INFECTION AND IMMUNITY, July 2003, p. 3714–3723 Vol. 71, No. 7
0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.7.3714–3723.2003

Functional Similarities between the *icm*/*dot* Pathogenesis Systems of *Coxiella burnetii* and *Legionella pneumophila*

Tal Zusman, Gal Yerushalmi, and Gil Segal*

Department of Molecular Microbiology & Biotechnology, George S. Wise Faculty of Life Sciences, Tel-Aviv University, Ramat-Aviv, Tel-Aviv 69978, Israel

Received 29 January 2003/Returned for modification 17 March 2003/Accepted 31 March 2003

***Coxiella burnetii*, the etiological agent of Q fever, is an obligate intracellular pathogen, whereas *Legionella pneumophila*, the causative agent of Legionnaires' disease, is a facultative intracellular pathogen. During infection of humans both of these pathogens multiply in alveolar macrophages inside a closed phagosome. *L. pneumophila* intracellular multiplication was shown to be dependent on the *icm*/*dot* system, which probably encodes a type IV-related translocation apparatus. Recently, genes homologous to all of the *L. pneumophila* *icm*/*dot* genes (besides *icmR*) were found in *C. burnetii*. To explore the similarities and differences between the *icm*/*dot* pathogenesis systems of these two pathogens, interspecies complementation analysis was performed. Nine *C. burnetii icm* homologous genes (*icmT*, *icmS*, *icmQ*, *icmP*, *icmO*, *icmJ*, *icmB*, *icmW*, and *icmX*) were cloned under regulation of the corresponding *L. pneumophila icm* genes and examined for the ability to complement *L. pneumophila* mutants with mutations in these genes. The *C. burnetii icmS* and *icmW* homologous genes were found to complement the corresponding *L. pneumophila icm* mutants to wild-type levels of intracellular growth in both HL-60-derived human macrophages and *Acanthamoeba castellanii*. In addition, the *C. burnetii icmT* homologous gene was found to completely complement an *L. pneumophila* insertion mutant for intracellular growth in HL-60-derived human macrophages, but it only partially complemented the same mutant for intracellular growth in *A. castellanii*. Moreover, as previously shown for *L. pneumophila*, the proteins encoded by the *C. burnetii icmS* and *icmW* homologous genes were found to interact with one another, and interspecies protein interaction was observed as well. Our results strongly indicate that the Icm/Dot pathogenesis systems of *C. burnetii* and *L. pneumophila* have common features.**

Coxiella burnetii, the etiological agent of Q fever, is an obligate intracellular pathogen (34). The reservoir host range of *C. burnetii* is extensive and includes livestock, pets, and wildlife, and the primary route of human infection is via inhalation of contaminated aerosols (42). When growing inside human macrophages, *C. burnetii* is found in a phagosome that has been shown to delay phagosome-lysosome fusion at early times during infection (28). However, later during infection the *C. burnetii*-containing phagosome fuses with many cell vesicles and forms a phagolysosome (17–19). This gram-negative bacterium is classified in the gamma subdivision of the class *Proteobacteria* and is evolutionarily closely related to *Legionella* (66).

Legionella pneumophila, the causative agent of Legionnaires' disease, is a facultative intracellular pathogen that multiplies within and kills human macrophages, as well as free-living amoebae (27, 47). After phagocytosis, *L. pneumophila* inhibits phagosome-lysosome fusion early during infection (4, 25, 26, 48, 58, 61, 67), but the phagosome has also been shown to acidify after several hours of infection (58). In addition, the *L. pneumophila* phagosome undergoes several recruitment events that include association with smooth vesicles, mitochondria, and the rough endoplasmic reticulum (4, 24, 29, 41). Both *C. burnetii* and *L. pneumophila* have been shown previously to reside in a phagosome that has characteristics of an autophagosome (3, 60), and it was suggested recently that these two bacteria use similar virulence strategies (55, 59).

* Corresponding author. Mailing address: Department of Molecular Microbiology & Biotechnology, George S. Wise Faculty of Life Sciences, Tel-Aviv University, Ramat-Aviv, Tel-Aviv 69978, Israel. Phone: 972-3-6405287. Fax: 972-3-6409407. E-mail: GilS@tauex.tau.ac.il.

Two regions of genes required for human macrophage killing and intracellular multiplication have been discovered in *L. pneumophila* (1, 2, 5, 44, 50, 51, 63). Region I contains seven genes (*icmV*, *icmW*, *icmX*, *dotA*, *dotB*, *dotC*, and *dotD*), and region II contains 18 genes (*icmT*, *icmS*, *icmR*, *icmQ*, *icmP*, *icmO*, *icmN*, *icmM*, *icmL*, *icmK*, *icmE*, *icmG*, *icmC*, *icmD*, *icmJ*, *icmB*, *icmF*, and *icmH*). Most of these genes have also been shown to be required for intracellular growth in the protozoan host *Acanthamoeba castellanii* (54). The *icm*/*dot* genes have been shown to participate in many aspects related to *L. pneumophila* pathogenesis, such as phagocytosis (21, 65), immediate cytotoxicity (31, 72), inhibition of phagosome lysosome fusion at early times during infection (10, 58, 67), association of the phagosome with the rough endoplasmic reticulum (29, 41), apoptosis (71), and exit from the phagosome (38). As a consequence of all these features the *icm*/*dot* genes are essential for intracellular multiplication and host cell killing by *L. pneumophila* (1, 2, 5, 44, 50, 51, 63). Eighteen proteins encoded by the *icm*/*dot* genes (IcmT, IcmP, IcmO, IcmM, IcmL, IcmK, IcmE, IcmG, IcmC, IcmD, IcmJ, IcmB, IcmV, IcmX, DotA, DotB, DotC, and DotD) exhibit significant sequence homology to conjugation-related proteins from plasmid R64 (32, 55), and homologous proteins were also found in *Pseudomonas syringae* (57). In addition, *L. pneumophila* can conjugate RSF1010-related plasmids between bacteria in an *icm*/*dot*-dependent manner (50, 63), and *L. pneumophila* intracellular growth and human macrophage killing have been shown to be inhibited by an active RSF1010 conjugation system (53). Therefore, it is believed that the *L. pneumophila* Icm/Dot system forms a type IV-related transport system lo-

TABLE 1. Bacterial strains

Strain	Genotype and features	Reference or source
L. pneumophila strains		
25D	Icm$^-$ avirulent mutant	23
GS3001	JR32 *icmS3001*::Kan	51
GS3003	JR32 *icmO3003*::Kan	51
GS3011	JR32 *icmT3011*::Kan	54
GS3013	JR32 *icmQ3013*::Kan	This study
GS3014	JR32 *icmP3014* (in-frame nonpolar deletion)	This study
GS3017	JR32 *icmJ3017* (in-frame nonpolar deletion)	This study
GY141	JR32 *icmW141* (in-frame nonpolar deletion)	This study
JR32	Homogeneous salt-sensitive isolate of AM511	49
LELA3393	JR32 *icmB3393*::Tn*903*dII*lacZ*	49
LELA3993	JR32 *icmX3993*::Tn*903*dII*lacZ*	49
E. coli strains		
IO-7012D	*trp his metE ilv cyaA*	6
MC1022	*araD139* Δ(*ara leu)7697* Δ(*lacZ*)M15 *galU galK strA*	7
SY327λpir	(*lac pro) argE*(Am) *rif nalA recA56 pir*	13

cated in the bacterial cell membrane, which translocates effector proteins into the host cells during infection (41, 52, 64).

Previously, it was reported that *C. burnetii* contains proteins homologous to three of the *L. pneumophila* Icm proteins (IcmT, IcmS, and IcmK) (55). Recently, the genome sequence of *C. burnetii* (http://www.tigr.org) revealed that this bacterium contains proteins homologous to all of the *L. pneumophila* Icm/Dot proteins except IcmR (57). Nevertheless, there is no information regarding the contribution of these Icm/Dot homologous proteins to *C. burnetii* pathogenesis, mainly due to the limited genetic tools available for this bacterium. To increase our understanding of the pathogenesis system of this important obligate intracellular pathogen, several of its *icm*/*dot* homologous genes were analyzed by using *L. pneumophila* genetic tools. Our results clearly show that the pathogenesis systems of these two bacteria have common features, as well as differences.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and media. The *L. pneumophila* and *Escherichia coli* strains used in this study are listed in Table 1. The plasmids and primers used in this study are shown in Tables 2 and 3, respectively. Bacterial media, plates, and antibiotic concentrations were used as described previously (51).

Plasmid construction for complementation with *L. pneumophila icm* genes. Plasmid pGS-VWX-01 contained a 12.4-kb *Eco*RI fragment cloned in pMMB207αb, which contained part of *icm* region I (*icmW*, *icmX*, *icmV*, and *dotA*), and was used for construction of two complementing plasmids. Plasmid pGS-VWX-01 was digested with *Bgl*II and *Bam*HI, and a 3,587-bp fragment that contained the *icmV* gene and the *icmWX* operon was cloned into the *Bam*HI site of pMMB207αb-km-14 to generate pZT-WX-01. In addition, plasmid pGS-VWX-01 was digested with *Hin*dIII and *Eco*RV, and a 1,051-bp fragment that contained the *icmW* gene by itself was filled in and cloned into the *Sma*I site of pMMB207αb-km-14 to generate pZT-W-01. Plasmid pMW100, which contained the *icmG*, *icmC*, *icmD*, *icmJ*, *icmB*, and *icmF* genes, was digested with *Pst*I, and a 1,864-bp fragment that contained the *icmD* and *icmJ* genes was treated with T4 DNA polymerase and cloned into pMMB207αb-km-14 partially digested with *Sma*I to generate pGS-Lc-84-14. In addition, plasmid pMW100 was digested with *Kpn*I, and a 6,856-bp fragment that contained the *icmG*, *icmC*, *icmD*, *icmJ*, and *icmB* genes was treated with T4 DNA polymerase and cloned into the *Sma*I site of pMMB207αb-km-14 to generate pGS-Lc-85-14. Plasmid pGS-Lc-34-14, which contained the *icmP* and *icmO* genes, was used to construct a plasmid that contained the *icmP* gene by itself. Plasmid pGS-Lc-34-14 was digested with *Swa*I, and the 12.7-kb fragment that contained the whole vector DNA and the *icmP* gene was self-ligated to generate pGS-Lc-34-D2-14. Plasmid pGS-Lc-35 was digested with *Eco*RI and *Hin*dIII, and the insert containing the *icmQ* gene was gel purified. The vector pMMB207αb-Km-14 was partially digested with *Hin*dIII and completely digested with *Eco*RI and then gel purified. The purified insert from pGS-Lc-35 was cloned into pMMB207αb-Km-14 to generate pGS-Lc-35-14.

Plasmid construction for complementation with *C. burnetii icm* homologous genes. The *C. burnetii icm* homologous genes were amplified from *C. burnetii* Nine-Mile phase I (RSA 493) chromosomal DNA, kindly provided by Hermann Willems (The Institute of Hygiene and Infectious Diseases of Animals, Justus Liebig University, Giessen, Germany). The *C. burnetii* sequence information was obtained from The Institute for Genomic Research website (http://www.tigr.org). Four operons (*icmTS*, *icmPO*, *icmJB*, and *icmWX)* and one gene (*icmQ)* were amplified by using an upstream primer that contained an *Nde*I site at the first ATG codon of the first gene of an operon (Cox-Nde-TS, Cox-Nde-PO, Cox-Nde-JB, Cox-Nde-WX, or Cox-Nde-Q [Table 3]) and a downstream primer that was located at the end of the coding sequence and contained a *Bam*HI site (Cox-Bam-TS, Cox-Bam-PO, Cox-Bam-JB, Cox-Bam-WX, or Cox-Bam-Q [Table 3]). The resulting fragments were cloned into pUC-18 digested with *Hin*cII to generate pZT-cox-TS, pZT-cox-PO, pZT-cox-JB, pZT-cox-WX, and pZT-cox-Q, respectively. The regulatory regions of the five corresponding *L. pneumophila icm* genes and operons (*icmTS*, *icmPO*, *icmJB*, *icmWX*, and *icmQ*) were amplified by PCR by using a downstream primer that contained an *Nde*I site at the first ATG codon of the relevant gene (Lpn-Nde-TS, Lpn-Nde-PO, Lpn-Nde-JB, Lpn-Nde-WX, or Lpn-Nde-Q [Table-3]) and an upstream primer containing an *Eco*RI site (Lpn-EI-T, Lpn-EI-P, Lpn-EI-J, Lpn-EI-W, or Lpn-EI-Q [Table 3]). The resulting fragments were cloned into pUC-18 digested with *Hin*cII to generate pZT-lpn-TS, pZT-lpn-PO, pZT-lpn-JB, pZT-lpn-WX, and pZT-lpn-Q, respectively. The inserts of all these plasmids were sequenced to confirm that no mistakes were incorporated during PCR amplification. The 10 plasmids described above were digested at the restriction sites incorporated into the primers (with *Bam*HI and *Nde*I for the plasmids containing *C. burnetii icm* homologous genes and with *Eco*RI and *Nde*I for the plasmids containing the *L. pneumophila icm* regulatory regions), and the members of each pair of fragments (a *C. burnetii icm* homologous gene and the corresponding *L. pneumophila icm* regulatory region) were cloned into the pMMB207αb-km-14 vector digested with *Eco*RI and *Bam*HI to generate pZT-lpncox-TS, pZT-lpncox-PO, pZT-lpncox-JB, pZT-lpncox-WX, and pZT-lpncox-Q. These plasmids were used for complementation of *L. pneumophila icm* mutants.

Plasmid construction for the two-hybrid analysis. To clone the *C. burnetii icmS* and *icmW* homologous genes into the pT18 and pT25 vectors of the *Bordetella pertussis cyaA* two-hybrid system (30), both genes were amplified by PCR with primers that were designed to form in-frame fusions with the *cyaA* gene product. To clone these genes into both plasmids, both genes were amplified with primers S-T18-F, S-T18-R, S-T25-F, S-T25-R, W-T18-F, W-T18-R, W-T25-F, and W-T25-R (Table 3). The four fragments generated were cloned into pUC-18 digested with *Hin*cII to generate pZT-coxS-T18, pZT-T25-coxS, pZT-coxW-T18, and pZT-T25-coxW and sequenced. Plasmids pZT-coxS-T18 and pZT-coxW-T18 were digested with *Xho*I and *Hin*dIII and cloned into the pT18 vector digested with the same enzymes to generate pZT-18-coxS and pZT-18-coxW. Plasmids pZT-T25-coxS and pZT-T25-coxW were digested with *Pst*I and *Bam*HI and cloned into the pT25 vector digested with the same enzymes to generate pZT-25-coxS and pZT-25-coxW. These four plasmids contained the *C. burnetii icmS* and *icmW* homologous genes fused to either the T18 or T25 fragments of the *B. pertusis cyaA* toxin. To analyze the *L. pneumophila icmS* and *icmW* genes by using the same system, we used eight primers (Ls-F-T18, Ls-R-T18, Ls-F-T25, Ls-R-T25, Lw-F-T18, Lw-R-T18, Lw-F-T25, and Lw-R-T25 [Table 3]), and four plasmids (pKP-18S, pKP-25S, pKP-18W, and pKP-25W [Table 2]) were constructed in a manner similar to the manner described above for the *C. burnetii icmS* and *icmW* homologous genes and used for the two-hybrid analysis.

Plasmid construction for allelic exchange. In order to generate in-frame nonpolar deletions in the *L. pneumophila* chromosome, a vector containing an R6K origin of replication, a gene conferring resistance to chloramphenicol (*cat*), and the *sacB* counterselectable marker was constructed. Plasmid pGP704, which contained an R6K origin of replication, was digested with *Sma*I, and a *Hin*cII-*Xmn*I fragment from pACYC184 that contained the *cat* gene was cloned into it to generate pGY-R6K-01. The resulting plasmid was digested with *Bam*HI and self-ligated to obtain a 2.1-kb plasmid (pGY-R6K-Cm-01) that contained the *cat* gene and the R6K origin of replication. The resulting plasmid was digested with *Eco*RV and *Xba*I, and a *Sca*I-*Xba*I fragment from pLAW-344 that contained the *sacB* gene was cloned into it to generate pGY-100. To generate in-frame non-

TABLE 2. Plasmids used in this study

Plasmid	Characteristic(s)	Reference or source
pACYC-184	*ori15A* Cm^r Tc^r	8
pGP-704	*oriR6K* Ap^r	36
pGS-Lc-34-14	*icmP-icmO* operon in pMMB207αb-Km-14	53
pGS-Lc-34-D2-14	*icmP* gene in pMMB207αb-Km-14	This study
pGS-Lc-35	*icmQ* gene in pMMB207αb	51
pGS-Lc-35-14	*icmQ* gene in pMMB207αb-Km-14	This study
pGS-Lc-37-14	*icmT-icmS* operon in pMMB207αb-Km-14	53
pGS-Lc-84-14	*icmD* and *icmJ* genes in pMMB207αb-Km-14	This study
pGS-Lc-85-14	*icmG*, *icmC*, *icmD*, *icmJ*, and *icmB* genes in pMMB207αb-Km-14	This study
pGS-R6K-J-Km	Insert of pZT-icmJ-Km in pGY-100	This study
pGS-R6K-J	pGS-R6K-J-Km without the kanamycin cassette	This study
pGS-R6K-P-Km	Insert of pZT-icmP-Km in pGY-100	This study
pGS-R6K-P	pGS-R6K-P-Km without the kanamycin cassette	This study
pGS-VWX-01	*icmWX* and *icmV-dotA* operons in pMMB207αb	This study
pGY-100	pGY-R6K-Cm-01 with the *sacB* gene from pLAW344	This study
pGY-100-dW-Km	Insert of pGY-dW-Km in pGY-100	This study
pGY-100-dW	pGY-100-dW-Km without the kanamycin cassette	This study
pGY-R6K-01	pGP704 with Cm^r gene from pACYC184	This study
pGY-R6K-Cm-01	pGY-R6K-01 without the Ap^r gene	This study
pGY-W-ES	1 kb of *icmW* upstream region in pUC-18	This study
pGY-W-SH	1 kb of *icmW* downstream region in pUC-18	This study
pGY-dW-Km	*icmW* upstream and downstream regions with the kanamycin cassette between them in pUC-18	This study
pKP-18S	*L. pneumophila icmS* in pT18	This study
pKP-18W	*L. pneumophila icmW* in pT18	This study
pKP-25S	*L. pneumophila icmS* in pT25	This study
pKP-25W	*L. pneumophila icmW* in pT25	This study
pLAW344	*sacB* MCS *oriT*(RK2) Cm^r *oriR*(ColE1) Ap^r	68
pMMB207	RSF1010 derivative, IncQ $lacI^q$ Cm^r *oriT*	40
pMMB207αb	pMMB207 with α-complementation	51
pMMB207αb-Km-14	pMMB207αb with *mobA*::*kan*	53
pMW100	*icmGCDJBF* in pMMB207	44
pT18	Amino acids 225 to 339 of CyaA, *ori*(ColE1) Ap^r	30
pT25	Amino acids 1 to 224 of CyaA, *ori15A* Cm^r	30
pUC18	*oriR*(ColE1) MCS Ap^r	69
pZT-cox-JB	*C. burnetii icmJB* operon in pUC-18	This study
pZT-cox-PO	*C. burnetii icmPO* operon in pUC-18	This study
pZT-cox-Q	*C. burnetii icmQ* gene in pUC-18	This study
pZT-cox-TS	*C. burnetii icmTS* operon in pUC-18	This study
pZT-cox-WX	*C. burnetii icmWX* operon in pUC-18	This study
pZT-coxS-T18	*C. burnetii icmS* homolog in pUC-18	This study
pZT-coxW-T18	*C. burnetii icmW* homolog in pUC-18	This study
pZT-icmJ-Km	*icmJ* upstream and downstream regions with the kanamycin cassette between them in pUC-18	This study
pZT-icmP-Km	*icmP* upstream and downstream regions with the kanamycin cassette between them in pUC-18	This study
pZT-lpncox-JB	*L. pneumophila icmJ* regulatory region and *C. burnetii icmJB* operon in pMMB207αb-Km-14	This study
pZT-lpncox-PO	*L. pneumophila icmP* regulatory region and *C. burnetii icmPO* operon in pMMB207αb-Km-14	This study
pZT-lpncox-Q	*L. pneumophila icmQ* regulatory region and *C. burnetii icmQ* gene in pMMB207αb-Km-14	This study
pZT-lpncox-TS	*L. pneumophila icmT* regulatory region and *C. burnetii icmTS* operon in pMMB207αb-Km-14	This study
pZT-lpncox-WX	*L. pneumophila icmW* regulatory region and *C. burnetii icmWX* operon in pMMB207αb-Km-14	This study
pZT-lpn-JB	*L. pneumophila icmJ* regulatory region in pUC-18	This study
pZT-lpn-PO	*L. pneumophila icmP* regulatory region in pUC-18	This study
pZT-lpn-Q	*L. pneumophila icmQ* regulatory region in pUC-18	This study
pZT-lpn-TS	*L. pneumophila icmT* regulatory region in pUC-18	This study
pZT-lpn-WX	*L. pneumophila icmW* regulatory region in pUC-18	This study
pZT-P-up	1 kb of *icmP* upstream region in pUC-18	This study
pZT-P-down	1 kb of *icmP* downstream region in pUC-18	This study
pZT-J-up	1 kb of *icmJ* upstream region in pUC-18	This study
pZT-J-down	1 kb of *icmJ* downstream region in pUC-18	This study
pZT-T25-coxS	*C. burnetii icmS* homolog in pUC-18	This study
pZT-T25-coxW	*C. burnetii icmW* homolog in pUC-18	This study
pZT-W-01	*icmW* gene in pMMB207αb-Km-14	This study
pZT-WX-01	*icmW-icmX* operon in pMMB207αb-Km-14	This study
pZT-18-coxS	*C. burnetii icmS* homolog in pT18	This study
pZT-18-coxW	*C. burnetii icmW* homolog in pT18	This study
pZT-25-coxS	*C. burnetii icmS* homolog in pT25	This study
pZT-25-coxW	*C. burnetii icmW* homolog in pT25	This study

polar deletions in the *L. pneumophila icmJ*, *icmP*, and *icmW* genes, a 1-kb DNA fragment located on each side of the planned deletion was amplified by PCR. The primers were designed to contain a *Sal*I site at the place where the deletion occurred. Six fragments were amplified by using primers P-Up-F, P-Up-R, P-Down-F, P-Down-R, J-Up-F, J-Up-R, J-Down-F, J-Down-R, W-Up-F, W-Up-R, W-Down-F, and W-Down-R (Table 3) and cloned into pUC-18 digested with *Hin*cII to generate pZT-J-up, pZT-J-down, pZT-P-up, pZT-P-down, pGY-W-ES, and pGY-W-SH. The inserts of all these plasmids were sequenced to confirm

TABLE 3. Primers used in this study

Primer	Sequence (5′-3′)
Cox-Bam-JB	CGGGGGATCCAGCTAATTGGATAAACCGCGAG
Cox-Bam-PO	CGGGGGATCCACGTCTGCTTCACGGAAAGGAC
Cox-Bam-Q	CGGGGGATCCATTAGCGGTTTGATCCGTTGCG
Cox-Bam-TS	CGGGGGATCCAGGGAATTTTCCTCATTATGCG
Cox-Bam-WX	CGGGGGATCCGCTCCTTGAAAGTTGATGTCCG
Cox-Nde-JB	CTAGCCGCATATGGCTTTGCGCAACATTCAAT
Cox-Nde-PO	CTAGCCGCATATGTATCCAGCGCAACAAAACG
Cox-Nde-Q	CTAGCCGCATATGACATCATTTGACTTGTCCAAC
Cox-Nde-TS	CTAGCCGCATATGAAATCTCTCGATGAGGCGC
Cox-Nde-WX	CTAGCCGCATATGCCAGATCTGTCGCATAAAG
Lpn-EI-J	GCCGGAATTCGGCATTATTATTCCTTCCTTC
Lpn-EI-P	GCCGGAATTCTATATCGATACTCCAATGGCC
Lpn-EI-Q	GCCGGAATTCAGCCATGATGAACGTGGTTTC
Lpn-EI-T	GCCGGAATTCGTTAGCTAAAATGCAAGGGAAC
Lpn-EI-W	GCCGGAATTCTGATCCTGATTTCTTTTTCATATT
Lpn-Nde-JB	CTAGCCGCATATGTTGGTTCACATTCAGTCAATTTTC
Lpn-Nde-PO	CTAGCCGCATATGTATATACAATTAGTTAATAGATTC TATATA
Lpn-Nde-Q	CTAGCCGCATATGTTCAATACTTATGGGAACCAAG
Lpn-Nde-TS	CTAGCCGCATATGTAAACCTCAAAAAATCATCCTTTA
Lpn-Nde-WX	CTAGCCGCATATGTTACTCTTTTTTTACTGAGATACG
Ls-F-T18	CCAACTCGAGAATGGAGCGAGATATTAGCAAG
Ls-F-T25	GGAACTGCAGGGATGGAGCGAGATATTAGCAAG
Ls-R-T18	GGAAAAGCTTATATCATACATTAACTCATCCAGGGG
Ls-R-T25	GGAAGGTACCCTAATCATACATTAACTCATCCAGGG
Lw-F-T18	CCTTCTCGAGAATGCCTGATTAAGCCATGAAGCC
Lw-F-T25	GGAACTGCAGGGATGCCTGATTTAAGCCATGAAGCC
Lw-R-T18	GGAAAAGCTTATTTCATCCCCTTCGAGTGCTCG
Lw-R-T25	GGAAGGTACCTTATTCATCCCCTTCGAGTGCTCG
J-Down-F	GTCGACAGGGCAAAATTCAGGAAGC
J-Down-R	CACATGTGAAGGAAGAATACGAG
J-Up-F	GTTTCCTCCGTACTGCAAAC
J-Up-R	GTCGACAGAATATAAACGCCAGGAACC
P-Down-F	CCAGTCGACAGACGTTTATG
P-Down-R	AGCGCTCGCATCAAGAAGAATTGCCC
P-Up-F	AGCGCTGTTTGGTGCTGCTGAATCTG
P-Up-R	GTCGACTATGACGATCCATACTGGCG
S-T18-F	CCCTCGAGGATGCAACTTGCGAATAAATTAACGG
S-T18-R	TCAAGCTTATATCGTACATCAGTTCATCCAGCG
S-T25-F	GGCTGCAGGGATGCAACTTGCGAATAAATTAACGG
S-T25-R	GGGGATCCTCAATCGTACATCAGTTCATCCAGC
W-Down-F	GTCGACGTTTTACGAGCACTCGAAGG
W-Down-R	AAGCTTGCGGCTGCTTGTTTTTGTTG
W-T18-F	CCCTCGAGGATGCCAGATCTGTCGCATAAAG
W-T18-R	TCAAGCTTATTAAACCACCTTCCTCAAGAGTTT
W-T25-F	GGCTGCAGGGATGCCAGATCTGTCGCATAAAG
W-T25-R	GGGGATCCTCATAAACCACCTTCCTCAAGAGT
W-Up-F	GAATTCCGCCTTGCACGATAACCCAC
W-Up-R	GTCGACTGCGGAGGCTTCATGGCTTA

that no mutations were incorporated during the PCR. The resulting plasmids were digested with *Eco*RI and *Sal*I (for plasmids that contained the fragment located upstream of the deletion) or with *Hin*dIII and *Sal*I (for plasmids that contained the fragment located downstream of the deletion). Pairs of the fragments were part of a four-way ligation that contained a kanamycin resistance cassette (Pharmacia) digested with *Sal*I and the pUC-18 vector digested with *Eco*RI and *Hin*dIII to generate pZT-icmJ-Km, pZT-icmP-Km, and pGY-dW-Km. These three plasmids were digested with *Pvu*II (this enzyme cut on both sides of the pUC-18 polylinker), and the resulting fragments were cloned into the pGY-100 vector digested with *Xmn*I to generate pGS-R6K-J-Km, pGS-R6K-P-Km, and pGY-100-dW-Km. These three plasmids were digested with *Sal*I and self-ligated to form pGS-R6K-J, pGS-R6K-P, and pGY-100-dW, which were used for allelic exchange as described below.

***L. pneumophila* allelic exchange.** Plasmids, pGS-R6K-J, pGS-R6K-P, and pGY-100-dW (see above) were introduced into *L. pneumophila* JR32 by electroporation, grown in AYE (*N*-[2-acetamino]-2-aminoethane-sulfonic acid–yeast extract) for 5 h, and plated on ABCYE (ACES-buffered charcoal yeast extract) plates containing chloramphenicol. Transformants were patched onto ABCYE plates containing chloramphenicol and then streaked on ABCYE plates containing 2% (wt/vol) sucrose (Suc) to select for cells that no longer contained vector pGY-100 sequences (Cm^s and Suc^r). Single isolates that grew on the Suc-containing plates were patched onto ABCYE plates containing chloramphenicol and plain ABCYE plates. Cm^s and Suc^r isolates were tested by PCR to confirm that the correct change had occurred. At least six independent isolates were tested for each allelic exchange.

Intracellular growth in *A. castellanii*. Intracellular growth assays were performed in a way similar to the way described previously (54). A total of 1.5×10^5 amoebae in proteose-yeast extract-glucose (PYG) were added to wells of a 24-well microtiter dish, and the amoebae were incubated for 1 h at 37°C so that they could adhere. Then the PYG was aspirated, the wells were washed once with 0.5 ml of warm (37°C) *Acanthamoeba* buffer (Ac buffer), and 0.5 ml of warm Ac buffer was added to the wells. Then *L. pneumophila* in Ac buffer was added to the wells at a multiplicity of infection (MOI) of approximately 0.1. The plate was incubated for 30 min at 37°C; then the Ac buffer was aspirated, the wells were washed three times with 0.5 ml of warm Ac buffer, and 0.6 ml of warm Ac buffer was added to the wells. The supernatant of each well was sampled (50 μl) at approximately 24-h intervals, and numbers of CFU were determined by plating samples on ABCYE plates.

Intracellular growth in HL-60-derived human macrophages. Intracellular growth assays were performed in a way similar to the way described previously (54). Wells of a 24-well microtiter dish containing 6×10^6 differentiated HL-60-derived macrophages were used for infection. *L. pneumophila* was added to the wells at an MOI of approximately 0.1, and the infected HL-60-derived macrophages were incubated for 1 h at 37°C under 5% CO_2. Then the wells were washed three times, and 0.6 ml of RPMI containing 2 mM glutamine and 10% normal human serum was added to the wells. The supernatant of each well was sampled (50 μl) at approximately 24-h intervals, and numbers of CFU were determined by plating on ABCYE plates.

Cytotoxicity assay. A cytotoxicity assay was performed as described previously (33). Briefly, wells of a 96-well microtiter dish containing 4×10^5 differentiated HL-60-derived macrophages were infected with 10-fold serial dilutions of *L. pneumophila* in RPMI, starting with about 10^8 bacteria/well. After 5 days of incubation at 37°C, the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide was added to each well at a concentration of 0.5 mg/ml. After incubation at 37°C for 4 h, the culture medium was removed, and the remaining reduced formazan dye was suspended in 100 μl of isopropanol containing 0.04 M HCl and 1% sodium dodecyl sulfate. The optical densities at 570 nm of three wells containing bacteria at the same MOI were averaged to determine the extent of macrophage killing.

β-Galactosidase assay. β-Galactosidase assays were performed as described previously (35). *E. coli* strains were grown on Luria-Bertani medium plates containing ampicillin and chloramphenicol for 18 h. Three individual colonies from each clone were selected and grown overnight at 30°C in Luria-Bertani broth containing ampicillin and chloramphenicol. The assays were performed with 50 μl of culture, and the substrate used for β-galactosidase hydrolysis was *o*-nitrophenyl-β-D-galactopyranoside.

RESULTS

Recently, it was reported that *C. burnetii* contains genes homologous to all the *icm*/*dot* genes that were found in *L. pneumophila* except *icmR* (57). Analysis of the levels of homology between the *L. pneumophila* Icm/Dot proteins and their homologs in *C. burnetii*, as well as on the R64 plasmid, led us to classify the Icm/Dot proteins into five groups (Table 4). Icm/Dot proteins that exhibit high levels of homology between *L. pneumophila* and *C. burnetii* (more than 36% identity and 48% similarity) were divided into two groups on the basis of the presence or absence of a homologous protein on the R64 plasmid (groups 1 and 2, respectively). The Icm/Dot proteins that exhibit low levels of homology between *L. pneumophila* and *C. burnetii* (less than 36% identity and 48% similarity) were also divided into two groups on the basis of the same criteria (groups 3 and 4). The IcmR gene product constitutes the last group (group 5) as it does not have a homologous protein in either system (Table 4).

To gain insight into the possible involvement of the *icm*/*dot* genes in *C. burnetii* pathogenesis, we examined whether the *C. burnetii icm* homologs can replace the *L. pneumophila icm*

TABLE 4. Groups of *L. pneumophila* lcm/Dot proteins classified according to homology

Group	Level of homology between *L. pneumophila* and *C. burnetii*[a]	Homolog on the R64 plasmid	Icm/Dot protein(s)
1	High	+	IcmT, IcmP, IcmO, IcmL, IcmK, IcmE, IcmJ, IcmB, DotB, DotC, DotD
2	High	−	IcmS, IcmW
3	Low	+	IcmG, IcmC, IcmD, IcmM, DotA, IcmV, IcmX
4	Low	−	IcmQ, IcmN, IcmF, IcmH
5	No	−	IcmR

[a] Proteins classified as having high levels of homology are more than 36% identical and 48% similar. Proteins classified as having low levels of homology are from 19% identical and 27% similar to 35% identical and 47% similar.

genes and provide their functions during pathogenesis. The analysis included eight *icm* genes that are part of four operons (*icmTS*, *icmPO*, *icmJB*, and *icmWX*) and one individual gene (*icmQ*). The genes were chosen so that they encoded at least one protein belonging to each of the Icm/Dot groups described above (Table 4). We examined a larger number of genes that exhibit high levels of homology between *C. burnetii* and *L. pneumophila* because the chance of interspecies complementation was obviously greater. Recently, functional complementation of an *E. coli rpoS* mutant strain by the *C. burnetii* RpoS sigma factor indicated that such an analysis can be performed with *C. burnetii* genes (56).

Concept of complementation analysis. To perform the complementation analysis, we had to choose the promoter from which the *C. burnetii icm* homologous genes were expressed in *L. pneumophila*, taking into account the possibility that correct timing and levels of expression might be required in order to observe complementation. Previous findings about the regulation of the *L. pneumophila icm/dot* genes indicated that there are considerable differences in the levels of expression and the patterns of expression of these genes (16). Because of this, each of the *C. burnetii icm* homologs examined was cloned downstream from the regulatory regions of the corresponding *L. pneumophila icm* gene or operon. The resulting complicated constructs were prepared in order to ensure that the timing and levels of expression of the *C. burnetii icm* homologs in *L. pneumophila* during intracellular growth were correct.

Analysis of the *C. burnetii icmT* homologous gene. The *C. burnetii* IcmT homologous protein that belongs to Icm/Dot group 1 was examined first. Previously, the *L. pneumophila icmT* gene was shown to be located on the same transcriptional unit as *icmS*, and it was found to be required for intracellular multiplication in both HL-60-derived human macrophages and amoebae (51, 54). Recently, it was found that a subset of *icmT* mutants (*rib* mutants) are defective at the exit stage from the phagosome during growth in amoebae but not in human macrophages (37–39).

To examine the ability of the *C. burnetii icmT* homologous gene to complement the corresponding *L. pneumophila* mutant, a plasmid that contained the *C. burnetii icmTS* homologous genes cloned downstream from the *L. pneumophila icmTS* regulatory region was constructed (pZT-lpncox-TS). This plasmid was examined to determine its ability to complement an *L. pneumophila* deletion substitution mutant with a mutation in *icmT* (GS3011) compared to the ability of a plasmid that contained the native *L. pneumophila icmTS* operon (pGS-Lc-37-14) (Fig. 1). The *icmT* homologous gene from *C. burnetii* was found to completely complement the *L. pneumophila icmT* insertion mutant for intracellular growth in HL-60-derived human macrophages (Fig. 1B), as well as for killing these cells (data not shown). However, when this plasmid was examined to determine its ability to complement the same strain for intracellular growth in *A. castellanii*, only partial complementation was observed (Fig. 1A) (this result did not occur due to polarity of the insertion located in *icmT* on the downstream *icmS* gene [see below]). This information was consistent with previous data indicating that a subset of *icmT* mutants have different phenotypes in human macrophages and amoebae (37–39). Importantly, the complementation results obtained with the *C. burnetii icmT* homologous gene clearly indicate that the pathogenesis systems of *C. burnetii* and *L. pneumophila* have common features.

Analysis of the *C. burnetii icmPO* and *icmJB* operons. The interesting results obtained with the *C. burnetii icmT* homologous gene encouraged us to test four additional genes that belong to Icm/Dot group 1 (Table 4). The *C. burnetii icmP*, *icmO*, *icmJ*, and *icmB* homologous genes that are organized in *L. pneumophila* as two transcriptional units (*icmPO* and *icmJB*) were examined to determine their abilities to complement the corresponding *L. pneumophila* mutants. Since two of these genes (*icmP* and *icmJ*) are located first in a transcriptional unit, in-frame nonpolar deletions were constructed in them on the *L. pneumophila* chromosome (GS3014 and GS3017, respectively), and two previously constructed insertion mutants with mutations in the *L. pneumophila icmO* and *icmB* genes (GS3003 and LELA3393, respectively) were used. Two complementing plasmids that contained the *C. burnetii icmPO* and *icmJB* homologous genes cloned downstream from the *L.*

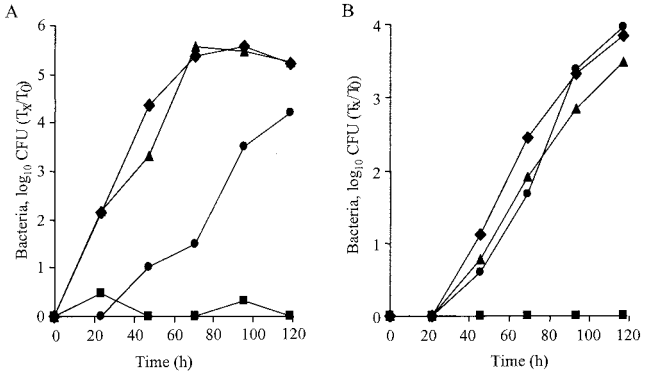

FIG. 1. Complementation analysis with the *C. burnetii icmT* homologous gene. Intracellular growth experiments in the protozoan host *A. castellanii* (A) and HL-60-derived human macrophages (B) were performed as described in Materials and Methods. Wild-type *L. pneumophila* JR-32 (◆) and the *icmT* mutant (GS3011) containing the vector pMMB207αb-Km-14 (■), the *L. pneumophila icmTS* operon (pGS-Lc-37-14) (▲), and the *C. burnetii icmTS* homologous genes under regulation of the *L. pneumophila icmTS* regulatory region (pZT-lpncox-TS) (●) were used. The experiments were performed at least three times, and similar results were obtained each time.

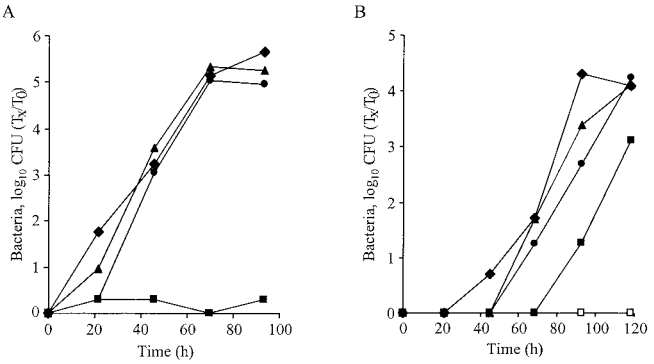

FIG. 2. *C. burnetii icmS* homologous gene complements an *L. pneumophila icmS* mutant for intracellular growth in two hosts. Intracellular growth experiments in the protozoan host *A. castellanii* (A) and in HL-60-derived human macrophages (B) were performed as described in Materials and Methods. Wild-type *L. pneumophila* JR-32 (◆) the *icmS* mutant (GS3001) containing the vector pMMB207αb-Km-14 (■), the *L. pneumophila icmTS* operon (pGS-Lc-37-14) (▲), and the *C. burnetii icmTS* homologous genes under regulation of the *L. pneumophila icmTS* regulatory region (pZT-lpncox-TS) (●), and the 25D mutant (□) were used. The experiments were performed three times, and similar results were obtained each time.

pneumophila icmPO and *icmJB* regulatory regions, respectively, were constructed as well (pZT-lpncox-PO and pZT-lpncox-JB, respectively). As positive controls, plasmids containing the native *L. pneumophila icmPO* and *icmJB* operons (pGS-Lc-34-14 and pGS-Lc-85-14, respectively), as well as plasmids that contained the *L. pneumophila icmP* and *icmJ* genes by themselves (pGS-Lc-34-D2-14 and pGS-Lc-84-14, respectively), were used. No complementation was observed with the plasmids that contained the *C. burnetii* homologous genes, and complete complementation was observed with the *L. pneumophila icm* genes in both hosts (data not shown). These results are surprising due to the high levels of homology found between the *C. burnetii* and *L. pneumophila* Icm proteins (the *L. pneumophila* IcmB and IcmO proteins are about 60% identical and 70% similar to the homologous proteins of *C. burnetii*).

Analysis of the *C. burnetii icmS* homologous gene. The *C. burnetii* IcmS homologous protein exhibited a high level of homology with the *L. pneumophila* IcmS protein (52.6% identity and 67.5% similarity), and it belongs to Icm/Dot group 2 (Table 4). Previously, an *L. pneumophila icmS* insertion mutant (GS3001) was shown to retain some ability to grow in HL-60-derived human macrophages but not in *A. castellanii* (51, 54). In addition, an *icmS* mutant was shown to be dispensable for pore formation in U937-derived human macrophages (9). As described above, the *icmS* gene was shown to be located second in the same transcriptional unit as *icmT* (51).

To examine the ability of the *C. burnetii icmS* homologous gene to complement the corresponding *L. pneumophila* mutant, the plasmid described above containing the *C. burnetii icmTS* homologous genes cloned downstream from the *L. pneumophila icmTS* regulatory region (pZT-lpncox-TS) was used. This plasmid was examined to determine its ability to complement a *L. pneumophila* deletion substitution mutant with a mutation in *icmS* (GS3001) in comparison to the ability of a plasmid that contained the native *L. pneumophila icmTS* operon (pGS-Lc-37-14) (Fig. 2). As shown in Fig. 2A, the *icmS* homologous gene from *C. burnetii* was able to complement an *L. pneumophila icmS* insertion mutant for intracellular growth in *A. castellanii* (a slight delay in complementation was observed 24 h postinfection, but identical yields of bacteria were obtained after 72 and 96 h; the reason for the slight delay is not known). The ability of the *C. burnetii icmS* homologous gene to complement the *L. pneumophila icmS* insertion mutant was also examined in HL-60-derived human macrophages, and complementation was also observed with this host in an intracellular growth experiment (Fig. 2B) and in a cytotoxicity assay (data not shown). These results support the hypothesis that the pathogenesis systems of *C. burnetii* and *L. pneumophila* have common features.

Analysis of the *C. burnetii icmW* homologous gene. Of all the *L. pneumophila icm/dot* genes, only the *icmW* gene was shown to result in intracellular growth phenotypes like those of the *icmS* gene. This gene has been shown to be dispensable for pore formation and to be partially required for intracellular growth in U937-derived human macrophages (9). IcmW does not have a homologous protein encoded on the R64 plasmid, and there is high level of homology between the *L. pneumophila* and *C. burnetii* homologs (58.6% identity and 68.4% similarity). IcmS and IcmW are the only members of Icm/Dot group 2 described above (Table 4). In addition, the *icmW* gene has been shown previously to be located first on the same transcriptional unit as *icmX* (5).

To analyze the *C. burnetii icmW* homologous gene, an in-frame nonpolar deletion was constructed in the *L. pneumophila icmW* gene (GY141). In addition, a plasmid that contained the *C. burnetii icmWX* homologous genes cloned downstream from the *L. pneumophila icmWX* regulatory region (pZT-lpncox-WX) was constructed. As shown in Fig. 3A, the *C. burnetii icmW* homologous gene complemented the *L. pneumophila icmW* mutant for intracellular growth in *A. cas-*

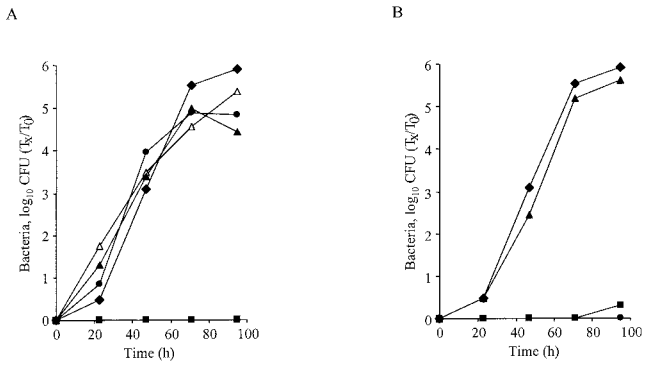

FIG. 3. *C. burnetii icmW* homologous gene complements an *L. pneumophila icmW* mutant for intracellular growth. Intracellular growth experiments in the protozoan host *A. castellanii* were performed as described in Materials and Methods. (A) Wild-type *L. pneumophila* JR-32 (◆) and the *icmW* mutant (GY141) containing the vector pMMB207αb-Km-14 (■), the *L. pneumophila icmW* gene (pZT-W-01) (△), the *L. pneumophila icmWX* operon (pZT-WX-01) (▲), and the *C. burnetii icmWX* homologous genes under regulation of the *L. pneumophila icmWX* regulatory region (pZT-lpncox-WX) (●) were used. (B) *icmX* mutant LELA3993 containing the same plasmids (without pZT-W-01) was used. The experiments were performed three times, and similar results were obtained each time.

tellanii. Similar results were obtained with HL-60-derived human macrophages (data not shown).

Analysis of the *C. burnetii icmX* and *icmQ* homologous genes. The Icm/Dot proteins of *L. pneumophila* and *C. burnetii* that constitute groups 3 and 4 exhibit low levels of homology, and one gene from each of these groups was examined (*icmX* and *icmQ*, respectively). The *C. burnetii* IcmX homologous protein that has a homologous protein encoded on the R64 plasmid was chosen as a representative of Icm/Dot group 3 (Table 4). The plasmid described above that contained the *C. burnetii icmWX* homologous genes cloned downstream from the *L. pneumophila icmWX* regulatory region (pZT-lpncox-WX) was used to complement an *icmX* insertion mutant (LELA3993) for intracellular growth. As shown in Fig. 3B, no complementation was observed when experiments were performed with *A. castellanii* or with HL-60-derived human macrophages (data not shown).

The *C. burnetii* IcmQ homologous protein that exhibits a low level of homology (22.6% identity and 34.0% similarity) to the *L. pneumophila* IcmQ protein and does not have a homologous protein encoded on the R64 plasmid was chosen as a representative of Icm/Dot group 4 (Table 4). Previously, it was reported that the *L. pneumophila* IcmQ and IcmR proteins interact with one another (9, 14), and since there is no protein homologous to the *L. pneumophila* IcmR protein in *C. burnetii*, it was interesting to analyze the *C. burnetii icmQ* gene. Similar to the results obtained with *icmX*, the *C. burnetii icmQ* homologous gene cloned under control of the *L. pneumophila icmQ* regulatory region (pZT-lpncox-Q) did not complement an *L. pneumophila icmQ* deletion substitution mutant (GS3013) for intracellular growth in HL-60-derived human macrophages or in amoebae (data not shown).

***C. burnetii* IcmW and IcmS interact with one another.** A previous report indicated that *L. pneumophila* IcmS and IcmW interact with one another (9). To further examine the similarities between the pathogenesis systems of *L. pneumophila* and *C. burnetii*, we tested whether the corresponding *C. burnetii* proteins also interact. To do this, we used a bacterial two-hybrid system that is based on the CyaA toxin of *B. pertussis* (30). The *C. burnetii icmS* and *icmW* homologous genes were fused to the T18 and T25 fragments of the CyaA toxin and transformed as pairs into an *E. coli* strain that lacked the *cyaA* gene. In this system, the interaction of the two proteins that were fused to the CyaA fragments led to formation of an active adenylate cyclase enzyme that resulted in production of cAMP from ATP. The production of cAMP was monitored by determining the increase in the level of expression of the *lacZ* gene product. As shown in Fig. 4A, the results of this analysis were very clear; like the IcmS and IcmW proteins from *L. pneumophila*, the IcmS and IcmW proteins from *C. burnetii* were found to interact with one another. High levels of β-galactosidase activity were obtained with the combination of the *icmS* and *icmW* genes fused to either of the *cyaA* fragments (18CS-25CW and 18CW-25CS in Fig. 4A). The β-galactosidase levels of expression were clearly higher than the levels of expression in the controls (combinations of each gene with the empty vector and the same gene fused to both CyaA fragments [Fig. 4A]).

Interspecies protein interaction between IcmS and IcmW. To further characterize the similarities between the IcmS and IcmW proteins from *L. pneumophila* and *C. burnetii*, we examined whether proteins from the two bacteria can interact with one another. To do this, the *L. pneumophila icmS* and *icmW* genes were fused to the *cyaA* reporter in a way similar to the way described for the corresponding genes from *C. burnetii.* First, we determined if the *L. pneumophila* IcmS and IcmW proteins interact with one another in this system, and very good interaction was observed (18LS-25LW and 18LW-25LS in Fig. 4B). All the controls that involved the *C. burnetii* IcmS and IcmW proteins (Fig. 4A) were also examined with the *L. pneumophila* IcmS and IcmW proteins, and the same results were obtained (data not shown). Subsequently, the four possible interspecies combinations were analyzed; in these analyses the IcmS-T18/T25 CyaA fusions from one bacterium and the IcmW-T18/T25 CyaA fusions from the other bacterium (and vice versa) were introduced into the *cyaA* mutant strain. As shown in Fig. 4B, protein interactions were observed with most of the combinations tested, and two of the combinations resulted in similar high levels of expression of β-galactosidase like the levels obtained with the *L. pneumophila icmS* and

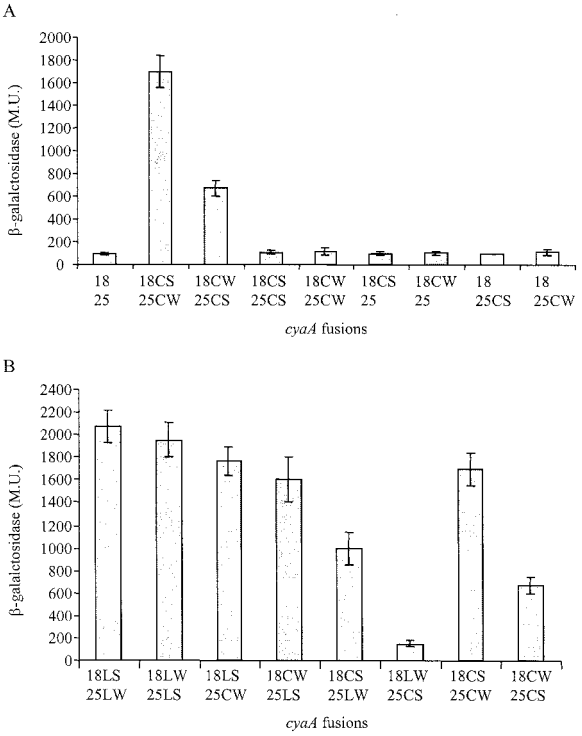

FIG. 4. Two-hybrid analysis of the interaction between the *C. burnetii* and *L. pneumophila* IcmW and IcmS proteins. An *E. coli cyaA* deletion strain containing various pairs of plasmids was examined for β-galactosidase activity, as described in Materials and Methods. (A) Analysis of the *C. burnetii* IcmS-IcmW homologous protein interaction; (B) analysis of the *L. pneumophila* IcmS-IcmW protein interaction and interspecies protein interaction. The plasmids used were the vector pT18 (18) the vector pT25 (25), the T18 fragment fused to the *C. burnetii icmS* gene (18CS), the T18 fragment fused to the *C. burnetii icmW* gene (18CW), the T25 fragment fused to the *C. burnetii icmS* gene (25CS), and the T25 fragment fused to the *C. burnetii icmW* gene (25CW). Fusions with the *L. pneumophila icmS* and *icmW* genes are indicated by L instead of C before the letter that indicates the gene. The data are expressed in Miller units (M.U.) and are the averages ± standard deviations (error bars) of at least three different experiments.

TABLE 5. Analysis of *C. burnetii icm* homologous genes in *L. pneumophila icm* mutants

Group	Protein	Predicted location[a]	Homology (% identity/% similarity)[b]		Intracellular growth[c]		Pore formation	*C. burnetii* complementation[d]	
			C. burnetii	R64	HL-60	*A. castellanii*		HL-60	*A. castellanii*
1	IcmT[e]	IM	47.1/63.2	24.5/32.3	−	−	−	+	±
	IcmP	IM	36.5/50.3	20.0/33.3	−	−	−	−	−
	IcmO	IM	59.6/70.9	23.8/35.5	−	−	−	−	−
	IcmJ	Cyt	51.2/61.9	18.7/29.8	−	−	−	−	−
	IcmB	Cyt	62.2/73.7	26.2/39.6	−	−	−	−	−
2	IcmS	Cyt	52.6/67.5	—	±	−	+	+	+
	IcmW	Cyt	58.6/68.4	—	±	−	+	+	+
3	IcmX	Perp	21.9/30.2	19.3/26.9	−	−	−	−	−
4	IcmQ	Cyt	22.6/34.0	—	−	−	−	−	−

[a] Predicted bacterial cellular locations of the Icm proteins. IM, inner membrane; Cyt, cytoplasm; Perp, periplasm.
[b] The levels of homology between the *L. pneumophila* and *C. burnetii* Icm proteins and between the *L. pneumophila* Icm proteins and the R64 plasmid Tra/Trb proteins were calculated. Similar amino acids are I, L, V, and M; H, K, and R; D and E; T and S; and Q and N. —, no homologous protein was found.
[c] The sources of the intracellular growth phenotypes are *icmT*, *icmS*, *icmQ*, *icmP*, and *icmO* (51, 54); *icmJ* and *icmB* (44, 54); *icmW* (72; this study); and *icmX* (5, 54). −, mutants cannot grow intracellulalrly ±, partial phenotype for intracellular growth.
[d] −, no complementation (Fig. 3B); ±, partial complementation (Fig. 1A); +, complete complementation (Fig. 1B, 2, and 3A).
[e] The *icmT rib* mutants were shown to be defective in exiting from the phagosome in amoebae (38).

icmW fusions (interactions between the *L. pneumophila* IcmS protein and the *C. burnetii* IcmW protein fused to either CyaA fragment). No interaction was observed with the strain containing the *C. burnetii icmS*-T25 fusion together with the *L. pneumophila icmW*-T18 fusion (18LW-25CS in Fig. 4B). However, the *C. burnetii icmS*-T25 fusion did not show a good interaction with the *C. burnetii icmW*-T18 fusion (18CW-25CS in Fig. 4A) as well. We think that the fusion between the *C. burnetii icmS* homologous gene and the T25 fragment was not properly oriented to result in a good interaction with either the *L. pneumophila* or *C. burnetii icmW*-T18 fusion. The interaction between the *C. burnetii* and *L. pneumophila* IcmS and IcmW proteins and the interspecies interactions between these proteins from the two bacteria strongly indicate that the functions of IcmS and IcmW in *C. burnetii* and *L. pneumophila* are similar.

DISCUSSION

Many bacterial pathogens contain homologous virulence systems that are used by the bacteria to subvert the host during infection. One well-documented example for such a system is the type III secretion system that has been described for many bacterial pathogens, such as *Salmonella enterica* (15), *Shigella flexneri* (62), *Yersinia* sp. (11), and many other gram-negative bacteria (70). Many components of the type III secretion complex were found to be homologous in different pathogens, but different effector proteins were shown to be delivered into the host cells and to determine the outcome of the infection (12). The type III secretion system was studied by using many different approaches, one of which was interspecies complementation. The functional conservation of the type III secretion system was demonstrated by translocation of *S. flexneri* effectors by using the *Yersinia pseudotuberculosis* translocation system, as well as translocation of *Y. pseudotuberculosis* effectors by the *S. enterica* system (45). Moreover, interspecies complementation performed with the *Pseudomonas aeruginosa pcrV* gene and a *Y. pseudotuberculosis lcrV* mutant (the proteins encoded by these two genes are homologous to one another) revealed that this component determines the size of the translocation channel (22). Additional studies in which such interspecies complementation experiments were performed revealed important information about the functions of different *S. flexneri* and *S. enterica* effectors (20, 43).

The important information about the type III secretion system that was obtained by using interspecies complementation led us to use this approach with the *icm*/*dot* homologous genes from *C. burnetii*. Because *C. burnetii* is an obligate intracellular pathogen that has limited genetic tools, we were able to perform the complementation experiments in only one direction, using *C. burnetii icm*/*dot* homologous genes to complement the corresponding *L. pneumophila* mutants. The goals of the interspecies complementation analysis were as follows: (i) to learn about the pathogenesis system of *C. burnetii*, which is an important obligate intracellular pathogen that was listed recently as a potential bioterrorism agent (46), and (ii) to increase our knowledge about the function of specific components of the *icm*/*dot* pathogenesis system in *L. pneumophila*.

To do this, we analyzed the nine *C. burnetii icm*/*dot* homologous genes summarized in Table 5. Our analysis clearly demonstrated that the pathogenesis systems of *L. pneumophila* and *C. burnetii* have similar features. The complementation of the *L. pneumophila icmS* and *icmW* mutants by the corresponding *C. burnetii* homologous genes, the interaction between the IcmS and IcmW proteins in the two bacteria, and the interspecies protein interactions strongly indicate that these proteins have similar functions in the two bacteria. Moreover, the interesting results obtained with the *C. burnetii* IcmT homologous protein might suggest that the similarities between the two systems are also located beyond the unique IcmS and IcmW proteins. Our results strongly support the hypothesis that *C. burnetii* uses the homologous Icm/Dot type IV secretion system during interactions with its hosts, a system that has common features with the *L. pneumophila* Icm/Dot type IV secretion system.

The *L. pneumophila icmS* and *icmW* genes were demonstrated previously to result in similar phenotypes with regard to intracellular growth and pore formation (9). Here, we showed that these two genes are the only *C. burnetii icm*/*dot* homologs

(of the nine homologs examined) that can replace the *L. pneumophila icmS* and *icmW* genes for intracellular growth. The only additional *C. burnetii icm* homologous gene that was found to complement the corresponding *L. pneumophila icm* mutant gene was *icmT*. However, full complementation was observed with this gene only in HL-60-derived human macrophages, and only partial complementation was observed in amoebae. This unique observation was consistent with previous results obtained for a subset of *icmT* mutants that were shown to be defective at the exit stage from the phagosome only in amoebae (37–39). Four additional *C. burnetii* Icm/Dot homologous proteins (IcmP, IcmO, IcmJ, and IcmB) that exhibit high levels of homology in the two bacteria (Table 5) were examined for interspecies complementation, but no complementation was observed. These results might indicate that the multisubunit complex that the Icm/Dot proteins that belong to groups 1 and 3 are expected to be part of (55) changed during evolution and that the two bacteria contain somewhat different complexes. In this situation, it may be that changing one or two of the complex building blocks did not result in a functional complex. Perhaps replacing several of the Icm/Dot proteins simultaneously would result in complementation.

The information presented here strongly support the idea that it is possible to obtain important information about the pathogenesis systems of obligate intracellular bacterial pathogens by using the genetic tools available for evolutionarily closely related facultative intracellular bacterial pathogens. We think that our results revealed valuable information about the components of the *C. burnetii* and *L. pneumophila icm*/*dot* pathogenesis systems that have similar functions. These results might make it possible to develop drugs that are directed specifically against the *C. burnetii* pathogenesis system.

ACKNOWLEDGMENTS

We are grateful to Howard A. Shuman for carefully reading the manuscript. We thank Hermann Willems for the *C. burnetii* genomic DNA. We thank Karen Pomeranz for plasmid construction. Preliminary sequence data were obtained from The Institute for Genomic Research website (http://www.tigr.org).

This work was supported by The Center for the Study of Emerging Diseases. G. Segal was supported by an Alon fellowship awarded by the Israeli Ministry of Education.

REFERENCES

1. **Andrews, H. L., J. P. Vogel, and R. R. Isberg.** 1998. Identification of linked *Legionella pneumophila* genes essential for intracellular growth and evasion of the endocytic pathway. Infect. Immun. **66:**950–958.
2. **Berger, K. H., J. J. Merriam, and R. R. Isberg.** 1994. Altered intracellular targeting properties associated with mutations in the *Legionella dotA* gene. Mol. Microbiol. **14:**809–822.
3. **Beron, W., M. G. Gutierrez, M. Rabinovitch, and M. I. Colombo.** 2002. *Coxiella burnetii* localizes in a Rab7-labeled compartment with autophagic characteristics. Infect. Immun. **70:**5816–5821.
4. **Bozue, J. A., and W. Johnson.** 1996. Interaction of *Legionella pneumophila* with *Acanthamoeba castellanii*: uptake by coiling phagocytosis and inhibition of phagosome-lysosome fusion. Infect. Immun. **64:**668–673.
5. **Brand, B. C., A. B. Sadosky, and H. A. Shuman.** 1994. The *Legionella pneumophila icm* locus: a set of genes required for intracellular multiplication in human macrophages. Mol. Microbiol. **14:**797–808.
6. **Brickman, E., L. Soll, and J. Beckwith.** 1973. Genetic characterization of mutations which affect catabolite-sensitive operons in *Escherichia coli*, including deletions of the gene for adenyl cyclase. J. Bacteriol. **116:**582–587.
7. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138:**179–207.
8. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from P15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
9. **Coers, J., J. C. Kagan, M. Matthews, H. Nagai, D. M. Zuckman, and C. R. Roy.** 2000. Identification of Icm protein complexes that play distinct roles in the biogenesis of an organelle permissive for *Legionella pneumophila* intracellular growth. Mol. Microbiol. **38:**719–736.
10. **Coers, J., C. Monahan, and C. R. Roy.** 1999. Modulation of phagosome biogenesis by *Legionella pneumophila* creates an organelle permissive for intracellular growth. Nat. Cell Biol. **1:**451–453.
11. **Cornelis, G. R.** 2002. *Yersinia* type III secretion: send in the effectors. J. Cell Biol. **158:**401–408.
12. **Cornelis, G. R., and F. Van Gijsegem.** 2000. Assembly and function of type III secretory systems. Annu. Rev. Microbiol. **54:**735–774.
13. **Donnenberg, M. S., and J. B. Kaper.** 1991. Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infect. Immun. **59:**4310–4317.
14. **Dumenil, G., and R. R. Isberg.** 2001. The *Legionella pneumophila* IcmR protein exhibits chaperone activity for IcmQ by preventing its participation in high-molecular-weight complexes. Mol. Microbiol. **40:**1113–1127.
15. **Galan, J. E.** 2001. *Salmonella* interactions with host cells: type III secretion at work. Annu. Rev. Cell. Dev. Biol. **17:**53–86.
16. **Gal-Mor, O., T. Zusman, and G. Segal.** 2002. Analysis of DNA regulatory elements required for expression of the *Legionella pneumophila icm* and *dot* virulence genes. J. Bacteriol. **184:**3823–3833.
17. **Hackstadt, T., and J. C. Williams.** 1981. Biochemical stratagem for obligate parasitism of eukaryotic cells by *Coxiella burnetii*. Proc. Natl. Acad. Sci. USA **78:**3240–3244.
18. **Heinzen, R. A., T. Hackstadt, and J. E. Samuel.** 1999. Developmental biology of *Coxiella burnetii*. Trends Microbiol. **7:**149–154.
19. **Heinzen, R. A., M. A. Scidmore, D. D. Rockey, and T. Hackstadt.** 1996. Differential interaction with endocytic and exocytic pathways distinguish parasitophorous vacuoles of *Coxiella burnetii* and *Chlamydia trachomatis*. Infect. Immun. **64:**796–809.
20. **Hermant, D., R. Menard, N. Arricau, C. Parsot, and M. Y. Popoff.** 1995. Functional conservation of the *Salmonella* and *Shigella* effectors of entry into epithelial cells. Mol. Microbiol. **17:**781–789.
21. **Hilbi, H., G. Segal, and H. A. Shuman.** 2001. *icm*/*dot*-dependent upregulation of phagocytosis by *Legionella pneumophila*. Mol. Microbiol. **42:**603–617.
22. **Holmstrom, A., J. Olsson, P. Cherepanov, E. Maier, R. Nordfelth, J. Pettersson, R. Benz, H. Wolf-Watz, and A. Forsberg.** 2001. LcrV is a channel size-determining component of the Yop effector translocon of *Yersinia*. Mol. Microbiol. **39:**620–632.
23. **Horwitz, M. A.** 1987. Characterization of avirulent mutant *Legionella pneumophila* that survive but do not multiply within human monocytes. J. Exp. Med. **166:**1310–1328.
24. **Horwitz, M. A.** 1983. Formation of a novel phagosome by the Legionnaires' disease bacterium (*Legionella pneumophila*) in human monocytes. J. Exp. Med. **158:**1319–1331.
25. **Horwitz, M. A.** 1983. The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. J. Exp. Med. **158:**2108–2126.
26. **Horwitz, M. A., and F. R. Maxfield.** 1984. *Legionella pneumophila* inhibits acidification of its phagosome in human monocytes. J. Cell Biol. **99:**1936–1943.
27. **Horwitz, M. A., and S. C. Silverstein.** 1980. Legionnaires' disease bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. J. Clin. Investig. **60:**441–450.
28. **Howe, D., and L. P. Mallavia.** 2000. *Coxiella burnetii* exhibits morphological change and delays phagolysosomal fusion after internalization by J774A.1 cells. Infect. Immun. **68:**3815–3821.
29. **Kagan, J. C., and C. R. Roy.** 2002. *Legionella* phagosomes intercept vesicular traffic from endoplasmic reticulum exit sites. Nat. Cell Biol. **4:**945–954.
30. **Karimova, G., J. Pidoux, A. Ullmann, and D. Ladant.** 1998. A bacterial two-hybrid system based on a reconstituted signal transduction pathway. Proc. Natl. Acad. Sci. USA **95:**5752–5756.
31. **Kirby, J. E., J. P. Vogel, H. L. Andrews, and R. R. Isberg.** 1998. Evidence for pore-forming ability by *Legionella pneumophila*. Mol. Microbiol. **27:**323–336.
32. **Komano, T., T. Yoshida, K. Narahara, and N. Furuya.** 2000. The transfer region of IncI1 plasmid R64: similarities between R64 *tra* and *Legionella icm*/*dot* genes. Mol. Microbiol. **35:**1348–1359.
33. **Marra, A., M. A. Horwitz, and H. A. Shuman.** 1990. The HL-60 model for the interaction of human macrophages with the Legionnaires' disease bacterium. J. Immunol. **144:**2738–2744.
34. **Maurin, M., and D. Raoult.** 1999. Q fever. Clin. Microbiol. Rev. **12:**518–553.
35. **Miller, J. H.** 1972. Experiments in molecular biology. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
37. **Molmeret, M., and Y. Abu Kwaik.** 2002. How does *Legionella pneumophila* exit the host cell? Trends Microbiol. **10:**258–260.
38. **Molmeret, M., O. A. Alli, M. Radulic, M. Susa, M. Doric, and Y. Abu Kwaik.** 2002. The C-terminus of IcmT is essential for pore formation and for intra-

cellular trafficking of *Legionella pneumophila* within *Acanthamoeba polyphaga*. Mol. Microbiol. **43:**1139–1150.
39. **Molmeret, M., O. A. Alli, S. Zink, A. Flieger, N. P. Cianciotto, and Y. Abu Kwaik.** 2002. *icmT* is essential for pore formation-mediated egress of *Legionella pneumophila* from mammalian and protozoan cells. Infect. Immun. **70:**69–78.
40. **Morales, V. M., A. Backman, and M. Bagdasarian.** 1991. A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. Gene **97:**39–47.
41. **Nagai, H., J. C. Kagan, X. Zhu, R. A. Kahn, and C. R. Roy.** 2002. A bacterial guanine nucleotide exchange factor activates ARF on *Legionella* phagosomes. Science **295:**679–682.
42. **Norlander, L.** 2000. Q fever epidemiology and pathogenesis. Microbes Infect. **2:**417–424.
43. **Osiecki, J. C., J. Barker, W. L. Picking, A. B. Serfis, E. Berring, S. Shah, A. Harrington, and W. D. Picking.** 2001. IpaC from *Shigella* and SipC from *Salmonella* possess similar biochemical properties but are functionally distinct. Mol. Microbiol. **42:**469–481.
44. **Purcell, M. W., and H. A. Shuman.** 1998. The *Legionella pneumophila icmGCDJBF* genes are required for killing of human macrophages. Infect. Immun. **66:**2245–2255.
45. **Rosqvist, R., S. Hakansson, A. Forsberg, and H. Wolf-Watz.** 1995. Functional conservation of the secretion and translocation machinery for virulence proteins of yersiniae, salmonellae and shigellae. EMBO J. **14:**4187–4195.
46. **Rotz, L. D., A. S. Khan, S. R. Lillibridge, S. M. Ostroff, and J. M. Hughes.** 2002. Public health assessment of potential biological terrorism agents. Emerg. Infect. Dis. **8:**225–230.
47. **Rowbotham, T. J.** 1980. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. J. Clin. Pathol. **33:**1179–1183.
48. **Roy, C. R., K. H. Berger, and R. R. Isberg.** 1998. *Legionella pneumophila* DotA protein is required for early phagosome trafficking decisions that occur within minutes of bacterial uptake. Mol. Microbiol. **28:**663–674.
49. **Sadosky, A. B., L. A. Wiater, and H. A. Shuman.** 1993. Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. Infect. Immun. **61:**5361–5373.
50. **Segal, G., M. Purcell, and H. A. Shuman.** 1998. Host cell killing and bacterial conjugation require overlapping sets of genes within a 22-kb region of the *Legionella pneumophila* genome. Proc. Natl. Acad. Sci. USA **95:**1669–1674.
51. **Segal, G., and H. A. Shuman.** 1997. Characterization of a new region required for macrophage killing by *Legionella pneumophila*. Infect. Immun. **65:**5057–5066.
52. **Segal, G., and H. A. Shuman.** 1998. How is the intracellular fate of the *Legionella pneumophila* phagosome determined? Trends Microbiol. **6:**253–255.
53. **Segal, G., and H. A. Shuman.** 1998. Intracellular multiplication and human macrophage killing by *Legionella pneumophila* are inhibited by conjugal components on IncQ plasmid RSF1010. Mol. Microbiol. **30:**197–208.
54. **Segal, G., and H. A. Shuman.** 1999. *Legionella pneumophila* utilize the same genes to multiply within *Acanthamoeba castellanii* and human macrophages. Infect. Immun. **67:**2117–2124.
55. **Segal, G., and H. A. Shuman.** 1999. Possible origin of the *Legionella pneumophila* virulence genes and their relation to *Coxiella burnetii*. Mol. Microbiol. **33:**669–670.
56. **Seshadri, R., and J. E. Samuel.** 2001. Characterization of a stress-induced alternate sigma factor, RpoS, of *Coxiella burnetii* and its expression during the development cycle. Infect. Immun. **69:**4874–4883.
57. **Sexton, J. A., and J. P. Vogel.** 2002. Type IVB secretion by intracellular pathogens. Traffic **3:**178–185.
58. **Sturgill-Koszycki, S., and M. S. Swanson.** 2000. *Legionella pneumophila* replication vacuoles mature into acidic, endocytic organelles. J. Exp. Med. **192:**1261–1272.
59. **Swanson, M. S., and E. Fernandez-Moreia.** 2002. A microbial strategy to multiply in macrophages: the pregnant pause. Traffic **3:**170–177.
60. **Swanson, M. S., and R. R. Isberg.** 1995. Association of *Legionella pneumophila* with the macrophage endoplasmic reticulum. Infect. Immun. **63:**3609–3620.
61. **Swanson, M. S., and R. R. Isberg.** 1996. Identification of *Legionella pneumophila* mutants that have aberrant intracellular fates. Infect. Immun. **64:**2585–2594.
62. **Tran Van Nhieu, G., R. Bourdet-Sicard, G. Dumenil, A. Blocker, and P. J. Sansonetti.** 2000. Bacterial signals and cell responses during *Shigella* entry into epithelial cells. Cell. Microbiol. **2:**187–193.
63. **Vogel, J. P., H. L. Andrews, S. K. Wong, and R. R. Isberg.** 1998. Conjugative transfer by the virulence system of *Legionella pneumophila*. Science **279:**873–876.
64. **Vogel, J. P., and R. R. Isberg.** 1999. Cell biology of *Legionella pneumophila*. Curr. Opin. Microbiol. **2:**30–34.
65. **Watarai, M., I. Derre, J. Kirby, J. D. Growney, W. F. Dietrich, and R. R. Isberg.** 2001. *Legionella pneumophila* is internalized by a macropinocytotic uptake pathway controlled by the Dot/Icm system and the mouse Lgn1 locus. J. Exp. Med. **194:**1081–1096.
66. **Weisburg, W. G., M. E. Dobson, J. E. Samuel, G. A. Dasch, L. P. Mallavia, O. Baca, L. Mandelco, J. E. Sechrest, E. Weiss, and C. R. Woese.** 1989. Phylogenetic diversity of the rickettsiae. J. Bacteriol. **171:**4202–4206.
67. **Wiater, L. A., K. Dunn, F. R. Maxfield, and H. A. Shuman.** 1998. Early events in phagosome establishment are required for intracellular survival of *Legionella pneumophila*. Infect. Immun. **66:**4450–4460.
68. **Wiater, L. A., A. B. Sadosky, and H. A. Shuman.** 1994. Mutagenesis of *Legionella pneumophila* using Tn*903*dll*lacZ*: identification of a growth-phase-regulated pigmentation gene. Mol. Microbiol. **11:**641–653.
69. **Yanish-Perron, C., J. Viera, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
70. **Zaharik, M. L., S. Gruenheid, A. J. Perrin, and B. B. Finlay.** 2002. Delivery of dangerous goods: type III secretion in enteric pathogens. Int. J. Med. Microbiol. **291:**593–603.
71. **Zink, S. D., L. Pedersen, N. P. Cianciotto, and Y. Abu-Kwaik.** 2002. The Dot/Icm type IV secretion system of *Legionella pneumophila* is essential for the induction of apoptosis in human macrophages. Infect. Immun. **70:**1657–1663.
72. **Zuckman, D. M., J. B. Hung, and C. R. Roy.** 1999. Pore-forming activity is not sufficient for *Legionella pneumophila* phagosome trafficking and intracellular growth. Mol. Microbiol. **32:**990–1001.

Editor: J. T. Barbieri