

## Reduced expression of the global regulator protein CsrA in *Legionella pneumophila* affects virulence-associated regulators and growth in *Acanthamoeba castellanii*

Vera Forsbach-Birk, Tamara McNealy, Chunwei Shi, Damien Lynch, Reinhard Marre\*

Department of Medical Microbiology and Hygiene, University of Ulm, Robert-Koch-Str.8, D-89081 Ulm, Germany

Received 1 September 2003; received in revised form 1 December 2003; accepted 5 December 2003

### Abstract

*Legionella* bacteria have a developmental cycle in which they go from existing in the aquatic environment to replicating inside eukaryotic host cells. The adaptation to the new environment requires an efficient regulatory system. Overexpression of CsrA, a global regulatory protein found in a variety of Gram-negative bacteria has been shown to suppress virulence-associated traits in *Legionella pneumophila*. Since evidence resulting only from overproduction may not be sufficient to validate the role of a regulatory protein, a *csrA* mutant strain, CsrA(–), with a drastically reduced production of CsrA, was created. Using RNA slot blots and Western blotting it was shown that *fliA* and *flaA*, genes which contribute to flagellation, were expressed early in the mutant. Additionally, in CsrA(–) the levels of the stationary-phase sigma factor, RpoS, and a recently described regulator of virulence traits, LetE, were increased. Growth curves of CsrA(–) bacteria were delayed with pigment production occurring at the same OD<sub>578</sub> but at reduced levels in the mutant. Replication ability of the CsrA(–) mutant in amoebae was also affected. Based on these results, we could show that CsrA is involved in the regulation of the bacterial switch from the replicative to the transmissible form.

© 2004 Elsevier GmbH. All rights reserved.

**Keywords:** *Legionella pneumophila*; Regulation; Stationary phase; Amoebae

### Introduction

Bacteria have evolved many mechanisms in order to survive and multiply in different environments. Bacteria such as *Legionella pneumophila*, *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Erwinia carotovora*, *Pseudomonas fluorescens*, and others use numerous regulatory systems in order to control the expression and repression of virulence genes during the developmental cycle. Two systems, which have been shown to

play roles in this control are the LetA/LetS (known as UvrY/BarA, SirA/BarA and GacA/GacS, respectively) and the CsrA/CsrB (known in *Erwinia* as RsmA/RsmB and in *Pseudomonas* as RsmA/PrrB) systems (Chatterjee et al., 2002; Cui et al., 2001; Fettes et al., 2001; Goodier and Ahmer, 2001; Hammer et al., 2002; Heeb et al., 2002; Lawhon et al., 2003; Suzuki et al., 2002). During the developmental cycle of *L. pneumophila*, the bacteria differentiate from an intracellular, replicative form to an extracellular, motile, infectious form. The two-component system LetA/LetS in conjunction with CsrA and RpoS seems to determine the precise control of the switch. The interaction of these genes appears to allow for the adjustment to multiple stimuli received from a

\*Corresponding author. Tel.: +49-731-5002-4601; fax: +49-731-5002-4619.

E-mail address: [r.marre@medizin.uni-ulm.de](mailto:r.marre@medizin.uni-ulm.de) (R. Marre).

variety of microenvironments during infection, multiplication and release from the host cell.

As the ability of *L. pneumophila* to replicate in human alveolar macrophages allows the bacteria to be a potentially deadly human pathogen, understanding of the cellular regulation of virulence gene expression during the developmental cycle of the bacteria could provide crucial information for control of these microbes. The natural hosts of *Legionella* bacteria, however, are freshwater protozoa (for review see Fields et al., 2002; Harb et al., 2000). During intracellular replication within protozoa, the bacteria are shielded from the adverse effects of environmental stresses such as chlorination and biocidal compounds. Protozoa are also responsible for amplifying the numbers of this bacterium in the environment thus increasing the chances of *L. pneumophila* encountering a suitable human host. There is also evidence that intracellular growth within protozoa amplifies the virulence of *L. pneumophila* (Cirillo et al., 1999; Swanson and Hammer, 2000). The infection of a eukaryotic host cell by *L. pneumophila* is facilitated by the controlled expression of specific genes or virulence factors at specific stages of the infection cycle (Cianciotto and Fields, 1992; Fettes et al., 2000; Harb and Abu Kwaik, 2000; Heuner and Steinert, 2003; Liles et al., 1999; Robey et al., 2001; Rossier and Cianciotto, 2001; Sadosky et al., 1994). Initial contact between the bacterium and host cell is promoted by the motility of the bacterium, which is dependent upon the production of flagella. Within the host cell, *L. pneumophila* replicates within a phagosome modified by the action of the *dot/iam* genes to prevent fusion with lysosomal compartments (Hilbi et al., 2001). After replicating to high numbers, *L. pneumophila* induces lysis of the host cell by the production of a pore-forming protein allowing the transmission of the microbes to a new host (Alli et al., 2000).

The growth phase-dependent regulation of *Legionella* virulence gene expression has been shown to be mediated to some extent by RpoS, the stringent response and the LetA/LetS two-component system (Hales and Shuman, 1999; Hammer and Swanson, 1999; Hammer et al., 2002). In a number of Gram-negative bacteria, the small regulatory protein, CsrA, has been shown to be an important component of regulatory cascades involving RpoS, and the LetA/LetS two-component system (Fettes et al., 2001; Hammer et al., 2002; Lynch et al., 2003). Recently, it was reported that a small protein, LetE, also affects virulence gene expression (Hammer et al., 2002). However, it is not clear if this protein functions directly or indirectly with members of the LetA/LetS and/or CsrA systems. It has been seen that mutations in the regulatory genes have differing effects on bacterial virulence in macrophages and amoebae and are depen-

dent on the *Legionella* wild-type strain used in the experiment (Hales and Shuman, 1999; Hammer et al., 2002; Lynch et al., 2003).

We previously isolated an *L. pneumophila* homologue of the *csrA* gene, overexpression of which altered the stationary-phase phenotype of *L. pneumophila* (Fettes et al., 2001). Here we report the isolation of a mutant exhibiting greatly reduced expression of the *L. pneumophila csrA* gene. Characterisation of this mutant strain expands investigation of the role of *csrA* in the regulation of *L. pneumophila* gene expression.

## Materials and methods

### Bacterial strains and plasmids

Bacterial strains and plasmids used in this study, their sources, and relevant genotypes are described in Table 1. *E. coli* DH5 $\alpha$  was used for the cloning experiments.

### Media and growth conditions

*E. coli* cultures were grown in Luria–Bertani (LB) medium with or without agar (Ausubel, 1987). When required, antibiotics were added as follows: ampicillin (Ap), 50  $\mu$ g/ml; kanamycin (Km), 25  $\mu$ g/ml (*L. pneumophila*) and 50  $\mu$ g/ml (*E. coli*); chloramphenicol (Cm), 7.5  $\mu$ g/ml (*L. pneumophila*) and 20  $\mu$ g/ml (*E. coli*). *L. pneumophila* was cultured on buffered charcoal yeast extract (BCYE) agar plates or was grown in buffered yeast extract (BYE) broth supplemented with ACES,  $\alpha$ -ketoglutarate, L-cysteine and ferric pyrophosphate (Edelstein, 1981) or in a complete defined medium designated LCDM (Abu Kwaik et al., 1993). All experiments described in this report were performed with the same batch of *Legionella* frozen in stocks. For each experiment, the bacteria were subcultured twice and then processed as described.

For growth kinetic experiments, an overnight culture in BYE (LCDM) medium was used to inoculate 20 ml of BYE (LCDM) to an OD<sub>578 nm</sub> of 0.1. The bacteria were grown at 30°C and 37°C with shaking. Samples of 1 ml were removed at times indicated for determination of optical density at 578 nm.

### Cell morphology

Bacterial morphology was studied by NanoOrange staining as described elsewhere (Grossart et al., 2000). Bacteria were viewed with a Zeiss Axioplan 2 fluorescence microscope.

**Table 1.** Bacterial strains and plasmids used

Strain or plasmid	Genotype or features	Reference or source(s)
<i>L. pneumophila</i>		
JR32	AM511 salt-sensitive isolate	Sadosky et al. (1993)
JRC	JR32 containing the plasmid pBC-CsrA	This study
CsrA(-)	JR32 <i>csrA</i> <sub>promoter</sub> : Kana	This study
CsrA(-)/R	CsrA(-) containing the plasmid pBC-CsrA	This study
CsrA(-)/BC	CsrA(-) containing the plasmid pBCKS(+)	This study
CsrA(-)/Rc	CsrA(-) containing the plasmid pBBR-CsrA	This study
<i>E. coli</i>		
DH5 $\alpha$	F <sup>-</sup> , $\phi$ 80dlacZDM15, D(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk <sup>-</sup> , mk <sup>+</sup> ), phoA, supE44, l <sup>-</sup> , thi-1, gyrA96, relA1	Woodcock et al. (1989)
<i>Plasmids</i>		
pBCKS(+)	OriR(f1), MCS, oriR (ColE1), <i>lacZ</i> , Cm <sup>r</sup>	Stratagene
pBC-CsrA	PCR product containing the <i>csrA</i> gene cloned into plasmid pBCKS(+)	This study
pBBR1MCS-5	Broad-host-range cloning vector, Gm <sup>r</sup>	Kovach et al. 1995)
pBBR-CsrA	PCR product containing the <i>csrA</i> gene cloned into plasmid pBBR1MCS-5	This study
pBCluc2	pBCKS (+) containing the luciferase gene from pFW11-luc	Lynch et al. (2003)
pBCluc2-RpoS	pBCluc2 containing the <i>L. pneumophila rpoS</i> promoter region	Lynch et al. 2003)
pBOC 20	OriR (ColE1), <i>sacB</i> , Cm <sup>r</sup>	O'Connell et al. (1995)
pBSL99	Source of kanamycin cassette	Alexeyev et al. (1995)

## Pigment production

Pigmentation kinetics were studied as described previously (Wiater et al., 1994). Overnight broth cultures were diluted to an OD 0.1 in BYE medium. Samples of 1 ml were removed at the times indicated, centrifuged at 4000g and the supernatant was used for spectrophotometral analysis at 550 nm. Pigmentation experiments were conducted at 30°C and 37°C.

## Recombinant DNA

Amplification of DNA by PCR was performed according to standard protocols (Ausubel, 1987). For the generation of DNA probes for DNA–DNA hybridisation, 0.5  $\mu$ l of DIG-dUTP (5 mM) (Roche) was added to the reaction mixture. The *L. pneumophila* JR32 insertion mutant CsrA(-) was generated as follows: The plasmid pVF4-Kan, a pBluescript KS(+) derivative containing a 3700-bp fragment with the *csrA*<sub>orf</sub> replaced by the kan<sup>r</sup> cassette (Fettes et al., 2001) was digested with EcoRV and SpeI and the fragment, consisting of *L. pneumophila* JR32 DNA containing the Km<sup>r</sup> cassette, was cloned into pBOC20 (O'Connell et al., 1995) generating pVFboc-Kana.

The plasmid pVFboc-Kana was electroporated into *L. pneumophila* JR32 using methods described previously (Cianciotto and Fields, 1992). Transformants were selected on BCYE plates containing kanamycin.

Transformants were then grown overnight in BYE liquid medium and plated on BCYE plates containing kanamycin and 6% sucrose. The resulting colonies were screened for correct insertion of the Km<sup>r</sup> cassette using PCR and Southern analysis. The resulting mutant was named *L. pneumophila* CsrA(-).

In order to construct a *csrA*-complementing clone, the *csrA* gene was amplified by PCR from *L. pneumophila* JR32 genomic DNA using the primers CsrAcomp\_F (5'-CTTGAGCTCCAGATAATTTAAGGAACAGAAT-3') and CsrAcomp\_R (5'-CCCAAGCTTATAATATGC GACAAATCAAATGC-3'). This PCR product is 940 bp in length and consists of the *csrA* gene plus 450 bp upstream of the *csrA* gene and 300 bp downstream of the *csrA* gene. The resulting fragment was digested with the enzymes HindIII and SacI and cloned into the vector pBCKS(+) generating the plasmid pBC-CsrA. This plasmid was then introduced into *L. pneumophila* CsrA(-) by electroporation generating *L. pneumophila* CsrA(-)/R. Plasmid pBBR-CsrA with an even more elevated expression of *csrA* in comparison to pBC-CsrA was constructed as well. The open reading frame of *csrA* was amplified by PCR from *L. pneumophila* JR32 genomic DNA using the primers CsrAcomp\_F' (5'-CCGCTCGAGCATGTTGATTTGACTCGGCG-3') and CsrAcomp\_R. The fragment was digested with XhoI and HindIII and cloned into the vector pBBR1MCS-5 (Kovach et al., 1995) generating the plasmid pBBR-CsrA\_orf. The *mip*-promoter was inserted upstream of the *csrA* open reading frame.

The promoter region was amplified by PCR from *L. pneumophila* JR32 genomic DNA using the primers Mip\_prom\_F (5'-CGGGGTACCCACTAATG TTCATCGCCGTT-3') and Mip\_prom\_R (5'-CCGC TCGAGCAATCCCCTTTTAGTCTTACAC-3'), the resulting PCR product was then digested with KpnI and XhoI and cloned into pBBR-CsrA\_orf generating the plasmid pBBR-CsrA. When this plasmid was introduced into wild-type JR32 an overproduction of *csrA* at the transcriptional level was observed at OD<sub>578 nm</sub> 1.2 and OD<sub>578 nm</sub> 1.8 (Fig. 1). The plasmid was introduced into *L. pneumophila* CsrA(-) by electroporation generating *L. pneumophila* CsrA(-)/Rc.

### RNA isolation, Northern blot and slot blot analysis

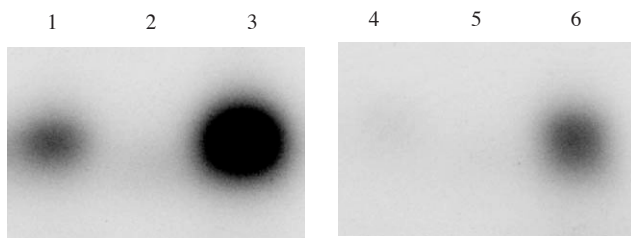
*L. pneumophila* strains were grown in BYE medium at 37°C. At specified time points a sample was removed and the bacterial cells were pelleted by centrifugation. Total RNA was isolated from the bacterial pellets using Trizol reagent (Life Technologies) according to the manufacturer's instructions. The RNA was aliquoted and stored at -70°C. Slot blots were performed with samples of 10 µg of RNA according to the manufacturer's instructions (Consort). For Northern blot analysis, 10 µg of RNA was size fractionated by

electrophoresis through a 1.2% agarose gel containing formaldehyde (Sambrook et al., 1989). RNA was transferred to a nylon membrane using vacuum transfer, and hybridisation was carried out overnight with PCR-generated probes labelled with digoxigenin (DIG)-dUTP (Roche). Dig-labelled probes were visualised using the DIG Luminescent Detection Kit (Roche). Northern blot and slot blot experiments were repeated three times with independent RNA isolates.

The primers, which were used to generate DIG-labeled probes specific for the *L. pneumophila* genes *csrA*, *flaA*, *fliA* and *letE*, are described in Table 2.

### Luciferase assay

In order to quantify luciferase activity, *L. pneumophila* strains harbouring the *rpoS* promoter-*luc* fusion were inoculated at an OD<sub>578 nm</sub> of 0.1 in BYE medium. The culture was then grown in an orbital shaker at 37°C. Samples were taken at 2-h intervals and the OD<sub>578 nm</sub> was measured. A 150-µl aliquot was held on ice until measured for luciferase activity. The reservoirs of the "Flash-n-Glow" luminometer (Berthold) were filled with 2.5 × assay buffer (62.5 mM glycyl-glycine, pH 7.8, 25 mM MgCl<sub>2</sub>, 12.5 mM ATP, 2.4 mM acetyl-CoA, 0.025% H<sub>2</sub>O<sub>2</sub>) and 330 mM D-luciferin, freshly dissolved in water. After placing the sample tube with the cell suspension into the luminometer, 200 µl of 2.5 × assay buffer followed by 200 µl of the luciferin solution were automatically added. Luminescence was immediately measured for 15 s at 22°C. Adjustments for pumping of liquids and washing of tubing were according to the manufacturer's instruction. The reporter gene fusion used for the luciferase assay has been previously described (Lynch et al., 2003). The luciferase activity of an *L. pneumophila* strain containing the pBCLuc2 plasmid, which was just above background levels, was subtracted from each measurement resulting in luciferase activity expressed as relative light units (RLUs).



**Fig. 1.** Determination of *csrA* transcription using RNA Northern blot. Total RNA (10 µg), isolated from JR32 (1), CsrA(-) (2), CsrA(-)/Rc (3) at OD<sub>578 nm</sub> 1.2 and from JR32 (4), CsrA(-) (5), CsrA(-)/Rc (6) at OD<sub>578 nm</sub> 1.8, was applied to a membrane and hybridised with a *csrA*-specific probe. All strains were grown at 37°C.

**Table 2.** Primers

Gene	Sequence	Reference
<i>csrA</i> uni	5'-ttgattttgactcgccgtatag-3'	Fettes et al. (2001)
<i>csrA</i> rev	5'-gattcttttctgtgtatgcgta-3'	Fettes et al. (2001)
<i>flaA</i> uni	5'-gtaatcaactaatgtggc-3'	Heuner et al. (1995)
<i>flaA</i> rev	5'-gttgacagaatttggttttggtc-3'	Heuner et al. (1995)
<i>fliA</i> -U3	5'-ttagctgactctgttg-3'	Heuner et al. (1995)
<i>fliA</i> -R5	5'-tttattccggaatcttgatc-3'	Heuner et al. (1995)
<i>letE</i> uni	5'-tacatgcactaaaagcggttctg-3'	Personal communication by M. Swanson
<i>letE</i> rev	5'gaacgcgatgatcatgctac-3'	This study

## Protein isolation and Western blotting

Total cell extracts of *L. pneumophila* were prepared after growth at 37°C. Briefly, bacterial cell pellets were resuspended in 250 µl of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40 and 0.5% Na-Deoxycholate) and sonicated for 10 s. The cells were then shaken for 2 h at 4°C and centrifuged for 10 min at 7600g. The protein-containing supernatant was removed and the protein concentration was estimated using a commercial kit (Biorad). SDS-PAGE was performed as described previously (Laemmli, 1970). Western blots were carried out as described elsewhere (Towbin et al., 1979). The major flagellar protein, FlaA, was detected using a polyclonal monospecific anti-flagellin antibody as described previously (Heuner et al., 1995). The CsrA protein was detected using an anti-*E. coli* CsrA antibody provided by Tony Romeo of Emory University (Atlanta, GA). Detection of RpoS was performed using an anti-*E. coli* RpoS antibody provided by Regina Hengge-Aronis (Freie Universität Berlin).

## Intracellular growth assays

Infection of *Acanthamoeba castellanii* (ATCC 30010) was carried out as described previously (Fettes et al., 2000) using a multiplicity of infection of 10. *A. castellanii* was grown in peptone–yeast extract–glucose broth as described elsewhere (Essig et al., 1997). Incubation was performed at 37°C.

## Results

### Isolation of a mutant defective in the transcription of *csrA*

We previously isolated and characterised the *L. pneumophila* homologue of the *E. coli* *csrA* gene (Fettes et al., 2001). However, numerous attempts to construct a *csrA* insertion mutant by homologous recombination proved unsuccessful. In an attempt to create a *csrA* deletion-substitution mutant the entire *csrA* open reading frame was replaced by a kanamycin resistance

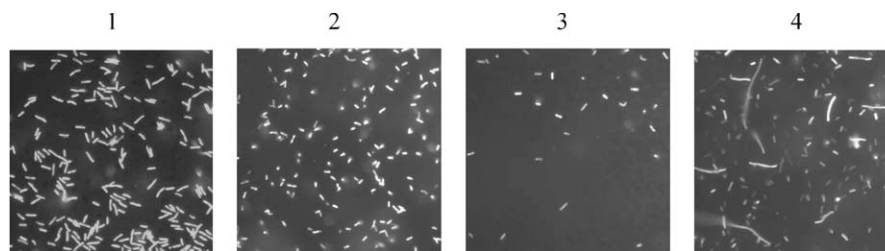
cassette and this fragment was cloned into the vector pBOC20. This construct was transformed into *L. pneumophila* JR32 and a number of screening methods were used in an attempt to isolate a *csrA* insertion mutant. Screening of more than 200 potential mutants failed to isolate a *csrA* deletion-substitution mutant. However, a single clone was isolated which contained the kanamycin gene inserted into the Nco site within the promoter region of the *csrA* gene, 50 bp upstream of the start codon. The insertion site was confirmed by sequencing (data not shown). The transcription level of the *csrA* gene in this clone was evaluated using Northern blot analysis and found to be greatly reduced compared to the wild type indicating that insertion of the kanamycin cassette into the promoter region attenuates the transcription of the *csrA* gene (Fig. 1). Reduced levels of CsrA protein in the mutant could be confirmed by Western blot analysis using an anti-*E. coli* CsrA antibody (data not shown). This clone was characterised to determine the effects of reduced *csrA* expression on *L. pneumophila*.

## Bacterial morphology

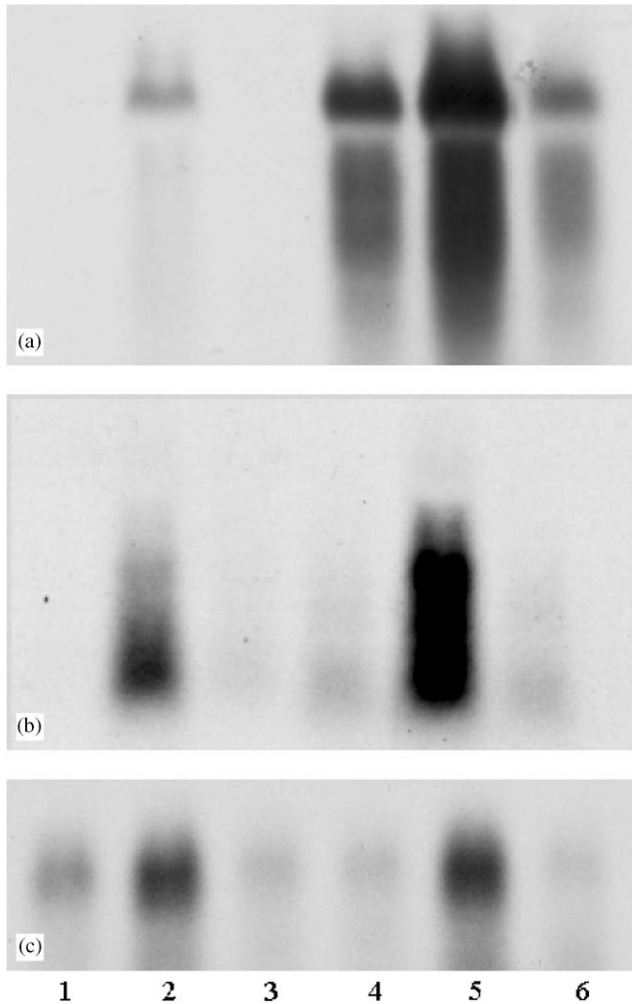
At the transition from exponential to post-exponential growth phase, *Legionella* bacteria undergo morphological changes from elongated forms to coccoid cell shapes (Fig. 2). CsrA appears to influence the morphological switch as the mutant strain CsrA(–) demonstrates the presence of coccoid cells in the exponential phase. The CsrA overproducing wild-type strain JRC bacteria predominantly shorten at the post-exponential phase but, some of the bacteria elongate at this stage of growth and appear as long, filamentous forms.

### Reduced expression of *csrA* alters the expression kinetics of the *flaA* and *fliA* genes

We recently described an effect of overexpression of *L. pneumophila* *csrA* on flagellation (Fettes et al., 2001). In order to determine if flagellation was also affected in the mutant strain CsrA(–), we analysed the expression of the *flaA* and *fliA* genes in *L. pneumophila*. *FlaA* encodes the major subunit of the flagella, flagellin, and *FliA*, the



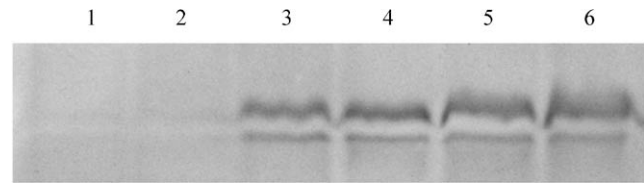
**Fig. 2.** Bacterial morphology of NanoOrange-stained microbes at the exponential (E) and the post-exponential (PE) growth phase respectively: JR32, E (1); JR32, PE (2); CsrA(–), E (3); JRC (4). All strains were grown at 37°C.



**Fig. 3.** Northern analysis of (a) *flaA*, (b) *fliA* and (c) *letE* transcription at 37°C. Total RNA (10 µg), isolated from (1) JR32, (2) *CsrA*(–), (3) *CsrA*(–)/Rc in (a), (b) respectively *CsrA*(–)/R in (c) at OD<sub>578 nm</sub> 1.2 and from (4) JR32, (5) *CsrA*(–), (6) *CsrA*(–)/Rc in (a), (b) respectively *CsrA*(–)/R in (c) at OD<sub>578 nm</sub> 1.8, was applied to a membrane and hybridised with the respective specific probe.

alternative  $\sigma^{28}$  factor, has been shown to regulate *flaA* expression at the transcriptional level (Heuner et al., 2002). The presence of *fliA* and *flaA* transcripts was analysed at 37°C by Northern blot (Fig. 3a and b). As expected in the wild-type JR32 bacteria, the expression of the flagellation genes was growth phase dependent and could be detected first in early stationary-phase cultures (OD<sub>578 nm</sub> 1.8). In contrast, the *CsrA*(–) mutant strain began transcription of *flaA* and *fliA* in the exponential phase (OD<sub>578 nm</sub> 1.2). These results were confirmed by Western blot analysis using an anti-flagellin antibody (Fig. 4). The data indicate a regulatory control mechanism by *CsrA* on *flaA* expression via the alternative  $\sigma^{28}$  factor FliA.

The mutant phenotype was complemented in Northern blot, as well as in Western blot analysis by



**Fig. 4.** Western blot analysis of total bacterial cell extracts using anti-flagellin antiserum. Proteins were isolated from JR32 (1), *CsrA*(–)/Rc (2), *CsrA*(–) (3) at OD<sub>578 nm</sub> 1.2 and from JR32 (4), *CsrA*(–)/Rc (5), *CsrA*(–) (6) at OD<sub>578 nm</sub> 1.8. Ten µg protein was loaded per lane. All strains were grown at 37°C.

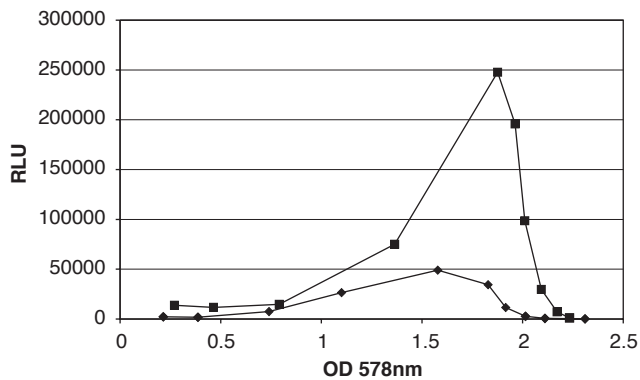
introduction of the plasmid pBBR-*CsrA*. In this plasmid *csrA* is expressed under the control of the *mip*-promotor and greatly overexpressed in comparison to the wild type. The complemented *CsrA*(–) mutant as well as the wild-type strain JR32 transformed with pBBR-*CsrA* (data not shown) showed wild-type expression patterns with respect to flagellation.

### Reduced expression of *csrA* affects the expression of the *rpoS* and *letE* genes

The expression of several stationary-phase traits has been shown to be coordinated by the sigma factor RpoS (Bachman and Swanson, 2001; Hales and Shuman, 1999). We therefore investigated the effect of the *csrA* mutation on the transcription kinetics of this gene in *L. pneumophila*. We used a luciferase reporter gene fusion system for the *rpoS* gene in which the predicted promoter was cloned upstream of the luciferase open reading frame (Lynch et al., 2003). Control strains containing the plasmid without an upstream promoter region produced negligible amounts of luciferase activity.

In the wild-type background the gene was transcribed beginning at the transition between exponential and stationary phase with expression dropping after entry into stationary phase. In the *CsrA*(–) mutant the *rpoS* transcription was found to be increased by the factor five compared to the wild type (Fig. 5). When confirmation of these results was attempted by Northern blot analysis, no differences were found in *rpoS* transcript level in the *CsrA*(–) mutant compared to the wild-type strain JR32, neither in exponential nor in stationary phase. In contrast, Western blotting using an anti-*E. coli* RpoS antibody detected a high RpoS protein level in the *CsrA*(–) mutant at the beginning of the stationary phase (data not shown). Therefore, the observed changes of RpoS expression seem to be the result of a regulatory mechanism at the post-transcriptional level.

Another recently described gene product influences the expression of virulence-associated traits is LetE

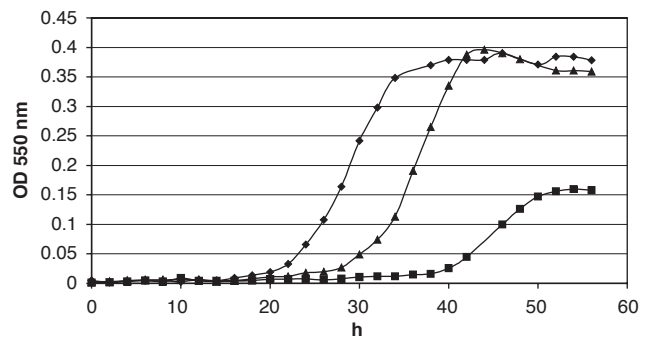


**Fig. 5.** Luciferase production (RLU) of JR32 (◆) and CsrA(-) (■) containing an *rpoS*-luciferase fusion (pBCluc2-RpoS). The bacterial strains were grown in BYE medium at 37°C and samples were taken every 2 h for determination of optical density at 578 nm and luciferase activity. The experiment was performed three times and the results of a representative experiment are shown. Bacteria containing the control plasmid, pBCluc2, produced no significant luciferase activity (data not shown).

(Hammer et al., 2002). Northern blot experiments were performed to examine the expression of *letE* in the CsrA(-) mutant. Abundant levels of the *letE* transcript were found in the CsrA(-) mutant strain in the exponential phase as well as in the idiophase compared to wild type (Fig. 3c). The abundance may be the result of an enhanced *letE* transcription rate or an increased transcript stability in CsrA(-). The results indicate that CsrA acts as a negative regulator of *letE* expression in either a direct or indirect way.

### Reduced expression of *csrA* affects growth kinetics and pigmentation at 30°C

As a *S. enterica* serovar Typhimurium *csrA* mutant was shown to be deficient in growth (Altier et al., 2000a), growth kinetics of *L. pneumophila* CsrA(-) were determined. Growth kinetics in BYE as well as pigmentation at 37°C were unchanged in the *L. pneumophila* CsrA(-) mutant compared to the wild type (data not shown). However, at 30°C JR32 reached a plateau of extinction approximately 20 h after inoculation. The CsrA(-) mutant strain reached this plateau at 38 h, a delay of 18 h compared to the wild type (data not shown). In both, JR32 and the CsrA(-) mutant, pigmentation was induced at an OD<sub>578 nm</sub> of approximately 2.0; however, the maximum production of pigmentation in CsrA(-) was 50% less than that of JR32 (Fig. 6). Therefore, the reduced pigment production in the mutant is not the result of its slower multiplication at 30°C. The growth defect, as well as the reduction in pigmentation, were complemented by the introduction of plasmid pBC-CsrA, which carries *csrA*



**Fig. 6.** Pigment production by *L. pneumophila* JR32 (◆), CsrA(-) (■) and CsrA(-)/R (▲) at 30°C as measured by optical density at 550 nm of culture supernatants (one representative graph out of three experiments).

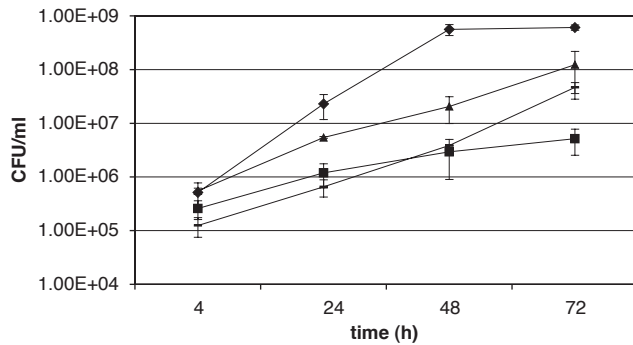
under the control of its native promoter. These data suggest participation of CsrA in a temperature-dependent control of bacterial growth and pigmentation.

CsrA has been found to be involved in the regulation of carbon metabolism in *E. coli* and *S. enterica* serovar Typhimurium (Lawhon et al., 2003; Liu et al., 1995; Romeo et al., 1993). However, *Legionella* does not utilise glucose and polysaccharides, but rather amino acids as carbon source (Pine et al., 1979). Therefore, the growth kinetics of the wild-type JR32 and the CsrA(-) mutant in a complete defined medium (LCDM) containing amino acids (Abu Kwaik et al., 1993) versus the normal growth medium BCYE were examined. It has been shown that a slow growth rate of *L. pneumophila* in LCDM is characteristic of this medium (Abu Kwaik et al., 1993). Nevertheless, in comparison to the wild type the growth rate and the pigmentation of the mutant strain is decreased by approximately 50% at both 30°C and 37°C (data not shown).

### Reduced expression of *csrA* affects the ability of *L. pneumophila* to replicate within protozoa

*L. pneumophila* normally inhabits aquatic environments where it survives as an intracellular parasite of protozoan hosts such as *A. castellanii*. Therefore, we were interested in examining the ability of the mutant strain to infect and multiply within these amoebae. While the wild-type strain JR32 could replicate 10<sup>3</sup>-fold within *A. castellanii*, CsrA(-) showed a reduced rate of multiplication compared to the wild type (Fig. 7). In contrast, JR32 and CsrA(-) showed no significant difference in the initial infection of amoebae.

In a complementation experiment amoebae were infected with the mutant strain CsrA(-) containing the plasmid pBC-CsrA. Infection of CsrA(-) with the vector, pBCKS (+), alone demonstrated a currently unexplainable positive effect on the replication ability of



**Fig. 7.** Intracellular growth kinetics of CsrA(-) (■), CsrA(-)/R (▲), CsrA(-)/BC (●) and the wild-type JR32 (◆) in *A. castellanii*. The number of viable bacteria within amoebae was evaluated by the standard plate count assay. Each time point represents the mean  $\pm$  SD of two independent experiments. Error bars may not be visible where the error is too low to display. Infections were performed at 37°C.

the mutant at 72 h. Nevertheless, the growth defect of the mutant was only partially cured by the recombination vector pBC-CsrA.

## Discussion

Numerous attempts to construct an *L. pneumophila* *csrA* insertion mutant proved unsuccessful indicating that the *csrA* gene may be essential for the proper growth and replication of this species. A similar situation exists for *S. enterica* serovar Typhimurium and *Proteus mirabilis* (Altier et al., 2000b; Liaw et al., 2003). Here, we report the isolation of a mutant exhibiting greatly reduced expression of the *csrA* gene due to the insertion of a kanamycin cassette into the predicted promoter region of the gene. The characterisation of this mutant helps to understand the role of CsrA in *L. pneumophila* and complements data from studies with a mutant overproducing CsrA (Fettes et al., 2001). Overproduction of CsrA resulted in a phenotype, which was less pigmented, less flagellated and elongated and therefore appeared as a *Legionella* population arrested in the logarithmic growth phase. This observation suggested that the mutant strain might exhibit a stationary-phase-like phenotype. As shown in this paper, CsrA(-) indeed showed coccoid cells already present in the logarithmic growth phase complete with premature flagellation.

In order to better understand the role of CsrA in the regulatory cascades, transcription of known regulatory genes was studied. These experiments showed that a severe reduction of CsrA production is associated with an increased expression of *rpoS* and *letE* and an earlier onset of *fliA* transcription, which leads to a premature switch into the stationary growth phase phenotype.

Together with the experiments published by Bachman and Swanson (2001), this allows the proposal of a tentative scheme of the time course of the activity of different regulatory genes. CsrA is produced early during the logarithmic growth phase (Fettes et al., 2001), which keeps down the activity of *fliA*, *rpoS* and *letE*. At the end of this growth phase, accumulation of ppGpp as a cellular response to amino acid starvation as shown by Bachman and Swanson (2001) could trigger the switch by down-regulating CsrA. Then *FliA*, up-regulated directly or by means of *rpoS*, leads to increased transcription of *flaA* and subsequently a state of flagellation. The resulting phenotype should be, as described by Bachman and Swanson (2001), sodium sensitive, motile, capable of infection and endosome evasion, a phenotype which corresponds to virulence. The CsrA(-) mutant as characterised by virulence-associated traits demonstrates premature flagellation and coccoid cell shape and infects amoebae with the same efficiency as the wild-type strain. However, replication of CsrA(-) within amoebae is reduced consistent with our finding that CsrA is necessary for proper growth of exponential phase bacteria.

The complementation of CsrA(-) in the infection experiments was only partially successful. Interestingly, similar effects were found by Hales and Shuman (1999) when examining the ability of an *rpoS* mutant strain to infect *A. castellanii*. The lack of RpoS in the mutant as well as the plasmid-mediated overproduction of the alternative sigma factor resulted in the inability of *Legionella* to grow in *A. castellanii*. Furthermore, it has also been shown in *S. enterica* serovar Typhimurium that loss of CsrA, as well as its overproduction, decreases the ability of mutant bacteria to invade cultured epithelial cells (Altier et al., 2000a).

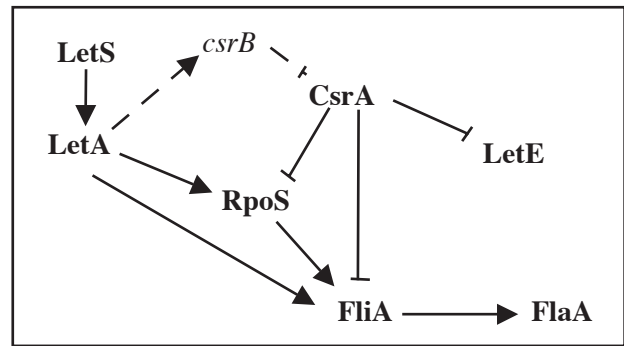
The precise nature of *rpoS* expression control in *Legionella* is not known. The sensor kinase homologue of the two-component transduction system LetA/LetS was shown to be required for the transcription of *rpoS* in *E. coli* and *P. fluorescens* (Mukhopadhyay et al., 2000; Whistler et al., 1998). Recently, several studies have provided evidence that the control of gene expression by the two-component system homologues of LetA/LetS in other Gram-negative bacteria, such as *E. coli*, *Erwinia* and *Pseudomonas*, is mediated in part by a CsrA/CsrB-like system (Blumer and Haas, 2000; Cui et al., 1995; Hyytiainen et al., 2001; Suzuki et al., 2002). CsrB, an untranslated RNA molecule, neutralises CsrA function by sequestering this regulatory protein and has been demonstrated to be under the control of the two-component system. In *L. pneumophila* a *letA* mutant was found to have an effect on the kinetics of *rpoS* transcription (Lynch et al., 2003), in that the gene is required for maximal transcription of *rpoS*. We hypothesise that the LetA-dependent control may be

mediated via CsrA. Using reporter gene fusions, *rpoS* transcription was found to be stimulated in the CsrA(–) mutant strain by a factor five compared to the wild type.

Interestingly, overexpression of *rpoS* in the CsrA(–) mutant analysed by a luciferase reporter gene fusion could not be confirmed at the transcriptional level. However at the protein level, by Western blot analysis, an increase in stationary RpoS levels in the mutant versus wild type was observed. In *E. coli*, RpoS levels increase dramatically at the onset of the stationary growth phase in spite of modest effects on *rpoS* transcription (Hengge-Aronis, 2002). Instead, the observed changes in the RpoS levels are the result of an increased translational efficiency and changes in the degradation of the RpoS protein. Factors such as the RNA-binding protein Hfq and the small regulatory RNA DsrA have been shown to be involved in unfolding the *rpoS* mRNA in *E. coli* thereby facilitating the transcript interaction with ribosomes. Other factors such as the small RNAs RprA and OxyS and the ClpXP protease also play important roles in the induction or the repression of RpoS activity at the post-transcriptional level (for review see Venturi, 2003). Therefore, we speculate that in *L. pneumophila* CsrA might control *rpoS* gene expression by repressing its translational activation.

Extrapolating from experiments with *E. coli csrA*, control in *Legionella* could be achieved by a counter-regulatory RNA molecule that binds CsrA. According to this model, LetA could up-regulate the expression of *csrB* in the stationary phase to neutralise the function of CsrA. This neutralisation would lead to an elevated expression of *rpoS*, which in turn induces stationary-phase functions. This model is supported by the finding of reduced *rpoS* transcription in a *letA* mutant (Lynch et al., 2003) and elevated levels in the CsrA(–) mutant strain. Experiments are currently underway in our laboratory to isolate a putative homologue of *csrB* in *L. pneumophila*. A proposed schematic outline of the virulence gene regulation is presented in Fig. 8.

The study of CsrA in the *L. pneumophila* strain JR32 and *L. pneumophila* ATCC 33125 showed differences in the effect of the regulatory protein on pigmentation and flagellation. Reduced pigmentation was observed in the CsrA(–) mutant (parent strain: JR32) as well as in the CsrA-overproducing strain BC1 (parent strain: *L. pneumophila* ATCC 33125, Philadelphia-1). With respect to flagellation, the effects of CsrA differed in *flaA* and *fliA* gene expression control. Under the conditions of CsrA overproduction these genes were not expressed in the Philadelphia 1 strain. However, in JR32 they showed wild-type expression patterns while reduction of CsrA in JR32 led to premature expression of flagellation. Therefore, our data indicate a role of CsrA as a negative regulator of flagellation in both strains while fine-tuning of the expression of CsrA-dependent genes appears to be



**Fig. 8.** Schematic overview of virulence gene regulation. Arrows indicate positive direct or indirect regulation, lines indicate negative direct or indirect regulation. Dotted lines indicate proposed regulation. CsrB has not been identified in *Legionella*.

strain dependent. According to Brassinga et al. (2003) *L. pneumophila* ATCC 33125 possess three copies of related but not identical *csrA* genes while *L. pneumophila* JR32 has lost the LpPI-1 LvrC together with 55 kb of a region described as a pathogenicity island LpPI-1. Although the LpPI-1 LvrC gene has only 63% homology with the *Legionella csrA* gene it may have some, albeit unknown, regulatory functions.

Interestingly, in comparing CsrA control in different species different effects can be observed. In *Erwinia* species CsrA has been shown to repress motility (Cui et al., 1995), while in *E. coli* and *P. mirabilis* CsrA up-regulates motility (Wei et al., 2001; Liaw et al., 2003). Length of *P. mirabilis* cells is reduced when CsrA is up-regulated, while *Legionella* bacteria appear longer. The same contradiction appears with respect to virulence of *Erwinia* and *Legionella* (Chatterjee et al., 1995). Thus, the genetic background is an important modulator of the effects of CsrA on its target genes and not only presence and absence, but a fine-tuned regulation of CsrA is important.

## Acknowledgements

The authors are grateful to A. Flosdorff and K. Müller for excellent technical assistance. This work was supported by the BMBF Community Acquired Pneumonia Network (CAPNETZ) program.

## References

- Abu Kwaik, Y., Eisenstein, B.I., Engleberg, N.C., 1993. Phenotypic modulation by *Legionella pneumophila* upon infection of macrophages. *Infect. Immun.* 61, 1320–1329.

- Alexeyev, M.F., Shokolenko, I.N., Croughan, T.P., 1995. Improved antibiotic-resistance gene cassettes and omega elements for *E. coli* vector construction and in vitro deletion/insertion mutagenesis. *Gene* 160, 63–67.
- Alli, O.A., Gao, L.Y., Pedersen, L.L., Zink, S., Radulic, M., Doric, M., Abu Kwaik, Y., 2000. Temporal pore formation-mediated egress from macrophages and alveolar epithelial cells by *Legionella pneumophila*. *Infect. Immun.* 68, 6431–6440.
- Altier, C., Suyemoto, M., Lawhon, S.D., 2000a. Regulation of *Salmonella enterica* serovar Typhimurium invasion genes by *csrA*. *Infect. Immun.* 68, 6790–6797.
- Altier, C., Suyemoto, M., Ruiz, A., Burnham, K., Maurer, R., 2000b. Characterization of two novel regulatory genes affecting *Salmonella* invasion gene expression. *Mol. Microbiol.* 35, 635–646.
- Ausubel, F.M., 1987. *Current Protocols in Molecular Biology*. Greene Publ. Associates and Wiley-Interscience, New York.
- Bachman, M.A., Swanson, M.S., 2001. RpoS co-operates with other factors to induce *Legionella pneumophila* virulence in the stationary phase. *Mol. Microbiol.* 40, 1201–1214.
- Blumer, C., Haas, D., 2000. Multicopy suppression of a *gacA* mutation by the *infC* operon in *Pseudomonas fluorescens* CHA0: competition with the global translational regulator RsmA. *FEMS Microbiol. Lett.* 187, 53–58.
- Brassinga, A.K., Hiltz, M.F., Sisson, G.R., Morash, M.G., Hill, N., Garduno, E., Edelstein, P.H., Garduno, R.A., Hoffman, P.S., 2003. A 65-kilobase pathogenicity island is unique to Philadelphia-1 strains of *Legionella pneumophila*. *J. Bacteriol.* 185, 4630–4637.
- Chatterjee, A., Cui, Y., Liu, Y., Dumenyo, C.K., Chatterjee, A.K., 1995. Inactivation of *rsmA* leads to overproduction of extracellular pectinases, cellulases, and proteases in *Erwinia carotovora* subsp. *carotovora* in the absence of the starvation/cell density-sensing signal, N-(3-oxohexanoyl)-L-homoserine lactone. *Appl. Environ. Microbiol.* 61, 1959–1967.
- Chatterjee, A., Cui, Y., Chatterjee, A.K., 2002. RsmA and the quorum-sensing signal, N-(3-oxohexanoyl)-L-homoserine lactone, control the levels of *rsmB* RNA in *Erwinia carotovora* subsp. *carotovora* by affecting its stability. *J. Bacteriol.* 184, 4089–4095.
- Cianciotto, N.P., Fields, B.S., 1992. *Legionella pneumophila mip* gene potentiates intracellular infection of protozoa and human macrophages. *Proc. Natl. Acad. Sci. USA* 89, 5188–5191.
- Cirillo, J.D., Cirillo, S.L., Yan, L., Bermudez, L.E., Falkow, S., Tompkins, L.S., 1999. Intracellular growth in *Acanthamoeba castellanii* affects monocyte entry mechanisms and enhances virulence of *Legionella pneumophila*. *Infect. Immun.* 67, 4427–4434.
- Cui, Y., Chatterjee, A., Liu, Y., Dumenyo, C.K., Chatterjee, A.K., 1995. Identification of a global repressor gene, *rsmA*, of *Erwinia carotovora* subsp. *carotovora* that controls extracellular enzymes, N-(3-oxohexanoyl)-L-homoserine lactone, and pathogenicity in soft-rotting *Erwinia* spp. *J. Bacteriol.* 177, 5108–5115.
- Cui, Y., Chatterjee, A., Chatterjee, A.K., 2001. Effects of the two-component system comprising GacA and GacS of *Erwinia carotovora* subsp. *carotovora* on the production of global regulatory *rsmB* RNA, extracellular enzymes, and harpinEcc. *Mol. Plant Microbe Interact.* 14, 516–526.
- Edelstein, P.H., 1981. Improved semiselective medium for isolation of *Legionella pneumophila* from contaminated clinical and environmental specimens. *J. Clin. Microbiol.* 14, 298–303.
- Essig, A., Heinemann, M., Simnacher, U., Marre, R., 1997. Infection of *Acanthamoeba castellanii* by *Chlamydia pneumoniae*. *Appl. Environ. Microbiol.* 63, 1396–1399.
- Fettes, P.S., Susa, M., Hacker, J., Marre, R., 2000. Characterization of the *Legionella pneumophila* gene *ligA*. *Int. J. Med. Microbiol.* 290, 239–250.
- Fettes, P.S., Forsbach-Birk, V., Lynch, D., Marre, R., 2001. Overexpression of a *Legionella pneumophila* homologue of the *E. coli* regulator *csrA* affects cell size, flagellation, and pigmentation. *Int. J. Med. Microbiol.* 291, 353–360.
- Fields, B.S., Benson, R.F., Besser, R.E., 2002. Legionella and Legionnaires' disease: 25 years of investigation. *Clin. Microbiol. Rev.* 15, 506–526.
- Goodier, R.I., Ahmer, B.M., 2001. SirA orthologs affect both motility and virulence. *J. Bacteriol.* 183, 2249–2258.
- Grossart, H.P., Steward, G.F., Martinez, J., Azam, F., 2000. A simple, rapid method for demonstrating bacterial flagella. *Appl. Environ. Microbiol.* 66, 3632–3636.
- Hales, L.M., Shuman, H.A., 1999. The *Legionella pneumophila rpoS* gene is required for growth within *Acanthamoeba castellanii*. *J. Bacteriol.* 181, 4879–4889.
- Hammer, B.K., Swanson, M.S., 1999. Co-ordination of *Legionella pneumophila* virulence with entry into stationary phase by ppGpp. *Mol. Microbiol.* 33, 721–731.
- Hammer, B.K., Tateda, E.S., Swanson, M.S., 2002. A two-component regulator induces the transmission phenotype of stationary-phase *Legionella pneumophila*. *Mol. Microbiol.* 44, 107–118.
- Harb, O.S., Abu Kwaik, Y., 2000. Essential role for the *Legionella pneumophila* *rep* helicase homologue in intracellular infection of mammalian cells. *Infect. Immun.* 68, 6970–6978.
- Harb, O.S., Gao, L.Y., Abu Kwaik, Y., 2000. From protozoa to mammalian cells: a new paradigm in the life cycle of intracellular bacterial pathogens. *Environ. Microbiol.* 2, 251–265.
- Heeb, S., Blumer, C., Haas, D., 2002. Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. *J. Bacteriol.* 184, 1046–1056.
- Hengge-Aronis, R., 2002. Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase. *Microbiol. Mol. Biol. Rev.* 66, 373–395.
- Heuner, K., Bender-Beck, L., Brand, B.C., Luck, P.C., Mann, K.H., Marre, R., Ott, M., Hacker, J., 1995. Cloning and genetic characterization of the flagellum subunit gene (*flaA*) of *Legionella pneumophila* serogroup 1. *Infect. Immun.* 63, 2499–2507.
- Heuner, K., Dietrich, C., Skriwan, C., Steinert, M., Hacker, J., 2002. Influence of the alternative sigma(28) factor on virulence and flagellum expression of *Legionella pneumophila*. *Infect. Immun.* 70, 1604–1608.

- Heuner, K., Steinert, M., 2003. The flagellum of *Legionella pneumophila* and its link to the expression of the virulent phenotype. *Int. J. Med. Microbiol.* 293, 133–143.
- Hilbi, H., Segal, G., Shuman, H.A., 2001. Icm/dot-dependent upregulation of phagocytosis by *Legionella pneumophila*. *Mol. Microbiol.* 42, 603–617.
- Hyytiäinen, H., Montesano, M., Palva, E.T., 2001. Global regulators ExpA (GacA) and KdgR modulate extracellular enzyme gene expression through the RsmA–rsmB system in *Erwinia carotovora* subsp. *carotovora*. *Mol. Plant Microbe Interact.* 14, 931–938.
- Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop, R.M., Peterson, K.M., 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166, 175–176.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lawhon, S.D., Frye, J.G., Suyemoto, M., Porwollik, S., McClelland, M., Altier, C., 2003. Global regulation by *CsrA* in *Salmonella typhimurium*. *Mol. Microbiol.* 48, 1633–1645.
- Liaw, S.J., Lai, H.C., Ho, S.W., Luh, K.T., Wang, W.B., 2003. Role of RsmA in the regulation of swarming motility and virulence factor expression in *Proteus mirabilis*. *J. Med. Microbiol.* 52, 19–28.
- Liles, M.R., Edelstein, P.H., Cianciotto, N.P., 1999. The prepilin peptidase is required for protein secretion by and the virulence of the intracellular pathogen *Legionella pneumophila*. *Mol. Microbiol.* 31, 959–970.
- Liu, M., Yang, H., Romeo, T., 1995. The product of the pleiotropic *E. coli* gene *csrA* modulates glycogen biosynthesis via effects on mRNA stability. *J. Bacteriol.* 177, 2663–2672.
- Lynch, D., Fieser, N., Glogglar, K., Forsbach-Birk, V., Marre, R., 2003. The response regulator LetA regulates the stationary-phase stress response in *Legionella pneumophila* and is required for efficient infection of *Acanthamoeba castellanii*. *FEMS Microbiol. Lett.* 219, 241–248.
- Mukhopadhyay, S., Audia, J.P., Roy, R.N., Schellhorn, H.E., 2000. Transcriptional induction of the conserved alternative sigma factor RpoS in *E. coli* is dependent on BarA, a probable two-component regulator. *Mol. Microbiol.* 37, 371–381.
- O'Connell, W.A., Bangsberg, J.M., Cianciotto, N.P., 1995. Characterization of a *Legionella micdadei mip* mutant. *Infect. Immun.* 63, 2840–2845.
- Pine, L., George, J.R., Reeves, M.W., Harrell, W.K., 1979. Development of a chemically defined liquid medium for growth of *Legionella pneumophila*. *J. Clin. Microbiol.* 9, 615–626.
- Robey, M., O'Connell, W., Cianciotto, N.P., 2001. Identification of *Legionella pneumophila rep*, a *pagP*-like gene that confers resistance to cationic antimicrobial peptides and promotes intracellular infection. *Infect. Immun.* 69, 4276–4286.
- Romeo, T., Gong, M., Liu, M., Brun-Zinkernagel, A., 1993. Identification and molecular characterization of *csrA*, a pleiotropic gene from *E. coli* that affects glycogen biosynthesis, gluconeogenesis, cell size, and surface properties. *J. Bacteriol.* 175, 4744–4755.
- Rossier, O., Cianciotto, N.P., 2001. Type II protein secretion is a subset of the PilD-dependent processes that facilitate intracellular infection by *Legionella pneumophila*. *Infect. Immun.* 69, 2092–2098.
- Sadosky, A.B., Wiater, L.A., Shuman, H.A., 1993. Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. *Infect. Immun.* 61, 5361–5373.
- Sadosky, A.B., Wilson, J.W., Steinman, H.M., Shuman, H.A., 1994. The iron superoxide dismutase of *Legionella pneumophila* is essential for viability. *J. Bacteriol.* 176, 3790–3799.
- Sambrook, J., Maniatis, T., Fritsch, E.F., 1989. *Molecular Cloning a Laboratory Manual 2nd Edition*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Suzuki, K., Wang, X., Weilbacher, T., Pernestig, A.K., Melefors, O., Georgellis, D., Babitzke, P., Romeo, T., 2002. Regulatory circuitry of the *CsrA/CsrB* and *BarA/UvrY* systems of *E. coli*. *J. Bacteriol.* 184, 5130–5140.
- Swanson, M.S., Hammer, B.K., 2000. *Legionella pneumophila* pathogenesis: a fateful journey from amoebae to macrophages. *Annu. Rev. Microbiol.* 54, 567–613.
- Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76, 4350–4354.
- Venturi, V., 2003. Control of *rpoS* transcription in *E. coli* and *Pseudomonas*: why so different? *Mol. Microbiol.* 49, 1–9.
- Wei, B., Brun-Zinkernagel, A., Simecka, J., Prüß, B., Babitzke, P., Romeo, T., 2001. Positive regulation of motility and *flhDC* expression by the RNA-binding protein *CsrA* of *E. coli*. *Mol. Microbiol.* 40, 245–256.
- Whistler, C.A., Corbell, N.A., Sarniguet, A., Ream, W., Loper, J.E., 1998. The two-component regulators GacS and GacA influence accumulation of the stationary-phase sigma factor sigma<sup>S</sup> and the stress response in *Pseudomonas fluorescens* PF-5. *J. Bacteriol.* 180, 6635–6641.
- Wiater, L.A., Sadosky, A.B., Shuman, H.A., 1994. Mutagenesis of *Legionella pneumophila* using Tn903 *dlllacZ*: identification of a growth-phase-regulated pigmentation gene. *Mol. Microbiol.* 11, 641–653.
- Woodcock, D.M., Crowther, P.J., Doherty, J., Jefferson, S., DeCruz, E., Noyer-Weidner, M., Smith, S.S., Michael, M.Z., Graham, M.W., 1989. Quantitative evaluation of *E. coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucleic Acids Res.* 17, 3469–3478.