spp. and their intracellular growth in *A. castellanii*. Moreover, LED209 was also found to reduce the intracellular growth of *M. marinum* in *A. castellanii*, but not the extracellular growth. LED209 inhibited the extracellular growth of *L. pneumophila* over 10-fold more effectively than naftopidil, whereas intracellular growth in *A. castellanii* was reduced with similar efficacy. These results are in agreement with the notion that LED209 might preferentially target a bacterial factor, while naftopidil delays intracellular bacterial replication by targeting a host receptor. On the other hand, LED209 as well as naftopidil inhibited growth of *L. pneumophila* in *A. castellanii*, while both compounds promoted replication in macrophages. Thus, the molecules might also target the host cells and modulate bacterial uptake and/or the formation of a replication-permissive LCV in amoebae and macrophages in a different manner. Alternatively, the exposure of intracellular *L. pneumophila* to LED209 or naftopidil might lead to the expression of sets of effector proteins determining the fate of the bacteria in different host cells. Accordingly, the compounds might downregulate effectors important for intracellular replication in amoebae and upregulate effectors crucial for intracellular replication in macrophages. Finally, if the compounds do not modulate LCV formation, they might modulate bacterial growth in the intracellular environments per se. In this case, the requirements for intracellular replication in amoebae or macrophages would (rather unlikely) have to be fundamentally different, thus explaining the anti- or pro-proliferative effects of the compounds, respectively.

LED209 seems to have a bactericidal effect on *L. pneumophila*, while naftopidil has a bacteriostatic effect, suggesting that the target(s) of these compounds are not identical. Accordingly, LED209 but not naftopidil acts partially through QseBC (Fig. 6). If the compounds indeed bind to different receptors, this might significantly alter downstream signalling and gene expression patterns. Thus, different responses of *L. pneumophila* to LED209 and naftopidil might provide an explanation for bactericidal versus bacteriostatic effects. Moreover, while LED209 was found to inhibit the virulence of several pathogens, the compound did not interfere with the growth of non-*Legionella* species (Hughes & Sperandio, 2008; Curtis et al., 2014) (Fig. 3d). Collectively, these findings are in agreement with the notion that in *L. pneumophila* LED209 targets receptors other than QseC.

*L. pneumophila* qseB and qseBC deletion mutants exhibited only minor changes in their response to the QseC inhibitor LED209, and the mutants remained sensitive to the growth-inhibitory effects of adrenergic compounds. This might indicate the existence of a further system that responds to adrenergic molecules while acting in concert with QseBC to respond to LED209 treatment. As yet we have not identified this system, although several potential candidates have been described in other bacteria such as *S. typhimurium* and *E. coli*, including the TCSs QseEF, BasSR and CpxAR.

QseE has been shown to respond to adrenalin, sulfate and phosphate in EHEC, regulating virulence factors and induction of pedestal formation by the host cell (Reading et al., 2009). BasSR controls the pmr operon, involved in lipopolysaccharide modification granting resistance to the antibiotic polymyxin B. This resistance has been shown to be altered by exposure to adrenalin or the β-adrenergic blocker propranolol (Karavolos et al., 2008). CpxAR regulates a broad range of genes, including the haemolytic activity of *S. typhi*, which has been shown to be induced by adrenalin and blocked by propranolol, but not the α-adrenergic inhibitor phenotamine (Karavolos et al., 2011a, b). While binding of adrenergic compounds to BasS or CpxA has not yet been conclusively demonstrated, these effects seem to be independent of QseC.

*L. pneumophila* does not appear to contain homologues of QseE or BasS; however, it does contain a homologue of CpxA (lpg1437, annotated as CpxA). Detection of adrenergic signalling is expected to be important in virulence of *L. pneumophila*, and indeed it has been shown that the QseBC (PmrAB) and CpxAR systems both control virulence and are upregulated in stationary growth phase (Faucher et al., 2011). Both QseBC and CpxAR have been described as controlling expression of components of the Icm/Dot T4SS as well as Icm/Dot-translocated effectors, indicating their importance in the transition into the virulent phase of the life cycle (Altman & Segal, 2008; Gal-Mor & Segal, 2003; Vincent et al., 2006; Zusman et al., 2007). As yet, the effect of adrenalin on CpxAR-mediated gene expression and virulence of *L. pneumophila* has not been determined.

Other candidates for target proteins of adrenergic compounds include ‘LuxR solo’ family homologues, which detect quorum-sensing compounds or other small-molecule signals, but have no cognate autoinducer synthase. Members of this family include VirA, detecting plant flavonoid molecules in *Agrobacterium tumefaciens* (Lee et al., 1995), or PsOR, which serves to detect host factor(s) in plant extracts.
in *P. fluorescens* (Subramoni *et al.*, 2011). Several LuxR homologues exist in *L. pneumophila*, such as LetA (Lynch *et al.*, 2003), LpnR1, LpnR2 and LpnR3 (Lebeau *et al.*, 2004), and are known to alter expression of virulence-associated genes such as flaA and rpoS. The role of these *L. pneumophila* LuxR homologues in detection and signalling of adrenergics has not been determined.

Knockout of the *L. pneumophila* qseB and qseBC genes appeared to alter entry into stationary phase, as seen both by reduced growth rates and decreased production of the pyomelanin brown pigment, itself associated with *L. pneumophila* qseB gene product (Steinert *et al.*, 2001). The QseBC (PmrAB) system is known to regulate the Icm/Dot T4SS (Zusman *et al.*, 2007), and deletions reduce the survival of intracellular bacteria (Al-Khodor *et al.*, 2009). The role that QseBC appears to play in the switch from the replicative to the infectious phase may thus link to the observed lack of an effect of LED209 or naftopidil on stationary-phase bacteria, which are already in the virulent, non-replicative phase.

Furthermore, the bacterial response to host catecholamines is not necessarily mediated by TCSs. Rather, catecholamines stimulate growth and virulence of *E. coli* (Burton *et al.*, 2002; Freestone *et al.*, 2007a), *Bordetella bronchiseptica* (Anderson & Armstrong, 2006; Armstrong *et al.*, 2012) and other bacteria by increasing the bioavailability of host-sequestered iron and subsequent uptake by catecholate siderophore transport systems (Freestone *et al.*, 2008; Sandrini *et al.*, 2010). While catecholamines stimulated the growth of enteropathogenic bacteria in iron-restricted medium, the compounds inhibited bacterial growth in iron-replete medium (Freestone *et al.*, 2007b). We analysed the effects of adrenergic compounds on the growth of *Legionella* and *Mycobacterium* spp. under rich conditions, i.e. in AYE or 7H9 medium, respectively. Under these conditions, adrenaline and noradrenaline did not significantly affect the growth of *Legionella* and *Mycobacterium* spp., while the adrenergic antagonists benoxathian, naftopidil and propranolol inhibited growth (Fig. 2).

The observations documented in this study raise the question as to under which circumstances *L. pneumophila* naturally senses adrenergic signals. In the majority of bacterial species for which QseBC-based adrenergic signalling has been described, the pathogen’s primary hosts are mammals, with corresponding catecholine signalling pathways. *Legionella* spp., by contrast, are best described as ‘accidental’ pathogens, naturally infecting freshwater amoebae. Interestingly, however, there is evidence that protozoa also use catecholamine molecules for signalling. Enteropathogenic amoebae of the genus *Entamoeba* have been noted to respond to stimulation via the β1- (but not β2-) adrenergic receptor pathway, inducing differentiation into infectious cyst stages, and catecholamines were detected in cell lysates, suggesting that the amoebae endogenously produce the compounds (Coppi *et al.*, 2002). Notably, in this study adrenaline was found to be released into the surrounding medium by amoebae undergoing encystation, acting as a signalling molecule to induce encystation of surrounding cells. Encystation of amoebae occurs under stress situations, and has been shown to lead to the expulsion of LCVs into the surrounding medium (Bouyer *et al.*, 2007). By eavesdropping on the adrenergic signals associated with this process and reducing their growth rate, *Legionella* spp. may be able to survive in conditions that are temporarily devoid of suitable hosts.

In summary, we have shown that α-adrenergic inhibitors such as naftopidil or the QseC sensor kinase inhibitor LED209 modulate the extra- and intracellular growth of *Legionella* spp. These effects are linked to the biphasic growth cycle of *L. pneumophila* and only observed in the replicative and not in the stationary phase. Signalling through LED209 appears to partially proceed through the QseBC TCS. This study identifies adrenergic molecules as inhibitors selectively targeting *Legionella* and *Mycobacterium* spp. Further studies on the mode of action of these compounds should contribute to a better understanding of how the bacteria form their unique intracellular vacuolar replication niche. Moreover, adrenergic antagonists as well as the QseC sensor kinase inhibitor LED209 were found to reduce intracellular replication of *Legionella* as well as *Mycobacterium* spp., thus representing a promising lead compound to combat infections with these vacuolar pathogens.

**Acknowledgements**

We thank Dr G. Segal (Tel Aviv University) and Dr R. Isberg (Tufts University) for providing the *L. pneumophila* pmrA/qseB and the ‘pentuple’ mutant strains. We thank Dr P. Cosson for critically reading the manuscript. This work was supported by grants from the Swiss National Science Foundation (SNF): ‘Sinergia’ CRSII3_130016 (awarded to T. S., L. S. and H. H.) and ‘SystemsX – HostPathX’ (awarded to T. S. and H. H.). H. H. was also supported by the Max von Pettenkofer Institute, Ludwig-Maximilians University Munich, the German Research Foundation (DFG: SPP1580) and the Bundesministerium fur Bildung und Forschung (BMBF: 0315834C). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

**References**


Inhibition of Legionella by adrenergics


Edited by: P. Langford