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Regulatory circuits involved in *Legionella pneumophila* virulence: the role of Hfq and the cis-encoded sRNA

Anti-hfq

Giulia Oliva

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Présentée par

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Réseaux de régulation impliqués dans la virulence de *Legionella pneumophila*: le rôle de Hfq et du petit ARN non-codant Anti-hfq

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LIST OF ABBREVIATIONS

BCYE	Buffered charcoal yeast extract
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
INVS	Institut de Veille Sanitaire
LCV	<i>Legionella</i> -containing vacuole
LLAP	<i>Legionella</i> -like amoebal pathogen
MIF	Mature intracellular form
mRNA	Messenger ribonucleic acid
RBS	Ribosome binding site
RF	Replicating form
RNA	Ribonucleic acid
RNAP	RNA polymerase
Sg	Serogroup
sRNA	Small RNA
Spp	Sub-Species
T4SS	Type IV secretion system
T4BSS	Type IVB secretion system
TCS	Two- component system
UTR	Untranslated region
VBNC	Viable but no-cultivable

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Adaptation to environmental changes

In the course of evolution, microbial pathogens have evolved a variety of mechanisms to replicate in diverse niches in the extra- or intracellular environment of a host to establish a successful infection. Throughout the infection process, they encounter many critical situations they have to overcome, such as invading a host, escaping the innate immune response and succeeding to replicate in a specific niche within their hosts. Most pathogens share common strategies to interact with the cellular hosts, however each bacterial species has also imprinted a unique repertoire of molecular mechanism to avoid the host defenses (Cossart & Sansonetti, 2004; Finlay & Cossart, 1997). Thus one may classify pathogens according to their lifestyles in the host as extracellular, which are restricted *in vivo* to extracellular habitats, facultative intracellular, which in addition are capable to invade and grow within a variety of host cells, and obligate intracellular pathogens, which require susceptible host cells for multiplication, even though they may be able to survive for extended periods of times in extracellular regions of the host (Brubaker, 1985; Silva, 2012). Facultative and obligate intracellular pathogens are able to invade a variety of host cells both macrophages and non-professional phagocytes like epithelial and endothelial cells and hepatocytes, to subvert or resist the host antimicrobial defenses, adapt to a new host environment and modulate the host immune responses to develop a new infectious cycle in a novel intracellular niche (Brubaker, 1985; Ribet & Cossart, 2015). Bacteria that transit between extracellular environments and host cells to replicate have a dual intracellular/extracellular lifestyle that allows those bacteria also to survive and multiply in a cell-free environment. Classical examples of facultative intracellular pathogens are the bacterial genera *Legionella*, *Chlamydia*, *Listeria*, *Coxiella*, *Mycobacterium*, *Shigella* or *Francisella*, which regularly switch from intracellular to extracellular variants (M. R. Brown & Barker, 1999; Molmeret, Horn, Wagner, Santic, & Abu Kwaik, 2005; Samuel, Kiss, & Varghees, 2003). Those bacteria exhibit two main survival strategies within the host cell: i) the rupture of the phagolysosome to reach and multiply in the host cell cytoplasm like *Listeria*, *Shigella*, *Francisella* or *Mycobacterium*, or ii) preventing the fusion with the lysosome and formation of a membrane-bound compartment like *Legionella*, *Salmonella* and *Coxiella* (Fredlund & Enninga, 2014; A. Haas, 2007; Simeone et al., 2012). Among those, the intracellular pathogen *Legionella pneumophila* is adapted to survive and spread in the environment as a free-living microbe and to replicate inside eukaryotic phagocytic cells like *Acanthamoeba castellanii* or human alveolar macrophages (Fields, 1996b; Rowbotham, 1980; Steinert, Hentschel, & Hacker, 2002). Thus *L. pneumophila* encounters various environmental

conditions throughout its life cycle with respect to nutrient access and availability, temperature, pH, intracellular environment of different eukaryotic cells, and host defenses during intracellular replication. To adapt to these multiple fluctuations in the natural and host environments, *L. pneumophila* evolved the ability to differentiate into multiple forms such as a replicating form (RF), a transmissive/virulent form and a cyst-like mature intracellular form (MIF) (R. A. Garduno, Garduno, Hiltz, & Hoffman, 2002). Furthermore filamentous forms associated with biofilms have been observed as an extracellular planktonic form (Piao, Sze, Barysheva, Iida, & Yoshida, 2006; Steinert, Emödy, Amann, & Hacker, 1997). The ability to differentiate into these different forms is crucial for *L. pneumophila* in order to colonize and multiply in diverse environmental niches and to ensure a successful infection. The capacities of *L. pneumophila* to adapt to intra- and extracellular growth conditions is coordinated by sophisticated regulatory circuits and diverse regulatory elements that fine tune the adaptive response of this pathogen.

1.2 The genus *Legionella*

The genus *Legionella* was established in 1979 after a large outbreak of pneumonia among members of the American Legion that had occurred in 1976 in Philadelphia, where at least 182 cases were reported along with 40 fatal ones (McDade et al., 1977). In honour of the Legionnaires, this bacterium was named *Legionella pneumophila* and the disease Legionnaires' disease. However, retrospectively it was found that Legionnaires' disease of course existed already before the 1976 outbreak. Indeed, two years before the epidemic in Philadelphia, approximately 1500 members of the Independent Order of Odd Fellows attended a convention in the same hotel and 20 persons developed pneumonia after the meeting, two of which died (Terranova, Cohen, & Fraser, 1978). Furthermore, other retrospective studies identified a frozen isolate of a pneumonia patient isolated in 1947 as *L. pneumophila* (David et al., 2016; Mercante, Morrison, Raphael, & Winchell, 2016, Jackson et al., 1952).

1.2.1 Characteristics of the genus *Legionella*

Bacteria belonging to the genus *Legionella* are Gram-negative, aerobic rods measuring ~2 µm in length and 0.3-0.9 µm in width. *L. pneumophila* is a catalase-positive motile rod with a mono- lateral or polar flagella. This bacterium exhibit a strictly aerobic metabolism and is

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grown on buffered charcoal yeast extract (BCYE), a nutrient rich medium where the amino acid L-cystein and iron supply are added (Fields, 1996a) (Figure 1).

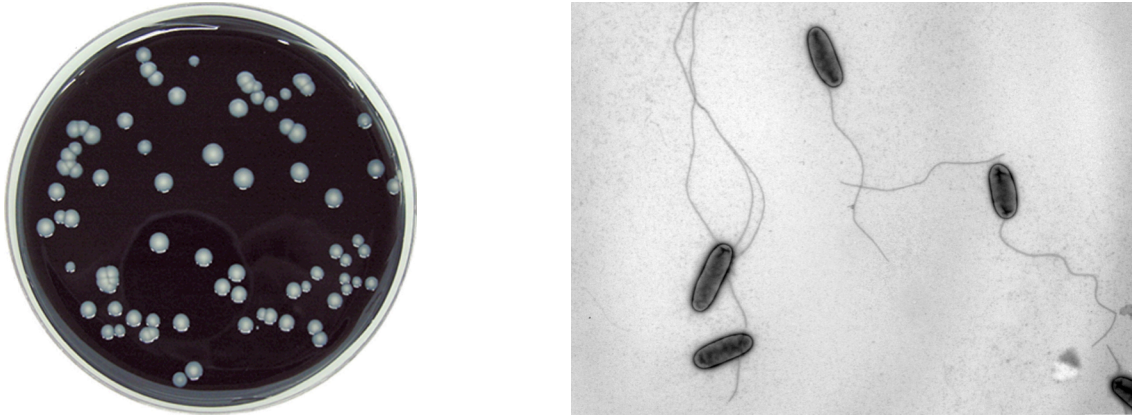


Figure 1. *L. pneumophila* growth on agar and microscopic view. Left panel *L. pneumophila* growing on BCYE agar, right panel, electron microscopy image of *L. pneumophila* in liquid.

They utilize mainly organic acids and amino acids as carbon and energy sources (Fields, 1996a; George, Pine, Reeves, & Harrell, 1980; Newton, Ang, van Driel, & Hartland, 2010). Different to what was thought previously, it was shown recently that carbohydrate usage is also important for the *L. pneumophila* life cycle (Eisenreich & Heuner, 2016; Eylert et al., 2010). Furthermore, *L. pneumophila* encodes a glycoamylase conferring glycogen- and starch-degrading activity (Herrmann et al., 2011).

The number of species and serogroups constituting the genus *Legionella* continuously increases; the genus is currently composed of 58 species and more than 70 serogroups which are colonizing natural aquatic environments like lakes, rivers, ponds and are often found in biofilms (Fields, Benson, & Besser, 2002; Newton et al., 2010; Steinert et al., 2002). An exception is *Legionella longbeachae* that is found predominantly in potting soil and is thought to be transmitted by inhalation of dust from contaminated soils (Cameron, Roder, Walker, & Feldheim, 1991; Newton et al., 2010). Despite the fact that *Legionella* have not co-evolved with humans but in aquatic habitats, bacteria belonging to the genus *Legionella* can cause severe, often fatal disease in humans. At least 20 *Legionella* species have been associated to human disease while others have been isolated only from the environment (Newton et al., 2010). In addition, a number of so-called *Legionella*-like amoebal pathogens (LLAPs) have been described. These *Legionella*-like strains do not grow without amoebae on routine growth

media and even though they show similar genetic composition, they are not associated with disease (Marrie et al., 2001; RF & BS, 1998). The most-studied *Legionella* species is *L. pneumophila*, as it is responsible for most cases of Legionnaires' disease (Newton et al., 2010; Yu et al., 2002).

1.2.2 Natural and man-made environmental reservoirs for *Legionella*

Several studies have analyzed the distribution of legionellae in natural habitats. *L. pneumophila* has been isolated from samples at temperature ranging from 10 to 42 °C, however they multiply at temperature between 20 and 42 °C, with an optimal growth temperature of 35 °C. Natural freshwater habitats are rarely associated with legionellosis, but most of the cases of legionellosis are due to man-made aquatic systems where the temperature is higher than the ambient temperature. Although osmolarity and pH appear to be environmental factors related to the prevalence of legionellae, temperature gradients influence the distribution of *Legionella* species and its multiplication (Flannery et al., 2006; Fliermans et al., 1981; Lee & West, 1991). In contrast to the wide spread distribution of *Legionella* in fresh water environments, the limiting nutrient levels of these habitats result mostly in low concentrations of non-replicating planktonic bacteria. Biofilm matrices, composed of heterogeneous communities, are known to provide shelter and nutrients required by this bacterium, thereby serving as an ecological niche for replication and persistence of *Legionella* outside a host cell (Al-Quadan, Price, & Abu Kwaik, 2012; Fields et al., 2002). Furthermore, biofilm colonization can be influenced by the symbiotic association with other microorganisms and more importantly by the presence of free-living amoebae (Declerck et al., 2009; Murga et al., 2001). Indeed, pioneering studies by Rowbotham showed that this facultative intracellular bacterium replicates and establishes a parasitic life style within free-living protozoa serving as their natural hosts (Rowbotham, 1980). Moreover, it had been reported that *Legionella* spp have the capacity to replicate within at least 14 different species of fresh water amoebae including *Acanthamoeba*, *Hartmannella*, *Neaglaria*, the ciliate *Tetrahymena pyriformis* and the slime mold *Dictyostelium discoideum* (Fields, 1996a; Hägele et al., 2000; Solomon & Isberg, 2000; Steinert & Heuner, 2005). Taken together, free-living amoebae and biofilms constitute the major environmental reservoirs for *L. pneumophila* and consequently a potential source of contamination for humans. Indeed, since the discovery that the Legionnaires' disease outbreak in Philadelphia in 1979 was caused by *L. pneumophila* that was present in the hotel's air conditioning system, the link between natural and man-made

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environmental reservoirs has been clearly shown. Furthermore, due to the elevated temperature, thermally altered aquatic environments can shift the equilibrium between free-living protozoa and bacteria, which may promote bacterial multiplication and the emergence of the disease. As human infection occurs by inhalation of contaminated aerosols, and more and more such devices are developed, it is generally thought that Legionnaires' disease may have emerged in the last decades as consequence of the industrialization and the alteration of many environments by and for human profit.

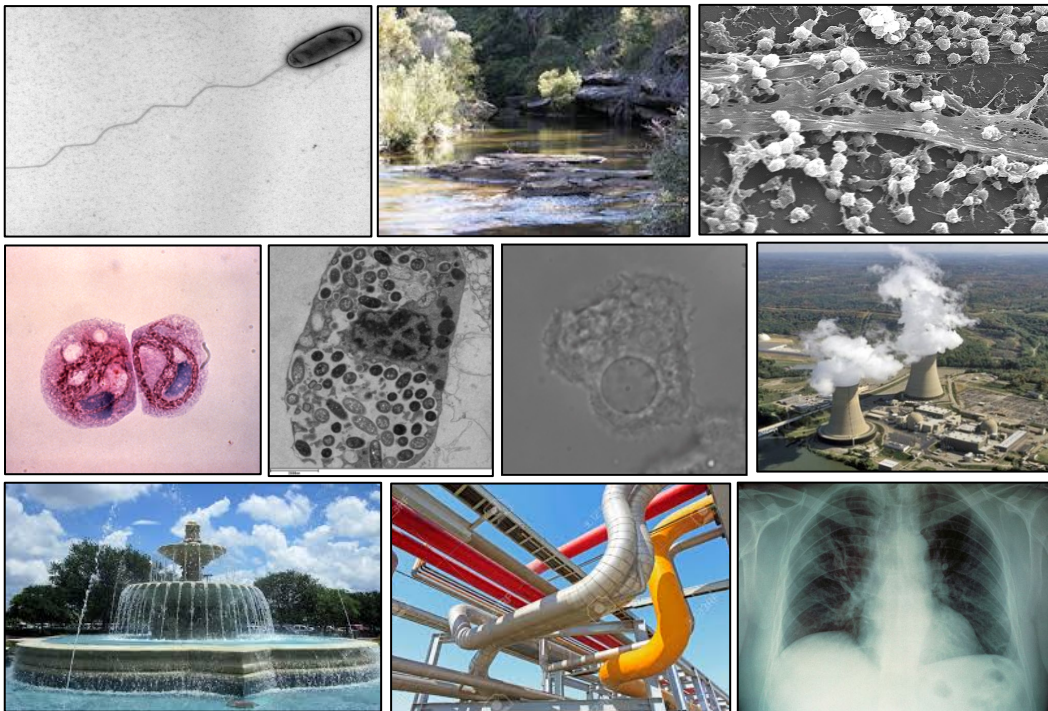


Figure 2. From the environment to humans. In the environment *Legionella* is able to survive as an intracellular parasite of protozoa or persist in biofilms. Upon transmission through man made devices (showers, air conditioning systems, cooling towers etc.) the bacteria may reach the lungs, where they can infect and colonize macrophages, which may lead to a severe pneumonia named Legionnaires' disease.

A great number of devices has been identified as source of aerosol transmission of *Legionella*, including potable water sources such as showers and taps, water faucets, and non-potable waters such as cooling towers and evaporative condensers, spas, fountains and humidifiers (Figure 2). The sources of outbreaks of Legionnaire's disease have been traced back to also to diverse locations, including hotels, homes, hospitals and cruise ships. Moreover, the material of the piping system, stagnation and periods of non-use of water pipes have been reported as pivotal factors in the occurrence of *Legionella* outbreaks (Atlas, 1999; Steinert, Ockert, Lück, & Hacker, 1998).

1.3 Legionnaires' disease

1.3.1 Symptoms and clinical manifestations

The term legionellosis includes collectively the diseases caused by *Legionella* that are transmitted by the inhalation of contaminated aerosols. Until recently it was thought that human to human transmission does not happen. However, a first possible evidence of person-to-person transmission of Legionnaires' Disease (LD) was recently reported to have occurred during a large outbreak in Portugal (Borges et al., 2016). Legionnaire's disease is a severe pneumonia with additional multisystem diseases (Diederer, 2008; McDade et al., 1977). Symptoms and signs of the disease are variable and range from mild fever, which is usually one of the first signs of illness, to respiratory and organ failure. Furthermore, non-productive cough, anorexia, chills, headache, chest pain, rigors, dyspnea and diarrhea are signs of the Legionnaires' disease. Interestingly, also extra pulmonary manifestations may occur such as confusion, memory loss, hallucinations, neurological manifestations, and metastatic or contiguous infections (Marrie, Haldane, & Bezanson, 1992; McClelland, Vaszar, & Kagawa, 2004). Legionnaire's disease occurs both as sporadic cases and as outbreaks and is either community or nosocomially acquired. The mild form of legionellosis is the so-called Pontiac fever. This non-pneumonic form is a self-limited influenza-like form of disease associated with exposure to *Legionella*. Symptoms include fever, headache and myalgia (Fields, Barbaree, Sanden, & Morrill, 1990; Newton et al., 2010).

1.3.2 Diagnosis and treatment

Legionnaires' disease is an atypical pneumonia that might clinically resemble pneumococcal or other bacterial pneumonias, thus a rapid and proper diagnosis is essential for starting the correct treatment since legionellosis has a poor prognosis when treatment starts late. However, the diagnosis of legionellosis is challenging and thus the disease is probably underreported. Legionnaires' disease can be diagnosed by both non-culture and culture techniques. Diagnosis methods are culturing from sputum, bronchial washing or autopsy/biopsy tissue, however although this was until recently the gold standard for diagnosis of legionellosis, it requires specific growth medium, takes time, and has a poor sensitivity. Since the development of the urinary antigen testing, early diagnosis and rapid initiation of appropriate antibiotic therapy has been achieved, thus it is now the most frequently used diagnostic test (Beauté, Zucs, de Jong, European Legionnaires' Disease Surveillance Network, 2013). Over the last decades the

number of cases diagnosed has remarkably increased because of the high sensitivity of this test. Nevertheless, this non-culture technique is limited solely to the detection of *L. pneumophila* Sg1 (Cunha, Burillo, & Bouza, 2016). Thus, the development of molecular techniques, such as nucleic acid amplification-based methods, can certainly provide diagnosis with specificity for the detection of other serogroups and higher sensitivity than culture methods. Indeed, several PCR based methods that rapidly allow the diagnosis of legionellosis including those due to non serogroup 1 *L. pneumophila* have been developed in the last years (Maze et al., 2014; Merault et al., 2011). *Legionella* are intracellular bacteria and as such antibiotics against the bacterium need to act and accumulate within infected cells. Beta-lactam and aminoglycoside antibiotics do not penetrate the cells and therefore are ineffective against *Legionella*. In the original outbreak of Legionnaires' disease the patients treated with the macrolide erythromycin have been reported to show lower mortality, however side effects were also reported. To date, most macrolides, tetracyclines, ketolides and quinolones are effective and particularly azithromycin, doxycycline or levofloxacin can be used as first-line treatment (Bruin, Ijzerman, Boer, Mouton, & Diederren, 2012; Roig & Rello, 2003).

1.3.3 Risk factors

Legionella spp are opportunistic pathogens thus persons presenting specific risk factors are mainly concerned. Risk factors for developing Legionnaires' disease include age older than 50 years, male gender, chronic lung disease, smoking, immunosuppression, cancer and AIDS (Newton et al., 2010; Sandkovsky et al., 2008). Moreover, the likelihood of death is increased in patients with hospital-acquired infections and in recipients of organ transplants (Kugler et al., 1983; Yu, 2000). However, recently, the infection of neonates from contaminated birthing pools has also gained significant attention since two independent cases and subsequent fatalities were reported (Fritschel, Sanyal, Threadgill, & Cervantes, 2015; Phin, Cresswell, Parry-Ford, Incident Control Team, 2014). Furthermore, repeated exposure to infectious sources represents a risk factor for Legionnaires' disease onset (Cunha et al., 2016; Fraser, 1980; J. T. Johnson et al., 1985; Marrie et al., 1991; Muder & Yu, 2002).

1.3.4 Epidemiology and incidence

Since the 1976 epidemic event, many sporadic cases and several outbreaks occurred worldwide. The precise incidence of Legionnaire's disease is not known, mostly due to the worldwide different awareness levels, diagnostic methods and surveillance methods, resulting

in a under-diagnosed and under-reported disease. In the United States about 5 000 cases of Legionnaires' disease are reported each year (Dooling et al., 2015). Data from the USA passive surveillance for Legionnaires' disease indicated a 286% increase in reported cases per 100,000 people during 2000-2014. This could be due to a true increase in the frequency of disease caused by a number of reasons such as an expanded susceptibility of the population, increased *Legionella* concentrations in the environment, or to an increased testing for Legionnaires' disease, (<http://www.cdc.gov/legionella/surv-reporting.html>). The yearly incidence shows seasonal variations, particularly more illness usually occur in the summer and early fall (<http://www.cdc.gov/legionella/health-depts/inv-tools-single/index.html>).

In 2013, 5851 cases of Legionnaires' disease were reported to the European Centre for Disease Prevention and Control. Within the years of 2005–2012 a notification rate of 11.4 cases per million people was observed. Most of the cases were community-acquired (73%), 19% were travel-associated, and 8% were linked to healthcare facilities. Most cases (88%) were confirmed by the urinary antigen test (http://ecdc.europa.eu/en/healthtopics/legionnaires_disease/pages/index.aspx). In France 1389 cases of Legionnaire's disease were reported in 2015 with a prevalence of 2.1 cases per 100 000 inhabitants (<http://invs.santepubliquefrance.fr/Dossiers-thematiques/Maladies-infectieuses/Infections-respiratoires/Legionellose>), a number of infection that remained quite stable over the last years as seen in Figure 3.

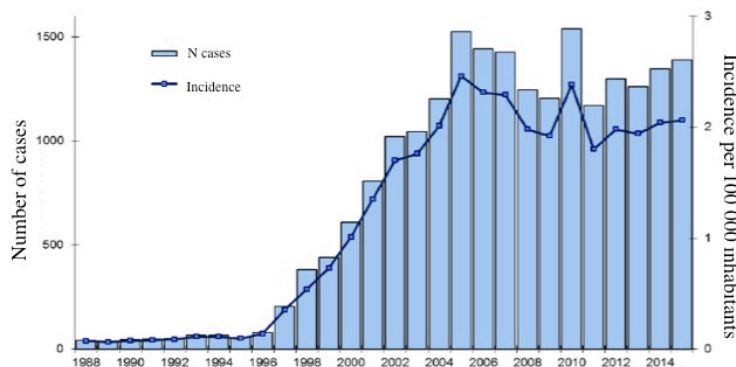


Figure 3. Evolution of the Legionnaires' disease incidence rate. Scheme representing the number of cases (left axis) and incidence rate (right axis) in France according to the mandatory reporting data from 1988 to 2015 (<http://invs.santepubliquefrance.fr/Dossiers-thematiques/Maladies-infectieuses/Infections-respiratoires/Legionellose/Donnees-de-surveillance>).

Quite interestingly, among the 58 *Legionella* species described until now, the strains belonging to the species *L. pneumophila* are responsible for over 90% of the Legionnaires' disease cases worldwide. Moreover, the strains belonging to the species *L. longbeachae* are

responsible for about 4% of human cases world (Yu et al., 2002), however, their distribution in Australia and New Zealand is different, as *L. longbeachae* accounts for 30.4% of the human Legionnaires' disease cases in this geographical region. Furthermore, among the strains causing Legionnaires' disease, *L. pneumophila* serogroup 1 (Lp1) alone is responsible for about 84% of cases although 15 serogroups are described within this species (Gomez-Valero, Rusniok, Cazalet, & Buchrieser, 2011a; Kozak-Muiznieks et al., 2014; Yu et al., 2002; Newton et al., 2010). Moreover, in the last years, molecular typing methods allowed to show, that some of the *L. pneumophila* Sg1 strains are worldwide distributed like *L. pneumophila* strain Paris (Cazalet et al., 2010; 2004), and others are emerging clones like the *L. pneumophila* Sg1 subclones ST47 (Lorraine) (Ginevra et al., 2008) or ST1, ST37, ST62 and ST23 (David et al., 2016).

1.4 Association with free-living amoebae

Many clinically relevant pathogens like *Listeria*, *Legionella*, *Vibrio*, *Mycobacterium* or *Burkholderia* are reported to survive within protozoa in the environment and to establish a symbiotic or parasitic relationship with these hosts (M. R. Brown & Barker, 1999; Greub & Raoult, 2004; Hilbi, Weber, Ragaz, Nyfeler, & Urwyler, 2007; Molmeret et al., 2005). However, *Legionella* has a special place among these pathogens, as protozoa are their natural hosts. Thus protozoa play a crucial role for the survival, transmission and virulence and their association with protozoa may explain the constant presence of *Legionella* in the environment. Within protozoa *L. pneumophila* displays a remarkable increased resistance to harsh conditions such as fluctuations in temperature, pH, acidity and osmolarity, facilitating their survival in the environment (Abu Kwaik, Gao, Harb, & Stone, 1997). In addition this intracellular niche provides *Legionella* with resistance to chemical disinfection and biocides, such as chloride, UV irradiation, compared to *in vitro*-grown bacteria, hindering thus the eradication of *L. pneumophila* from environmental sources of infections (Barker & Brown, 1994) (Barker, Scaife, & Brown, 1995; Cunha et al., 2016). In addition, protozoa have been reported to release respirable *L. pneumophila*- containing vesicles, which are resistant to freezing- thawing and sonication (Berk, Ting, Turner, & Ashburn, 1998). Other observations derived from the co-cultivation of viable but no-culturable (VBNC) *L. pneumophila* with amoebae, showed that these provided the adequate stimuli and nutrients necessary for resuscitating the hibernated *L. pneumophila* (García, Jones, Pelaz, Millar, & Abu Kwaik, 2007; Steinert et al., 1997). Thus, environmental protozoa represent not only a nutrition

resource for the survival, replication and distribution of *Legionella*, but also function as shelter affording protection against adverse environmental conditions (Barker et al., 1995; Berk et al., 1998). In addition to shelter and nutrient provided by amoebae to *Legionella*, it has been reported that *L. pneumophila* released from protozoan hosts display a striking increase in infectivity of mammalian cells *in vitro*. For instance, bacteria released from *H. vermiformis* are more infectious and kill host cells more efficiently than *in vitro* grown *Legionella* (Brieland et al., 1997). *L. pneumophila* bacteria grown within amoeba are also highly motile and survive and multiply more efficiently within human monocytes, (J. D. Cirillo et al., 1999; J. D. Cirillo, Falkow, & Tompkins, 1994; R. A. Garduno et al., 2002).

While protozoa are the natural hosts of *L. pneumophila*, the infection of human cells remains opportunistic, however the dual host specificity may be due to the fact that protozoa are primordial phagocytes and as such they share many features with mammalian phagocytes including conserved antimicrobial processes (Hilbi et al., 2007; Al-Quadan et al., 2012). Particularly, the intracellular life cycle of *L. pneumophila* has been shown to be very similar in both hosts (Fields et al., 2002). Thus it has been proposed that the million years of co-evolution with amoebae has equipped these bacteria with effective strategies to face antimicrobial effectors and the human immune system (Gao, Harb, & Abu Kwaik, 1997; Molmeret et al., 2005). The analyses of the *L. pneumophila* genome sequence in 2004 further supported this hypothesis (Cazalet et al., 2004). This sequence analyses was a major breakthrough and key to the further understanding of the strategies employed by *Legionella* to subvert host functions and the evolution of virulence as it led to the identification of a large number of bacterial proteins with eukaryotic-like properties as witness of the tight co-evolution between *L. pneumophila* and its protozoan hosts (Cazalet et al., 2004). It also led to a new concept in host pathogen interactions: bacteria parasitizing protozoa acquire genes from their hosts to subvert host functions. Later the work of several groups including ours, showed that these eukaryotic-like proteins are indeed secreted effectors that act in the host like their eukaryotic homologues – thus molecular mimicry is a major virulence strategy of *Legionella* (see below) (Nora, Lomma, Gomez-Valero, & Buchrieser, 2009). Taken together, the association of *Legionella* with environmental protozoa is essential for the ecology and pathogenesis of these bacteria.

1.5 Intracellular life cycle within phagocytic cells

The primary feature of *L. pneumophila* pathogenesis is related to its capability to multiply intracellularly. The intracellular cycle of legionellae has been characterized in both protozoan and mammalian cells. Microscopically, the two processes are identical, even though differences in entering and exiting the host cell steps have been described (Escoll, Rolando, Gomez-Valero, & Buchrieser, 2013). Our current understanding of the *L. pneumophila* infectious cycle is represented in Figure 4. Following the contact of *L. pneumophila* with phagocytic cells, such as macrophages or amoebae, the bacteria are internalized through conventional phagocytosis or coiling phagocytosis (Bozue & Johnson, 1996; J. A. Elliott & Winn, 1986; Horwitz, 1984). Conventional phagocytosis in macrophages was shown to be mediated through a complement- dependent mechanism by complement receptor CR1 and CR3. Following the CR3 binding to the major outer membrane protein MOMP, the association MOMP-CR3 is sufficient to induce the bacterial phagocytosis (Bellinger-Kawahara & Horwitz, 1990; N. R. Payne & Horwitz, 1987). By contrast, in the amoebae *Hartmannella veriformis*, the attachment and the induction of *L. pneumophila* uptake are mediated through the lectin receptor in a time-dependent manner (Escoll et al., 2013; Venkataraman, Haack, Bondada, & Abu Kwaik, 1997).

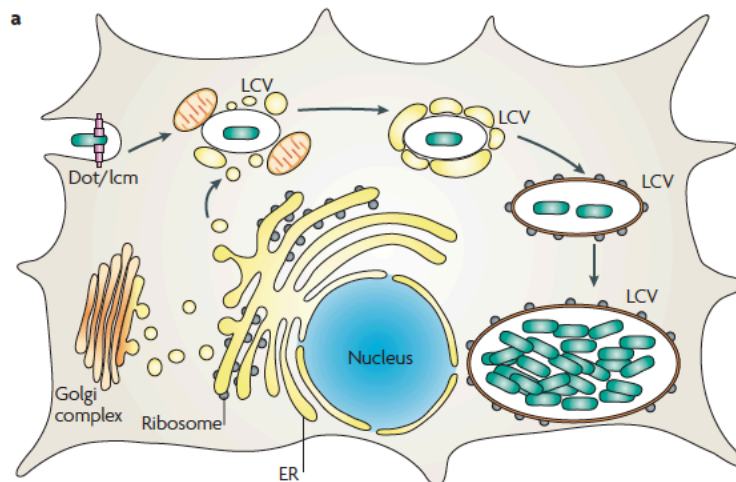


Figure 4. Schematic representation of the different steps of the intracellular life cycle of *L. pneumophila* in phagocytic cells. After uptake in protozoa or macrophages, the bacteria persist within the so-called *Legionella*-containing vacuole (LCV), which subsequently evades the lysosomal network. Within minutes of uptake, numerous vesicles derived from the mitochondria and the endoplasmic reticulum (ER) are docked onto the LCV membrane. Within this ER-like vacuole, the bacteria replicate and at the end of the replication cycle flagellated bacteria are released. Adapted from (Isberg, O'Connor, & Heidtman, 2008).

The non-complement mediated attachment and invasion of *L. pneumophila* in phagocytic cells depends on several factors such as type IV pili (Stone & Abu Kwaik, 1998), the 60 Kda heat shock protein Hsp60 (R. A. Garduño, Garduño, & Hoffman, 1998) and the enhanced entry protein RtxA (Repeats in structural Toxin) encoded by the *enhI* locus and the enhanced entry protein EnhC (S. L. Cirillo, Bermudez, El-Etr, Duhamel, & Cirillo, 2001; S. L. Cirillo, Lum, & Cirillo, 2000). Recently it has been shown that EnhC also interferes with immunostimulatory muramyl-peptide production to evade innate immunity (M. Liu et al., 2012). Furthermore, the secreted proteins LaiA, SidE and LpnE (Newton, Sansom, Bennett-Wood, & Hartland, 2006) are required for efficient host entry (Chang, Kura, Amemura-Maekawa, Koizumi, & Watanabe, 2005; Newton et al., 2006). However, LpnE like EnhC and LidL also influences trafficking of the *L. pneumophila* containing vacuole (Newton et al., 2007). In addition, the presence of the flagellum and generally motility also positively affects the establishment of infection. Comparative evidences have suggested that elaborate and diverse uptake processes exist in different hosts (Steinert et al., 2002).

After entry into phagocytes, *L. pneumophila* manipulates the maturation process of the nascent phagosome and forms a unique replication-permissive compartment that is resistant to acidification (Horwitz, 1983a; 1983b). This *Legionella*-containing vacuole (LCV) is a single-membrane compartment surrounded by numerous small vesicles on the cytoplasmic face. Distinct and temporal docking events follow the escape of the LCV from the lysosome, including the recruitment of mitochondria and within 4 hours vesicles derived from rough endoplasmic reticulum (ER) cluster near the LCV membrane (Isberg et al., 2008; Roy, 2002) (Figure 4). Fluorescence microscopy analysis of either intact cells or isolated LCVs has shown that ER-associated proteins, such as Sec22b and the GTPase Rab1, localize near the vacuole, elucidating the origin and the trafficking network of vesicles surrounding the LCVs (Kagan & Roy, 2002; Swanson & Isberg, 1995). Further studies demonstrated that the vesicles derived from the ER fuse with the LCV membrane, leading to the release of their soluble content into the LCV lumen (Robinson & Roy, 2006). Although the ER association with the LCV has been detected during intracellular replication of *Legionella*, additional membrane trafficking events may occur and modulate the intracellular life cycle of this bacterium. During the late replicative phase, the LCV was found in association with the late endosomal protein LAMP1 (lysosome-associated membrane protein 1), postulating that the lysosomal compartment may provide *Legionella* with a nutrient-rich environment necessary

for replication (Sturgill-Koszycki & Swanson, 2000). Approximately 20-24 hours post-infection, the host cell is extensively filled with bacteria (Figure 5).

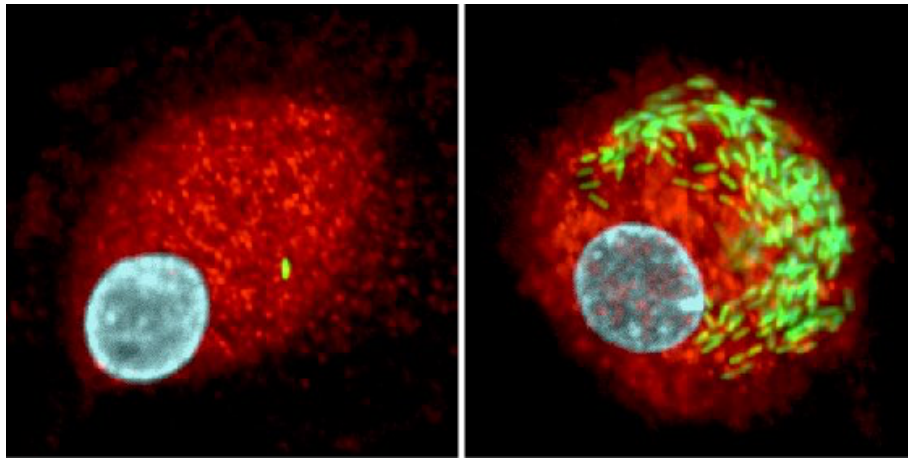


Figure 5. Intracellular multiplication of *L. pneumophila* within macrophages. Confocal images (60X objective) of human monocyte-derived macrophages (hMDMs) infected with *L. pneumophila* strain Philadelphia JR32 expressing constitutively GFP, at 2h (left) and 20h (right) post-infection. Cyan: nucleus (DAPI), Red: cytoplasm, Green: *L. pneumophila*, Bar: 10 μ m (Pictures kindly provided by P. Escoll, Institut Pasteur).

Following replication, depletion of nutrients drives the differentiation of *L. pneumophila* from a replicative form, where the bacteria are not motile and not cytotoxic, to a virulent form, characterized as motile, cytotoxic and flagellated bacteria (Molofsky & Swanson, 2004). This transition ensures that the bacteria activate the infectious traits for the escape and the following transmission into a new host (and & Hammer, 2003). During replication in the host cell *L. pneumophila* induces the expression of several host antiapoptotic genes *via* activation of the transcription factor NF κ B (Abu-Zant et al., 2007; Losick & Isberg, 2006). Only at the late stages of infection macrophages show increasingly apoptotic phenotypes, such as nuclear condensation and phosphatidylserine exposure but little is known about how *Legionella* may induce apoptosis to leave the host cell (Speir, Vince, & Naderer, 2014). Recently it has been proposed that inducing apoptosis of the host cell by inhibiting the host factors BCL-XL may help eliminating *L. pneumophila* during infection (Speir et al., 2016).

1.5.1 Hijacking host cell defenses by the Dot/Icm machinery

Genome sequencing and analysis led to an in depth understanding of genomic and but also pathogen-related features of *L. pneumophila*, like the presence of multiple pathogenicity islands and mobile genetic elements. This bacterium shows high plasticity and genetic diversity, considering that 7.5-10.5% of its genes are strain specific (Gomez-Valero, Rusniok,

Jarraud, Vacherie, Rouy, et al., 2011b). However, the most remarkable genomic feature of *L. pneumophila* is the presence of a high number and variety of eukaryotic-like proteins or eukaryotic-like domains, which are thought to have evolved during the bacterial co-evolution with fresh-water amoebae (Cazalet et al., 2004). Indeed, phylogenetic analyses of some of these eukaryotic-like proteins has been carried out revealing a clustering of the *Legionella* eukaryotic like proteins with eukaryotic sequences, further supporting the hypothesis that these were acquired by HGT from eukaryotic organisms like amoeba (Gomez-Valero & Buchrieser, 2013; Lurie-Weinberger et al., 2010; Nora et al., 2009). These eukaryotic-like proteins and proteins encoding domains normally only or mainly present in eukaryotic proteins are good candidates to manipulate host cell functions to the pathogens advantage. As such, these proteins need to be secreted in the host cells. Among the wide varieties of secretion systems identified in *L. pneumophila*, the type IVB secretion system (T4BSS) named *dot/icm* (defective organelle trafficking/intracellular multiplication) is essential for intracellular replication of *L. pneumophila* (Berger & Isberg, 1993; Marra, Blander, Horwitz, & Shuman, 1992). This macromolecular complex, shown in Figure 6, is encoded by 27 genes, which are located in two distinct genomic regions.

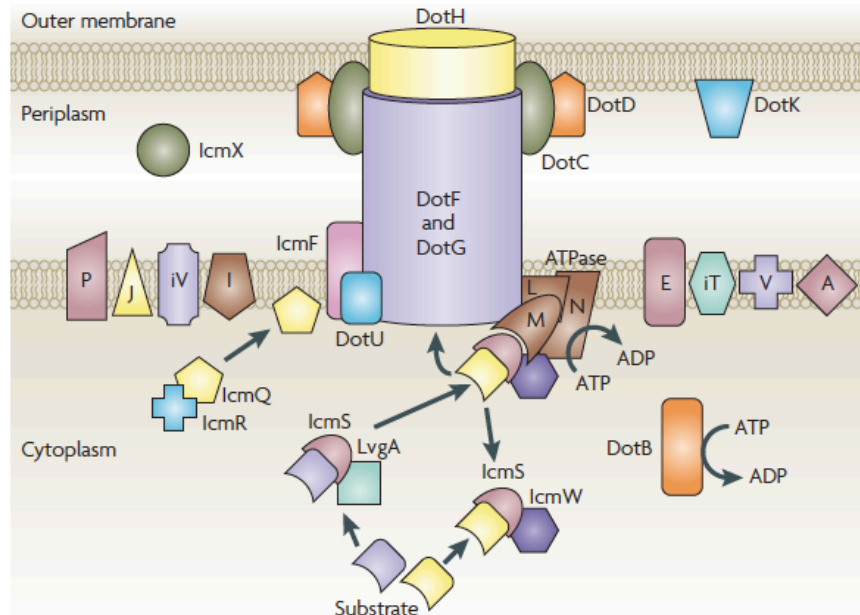


Figure 6. The Dot/Icm translocation system of *L. pneumophila*. Proposed location and interaction of the various Dot/Icm components in the *L. pneumophila* membrane, based on a protein stability study (Buscher et al., 2005). Individual letters represent Dot protein names, whereas letters preceded by an “I” indicate Icm protein names. Adapted from (Isberg et al., 2008).

GENERAL INTRODUCTION

The majority of the *dot/icm* genes are dedicated to the formation of the secretion complex (Vincent et al., 2006). Besides structural proteins, membrane-spanning proteins are associated and interact with the host cell membrane by forming a pore for the translocations of “effectors” into the cytoplasm (Segal, Feldman, & Zusman, 2005). In addition, the Dot/Icm secretion system includes chaperons, such as IcmS and IcmW/DotB which guide the effectors into the secretion system or provide the necessary energy for the assembly of the whole apparatus (Segal et al., 2005). The sequences of the *dot/icm* genes is 96-98% conserved across *L. pneumophila* strains and 62-79% conserved across *Legionella* species (Gomez-Valero, Rusniok, Cazalet, & Buchrieser, 2011a; Gomez-Valero et al., 2014; Morozova et al., 2004). The Dot/Icm system translocates the surprisingly high number of over 300 effectors into the host cytoplasm, allowing this bacterium to manipulate many host signalling and metabolic pathways (de Felipe et al., 2008; Heidtman, Chen, Moy, & Isberg, 2009; Ninio, Celli, & Roy, 2009; W. Zhu et al., 2011). This secretion apparatus is essential for the establishment of the LCV, the escape from lysosomal fusion and the multiplication in both, amoeba and macrophages (Burstein et al., 2009; de Felipe et al., 2008; Escoll et al., 2013; Heidtman et al., 2009; Shohdy, Efe, Emr, & Shuman, 2005). The first characterized effector of the Dot/Icm T4SS was RalF, a guanine nucleotide exchange factor (GEF) required for the activation and recruitment of the host GTPase ARF-1 to the LCV. ARF-1 is a highly conserved master regulator of membrane trafficking processes, particularly vesicle trafficking between the ER and Golgi complex and thus important for the intracellular growth of *L. pneumophila* (Nagai, Kagan, Zhu, Kahn, & Roy, 2002). Moreover, a second bacterial GEF named SidM (also known as DrrA) activates the small GTPase Rab1 by promoting nucleotide exchange and recruits it to the LVC, similarly to ARF1 recruitment (Murata et al., 2006). Despite the recruitment of Rab1 by SidM, it is not essential for intracellular growth, the coordinated activity of ARF-1, Rab 1 and Sar1 is required to guide the trafficking and fusion of ER-derived vesicles to the LCV (Machner & Isberg, 2006; Nagai & Roy, 2003). Another Dot/Icm secreted effector named VipA has been described to act as actin nucleator, polymerizing microfilaments. VipA alters host cell vesicle trafficking, however it is not essential for entry or replication in amoeba or macrophages (Franco, Shohdy, & Shuman, 2012). Several, functionally distinct translocated substrates have been identified and characterized such as LidA, involved in the recruitment of early secretory vesicles to the LCV in the integrity maintenance of the Dot/Icm complex, and LepA and LepB, involved in the bacterial non-lytic release from the LCV during the amoeba infection (J. Chen et al., 2004).

In recent years, the eukaryotic like proteins or proteins with eukaryotic like domains have been functionally analysed. This has shown that they are indeed Dot/Icm effectors that subvert host functions to the pathogens advantage. For example *L. pneumophila* subverts ubiquitin signalling by secreting F-box and U-box proteins into the host cell where they ubiquitinate proteins on the *Legionella* containing vacuole (Kubori, Hyakutake, & Nagai, 2008; Lomma et al., 2010; Price, Al-Khodor, Al-Quadani, & Abu Kwaik, 2010). Thanks to the eukaryotic CaaX motif that allows anchoring into membranes, the F-box protein named AnkB was reported to serve as platform for the docking of polyubiquitinated proteins to the LCV to generate aminoacids for intracellular replication of *L. pneumophila* (Ivanov, Charron, Hang, & Roy, 2010; Price et al., 2010). Furthermore autophagy is subverted by *L. pneumophila* by secreting different effectors like RavZ or Spl, a protein that shows a high degree of sequence similarity to the eukaryotic enzyme sphingosine-1 phosphate (SPL). RavZ is a protease that deconjugates Atg8 in the host cell (Choy et al., 2012), whereas Spl is an enzyme that directly targets the host sphingolipid metabolism (Rolando et al., 2016). Thus Spl inhibits autophagosome formation and RavZ autophagosome maturation, showing that *L. pneumophila*, like other intracellular pathogens, employs several strategies to counteract autophagy in the host cell (Escoll, Rolando, & Buchrieser, 2016). Furthermore, *L. pneumophila* modifies the chromatin landscape of its host by secreting the effector RomA that contains a eukaryotic SET-domain conferring methyltransferase activity to tri-methylate lysine 14 of histone H3. RomA promotes H3K14 methylation of 4870 promoter regions, including genes with roles in innate immunity (Rolando et al., 2013).

These examples show, that indeed, the acquisition of eukaryotic like genes by *L. pneumophila* from its hosts, has shaped its genome and its virulence strategies and many more functions are still to be discovered. However, not all the characterized Dot/Icm effectors are present in all *L. pneumophila* strains, suggesting that *L. pneumophila* has acquired strain-specific sets of effector proteins to face distinct host cell niches, to which they may be confronted during their evolution (Cazalet et al., 2004; Gomez-Valero & Buchrieser, 2013).

CHAPTER TWO

HFQ AND SMALL REGULATORY RNAs

2.1 Implication of small regulatory RNAs and their regulators in virulence and adaptation of intracellular bacteria

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Small RNAs, 5' UTR elements and RNA-binding proteins in intracellular bacteria: impact on metabolism and virulence

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One sentence summary: Small RNAs can base-pair target mRNAs, modulate protein activity, mimic other nucleic acids and exert their function in the regulation of a multitude of cellular processes of bacteria, including virulence gene expression in intracellular bacterial pathogens.

Keywords: intracellular bacteria; small RNAs; riboswitch; Hfq; CsrA; virulence

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REVIEW ARTICLE

Small RNAs, 5' UTR elements and RNA-binding proteins in intracellular bacteria: impact on metabolism and virulence

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One sentence summary: Small RNAs can base-pair target mRNAs, modulate protein activity, mimic other nucleic acids and exert their function in the regulation of a multitude of cellular processes of bacteria, including virulence gene expression in intracellular bacterial pathogens.

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ABSTRACT

Sequencing-based studies have illuminated increased transcriptional complexity within the genome structure of bacteria and have resulted in the identification of many small regulatory RNAs (sRNA) and a large amount of antisense transcription. It remains an open question whether these sRNAs all indeed play regulatory roles, but their identification led to an exponential increase in studies searching for their function. This allowed to show that sRNAs may modulate virulence gene expression, cellular differentiation, metabolic functions, adaptation to environmental conditions and pathogenesis. In this review we will provide mechanistic insights into how sRNAs bind mRNAs and/or proteins. Furthermore, the important roles of the RNA chaperone Hfq, the CsrA system and the CRISPR RNA will be discussed. We will then focus on sRNAs and 5' untranslated region (UTR) elements of intracellular bacteria like *Chlamydia*, *Listeria*, *Legionella*, or *Salmonella*, and place emphasis on those that are expressed during replication in host cells and are implicated in virulence and metabolism. In addition, sRNAs that regulate motility, iron homeostasis, and differentiation or stress responses will be highlighted. Taken together sRNAs constitute key elements in many major regulatory networks governing the intracellular life and virulence of pathogenic bacteria.

Keywords: intracellular bacteria; small RNAs; riboswitch; Hfq; CsrA; virulence

INTRODUCTION

Pathogenic bacteria have evolved a variety of strategies to replicate in many different niches in their hosts. They have learned how to counteract the host defences and to replicate in specific, normally sterile regions. There are bacteria that remain extracellular and those that are internalized via active or passive pathways (Cossart and Sansonetti 2004). Intracellular bacterial pathogens co-exist with the infected cell in an obligate intracellular state or transit between the extracellular and intracellular environment. However, all have evolved the ability to survive

and grow within eukaryotic cells such as professional phagocytes or other cell types by employing diverse mechanisms to manipulate the host cell for their own benefit. After entering the host cell, the bacteria are surrounded by a membrane-bound vacuole that is targeted to the lysosomal compartments to undergo proteolytic degradation. There, the bacteria are facing a changing and very hostile environment characterized by decreasing pH, elevated concentration of reactive oxygen and nitrogen species and poor nutrient content to which either they have to adapt or they have to avoid in order for infection to succeed. Two main strategies are observed: (i) survival in this

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niche either by preventing vacuole-lysosome fusion or by modifying the environment within the phagolysosome (vacuolar pathogens) like *Legionella*, *Coxiella*, *Brucella*, *Mycobacterium* and *Salmonella*; or (ii) escape from the vacuole to gain access to and proliferate within the host cell cytosol (cytosolic pathogens) like *Listeria*, *Shigella*, *Burkholderia*, *Francisella* and *Rickettsia* (Haas 2007; Ray, Marteyn, Sansonetti, et al. 2009; Fredlund and Enninga 2014).

Adaptation to these intracellular lifestyles is regulated in both space and time. Those bacteria that adapt most rapidly to the hostile conditions encountered in the host are the most successful. The adaptation process is accompanied by major changes in gene transcripts, post-transcriptional regulatory molecules and the protein levels of the bacteria. For intracellular bacteria this includes a temporal and spatial highly co-ordinated regulation of the production of specific effector proteins. These proteins are transferred to the host by dedicated secretory systems and are able to modify the immune response and the metabolism of the infected cell, bringing advantage for the pathogens (Hubber and Roy 2010; Agbor and McCormick 2011; Steiner, Furuya and Metzger 2014). The bacteria sense specific stimuli and respond with changes in gene expression, a process that is tightly controlled by various and complex regulatory networks. Involved therein are factors that are highly conserved in the microbial world including the stringent response alarmone (p)ppGpp (Dalebroux and Swanson 2012), two-component systems (Salazar and Laub 2015), the global regulator CsrA (Romeo, Vakulska and Babitzke 2013), the RNA chaperone Hfq (Sobrero and Valverde 2012) and the alternative sigma factor RpoS (Lange and Hengge-Aronis 1991; Schellhorn 2014). Key players in these important regulatory networks are small, non-coding RNAs (sRNA). In the last years, genome-wide expression studies using high-density tiling arrays or RNA deep sequencing (RNAseq) have uncovered the wide distribution and large number of non-coding RNAs present in bacterial genomes (Papenfert and Vogel 2010). Studies of their functionality attracted more and more attention, and meanwhile it is evident that they play a crucial role in many biological processes such as in environmental sensing and stress adaptation, virulence and infectivity of intracellular bacteria, as well as development and metabolism (Gottesman and Storz 2011). In this review we will focus on how intracellular pathogens regulate their adaptation to the invaded host, allowing their replication, by discussing the fascinating roles that sRNAs and the two major RNA-binding proteins Hfq and CsrA/RsmA play in these regulatory processes. After describing their mechanisms of function we will illustrate their impact by providing selected examples of their regulatory roles in intracellular bacteria.

MECHANISMS BY WHICH sRNAs FUNCTION IN BACTERIA

Regulatory RNAs in bacteria are usually not translated and comprise a size range between 50 and 400 nucleotides in length. They can modulate transcription, translation, mRNA stability, and DNA maintenance or silencing. These diverse functions are achieved through a variety of mechanisms, including changes in RNA conformation, protein binding, base pairing with other RNAs and interactions with DNA (Waters and Storz 2009; Gottesman and Storz 2011). Due to the recent development and improvement of genome-wide RNAseq methods, there has been an explosion in the amount of sRNAs identified, but the characterization of their function in the diverse regulatory networks is still in its infancy (Sorek and Cossart 2010). The major mechanisms of how sRNAs can act are as follows.

- Trans-encoded sRNA molecules are present on the chromosome in a location distinct from their targets and thus share only limited complementarity with their targets. The general mechanism of trans-acting sRNAs is to sequester the ribosome-binding site (RBS) of a target mRNA by base-pairing to the Shine-Dalgarno (SD) sequence or the start codon and may also interact with the coding sequence of the mRNAs (Storz, Vogel and Wassarman 2011). In order to exploit their regulatory functions, most of the already characterized trans sRNAs are tightly coupled with the activity of RNases, which are enzymes involved in RNA turnover through RNA cleavage (Viegas et al. 2007; Viegas et al. 2011; Saramago et al. 2014). Furthermore, taking into account the limited complementarity between sRNAs and their mRNA targets, many trans-encoded RNA molecules engage the RNA chaperone protein Hfq (Vogel and Luisi 2011) (Fig. 1A).
- Cis-encoded antisense RNAs are complementary to their target RNA as they are transcribed from the DNA strand opposite to the genes they regulate, hence they can interact autonomously. They are often located in the untranslated regions (UTRs) of the corresponding gene where the RNA duplex formation can affect ribosome-binding/translation, termination events or overall stability of the mRNA by rearranging the secondary structures, such as hairpins in the target RNA (Caldelari et al. 2013) (Fig. 1B).
- Alternatively to the RNA-RNA regulation, sRNA can also interact with regulatory proteins, influencing their activity directly. The best understood system in this context is the global carbon storage regulator of the CsrA/RsmA family. A common feature in this network present in numerous pathogenic and non-pathogenic bacteria is the transcription of at least one or more sRNAs named CsrBC or RsmYZ, depending on a two-component system (TCS). These sRNAs can interact with CsrA/RsmA and sequester it from its specific position on the target mRNA often in the immediate vicinity of the RBS (Babitzke and Romeo 2007). This leads to the translation of the previously blocked transcripts. Alternatively, it is discussed that CsrA might also have positive effects on the turnover of mRNAs due to stabilization of the target transcript and protection against RNase attacks.
- A more recently described class of sRNAs participates in the clustered regularly interspaced short palindromic repeats/CRISPR associated (CRISPR/Cas) system, which provides the bacteria with an RNA-mediated adaptive immune system against nucleic acids deriving, for example, from bacteriophages, plasmids or mobile genetic elements (Brouns et al. 2008; Jinek et al. 2014). The variable crRNAs originating from the foreign DNA are located in an array, each of which is flanked by an identical repeat sequence (Fig. 2). Together with the conserved Cas proteins, the crRNA can recognize the complementary DNA target and mediate its degradation.

By highlighting some of the recent studies about these different RNA-based regulations in intracellular bacteria we show the remarkable contribution of sRNAs and their broad diversity and functionality in regulatory networks (Table 1).

THE RNA CHAPERONE HFQ AND ITS INTERACTION WITH TRANS-ENCODED SMALL RNAs

Hfq was identified in *Escherichia coli* as a host factor required for replication of the RNA phage Q β > 40 years ago (Franze de

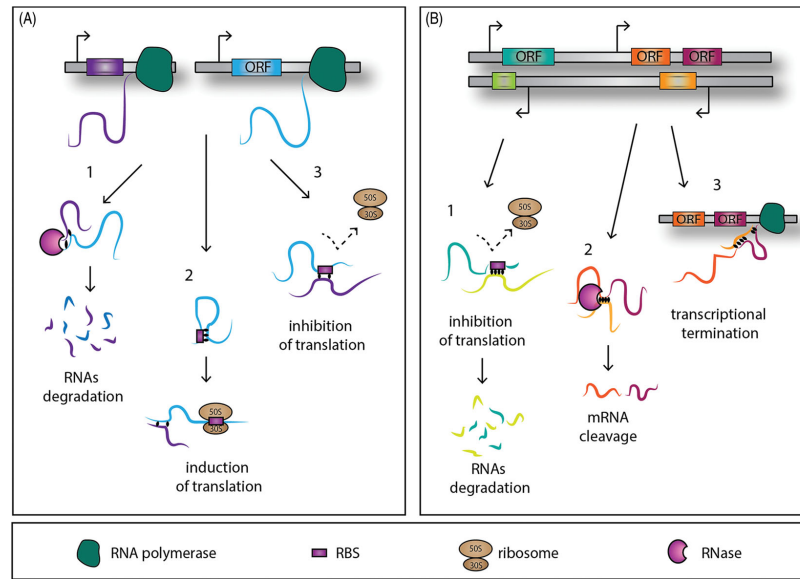


Figure 1. Simplified model of action of trans-encoded and cis-encoded RNAs. (A1) trans-encoded sRNAs interact with their target RNA through imperfect base-pairing and hence promote RNase degradation of the double-stranded RNA molecules. Alternatively the sRNAs might affect the target translation positively (A2) and negatively (A3) by releasing or masking the ribosome-binding site, respectively. Conversely the cis-encoded sRNAs bind through full sequence complementarity the mRNA target, affecting translation, and end in the degradation of the sRNA-target RNA complex (B1). A small cis-encoded RNA, antisense between two genes, can lead to mRNA cleavage (B2) or the transcriptional termination through a putative loop formation and consequently the cessation of the RNA polymerase activity (B3).

Fernandez, Eoyang and August 1968). Hfq is now known as a central mediator of sRNA-based gene regulation in bacteria (Aiba 2007; Waters and Storz 2009; Gottesman and Storz 2011; Storz, Vogel and Wassarman 2011; Vogel and Luisi 2011; Wagner 2013). It is a member of the Sm protein family that is ubiquitous in prokaryotes and in eukaryotes where it is implicated in RNA splicing and decay, suggesting an ancient origin (Moller et al. 2002; Wilusz and Wilusz 2005). However, in some cases there is no known Hfq homologue, as in the bacterial clades: *Chlamydia*-*Spirochaetes*, *Actinomycetes-Deinococcus-Cyanobacteria* and Green sulfur bacteria-*Cytophagales* and in those that have experienced a massive genome reduction due to their parasitic lifestyle, e.g. *Buchnera* sp., *Rickettsia prowazekii* and *Brucella melitensis* (Sun, Zhulin and Wartell 2002; Chao and Vogel 2010).

Briefly, Hfq in association with an sRNA might block the binding of 30S and 50S ribosomal subunits to the RBS of a target mRNA, leading to repression of protein synthesis (Fig. 3A). Conversely, Hfq can bolster protein translation by accompanying the sRNA to the 5' end of its mRNA target and disrupting a secondary structure which otherwise could mask the RBS and prevent mRNA translation (Fig. 3B). Moreover Hfq can act alone, protecting sRNAs from ribonuclease cleavage by RNase E by binding to many RNase E cleavage sites and prohibiting its degradation in a post-target recognition manner (Vogel and Luisi 2011). On the other hand, Hfq can also stimulate the cleavage of sRNAs and their target mRNAs by directly interacting with RNase E, forming a degradasome-like complex that digests the bound RNA (Hajnsdorf and Regnier 2000; Morita, Maki and Aiba 2005) (Fig. 3C and D). Furthermore, Hfq may induce 3' end polyadenylation of an mRNA by the enzyme poly(A)polymerase

(PAP) and the subsequent 3' to 5' degradation by the exoribonucleases, governing the RNA turnover (Fig. 3E). However, it should be mentioned that not all trans-encoded RNAs require Hfq (e.g. RprA or RNAII). These sRNAs free of Hfq binding are preferably degraded by polynucleotide phosphorylase (PNPase) (Andrade et al. 2012).

Taken together, Hfq is establishing dynamic interactions with a plethora of diverse RNA molecules and affects physiological functions and virulence of bacterial pathogens. Indeed, deletion of *hfq* led to growth defects and dramatic virulence phenotypes in several intracellular bacteria, such as *Francisella tularensis* (Meibom et al. 2009), *Legionella pneumophila* (McNealy et al. 2005), *Neisseria gonorrhoeae* (Dietrich et al. 2009), *S. enterica* serovar Typhimurium (Sittka et al. 2007) and *Listeria monocytogenes* (Christiansen et al. 2004). Intriguingly, in a growing number of bacteria such as *Burkholderia cenocepacia* (Ramos et al. 2013), multiple Hfq proteins indicating potential functional diversification have been identified. Studies of Hfq in these different organisms will help to uncover its full role in post-transcriptional regulation.

Genome-wide identification of Hfq targets in intracellular bacteria

With the advent of next-generation sequencing (NGS) and comprehensive transcriptome analysis techniques, increasing numbers of new sRNAs have been detected in bacteria (Albrecht et al. 2011; Wurtzel et al. 2012; Bilusic et al. 2014; Boudry et al. 2014). Using custom-made microarrays and transcriptome analyses of the intracellular pathogens *N. gonorrhoeae* (Dietrich et al. 2009) or *F. tularensis* (Meibom et al. 2009), it was reported that

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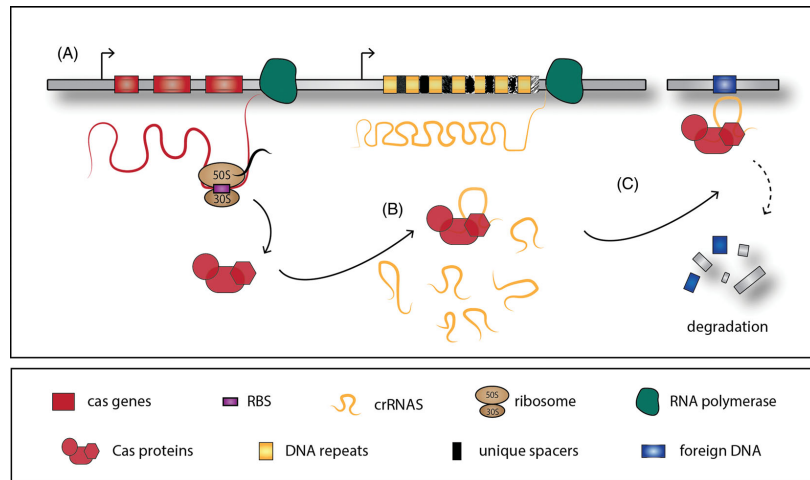


Figure 2. Model of CRISPR/Cas system function. (A) Basic structure of the CRISPR/Cas system: several Cas proteins are encoded by the cas genes, located typically in the close proximity of the CRISPR arrays, composed of a variable number of DNA repeats interrupted by unique spacer regions which originate from foreign DNA acquisition. (B) The Cas proteins are in charge of the crRNAs processing and interference with the cDNA region, promoting DNA degradation or silencing (C).

6–15% of their genes were affected by the deletion of *hfq* (Chao and Vogel 2010). For *S. enterica* serovar Typhimurium, the intracellular pathogen, where Hfq has been studied probably the most using several different techniques, it has been shown that Hfq might directly or indirectly regulate 20% of all *Salmonella* genes. These genes include those required for host cell invasion, motility, central metabolism, lipopolysaccharide biosynthesis, two-component regulatory systems and fatty acid metabolism (Sittka et al. 2008; Ansong et al. 2009). Thus Hfq causes diminished fitness and pleiotropic phenotypes and has been shown to be required for virulence of an increasing number of bacterial pathogens.

For many intracellular bacteria, it was reported that Hfq deficiency dramatically impacts on virulence and fitness. For example Hfq is required for the expression of the VirB Type IV secretion system that is indispensable for full virulence of *Brucella abortus* (Caswell, Gaines and Roop 2012). Hfq is also necessary for the stabilization of two small regulatory RNAs, called AbcR1 and AbcR2 (Table 1). Although single mutants are not defective in intracellular growth in macrophages, double mutants are significantly attenuated. Thus AbcR1 and AbcR2 together are important for survival of *B. abortus* in macrophages and for virulence in a mouse model of infection. Although less is known about their mechanism of action, AbcR1 and AbcR2 directly regulate gene expression by degradation of the mRNA targets, which may involve the recruitment of a ribonuclease (Caswell et al. 2012). These two sRNAs seem to perform redundant and compensatory regulatory functions probably as a consequence of an evolutionary adaptation. This is an example where functional redundancy of RNA molecules may ensure the proper expression of genes required for the pathogenic lifestyle and is reminiscent of the CsrA system of, for example, *L. pneumophila* where the two sRNAs RsmY and RsmZ have a very strong virulence phenotype only when both are missing (Sahr et al. 2009).

Furthermore, the Hfq RNA chaperone of *L. monocytogenes* was shown to contribute to stress tolerance and virulence.

Co-immunoprecipitation with *L. monocytogenes* Hfq followed by sequencing of the Hfq-binding RNA molecules identified initially three novel sRNAs (Christiansen et al. 2006). Interestingly, Hfq is dispensable for sRNA-mediated ribo-regulation in most Gram-positive bacteria. However, in the Gram-positive, intracellular pathogen *L. monocytogenes* there is evidence for Hfq-dependent translational repression. Hfq regulates the expression of the gene *lmo0850* in two ways. First, Hfq stabilizes the sRNA LhrA (Table 1), encoded antisense to its target gene, and consequently down-regulates the *lmo0850* mRNA in an LhrA-dependent manner. Secondly, Hfq facilitates the association of LhrA with its mRNA target, defining a structure amenable for RNA duplex formation. However, the exact biological role of the LhrA sRNAs in *L. monocytogenes* remains to be characterized (Nielsen et al. 2010).

In the last years several approaches have been developed to identify those sRNAs that are interacting with Hfq genome-wide. NGS of RNA enriched by co-immunoprecipitation with chromosomally encoded, epitope-tagged Hfq proteins has been used successfully for several bacteria (Sharma and Voge 2009). The first time it was applied to study sRNAs and mRNAs bound to Hfq of *S. enterica* serovar Typhimurium it allowed differentiation between transcriptional and post-transcriptional effects of Hfq and definition of the exact sequence of these RNA molecules (Sittka et al. 2008). This approach confirmed the previously described Hfq-associated RNAs identified by other techniques but revealed also that a fifth of all *Salmonella* genes are governed by the action of Hfq, including: the mRNAs of *hilD*, the master regulator of *Salmonella* invasion genes; *flhDC*, the flagellar master regulator; and two sigma factor regulons (Sittka et al. 2008). Recently this study was expanded by deep sequencing of Hfq-bound transcripts from multiple stages of growth of *S. enterica* serovar Typhimurium (Chao et al. 2012). This revealed a plethora of new small RNA species from within mRNA loci, and showed that the synthesis of the sRNA DapZ is controlled by *hilD*. Additionally, this study discovered that an mRNA locus might have a double functional output by producing both a

Table 1. Examples for the functional diversity of sRNAs in selected intracellular bacteria.

Species	sRNA	Property	Function	References
<i>Legionella pneumophila</i>	6S, 6S2	RNA polymerase-binding RNA	Required for intracellular replication in macrophages; implicated in stress response and nutrient acquisition	Faucher et al. 2010; Weissenmayer et al. 2011
	RsmY, Z, X	CsrA-binding RNA	Implicated in replication in macrophages; regulation of motility	Sahr et al. 2009; Rasis and Segal 2009
	Cas2-dependent crRNA	CRISPR/Cas system	Implicated in stress response and replication in amoeba	Sahr et al. 2009; Gunderson and Cianciotto 2013
<i>Listeria monocytogenes</i>	Anti0677	RNA excludon	Control of flagellum biosynthesis and motility	Toledo-Arana et al. 2009
	5' UTR- <i>prfA</i>	RNA thermometer	Control of the major virulence regulator PrfA	Johansson et al. 2002
	AspoC	RNA B ₁₂ riboswitch	Regulation of propanediol utilization	Mellin et al. 2013
	Rli55	RNA B ₁₂ riboswitch	Regulation of ethanolamine utilization	Mellin et al. 2014
	LhrA	Hfq-binding RNA	Unknown	Nielsen et al. 2010
	RliB-CrispR	CRISPR/Cas system	Implicated in virulence	Toledo-Arana et al. 2009; Sesto et al. 2014
	Rli27	Trans-acting RNA	Implicated in virulence; regulation of the cell wall-associated protein (Lmo0514)	Quereda et al. 2014
	SreA, SreB	Trans-acting riboswitches	Control of the major virulence regulator PrfA	Loh et al. 2009; Toledo-Arana et al. 2009
	<i>Salmonella enterica</i> serovar Typhimurium	6S	RNA polymerase-binding RNA	Related to intracellular replication
CsrB,C		CsrA-binding RNA	Control of pathogenesis; required for flagella expression	Jonas et al. 2010
IsrM		Trans-acting RNA	Implicated in virulence; required for intracellular replication in macrophages	Gong et al. 2011
RybB-1, RybB-2		Trans-acting RNA	Regulation of oxidative stress response	Calderon et al. 2014
lesR-1		Cis-encoded RNA	Control of the intracellular replication in eukaryotic cell lines; implicated in virulence	Gonzalo-Asensio et al. 2013
5' UTR- <i>agsA</i>		FourU thermometer sensor	Regulation of the small heat shock gene <i>agsA</i>	Waldminghaus, Gaubig and Narberhaus 2007
AmgR		Cis-encoded RNA	Implicated in virulence in mice	Lee and Groisman 2010
<i>Salmonella enterica</i> serovar Typhi	AsdA	Cis-encoded RNA	Regulation of intracellular replication	Dadzie et al. 2013
	RfrA, RfrB	Trans-acting RNA	Regulation of iron homeostasis	Leclerc, Dozois and Daigle 2013
<i>Chlamydia trachomatis</i>	lhtA	Trans-acting RNA	Inhibitor of the histone-like protein Hc1; required for the RB to EB differentiation programme	Tattersall et al. 2012
<i>Neisseria meningitidis</i>	NrrF	Trans-acting RNA	Iron homeostasis regulation	Mellin et al. 2007, Mellin et al. 2010
<i>Francisella novicida</i>	Cas9-dependent crRNA, tracrRNA, scaRNA	CRISPR/Cas system	Regulation of endogenous virulence factors	Sampson et al. 2013
<i>Brucella abortus</i>	AbcR-1, AbcR-2	Trans-acting RNA	Implicated in virulence in mice; important for survival in macrophages	Caswell et al. 2012
<i>Coxiella burnetii</i>	6S	RNA polymerase-binding RNA	Potential implication in regulating intracellular stress response	Warrier et al. 2014
<i>Mycobacterium tuberculosis</i>	Mcr7	Trans-acting RNA	Regulation of the TAT secretion system	Solans et al. 2014

protein and an Hfq-dependent trans-acting RNA. Furthermore, the genome-wide map established for Hfq targets suggested that the 3' regions of mRNA genes constitute a reservoir of regulatory small RNAs (Chao et al. 2012).

Despite the fact that not all bacteria encode an Hfq protein, the high percentage of sRNAs regulated by this RNA-binding protein in many bacteria and several bacterial pathogens led

to the explosion of studies aiming not only at identifying Hfq-dependent sRNAs and their targets but also at characterizing their roles to understand the diverse and often species-specific cellular functions and their implications in pathogenesis. However, not all trans-encoded sRNAs need Hfq. As examples of the versatility of trans-encoded sRNAs, Hfq-independent sRNAs and their role in the iron level and life cycle regulation

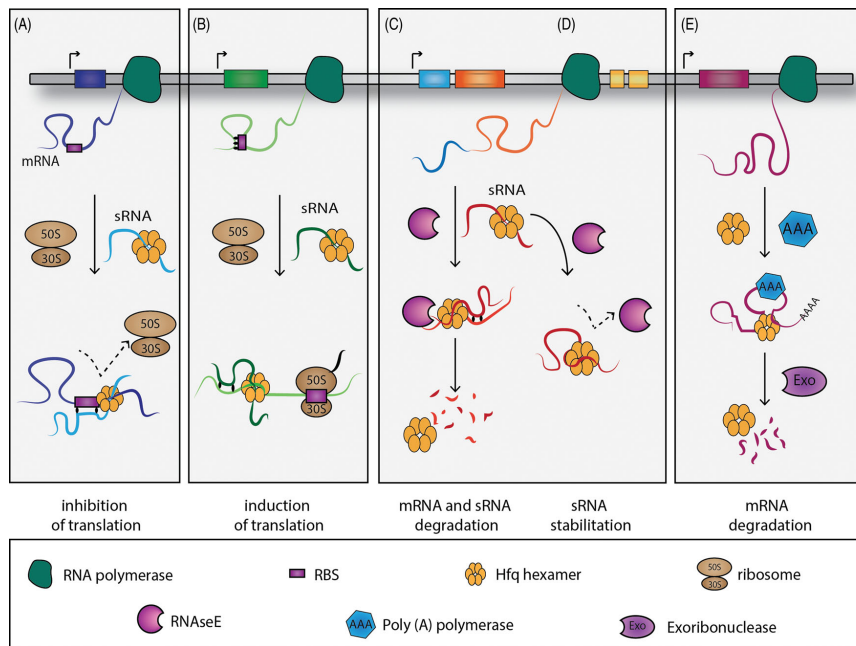


Figure 3. Schematic representation of the mechanisms of action employed by the RNA-binding protein, Hfq. (A) Inhibition at the translational level of the target mRNA by interaction of the sRNA-Hfq complex with the ribosome-binding site (RBS). (B) The complex sRNA-Hfq-mRNA favours the target translation by setting free the RBS, which alternatively would be locked by the formation of a secondary structure. (C) The RNA-binding protein Hfq modulates the mRNA and sRNA turnover rate by recruiting RNase E. (D) Hfq protects the sRNA cleavage site against RNase E attack. (E) Hfq facilitates the mRNA target polyadenylation by the poly(A) polymerase and degradation by exoribonucleases.

in different intracellular pathogens will be discussed in greater detail in the following paragraphs.

TRANS-ENCODED BACTERIAL sRNAs IMPLICATED IN THE INTRACELLULAR LIFE

Trans-encoded sRNAs respond to iron levels in the host cell

The impact of sRNAs on many aspects of bacterial physiology is known. Here we will concentrate on one important aspect, the regulation of iron levels in the cell. Iron participates in many biological functions and it is essential for bacterial growth but it is toxic at higher concentrations. Thus, iron homeostasis is tightly regulated. A bioinformatics screen identified the small RNA NrrF [for neisserial regulatory RNA responsive to iron (Fe)] in *Neisseria meningitidis*, which was demonstrated to be both responsive to iron in the environment as encountered in host tissues of fluids and Fur regulated. NrrF has a well-conserved orthologue in *Neisseria gonorrhoeae* (Mellin et al. 2007) (Table 1). In order to monitor the level of iron within the cells, bacteria engage a tight iron-responsive regulation, mediated by the ferric uptake repressor Fur, an iron-dependent transcriptional repressor. In *N. meningitidis* Fur can indirectly activate gene expression by repressing the regulatory sRNA NrrF. NrrF is able to bind to limited complementarity regions of the *sdh* transcripts and down-regulate the expression of the *sdhA* and *sdhC* genes, encoding

subunits of the succinate dehydrogenase complex. Thus, the sRNA NrrF regulates Fur-dependent, iron-activated gene transcription in *N. meningitidis*. Interestingly, the Fe-regulated sRNA RyhB, first characterized in *E. coli* (Masse and Gottesman 2002; Masse, Escorcia and Gottesman 2003; Masse, Vanderpool and Gottesman 2005), needs the binding of Hfq to exploit its function. In contrast, the NrrF-mediated iron regulation of the *sdhA* and *sdhC* genes in *Neisseria* does not require the chaperone Hfq (Mellin et al. 2010).

Two RyhB homologues, named RfrA and RfrB, predicted in *Salmonella enterica* serovar Typhi are important for optimal intracellular replication in macrophages (Leclerc, Dozois and Daigle 2013) (Table 1). Furthermore, Fur, a repressor of both of these sRNAs, was shown to play a role in phagocytosis and intracellular survival of *S. Typhi* in human macrophages. Interestingly, loss of either RfrA or RfrB resulted in distinct phenotypes, with respect to siderophore production, salmochelin production, or *fur* expression in low iron conditions, suggesting a non-redundant role for these regulatory RNAs (Leclerc, Dozois and Daigle 2013). Similarly, deletion of either *rfrA* or *rfrB* did not affect bacterial uptake, survival or replication; however, deletion of both sRNAs resulted in a significantly reduced intracellular replication ability, suggesting a complementary role for these two sRNAs for intracellular replication (Leclerc, Dozois and Daigle 2013). Indeed, RfrA and RfrB were induced inside THP-1 macrophages (Leclerc, Dozois and Daigle 2013), inside murine macrophages (Padalon-Brauch et al. 2008) and inside

fibroblasts (Ortega, Gonzalo-Asensio and Garcia-del Portillo 2012). In addition to their central role in iron homeostasis regulation, these two sRNA molecules and their homologues have additional functions such as protecting against oxidative stress, bactericidal antibiotics and acid resistance (Kim and Kwon 2013). Indeed, it was shown that the homologous sRNAs in *S. enterica*, named RyhB-1 and RyhB-2, are controlled by OxyR and are important for the oxidative stress response of this pathogen (Calderon et al. 2014) (Table 1). This suggests that multiple bacterial pathogens that share these conserved sRNAs may employ a similar mechanism to regulate iron and/or oxidative stress within the host cells.

Trans-encoded sRNAs regulate intracellular differentiation of *Chlamydia trachomatis*

Another example of regulation of important functions of intracellular life by trans-encoded sRNAs is found in the obligate intracellular bacterium *Chlamydia trachomatis*. This pathogen employs the sRNA IhtA (inhibitor of *hctA* translation) to regulate its differentiation processes (Grieshaber et al. 2006) (Table 1). IhtA is a trans-encoded RNA, which is implicated in controlling the differentiation from the replicative reticulate bodies (RBs) to the infectious elementary bodies (EBs). IhtA is highly expressed in the replicative phase where it represses the translation of the *hctA* gene encoding the histone-like protein Hc1. Hc1 binds to and compacts the bacterial chromosome, making the EBs transcriptionally and translationally inactive. In contrast, as soon as IhtA transcription decreases during the late infectious phase, the *hctA* transcript is highly expressed, allowing the pathogen to transit and differentiate to EBs, where it endures in a metabolically inert stage (Grieshaber et al. 2006; Tattersall et al. 2012; Ortega et al. 2014). Thus, the sRNA IhtA is part of a global regulatory circuit that controls differentiation of RBs to EBs during the chlamydial life cycle. Recently it was shown that regulation of HctA by IhtA is a conserved mechanism found across pathogenic chlamydial species (Tattersall et al. 2012).

Trans-encoded sRNAs are implicated in pathogen entry into and replication within host cells

The advances in RNA sequencing and the possibility to undertake these analyses in infection-relevant conditions led to the discovery of several sRNAs implicated in virulence in cellular and animal infection models. An excellent example is the Rli27 sRNA that was identified by whole-genome tiling array analyses as a sRNA induced in *L. monocytogenes* in vivo (Toledo-Arana et al. 2009) (Table 1). Further functional analysis showed that Rli27 mediates the regulation of the cell wall-associated protein, Lmo0514 during the intracellular infection cycle (Quereda et al. 2014). Interestingly, in response to environmental cues sensed in the eukaryotic intracellular niche, distinct promoters located upstream of the *lmo0514* gene generate two alternative *lmo0514* transcript isoforms containing 5' UTRs of different length. These two transcript isoforms, which display two distinct target sites within the 5' UTR for sRNAs, are differentially expressed in intracellular and extracellular bacteria. During the intracellular infection cycle, the long 5' UTR *lmo0514* transcript isoform, bearing the Rli27-binding site, is targeted by the Rli27 sRNA, leading to the release of the occluded SD sequence and allows therewith Lmo0514 protein production. Thus, *L. monocytogenes* promotes Rli27-mediated post-transcriptional regulation of its specific target, a cell wall-associated protein Lmo0154, only in response to its entry into eukaryotic cells (Quereda et al. 2014).

Another sRNA-dependent temporal and tissue-specific regulation of virulence has been reported in the *S. enterica* serovar Typhimurium pathogenicity islands (SPIs) (Gong et al. 2011). This sRNA, called IsrM, is expressed *in vitro* under conditions comparable with those during infection in the gastrointestinal tract (Table 1). Moreover, *in vivo* assays revealed a higher expression of IsrM in the ileum, suggesting an involvement of this sRNA in *Salmonella* pathogenicity. Indeed, IsrM targets the SPI-effector SopA and the global regulator of the SPI-1 genes, HliE. HliE is the major regulator of *Salmonella* virulence crucial for bacterial evasion. Specifically IsrM targets the 5' UTRs and masks the nearby SD sequence of both *hliE* and *sopA* mRNAs, hindering the production of the two proteins. Thus, *Salmonella* probably uses the fine-tuned expression of IsrM as a mechanism to regulate specifically SPI-1 protein expression *in vivo* temporally and spatially.

REGULATORY 5' UTR ELEMENTS

Two diverse classes of 5' UTRs of mRNAs have gained recent attention: RNA thermometers and RNA riboswitches. To some extent, these two types of regulatory molecules share common features but, most importantly, both are complex RNA molecules that sense a particular chemical or physical signal and accordingly alter their conformation to control the expression of downstream genes. 5' UTR elements are another striking example of a refined RNA-dependent mechanism, allowing bacteria to respond rapidly and cost-effectively to physical alterations. Riboswitches are regulating the use of different nutrient sources that seem to be important for growth of bacterial pathogens inside host cells. Thermosensors may allow a pathogen to recognize the host environment by sensing the temperature shift encountered when extracellular bacteria invade host cells to replicate intracellularly. An example is *L. monocytogenes* (discussed below) that regulates virulence gene expression indispensable for intracellular growth in response to temperature by a thermosensor.

RNA thermometers

RNA thermometers control gene expression by temperature-induced conformational changes. All cellular processes are temperature dependent, but virulence genes, cold shock genes and heat shock genes are particularly disposed to thermoregulation. Specifically, the response to an increase in temperature is often used by pathogenic bacteria when they enter their mammalian host. The temperature of 37°C causes induction of virulence genes whose products are only needed in the host environment. The advantage of regulation via RNA thermometers lies in the fact that they respond to temperature changes in a more immediate manner because they control the translation of already existing or nascent mRNAs (Kortmann and Narberhaus 2012). Despite the fact that cis-acting regulatory 5' UTR elements modulate gene expression either at the transcriptional or translational level (Mandal and Breaker 2004), all RNA thermometers described so far act at the level of translational initiation. In most of the cases, the SD region and/or the initiation codon are involved in base-pairing interactions forming a secondary structure at low temperature. An increase in temperature facilitates the mRNA-ribosome interaction after the full liberation of the SD sequence and the AUG start codon, leading to the formation of the translation initiation complex (Narberhaus, Waldminghaus and Chowdhury 2006). The first RNA thermometer described controls the expression of the *cIII* protein and also governs the development of phage λ . Strikingly, it

represents the only thermometer which turns on the translation of the *cIII* protein when the temperature is decreased and, furthermore, it does not operate by gradual melting but switches between two exclusive conformations (Altuvia *et al.* 1989). However, RNA thermometers have little or no sequence conservation and are thus difficult to predict from genome sequences. Therefore, the bioinformatics prediction has remained a major challenge (Waldminghaus, Gaubig and Narberhaus 2007). The mechanisms of function of the different RNA thermometers known to date have recently been reviewed in great detail (Kortmann and Narberhaus 2012), and thus here we will give a brief overview of those that are implicated in regulatory processes in intracellular bacteria.

A specific family of RNA thermometers is characterized by the presence of a short sequence motif composed of four uridines, also known as fourU. The fourU element is located at the 5' UTRs of several virulence and heat shock genes and pairs particularly with AGGA in the SD sequence. FourU elements have been identified and characterized in several pathogenic bacteria such as the *lcrF* thermometer in *Yersinia pestis*, the *agsA* fourU thermometer sensor of *Salmonella Typhimurium* and the *prfA* sensor of the intracellular pathogen *L. monocytogenes* (Waldminghaus *et al.* 2007; Johansson *et al.* 2002; Bohme *et al.* 2012). *PrfA* of *L. monocytogenes*, the master regulator of virulence gene expression, is expressed at 37°C, the temperature of mammalian host cells, but not at lower temperatures that *L. monocytogenes* encounters mainly when growing extracellularly in the environment. Indeed the 5' UTR of the *prfA* mRNA adopts a stable stem-loop structure at low temperature, thereby blocking the SD sequence and preventing binding of the ribosome (Table 1). At 37°C, the temperature encountered when infecting humans, the stem-loop melts into an alternative secondary structure, allowing the ribosome to access the SD sequence, leading to the translation of the *prfA* mRNA and to the subsequent induction of a number of virulence genes (Johansson *et al.* 2002; Johansson and Cossart 2003). Another fourU RNA thermometer was identified in the 5' UTR of the small heat shock *agsA* gene in *S. enterica* serovar Typhimurium. The 5' UTR of the *agsA* gene, which codes for a small heat shock protein, consists of only two stem-loop structures, one of which contains the fourU motifs with imperfect complementarity to the SD sequence. As all RNA thermometers, the stability of this structure was calculated to decrease with increasing temperatures, thereby leading to a high level of *agsA* expression and subsequent availability of the small heat shock protein (Waldminghaus, Gaubig and Narberhaus 2007).

RNA riboswitches regulating metabolic function during intracellular growth

Riboswitches are widespread *cis*-acting RNA elements in mRNAs that sense metabolites and modulate transcription or translation of mRNAs in response to these metabolites by forming alternative metabolite-free and metabolite-bound conformations (Serganov and Nudler 2013; Peselis and Serganov 2014). In prokaryotes, most of the riboswitches are at the 5' UTR of the main coding region of a particular mRNA and typically consist of two distinct functional domains. The evolutionarily conserved aptamer element serves as the ligand-binding domain (sensor) whereas a variable sequence, termed the expression platform, is responsible for exerting an influence on the downstream coding sequences, regulating their expression (Serganov and Nudler 2013). Riboswitches can control a broad range of genes including those involved in the biosynthesis and transport of prokaryotic

metabolites such as amino acids, nucleotides, metal ions and cofactors (Henkin 2008). Therefore, to some extent the ligand represents not only the stimulus but also the final product of the biosynthetic pathway regulated by the downstream genes of the riboswitch, leading to a feedback control.

Riboswitches have been extensively studied in the last decade and many classical ones have been identified in prokaryotes. However, recently, the first riboswitch controlling the transcription of non-coding RNAs was described in the intracellular pathogen *L. monocytogenes* (Mellin *et al.* 2013; Mellin *et al.* 2014). In these studies two vitamin B₁₂-binding (B₁₂) riboswitch-dependent mechanisms were identified that control the catabolism of propanediol and ethanolamine molecules (Mellin *et al.* 2013; Mellin *et al.* 2014). They showed that the B₁₂ riboswitch was transcribed as part of a non-coding antisense RNA, named *AspC*, and surprisingly was located downstream and in the antisense orientation of the adjacent gene, *pocR* (Table 1). *PocR* is a transcriptional factor that is activated by propanediol, a molecule produced in the intestine by commensal bacteria as a byproduct of the fermentation of rhamnose and fucose. Propanediol catabolism requires a B₁₂-dependent diol dehydratase encoded by the *pduCDE* genes whose expression is induced by the transcriptional regulator *PocR* (Bobik *et al.* 1997). In the absence of B₁₂, the riboswitch induces the formation of an *aspocR* long transcript, which in turn inhibits *trans pocR* expression. Furthermore it was suggested that this regulation relies on binding of *AspocR* to *pocR* mRNA. As such, the *pdu* genes are not expressed when vitamin B₁₂ is not available. In contrast, low B₁₂ conditions partially repress *pocR* expression and appear sufficient to induce the expression of the B₁₂ biosynthesis genes; however, to induce the *pdu* gene expression maximally, accumulation of vitamin B₁₂ is required. In response to sufficient accumulation of B₁₂ and upon the ligand-aptamer interaction, the riboswitch leads to the production of a short *aspocR* truncated transcript that does not prevent the expression of the *pocR* mRNA, whose product enables propanediol catabolism. Thus, the B₁₂ riboswitch does not operate in a black-or-white switch but acts more as a fine-tuning regulatory device. Markedly, regulation by the B₁₂ riboswitch-dependent *AspocR* allows the propanediol catabolism pathway to be highly activated only when both propanediol and B₁₂ are available (Mellin *et al.* 2013).

Furthermore, in *L. monocytogenes*, vitamin B₁₂ is also required for the utilization of ethanolamine (Mellin *et al.* 2014). Ethanolamine, like the closely related propanediol molecule, is an important nutrient source for pathogens during infection (Buchrieser *et al.* 2003). Recent studies suggested that propanediol and ethanolamine both play a role in *L. monocytogenes* pathogenesis as *L. monocytogenes* up-regulates the *pdu* and ethanolamine utilization (*eut*) genes during intracellular growth in intestinal epithelial cells (Joseph *et al.* 2006), and in the intestine of germ-free mice pre-treated with lactobacilli (Archambaud *et al.* 2012). Related to these findings, an in-depth whole-genome transcriptome analysis revealed the presence of a B₁₂ riboswitch upstream of the first gene of the *eut* locus, coding for the ethanolamine utilization pathway (Toledo-Arana *et al.* 2009). Indeed, according to the availability of the B₁₂ cofactor, the riboswitch modulates *eut* gene expression through the production of a long or a short truncated sRNA transcript, called *Rli55* (Table 1). Furthermore, four ANTAR (AmiR and NasR transcriptional antiterminator regulator) elements were found upstream of the *eutA* and *eutV* genes, and two in the *Rli55* locus, which are transcribed as part of the 3' end of the *Rli55* transcript. Very elegant experiments showed that in the presence of ethanolamine

but absence of the B₁₂ cofactor, a long Rli55 together with the first ANTAR element is transcribed to sequester the two-component response regulator EutV, which consecutively leads to the down-regulation of the *eut* genes. Conversely, the accumulation of B₁₂ and the successive interaction with the riboswitch triggers the production of a truncated Rli55 transcript lacking the first ANTAR element, which prevents binding to the EutV regulator. As such, the EutV protein co-ordinates the activation of the *eut* genes, leading to the utilization of ethanolamine (Mellin, Koutero, Dar, 2014). Thus, these studies highlighted a new, non-classical role for regulatory sRNAs, which is sequestering of a two-component system element dependent on a B₁₂ riboswitch.

Recently *trans*-regulatory riboswitch elements that can function as sRNAs and link virulence and nutrient availability have been discovered in *L. monocytogenes* (Loh et al. 2009). *Listeria monocytogenes* harbours seven putative S-adenosylmethionine (SAM) riboswitches that may, when SAM binds, form a termination structure that terminates transcription and inhibits synthesis of the downstream mRNA (Toledo-Arana et al. 2009). In *L. monocytogenes*, two riboswitches, named SreA and SreB, have been shown to function *in trans* and to act as sRNAs controlling expression of PrfA, the major regulator of virulence gene expression (Table 1). Elegant *in vitro* and *in vivo* experiments suggest a non-coding RNA function for SreA and SreB. They bind to the 5' UTR of *prfA*, and thereby reduce *prfA* transcript stability and/or *prfA* mRNA translation. Thus, both SreA and SreB act together to control expression of *prfA* and thus the virulence of this intracellular pathogen. The identification of many diverse riboswitches, facilitated by the rapid expansion of computational data analysis together with experimental analyses will reveal new mechanisms employed by bacteria to regulate their transcriptional response when growing inside host cells.

CIS-ENCODED SMALL RNAs

In contrast to the *trans*-encoded sRNAs, the *cis*-encoded sRNAs are located in the antisense strand of their target RNA regions and their mechanism of action leads to the post-transcriptional down-regulation of the target gene(s) through a high degree of sequence complementarity. In general, this class of sRNAs does not require the help of the RNA-binding protein Hfq (Caldelari et al. 2013). However, some *cis*-encoded sRNAs may also stabilize their mRNA target, such as, for example, the AsdA sRNA discussed below. Although the first described *cis*-antisense RNAs were mobile elements such as phages, transposons and plasmids (Wagner and Simons 1994), few chromosomally encoded *cis*-antisense transcripts had been reported by 2007 (Brantl 2007). With the use of tiling arrays and RNAseq to analyse the transcriptional landscape of a bacterial genome widely and at a high level of depth, antisense transcription that was thought to be simply an irregularity turned into a rule. Antisense sRNAs (asRNAs) are present in Gram-positive and Gram-negative bacterial species, but a high variability in their prevalence and genomic density was reported (Georg and Hess 2011; Raghavan, Sloan and Ochman 2012; Lybecker et al. 2014). Thus new attention to asRNAs within the bacterial kingdom emerged. AsRNAs can be classified according to their position as internally located, 5'-overlapping or 3'-overlapping asRNAs, or according to their size as short asRNAs (ranging from 100 to 300 nucleotides) or as long asRNAs reaching several kilobases in size (Georg and Hess 2011). However, the lack of functional characterization of many of the identified asRNAs hinders their

annotation according to their role in cells. To date, asRNAs have been reported to act at the level of transcription, mRNA stability or translation. Their diverse regulatory mechanisms, asRNA regulation has been reviewed recently in several articles (Thomason and Storz 2010; Georg and Hess 2011). We will thus concentrate here on a particular asRNA mechanism newly described in *Listeria*, the *excludon*, and asRNAs important for *Salmonella* replication.

A new concept in antisense RNA-mediated regulation: the *excludon*

Recently a sophisticated asRNA-dependent mechanism that achieves a regulatory connection between neighbouring genes that often have opposite functions was reported in *L. monocytogenes*. The name '*excludon*' has been coined to describe this new regulatory mechanism. A single transcript serves in parallel as an antisense repressor of one gene or group of genes and as an mRNA promoting the expression of the adjacent genes. This results in the regulation of adjacent genes of opposite functions (Sesto et al. 2013).

The '*excludon*' in *L. monocytogenes* takes part in the control of flagellum biosynthesis. The asRNA, Anti0677 has a proximal region that is antisense to the *lmo0675-lmo0676-lmo0677* locus and a distal region that is part of the gene encoding the regulator MogR (Toledo-Arana et al. 2009) (Table 1). The *lmo0676-lmo0677* locus encodes FlIP and FlIQ that together with FlIR (encoded by the adjacent gene *lmo0678*) constitute the flagellum export apparatus. The MogR regulator binds the *fli* operon promoter and down-regulates the flagellum and thus motility in *L. monocytogenes*. When Anti0677 is expressed, it inhibits the synthesis of *lmo0675* (its function is not known yet) and the entire flagellum export apparatus and leads simultaneously to the expression of MogR. Thus the flagellum apparatus is concurrently turned off by the increasing expression of MogR and by the repression of the *lmo0676-lmo0677* locus driven by the asRNA Anti0677. Furthermore, the inhibition of the flagellum export apparatus is likely to involve the formation of a double-stranded RNA when Anti0677 is transcribed and the subsequent cleavage by RNase III (Wurtzel et al. 2012). The above-described asRNA Anti0677 was the first example for the *excludon* concept; however, at least 13 additional, putatively *excludon*-like regulated genomic loci were detected through comparative transcriptomic analysis of *L. monocytogenes*, suggesting that *excludon*-mediated regulation might represent a frequent mechanism. Furthermore, similar organizations have been found in other bacteria such as *L. pneumophila* (unpublished data), thus this mechanism of antisense regulation is probably not restricted to the genus *Listeria* but extends to other bacteria.

Cis-encoded sRNAs: regulation of replication

Although transcription of asRNAs occurs genome-wide in many bacteria, only a small fraction of asRNAs is shared across species. Moreover, the promoters associated with asRNAs show no evidence of sequence conservation between, or even within, species. Thus researchers are questioning whether these asRNAs are all functional or not (Raghavan, Sloan and Ochman 2012). Further functional analyses and in-depth characterization of these asRNAs will be necessary to answer this open question. For example, one of the *cis*-encoded RNAs, termed AsdA, was characterized in *S. enterica* serovar Typhi where it was shown to be involved in regulating the replication process (Table 1). The AsdA sRNA is located in the complementary strand to the *dnaA*

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gene that encodes a site-specific DNA-binding protein (Dadzie et al. 2013). DnaA serves as a bacterial replication initiation factor and is capable of recognizing the origin of replication (*oriC*), mediating the open complex formation, and therefore it induces the assembly of the DNA replication machinery. Moreover DnaA acts as a transcriptional factor, promoting the transcription of target genes (Messer and Weigel 2003). Non-coding RNA elements are often transcribed in response to specific conditions. Indeed, the *AsdA* sRNA is highly expressed during the stationary growth phase after growth in rich medium but it is already expressed in very early exponential growth when iron limitation or osmotic stresses are exerted. In these conditions, the expression of the *AsdA* antisense transcript enhances the stability of the *dnaA* mRNA, which in turn increases its translational rate (Dadzie et al. 2013). These observations indicated that the *AsdA* sRNA, through stabilization of the *dnaA* mRNA, is a crucial regulator of DNA replication in *S. enterica* serovar Typhi.

Cis-encoded sRNAs: regulation of virulence gene expression

Interestingly, a long asRNA of 1.2 kb, named *AmgR*, was identified in the antisense strand of the *mgtC* operon and complementary to the 5' terminus of this polycistronic mRNA in *S. enterica* serovar Typhimurium (Table 1). *AmgR* is transcribed from a promoter that is located in the intergenic region between the *mgtC* and *mgtB* genes. The aforementioned operon encodes the MgtC protein implicated in Mg^{2+} homeostasis and virulence, the Mg^{2+} transporter MgtB and the 30 amino acid peptide MgtR that mediates degradation of MgtC by the FtsH protease. Intriguingly, the transcription of the polycistronic *mgtC* mRNA and the *AmgR* sRNA is under the positive control of the two-component regulatory system PhoP/PhoQ. In detail, when PhoQ senses low extracytoplasmic Mg^{2+} , it phosphorylates PhoP, which then binds to the *mgtC* and *amgR* promoters, mediating the transcription of the *mgtC* mRNA and the long sRNA. As such, the long RNA regulatory element limits the MgtC and MgtB protein levels, promoting the MgtR binding to the MgtC protein and the subsequent degradation by the FtsH protease. Markedly, *AmgR* serves as a timing device to attenuate virulence in mice mediated by the sense-encoded MgtC protein (Lee and Groisman 2010). The finding that the *AmgR* asRNA is located inside a polycistronic region revealed that the repertoire of regulatory sRNA elements might be larger than thought and raises the possibility that additional asRNAs exist which have been overlooked.

Another example of an asRNA, named *lesR-1*, encoded within the PSLT virulence plasmid has been reported recently in the bacterial pathogen *S. enterica* serovar Typhimurium (Gonzalo-Asensio et al. 2013) (Table 1). RNA expression has been studied using oligonucleotide microarrays containing probes in each of the two strands of every intergenic region testing a variety of culture conditions. Remarkably, *lesR-1* was preferentially expressed in non-growing dormant bacteria during colonization of fibroblasts. The deletion of this asRNA not only influenced the control of bacterial growth within the fibroblast cell lines but also impaired virulence in a mouse infection model. As an asRNA that is overlapping the PSLT047 transcript, the *lesR-1* regulatory mechanism involves the direct interaction of the 3' end of the RNA molecule leading to the subsequent remodelling of the PSLT047 protein levels. Given the virulence phenotype, this suggests that PSLT047 is important for the intracellular lifestyle of *S. enterica* serovar Typhimurium (Gonzalo-Asensio et al. 2013).

DYNAMIC EXPRESSION CHANGES OF sRNAs DURING INTRA- AND EXTRACELLULAR GROWTH

The use of whole-genome tiling arrays and RNA deep sequencing has revealed that sRNAs, similar to protein-coding genes, show dynamically changing expression profiles during growth in laboratory media but also during infection. An example is *L. pneumophila* as this intracellular pathogen is known to have a pronounced biphasic life cycle. Schematically, pathogenic *L. pneumophila* encode one set of genes dedicated to transmission that includes all major virulence traits and another set that promotes replication in phagocyte vacuoles, a characteristic that is reflected in a major shift of gene expression during the switch from replicative to transmissible bacteria (Molofsky and Swanson 2004; Bruggemann et al. 2006). Recently, RNAseq analyses showed that ~700 sRNAs are expressed in the *L. pneumophila* genome. Similar to the protein-coding genes, >60% of the sRNAs are growth phase dependently regulated (Sahr et al. 2012). Most interestingly, transcriptional start site mapping identified in addition a high number of sRNAs with tandem promoters, 30% of which are used growth phase dependently (Sahr et al. 2012). These strong changes in sRNA expression profiles indicate an important role for sRNAs in regulating the biphasic life cycle of *L. pneumophila* and thus virulence.

Similarly, transcriptome data using a 70mer oligonucleotide array of the *S. enterica* Typhimurium genome revealed that ~2% (98 genes) of all genes are differentially expressed in non-growing intracellular bacteria, when the fibroblast infection model was used (Nunez-Hernandez et al. 2013). Later work also identified sRNAs that are differentially expressed. As an example, *Sral* shows higher expression levels in non-growing bacteria than in actively growing ones (Ortega, Gonzalo-Asensio and Garcia-del Portillo 2012).

A comprehensive picture of growth phase- and condition-dependent expression of sRNAs was reported for *L. monocytogenes*. The analyses of *L. monocytogenes* expression profiles using tiling arrays and bacteria grown in exponential phase, stationary phase, hypoxia and low temperature or isolated from the intestine of axenic mice or bacteria grown in the blood of human donors provided important information about their expression conditions and thus hints of their putative functions (Toledo-Arana et al. 2009). Similarly, RNA sequencing of *L. monocytogenes* grown in macrophages showed that 29 of the 150 described sRNAs are specifically induced during intracellular growth, pointing to functional roles during infection. Indeed, knockout mutants of three of these RNAs, named *rli31*, *rli33-1* and *rli50*, were defective in intracellular growth (Mraheil et al. 2011). Recently the non-coding genome was characterized in multiple growth conditions that are relevant for the infection process using RNA deep sequencing. This led not only to the characterization of condition-dependent expression of sRNAs but also to the identification of new regulatory mechanisms such as the *excludon* described above (Wurtzel et al. 2012).

Using a combined experimental and *in silico* approach, 34 sRNAs have been identified in *Mycobacterium bovis* BCG, many of which are responsive to changing environmental growth conditions. Homologues of several of these sRNAs are also present in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* (DiChiara et al. 2010). Recently one of these, the sRNA homologous to *Mcr7*, has been functionally characterized in *M. tuberculosis*. To analyse the regulon of PhoPR, a two-component system essential for virulence of *M. tuberculosis*, a ChIPseq analyses using PhoP antibodies was undertaken. This allowed identification

of all directly PhoP-regulated genes, but also a PhoP-regulated sRNA named Mcr7. In elegant experiments, it was shown that Mcr7 modulates the TAT secretion system of *M. tuberculosis* and impacts its activity. This leads to a diminished secretion of the immunodominant AG85 complex, implicated in successful infection of the host (Solans et al. 2014). Taken together, sRNAs show dynamic changes of expression according to the conditions in which they are functioning and many are crucial contributors to the virulence process of intracellular bacteria.

PROTEINS REGULATING SMALL RNAs

RNA-binding proteins are major players in the regulation of gene expression. Certain of these proteins involve an association with sRNAs. One of the best known is the 6S RNA that interacts with σ^{70} -containing RNA polymerase and regulates transcription at specific promoters and that recently has also been shown to be involved in virulence regulation of intracellular bacteria. Another well-known system present in many bacteria is the CsrA/RsmA protein, whose activity is regulated by sRNAs.

The RNA polymerase-interacting 6S RNA

First identified in *E. coli* in the late 1960s, the 6S RNA is a double-stranded sRNA transcribed by the *ssrS* gene and ubiquitously expressed, with a remarkable accumulation during the late stationary phase of the bacterial growth cycle (Hindley 1967; Wassarman and Storz 2000). The conserved secondary structure consisting of two irregular stem structures and a single-stranded central bulge has led to the prediction of the presence of this sRNA in >100 bacterial species so far (Barrick et al. 2005). Although the characterization and the identification of physiological functions of 6S RNA remained elusive for three decades as no appreciable growth phenotype in cells depleted or overexpressing 6S RNA was observed, the conserved secondary structure suggested an important role for this sRNA. The first time, a detectable phenotype was observed when *E. coli* was grown under stringent stress and limiting nutrient conditions, suggesting that the bacteria, under these conditions, engage 6S RNA to allocate the nutrients (Trotchaud and Wassarman 2004). Strikingly, the 6S RNA is the first sRNA that was shown to alter gene expression by binding directly to the active site of the house-keeping holoenzyme form of the RNA polymerase, the σ^{70} -RNA polymerase, to hinder the access to the promoter DNA. In *E. coli* it was shown that the 6S RNA contributes to the regulation of a large number of genes, by inhibiting their transcription during the late stationary phase (Wassarman and Storz 2000; Willkomm and Hartmann 2005). The specificity of the binding strictly resides in the conserved secondary structure and in the internal stem bulge region of the 6S RNA (Trotchaud and Wassarman 2004). Secondly, further investigations on the nature of the secondary structure of the 6S RNA revealed a remarkable resemblance to the conformation of DNA, suggesting a shared mechanism of binding. Additional structural requirements, related to the 6S-dependent inhibition, involve the presence of a promoter with an extended -10 element and/or a weak -35 element (Cavanagh, Sperger and Wassarman 2012). Furthermore, Wassarman and Saecker showed that 6S RNA can be used as a template for RNA synthesis during the stationary phase, generating a 14–20 nucleotide long RNA product, named pRNA (Wassarman and Saecker 2006). The synthesis of the pRNA seems to be required to induce the release of the 6S–RNA polymerase complex, which consequently would lead to the formation of the 6S RNA–pRNA duplex. Thus, during outgrowth, the inhibitory machin-

ery is switched off when necessary and the 6S RNA is destabilized and alternatively degraded post-pRNA synthesis (Wassarman and Saecker 2006). Although a down-regulation of the pRNA depending on the NTP concentration is suggested, many questions concerning the potential cellular function of this pRNA still need to be answered.

The function of the 6S RNA has been studied in the intracellular pathogens *L. pneumophila*, *S. typhimurium* and *Coxiella burnetii* (Ortega, Gonzalo-Asensio and Garcia-del Portillo 2012; Ortega et al. 2014; Warrier et al. 2014). In *L. pneumophila* the exact role of the 6S RNA (*ssrS*) is not known but it has been shown that it is important for intracellular multiplication in human macrophages and in *Acanthamoeba castellanii* but not for growth in laboratory media (Table 1). In contrast to the above-discussed function of the 6S RNA as a repressor of the transcription of genes during stationary phase of growth through the binding to the σ^{70} -containing RNA polymerase, the 6S RNA of *L. pneumophila* serves mainly as a positive regulator. Several genes including those involved in stress adaptation, amino acid metabolism and genes encoding Dot/Icm effectors, important for *Legionella*'s ability to survive in the host, were identified (Faucher et al. 2010). This different regulatory mechanism of the *L. pneumophila* 6S RNA could be due to an only weak binding to the RNA polymerase, adding another regulatory level to the current knowledge of the regulatory mechanism by which 6S RNAs function (Faucher et al. 2010). Interestingly, a second 6S RNA copy, named 6S2 RNA, was reported to be present in *L. pneumophila* strain Philadelphia (Weissenmayer et al. 2011). According to their results these two 6S RNAs are expressed at different stages during the biphasic life cycle of *L. pneumophila* (Weissenmayer et al. 2011). However, this second copy does not seem to be present in *L. pneumophila* strain Paris (Cazalet et al. 2004), indicating strain-specific differences (Cazalet et al. 2004; Sahr, Rusniok, Dervins-Ravault et al. 2012). Similarly, the 6S RNA homologues of *S. enterica* serovar Typhimurium and *Coxiella burnetii* were described as important for intracellular growth especially during stress conditions (Ortega, Gonzalo-Asensio and Garcia-del Portillo 2012; Ortega et al. 2014; Warrier et al. 2014). In *C. burnetii* the 6S RNA also interacts with the σ^{70} -containing RNA polymerase, but it can serve as both a positive and a negative regulator during stationary phase growth (Table 1).

In conclusion, the different homologues of 6S RNA have multiple regulatory functions in intracellular pathogens with respect to both the genes they regulate and the mode of action, as they may induce or repress gene expression. Further investigations are necessary to determine when it acts as a negative or positive regulator and how this different mechanism is achieved.

The Csr/Rsm network and its interacting sRNAs

One of the best described RNA-binding proteins is the CsrA/RsmA (Carbon storage regulator/Regulator of secondary metabolism) ribonucleoprotein complex which controls different metabolic pathways and the repression of a variety of stationary-phase genes, including virulence-linked traits in pathogenic bacteria. CsrA was identified first in *E. coli* and defined as a carbon storage regulator system as the mutant strain of the *csrA* gene exhibited increased levels of glycogen compared with the wild-type strain (Romeo et al. 1993). CsrA is a global bacterial regulator, as many homologous highly conserved proteins have now been identified in numerous bacteria but not in eukaryotes or Archaea. CsrA is a homodimer, in which each subunit is composed of five β -strands, one α -helix and an unstructured

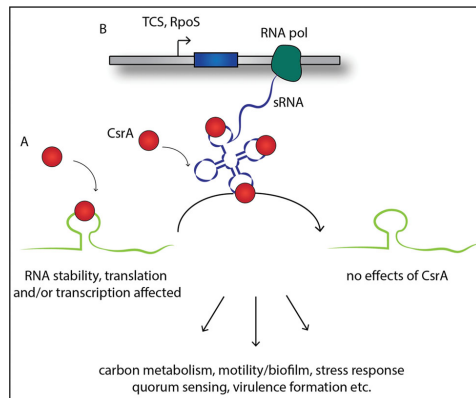


Figure 4. Simplified schematics of the general function of the Csr system. (A) The dimeric RNA-binding protein CsrA interacts with one or more A(N)GGA motifs of the target RNA, typically represented in a hairpin loop structure, leading to an alteration of the accessibility of the translation machinery, transcription (anti-)termination and/or the stability of the RNA. (B) Production of regulatory sRNA under the control of a two-component system and the alternative sigma-factor RpoS antagonizes CsrA function by binding CsrA with high affinity and hence abolishing the interaction with their target RNAs. This results in a global metabolic shift within the pathogen and amongst others to the activation of virulence-related proteins.

C-terminal part (Gutierrez et al. 2005; Rife et al. 2005). The major RNA-binding site is predicted to be the GxxG motif between strands $\beta 3$ and $\beta 4$, but other structures such as the $\beta 4$ strand, the region between $\beta 2$ and $\beta 3$ and several conserved surface-exposed residues are also most probably involved in the interaction. This RNA-binding site allows CsrA to recognize specific A(N)GGA motifs in the RNA sequence, apparently with much higher affinity if these motifs are represented in the loop of a hairpin structure (Duss et al. 2014). Typically, the SD sequence in the RNA leader region is rich in GA motifs; therefore, it is assumed that CsrA is predominantly regulating translation initiation by inhibiting the ribosome binding and furthermore favouring the degradation of the transcript (Seyl and Van Melderen 2013) (Fig. 4). However, there is also evidence that CsrA interactions influence the fate of the RNA not only by blocking translation but also in various other ways, such as stabilization of the RNA by protecting it from RNase degradation, affecting transcription elongation/termination or also as a positive regulator of translation by forming alternative RNA structures which even support ribosome binding (Liu, Yang and Romeo 1995; Figueroa-Bossi et al. 2014). Interestingly, the regulation of the intracellular level of CsrA itself is also controlled predominantly at the post-transcriptional level, in particular by two or more sRNAs that efficiently bind and sequester the CsrA protein (Romeo 1998; Babitzke and Romeo 2007). These specific sRNAs, named CsrB/C or RsmY/Z in different bacteria, are composed of several repetitive sequence elements containing numerous conserved A(N)GGA motifs, most of which are located in hairpin loops (Table 1). Hence, almost the entire sRNA becomes covered with protein, making them a highly efficient antagonist to the interaction between CsrA and its target RNAs. Thus, the transcript level of these specific, small, untranslated RNAs is the key determinant of CsrA activity in the cell and already their moderate increase in

expression can dramatically influence the equilibrium between target RNA- or sRNA-bound CsrA (Fig. 4).

In all Csr/Rsm-regulatory networks known to date, the transcription of the small RNAs sequestering CsrA is regulated by a two-component system (TCS) that is homologous to the BarA/UvrY system in *E. coli*, and at least partially also by the alternative stress sigma factor RpoS (Romeo, Vakulskas and Babitzke 2013). This cascade of TCS/sRNA/CsrA is involved in the regulation of numerous cellular functions including carbon metabolism, motility, biofilm formation and adaptation to environmental stress responses, and production of virulence traits in the pathogen (Lucchetti-Miganeh et al. 2008; Timmermans and Van Melderen 2010; Romeo, Vakulskas and Babitzke 2013) (Fig. 4). Even though the overall processes of CsrA regulation and its influence on regulation of the stationary phase and virulence-related pathways are better and better understood, not many direct target transcripts of CsrA, except its two regulatory sRNAs, have been identified to date in pathogenic bacteria. Most of the studies concentrated so far on indirect effects due to the loss of CsrA expression or overexpression of CsrA but rarely on physical interaction between the protein and the RNA.

The Csr/Rsm system is implicated in regulation of growth and motility

Among intracellular bacteria, the Csr-type regulatory system is well characterized in *L. pneumophila* and *S. enterica* serovar Typhimurium (Altier, Suyemoto and Lawhon 2000; Fettes et al. 2001; Molofsky and Swanson 2003; Jonas et al. 2008; Rasis and Segal 2009; Sahr et al. 2009; Jonas et al. 2010; Edwards et al. 2011; Knudsen et al. 2014; Nevo et al. 2014). The Csr system is very versatile, as even though the regulatory impact observed for *Legionella* and *Salmonella* is similar to what is known for other pathogens such as *Pseudomonas*, *Yersinia*, *Vibrio* or *E. coli*, there are also striking differences. One commonly observed feature of the CsrA cascade is its strong effect on the cell morphology of the bacteria. Due to the loss of the *csrA* gene, the cells are converted during replication into smaller sized cells with coccoid shape instead of the typical rod-shaped structure of *Salmonella* and *Legionella*, while overexpression of CsrA resulted in highly elongated cells. Furthermore, mutation in *csrA* causes a drastic reduction in growth rate in particular at lower temperature (Forsbach-Birk et al. 2004; Knudsen et al. 2014). Also the transition from a sessile to a motile life form of *S. enterica* serovar Typhimurium is strongly affected by CsrA (Jonas et al. 2010). In *S. enterica* serovar Typhimurium CsrA seems to act positively on swarming motility and it is required for accurate flagella expression by stabilizing the *flhDC* and the *fljA* mRNAs, which are the regulators of the flagella operon. This stabilizing effect leads to an increased production of flagellar proteins (Jonas et al. 2010). In *Legionella*, CsrA also affects the flagella sigma factor FliA, but in contrast to *S. enterica* serovar Typhimurium, overexpression of CsrA in *L. pneumophila* resulted in lower *fljA* transcription and subsequently to reduced levels of FlaA, the major structural flagellar protein controlled by FliA (Forsbach-Birk et al. 2004; Bruggemann et al. 2006). Additionally, the knockout mutation of the TCS LetA/LetS, which is necessary for the transcription of the CsrA antagonists RsmY/RsmZ, completely abolishes motility in the post-exponential phase (Molofsky and Swanson 2003; Rasis and Segal 2009; Sahr et al. 2009). Taken together, although CsrA is apparently a common regulator for flagella expression in different bacteria, the regulatory function differs significantly between them, as it can be either positive or negative.

The Csr/Rsm system is implicated in regulation of virulence and stress response

The control of stress responses and expression of virulence traits by the Csr system is a common feature among pathogens (Lucchetti-Miganeh et al. 2008). The drastic changes in morphology and motility are closely related to the expression of genes which are important during stress response, such as heat/cold shock, acid resistance, osmotic stress and iron starvation. For example, in *L. pneumophila*, the inhibition of CsrA expression plays a major role during the transition from the replicative to transmissive phase, which is associated with adaptation to environmental stress and virulence formation (Molofsky and Swanson 2004; Sahr et al. 2009). This finding was also supported by the recent identification of 26 effectors under the control of the Leta-RsmYZ-CsrA cascade (Nevo, Zusman, Rasis, et al. 2014). These effectors were identified by comparing the expression levels of several effector-encoding genes in the wild type and a letA knockout mutant strain, and selecting those that showed a strong reduction of the expression level in the mutant strain. Then the presence of a putative CsrA-binding site was searched for by using the consensus sequence A(N)GGA. To confirm the regulation by CsrA, mutation of these putative CsrA-binding sites was performed. Indeed, during the exponential phase elevated expression levels of 22 of the 26 tested effector genes were observed. In contrast, in the wild-type strain, these effectors were more highly expressed in the stationary phase, suggesting that they might participate in the early steps of a new infection cycle most probably during the establishment of the *Legionella*-containing vacuole (LCV). Indeed, at least 19 of these 26 *L. pneumophila* effectors under the control of CsrA are involved in the modulation of endoplasmic reticulum (ER)-Golgi vesicular trafficking in *S. cerevisiae* (Nevo et al. 2014). The effectors RalF, YlfA and VipA had already been shown to cause growth defects in *S. cerevisiae* and to affect vesicular trafficking in the host cell directly (Shohdy et al. 2005; Heidtman et al. 2009). Nevo and colleagues proposed that these effectors are probably the first to translocate into the host cell when *L. pneumophila* begins a new infection cycle and they then participate in the establishment of the LCV, by modulating different components of the ER-Golgi trafficking pathway. The finding that secreted effectors important for forming the LCV are under the control of CsrA further highlights the important role of this RNA-binding protein in survival and replication of intracellular bacteria.

In *Salmonella*, virulence gene expression is also under the control of CsrA as it binds near to the SD sequence of the *hilD* mRNA. HilD is the master regulator of the expression of virulence genes located on the SPIs. This negative regulation of HilD translation results in an overall down-regulation of the two pathogenicity islands SPI-1 and SPI-2, both required for intestinal infection in animal models (Martinez et al. 2011). This effect is abolished by the activation of the SirA/BarA two-component system, resulting in a high level of transcription of the sRNAs CsrB/CsrC that sequester the CsrA binding to the *hilD* untranslated region (Table 1).

Given that CsrA is a very important post-transcriptional regulator, several attempts have been made to develop bioinformatics approaches to identify the RNAs that CsrA is targeting. A sequence-based approach to predict genes directly regulated by CsrA, CSRA.TARGET, was published recently (Kulkarni et al. 2014). This computational algorithm predicted >100 mRNA targets in *E. coli*, *S. enterica* serovar Typhimurium, *P. aeruginosa* and *L. pneumophila*. In addition to new targets, already known and confirmed CsrA targets of these pathogens were identified,

giving high confidence in its predictions. Furthermore, some predicted targets were tested and confirmed experimentally (Kulkarni et al. 2014). The predicted CsrA targets indicated the important roles of CsrA in diverse processes such as stress response, quorum sensing and virulence factor regulation or metabolism. Also CsrA targets specific for intracellular pathogens, such as, for example, *hilD*, encoding the master regulator for the induction of invasion genes located on SPI-1 or *sipA* encoding a type III effector protein of *S. enterica* serovar Typhimurium necessary to induce a proinflammatory response in epithelial cells, were identified (Kulkarni et al. 2014).

Taken together, the Csr-type regulatory system is important for the expression of the virulence phenotype of intracellular bacteria by controlling a large variety of physiological and stress responses that are implicated directly or indirectly in host cell infection and the overall biological fitness during changing environmental conditions. However, although very similar cellular functions are regulated by this post-transcriptional control mechanism in many pathogens, the impact of the regulation, whether inducing or inhibiting, differs considerably between different bacterial species.

THE CRISPR/CAS SYSTEM AND THE IMPLICATION OF crRNAs IN VIRULENCE

Recently, an adaptive microbial immune system, named clustered regularly interspaced short palindromic repeats (CRISPR), has been identified which provides acquired immunity against viruses and plasmids in bacteria and archaea (Barrangou et al. 2007). Unlike the restriction-based defence mechanism, the CRISPR/Cas system is highly specific, adaptive and heritable. It consists of an array of conserved short DNA repeat sequences, which are interspaced by variable spacer regions, originating from foreign DNA. Additionally, several *cas* (CRISPR-associated) genes are located in close proximity to the repeat-spacer array. The composition of the CRISPR/Cas locus can vary greatly between different microbial species and accordingly can be divided into types I–III. However, the basic mechanism to mediate adaptive immunity is common to all types of CRISPR/Cas systems and is composed of three main steps: the acquisition of foreign DNA, the processing and crRNA generation and the interference or silencing (for a review, see Sorek, Lawrence and Wiedenheft 2013) (Fig. 2). Thus CRISPR/Cas is a DNA-encoded, RNA-mediated defence system that provides sequence-specific recognition, targeting and degradation of exogenous nucleic acids. Going into the details of functionality and mechanistic differences between species would exceed the scope of this review, thus we place our main focus here on a newly described role for the CRISPR/Cas systems during infection and virulence formation of pathogens.

In a model it was proposed that the pathogenic potential of bacteria is associated with or even controlled by bacteriophage infections as CRISPR activity might interfere with the uptake of foreign DNA potentially carrying virulence traits, such as genes coding for toxins or antibiotic resistance (Mojica et al. 2005). Furthermore, it was found that the spacer regions contain sequence homologous not only to foreign DNA but also, even if in minor quantity, to endogenous chromosomal regions of the bacteria itself. This self-targeting was proposed to lead to a form of autoimmunity, suggesting a role in regulation of endogenous gene expression (Stern et al. 2010). Furthermore, changes in *cas* gene expression seem to be correlated with stress response, e.g. observed in *L. pneumophila* where an induction of the *cas* operon is dependent on the growth phase and typically up-regulated

in the transmissive phase (Sahr et al. 2009). This phenomenon is abolished in a $\Delta letA$ and a $\Delta rpoS$ knockout mutant, suggesting a connection with the stringent response. Assuming an increased transcript level of RNAs in conditions relevant for their biological role, it suggests that CRISPR/Cas might have relevance for *Legionella* virulence formation. Indeed, similar results were observed *in vivo* during intracellular growth in macrophages and amoeba. Furthermore, *cas2* mutants were significantly impaired during intracellular growth in amoebae, indicating a pivotal role for the CRISPR/Cas system during amoeba infection (Gunderson and Cianciotto 2013) (Table 1). Also in *Listeria*, the *rliB* locus composed of five small copies of expressed CRISPR repeats was shown to be involved in virulence (Toledo-Arana et al. 2009). Interestingly, in *L. monocytogenes*, *RliB*-CRISPR is not related to an adjacent *cas* operon but is instead expressed and processed by the PNPase that is needed for *RliB*-CRISPR-mediated DNA interference (Sesto et al. 2014) (Table 1).

In both examples, a strong correlation between CRISPR expression and pathogenicity is evident. However, the functionality and the role are not yet established. CRISPR/Cas can promote bacterial fitness through protection against bacteriophages and thus increase indirectly the survival rate and ability to infect host cells. Alternatively, it might have a direct influence by regulating endogenous virulence factors and effectors similar to a mechanism identified recently in *Francisella novicida* (Sampson et al. 2013). Therein it was shown for the first time that the Cas9 complex of this intracellular pathogen directly represses the production of an immunogenic lipoprotein, BLP, by stimulating its mRNA degradation. The BLP protein is recognized by the Toll-like receptor 2, triggering the antibacterial proinflammatory immune response. Therefore, Cas9-dependent repression of BLP production is essential for innate immune evasion and intracellular survival of *F. novicida* during infection of eukaryotic host cells. This process involves additionally two other newly described small RNAs that mediate the sequence-specific recognition of the *blp* transcript (Sampson et al. 2013). These two sRNAs, named *trans*-activating crRNA (*tracrRNA*) and CRISPR/Cas-associated RNA (*scaRNA*), are not part of the crRNA cluster but closely related to the CRISPR/Cas region (Table 1). The knockout mutants of either one of them were not capable of establishing infection in a mouse model.

This outstanding observation demonstrates that the CRISPR/Cas system can also have an endogenous function independent from classical bacterial defence via an antisense RNA silencing mechanism. As CRISPR/Cas is widespread in bacteria, a similar mechanism might exist in numerous other pathogens, suggesting that this system might have a more general involvement in gene regulation, particularly during stress response and virulence formation. Unravelling this phenomenon and a deeper knowledge of the functionality of the CRISPR/Cas system will lead to a better understanding of gene regulation and virulence formation in pathogens and possibly to a new chance to fight bacterial infection independently of preceding antibiotic multiresistance.

CONCLUDING REMARKS

Several decades of research revealed the high variability, wide distribution and diverse functionality of sRNAs and demonstrated their crucial role in many biological processes such as in environmental sensing and stress adaptation, virulence and infectivity of intracellular bacteria, as well as development and metabolism. Here we aimed to highlight some of the most

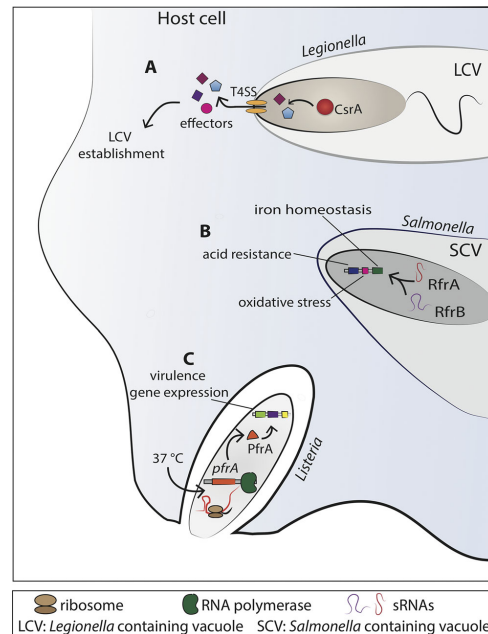


Figure 5. Examples of RNA-mediated regulation implicated in adaptation to the host environment in intracellular bacteria. (A) The regulatory cascade comprising CsrA and two sRNAs (RsmY and RsmZ) regulates the expression of secreted effectors of *L. pneumophila* that are released into the host cell cytosol. These effectors manipulate different processes of the secretory pathway to help in establishing the *Legionella*-containing vacuole (LCV). (B) The *trans*-encoded regulatory sRNAs RfrA and RfrB of *Salmonella enterica* serovar Typhi play a central role in iron homeostasis regulation, and are implicated in protecting the bacteria against oxidative stress, bactericidal antibiotics and acid resistance when replicating in the host cell. (C) *Listeria monocytogenes* virulence genes are under the control of PrfA, the master regulator of virulence gene expression. A thermosensor present in the 5' UTR of *prfA* senses the variation of the temperature when *L. monocytogenes* changes from the extracellular environment (lower temperature) to invade a mammalian host cell (37°C). The increase in the temperature changes its conformation, freeing the ribosome-binding site, which in turn allows the translation of PrfA and subsequent virulence gene expression necessary for invasion of eukaryotic cells and the rapid escape from the vacuole to the cytosol.

striking aspects that make sRNAs such versatile regulators in bacteria in particular for intracellular bacteria during the interaction, infection and growth within their eukaryotic host cells. However, considering the outstanding plethora and variety of sRNA regulatory elements, one may speculate about their functional redundancy, mainly as a consequence of their nature, within pathogenic and non-pathogenic bacterial species. In this context we highlighted several and very diverse sRNAs that can act independently or simultaneously to ensure the bacteria a favourable adaptation to adverse conditions and a benefit during the infection of the host cells (Fig. 5). On the other hand, only little is known about the opposite scenario, that one single sRNA may combine multiple regulatory mechanisms. Excitingly, it was recently shown that the same sRNA might indeed have several functions. The sRNA Qrr is able to fulfil four distinct regulatory mechanisms to fine-tune the regulation of quorum sensing in *Vibrio* (Feng et al. 2015). Qrr can act as a

negative control element to repress its targets through sequestration, catalytic degradation or coupled degradation, and at the same time as a positive regulatory element by setting free the RBS, allowing the target expression to occur while promoting its own degradation. The four different mechanisms are the results of diverse base-pairing strategies, employed by the Qrr sRNA to target defined quorum-sensing mRNAs (Feng et al. 2015). Thus, this new view of multifunctional sRNA may be employed by other bacterial pathogens, representing more than a mere peculiarity but a common role not yet thoroughly examined. Recently it has been shown that sRNAs of bacteria can have an interspecies role, as two sRNAs, OxyS and DsrA of *E. coli*, have been reported to have physiological effects on *Caenorhabditis elegans* via regulating its gene expression (Liu et al. 2012), and that plant microRNAs in the food of animals could have interspecies roles in mammalian cells (Zhang et al. 2012). It also has been shown that *L. monocytogenes* releases nucleic acids and that such secreted bacterial RNA/DNA is recognized by cytosolic sensors such as RIG-I, MDA5 and STING, leading to the triggering of interferon- β production (Abdullah et al. 2012). However, until now the main virulence factors identified, which are secreted into the host cell by intracellular bacteria, are proteins. Thus another current challenge might be to discover whether specific sRNAs of intracellular bacteria might play a role in the host cells they infect. Taking into account the huge flexibility and evolutionary variety of sRNA within pathogenic bacteria one can imagine that bacterial sRNAs may also be secreted during infection to manipulate host cell function. In particular these bacterial sRNAs might mimic eukaryotic miRNAs to subvert their functions in the cells they infect. The identification of sRNAs secreted or released into the host cell to subvert its functions may emerge as a new, exciting topic of research in host-pathogen interactions. Thus, even though the analyses of bacterial RNAs is an ever-growing field of study, many questions still remain unanswered and new regulatory mechanisms might be discovered allowing us to appreciate more and more the diversified world of sRNA and its regulatory potential.

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2.2 The RNA chaperone Hfq

2.2.1 General properties and structure of Hfq

As mentioned before, Hfq was identified in the late 1960s in *E. coli* as host factor for the efficient replication of the RNA bacteriophage Q β (Franze de Fernandez, Eoyang, & August, 1968). It was demonstrated that Hfq binds at the cytosine-rich 3' end of the plus-strand of the viral RNA genome, thus allowing the initiation of the minus-strand RNA synthesis by the Q β -replicase (Schuppli, Georgijevic, & Weber, 2000). Franze de Fernandez and collaborators purified and biochemically characterized a hexameric protein, first named HF₁, capable of binding different AU-rich single-stranded RNAs (Franze de Fernandez, Hayward, & August, 1972). The following studies on the HF₁ protein focused mainly on its binding properties and only in the early 1990s the *E. coli* gene encoding HF₁ was identified and designated as *hfq* (Carmichael, Weber, Niveleau, & Wahba, 1975; de Haseth & Uhlenbeck, 1980a; 1980b; Senear & Steitz, 1976). Since then, the attention has changed to the role of Hfq in the control of gene expression within bacterial cells. Hfq has been characterized as a member of the conserved family of RNA binding proteins named LSm (like-Sm)/Sm, that is present in all three domains of life. In eukaryotes, the Sm and LSm proteins have been associated with mRNA splicing, RNA decapping and RNA stabilization (reviewed in (C. J. Wilusz & Wilusz, 2005). Hfq is missing from three bacterial clades, the Chlamydia-Spirochaetes, Actinomycetes-Deinococcus- Cyanobacteria and Green sulfur bacteria-Cytophagales but is present in all alpha, beta, gamma and delta proteobacteria except some that have undergone massive genome reduction due to their parasitic lifestyle, like *Buchnera* sp., *Rickettsia prowazekii* and *Brucella melitensis* (Sun, Zhulin, & Wartell, 2002).

In terms of topology, (L)Sm proteins are characterized by forming a ring-like multimeric complex that binds RNA and by the presence of a conserved protein fold, named LSm-domain (Achsel, Stark, & Lührmann, 2001). In detail, the LSm-fold structure consists of an N-terminal α -helix (α 1), followed by five β -strands (β 1-5), which are separated by five loops (L1-5) of variable length. The antiparallel β -strands form a half-open barrel structure with the N-terminal α -helix on top (Figure 7A). At the primary sequence level, the LSm proteins contain two conserved sequence motifs, named Sm1 and Sm2. The Sm1 signature resides in the first three β -strands and is more similar to the one of the eukaryotic LSm-proteins, whereas the Sm2 motif is located in the strands β 4 and β 5 (Figure 7C).

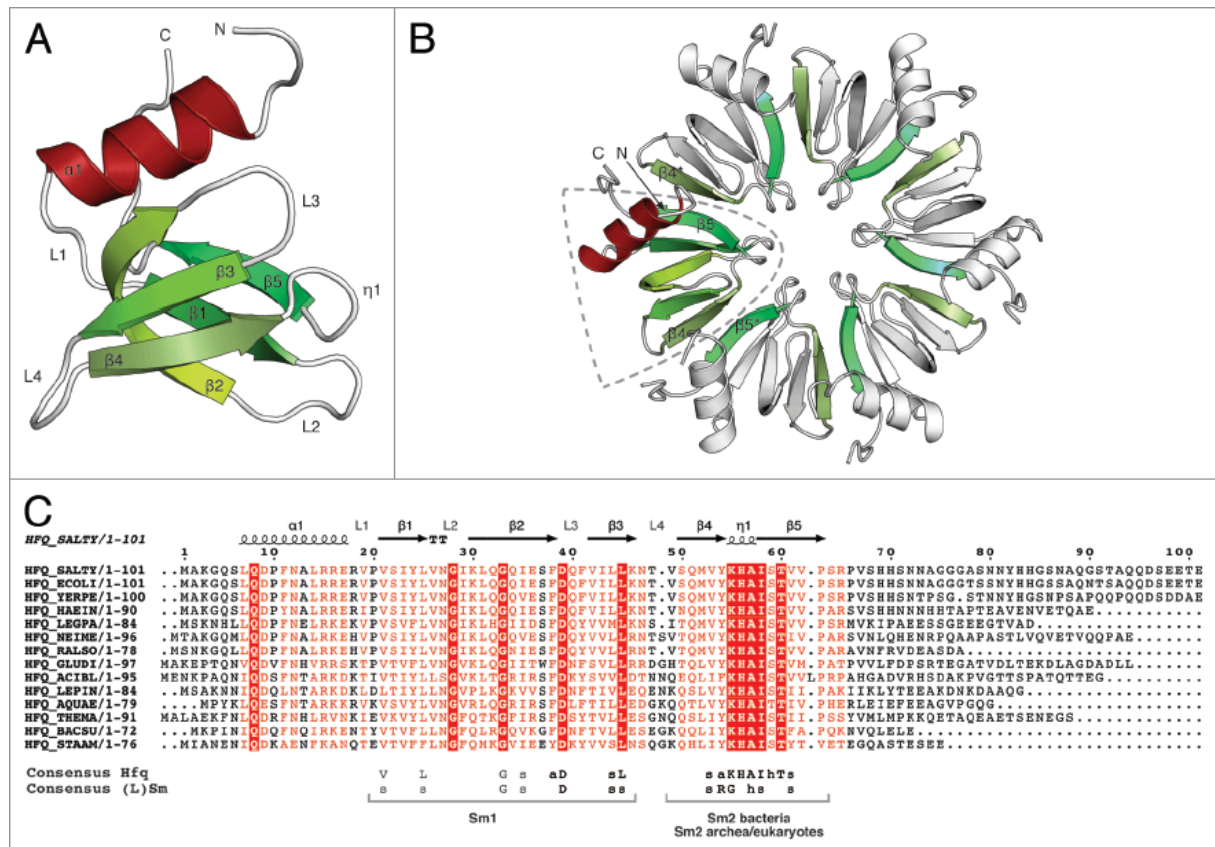


Figure 7. Schematic representation of the Sm fold and the hexameric structure of the Hfq protein. (A) Fold of the LSm domain of *Salmonella typhimurium* Hfq. The five β -strands (β 1-5) are depicted in green and form an open barrel with the α -helix on top (α 1 in red). (B) Hexameric association of the monomeric Hfq showing a typical doughnut-like structure. Intersubunit interactions are shown in green and are provided by backbone interactions between strands β 4 and β 5 to strands β 4* and β 5* in the neighbouring monomers, respectively. (C) Sequence alignment of Hfq proteins from diverse bacterial species. The secondary structure of *Salmonella typhimurium* Hfq (PDB-ID: 2YLB9) is superimposed on the primary sequence. The Sm consensus sequences are shown below the alignment (the nature of the amino acid side-chains is: s = small hydrophobic, I, L, V; h = hydrophilic, S, T; a = aromatic, Y, F). Highly conserved residues are red (> 70% conservation) or white in red boxes (100% conservation). While the Sm1 signature is conserved in all domains of life, Sm2 is divergent in bacteria. The species abbreviations and UniProt-IDs are: γ -Proteobacteria: SAL TY, *Salmonella typhimurium* (P0A1R0); ECOLI *Escherichia coli* (P0A6X3); YERPE, *Yersinia pestis* (A4TRN9); HAEI N, *Hemophilus influenzae*, (P44437); LE GPA *Legionella pneumophila* (Q5X982). β -Proteobacteria: NEI ME, *Neisseria meningitidis* (B9VV 05); RAL SO, *Ralstonia solanacearum* (Q8Y025). α -Proteobacteria: GLU DI, *Gluconacetobacter diazotrophicus* (Q8RMG6); Acidobacteria: ACI BL, *Acidobacteria bacterium* (Q1II F9). Spirochaetales: LEPI N, *Leptospira interrogans* (Q8F5Z7). Aquafecales: AQUAE *Aquifex aeolicus*, (O66512). Thermotogales: THEMA, *Thermotoga maritime* (Q9WYZ6). Firmicutes: BAC SU, *Bacillus subtilis* (O31796); STAA M, *Staphylococcus aureus* (Q99UG9). Adapted from (Sauer, 2013) .

The Sm1 motif is composed of diverse highly phylogenetically conserved residues such as acidic aspartate and glycine, which are involved in the fold maintenance. In contrast the Sm2 signature is divergent in the bacterial Hfq proteins and is composed of several residues, which

are essential for the protein stability (Moskaleva et al., 2010; Møller et al., 2002; Sobrero & Valverde, 2012). The eukaryotic Sm proteins classically form heteromultimeric rings, whereas in bacteria there is usually only one Hfq protein, which assembles into ring-shaped homo-hexamers, as revealed from the crystal structures of several bacterial Hfq proteins (Baba, Someya, Kawai, Nakamura, & Kumasaka, 2010; Beich-Frandsen, Vecerek, Sjöblom, Bläsi, & Djinovic-Carugo, 2011b; Bøggild, Overgaard, Valentin-Hansen, & Brodersen, 2009; Nikulin et al., 2005; Sauer & Weichenrieder, 2011; Sauter, Basquin, & Suck, 2003; Schumacher, Pearson, Møller, Valentin-Hansen, & Brennan, 2002). The intersubunit interactions are made up from residues in the β 4 strand of one subunit and the β 5 strand of the neighboring subunits. Adjacent monomers interact in an oriented way so that the doughnut-like structure assembles with N-terminal α -helices on the same face of the oligomer (Figure 7B). Interestingly, Hfq of enterobacteria contains unusually long C-terminal extensions (Figure 7C). Moreover, the sequence of these disordered C-terminal regions, which extend outwards from the hexameric ring, is not conserved and its biological function in *E. coli* is associated with protein stability but this is controversial in other microorganisms (Arluison et al., 2004; Beich-Frandsen, Vecerek, Konarev, Sjöblom, Kloiber, Hämmerle, et al., 2011a; Beich-Frandsen, Vecerek, Sjöblom, Bläsi, & Djinovic-Carugo, 2011b; Olsen, Møller-Jensen, Brennan, & Valentin-Hansen, 2010; Vecerek, Rajkowitsch, Sonnleitner, Schroeder, & Bläsi, 2008).

2.2.2 The RNA binding-features of Hfq

Hfq works as chaperone and RNA-binder. Its surface properties are crucial for the binding and the discrimination of different targets. Hfq proteins are circular hexamers that delimit a central pore (Figure 8) and display two asymmetric faces, usually with a net positive electrostatic potential, an expected feature for a nucleic acid-binding protein. Thus, the two exposed faces – denoted distal and proximal – show distinct properties and binding specificities (Vogel & Ben F Luisi, 2011a) (Figure 8). Co-crystal structures of *S. aureus*, *E. coli* and *L. monocytogenes* Hfq in complex with short oligonucleotides demonstrated that the proximal face binds U-rich sequences with uridines accommodated around the pore in a constricted conformation that is stabilized by water molecules (Sauer & Weichenrieder, 2011; Schumacher et al., 2002; W. Wang, Wang, Wu, Gong, & Shi, 2013). Analysis of *E. coli* strains expressing Hfq proteins with mutations in conserved proximal face residues, such as Q8, F42 and K56, have highlighted the relevance of these residues for selection of U-rich

segments (Mikulecky et al., 2004; Sauer & Weichenrieder, 2011). On the target side, sRNAs contain typical Rho-independent terminators, whose stretches usually have a poly-U 3' terminus, thus *in vitro* and *in vivo* elegant data have demonstrated that the specificity and affinity of the so-called proximal face relies on the recognition of the 3' hydroxyl group in the RNA conformation. As such, it is likely that binding in the proximity of the sRNA terminators affects the sRNA stability and turnover (Otaka, Ishikawa, Morita, & Aiba, 2011; Wilson & Hippel, 1995). The model proposed is that the interaction of Hfq with the sRNA 3' end anchors the sRNA on the proximal face, whereas the U-rich sequences stabilize the complex by binding the lateral surface of the hexamer. This so-called lateral surface comprises six patches of conserved polar residues (R16, R17 and R19), constituting the lateral RNA binding motif. The number of the arginine residues affects the ability of Hfq to facilitate RNA interactions and defines different chaperone functions depending on the host organism (Zheng et al., 2016). Strong arginine patches such as the ones in *E. coli*, *Salmonella* or *Pseudomonas* correlate with strong RNA activity of Hfq, typical for Gram-Negative bacteria. In contrast, low arginine content in the lateral motif has little effect on the physiology of Gram-positive bacteria (Zheng et al., 2016). Furthermore, in *E. coli* it has been shown that acidic residues adjacent to this arginine patch affect the annealing and thus the selective recognition of the complementary RNA molecules (Panja et al., 2015). In addition to the lateral motif, another structural element, the C-terminal domain, modulates Hfq activity. This domain in *E. coli* is required for the dissociation of the double stranded RNA product and consequently stimulates the cycling of the sRNA and the duplex RNA on the ring (Santiago-Frangos et al., 2016). Moreover, possible interaction sites could be associated with the flexible C-terminal extensions of Hfq and the groove of the sRNA helix, which would change the view of how sRNAs and Hfq interact (Beich-Frandsen, Vecerek, Konarev, Sjöblom, Kloiber, Hämmerle, et al., 2011a; Ishikawa, Otaka, Maki, Morita, & Aiba, 2012; Salim, Faner, Philip, & Feig, 2012; Sauer, Schmidt, & Weichenrieder, 2012).

In contrast, the so-called distal face has preference for A-rich sequences, which avidly bind in a circular conformation the Hfq surface (Link, Valentin-Hansen, & Brennan, 2009). The distal face seems to be important for the interaction with internal adenosine-rich sequences of mRNAs and in addition with poly-(A)_n sequences of the 3' end of RNA degradation molecules (Hajnsdorf & Régnier, 2000; Salim et al., 2012; Salim & Feig, 2010; Soper & Woodson, 2008). Particularly, crystal structures of the *E. coli* Hfq protein with poly -(A)₁₅ RNA showed

the each Hfq monomer contains a tripartite RNA binding motif (A-R-E motif) with the 5'-adenosine binding to Site A (specific for adenosines), the second nucleotide binding to the R site (specific for purine) and the following to a nondiscriminatory RNA-entrance/exit (E) site (Link et al., 2009). Interestingly, Hfq of *S. aureus* binds to the distal surface to an (AA)₃-A oligonucleotide, whereas the *B. subtilis* Hfq showed a more strictly preference for AG repeated sequences, suggesting the existence of species-specific RNA binding motifs (Horstmann, Orans, Valentin-Hansen, Shelburne, & Brennan, 2012; Someya et al., 2012). Generally, Hfq proteins of Gram-negative bacteria comprise a tripartite binding motif for (ARN)_n sequences, whereas the Hfq homologs in Gram-positive bacteria have propensity for (AN)_n repeats (Horstmann et al., 2012; Sauer, 2013). A typical Hfq binding scenario comprises sRNAs carrying U-rich sequences preferentially binding the Hfq proximal face, on the other hand mRNA targets with A-rich motifs often found in the 5'UTRs bind preferentially to the distal face. However, additional evidence indicated that a simple two-face model does not necessarily occur (Sauer, 2013).

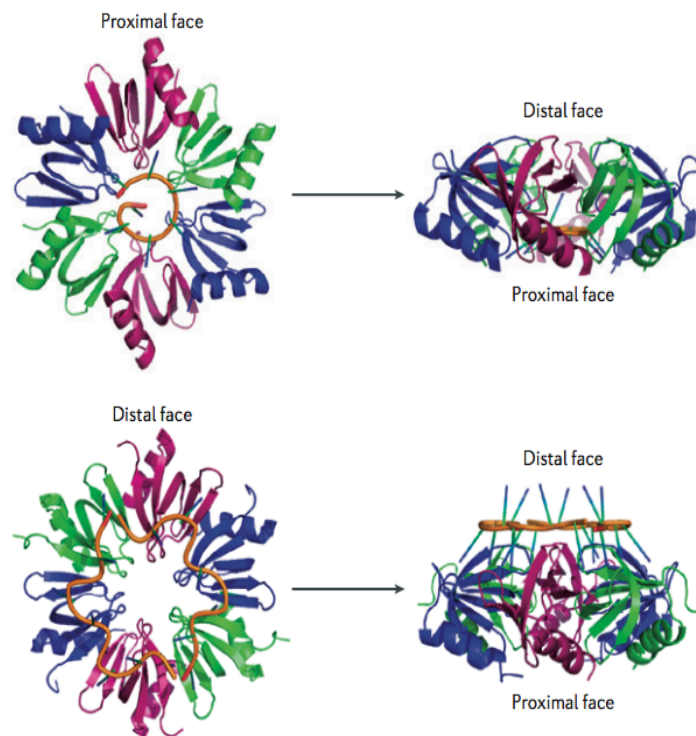


Figure 8. Structure of the Hfq hexamers showing its main surfaces. The Hfq proximal face and distal face with the RNAs (orange) displayed on opposite sides of the hexamer. The proximal face with the exposed amino-terminal α -helix includes residues in the Sm2 sequence motif. The single monomers are presented in different colours. Adapted from (Vogel & Ben F Luisi, 2011a).

2.2.3 Mechanisms of Hfq riboregulation

The systematic discovery of sRNAs and the growing understanding of their Hfq-dependent action in bacterial pathogens, paved the way to analyze in molecular details the specific interactions of Hfq with sRNAs. Indeed, first *in-vitro* data demonstrated that the Hfq protein is facilitating sRNA-mRNA target interactions (Møller et al., 2002; Sittka, Sharma, Rolle, & Vogel, 2009; Soper & Woodson, 2008). Interestingly, the sRNA-mRNA complex could be assembled also in absence of the Hfq RNA-binder, however its presence strongly accelerated the RNA duplex formation (Kawamoto, Koide, Morita, & Aiba, 2006; Soper, Mandin, Majdalani, Gottesman, & Woodson, 2010). Moreover, due to the fact that Hfq has been shown to bind *in-vitro* almost any single-stranded, unstructured RNA molecule with high affinity, an intriguing question to address is how Hfq can bind and affect the function of so many RNAs. The debate is still under way; however two main mechanisms how Hfq-facilitated base pairing may work have been established. The first proposed mechanism is a simultaneous binding of two RNAs to Hfq through the formation of a ternary sRNA-mRNA-Hfq complex, in which Hfq “passively” serves as platform for the RNA interactions (Mikulecky et al., 2004). This mechanism has been shown for *E. coli* Hfq, which forms a stable complex with the DsrA sRNA and its *rpoS* mRNA target (Soper & Woodson, 2008). Although the simultaneous interaction with Hfq has been reported also for *fhlA* mRNA and OxyS sRNA, whether the ternary complexes, for both *fhlA* and *rpoS*, is a prerequisite for the mRNA-sRNA duplex formation remains still to be clarified (Salim & Feig, 2010). The second proposed effect concerns the metabolic stability of the bound RNA molecules. Indeed, Hfq impacts multiple steps by changing the secondary structure of bound RNAs, placing the RNAs in the proximity, neutralizing the charge of the RNAs or promoting the annealing of the first base pairs (Updegrove, Zhang, & Storz, 2016; E. G. H. Wagner, 2013). Moreover, several sRNAs are unstable in the absence of Hfq, due to the lack of protection from degradation by RNase E and other exoribonucleases (Chao, Papenfort, Reinhardt, Sharma, & Vogel, 2012; Moon & Gottesman, 2011). Intriguingly, Hfq and RNase E display similar binding preferences for AU-rich sequences and the interaction of the proximal face of Hfq with the 3'-terminal U of a terminator eventually makes the sRNA inaccessible for RNase E-dependent cleavage (Folichon et al., 2003; Moll, Afonyushkin, Vytvytska, Kaberdin, & Bläsi, 2003; Sauer & Weichenrieder, 2011).

Overall, Hfq-facilitated sRNA-mRNA target pairing leads to distinct fates and activities. On

one hand, Hfq facilitates sRNA binding to the mRNA ribosome-binding site (RBS) on its 5' untranslated region, thus arresting the binding of the ribosome subunits and consequently the translation initiation. On the other hand, Hfq favours the interaction of the sRNA with the 5' region of the mRNA target and its translation by setting free the RBS, which alternatively would be locked by the formation of a secondary structure. In some cases, the formation of the sRNA-mRNA target duplex bound by Hfq results in an accelerated degradation due to RNase E cleavage of the mRNA or both RNA molecules (Aiba, 2007; Caron, Lafontaine, & Massé, 2010; Morita, Maki, & Aiba, 2005). In addition, mRNA cleavage can occur by promoting the adenylation of the 3' ends of the mRNA by the enzyme poly(A)polymerase (PAP) and consequently triggering the 3' to 5' exonucleolytic cleavage, governing the RNA turnover (Hajnsdorf & Régnier, 2000). As such, Hfq acts as a central mediator of sRNA-based gene regulation in bacteria (Aiba, 2007; Storz, Vogel, & Wassarman, 2011; Vogel & Ben F Luisi, 2011b; Waters & Storz, 2009). Thus the loss of Hfq has strong effects on the physiology and fitness of bacteria, however Hfq effects are not the same in all bacteria, but species-specific phenotypes have been observed. With the exception of *F. tularensis*, where Hfq acts only as repressor, Hfq may be an activator or repressor of gene expression (Meibom et al., 2009). Hfq as pleiotropic regulator is involved in the altered expression of genes of diverse functions such as in metabolism, transport, energy production and conversion or membrane proteins (Boudry et al., 2014; Chiang, Lu, Liu, Lin, & Lai, 2011; M. Cui et al., 2013; Geng et al., 2009; Kendall, Gruber, Rasko, Hughes, & Sperandio, 2011).

2.2.4 Regulation of *hfq* expression

Despite the fact that it has been shown that the activity of a large plethora of sRNAs and their mRNA targets is governed by Hfq, how *hfq* gene and protein expression itself is regulated remains still poorly understood. The *E. coli hfq* gene is part of the *amiB-mutL-miaA-hfq-hflX-hflK-hflC* superoperon, which contains four σ^{70} -dependent promoters and three σ^{32} -dependent heat-shock promoters (Tsui, Feng, & Winkler, 1996). This superoperon organization is well conserved in most γ -proteobacteria (Sobrero & Valverde, 2012). Within the α - and β -proteobacteria and in some bacillales, the operon is composed of only the *hfq-hflX* tandem, the same organization that is found in *L. pneumophila* (described below). Only in few bacterial species such as *E. coli* and *Francisella tularensis* evidence of *hfq-hflX* co-transcription has been shown (Meibom et al., 2009; Tsui, Leung, & Winkler, 1994). *hfq* expression has been best studied in *E. coli*, in which Hfq negatively regulates its own expression through binding

of the protein to its own mRNA at two distinct sites of the 5' UTR, competing with the translation machinery for the ribosome-binding site (Vecerek, Moll, & Bläsi, 2005). In addition, RNase E is involved in the regulation of the *hfq* transcript level, which accumulates 3-fold in the *rne* mutant strain compared to the transcript level detected in the wild type strain. In detail, the binding of Hfq to its own transcript results in the exposition of the RNase E binding sites on the *hfq* mRNA and consequently to the cleavage by the endonuclease RNase E (Tsui et al., 1994). In this context, the Carbon Storage Regulator CsrA was demonstrated to inhibit the formation of the translational initiation complex by blocking the RBS and overlapping the *hfq* mRNA Shine-Dalgarno sequence (Baker et al., 2007). Despite the evolutionary divergence in the tree of life of the bacterial species *E. coli* and *Sinorhizobium meliloti*, in the latter the Hfq protein has also been reported to govern its own expression by analyzing a *hfq*'-*lacZ* translational fusion (Sobrero & Valverde, 2011). However, whether this process involves also sRNAs targeting of the *hfq* mRNA is not known. This regulatory circuit might control the expression of *hfq* precisely to keep the concentration of this global regulator within a defined range. Furthermore, the analysis of RNAs co-immunoprecipitated with Hfq in the γ -proteobacterium *Rhodobacter sphaeroides* has revealed the presence of the *hfq* mRNA, which might suggest a evolutionary conservation of the autoregulation of Hfq in other bacteria (Berghoff et al., 2011). In addition to the abovementioned Hfq translational autocontrol, evidence has emerged that Hfq is post-translationally regulated by titration mechanisms. Examples for such a mechanism have been described in diverse bacteria such as the titration of the *Pseudomonas aeruginosa* Hfq by the sRNA CrcZ (Sonnleitner & Bläsi, 2014) or the altered number of Hfq-bound mRNAs upon overexpression of the sRNA ArcZ in *Salmonella* or the competitive binding of the *E.coli* sRNAs on Hfq, leading to the modification of protein availability (Moon & Gottesman, 2011).

In some bacterial species, *hfq* expression is growth phase dependently regulated by pleiotropic regulators or sigma factors. An example is Hfq of *Shigella flexneri*, which is positively controlled by the pleiotropic regulator DksA during the exponential phase of growth and also under stringent conditions (A. K. Sharma & Payne, 2006). In *L. pneumophila* *hfq* expression was reported to be upregulated by the sigma factor σ^S during exponential growth and downregulated in stationary phase by the two component response regulator LetA (McNealy, Forsbach-Birk, Shi, & Marre, 2005) In contrast in *P. aeruginosa*, Hfq is expressed twice as much during stationary phase as compared to earlier growth phases (Sonnleitner, Sorger-

Domenigg, & Bläsi, 2006). Similarly, in *Listeria monocytogenes*, *hfq* transcript levels are strongly upregulated by the alternative stress sigma factor in stationary phase of growth (Christiansen, Larsen, Ingmer, Søgaaard-Andersen, & Kallipolitis, 2004). The expression of *hfq* has also been studied in the Gram-positive bacterium *Staphylococcus aureus*. The work of Liu and colleagues demonstrated that under their conditions, Hfq is a pleiotropic regulator of gene expression that is involved in pigmentation, one of the different facets influencing *S. aureus* pathogenicity (Y. Liu et al., 2010). Moreover, the transcriptome data from the wt strain and the *hfq* deletion mutant strain revealed that the expression of many genes, most of which were related to virulence, was altered. Nevertheless, an in depth investigation on the regulation of *hfq* is still missing what would probably explain why Hfq could not be detected in some strains. A particular case are bacteria from the *Burkholderia cepacia* complex, which encode two distinct and functional Hfq-like proteins that are differentially regulated in a growth-phase dependent manner, however the mechanism is not known (Sousa, Ramos, Moreira, & Leitão, 2010). The *hfq1* transcript is maximally expressed at the early exponential growth phase, whereas *hfq2* expression is highly active during the stationary phase (Ramos et al., 2014; Sousa et al., 2010). Similarly, Panda and colleagues reported recently the presence of three *hfq*-like genes in *Bacillus anthracis*, of which *hfq1* and *hfq2* are encoded on the chromosome, while *hfq3* is encoded on a plasmid (Panda, Tanwer, Ansari, Khare, & Bhatnagar, 2015). The expression of *hfq1* and *hfq2* is also growth-phase dependent but again, the regulators responsible are not described (Panda et al., 2015).

2.2.5 Role of Hfq in bacterial pathogens

Hfq is implicated in the regulation of virulence in several bacteria as described in chapter two. Another not described example for the implication in virulence gene regulation of Hfq is *Yersinia enterocolitica*. Indeed, it was shown that Hfq governs the production of the heat-stable enterotoxin, a urease, the siderophore yersiniabactin and the LpxR/SfpA lipopolysaccharide deacylase, all of which are important virulence factors (Kakoschke et al., 2014). Another example is *Bordetella pertussis*, where Hfq is required for the expression of the adenylate cyclase toxin, the pertussis toxin and the hemagglutinin virulence factors (Bibova et al., 2013). Hfq activity is often associated with alternative sigma factors, which redirect gene expression to accomplish stress and virulence responses. In some pathogens like *Salmonella Typhimurium* and *E. coli*, Hfq was reported to be required for the efficient translation derepression of the alternative sigma factor RpoS, leading to increased availability

of the protein and activating its targets (L. Brown & Elliott, 1996; Muffler, Fischer, & Hengge-Aronis, 1996). Also in *P. aeruginosa*, about two-thirds of the RpoS-dependent genes are regulated by Hfq (Sonnleitner et al., 2003). In *K. pneumoniae*, the Hfq-dependent regulation of *rpoS* appears more elaborate than the one in *E. coli* and *S. Typhimurium* (Chiang et al., 2011). Particularly the absence of Hfq not only affects the transcript level of *rpoS* but the loss of Hfq causes also a reduced translation efficiency of *rpoS* probably due to the absence of a positive regulation by sRNAs. Besides RpoS, the expression of the *K. pneumoniae* envelope stress sigma factor RpoE was also affected by the absence of Hfq. Overall, 19,5 and 17,3% of Hfq-dependent target genes are part of the RpoE and RpoS regulons, respectively.

In *S. Typhimurium*, the Hfq-dependent sRNA SdsR, which is an enterobacterial “core” sRNA, is part of the large operon controlled by the stationary phase sigma factor σ^S . σ^S engages the sRNA SdsR to inhibit the synthesis of the OmpD, StcD, EnvE and TolC membrane proteins in a Hfq-mediated manner (Fröhlich, Haneke, Papenfort, & Vogel, 2016). In addition, *Salmonella* utilizes further sRNAs to control the expression of outer membrane proteins (OMPs). Some of them are under the control of the sigma factor RpoE, which in order to avoid a compromised envelope integrity, induces the expression of MicA, RybB or MicL for the repression of the OMP synthesis (Gottesman & Storz, 2011; Papenfort et al., 2006; Vogel & Papenfort, 2006).

2.2.6 The RNA binding proteins Hfq and CsrA may work together

Besides the connection of Hfq with sigma factors, there is now evidence of an association of Hfq-regulated sRNAs also with other RNA binders. For example, a small RNA in *E. coli* named McaS has been demonstrated to serve both the Hfq and CsrA regulons (Jørgensen, Thomason, Havelund, Valentin-Hansen, & Storz, 2013). Like Hfq, the post-transcriptional regulator CsrA was reported to impact more than 20% of all mRNAs in *E. coli*, including those involved in motility and biofilm formation (A. N. Edwards et al., 2011; Lawhon et al., 2003; Wei et al., 2001; Yakhnin et al., 2013). Particularly, the McaS sRNA was shown to display a conserved region as potential hotspot of sRNA-based regulation, which base-pairs the *csgD* mRNA, leading to its inhibition of the translational initiation in an Hfq-dependent manner (Holmqvist et al., 2010; Jørgensen et al., 2012; Mika et al., 2012; Thomason, Fontaine, De Lay, & Storz, 2012). CsgD is a transcriptional activator of genes required for the formation of the biofilm matrix (Povolotsky & Hengge, 2012). In contrast, McaS activates the

translation of the FlhD₂C₂ master regulator of flagellar synthesis, also in an Hfq-dependent mechanism (Thomason et al., 2012). Thus, this sRNA displays a dual function governing the bacterial biofilm and its motility. In addition, CsrA was previously shown to repress translation of the *pgaA* mRNA, whose protein is required for the production of the biofilm-promoting adhesion poly- β -1,6-N-acetyl-d-glucosamine (PGA)(X. Wang, Preston, & Romeo, 2004) and more recent evidence indicated that McaS is implicated in the activation of the PGA production by decoying the CsrA protein (Jørgensen et al., 2013). Thus, McaA through Hfq and CsrA, represses one type of biofilm while promoting the other one (Holmqvist & Vogel, 2013; Jørgensen et al., 2013). Taken together, among the displayed pleiotropic functions, this chaperone regulates a plethora of sRNAs, which are implicated in diverse post-transcriptional regulatory circuits, including metabolism, virulence or biofilm formation.

CHAPTER THREE

REGULATION OF *L. PNEUMOPHILA* VIRULENCE

3.1 The *L. pneumophila* life cycle

In the natural environment, *L. pneumophila* resides in biofilm communities together with grazing amoebae. When engulfed by these protozoa, the bacterium is not digested as food, but is able to protect itself from the lysosomal digestion. It builds a protected vacuole, called *Legionella* containing vacuole (LCV) where it replicates to high numbers before escaping the spent host to search for a new one. As a facultative intracellular microbe, *L. pneumophila* transits between an intracellular and an extracellular habitat, displaying a multiphasic life cycle composed of at least two distinct stages. Besides an extracellular form when persisting in aquatic environments, *L. pneumophila* alternates between two morphologically distinct and reversible forms within the host cell: a non-infectious replicative form where virulence genes are down regulated, and a virulent, transmissible form where virulence genes are upregulated and flagella are expressed leading to high motility (Molofsky & Swanson, 2004) (Figure 9).

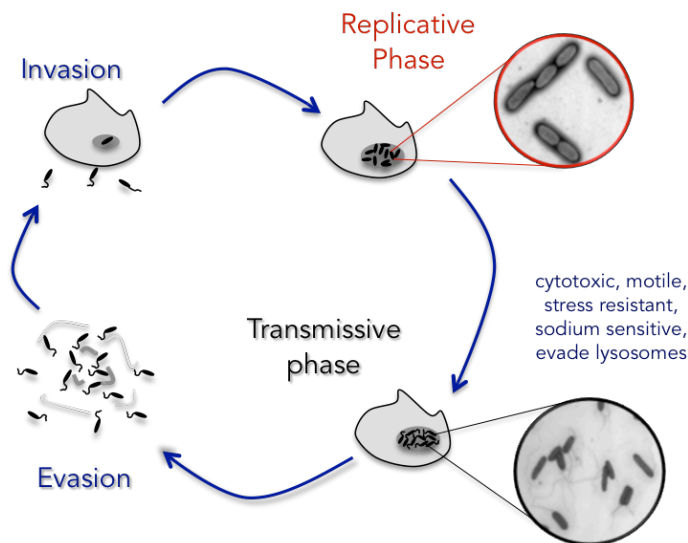


Figure 9. The *L. pneumophila* life cycle. Free-swimming, motile, transmissive bacteria reside in the extracellular environment before being engulfed by phagocytic cells, in which they replicate within a vacuole. In this phase the bacteria are not virulent and not motile. When the nutrients become limiting, the bacteria stop multiplying and the expression of traits for motility, cytotoxicity, survival in the environment and the transmission into a new host is promoted. After they left the host cell a new phagocyte will be infected and, the cycle starts again.

Many aspects of the pathogen's life cycle like the growth phase-dependent regulation of numerous virulence traits are similar to what is seen during infection. Thus the biphasic life cycle of *L. pneumophila* can be mimicked in synchronous broth cultures (Byrne & Swanson, 1998). In detail, at the beginning of the infectious cycle, nutrients are abundant and thus the conditions are favourable for the replication of *L. pneumophila* comparable to exponential

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growth in rich medium; now replicative traits are expressed, whereas traits that promote transmission are repressed. In contrast, when nutrients become limiting (end of the infection cycle inside the host cell or in broth culture when the bacteria reach the post exponential growth phase) this leads to the induction of the transmissive phase. This phase is characterized by bacteria that become highly motile, express many virulence traits allowing them to survive osmotic stress, to lyse the spent host cell and to leave the host cell and to be infection competent for a new host. This transition from the replicative phase to the transmissive phase is reversible as the infection of a new host stimulates the repression of the virulent traits and the return to the replicative phenotype (Figure 10).

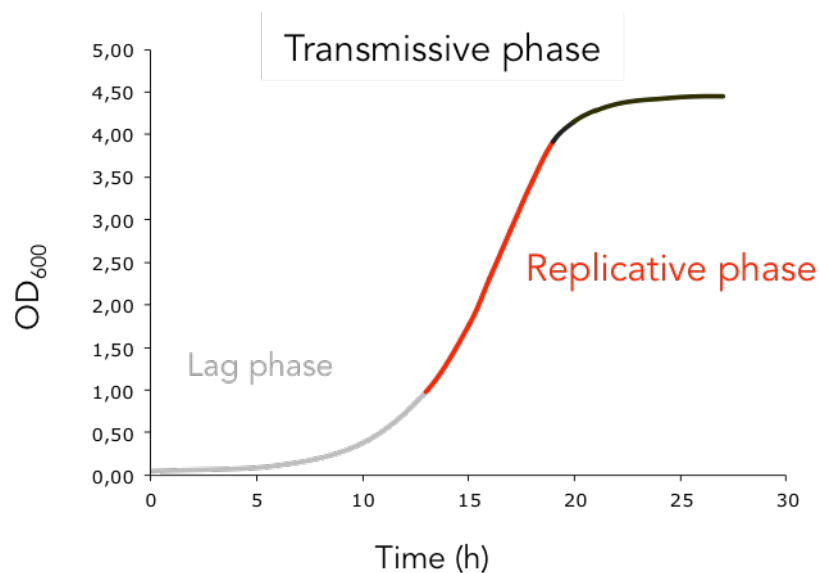


Figure 10. Life cycle of *L. pneumophila* in broth culture. The exponential phase of *L. pneumophila* growth is coupled with intracellular multiplication and the induction of replicative traits (red line). The entry into the post-exponential, mostly stationary phase, is correlated with the expression of virulence and transmissive traits.

The reciprocal expression of replicative or transmissive traits is a rational strategy for this intracellular pathogen to limit energy costs. In agreement, in a nutrient-rich environment, the infectious traits are not required or built. Conversely, during the transmissive phase, the biochemical pathways dedicated to the expression of the replicative aspects are neither demanded nor activated (Molofsky & Swanson, 2004). The transition between the mutually exclusive phases is reflected by a major change in the gene expression (Brüggemann, Cazalet, & Buchrieser, 2006). In particular, the bacteria coordinately fine-tune in a growth phase dependent manner the expression of motility, cytotoxicity, sodium sensitivity, resistance to stresses such as UV light, heat or osmotic pressure, metabolic pathways and evasion from the

lysosomal digestion (Brüggemann et al., 2006; Byrne & Swanson, 1998; Hammer & Swanson, 1999; Hammer, Tateda, & Swanson, 2002; Molofsky & Swanson, 2003; 2004).

3.2 Regulatory network governing *Legionella* differentiation

3.2.1 Metabolic triggers

The transition from the replicative to the transmissive phase is triggered by a stringent response like mechanism. In *E. coli* the stringent response is induced by a limited amino acid supply to allow a long-term survival in adverse conditions. This includes the rapid arrest of growth and the inhibition of ribosome, protein and stable RNA synthesis by guanosine tetraphosphate (p)ppGpp. The alarmone (p)ppGpp is converted from GTP by the (p)ppGpp synthetase RelA, which in turn is activated by the binding of uncharged transfer RNAs (tRNAs) to ribosomes. As result, the accumulation of the second messenger (p)ppGpp leads to the activation of the stationary-phase σ factor RpoS and the expression of the stationary-phase genes. Thus the cellular (p)ppGpp level inversely correlates with the growth rate and increasing its concentration decreases the steady state growth rate in a defined growth medium (Nazir & Harinarayanan, 2016). By analogy to *E. coli*, in *L. pneumophila* two observations supported the activation of a similar stringent response pathway for the entry into the virulent phase. Firstly, in response to amino acid starvation and upon entry into the post-exponential phase of growth, the bacteria answer with accumulation of the second messenger (p)ppGpp. Secondly, upon expression of the *E. coli relA* gene and in nutrient-independent manner, *L. pneumophila* accumulates (p)ppGpp and activates different virulence traits (Hammer & Swanson, 1999). In support to this hypothesis, genetic data demonstrated that a *L. pneumophila relA* mutant replicates efficiently within amoeba and macrophages and upon exit from the exponential phase, the mutant strain does not accumulate (p)ppGpp and the infectious traits are poorly expressed (Zusman, Gal-Mor, & Segal, 2002). However, the fact that the *relA* mutant displays milder effects on the transmissive traits than those of other regulatory mutants such as *letA* and *rpoS* (described in the following paragraph) suggests that beside the stringent response, additional signals and redundant strategies are employed by *L. pneumophila* to govern its differentiation (Zusman et al., 2002). Accordingly, other factors besides RelA, might trigger the microbial switch to the virulent phase. Indeed, in response to fatty acid biosynthesis perturbations, the bifunctional and essential enzyme SpoT mediates (p)ppGpp turnover via its hydrolase activity and weak synthase activity (Dalebroux,

Svensson, Gaynor, & Swanson, 2010a; Potrykus & Cashel, 2008). Moreover, In *E. coli*, a *relA* and *spoT* double mutant does not lead to the production and accumulation of (p)ppGpp, resulting consequently in a state of rRNA transcription activation and in the synthesis of stable RNA (Paul, Ross, Gaal, & Gourse, 2004; STENT & BRENNER, 1961). By analogy to *E. coli*, the *L. pneumophila* strain depleted of *relA* and *spoT* lacks (p)ppGpp synthetase activity (Dalebroux, Edwards, & Swanson, 2009; Trigui, Dudyk, Oh, Hong, & Faucher, 2015). Taken together, *in vitro* analyses demonstrated that the two (p)ppGpp synthetases confer plasticity to *L. pneumophila*, enabling transition to the transmissive phase when either amino acid or fatty acid synthesis is compromised. In addition, after the conditions are again favorable for establishing a protective vacuole, *L. pneumophila* employs SpoT to hydrolyse (p)ppGpp to tightly regulate the alarmone abundance and reverses the state from the transmissive to the replicative form (Dalebroux et al., 2009).

3.2.2 Transcriptional control by sigma factors

The signaling alarmone (p)ppGpp is a key player at the top of the regulatory network governing the transition of *L. pneumophila* from the replicative state to the transmissive state. Generally, bacteria dealing with changing conditions reorganize their transcriptome in order to activate genes necessary for the new condition and repress the ones no longer required (Ishihama, 2000). This is often achieved by the activity of sigma factors that are essential for modulating gene expression upon entry into a particular state or condition (T. M. Gruber & Gross, 2003; Ishihama, 2000; Navarro Llorens, Tormo, & Martínez-García, 2010). For example, the alternative sigma factor RpoS (σ^S/σ^{38}), the master regulator of the stress response in *E. coli*, is implicated in resistance to heat, high osmolarity, acid and oxidative stress and in the expression of virulence traits (Dong & Schellhorn, 2010). Similarly, *L. pneumophila* RpoS controls multiple pathways associated with motility and pathogenic functions as well as the activity of transcriptional regulators and of Dot/Icm effectors leading to a global influence on intracellular multiplication (Bachman & Swanson, 2001; Hales & Shuman, 1999; Hovel-Miner et al., 2009; Trigui et al., 2015). The accumulation of (p)ppGpp increases the amount of *L. pneumophila* RpoS, however the molecular mechanism remains to be elucidated. Biochemical studies on *E. coli* (p)ppGpp demonstrated that this alarmone, by biasing the competition among sigma factors for the binding to the RNA core polymerase, acts as global regulator of transcription (Farewell, Kvint, & Nyström, 1998; Jishage, Kvint, Shingler, & Nyström, 2002; Laurie et al., 2003). In detail, (p)ppGpp is suggested to destabilize the binding

of the vegetative sigma factor $\sigma^{D/70}$ to the core and endorses the recruitment of alternative sigma factors and the expression of their targets (Hales & Shuman, 1999; Jishage et al., 2002; Molofsky & Swanson, 2004).

Many of the physiological effects of the alarmone (p)ppGpp are mediated in cooperation with the RNA polymerase (RNAP) secondary channel interacting protein DksA (Haugen, Ross, & Gourse, 2008; Potrykus & Cashel, 2008). This protein was shown in *E. coli* and *S. flexneri* to act as cofactor for the (p)ppGpp-dependent transcriptional regulation, activating the transcription of critical virulence regulators and inhibiting some others such as the flagellar regulon (Aberg, Shingler, & Balsalobre, 2008; Lemke, Durfee, & Gourse, 2009; Nakanishi et al., 2006; A. K. Sharma & Payne, 2006). In *E. coli*, DksA can additionally compensate the lack of the alarmone, indicating that DksA works independently of (p)ppGpp (Potrykus & Cashel, 2008). *In vitro* evidences have shown that DksA and (p)ppGpp oppositely regulate some process and promoters (Aberg et al., 2008; Aberg, Fernández-Vázquez, Cabrer-Panes, Sánchez, & Balsalobre, 2009; Magnusson, Gummesson, Joksimović, Farewell, & Nyström, 2007; Łyzen, Kochanowska, Wegrzyn, & Szalewska-Palasz, 2009). Similarly to *E. coli* it has been shown that in *L. pneumophila*, the effect of the second messenger (p)ppGpp and DksA depends on the context. In particular, DksA seems to respond to fatty acid stress to promote the bacterial differentiation in a (p)ppGpp-independent manner, as judged by the expression of flagellin and the evasion of the lysosomal degradation by macrophages (Dalebroux, Yagi, Sahr, Buchrieser, & Swanson, 2010b). However, when the (p)ppGpp levels increase, DksA and (p)ppGpp itself coordinately regulate the hierarchical cascade for flagellar expression (see below). Thus *L. pneumophila* employs both (p)ppGpp and DksA in order to act independently or cooperatively during the bacterial differentiation (Dalebroux, Svensson, Gaynor, & Swanson, 2010a).

One of the most important features of *L. pneumophila* in the late phase of its life cycle is the formation of a flagellum, whose coordinated expression is crucial for efficient and maximal virulence of the bacterium (Molofsky & Swanson, 2004). The flagellar regulon of *L. pneumophila* is composed of four different classes of genes, regulated in a cascade like manner. First the flagellar master regulator and σ^{54} activator protein FleQ together with the alternative sigma factor RpoN (σ^{54}) (class I genes) simultaneously enhance the expression of the class II genes that code for proteins forming the flagellar basal body, hook and regulatory proteins (Albert-Weissenberger et al., 2010). Finally, the flagellar sigma factor FliA (σ^{28})

(encoded by a class III gene and regulated by DksA) is directly controlling the flagellar class IV genes such as *flaA*, encoding the flagellin, and *fliDS*, encoding the filament cap, leading to the complete formation of the flagellum (Albert-Weissenberger et al., 2010; Brüggemann et al., 2006; Dalebroux, Yagi, Sahr, Buchrieser, & Swanson, 2010b; Heuner & Steinert, 2003; Jacobi, Schade, & Heuner, 2004). Moreover, *L. pneumophila* engages the flagellar sigma factor FliA not only for the expression and synthesis of the flagellum, but also for the expression of pathways related to cytotoxicity, lysosome evasion and replication (Heuner, Dietrich, Skriwan, Steinert, & Hacker, 2002). Thus FliA can act both as regulator of virulence factors and together with FleQ and RpoN to coordinately initiate the expression of the flagellum machinery (Hammer et al., 2002; Heuner et al., 2002; Heuner & Steinert, 2003; Molofsky & Swanson, 2004).

3.2.3 Post-transcriptional regulation of the transmissive traits

An additional layer of regulation of the *L. pneumophila* biphasic life cycle occurs at post-transcriptional level. Post-transcriptional regulation is often controlled by two-component systems (TCS), which use protein phosphorylation cascades for signal transduction (J. Stock & Da Re, 2000). TCS control diverse cellular pathways such as chemotaxis, stress and metabolic responses, virulence and differentiation (Mascher, Helmann, & Uden, 2006). Typical TCS are composed of two elements: a membrane-bound sensor histidine kinase, which detects the external signal and a cytoplasmatic protein, which upon activation by the cognate kinase induces the cellular response. The mechanism of activation of the TCS is triggered by the binding of the physiological signal, which leads to a conformational change and the autophosphorylation on a conserved histine residue of the sensor kinase. The cognate response regulator is then stabilized and phosphorylated by the kinase leading to its activation and the subsequent adaptive response (A. H. West & Stock, 2001). Indeed, the TCS LetA/LetS (*Legionella* transmission activator and sensor, respectively) of *L. pneumophila* is an important regulator in its life cycle switch. Originally, the LetA/LetS TCS of *L. pneumophila* was identified in a genetic screen for mutants lacking the flagellum and only later the adaptive response induced by the LetA/LetS pathway was reported to include also the activation of a large set of virulence phenotypes and the control of the progression into the transmissive state (Gal-Mor & Segal, 2003b; Hammer et al., 2002; Lynch, Fieser, Glöggler, Forsbach-Birk, & Marre, 2003). Whether, the alarmone (p)ppGpp directly activates the sensor

histidine kinase LetS is not yet known, however it has been reported that during the stationary phase LetS activates LetA by a multi-step phosphorelay (R. L. Edwards, Jules, Sahr, & Buchrieser, 2010). By analogy to other bacteria harboring this TCS, it was postulated that the *L. pneumophila* LetA/S TCS modulates the carbon metabolism when the conditions are not favorable, which are genes that are regulated in parallel by the small RNA binding protein CsrA (Heeb & Haas, 2001). Indeed, genetic data later demonstrated that the major function of LetA is to relieve the repression exerted by the global regulatory RNA-binder CsrA, ensuring thereby the expression of the transmissive traits (Hovel-Miner et al., 2009; Rasis & Segal, 2009; Sahr et al., 2009). Thus the TCS LetA/LetS has a central role in the regulatory network governing the *L. pneumophila* biphasic life cycle and virulence.

CsrA belongs to a family of global regulators that in *L. pneumophila* repress diverse traits related to virulence, including motility, pigmentation, sodium resistance or cytotoxicity (Fettes, Forsbach-Birk, Lynch, & Marre, 2001; Molofsky & Swanson, 2003). *L. pneumophila* CsrA is essential thus only conditional mutants or partial mutants have been obtained, which are all strongly attenuated for intracellular multiplication. First, four effector-encoding genes have been shown to be regulated by the LetAS-CsrA regulatory cascade (Rasis & Segal, 2009; C. Shi, Forsbach-Birk, Marre, & McNealy, 2006). These four effectors, named RalF, VipA, YlfA and YlfB, are involved in vesicular trafficking during the establishment of the LCV (de Felipe et al., 2008; Franco et al., 2012; Nagai et al., 2002). Recently, by applying a bioinformatics approach and validation by expression analyses, 26 effector-encoding genes have been identified to be regulated by the LetAS-CsrA regulatory cascade, all of which were expressed at higher levels during the stationary phase (Nevo, Zusman, Rasis, Lifshitz, & Segal, 2014), further supporting the important role of CsrA in the expression of the virulent phenotype.

In addition, another TCS, named PmrA/B, has been described to be important for virulence of *L. pneumophila* (Zusman et al., 2007). *L. pneumophila* PmrA/B activates the expression of 43 effector-encoding genes, representing the largest effector regulon known (Al-Khodori, Kalachikov, Morozova, Price, & Abu Kwaik, 2009; Zusman et al., 2007). In addition an interplay between PmrA/B TCS and CsrA in a growth-dependent manner has been reported (Rasis & Segal, 2009), as demonstrated by the direct activation of the CsrA encoding gene by PmrA. Thus, PmrA activates CsrA and consequently post-transcriptional repression of the CsrA-regulated effectors. As such, it is likely that a regulatory switch between two sets of

effectors occurs: one set of effectors, activated by PmrA/B and expressed in the replicative/exponential phase of growth and the second group of effectors which is regulated by LetA/S TCS during the entry into the transmissive/stationary phase of *L. pneumophila*.

Another player in this complex regulatory network, is the Lqs (*Legionella* quorum sensing) system that also plays a role in the regulation of gene expression during the transmissive phase (Spirig et al., 2008). The Lqs system consists of the LqsR response regulator and the LqsS sensor histidine kinase (Tiaden et al., 2007). The regulation is initiated by the production of the diffusible signaling molecule 3- hydroxypentadecan-4-one (*Legionella* auto inducer-1-LAI-1) by the autoinducer synthase LqsA, which is detected by the sensor kinase LqsS, which in turn activates the response regulator LqsR (Spirig et al., 2008; Tiaden et al., 2010). Interestingly, this system contains two histidine kinases, as a homologue of LqsS, named LqsT, was identified and shown to also respond to LAI-1 (Kessler et al., 2013). Although the mechanism of how LqsR affects gene expression is currently not known, it has been reported that LqsR influences the expression of genes involved in virulence including 12 effector-encoding genes (Kessler et al., 2013; Tiaden et al., 2007). Furthermore, LqsR expression depends on the alternative sigma factor RpoS and to a smaller extent on the response regulator LetA (Tiaden et al., 2007). Considering that in *L. pneumophila* the transmissive traits are repressed by CsrA, it is not surprising that the production of LqsR is regulated at the post-transcriptional level by the global repressor CsrA (Sahr et al., 2009). In addition to the abovementioned systems, a fourth TCS, named CpxR/A has been analyzed in *L. pneumophila* (Gal-Mor & Segal, 2003a). CpxR/A was shown to repress few Dot/Icm effector genes and to activate few others (Altman & Segal, 2008; Gal-Mor & Segal, 2003a). Taken together, the study of the complex regulatory system governing the *L. pneumophila* life cycle uncovered four TCS (LetA/S, PmrA/B, LqsR/Q and CpxR/A), sigma factors (RpoS) and RNA-binding proteins (CsrA) as important regulatory elements which act at the post-transcriptional level to fine tune gene expression according to the environmental conditions.

3.2.4 Implication of regulatory sRNAs on *L. pneumophila* virulence

This fine-tuned and hierarchical regulation of the *L. pneumophila* life cycle includes also small RNAs, which ensure a fast and more cost-effective regulation compared to the one controlled by polypeptides (Altuvia, Weinstein-Fischer, Zhang, Postow, & Storz, 1997). Previous evidences in *E. coli* showed that the BarA/UvrY TCS (the *L. pneumophila* LetA/S

homolog) controls the expression of two sRNAs, named CsrB and CsrC. Within these sRNAs GGA motifs were identified, which are the characteristic binding sequences for CsrA. Thus it was thought that such sRNAs might also exist in *L. pneumophila*. Indeed, two homologs of CsrB, named RsmY and RsmZ were identified by a bioinformatics search (P. R. Kulkarni, Cui, Williams, Stevens, & Kulkarni, 2006). Functional analyses confirmed that these sRNAs were the missing regulatory elements between the LetA/S TCS and the RNA binding protein CsrA in *L. pneumophila* and that LetA induces the expression of these small RNAs (Rasis & Segal, 2009; Sahr et al., 2009). Deep RNA sequencing from exponentially (replicative) and post exponentially (virulent) *in vitro* grown *L. pneumophila* have identified more than 700 sRNAs, 60% of which are growth-phase dependently regulated, suggesting that a set of these yet uncharacterized sRNAs, coordinately with RsmY/Z/X might regulate gene expression and influence the expression of virulence determinants (Sahr et al., 2012). Probably Hfq, acting as RNA chaperone regulates a number of these sRNAs implicated in bacterial virulence.

Indeed, Hfq seems to play a role in this regulatory network. However, to date little is known of the functional role and the regulation of *L. pneumophila* Hfq. First results published in 2005 suggested that Hfq plays a role the iron uptake, and that it interacts not only with the exponential gene *fur*, but also with the global regulator gene *csrA*, influencing thus genes under the control of this RNA-binding protein. The authors reported that the *hfq* transcript is highly expressed in the mid-exponential phase of growth and that it is under the control of the sigma factor RpoS. By contrast, *hfq* transcription was shown to be down regulated in the stationary phase of *L. pneumophila* growth probably due to a regulatory role of the two-component regulator LetA (McNealy et al., 2005). Recently, Tiaden and colleagues have shown that the quorum sensing regulator LqsR, influences the expression of *hfq* upon entry into the stationary growth phase, as *hfq* expression was upregulated in an *lqsR* mutant strain (Tiaden et al., 2007). Thus *L. pneumophila* Hfq seems also to play a role in the regulatory network governing *L. pneumophila* differentiation from the replicative/non infectious to the transmissive/infectious phase.

3.2.5 Regulatory network governing *L. pneumophila* bi-phasic life cycle

The present view on the regulatory cascade governing *L. pneumophila* virulence is as follows: Upon entry into the post-exponential phase, the detection of uncharged tRNAs due to amino acid starvation leads to the activation of RelA, whereas SpoT recognizes fatty acid starvation.

Together RelA and SpoT activation leads to the synthesis of (p)ppGpp which by an unknown mechanism results in the activation of the LetA/S system. LetA binds directly to a conserved consensus sequence upstream the *rsmX/Y/Z* genes, leading to their expression (Rasis & Segal, 2009; Sahr et al., 2009). These sRNAs contain multiple repeated CsrA binding motifs and act as sponge to bind and sequester CsrA from their target mRNAs, leading to the expression of virulence traits. The expression of RsmX, RsmY and RsmZ is strongly reduced in *letS* or *rpoS* deletion mutants, suggesting that these two regulators might coordinately regulate expression of these sRNAs positively (Hovel-Miner et al., 2009; Rasis & Segal, 2009). Moreover, it is important to mention that although Hfq was reported to influence *L. pneumophila* differentiation by interacting with the major regulatory elements of the cascade, it is expected that Hfq, acting as RNA chaperone and RNA binder might regulate a number of sRNAs implicated in bacterial virulence (Figure 11). However, the role of Hfq in this regulatory network is not known yet. Taken together, the expression of *L. pneumophila* virulent determinants is fine-tuned both at the transcriptional and post-transcriptional level, by a complex regulatory network ensuring a successful infection cycle.

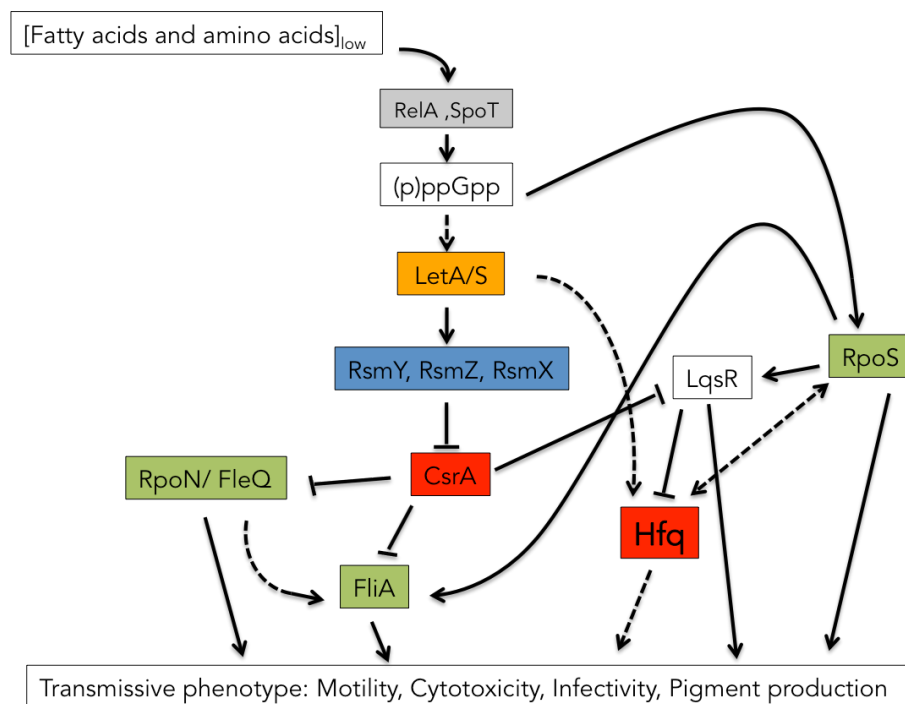


Figure 11. Schematic representaion of the regulatory network controlling the *L. pneumophila* life cycle. During transmissive phase, amino acid starvation triggers the ppGpp synthetase RelA and fatty acid starvation triggers the ppGpp synthetase/hydrolase SpoT to produce the alarmone (p)ppGpp. (p)ppGpp may be sensed by the sensor kinase LetS which then phosphorylates LetA. Phosphorylated LetA binds upstream of the small ncRNAs RsmX, RsmY and RsmZ and activates their transcription. CsrA that is bound near the ribosomal binding site of its mRNA targets inhibits their translation. RsmX, RsmY and RsmZ titrate CsrA away from its targets, enabling translation of the mRNAs, leading to expression of transmissive phenotypes. Hfq also influences the different phenotypes, however its exact role is not known.

Aim of the Ph.D. thesis

The elucidation of the complex regulatory network and its components that govern the *L. pneumophila* differentiation from the replicative phase to the transmissive phase is a major focus of *Legionella* research. Deep RNA sequencing analysis comparing the transcriptional profiles of *L. pneumophila* grown at the exponential (replicative) and post- exponential (virulent) phases defined the complete operon map of this pathogen and the presence of more than 700 sRNAs, many of which are growth phase dependently regulated (Sahr et al., 2012). As reported for many pathogenic bacteria, a key factor in the regulation of small regulatory RNAs is the global posttranscriptional regulator Hfq. This RNA binder and chaperone is also growth phase-dependently regulated in *L. pneumophila*, however how it is regulated is not known. Our transcriptional start site mapping of the *L. pneumophila* genome lead to the identification of a putative sRNA transcribed at the antisense strand that overlaps the 5' untranslated region of the *hfq* transcript including its ribosome binding site (Sahr et al., 2012). This sRNA, named Anti-hfq seemed to be more abundant in the replicative phase and its expression decreased at the beginning of the transmissive phase.

Based on these findings the aim of this work was

- I) to elucidate how the growth phase dependent expression of Hfq is regulated in *L. pneumophila*;
- II) to analyze the role of Hfq and its newly identified putative antisense RNA in *L. pneumophila* virulence;
- III) to decipher the regulatory mechanism by which this sRNA functions.

In the first part of the thesis a *L. pneumophila* deletion mutant of the *hfq* gene and a knock-down mutant of the sRNA were constructed and investigated by applying cell biological assays to characterize their regulation and intracellular growth phenotypes.

In the second part of the thesis RNA binding and stability assays were performed to provide insight into the mechanism of regulation by which the sRNA regulates Hfq expression.

CHAPTER FOUR

RESULTS- REGULATION OF HFQ IN *L. PNEUMOPHILA*

4.1 *L. pneumophila* *hfq*- chromosomal organization

In *L. pneumophila* *lpp0009* encodes Hfq, an 85 amino acid protein. It is part of an operon together with the gene *lpp0010*, putatively coding a GTP-binding protein HflX (Figure 12A). Hfq protein has homologs in all the *L. pneumophila* genomes sequenced to date, with at least 80% of conservation. Our transcriptional starting site analysis revealed the presence of a putative small RNA, which is divergently transcribed on the antisense strand of the *hfq* gene and overlaps its 5' UTR region as shown in the Figure 12.

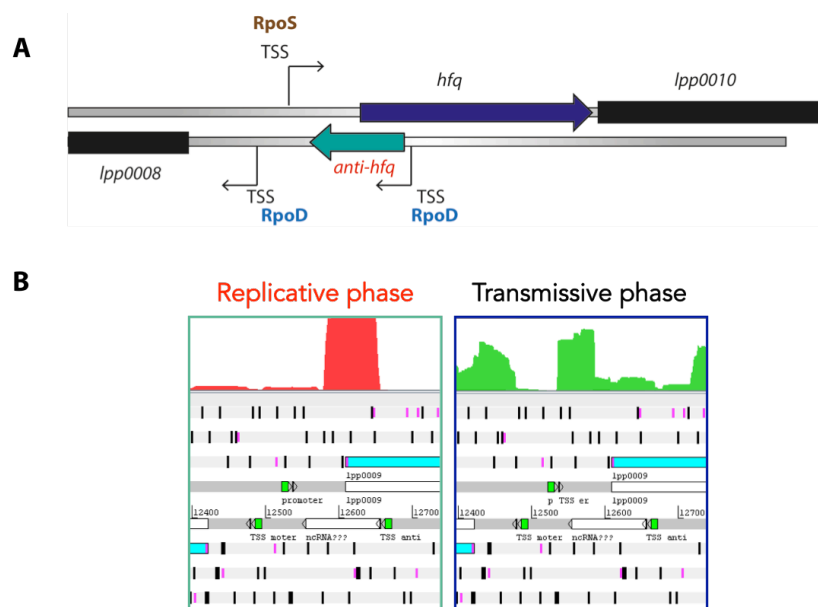


Figure 12. Schematic representation of the *L. pneumophila* *hfq* and *anti-hfq* locus. (A) Schematic representation of the transcriptional starting site (TSS) of the *hfq* and *anti-hfq* gene of *L. pneumophila* strain Paris. (B) Representation of the deep sequencing data obtained from bacteria cultured at the replicative phase of growth (OD=2; left panel) and transmissive phase of growth (OD=4; right panel), indicating when the two transcripts are expressed. Red colour, transcription of the sRNA *anti-hfq* during the replicative phase; green colour, expression of the *hfq* transcript during the transmissive phase.

Further analysis of the deep sequencing data performed from bacteria grown at the replicative phase and transmissive phase indicated that the *hfq* transcript is highly expressed in transmissive phase and less in replicative phase, whereas the sRNA, later named Anti-hfq, seems to be highly abundant in replicative phase and less in transmissive phase of growth. Thus, we hypothesized that the putative sRNA might act as a *cis*- encoded sRNA, regulating the expression of the *hfq* transcript in a growth phase-dependent manner.

4.2 Article published in the journal mBIO

A Unique *cis*-Encoded Small Noncoding RNA Is Regulating *Legionella pneumophila* Hfq Expression in a Life Cycle- Dependent Manner

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Running Head: An antisense RNA regulates Hfq in *L. pneumophila*

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ABSTRACT *Legionella pneumophila* is an environmental bacterium that parasitizes protozoa, but it may also infect humans, thereby causing a severe pneumonia called Legionnaires' disease. To cycle between the environment and a eukaryotic host, *L. pneumophila* is regulating the expression of virulence factors in a life cycle-dependent manner: replicating bacteria do not express virulence factors, whereas transmissible bacteria are highly motile and infective. Here we show that Hfq is an important regulator in this network. Hfq is highly expressed in transmissible bacteria but is expressed at very low levels in replicating bacteria. A *L. pneumophila* *hfq* deletion mutant exhibits reduced abilities to infect and multiply in *Acanthamoeba castellanii* at environmental temperatures. The life cycle-dependent regulation of Hfq expression depends on a unique *cis*-encoded small RNA named Anti-*hfq* that is transcribed antisense of the *hfq* transcript and overlaps its 5' untranslated region. The Anti-*hfq* sRNA is highly expressed only in replicating *L. pneumophila* where it regulates *hfq* expression through binding to the complementary regions of the *hfq* transcripts. This results in reduced Hfq protein levels in exponentially growing cells. Both the small noncoding RNA (sRNA) and *hfq* mRNA are bound and stabilized by the Hfq protein, likely leading to the cleavage of the RNA duplex by the endoribonuclease RNase III. In contrast, after the switch to transmissible bacteria, the sRNA is not expressed, allowing now an efficient expression of the *hfq* gene and consequently Hfq. Our results place Hfq and its newly identified sRNA anti-*hfq* in the center of the regulatory network governing *L. pneumophila* differentiation from nonvirulent to virulent bacteria.

IMPORTANCE The abilities of *L. pneumophila* to replicate intracellularly and to cause disease depend on its capacity to adapt to different extra- and intracellular environmental conditions. Therefore, a timely and fine-tuned expression of virulence factors and adaptation traits is crucial. Yet, the regulatory circuits governing the life cycle of *L. pneumophila* from replicating to virulent bacteria are only partly uncovered. Here we show that the life cycle-dependent regulation of the RNA chaperone Hfq relies on a small regulatory RNA encoded antisense to the *hfq*-encoding gene through a base pairing mechanism. Furthermore, Hfq regulates its own expression in an auto-regulatory loop. The discovery of this RNA regulatory mechanism in *L. pneumophila* is an important step forward in the understanding of how the switch from inoffensive, replicating to highly virulent, transmissible *L. pneumophila* is regulated.

In recent years, the discovery of a class of regulatory elements, called small noncoding RNAs (sRNAs) revealed a high complexity of posttranscriptional gene regulation in prokaryotes and eukaryotes (1). sRNAs were reported to exert a wide range of cellular functions in bacterial physiology, in which rapid and fine-tuned adaptations in response to environmental changes are required (2, 3). sRNAs are classified as *cis*- or *trans*-

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encoded sRNAs that modulate gene expression through complementarity to their adjacent or distant mRNA targets, respectively. In bacteria, *trans*-encoded sRNAs commonly require the assistance of the RNA chaperone Hfq to promote their interaction with the cognate mRNA targets. Although *cis*-encoded sRNAs share extended base pairing complementarity to their counterpart mRNAs, in a few cases, Hfq is required for their function (4). First identified in *Escherichia coli* as a host factor essential for the replication of the Q β RNA phage, Hfq is now recognized as a global regulator of gene expression present in a wide variety of bacteria that impacts many molecular processes in bacterial physiology, stress response, and virulence (5, 6). The importance of the RNA-binding protein Hfq was uncovered by the characterization of *hfq* null mutants in diverse bacterial pathogens (7, 8). Further detailed research in its function in different bacteria showed that Hfq is a key posttranscriptional regulator, stabilizing sRNAs or facilitating sRNA/mRNA interactions that inhibit or enhance translation initiation. Furthermore, Hfq can act independently to modulate gene expression by affecting mRNA translation (for reviews, see references 6 and 9). Although deep sequencing approaches have revealed a high number and broad spectrum of sRNAs in diverse pathogens, such as *Salmonella enterica* serotype Typhimurium (10), *Pseudomonas aeruginosa* (11), *Yersinia pseudotuberculosis* (12), or *Legionella pneumophila* (13), the extent of Hfq-mediated riboregulation is highly complex and variable for each RNA type and in each organism. Furthermore, Hfq-associated sRNAs have been reported to control gene expression of multiple targets, thus regulating diverse cellular pathways, such as biofilm formation (14), catabolite repression (15), quorum sensing (16), or the control of transcriptional factors (17). Hfq is closely related to the Sm family of RNA-binding proteins in archaea and eukaryotes and phylogenetically widespread among bacteria, as about half of the sequenced bacterial genomes harbor at least one copy of the *hfq* gene (4, 18).

Legionella pneumophila is an intracellular bacterium that inhabits environmental aquatic systems, like lakes and rivers where it replicates in aquatic protozoa, but it can also infect humans to cause a severe pneumonia, and it also carries a gene that encodes Hfq (19, 20). However, little is known about the role of Hfq in the *L. pneumophila* life cycle or its regulatory function. The change between extra- and intracellular life and between replication in a host (replicative phase) and transmission to a new host (transmissive/virulent phase) demands a highly fine-tuned regulatory network (21). Indeed, the life cycle switch from replicating to transmissive/virulent *L. pneumophila* is governed through the function of several key regulators. Probably the most important ones are the two-component system (TCS) LetA/LetS (*Legionella* transmission activator and sensor, respectively) that induces traits necessary for efficient host transmission (22–24) and CsrA (carbon storage regulator) that is a posttranscriptional regulator, repressing transmissive/virulent traits during replication of *L. pneumophila* and releasing them in later stages of infection (25, 26; T. Sahr, C. Rusniok, F. Impenes, G. Oliva, O. Sismeyro, J. Y. Coppee, and C. Buchrieser, unpublished data). Moreover, the three sRNAs RsmX, RsmY, and RsmZ that are sequestering CsrA in transmissive phase to allow virulence traits to be translated are indispensable in this regulatory cascade (27, 28).

Here we report that *L. pneumophila* Hfq is regulated in a life cycle-dependent manner by a unique sRNA, named Anti-*hfq* that is transcribed in the early phase of the *L. pneumophila* life cycle. Our data support a complex model of regulation of the *hfq* transcript by the Anti-*hfq* sRNA, in which the Hfq chaperone together with RNase III are engaged to ensure the growth phase-dependent expression of this RNA-binding protein. Moreover, our results show that Hfq affects intracellular multiplication in amoebae, and consequently *L. pneumophila* virulence.

RESULTS

Hfq is highly conserved within the genus *Legionella* and other bacterial species. In *L. pneumophila*, Hfq is an 85-amino-acid protein encoded by the gene *lpp0009*. The *hfq* gene is organized in an operon with the putative GTP-binding protein HflX encoded by gene *lpp0010* (Fig. 1A). Although the *L. pneumophila* *hfq* gene shares the conserved chromosomal gene arrangement typical of other organisms like *E. coli* or

An Antisense RNA Regulates Hfq in *L. pneumophila*

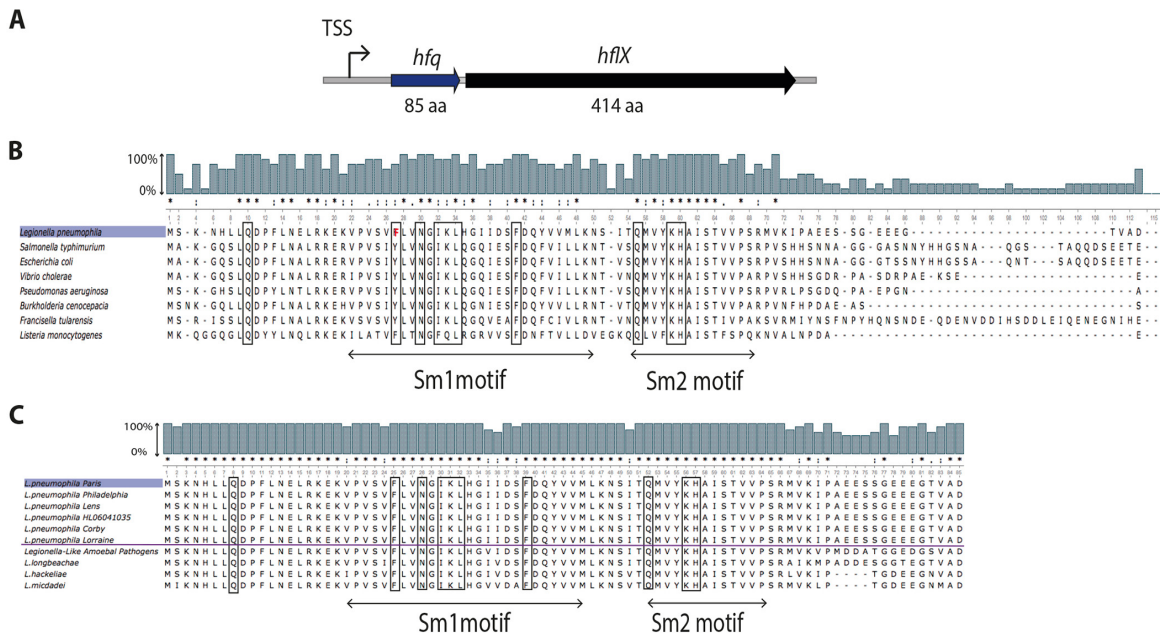


FIG 1 *Legionella* Hfq is conserved across the genus and other bacterial species. (A) Schematic organization of the *L. pneumophila* *hfq* locus. TSS, transcription start site; aa, amino acids. (B) Alignment of the *L. pneumophila* Hfq protein sequence with other bacterial Hfq protein sequences reveals high sequence and RNA binding site conservation. (C) Alignment of the *L. pneumophila* Paris Hfq protein sequence with the Hfq protein sequences from different *L. pneumophila* strains and other *Legionella* or *Legionella*-like species. Amino acids involved in RNA binding are boxed. Conserved amino acid residues (asterisks) and semiconservative substitutions (dots) and conservative substitutions (colons) are indicated. The bars above the sequence alignment indicate the sequence percentage of sequence conservation.

Vibrio cholerae only partly, it shows high nucleotide and amino acid identity with Hfq of many Gram-negative bacteria (up to 70%) and Gram-positive bacteria (up to 50%). Furthermore, all residues that contribute to RNA binding are conserved in *L. pneumophila* (Fig. 1B). Comparison of the Hfq amino acid sequence among more than 300 *L. pneumophila* strains sequenced in the last years (19, 29–32) revealed that Hfq is 100% conserved across the different *L. pneumophila* strains. Analyses of four non-*pneumophila* *Legionella* species (33) showed that Hfq is 80% conserved (Fig. 1C).

Hfq is highly expressed during postexponential/transmissive growth phase. In several pathogens, the level of expression of Hfq is growth phase dependent. In order to assess the transcriptional and posttranscriptional level of Hfq at different growth phases, we performed Northern and Western blot analyses of total RNA and whole protein lysates obtained from cultures of *L. pneumophila* (wild type [wt]) grown in liquid medium at 37°C. Northern blots using an *hfq*-specific probe showed very low *hfq* transcripts during exponential growth (optical density at 600 nm [OD₆₀₀] of 2), but high transcript levels upon entry into postexponential growth (OD₆₀₀ of 4). Protein expression followed the same pattern as shown by immunoblotting using anti-Hfq antibodies (Fig. 2A).

Hfq is necessary for efficient intracellular replication at environmental temperatures. In order to analyze the role and regulation of Hfq of *L. pneumophila*, we constructed an *hfq* deletion mutant (Δhfq) by the insertion of an in-frame apramycin resistance cassette (Fig. 2B). The resistance cassette used does not contain a transcriptional terminator; thus, transcription of the downstream gene, *hflX*, was not negatively affected as verified by transcriptome analyses (described below). Furthermore, the Δhfq mutant strain was completely sequenced using the Illumina technique, which ascertained that no secondary mutations had been introduced during the mutant construction. Analyses of the Δhfq mutant confirmed that the expression of Hfq was indeed abolished (Fig. 2C). To complement the Δhfq mutant, a plasmid harboring the entire *hfq* gene and its own promoter was transformed into the Δhfq mutant, generating the

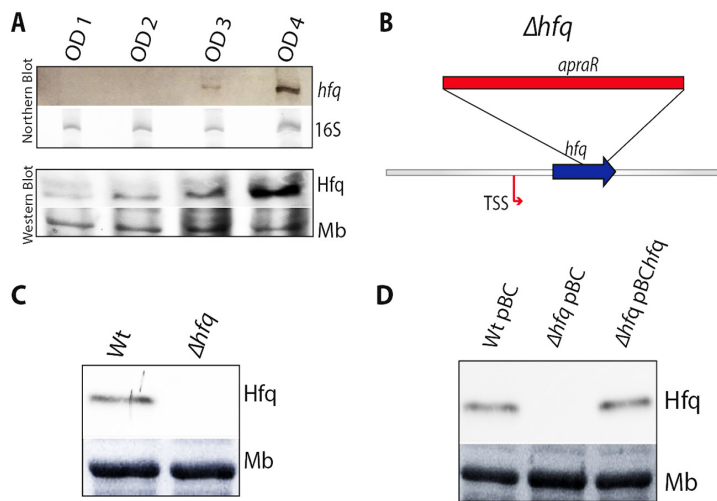


FIG 2 Transcript and protein expression of *hfq* are growth phase dependently regulated. (A) Northern blot and Western blot analyses of bacterial lysates from wild-type *L. pneumophila* Paris strain during growth (OD₆₀₀s of 1, 2, 3, and 4) using an *hfq* probe and anti-Hfq antiserum, respectively. 16S RNA and the stained membrane (Mb) signals are shown as loading controls. (B) Schematic representation of the insertion of the apramycin resistance cassette (*apraR*) in the Δhfq mutant. (C) Detection of Hfq by Western blotting in the wild-type (wt) and Δhfq mutant strains grown to an OD₆₀₀ of 4. (D) Detection of Hfq by Western blotting in the wild-type, Δhfq mutant, and complemented strain Δhfq pBChfq (Wt and Δhfq carrying the empty plasmid pBC-KS) grown to an OD₆₀₀ of 4.

complemented strain Δhfq pBChfq. Western blot analyses using anti-Hfq antibodies confirmed the expression of Hfq in Δhfq pBChfq (Fig. 2D). In contrast to a previous report where the Δhfq mutant in another *L. pneumophila* strain showed a prolonged lag phase (20), the growth pattern of the Δhfq mutant analyzed here was very similar to that of the wt strain at 37°C (see Fig. S1A in the supplemental material) and at 20°C (Fig. S1B), indicating that the growth defect of a strain lacking Hfq is due to the intracellular environment in amoeba, and not to a general growth defect at lower temperatures.

To learn whether Hfq is implicated in virulence of *L. pneumophila* as reported for other bacterial pathogens, we compared the ability of the wt *L. pneumophila* and Δhfq mutant to infect and multiply in *Acanthamoeba castellanii* and in the human monocyte-derived cell line THP-1. Similarly to what was reported previously, the Δhfq mutant strain showed only a minimal growth defect in *A. castellanii* and THP-1 cells at 37°C (Fig. 3A and B). In contrast, when replication in *A. castellanii* was monitored at 20°C, the Δhfq mutant showed a clear replication defect compared to the wt strain (Fig. 3C). Furthermore, complementation of the Δhfq mutant restored the intracellular replication pattern (Fig. 3D). Taken together, our data imply that Hfq plays a role in intracellular replication in amoeba at environmental temperatures and thus on the virulence of *L. pneumophila*.

Hfq expression is affected by RpoS and LetA. The activation of virulence traits of *L. pneumophila* is highly regulated at the transcriptional and posttranscriptional levels. Major regulators implicated are the sigma factor RpoS and the two-component system LetA/LetS (21) (Fig. 4D). Hfq is another candidate, as the mutant showed a replication defect (Fig. 3C). To determine the role and place of Hfq in this regulatory network, we analyzed the *hfq* transcript and protein levels in *rpoS* and *letA* mutants. Northern blot analysis showed that *hfq* transcripts were abolished in $\Delta rpoS$ and $\Delta letA$ mutants, confirming that RpoS and LetA are implicated in the regulation of *hfq* expression (Fig. 4A). This was also reflected in the protein level, as observed by immunoblot analysis, where Hfq expression in the $\Delta rpoS$ and $\Delta letA$ mutants was strongly decreased compared to the Hfq levels in the wt strain (Fig. 4B). Thus, RpoS and LetA are strongly

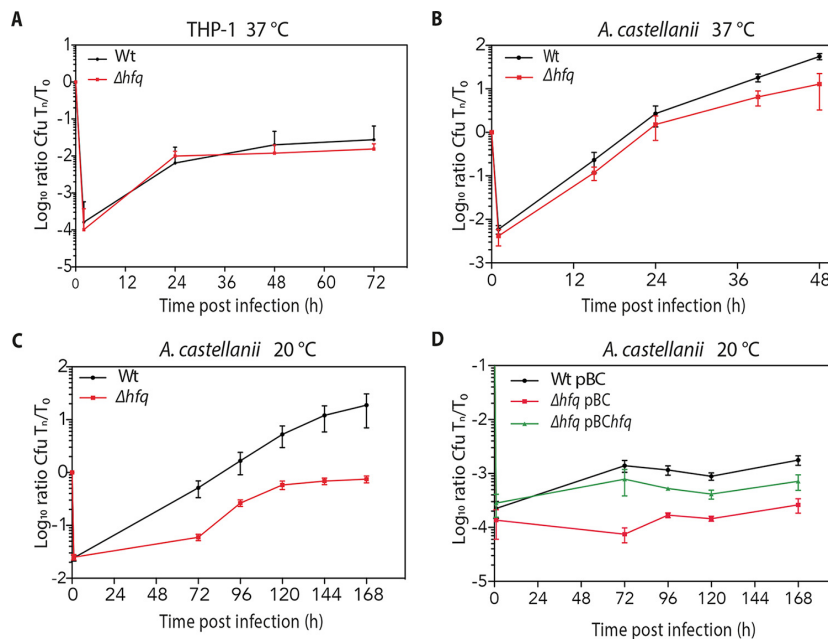


FIG 3 Efficient intracellular replication of *L. pneumophila* in *A. castellanii* and THP-1 macrophages is dependent on functional Hfq. (A) THP-1 cells were infected with wt and Δhfq mutant strains at an MOI of 10 at 37°C. The number of intracellular bacteria was monitored for 72 h, revealing a slightly diminished replication of the Δhfq mutant compared to the wt. (B and C) Monolayers of *A. castellanii* were infected with wt and Δhfq strains at an MOI of 0.1 at 37°C (B) and at an MOI of 1 at 20°C (C), showing a slight growth defect of the Δhfq mutant at 37°C but a clear defect at 20°C. (D) Infection of *A. castellanii* with the complemented Δhfq pBC $Chfq$ strain at an MOI of 1 at 20°C, showing complementation of the growth phenotype. The wt strain carrying plasmid pBC-KS, the Δhfq strain carrying the empty plasmid, and complemented strain Δhfq pBC $Chfq$ were examined. The number of intracellular bacteria was determined by recording the number of CFU per milliliter. Results are expressed at \log_{10} ratio of CFU at T_t/T_0 . Each time point represents the mean \pm standard deviation (SD) (error bar) from at least three independent experiments.

implicated in the regulation of Hfq expression at the transcript and protein levels. Flagella and consequently motility are hallmarks of the transmissive/virulent phase of *L. pneumophila*. We thus analyzed FlaA expression in the Δhfq mutant strain and the $\Delta rpoS$ and $\Delta letA$ mutants in which FlaA expression is known to be reduced. As expected, FlaA was highly expressed in the late postexponential phase in the wt but strongly reduced in the Δhfq mutant strain, suggesting its involvement in the regulatory cascade governing *L. pneumophila* differentiation, motility, and virulence (Fig. 4C). Taken together, the expression of Hfq in *L. pneumophila* is regulated in a growth phase-dependent manner and is influenced by RpoS and LetA. Furthermore, Hfq itself seems to be implicated in the activation of traits typical of the transmissive/virulent phase of *L. pneumophila*.

Transcriptome analyses of the Δhfq mutant strain reveal only few changes in gene expression. To analyze which genes Hfq is affecting that may lead to the decreased intracellular replication, transcriptome analysis at postexponential growth (OD_{600} of 4 grown *in vitro* in BYE medium and *in vivo* after 96 h of infection of *A. castellanii*) when Hfq is expressed the highest was performed. The comparison of the wt and Δhfq mutant transcriptomes *in vitro* identified only 18 differentially expressed genes (Table S1). This is in accordance with an *in vitro* transcriptome analysis of an *hfq* mutant in strain *L. pneumophila* JR32, where only a few genes and a mobile genetic element that excised upon the deletion of *hfq* were differentially expressed (34). *In vivo*, 74 genes were differentially transcribed due to the loss of Hfq, the majority of which (69 genes) was upregulated in the absence of Hfq, whereas only five genes were downregulated (Table S2). Interestingly, CsrA (0.43 \times), a major regulator of metabolic and regulatory functions during replication (Sahr et al., unpublished) was downregulated *in*

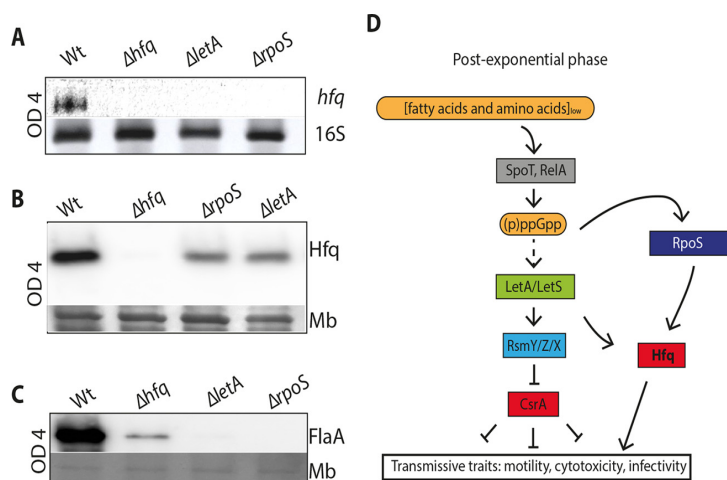


FIG 4 *hfq* transcript and protein expression are influenced by LetA and RpoS and impact flagellar expression. (A) Northern blot analyses of *hfq* transcripts in wt *L. pneumophila* and the Δhfq , $\Delta letA$, and $\Delta rpoS$ regulatory mutants grown until they reached an OD₆₀₀ of 4 show that the *hfq* transcript is under the control of LetA and RpoS and is abolished in the Δhfq mutant. (B) Western blot analysis of Hfq protein levels in wt *L. pneumophila* and Δhfq , $\Delta letA$, and $\Delta rpoS$ mutants grown until an OD₆₀₀ of 4 revealed a significantly decreased expression of Hfq in the regulatory mutants, indicating that RpoS and LetA influence Hfq expression. (C) Western blot analysis of FlaA protein levels in wt *L. pneumophila* and Δhfq , $\Delta letA$, and $\Delta rpoS$ mutants grown until an OD₆₀₀ of 4 revealed that expression of FlaA is strongly decreased in the Δhfq mutant and as expected missing in the $\Delta letA$ and $\Delta rpoS$ mutants, suggesting that Hfq also influences flagellar expression. Mb, stained membrane signal as a loading control. (D) Schematic overview of the major regulatory elements governing *L. pneumophila* virulence expression in transmissive/postexponential phase and the place and role of Hfq in this network.

vivo. In contrast, no effect of Hfq on other important regulators like RpoS or the two-component system LetA/LetS was seen on the transcript level, indicating no direct feedback cascade for this regulatory pathway. In total, eight genes were upregulated both *in vitro* and *in vivo*. Two of these genes are involved in flagellar assembly and motility (*flgG* and *flgH*), and two are coding for the enhanced entry protein EnhA (*lpp2693*) and EnhB (*lpp2694*), which are implicated in host cell infection (35). Additionally, the macrophage infectivity potentiator Mip, at least four Dot/Icm effector proteins, transcriptional regulators Fis1 and Fis2, and the DNA-binding protein HU-beta are differentially transcribed in the Δhfq mutant during *in vivo* growth. These data might suggest a direct influence of Hfq on virulence formation as seen in infection of *A. castellanii*.

An antisense RNA is present in the 5' untranslated region of *hfq*. We had previously established a complete transcriptional map of the *L. pneumophila* genome that revealed the presence of a dynamic pool of sRNAs regulated in a growth phase-dependent manner (13). Among these sRNAs, we identified a transcriptional start site (TSS) of a noncoding gene located in the reverse strand of the 5' untranslated region (5' UTR) of the *hfq* gene (Fig. 5A). In order to confirm experimentally the presence of a sRNA, we performed 3' rapid amplification of cDNA ends (RACE), which yielded only a single band around 100 bp from RNA samples isolated from a culture grown at the early exponential phase (OD₆₀₀ of 2). Cloning and sequencing of this cDNA amplicon that we named Anti-*hfq* showed that the noncoding RNA is 101 bp long (Fig. 5B and Fig. S2A). Using the program Mfold (36), the anti-*hfq* secondary structure was predicted to be composed of a duplex, with a 5' overhang of 1 nucleotide (5' C) and a 3' overhang of 3 nucleotides (3' UUA) containing a putative Rho-independent terminator identified by FindTerm (Softberry) (Fig. 5C). Although other programs did not confirm this terminator structure, the RACE PCR results showed that the transcript terminated at 101 bp where FindTerm predicted the terminator; hence, under the given conditions, Anti-*hfq* is indeed an sRNA. Bioinformatic analysis revealed the presence of an identical

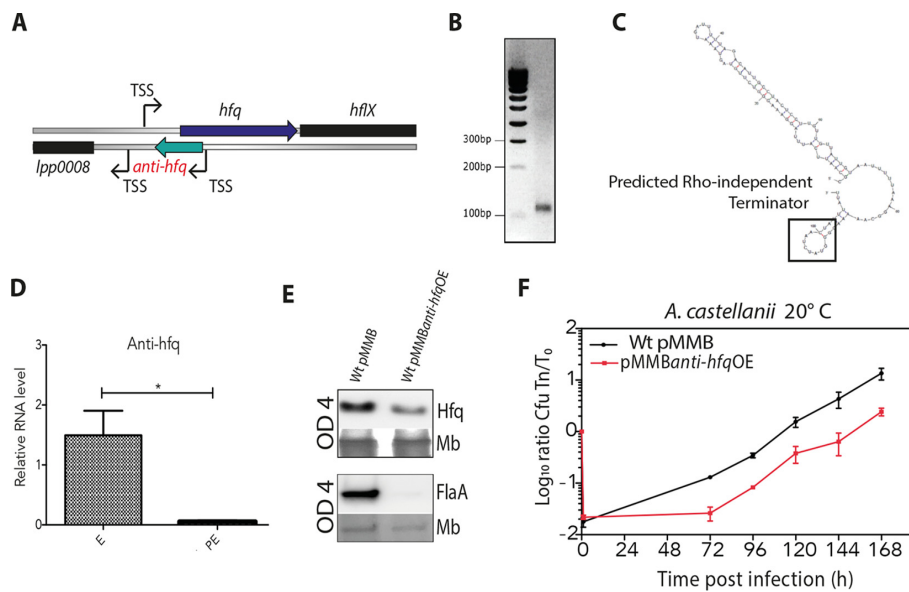


FIG 5 A small noncoding RNA named Anti-*hfq* is expressed antisense to *hfq* and influences Hfq expression and intracellular replication. (A) Schematic organization of the chromosomal organization of the *L. pneumophila* *hfq* and *anti-hfq* locus. (B) 3' RACE PCR product in a 2% agarose gel obtained from exponentially grown wt *L. pneumophila* confirms the presence of an sRNA of 101 bp, named Anti-*hfq*. (C) Structure of the Anti-*hfq* sRNA of *L. pneumophila* as predicted by the program FindTerm. (D) qPCR analyses of the expression of Anti-*hfq* in the wt strain grown to exponential (E) phase and to postexponential (PE) phase, showing that Anti-*hfq* is expressed about 1.5 times in the E phase and 0.05 in the PE phase normalized to an OD₆₀₀ of 1. *gyrB* and *tldD* were used as internal controls for normalization. Each time point represents the mean plus standard deviation from three independent experiments. The means for the wt strain at the E and PE phases were statistically significantly different ($P < 0.05$) by the *t* test as indicated by the bar and asterisk. (E) The anti-*hfq* sRNA influences Hfq and FlaA protein expression as evaluated by Western blotting analysis using the anti-Hfq or anti-FlaA antisera and lysates of wt and Anti-*hfq*-overexpressing (pMMBanti-*hfq*OE) strains grown to an OD₆₀₀ of 4. Membrane (Mb) signals are shown as loading controls. (F) Infection of *A. castellanii* with the pMMBanti-*hfq*OE strain shows a similar growth defect as the *hfq* mutant strain, indicating a role in intracellular replication. Monolayers of *A. castellanii* were infected with wt and the pMMBanti-*hfq*OE strain at an MOI of 1 at 20°C. Intracellular replication was determined by recording the number of CFU per milliliter. Results are expressed in log₁₀ ratio CFU T_n/T_0 . Each time point represents the mean \pm SD from three independent experiments.

anti-*hfq* sequence among all *L. pneumophila* strains investigated. Anti-*hfq* homologues were also found among other *Legionella* species with a sequence identity of at least 80%, but no homologous sequences were found in other bacterial genomes. Thus, Anti-*hfq* represents a unique sRNA element within the genus *Legionella*.

Anti-*hfq* is expressed at the early exponential phase of the *Legionella* growth cycle. To determine the pattern of the Anti-*hfq* transcripts during the *L. pneumophila* life cycle, total RNA was extracted at exponential growth (OD₆₀₀ of 1) and postexponential growth (OD₆₀₀ of 4) of wt *L. pneumophila* grown in liquid BYE medium. The total RNA was reverse transcribed, and quantitative PCR (qPCR) analysis on the obtained cDNA was performed. We used different primer pairs: primer pair 1 (*hfq*-qPCR-F [F stands for forward] and *hfq*-qPCR-R [R stands for reverse]) exclusively recognizing the *hfq* mRNA and primer pair 2 (anti-*hfq*-qPCR-F and anti-*hfq*-qPCR-R) recognizing both the *hfq* and Anti-*hfq* RNAs, as these two transcripts entirely overlap (Fig. 5A). To confirm the growth phase-dependent expression of Anti-*hfq*, we calculated the ratio between the *hfq* and Anti-*hfq* transcript levels in the two growth phases. This showed that in the exponential phase, the Anti-*hfq* transcript was expressed about 1.5-fold higher than the *hfq* transcript, whereas its expression levels decreased to 0.05-fold compared to *hfq* in the postexponential phase (Fig. 5D). This alternative expression of either *hfq* or Anti-*hfq* suggests a regulation in which the expression of the Anti-*hfq* transcript might inhibit the expression of the sense transcript due to the *cis* regulatory function of the Anti-*hfq* sRNA.

Anti-*hfq* affects intracellular replication. To analyze whether the Anti-*hfq* sRNA indeed impacts Hfq expression levels, we first constructed a strain overexpressing

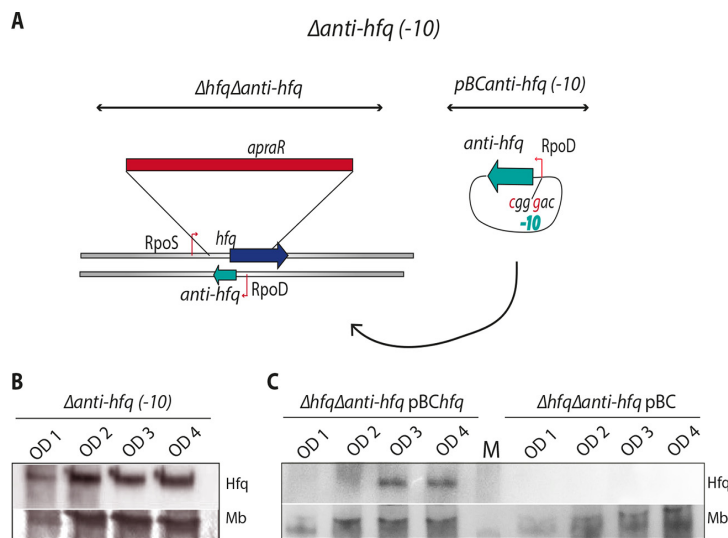


FIG 6 In an *anti-hfq* mutant, Hfq is already expressed during exponential growth. (A) Schematic presentation of the $\Delta hfq \Delta anti-hfq$ mutant and sequence changes introduced in the *anti-hfq* promoter region to construct the $\Delta anti-hfq(-10)$ mutant without disrupting the Hfq amino acid sequence. (B) The Anti-*hfq* sRNA influences Hfq protein expression as evaluated by Western blot analysis of Hfq in the $\Delta anti-hfq(-10)$ mutant strain. Stained membrane (Mb) signals are shown as a loading control. (C) Western blot analysis of Hfq protein levels in the $\Delta hfq \Delta anti-hfq$ mutant complemented with *hfq* and *anti-hfq* ($\Delta hfq \Delta anti-hfq$ pBChfq) shows that the growth phase-dependent Hfq expression pattern is restored. In contrast, the control strain carrying the empty plasmid ($\Delta hfq \Delta anti-hfq$ pBC) does not express Hfq. Stained membrane (Mb) signals are shown as loading control. M, molecular weight marker.

Anti-*hfq* sRNA, in which the *anti-hfq* gene was cloned under the control of an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible promoter. Upon induction with IPTG, the overexpression of the Anti-*hfq* sRNA decreased Hfq expression levels compared to the wt (Fig. 5E, top blot), supporting the idea that Anti-*hfq* sRNA is able to directly regulate Hfq expression. As the deletion of *hfq* resulted in a strongly decreased flagellin expression (Fig. 4C), we postulated that the overexpression of the Anti-*hfq* sRNA should also impact flagellin expression via the repression of Hfq. Indeed, when the Anti-*hfq* sRNA was overexpressed, the expression of FlaA was strongly reduced compared to the wt strain (Fig. 5E, bottom blot), further suggesting a *cis* regulatory function of the Anti-*hfq* sRNA on Hfq expression. This result is consistent with a model in which an antisense sRNA regulates the transcription of its sense protein-coding gene, here *hfq*. The *L. pneumophila* Δhfq mutant is attenuated in intracellular growth of *A. castellanii* (Fig. 3C), and flagellin is less well expressed in comparison to the wt strain (Fig. 4C). Thus, to test whether Anti-*hfq* has a role in intracellular replication of *L. pneumophila*, we infected *A. castellanii* with the strain overexpressing *anti-hfq*. At 72 h postinfection, 10-fold fewer intracellular bacteria were recovered from amoeba infected with the Anti-*hfq* sRNA-overexpressing strain (pMMB*anti-hfq*OE) compared to the wt, similar to the replication rate seen for the Δhfq mutant strain (Fig. 5F and 3C). Thus, Anti-*hfq* sRNA plays a role in intracellular replication of *L. pneumophila*.

Hfq expression is regulated by the Anti-*hfq* sRNA. In the Δhfq mutant used until now, the *anti-hfq* gene was still intact (Fig. 2B). Thus, to further study the function of Anti-*hfq* sRNA, we constructed a second mutant containing a larger deletion as the entire region spanning *hfq* and *anti-hfq* ($\Delta hfq \Delta anti-hfq$) was replaced with an apramycin cassette (Fig. 6A). By complementing this mutant with the plasmid pBC*anti-hfq(-10)* in which two single mutations in the *anti-hfq* -10 box had been introduced, we were able to study the role of the Anti-*hfq* sRNA without disturbing Hfq expression. This complemented strain was named the $\Delta anti-hfq(-10)$ strain (Fig. 6A). When analyzing the Hfq expression levels in the $\Delta anti-hfq(-10)$ mutant, the Hfq expression pattern differed

compared to the wt strain, as the expression of the *hfq* transcripts started already during exponential growth of *L. pneumophila* (Fig. 2A, bottom blot, and Fig. 6B), indicating that Anti-*hfq* sRNA indeed represses *hfq* transcripts in exponential growth. In contrast, in the complemented mutant strain ($\Delta hfq \Delta anti-hfq$ pBC hfq), Hfq expression was restored to wt levels (Fig. 6C), whereas in the control strain ($\Delta hfq \Delta anti-hfq$ mutant carrying the empty plasmid; $\Delta hfq \Delta anti-hfq$ pBC), no expression of Hfq was seen, as expected (Fig. 6C). Thus, the antisense RNA Anti-*hfq* regulates Hfq expression levels in a growth phase-dependent manner by functioning as a *cis*-complementary sRNA.

The *hfq* and Anti-*hfq* RNA transcripts interact *in vitro*. Our previous results suggest a regulation of the *hfq* transcript through binding of its Anti-*hfq* antisense sRNA. To investigate a direct interaction of Anti-*hfq* and *hfq* mRNA *in vitro*, we performed electrophoretic mobility shift assays (EMSAs). Incubation with a radioactively labeled Anti-*hfq* RNA probe and increasing concentrations of cold *hfq* mRNA resulted in a slower-migrating complex, suggesting a direct interaction of the two RNA molecules (Fig. 7A). In contrast, when the EMSA was performed with Anti-*hfq* sRNA and a truncated *hfq* mRNA probe spanning the nucleotides 78 to 255 missing the 5' UTR region and the first 26 codons (*hfq* OUT), no changes in terms of migration were observed, consistent with the absence of formation of a complex (Fig. 7A). Similar results were obtained when using the mRNA of an unrelated gene (*lpp0644* RNA probe) as a second negative control (Fig. S2B). Thus, Anti-*hfq* forms an RNA duplex with the *hfq* mRNA and most likely regulates *hfq* mRNA expression by direct binding due to complementarity.

Purified Hfq binds *hfq* and Anti-*hfq* sRNA with different affinity. Although the Hfq protein is known to facilitate the interaction between *trans*-encoded sRNAs and their mRNA targets, the Hfq chaperone may also function to stabilize/destabilize *cis*-encoded sRNAs and their complementary mRNA targets. Thus, we sought to determine whether the Hfq protein might be able to form complexes either with the *hfq* mRNA or with the Anti-*hfq* sRNA. The analysis of the *hfq* and anti-*hfq* sequences revealed the presence of (AAN)_n triplets and AU-rich regions, which could be Hfq binding regions, further suggesting the hypothesis of an Hfq autoregulatory loop. To assess the ability of Hfq to bind *hfq* and Anti-*hfq* transcripts separately, we evaluated binding *in vitro* by EMSAs using recombinant Hfq protein. As shown in Fig. 7B and C, Hfq interacts with both RNA molecules but with different affinities.

To study the inhibitor complex formed by the *hfq* mRNA, Anti-*hfq*, and the Hfq protein in more details, we employed a gel-shift kinetic assay (Fig. 7D). A radioactively labeled Anti-*hfq* RNA probe was incubated with 25 nM of cold *hfq* mRNA in the absence (Fig. 7D, lanes 1, 3, and 5) or presence (Fig. 7D, lanes 2, 4, and 6) of Hfq protein for 0.5 (lanes 1 and 2), 1.5 (lanes 3 and 4), and 2.5 (lanes 5 and 6) minutes. As shown above, the two RNA molecules were able to interact. Additionally, we detected a strong band corresponding to the formation of a ternary complex already after 0.5 min of incubation. Moreover, the intensities of the shifted bands indicated that the affinity of Hfq for the RNA-RNA complex might be much stronger than for the single RNAs alone. The super shift and thus the formation of the ternary complex was increasing with longer incubation time (after 1.5 and 2.5 minutes). To test the specificity of this complex, radioactively labeled *hfq* or Anti-*hfq* probes were incubated alone in parallel with increasing amounts of Hfq confirming that Hfq is indeed able to bind each of the RNA molecules separately (Fig. 7D, lanes 7 to 9 and 10 to 12). Therefore, although Anti-*hfq* is complementary to its own target and thus it should not require Hfq for binding, Hfq is able to bind the two RNA molecules, forming a ternary complex.

RNase III might participate in the double-strand RNA (dsRNA) regulation. One of the regulatory functions of the Hfq RNA chaperone is the recruitment of RNases for the degradation of sRNA and/or mRNA targets. Thus, we wondered whether RNases might be involved in the degradation of the ternary complex in *L. pneumophila*. To answer this question, we performed an RNA stability assay in the wt and an RNase III gene (*lpp1834*) deletion mutant that we constructed. Analysis of the *hfq* mRNA levels,

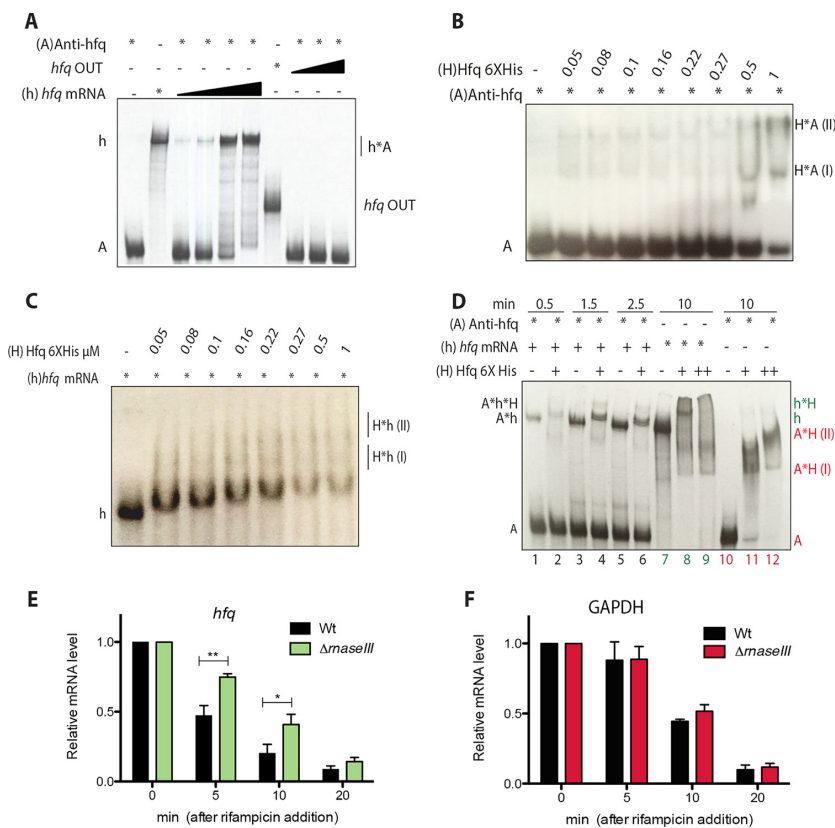


FIG 7 Anti-hfq regulates *hfq* expression through binding to its complementary region which is facilitated by Hfq. (A) EMSA using 25 nM radioactively labeled Anti-hfq and 0, 10, 15, 30, or 50 nM cold full *hfq* transcript or 0, 15, 30, or 50 nM *hfq*OUT as control RNA probes shows that Anti-hfq binds *hfq* mRNA. The amount of RNA probe is indicated by the height of the black triangle above the lane. (B and C) EMSAs using 25 nM radioactively labeled Anti-hfq (B) and *hfq* (C) RNA alone or with the indicated increasing molar amounts of Hfq protein, revealing that Anti-hfq and *hfq* bind Hfq. (D) A radioactively labeled Anti-hfq RNA probe and the cold *hfq* mRNA probe were incubated (lanes 1, 3, and 5), showing the formation of a duplex complex or with 1 μM Hfq protein (lanes 2, 4, and 6) showing the formation of a ternary complex. The ability of the protein to bind separately was evaluated by incubating radioactively labeled *hfq* (lanes 7 to 9) or Anti-hfq (lanes 10 to 12) RNA probes for 10 min. The duplex and ternary complexes were incubated for 0.5 min (lanes 1 and 2), 1.5 min (lanes 3 and 4), and 2.5 min (lanes 5 and 6) at room temperature. The resulting complexes were analyzed on an 8% native polyacrylamide gel as described in Materials and Methods. Abbreviations: (A), radioactively labeled Anti-hfq RNA probe; (h), radioactively labeled *hfq* mRNA probe; (H), Hfq6XHis; (I) and (II), formation of complexes. Symbols: *, radioactively labeled RNA probes; +, cold RNA probes; -, no RNA probes. (E and F) RNA stability assays reveal the RNase III dependence of the *hfq* transcript mRNA *in vivo*. Wt and RNase III deletion strains were grown in BYE medium before rifampin treatment, showing that RNase III-dependent *hfq* mRNA decay was favored. The graphs show the relative amount of *hfq* (E) and GAPDH (F) mRNA remaining at each time point in the wt and RNase III gene deletion strains. 16S was used as internal control for normalization. Each time point represents the mean plus standard deviation from three independent experiments. The quantitative data were analyzed using two-way analysis of variance (ANOVA) test with Bonferroni posttest. A *P* of <0.05 was considered to be statistically significant. The values that are significantly different are indicated by a bar and asterisk as follows: **, *P* < 0.01; *, *P* < 0.05.

after the addition of rifampin, showed a half-life of 4.1 min in the wt strain and of 8.2 min in the RNase III deletion mutant (Fig. 7E). In contrast, when the half-life of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript was determined in the same conditions, no significant differences were observed in the relative mRNA levels between the wt and the RNase III deletion mutant (Fig. 7F). This strongly suggests that RNase III is involved in the cleavage of the *hfq*-Anti-hfq RNA duplex and hence, affects the stability of the *hfq* mRNA, closing the Hfq regulation loop.

DISCUSSION

Legionella pneumophila needs to adapt to many different environmental conditions, including low-temperature and nutrient-poor aquatic and hostile intracellular environ-

ments of protozoa or human macrophages. To regulate the transition from one environment to another environment, *L. pneumophila* has evolved a complex regulatory cascade allowing it to switch from a replicative stage to a transmissive/virulent stage (21). This regulatory network is comprised of many global regulators like the RNA-binding protein CsrA and its small noncoding RNAs RsmX, RsmY, and RsmZ, the TCS LetA/LetS, and the stress sigma factor RpoS (22–28; Sahr et al, unpublished). Here we demonstrate that the RNA chaperone Hfq is another major player in the regulation of the switch to transmissive/virulent *L. pneumophila* and that life cycle-dependent Hfq expression is regulated by an antisense RNA named Anti-hfq.

Comparative sequence analyses showed that Hfq is highly conserved and present in all *L. pneumophila* strains sequenced thus far (Fig. 1B). Our observation that the *hfq* transcript and the Hfq protein are barely expressed at early stages of growth but highly expressed at the postexponential phase of growth (Fig. 2A) establishes Hfq as a growth phase-dependent regulated protein and suggests its implication in the regulation of the expression of virulence traits, a feature of postexponential bacteria. Interestingly, in 2005, McNealy and colleagues (20) had reported that Hfq of *L. pneumophila* JR32 is expressed in the exponential phase of growth and is positively regulated by the stationary-phase sigma factor RpoS. Furthermore, they proposed that upon entry into stationary phase, Hfq expression is abolished through the regulatory function of the two-component regulator LetA, thereby ensuring that *hfq* transcripts are off when the infectious traits need to be activated (20). The differences from our results might be due to the different strains used or perhaps to the excision of the 100-kb plasmid pL100 when *hfq* is deleted, as reported by Trigui and colleagues (34). However, our results are in agreement with the *hfq* expression pattern observed in several other bacterial pathogens such as *P. aeruginosa* and *Listeria monocytogenes* (37, 38) but also with the life cycle of *L. pneumophila* (21, 39). The regulation of virulence traits by Hfq, which demands its expression in the postexponential growth phase, is supported by the observation that the *hfq* mutant is defective in intracellular growth, a characteristic also reported by McNealy and colleagues, and the transcriptome results identifying virulence genes and virulence gene regulators to be differentially expressed upon deletion of *hfq* (see Table S1 and S2 in the supplemental material). By analyzing the protein and transcript levels of Hfq in different regulatory mutants, we show that Hfq expression is influenced by the stationary sigma factor RpoS and the response regulator LetA during the postexponential phase, as both directly or indirectly turn on *hfq* transcription (Fig. 4A and B). Thus, Hfq plays an important role in the regulatory cascade governing the switch to the transmissive phase of *L. pneumophila* (Fig. 4D).

In agreement with the position of Hfq in this regulatory network, the loss of Hfq impaired intracellular replication at 20°C, the optimal growth temperature of *A. castellanii* and a temperature that is close to environmental conditions (Fig. 3C). The transcriptome analysis of the Δhfq mutant during infection of *A. castellanii* supported this finding, as several secreted effector proteins, the enhanced entry proteins EnhABC, the global DNA-binding transcriptional regulators Fis1 and Fis2, and the DNA-binding protein HU-beta were differentially regulated in the *hfq* mutant. Moreover, the above-mentioned regulators are all related to environmental adaptation, virulence, and stress response regulation and fitness in different pathogenic bacteria (40). Furthermore, a hallmark of transmissive/virulent *L. pneumophila*, the expression of flagellar protein FlaA that is intimately linked to virulence, was strongly reduced in the Δhfq mutant at an OD₆₀₀ of 4, similar to what is seen in LetA and RpoS mutants (Fig. 4C). Collectively, these results indicate that *L. pneumophila* requires Hfq to promote motility and to efficiently multiply within *A. castellanii* at environmental temperatures.

Most studies of Hfq analyzed its role in the regulation of sRNAs and their mRNA targets, but not how Hfq expression itself is regulated. *L. pneumophila* Hfq is clearly growth phase dependently regulated, as transcript and protein levels are low during replicative/exponential growth but are strongly expressed in transmissive/postexponential growth (Fig. 2A). This growth phase-dependent regulation is achieved by an sRNA that we named Anti-hfq as it is transcribed on the antisense strand of the *hfq*

gene overlapping its 5' UTR (Fig. 5A). Anti-hfq is a 101-bp long sRNA that is highly expressed during exponential growth, but its expression is strongly decreased upon entry into the transmissive/postexponential growth phase. These opposite expression patterns of the *hfq* and Anti-*hfq* transcripts together with the fact that the sRNA is encoded antisense to *hfq* suggested that it has a role in regulating *hfq* expression. Furthermore, the identification of a partly conserved LetA binding site (two mismatches) suggested that the growth phase-dependent expression of Anti-*hfq* sRNA might be regulated by LetA. However, we could not firmly establish a specific interaction; thus, this regulatory pathway remains to be analyzed in the future. A detailed analysis of the anti-*hfq* sequence revealed the presence of a putative Rho-independent transcriptional terminator as described in a large part of functional Hfq binding modules of sRNAs (41). Furthermore, the ARN or ARNN (R is purine, and N is any nucleotide) motifs that are preferentially bound in the distal site of the Hfq homohexamer (42) were also present in the proximity of the *hfq* ribosome binding site (RBS), and we showed that Anti-*hfq* sRNA binds the complementary region of the *hfq* mRNA. Furthermore, Hfq is able to interact separately with both RNA molecules, *hfq* and Anti-*hfq* (Fig. 7B and C), but it also forms a ternary complex, suggesting an autoregulatory circuit (Fig. 7D). Finally, the ribonuclease RNase III takes part in the regulation of Hfq probably cleaving the double-strand RNA as suggested by RNA stability measurements in an RNase III mutant strain (Fig. 7E). Several studies of *E. coli* had suggested that Hfq binds two distinct sites of the 5' UTR of its own mRNA, hindering the formation of the translation initiation complex and thus negatively regulating its own expression. In *E. coli*, RNase E is recruited to exert its RNase function to degrade *hfq* mRNA (43). Thus, collectively, the data suggest that binding of the *cis*-encoded Anti-*hfq* sRNA obstructs Hfq translation in exponential growth (Fig. 7A).

The regulation of Hfq by a *cis*-encoded sRNA is an unusual feature. We propose that binding of the *cis*-encoded Anti-*hfq* sRNA to *hfq* mRNA in exponential growth leads to low translation of Hfq, whereas when the expression of Anti-*hfq* sRNA decreases in the transmissive phase, high expression of Hfq is possible. This leads to the expression of several Dot/Icm secreted substrates, global regulators like Fis1 and Fis2 that are implicated in the regulation of virulence traits (44) and probably of several of the many growth phase dependently regulated sRNAs that we identified earlier (13) (Fig. 8). Thus, *L. pneumophila* is equipped with a highly sophisticated regulatory mechanism further fine-tuning the regulation of the reciprocal expression of distinct sets of genes under different environmental conditions.

MATERIALS AND METHODS

Bacterial strains, growth media, and culture conditions used. The bacterial strains used in this study are listed in Table 1. *L. pneumophila* strain Paris and its derivatives were cultured in *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered yeast extract broth (BYE) or on ACES-buffered charcoal-yeast (BCYE) extract agar (45), and *E. coli* was grown in Luria-Bertani (LB) broth and agar. All strains were grown at 37°C. For the construction of knockout mutants and complementation plasmids, antibiotics were used at the following concentrations: ampicillin at 100 mg/ml, kanamycin at 50 mg/ml, and chloramphenicol at 20 mg/ml for *E. coli*; and kanamycin at 10 mg/ml, chloramphenicol at 20 mg/ml, and apramycin at 15 mg/ml for *L. pneumophila*. *A. castellanii* ATCC 50739 was cultured in PYG 712 medium [2% proteose peptone, 0.1% yeast extract, 0.1 M glucose, 4 mM MgSO₄, 0.4 M CaCl₂, 0.1% sodium citrate dihydrate, 0.05 mM Fe(NH₄)₂(SO₄)₂ · 6H₂O, 2.5 mM NaH₂PO₃, 2.5 mM K₂HPO₃] at 20°C. THP-1 human monocytes were grown in RPMI 1640 GlutaMAX medium (Gibco) supplemented with 10% fetal bovine serum at 37°C and 5% CO₂.

Mutant and plasmid constructions. The plasmids and oligonucleotide primers used in this study are listed in Table 1 and 2, respectively. Mutant strains of *L. pneumophila* were constructed as previously described (39, 46). In brief, the gene of interest was inactivated by introduction of an apramycin resistance (Apr^r) cassette. The mutant alleles were constructed using a three-step PCR. For the construction of the Δ *hfq* deletion mutant strain, three overlapping fragments (*lpp0009* upstream region primers *hfq*-Mut_F and *hfq*-apra_R, antibiotic cassette-primers *apra*_F and *apra*_R, *lpp0009* downstream region primers *hfq*-apra_F and *hfq*-Mut_R; Table 2) were amplified independently and purified on agarose gels. The three resulting PCR products were mixed at the same concentration (15 nM), and a second PCR with flanking primers (primers *hfq*-Mut_F and *hfq*-Mut_R) was performed. This PCR product, the resistance marker cassette flanked by 300-bp regions homologous to *lpp0009* was introduced into the *L. pneumophila* Paris strain by natural competence (47). Strains that had undergone allelic exchange were selected by plating on BCYE containing apramycin, and the mutant was verified by PCR and sequencing. For the

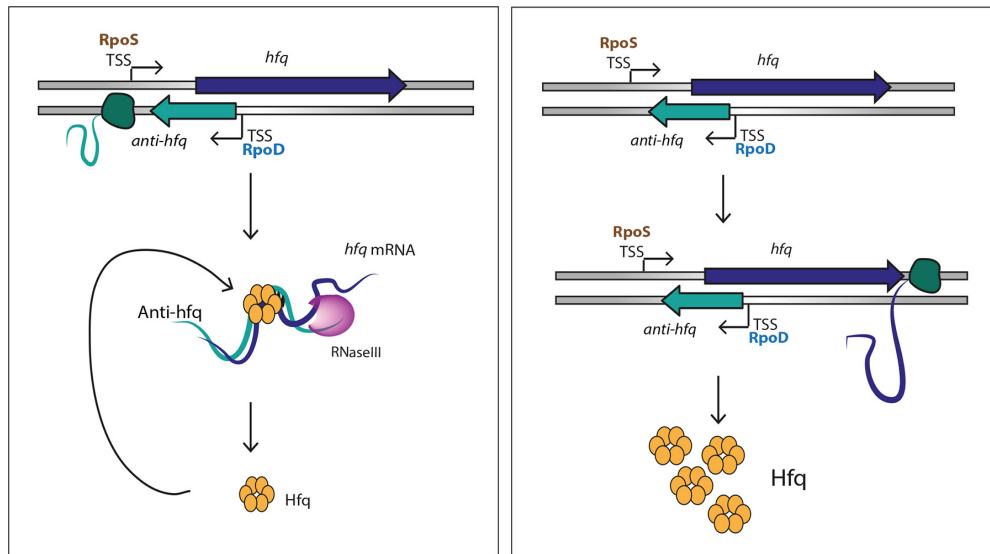


FIG 8 Model of the regulation of Hfq in replicative and transmissive *L. pneumophila*. During the replicative phase, the Anti-*hfq* sRNA is highly expressed and represses Hfq expression through binding to the *hfq* mRNA. This process also involves Hfq itself, which autoregulates its own expression and the ribonuclease RNase III that likely cleaves the *hfq* mRNA product. In contrast, upon entry into the transmissive phase, Anti-*hfq* is not expressed, leading to high Hfq expression that now influences the expression of motility and virulence traits of *L. pneumophila*.

construction of the Δhfq $\Delta anti-hfq$ double mutant strains and the RNase III mutant, the same cloning strategy was used, and the primers are listed in Table 2.

For complementation experiments, the region, including *lpp0009* and *lnc0003* was PCR amplified with primers containing HindIII and SalI restriction sites at their ends (*Hfq_compl_F* and *Hfq_compl_R*) and ligated to the pBC-KS plasmid, previously digested with the two restriction enzymes. The resulting plasmid, named pBC*hfq*, was introduced into the Δhfq and Δhfq $\Delta anti-hfq$ deletion mutant strains by electroporation. The wild-type (wt) *L. pneumophila* Paris, the Δhfq and the Δhfq $\Delta anti-hfq$ deletion

TABLE 1 Bacterial strains and plasmids used in the study

Strain or plasmid	Description ^a	Reference or source
Strains		
<i>L. pneumophila</i> CIP 107629	<i>L. pneumophila</i> serogroup 1 strain Paris (<i>LpP</i>)	19
<i>L. pneumophila</i> pBC	<i>LpP</i> carrying pBC-KS	54
<i>L. pneumophila</i> pMMB207C	<i>LpP</i> carrying pMMB207C	28
<i>L. pneumophila</i> Δhfq	<i>LpP hfq::Apr^r</i>	This study
<i>L. pneumophila</i> Δhfq pBC	<i>LpP hfq::Apr^r</i> carrying pBC-KS	This study
<i>L. pneumophila</i> Δhfq pBC <i>hfq</i>	<i>LpP hfq::Apr^r</i> carrying pBC <i>hfq</i>	This study
<i>L. pneumophila</i> Δhfq $\Delta anti-hfq$	<i>LpP hfq anti-hfq::Apr^r</i>	This study
<i>L. pneumophila</i> Δhfq $\Delta anti-hfq$ pBC	<i>LpP hfq anti-hfq::Apr^r</i> carrying pBC <i>hfq</i>	This study
<i>L. pneumophila</i> Δhfq $\Delta anti-hfq$ pBC <i>hfq</i>	<i>LpP hfq anti-hfq::Apr^r</i> carrying pBC <i>hfq</i>	This study
<i>L. pneumophila</i> $\Delta anti-hfq$ (-10)	<i>LpP hfq anti-hfq::Apr^r</i> carrying pBC <i>hfqanti-hfq(-10)</i>	This study
<i>L. pneumophila</i> pMMB <i>anti-hfq</i> OE	<i>LpP</i> carrying pMMB <i>anti-hfq</i> OE	This study
<i>L. pneumophila</i> $\Delta letA$	<i>LpP letA::Km^r</i>	28
<i>L. pneumophila</i> $\Delta rpoS$	<i>LpP rpoS::Km^r</i>	13
<i>L. pneumophila</i> $\Delta rnasell$	<i>LpP</i> carrying the RNase III gene fused to the <i>Apr^r</i> cassette	This study
<i>E. coli</i> DH5 α	F ⁻ ϕ 80dlacZ Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺ <i>phoA supE44</i> λ^- <i>thi-1 gyrA96 relA1</i>)	Invitrogen
Plasmids		
pGEM-T Easy	Cloning of PCR products; Amp ^r	Promega
pBC-KS	Expression vector; Cm ^r	Stratagene
pMMB207C	<i>Legionella</i> expression vector; $\Delta mobA$; Cm ^r	55
pBC <i>hfq</i>	pBC-KS containing <i>hfq</i> and <i>anti-hfq</i> genes; Cm ^r	This study
pMMB <i>anti-hfq</i> OE	pMMB207C containing <i>anti-hfq</i> gene under the <i>ptac</i> promoter; Cm ^r	This study
pBC <i>anti-hfq(-10)</i>	pBC <i>hfq</i> mutated in the -10 upstream region of <i>anti-hfq</i> ; Cm ^r	This study

^aAbbreviations: Amp^r, ampicillin resistance; Apr^r, apramycin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance.

TABLE 2 Primers used in this study

Primer	Primer sequence (5' –3')	Purpose	Reference
<i>hfq</i> -Mut_F	AAGAATTGATCAGGCCTGTC	Deletion of the <i>hfq</i> gene	This study
<i>hfq</i> -Mut_R	CCGACGATGCGTAAATGGGA	Deletion of the <i>hfq</i> gene	This study
<i>apra</i> _F	TTCATGTGCAGCTCCATCAGC	Deletion of the <i>hfq</i> gene	This study
<i>apra</i> _R	GAGCGGATCGGGGATTGTCTT	Deletion of the <i>hfq</i> gene	This study
<i>hfq</i> - <i>apra</i> _R	GCTGATGGAGCTGCACATGAATGCAATTAATACCATTGACCAGG	Deletion of the <i>hfq</i> gene	This study
<i>hfq</i> - <i>apra</i> _F	GAGCGGATCGGGGATTGTCTTTCTGGTGAGGAAGAAGGAACTG	Deletion of the <i>hfq</i> gene	This study
<i>hfqanti</i> - <i>hfq1</i> _F	ACACTCCAAAACGAGGCGGCTG	Deletion of the <i>hfq</i> and anti- <i>hfq</i> genes	This study
<i>hfqanti</i> - <i>hfq2</i> _R	GCTGATGGAGCTGCACATGAACGGGTATCTAACTATTATTTCGA	Deletion of the <i>hfq</i> and anti- <i>hfq</i> genes	This study
<i>hfqanti</i> - <i>hfq2</i> _F	GAGCGGATCGGGGATTGTCTTACTGTGCAGACTAATCAATTTA	Deletion of the <i>hfq</i> and anti- <i>hfq</i> genes	This study
<i>hfqanti</i> - <i>hfq1</i> _R	CGACATCCAAATAATCGCTCG	Deletion of the <i>hfq</i> and anti- <i>hfq</i> genes	This study
<i>Hfq</i> _comple_F	AAGCTTGCCAGCTCAATGCAATTGGC	Complementation of <i>hfq</i> and <i>hfqanti</i> - <i>hfq</i>	This study
<i>Hfq</i> _comple_R	GTCGACTTGATTAGTCTGCCACAGTTC	Complementation of <i>hfq</i> and <i>hfqanti</i> - <i>hfq</i>	This study
M-10 <i>anti</i> - <i>hfq</i> _F	ATTGACCAGGAACACTGAAACCCGGGACCTTTTCCTTGCGCAATTCATT	Mutation of the –10 promoter of anti- <i>hfq</i>	This study
M-10 <i>anti</i> - <i>hfq</i> _R	AATGAATTGCGCAAGGAAAAGGTCCCGTTTCAGTGTTCTGGTCAAT	Mutation of the –10 promoter of anti- <i>hfq</i>	This study
anti- <i>hfq</i> _OE_F	TCTAGAGCGCAATTCATTTAGGAAAGG	Overexpression of anti- <i>hfq</i>	This study
anti- <i>hfq</i> _OE_R	CTGCAGAAACCACGCTGTGCATGAAAATATAC	Overexpression of anti- <i>hfq</i>	This study
anti- <i>hfq</i> _3' RACE_F	TTTAGGAAAGGGTCTGTAGTAAATG	3' RACE anti- <i>hfq</i>	This study
anti- <i>hfq</i> _3' RACE_R	AATAGTTAGATACCCGTTTTTGCC	3' RACE anti- <i>hfq</i>	This study
<i>masellI</i> _Mut_F	ATGCGCTCAGCAATGAATTAGC	Deletion of the RNase III gene	This study
<i>masellI</i> _Mut_R	TCTGGTCTGGATGAGTTGGAATG	Deletion of the RNase III gene	This study
<i>masellI</i> _Inv_F	GAGCGGATCGGGGATTGTCTTTATCGCTACCAGCACTGCAATG	Deletion of the RNase III gene	This study
<i>masellI</i> _Inv_R	GCTGATGGAGCTGCACATGAATGTAACATGCACAATTGAGGGAG	Deletion of the RNase III gene	This study
anti- <i>hfq</i> RNA_T7_F	TAATACGACTCACTATAGGGCAATTCATTTAGGAAAAGGG	<i>In vitro</i> transcription of anti- <i>hfq</i>	This study
anti- <i>hfq</i> RNA_T7_R	T AGTTAGATAACCGTTTTTGCC	<i>In vitro</i> transcription of anti- <i>hfq</i>	This study
<i>hfq</i> mRNA_T7_F	TAATACGACTCACTATAGGGATAGGGTGTGCAATAAATAG	<i>In vitro</i> transcription of <i>hfq</i> mRNA	This study
<i>hfq</i> mRNA_T7_R	TTGATTAGTCTGCCACAGTTC	<i>In vitro</i> transcription of <i>hfq</i> mRNA	This study
<i>lpp0644</i> _T7_F	TAATACGACTCACTATAGGGGAATGTTATGAGTGACTTG	<i>In vitro</i> transcription of <i>lpp0644</i>	This study
<i>lpp0644</i> _T7_R	TCCAGTCTGTGCGCGCATCC	<i>In vitro</i> transcription of <i>lpp0644</i>	This study
<i>hfq</i> OUT_T7_F	TAATACGACTCACTATAGGGTCAATGTTATAAATTCATGGG	<i>In vitro</i> transcription of <i>hfq</i> mRNA missing the 5' UTR	This study
anti- <i>hfq</i> _qPCR_F	TTTAGGAAAGGGTCTGTAGTAA	qPCR analysis of the anti- <i>hfq</i> region overlapping <i>hfq</i> mRNA	This study
anti- <i>hfq</i> _qPCR_R	AATAGTTAGATACCCGTTTTTGCC	qPCR analysis of the anti- <i>hfq</i> region overlapping <i>hfq</i> mRNA	This study
<i>tldD</i> _qPCR_F	AATCGGAACGTCGATGATGCTG	qPCR analysis of the <i>tldD</i> mRNA	This study
<i>tldD</i> _qPCR_R	ATCCCTACCCCTTATCCAGAG	qPCR analysis of the <i>tldD</i> mRNA	This study
<i>gyrB</i> _qPCR_F	GAGCGTAGACGCCAGTTATGA	qPCR analysis of the <i>gyrB</i> mRNA	This study
<i>gyrB</i> _qPCR_R	TGATGCAAACCCGGTCCATCA	qPCR analysis of the <i>gyrB</i> mRNA	This study
<i>hfq</i> mRNA_NB_F	TAATACGACTCACTATAGGGGAACAAGTGTGAAATGGCGTG	Northern blot analysis of <i>hfq</i> mRNA	This study
<i>hfq</i> mRNA_NB_R	GTTTCAGTGTCTCTGGTCAATGG	Northern blot analysis of <i>hfq</i> mRNA	This study
<i>hfq</i> _qPCR_F	TCAGTGTCTCTGGTCAATGG	Determination of <i>hfq</i> mRNA half-life	This study
<i>hfq</i> _qPCR_R	AACAACCTGTTGAAATGGCGTG	Determination of <i>hfq</i> mRNA half-life	This study
<i>gapdH</i> _qPCR_F	TTGATACGACAGTGGTCTATGG	Determination of GAPDH mRNA half-life	This study
<i>gapdH</i> _qPCR_R	CATGGACAGTGTGACTAAGCC	Determination of GAPDH RNA half-life	This study
16S_qPCR_F	TTGTCTAGCTTGCTAGACAGATGG	Determination of 16S half-life	This study
16S_qPCR_R	AGCTTTCGCTCCTCAGACATTATGC	Determination of 16S half-life	This study

mutant strains containing the empty plasmid pBC-KS were used as control. For constructing the Anti-*hfq* mutant strain, site-directed mutagenesis of anti-*hfq* was performed on the pBC*hfq* plasmid as the template using the QuikChange site-directed mutagenesis kit (Qiagen) following the manufacturer's instructions. Two mutations were introduced in the –10 promoter region of the anti-*hfq* gene using the primers M-10*anti*-*hfq*_F and M-10*anti*-*hfq*_R. The resulting plasmid, pBC*anti*-*hfq*(-10), was introduced into the Δ *hfq* Δ anti-*hfq* deletion mutant, creating the Δ anti-*hfq*(-10) mutant strain.

For overexpression of Anti-*hfq* sRNA in *L. pneumophila*, we used the pMMB207C (derived from pTS-10, kindly provided by H. Hilbi [48]). The anti-*hfq* gene was amplified using primers containing XbaI and PstI restriction sites (*anti*-*hfq*_OE_F and *anti*-*hfq*_OE_R primers) and ligated into pMMB207C, linearized using the same restriction enzymes. The resulting plasmid (pMMB*anti*-*hfq*OE) and the control plasmid pMMB207C (here named pMMB) were introduced via electroporation into wt *L. pneumophila* Paris strain. For overexpression, IPTG (0.5 mM) was added at an OD₆₀₀ of 0.8.

Sequencing of the Δ *hfq* mutant strain. For whole-genome sequencing, paired-end sequences and a read length of 100 bases were obtained from an Illumina HiSeq platform (Biomics pole Institut Pasteur).

Sequence reads were mapped to a reference genome using SMALT v0.7.4, and single nucleotide polymorphisms (SNPs) were searched for using a standard approach.

A. castellanii and THP-1 infection assay. Infection of *A. castellanii* with *L. pneumophila* Paris and its derivatives was done as described previously (49). In brief, *A. castellanii* were washed once with infection buffer (PYG 712 medium without proteose peptone, glucose, and yeast extract) and seeded at a density of 4×10^6 cells per 25-cm² flask. Wild-type and mutant strains of *L. pneumophila* were grown on BCYE agar to stationary phase, diluted in infection buffer, and mixed with *A. castellanii* at a multiplicity of infection (MOI) of 0.1 or 1 (as indicated in the figure legends). Intracellular multiplication was monitored by plating a 100- μ l sample that was centrifuged at 14,500 rpm and vortexed to break up amoeba, at different time points on BCYE plates. The number of bacteria recovered was counted as CFU. In THP-1 cell infection assays, cells were seeded in 12-well tissue culture trays (TTP) at a density of 2×10^5 cells/well. THP-1 cells were pretreated with 10 to 8 M phorbol 12-myristate 13-acetate (PMA) (Sigma) for 72 h to induce differentiation into macrophage-like adherent cells. Stationary-phase *L. pneumophila* bacteria were resuspended in serum-free medium and added to cells at an MOI of 10. After 2 h of incubation, cells were washed with phosphate-buffered saline (PBS) before incubation with serum-free medium. At 2, 24, 48, and 72 h, the supernatant was collected and the cells were lysed with PBS–0.1% Triton X-100. The infection efficiency was monitored by determining the CFU of the different *L. pneumophila* strains recovered on BCYE agar plates. Each infection was carried out in triplicate.

RNA isolation and Northern blot analysis. Total RNA was extracted as previously described (50). Wild-type and mutant *L. pneumophila* Paris strains were grown in BYE medium and harvested for RNA isolation at exponential phase (OD₆₀₀ of 1.0 and 2.0) and postexponential phase (OD₆₀₀ of 3 and 4). Total RNA was treated with DNase I and purified using columns (Qiagen). Ten micrograms of total RNA isolated from different conditions (see above) were size separated on 10% denaturing polyacrylamide gels containing 8 M urea (Bio-Rad) and transferred onto positively charged nylon membranes (BrightStar-Plus; Ambion). The membranes were photographed under UV light to capture ethidium bromide staining of rRNA bands for loading controls. RNA was cross-linked to membranes by exposure to UV light for 2 min, and membranes were prehybridized in Ultrahyb buffer (catalog no. AM8670; Ambion) for 1 h. RNA probes radioactively labeled with [α -³²P]UTP (catalog no. BLU007X500UC; PerkinElmer) were generated using the T7 Maxiscript kit (catalog no. AM1314; Ambion), and PCR templates were amplified from genomic DNA using primers listed in Table 2. The membrane was then hybridized at 65°C by adding the radiolabeled probes overnight. Blots were washed twice at the hybridization temperature in 2 \times SSC–0.1% SDS (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then washed twice in 0.1 \times SSC–0.1% SDS. Membranes were wrapped in Saran Wrap and subsequently used to expose films (catalog no. 28906844; GE Healthcare).

RNA isolation, labeling, and microarray hybridization. For total RNA extraction, wild-type Paris and the Δ hfq mutant strains were grown in BYE medium *in vitro* and harvested for RNA isolation at postexponential growth phase (OD₆₀₀ of 4). For *in vivo* experiments, *A. castellanii* amoebae were infected with wt or Δ hfq mutant at an MOI of 100 as described above. Cells were cultivated at 20°C and harvested for RNA isolation after 96 h. RNA was prepared in biological triplicates for *in vitro* and biological duplicates for *in vivo* experiments as described above, and all samples were hybridized twice to the microarrays (dye swap). RNA was reverse transcribed with Superscript indirect cDNA kit (Invitrogen) and labeled with Cy5 or Cy3 (Amersham Biosciences, Inc.) according to the supplier's instructions. The design of microarrays containing gene-specific 70-mer oligonucleotides based on all predicted genes of the genome of *L. pneumophila* strain Paris (CR628336) and its plasmid (CR628338) was previously described (39). Hybridization was performed following the manufacturers' recommendations (Corning) using 250 pmol of Cy3- and Cy5-labeled cDNA. Slides were scanned on a GenePix 4000A scanner (Axon Instruments). Laser power and/or the photomultiplier tube (PMT) were adjusted to balance the two channels, and the resulting files were analyzed using GenePix Pro 4.0 software. Spots were excluded from analysis in case of high local background fluorescence, slide abnormalities, or weak intensity.

Data normalization and differential analysis were conducted using the R software (<http://www.R-project.org>). No background subtraction was performed, but a careful graphical examination of all the slides was conducted to ensure a homogeneous, low-level background in both channels. A loess normalization (51) was performed on a slide-by-slide basis (BioConductor package marray; <https://www.bioconductor.org/packages/release/bioc/html/marray.html>). Differential analysis was carried out separately for each comparison between two time points, using the VM method (VarMixt package [52]), together with the Benjamini and Yekutieli *P* value adjustment method (53). Empty and flagged spots were excluded from the data set, and only genes with no missing values for the comparison of interest were analyzed.

Determination of RNA half-life and quantitative RT-PCR. Wild-type and RNase III gene deletion mutant strains of *L. pneumophila* were grown to an OD₆₀₀ of 2.5 in BYE medium. Cells were subsequently treated with rifampin (final concentration of 500 μ g/ml). Aliquots were removed at time zero (just before treatment) or after 5, 10, or 20 min of treatment. Cells were harvested by centrifugation in a tabletop centrifuge at 13,000 rpm for 1 min. Pellets were flash frozen in liquid nitrogen, and subsequently RNA was isolated as described above. Quantitative reverse transcription-PCR (qPCR) was then performed as described previously (39) at cDNA concentrations ranging from 5 ng to 5×10^{-3} ng. Primers used are listed in Table 2. Primer efficiencies were evaluated by generating a standard curve with serial dilutions, which indicated an efficiency of 90% to 110% for all primers used. The specificity of the amplified product and primer dimer formation was verified for each primer set by the presence of a single peak in a dissociation step carried out after each run. The absence of contaminating DNA was verified using control samples for each RNA sample for which no prior reverse transcription reaction had been carried

out. Fold changes were calculated using the $\Delta\Delta C_T$ method. Values represent mean values of three biological replicate experiments \pm standard deviations (SD), normalized to the 16S loading controls.

Western blot analysis. Samples were denatured at 90°C for 10 min and separated on a 4 to 20% gradient SDS-PAGE gel (Bio-Rad) and transferred using a Trans-Blot Turbo transfer system (Bio-Rad). The membrane was stained with black amide or red ponceau solutions for loading controls and blocked in 1.2% bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (TBS-Tween) for 1 h at room temperature. Membranes were incubated overnight at 4°C with anti-Hfq or anti-FlaA primary antibodies that we generated. Briefly, Hfq and FlaA 6 \times His protein production was induced at an OD₆₀₀ of 0.5 by 0.4 mM IPTG at 37°C for 4 h. Hfq and FlaA-6 \times His proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose beads and a Poly-Prep chromatography column. The resulting proteins were injected into rabbits, and crude sera were recovered 90 days later (Thermo Fisher Custom Antibody Services). Specific immunoglobulins were purified from serum samples by using a 1.0-ml HiTrap affinity NHS column (GE Healthcare) according to the manufacturer's instructions. The antibody specificity and purity were assessed by Western blotting against the purified proteins. Membranes were incubated overnight at 4°C with Hfq or FlaA primary antibodies (diluted 1:2,000). The membranes were washed three times for 5 min each time in TBS-0.5% Tween at room temperature. The membrane was incubated for 1 h at room temperature with the secondary antibody, horseradish peroxidase (HRP)-labeled anti-rabbit (Dako) in TBS-0.5% Tween before the membrane was washed as described above. Signals were visualized using the ECL2 prime Western blot detection kit (Pierce) and the G-Box imaging system (Syngene).

Rapid amplification of the 3' end of cDNA (3' RACE). Amplification of the 3'-end region of anti-*hfq* was performed using total RNA purified from wt *L. pneumophila* Paris strain in the early exponential growth phase (OD₆₀₀ of 2) as described above. Total RNA was treated with DNase I (Roche), incubated at 37°C with 10 U tobacco acid pyrophosphatase (TAP) (Epicentre) as previously described (28), subjected to phenol-chloroform-isoamyl alcohol (IAA) extraction (25:24:1), and precipitated overnight at -20°C with 10% 3 M sodium acetate (pH 5.2), 2% glycogen (20 mg/ml), and 2.5 volume of ethanol. For the 3' adapter ligation, a mix of 3' RNA adapters P-UCGUAUGCCGUCUUCUGCUUG-UidT (100 μ M) was ligated to the processed RNA using the T4 RNA ligase (Epicentre) according to the manufacturer's instructions. cDNA was then synthesized as described above, and amplification (primers *anti-hfq_3'RACE_F* and *anti-hfq_3'RACE_R*) products were fractionated in a 2% agarose gel. After staining with ethidium bromide, the sole band obtained of about 100 nucleotides (nt) was cut from the gel and purified using the NucleoSpin Extract kit (Macherey-Nagel). The purified size-selected cDNA fragment was cloned into the pGEM-T Easy (Promega) plasmid, and the cloned fragment was sequenced.

RNA in vitro transcription and labeling. Anti-*hfq* (103-nt), *hfq* mRNA (335-nt), *lpp0644* (137-nt), and *hfqOUT* (180-nt) genes used for electrophoretic mobility shift assay (EMSA) gel mobility assays and *hfq* mRNA (128 nt) used for Northern blot analyses were amplified from bacterial DNA with primers containing the T7 promoter at the 5' end (Table 2). The resulting fragments were used as the templates to produce *in vitro* RNA (MEGAscript T7 kit; Ambion) and radioactively labeled with [α -³²P]UTP (PerkinElmer). The reaction mixture was incubated at 37°C for 30 min, and RNA was digested with Turbo DNase digestion (1 U, 15 min at 37°C) and purified using the Illustra Micro-Spin G-25 columns (GE Healthcare) according to the supplier's protocol.

EMSA gel mobility assay. RNA-RNA binding assays were performed to assess the binding affinity of the Anti-*hfq* transcript and *hfq* mRNA, *hfqOUT* (spanning the nucleotide sequence 78 to 255 and missing the 5' UTR and the first 26 codons of the *hfq* transcript), and *lpp0644* as a control. Briefly, 25 nM anti-*hfq*, together with 0, 10, 15, 30, or 50 nM *hfq* full transcript or 0, 15, 30, or 50 nM *hfqOUT* probes was incubated with buffer containing 10 mM Tris-Cl (pH 8.3) and 0.1 mM EDTA, denatured at 70°C for 5 min, and cooled down for 15 min at room temperature. *In vitro* formation of complexes between Hfq and *hfq* mRNA or Anti-*hfq* sRNA (25 nM) *in vitro* was analyzed by EMSA using 0.05, 0.08, 0.1, 0.16, 0.22, 0.27, 0.5, and 1 μ M His-tagged Hfq (Hfq6XHis) and supplemented with 5 \times structure buffer (50 mM Tris-HCl [pH 8.0], 250 mM NaCl, 250 mM KCl, 200 ng/ml tRNA) and incubated at 37°C for 15 min.

For the formation of ternary complexes, 25 nM radioactively labeled Anti-*hfq* and 25 nM cold *hfq* mRNA probes were incubated alone or with 1 μ M His-tagged Hfq (Hfq6XHis) protein for 0.5, 1.5, or 2.5 min and supplemented with 5 \times structure buffer (50 mM Tris-HCl [pH 8.0], 250 mM NaCl, 250 mM KCl, 200 ng/ml tRNA). For a control, 25 nM radioactively labeled Anti-*hfq* or *hfq* mRNA probes were incubated with 0.5 and 1 μ M (Hfq6XHis) for 10 min at room temperature. Prior to loading, reactions were mixed with native loading buffer, and samples were loaded onto 6 or 8% polyacrylamide 1 \times Tris-acetate-EDTA gel in 1 \times Tris-acetate EDTA running buffer. Following electrophoresis at 4°C, the gels were wrapped in Saran Wrap and subsequently exposed to films (GE Healthcare).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mBio.02182-16>.

FIG S1, TIF file, 3.2 MB.

FIG S2, TIF file, 9.4 MB.

TABLE S1, DOCX file, 0.1 MB.

TABLE S2, DOCX file, 0.1 MB.

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Supplemental Material

A unique *cis*-encoded RNA is regulating *Legionella pneumophila* Hfq expression in a life cycle dependent manner

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FIG S1. *In vitro* growth of the Δhfq mutant at 37°C and 20°C is similar to, that of the wild type strain

FIG S2. Sequence of *anti-hfq* and control EMSA showing that no ternary complex is formed when an unrelated RNA is used.

Table S1: Differentially expressed genes in *in vitro* analyses of wt and Δhfq grown in AYE broth at 37°C until OD 4 (PE phase)

Table S2: Differentially expressed genes according to transcriptome *in vivo* analyses of wt and Δhfq grown for 96h at 20°C within *A. castellanii*

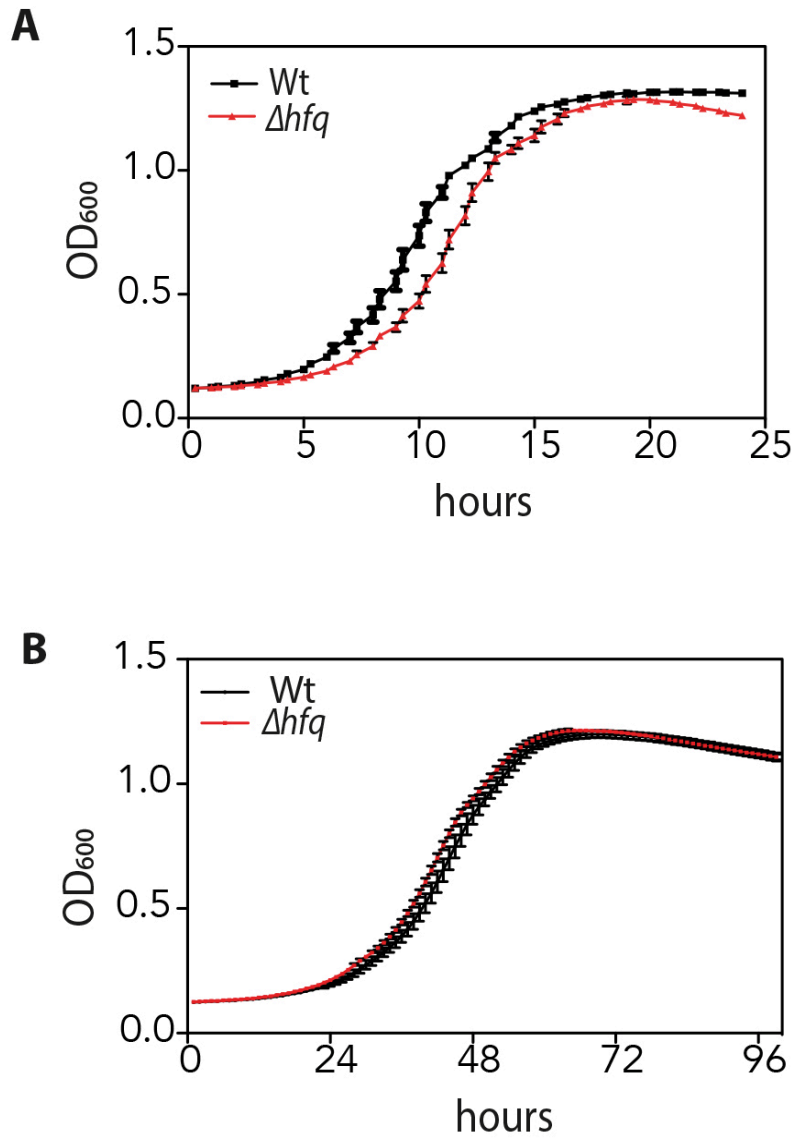
RESULTS

Figure Legends:

FIG S1. *In vitro* growth of the Δhfq mutant at 37°C and 20°C is similar to, that of the wild type strain. (A) Growth of the Δhfq deletion mutant at 37°C. (B) Growth of the Δhfq deletion mutant at 20°C. Optical density values at OD₆₀₀ of triplicate cultures in BCYE medium were determined for 24 hours (A) and for 96 hours (B) (Black, wild-type; red, Δhfq)

FIG S2. Sequence of *anti-hfq* and control EMSA showing that no ternary complex is formed when an unrelated RNA is used. A Sequence of the small RNA and the flanking regions, blue letters, the sequence of the small RNA, grey letters, promoter region. The arrow indicates the flanking *lpp0008* starting codon. Red letters, Hfq start codon. **B.** EMSA using 25 nM radioactively labelled Anti-hfq together with 0, 10, 15, 30, 50, nM cold *hfq* full transcript or 0, 15, 30, 50 nM of the cold *lpp0644* RNA probes. Symbols: (A) indicates radioactively labelled Anti-hfq RNA probe, (h) indicates radioactively labelled *hfq* mRNA probe. Asterisks indicate radioactively labelled RNA probes, - the absence of the RNA probes, triangles indicate increased amount of cold RNA probes.

Figure S1



RESULTS

Figure S2

A

5'- aaccatttgggttatggagtttttaagcatcaccacatattgatcaaaagaatcgattatcccatgca
 atttaataccattgaccaggaacactgaaacaggtaccttttccttgccaattcatttaggaaaggtctt
 gtagtaaatgatTTTTtagacattgcctactccttgttgttattgtaatTTTTaaaggcaaaaacgggtatct
 aactatTTtattogacaccctataataaaaacttttagtatatTTTcatgacagcgtggtttgaattcgttaata
 tagtcaatcaaaatTTTTTgcacatctagaatcaggctactgatgagttaataaggagattTTTaatgacgt
 ttacttgcgatgaattaaaaggattggagcaccctacgaagtacttggcaatggtgac -3'

B

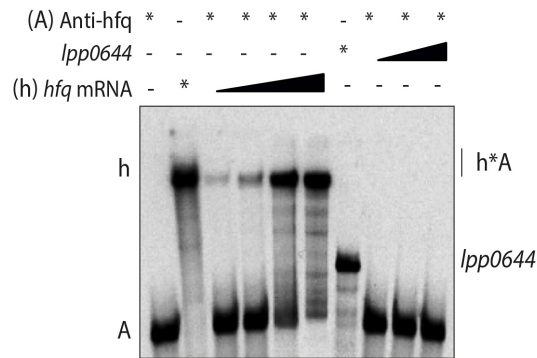


Table S1: Differentially expressed genes in *in vitro* analyses of wt and Δhfq grown in AYE broth at 37°C until OD 4 (PE phase)

Up-regulated in the Δhfq strain ($p < 0.05$, 8 up-regulated also *in vivo* analyses)

gene.ID	description	FC
<i>lpp0460</i>	hypothetical protein	2,04
<i>lpp0973</i>	pantothenate kinase type III (putative Bvg accessory factor family protein)	2,32
<i>lpp1229</i>	flagellar biosynthesis protein FlgG	3,29
<i>lpp1230</i>	flagellar L-ring protein precursor FlgH	3,36
<i>lpp1231</i>	flagellar P-ring protein precursor FlgI	2,01
<i>lpp1291</i>	flagellar protein FliS	2,49
<i>lpp1292</i>	flagellar hook-associated protein 2 (flagellar capping protein)	2,49
<i>lpp1974</i>	uncharacterized bacterial polysaccharide deacetylases, catalytic NodB homology domain	2,26
<i>lpp2350</i>	chemiosmotic efflux system C protein A	3,09
<i>lpp2351</i>	chemiosmotic efflux system protein A-like protein	2,21
<i>lpp2353</i>	chemiosmotic efflux system C protein C	5,49
<i>lpp2354</i>	domain of unknown function (DUF4156)	4,63
<i>lpp2693</i>	enhanced entry protein EnhB - Sel1-like repeats protein	2,99
<i>lpp2694</i>	enhanced entry protein EnhA - L,D-transpeptidase catalytic domain,	2,42
<i>lpp2988</i>	lytic murein transglycosylase, SLT domain	3,43

Down-regulated in the Δhfq strain ($p < 0.05$, 1 down-regulated also *in vivo* analyses)

gene.ID	description	FC
<i>lpp0009</i>	host factor-1 protein Hfq	0,07
<i>lpp0679</i>	StaR-like protein, TPR-domain - eukaryotic-like protein	0,48
<i>sRNA</i>	RsmX	0,48

RESULTS

Table S2: Differentially expressed genes according to transcriptome *in vivo* analyses of wt and Δhfq grown for 96h at 20°C within *A. castellanii*

Up-regulated in the Δhfq strain (p<0.05)

gene.ID	description	FC
<i>lpp0008</i>	substrate of the Dot/Icm secretion system RavA	1,91
<i>lpp0122</i>	protein of unknown function	1,88
<i>lpp0257</i>	chitin-binding protein CbpD	1,87
<i>lpp0381</i>	preprotein translocase secE subunit	1,93
<i>lpp0384</i>	50S ribosomal protein L1	2,07
<i>lpp0385</i>	50S ribosomal subunit protein L1	2,19
<i>lpp0386</i>	50S ribosomal subunit protein L7/L12	2,40
<i>lpp0389</i>	30S ribosomal protein S12	2,03
<i>lpp0392</i>	translation elongation factor Tu	2,39
<i>lpp0393</i>	30S ribosomal subunit protein S1	2,05
<i>lpp0394</i>	50S ribosomal subunit protein L3	1,88
<i>lpp0396</i>	50S ribosomal subunit protein L23	2,02
<i>lpp0398</i>	30S ribosomal subunit protein S19	2,08
<i>lpp0399</i>	50S ribosomal subunit protein L22	1,88
<i>lpp0400</i>	30S ribosomal protein S3	2,19
<i>lpp0401</i>	50S ribosomal protein L16	2,26
<i>lpp0402</i>	50S ribosomal subunit protein L29	1,96
<i>lpp0403</i>	30S ribosomal protein S17	1,98
<i>lpp0404</i>	50S ribosomal protein L14	2,14
<i>lpp0406</i>	50S ribosomal protein L5	1,96
<i>lpp0407</i>	30S ribosomal protein S14	2,46
<i>lpp0408</i>	30S ribosomal protein S8	2,12
<i>lpp0410</i>	50S ribosomal subunit protein L18	2,20
<i>lpp0411</i>	30S ribosomal subunit protein S5	2,17
<i>lpp0412</i>	50S ribosomal subunit protein L3	2,08
<i>lpp0418</i>	30S ribosomal subunit protein S4	2,03
<i>lpp0419</i>	DNA-directed RNA polymerase alpha chain	1,98
<i>lpp0460</i>	hypothetical protein	2,65
<i>lpp0466</i>	30S ribosomal protein S16	1,87
<i>lpp0493</i>	cold shock-like protein CspD	2,47
<i>lpp0544</i>	50S ribosomal protein L28	1,95
<i>lpp0570</i>	outer membrane protein (OmpH-like)	2,10
<i>lpp0605</i>	bacterial protein of unknown function (DUF945)	1,99
<i>lpp0606</i>	global DNA-binding transcriptional regulator Fis1	2,35
<i>lpp0688</i>	substrate of the Dot/Icm secretion system	2,21
<i>lpp0725</i>	predicted integral membrane protein (DUF2282)	2,08
<i>lpp0742</i>	chaperonin 10 subunit, Cpn10 or GroES	1,96
<i>lpp0743</i>	chaperonin 60, Cpn60 or GroEL	1,94
<i>lpp0855</i>	macrophage infectivity potentiator Mip	2,52
<i>lpp0972</i>	enhanced entry protein EnhA (L,D-transpeptidase catalytic domain)	2,56
<i>lpp1146</i>	substrate of the Dot/Icm secretion system	2,46
<i>lpp1207</i>	cold-shock protein (CSP)	2,24
<i>lpp1229</i>	flagellar biosynthesis protein FlgG	1,86
<i>lpp1230</i>	flagellar L-ring protein precursor FlgH	1,82
<i>lpp1324</i>	global DNA-binding transcriptional regulator Fis2	2,48
<i>lpp1772</i>	hypothetical protein	2,07
<i>lpp1805</i>	Com1-like membrane-associated immunoreactive protein, DsbA family	1,88

<i>lpp1826</i>	DNA-binding protein HU-beta	2,82
<i>lpp1958</i>	Legionella major outer membrane protein	2,45
<i>lpp1974</i>	uncharacterized polysaccharide deacetylases, catalytic NodB homology domain	1,86
<i>lpp2026</i>	peptidoglycan-associated lipoprotein, OmpA-like domain	2,07
<i>lpp2164</i>	heme-binding protein Hbp	1,92
<i>lpp2209</i>	hypothetical membrane protein	2,48
<i>lpp2276</i>	substrate of the Dot/Icm secretion system	2,09
<i>lpp2354</i>	domain of unknown function (DUF4156)	1,91
<i>lpp2675</i>	papain-like C1 peptidase	1,93
<i>lpp2689</i>	30S ribosomal subunit protein S2	2,08
<i>lpp2692</i>	enhanced entry protein EnhC - Sel1-like repeats protein	1,83
<i>lpp2693</i>	enhanced entry protein EnhB - Sel1-like repeats protein	1,97
<i>lpp2694</i>	enhanced entry protein EnhA - L,D-transpeptidase catalytic domain,	1,68
<i>lpp2768</i>	50S ribosomal protein L35	2,05
<i>lpp2817</i>	30S ribosomal protein S15	1,94
<i>lpp2866</i>	leucine aminopeptidase, Zn-peptidase M28 family	1,89
<i>lpp2968</i>	hypothetical protein	1,95
<i>lpp2988</i>	lytic murein transglycosylase, SLT domain	1,72
<i>lpp3021</i>	hypothetical protein	2,09
<i>lpp3031</i>	major outer membrane protein precursor	2,47
<i>lpp3032</i>	major outer membrane protein precursor	3,24
<i>lpp3033</i>	major outer membrane protein precursor	3,07

Down-regulated in the Δhfq strain ($p < 0.05$)

gene.ID	description	FC
<i>lpp0009</i>	host factor-1 protein Hfq	0,19
<i>lpp0034</i>	hypothetical protein	0,52
<i>lpp0045</i>	fatty acid hydroxylase	0,52
<i>lpp0845</i>	global regulator CsrA	0,43
<i>lpp1823</i>	hypothetical protein	0,48

4.3 Supplementary results

LetA might regulate *antihfq* expression

As LetA influences positively the expression of the *hfq* transcript, we sought to determine whether LetA directly regulates the expression of these two oppositely transcribed genes. Interestingly, a search for LetA binding sites in the *hfq* and *anti-hfq* sequences revealed the presence of a putative binding site within the *anti-hfq* sequence, very similar to the consensus-binding site deduced from the known LetA binding sites in the sRNAs RsmY, RsmZ and RsmX (Figure 12).

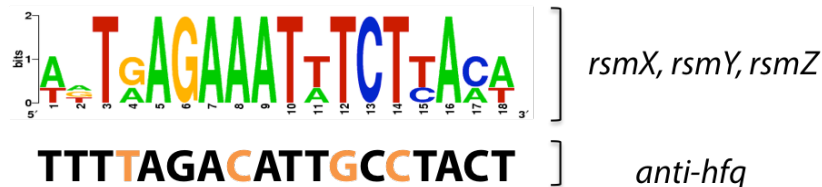


Figure 13. Consensus LetA binding site and putative LetA binding site of *anti-hfq*. A 18 bp consensus LetA binding site using the sequence of the already known LetA targets RsmY, RsmZ and RsmY was established (<http://mergealign.appspot.com/>) and compared to the putative LetA binding site found within the *anti-hfq* gene. Orange letters in the *anti-hfq* sequence indicate divergences with the highly conserved nucleotides in the LetA *Legionella* consensus.

Differently to the reported mechanism of action of this protein, we hypothesized that LetA binding to this sequence might lead to the repression of the expression of *anti-hfq* upon entry into the stationary phase, enabling thus the expression of the complementary target *hfq*. In order to investigate a direct interaction between LetA and the *anti-hfq* gene *in vitro*, I performed an electrophoretic mobility shift assays (EMSA). Incubation of a radioactive *anti-hfq* probe with increasing concentrations of the purified recombinant LetA protein resulted in a shift of a higher molecular weight, suggesting a direct interaction with the *anti-hfq* target. However, under our conditions, the LetA protein displayed the same pattern of slower migrating complexes also for a negative control, suggesting an unspecific binding (Figure 13).

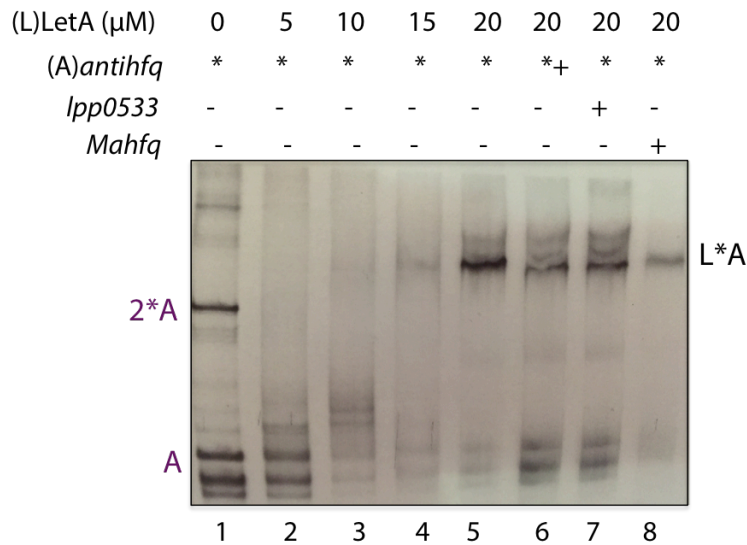


Figure 14. *In vitro* binding of a LetA and *anti-hfq* DNA probe. EMSA assays using 50nM radioactively labeled *anti-hfq* with the indicated increasing molar amounts of LetA protein showed that LetA indeed binds *anti-hfq*, however this may be an unspecific binding as the control was also binding. The duplex complexes between LetA and the competitors *lpp0533*, the cold *anti-hfq* a derivate *anti-hfq* probe in which highly conserved nucleotides in the putative LetA binding sites were mutated, were pre- incubated 20 minutes at room temperature. A binding reaction was carried out for 15 min at room temperature, and samples were then loaded onto 6% polyacrylamide 0.5X Tris-acetate-EDTA gel in 0.5X Tris-acetate-EDTA running buffer. Symbols: (L) LetA6X His, (A) radioactively labeled *anti-hfq* DNA probe. Asterisks, radioactively labeled DNA probes, + cold RNA probes; - no RNA probes.

To further investigate the relationship between LetA and Anti-hfq, a controlled system in which the *antihfq* gene, including the putative LetA binding site, was cloned in frame with the *blaM* gene into the pXDC61 plasmid, has been constructed (Figure 14A). We aimed to evaluate the levels of Anti-hfq expression at the exponential and post-exponential growth phases. Betalactamase activity analyses revealed a down regulation of Anti-hfq after the entry of the bacteria in the post-exponential phase, confirming our hypothesis (Figure 14B). Moreover the expression of the betalactamase, related to the Anti-hfq transcript level, was not affected in the ΔletA deletion mutant during bacterial growth, indicating that the lack of the DNA-binding protein LetA led to the accumulation of Anti-hfq even in the transmissive phase (Figure 14B). Although, this approach demonstrated that LetA has a negative influence on the *anti-hfq* gene in the post-exponential phase, I had problems with reproducibility and thus we could unfortunately not confirm our hypothesis.

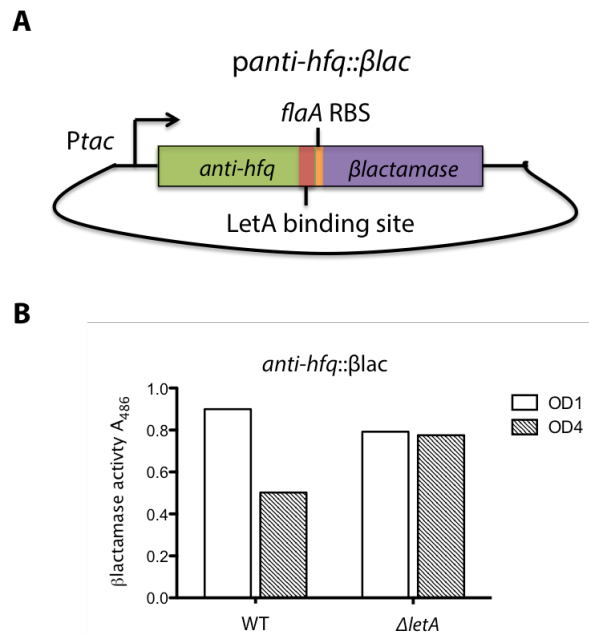


Figure 15. Activity assay to measure the level of *anti-hfq* expression (A) Schematic representation of the plasmid *panti-hfq::βlac*, in which the *anti-hfq* gene was cloned under the control of the inducible *Ptac* promoter and in fusion with the β lactamase gene. In order to evaluate the expression of the β lactamase the ribosome-binding site of the *flaA* gene was cloned upstream the β lac gene. The resulting plasmid was transformed in the *L. pneumophila* wild-type strain and in the *L. pneumophila letA* deletion mutant strains (B) The expression of the *anti-hfq::βlac* fusion was examined at the exponential phase, bacteria grown until OD=2 (white bars) and post-exponential phase, bacteria grown until OD=4 (grey bars), in the *L. pneumophila* wild-type strain (WT) and in the *L. pneumophila letA* deletion mutant strain ($\Delta letA$). The β lactamase activity assay shown represents one of the four replicates.

We then set up an *in vivo* approach using LetA Chromatin immunoprecipitation (ChIP) coupled to *antihfq* detection by quantitative real-time PCR. To generate a strain in which the LetA protein was overexpressed, the *L. pneumophila* wt strain was transformed with a plasmid in which the *letA* gene was under the control of a strong promoter and cloned in frame with 2 FLAG tags. Moreover, a strain in which the 2 FLAG tags were missing was used as negative control. Quantitative RT-PCR of the LetA-immunoprecipitated fractions revealed *anti-hfq* as direct target of the DNA-binding protein LetA. However, like for the other approaches used, several negative controls were also found among the LetA targets, and thus we could not confirm our hypothesis (Figure 15, data showing only one negative and one positive control). Taken together, the three different approaches used did suggest that LetA

indeed regulates *anti-hfq* expression, but did not firmly establish that a direct interaction between LetA and *anti-hfq* occurs during the post-exponential phase. Thus it is also possible that the influence of LetA is indirect.

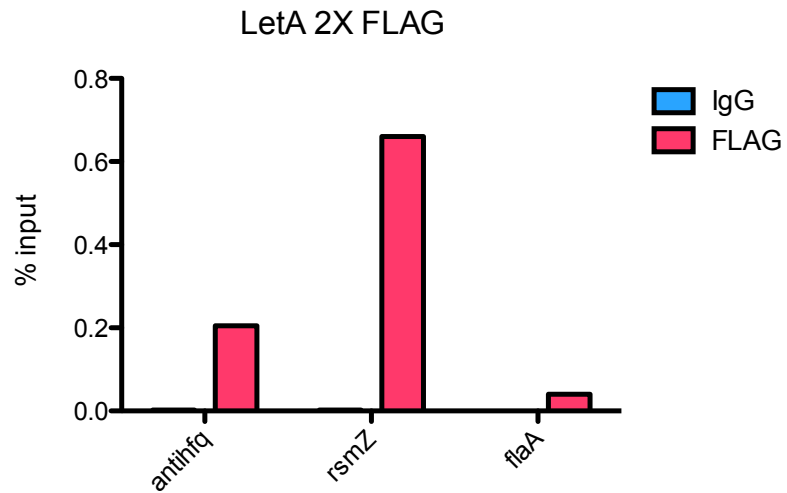


Figure 16. ChIP experiment in LetA2X FLAG clone using antibodies against IgG and FLAG. *anti-hfq*, *rsmZ* and *flaA* DNA enrichments were analyzed by qPCR (normalized as percent of input). ChIP experiment represents one of the three experimental replicates.

CHAPTER FIVE

CONCLUSIONS AND PERSPECTIVES

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Legionella pneumophila persists in the environment in diverse and multiple forms, such as the viable-but-non-cultivable state, the biofilm-associated sessile state or the free-living planktonic state. However, the primary site for *L. pneumophila* replication is within environmental protozoa (Fields et al., 2002). Exposure to contaminated aerosols generated by man-made water systems such as cooling towers, air-conditioning systems, humidifiers or even showers may lead to human infection, culminating eventually in Legionnaires' disease, mainly in susceptible individuals (Fields et al., 2002; Yiallourous et al., 2013). In order to survive and replicate in the environment, this intracellular bacterium must adapt to various stresses, including hostile intracellular as well as nutrient-limited extracellular habitats. Adaptation to these changing conditions is achieved by a tight regulation of its life cycle in which *L. pneumophila* alternates between a replicative phase in the host and a transmissive phase, when nutrient depletion triggers the escape of the bacterium from the spent host (Molofsky & Swanson, 2004). Thus, *L. pneumophila* represents a paradigm of highly adapted intracellular pathogens that have evolved sophisticated mechanism to evade the host cell defense but also to persist in the environment (Berjeaud et al., 2016). The bacterium interferes with the host signaling pathways by employing a large set of effector proteins, many of which contain eukaryotic-like domains that are translocated to the host cell cytoplasm by a dedicated type-IVB (T4BSS) secretion system (So, Mattheis, Tate, Frankel, & Schroeder, 2015). These effectors influence trafficking in the host cell, the signal transduction or the transcriptional regulation and ensure the establishment of the protective and replicative niche for the bacteria within the host cell (Heidtman et al., 2009; Hubber & Roy, 2010; J. S. Pearson, Zhang, Newton, & Hartland, 2015; Simon & Hilbi, 2015). Each infection stage requires the function of defined and specific sets of effectors and virulence traits to be produced, thus their expression needs also to be spatially and temporally controlled.

Indeed, transcriptome analysis of different *L. pneumophila* life cycle stages showed that in each phase *L. pneumophila* expresses a particular set of genes dependent on the environmental cues perceived (Brüggemann et al., 2006). To allow such a sophisticated regulation of its genes, *L. pneumophila* has evolved a complex and elaborated regulatory network, which ensures that global changes as well as temporal demands throughout the intracellular life cycle are rapidly answered. Although several of the key regulators and signals triggering the regulatory cascade controlling the transition from the replicative to the infectious form of

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L. pneumophila have been identified our understanding of the regulatory circuits and elements involved is far from being complete.

During my PhD thesis I focused on the characterization of the global regulator and sRNA chaperone Hfq, which is implicated in the regulatory circuit controlling *L. pneumophila* virulence. Nearly 45 years ago, Hfq was discovered in *E. coli* as the host factor for the replication of a coliphage (Franze de Fernandez et al., 1968) but nowadays Hfq represents one of the most extensively studied RNA-binding protein in prokaryotes. Hfq is recognized as a RNA chaperone, capable of mediating riboregulatory mechanisms. Comprehensive reviews and different works have reported the biochemical binding properties and preferences of Hfq for both sRNAs and their mRNA targets and the functional mechanisms of sRNA-mediated gene regulation (Feliciano, Grilo, Guerreiro, Sousa, & Leitão, 2016; Sauer, 2013; Sobrero & Valverde, 2012; Updegrove et al., 2016; Vogel & Ben F Luisi, 2011a). Global transcriptome and proteomic data of several pathogenic bacteria suggest a global impact of Hfq on the bacterial physiology as they revealed that Hfq directly or indirectly regulates for example 20% of all *Salmonella* genes, 18% of all *K. pneumoniae* genes or 6% of all *Francisella* genes (Chiang et al., 2011; Meibom et al., 2009; Sittka et al., 2008). Thus, it is not surprising that *hfq* mutations are associated with broadly pleiotropic phenotypes, with a remarkable impact on bacterial fitness, stress response and virulence (Sobrero & Valverde, 2012; Vogel & Ben F Luisi, 2011a). Moreover, the Hfq protein has been used as bait in many high-throughput RNA sequencing studies to pull down and identify new classes of regulatory Hfq-dependent sRNAs, so far the biggest class of sRNAs identified in prokaryotes. Although Hfq is a key player in the riboregulation network of several bacteria, a generalized model of which are Hfq targets cannot be established as most of our current knowledge on the role of Hfq is based on studies performed in *E. coli* and *Salmonella* (Sittka et al., 2008; Tsui et al., 1994). Thus only the in depth analysis of Hfq in a greater number of bacteria will allow to learn whether there are common regulatory pathways that are under the control of Hfq or whether these differ in each species.

In this study we showed that the *hfq* deletion mutant does not affect *L. pneumophila* growth in liquid medium as judged by the growth curve profiles comparing the wild type and the deletion mutant strains. When characterized in cell culture assays using THP-1 human monocyte-derived macrophages and *A. castellanii* cells Hfq was required for efficient replication within *A. castellanii* at 20° C, however no effect was observed when both THP-1

and amoebae were infected at 37° C. This observation suggests that Hfq is an important virulence factor in the environment, as temperatures in environmental waters rarely are much higher than 20°C. Thus, we suggest that Hfq regulates a defined set of transmissible traits, which this bacterium engages for an efficient replication in its natural host. Furthermore, we have shown that Hfq directly or indirectly influences bacterial motility, as judged by the loss of FlaA in the *hfq* deletion mutant compared to the wild-type in the post-exponential phase. This observation is in agreement with what is known from other pathogens that engage Hfq to regulate motility during host cell colonization (Kendall et al., 2011; Sonnleitner et al., 2003; Wilf et al., 2011). Thus, our data show, that the RNA-binding protein Hfq takes part in the regulatory network governing *L. pneumophila* virulence.

In agreement with a role of Hfq in virulence regulation, we showed that Hfq is regulated in a growth-phase dependent manner, with high expression of Hfq in post exponential/transmissive growth phase but low expression in exponential/replicative growth phase. Also our transcriptional analyses of the *L. pneumophila* genome showed that Hfq expression is high upon entry into the infectious form of *L. pneumophila*. This finding is different to what has been reported previously for Hfq of another *L. pneumophila* strain (McNealy et al., 2005). Their model proposed a growth-phase dependent regulation of Hfq, in which during the exponential phase RpoS controls *hfq* expression to allow the bacteria to adapt and efficiently use the replicative nutrients. Upon entry into the transmissive phase, induced by nutrient depletion, LetA switches off the *hfq* transcription through an RpoS-independent pathway, enabling transmissible traits to be activated (McNealy et al., 2005).

The differences with this study may be due to the different strain used by the two groups, or may also lie in an erroneous interpretation of the results by McNealy and colleagues. Hales and Shumann (Hales & Shuman, 1999) showed that RpoS is very poorly expressed in exponential growth but highly expressed in stationary phase cells, suggesting that it exerts its major regulatory role during stationary phase what is in agreement with our results but in contrast to the model proposed by McNealy where RpoS exerts its regulatory role in exponential phase. Furthermore, the clear impact on intracellular replication in *A. castellanii* also suggests that Hfq has a role in post-exponential phase and not in replicative/exponential growth. Thus the predominant production of Hfq upon onset of the late post-exponential phase as revealed in our study goes in hand with a role of this RNA-binding protein in the regulatory circuit that controls the switch from the replicative into the transmissive phase. We

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also show that the expression of Hfq is dependent on RpoS and LetA, but in contrast to McNealy and colleagues we found that RpoS regulates Hfq expression in post-exponential phase when RpoS is expressed the highest and in agreement with the role of Hfq in the regulation of the transmissive phase.

Our analysis of the *L. pneumophila* operon map lead also to the identification of a putative sRNA, later named Anti-hfq that is transcribed in the antisense strand of the *hfq* gene and overlaps its 5'UTR region. These observations supported a model in which the sRNA Anti-hfq may negatively regulate the *hfq* transcript, thus acting as a *cis*-encoded sRNA. Indeed, we show here that this sRNA, named Anti-hfq, is expressed and is 101 bp long as judged by amplification by 3' RACE PCR and sequencing. The Anti-hfq transcript is highly abundant in the early exponential phase and its expression level strongly decreases upon entry into the post-exponential phase, showing a reverse expression profile compared to the one of the *hfq* transcript. When Anti-hfq was overexpressed in *L. pneumophila* the expression of Hfq was downregulated whereas its knock-down resulted in the constitutive expression of Hfq throughout the *L. pneumophila* life cycle, suggesting a direct mechanism of regulation. Indeed, the *in-vitro* transcribed Anti-hfq is able to bind the *hfq* transcript through complementary base pairing, suggesting that Anti-hfq acts as a *cis*-encoded sRNA and directly regulates *hfq* transcription. This binding imposes a negative regulation on the mRNA translation due to the fact that the base-pairing region is in the proximity of the ribosome-binding site. The regulation of Hfq by an antisense RNA is an unusual feature, however a similar mechanism seems to exist in *Burkholderia cenocepacia*. This bacterium encodes two distinct *hfq* genes. The *hfq* gene encodes a 79- amino acid protein and the *hfq2* gene encodes an unusual Hfq-like protein of 188 amino acids (Sousa et al., 2010). The analysis of the two *hfq* transcripts showed that these two mRNA molecules are differently transcribed dependent on the growth phase. The mRNA levels corresponding to *hfq* are maximal at the early exponential phase of growth and decrease upon entry into stationary phase, whereas *hfq2* transcripts are strongly expressed in cells in the stationary phase (Ramos, Sousa, Grilo, Feliciano, & Leitão, 2011). This strict pattern of expression requires a tightly controlled regulation in the bacterial cell. Indeed, further studies proposed that the Hfq protein was negatively regulated by a sRNA named MtvR. This regulation is mediated by the binding of MtvR to the RBS of the *hfq* 5'UTR by an antisense mechanism, leading to the inhibition of the Hfq translation. Moreover, the binding of the MtvR on the 5'UTR of *hfq* is likely to be mediated through the help of the RNA chaperone Hfq2 (Ramos, Grilo, da Costa, Feliciano, &

Leitao, 2013). Furthermore, a sRNA, named h2cR present in the antisense strand of the *hfq2* gene and overlapping its 5' UTR region negatively regulates *hfq2* mRNA and a cross-regulatory loop has been suggested as the Hfq protein seems to stabilize the *hfq2* mRNA (Ramos, da Costa, Döring, & Leitão, 2012). However, as recently, three of the above-described articles have been retracted, we do not know what of these results is correct. However, the mechanism described resembles the *hfq* regulatory mechanism we found in *L. pneumophila*.

In our proposed model, during the exponential phase the abundantly transcribed sRNA ensures that the Hfq protein is not unnecessarily expressed. Indeed, we show that the RNase III is involved in the regulation of the *hfq* transcripts in exponential growth phase probably by a double-strand cleavage mechanism. Thus, as described in other bacterial species, also in *L. pneumophila* the sRNA-mRNA binding and the subsequent translational blockage results in the degradation of the RNA molecules by Hfq-dependent recruitment of RNase III (Aiba, 2007; Caron et al., 2010). Thus, the *L. pneumophila* Hfq protein might facilitate the binding of its own mRNA and *anti-hfq*, inhibiting its own translation. Such an Hfq auto regulatory loop has been described in *E. coli*, in which the binding of either Hfq or ribosomes to the *hfq* mRNA determines the fate of the *hfq* transcript (Vecerek et al., 2008). Indeed, in *E. coli* two binding sites in the 5'UTR, one of which overlaps the RBS of the *hfq* mRNA, act synergistically to bind Hfq and thereby repress its translation. This regulation renders the untranslated *hfq* mRNA a target for RNase E cleavage (Vecerek et al., 2008). In *L. pneumophila*, the presence of a (ARN)_n motif in the *hfq* 5'UTR and of an uridine-rich sequence in the 3' end of Anti-*hfq* that may represent putative Hfq-binding sites, suggested that also in *L. pneumophila* a Hfq auto regulatory mechanism may exist. Indeed, Hfq interacts *in vitro* with Anti-*hfq* and/or *hfq* mRNA transcripts as confirmed by EMSA assays. Thus Hfq does not only bind Anti-*hfq* RNA or its own mRNA but also forms a ternary complex with both RNA molecules. Since the sRNA-mRNA duplex appears to be formed *in vitro* also in absence of the protein, Hfq is not necessary but might contribute to the accelerated interaction with these RNAs.

Although the Hfq chaperone is known to mainly facilitate *trans*-encoded sRNA-mediated riboregulation, few examples of *cis*-encoded sRNA acting *in trans* have already been described. For example, the *cis*-encoded sRNA GadY of *E. coli* was reported to base pair with the 3' end of its target mRNA *gadX* and also to efficiently bind Hfq. Whether Hfq is required

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for the GadY and *gadX* mRNA interaction is not known, however the GadY RNA levels are strongly reduced in the *hfq* mutant, suggesting that this sRNA can act in *trans* (Opdyke, Kang, & Storz, 2004). Furthermore, the *cis*-encoded sRNA named ArrS targets and inhibits the *gadE* T3 mRNA, which is involved in acid resistance in *E. coli* (Aiso, Kamiya, Yonezawa, & Gamou, 2014). ArrS and GadX and their partners were later found to be enriched in an Hfq-immunoprecipitated pool of RNAs, confirming that these *cis*-encoded RNAs act as *trans*-sRNAs (Melamed et al., 2016). In addition, a model in which Hfq acts as “passive” platform for the sRNA and mRNA duplex formation has been described. In *E. coli*, Hfq forms a ternary complex with the sRNA DsrA and its mRNA target *rpoS*. This ternary complex is likely to be an intermediate state during inter-molecular annealing and it needs to be disrupted in order to allow the base pairing. This mechanism would explain the nature of an unstable ternary and intermediate complex, which still is necessary for the RNA duplex binding (W. Wang et al., 2013). Thus we can speculate that *L. pneumophila* engages Hfq for the efficient binding between *hfq* and Anti-*hfq*, allowing a temporal and spatial *hfq* regulation. Taken together, we have elucidating the tightly controlled regulation of the Hfq gene and protein expression during the bacterial life cycle and its role in the bacterial physiology. Thus the present work provides a novel and global comprehension of the regulatory mechanisms associated to virulence and intracellular adaptation of *L. pneumophila*.

To substantiate the proposed *in vitro* model a global analysis of the protein and RNA molecules bound by *L. pneumophila* Hfq will be performed in the future using RNA deep sequencing approaches. This analysis would not only confirm that the *hfq* mRNA and the Anti-*hfq* sRNA are direct targets of Hfq, supporting our Hfq auto regulatory model tested *in vitro* but it would also lead to the identification of all Hfq-dependent targets in *L. pneumophila*. Furthermore, as over 700 sRNAs are predicted to be present in *L. pneumophila* according to RNAseq analyses and transcriptional start site mapping (Sahr et al., 2012) we expect to identify many of these as bound by Hfq. Indeed, in analogy to the iPAR-CLIP or CLASH methodologies that were applied to detect protein-dependent miRNA-targets in eukaryotes, several RNA sequencing approaches have been established also in prokaryotes and provided a detailed and global knowledge about Hfq-binding sRNAs. For example the recently described RIL-seq approach (RNA interaction by ligand and sequencing) applied to *E. coli* led to the identification of Hfq-bound pairs of small RNAs showing sequence complementarity, revealing new insights into the sRNA-target interactions (Melamed et al., 2016). This approach resulted also in the expansion and re-wiring of the

sRNA-target networks, as additional targets of established sRNAs as well as of new Hfq-dependent sRNAs and their targets were identified. Moreover, RIL-seq contributed to a better understanding of the structure and dynamics of Hfq-bound sRNA interactions (Melamed et al., 2016). Thus applying this methodology to *L. pneumophila* at different stages of its life cycle should allow characterizing Hfq-mediated sRNA-mRNA interactions. Further analyses would uncover the roles of these RNA molecules in the physiology of *L. pneumophila*, including virulence.

Most interestingly, based on the bulk of experimental data collected on the functional characterization and properties of the RNA chaperone Hfq of several bacterial species, this protein has also been used as an innovative tool for practical applications. Indeed, comparable to the silencing strategy by small-interfering RNAs in eukaryotes, artificial sRNAs (atsRNAs) guided by the Hfq protein will lead to a stringent regulation of base pairing mRNA targets. As certain sRNAs directly mediate gene expression of multiple mRNA targets, the above-mentioned silencing strategy might represent a double-edged sword, which on one hand may globally switch off crucial processes but on the other hand might result in undesired altered effects (Nielsen et al., 2010; Papenfort & Vogel, 2010; Sobrero & Valverde, 2012). A successful example of using atsRNAs has been addressed in *E. coli*, in which a series of artificial sRNAs were used for targeting and silencing essential genes in an Hfq and RNase E-dependent manner, demonstrating that atsRNA was a powerful approach for specific gene silencing and thereby deciphering gene functions in prokaryotes (Man et al., 2011).

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Réseaux de régulation impliqués dans la virulence de *Legionella pneumophila* : le rôle de Hfq et du petit ARN non –codant Anti-hfq

RÉSUMÉ

Legionella pneumophila, responsable de la maladie du légionnaire, est une bactérie aquatique parasitant les amibes, mais aussi les macrophages alvéolaires humains. *Legionella* alterne entre une forme infectieuse non répliquative et une forme répliquative intracellulaire, qui n'exprime pas les facteurs de virulence. Ce cycle de vie biphasique est gouverné par un système de régulation complexe permettant son adaptation dans différents hôtes. Le but de mon projet de thèse était d'étudier un des facteurs clés dans la régulation des ARNm, le régulateur post-transcriptionnel global Hfq. L'expression de Hfq est régulée au cours du cycle infectieux chez *L. pneumophila*: Hfq est peu exprimée en phase répliquative, mais fortement exprimée lors de la phase de transmission, ce qui suggère un rôle dans la transition entre ces deux phases. J'ai identifié un petit ARN (sRNA) que j'ai nommé Anti-hfq puisqu'il est transcrit dans l'orientation antisens à Hfq et chevauche sa région 5' non traduite (UTR). Mes recherches ont mis en évidence un mécanisme sophistiqué par lequel Anti-hfq régule l'expression de Hfq: Anti-hfq interagit avec l'ARNm du gène *hfq* par sa région complémentaire et ainsi contrôle la stabilité de la protéine. De plus, j'ai montré que la protéine Hfq auto-réprime sa propre traduction en facilitant l'interaction entre Anti-hfq et son propre ARNm. Finalement, Hfq régule son propre renouvellement par le recrutement de la RNase III. De plus, des tests de répliquaison intracellulaire ont montré que Hfq et Anti-hfq sont nécessaires pour la multiplication intracellulaire de *L. pneumophila*, ce qui a mis en évidence un rôle important de Hfq et Anti-hfq dans la virulence de cette bactérie.

Mots clés: *Legionella pneumophila*; cycle biphasique; Hfq; virulence; Anti-hfq; régulation génique.

Regulatory circuits involved in *Legionella pneumophila* virulence : the role of Hfq and the cis-encoded sRNA Anti-hfq

SUMMARY

Legionella pneumophila, the causative agent of the pneumonia-like Legionnaires' disease, is commonly found in aquatic habitats worldwide where it multiplies within protozoa. To adapt between intra- and extracellular environments, *L. pneumophila* evolved a biphasic lifecycle wherein it alternates between an infectious and non-replicative form and an intracellular form, which does not express the virulent phenotypes. This biphasic life cycle is governed by a complex regulatory network that comprises transcriptional and post-transcriptional regulatory elements, enabling the bacteria to adapt in diverse hosts. During my Ph.D., I focused my attention on the post-transcriptional regulator Hfq, a hexameric, RNA-binding protein and chaperon of small RNAs (sRNA). The expression of this fascinating protein is life cycle regulated: poorly expressed during the replicative phase of growth, whereas significantly upregulated upon entry into the transmissive phase of growth. Moreover, my research lead to the identification of a sRNA transcribed antisense to the *hfq* gene overlapping its 5'UTR region. This antisense RNA, named Anti-hfq, was found to regulate *hfq* expression by base-pairing complementarity, describing a sophisticated mechanism of regulation. In addition, the Hfq protein controls its own translation by facilitating the interaction between Anti-hfq and its own mRNA. Thus, Hfq regulates its turnover, recruiting the endoribonuclease RNaseIII. Furthermore, infection assays revealed that Hfq and Anti-hfq are necessary for efficient replication of *L. pneumophila* in amoeba revealing an important role of both in bacterial virulence.

Key words: *Legionella pneumophila*; biphasic life cycle; Hfq; virulence; Anti-hfq; gene regulation.