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Icm/Dot-dependent upregulation of phagocytosis by *Legionella pneumophila*

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Summary

***Legionella pneumophila* is the causative agent of Legionnaires' disease, a severe pneumonia. Dependent on the *icm*/*dot* loci, *L. pneumophila* survives and replicates in macrophages and amoebae within a specialized phagosome that does not fuse with lysosomes. Here, we report that phagocytosis of wild-type *L. pneumophila* is more efficient than uptake of *icm*/*dot* mutants. Compared with the wild-type strain JR32, about 10 times fewer *icm*/*dot* mutant bacteria were recovered from HL-60 macrophages in a gentamicin protection assay. The defect in phagocytosis of the mutants could be complemented by supplying the corresponding genes on a plasmid. Using fluorescence microscopy and green fluorescent protein (GFP)-expressing strains, 10–20 times fewer *icm*/*dot* mutant bacteria were found to be internalized by HL-60 cells and human monocyte-derived macrophages (HMMΦ). Compared with *icm*/*dot* mutants, wild-type *L. pneumophila* infected two to three times more macrophages and yielded a population of highly infected host cells (15–70 bacteria per macrophage) that was not observed with *icm*/*dot* mutant strains. Wild-type and *icmT* mutant bacteria were found to adhere similarly and compete for binding to HMMΦ. In addition, wild-type *L. pneumophila* was also phagocytosed more efficiently by *Acanthamoeba castellanii*, indicating that the process is independent of adherence receptor(s). Wild-type *L. pneumophila* enhanced phagocytosis of an *icmT* mutant strain in a synchronous co-infection, suggesting that increased phagocytosis results from (a) secreted effector(s) acting *in trans*.**

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Introduction

Legionella pneumophila is the causative agent of a severe pneumonia known as Legionnaires' disease. The Gram-negative, facultative intracellular pathogen replicates within and ultimately kills human monocytes, alveolar macrophages and free-living amoebae (Horwitz and Silverstein, 1980; Rowbotham, 1980; Nash *et al*., 1984). Within host cells, *L. pneumophila* creates a specialized phagosome that is initially pH neutral and does not fuse with lysosomes (Horwitz, 1983a; Horwitz and Maxfield, 1984; Bozue and Johnson, 1996). Phagosomes containing *L. pneumophila* successively associate with smooth vesicles, mitochondria and endoplasmic reticulum (Horwitz, 1983b; Swanson and Isberg, 1995; Abu Kwaik, 1996) and, at late stages, may acidify and communicate with the endosomal/lysosomal network (Sturgill-Koszycki and Swanson, 2000).

The *L. pneumophila icm* (intracellular multiplication)/*dot* (defective organelle trafficking) genes, located in two chromosomal regions, are required for intracellular multiplication and host cell killing (reviewed by Kirby and Isberg, 1998; Segal and Shuman, 1998a). Chromosomal region I harbours the genes *icmV*, *-W*, *-X* and *dotA*, *-B*, *-C*, *-D* (Marra *et al*., 1992; Berger *et al*., 1994; Brand *et al*., 1994; Vogel *et al*., 1998), and region II contains the genes *icmT*, *-S*, *-R*, *-Q*, *-P*, *-O*, *-N*, *-M*, *-L*, *-K*, *-E*, *-G*, *-C*, *-D*, *-J*, *-B*, *tphA* and *icmF* (Segal and Shuman, 1997; Andrews *et al*., 1998; Purcell and Shuman, 1998; Segal *et al*., 1998; Vogel *et al*., 1998). The *icm*/*dot* genes are also indispensable for growth within the amoeba *Acanthamoeba castellanii* (Segal and Shuman, 1999a) and *Dictyostelium discoideum* (Solomon *et al*., 2000), indicating that *L. pneumophila*'s mechanism of intracellular growth is evolutionarily conserved. Biochemical analysis of soluble Icm proteins indicated that IcmQ and -R, as well as IcmS and -W, bind directly to each other (Coers *et al*., 2000), and that IcmX is secreted (Matthews and Roy, 2000). Mutant strains lacking these genes showed discernable phenotypes, as the *icmR*, *-Q* and *-X* but not the *icmS* and *-W* mutants were defective for immediate cytotoxicity caused by contact-dependent pore formation in the macrophage membrane (Zuckman *et al*., 1999; Coers *et al*., 2000).

Some of the *icm*/*dot* genes are required for conjugation of the mobilizable plasmid RSF1010 (Segal *et al*., 1998; Segal and Shuman, 1998b; Vogel *et al*., 1998). This result corresponds with the finding that a majority of the Icm/Dot

proteins share significant sequence similarity with plasmid-encoded conjugation systems (Segal and Shuman, 1998b; 1999b). Furthermore, IcmE and DotB are homologous to proteins of the *Agrobacterium tumefaciens* Vir type IV conjugation system (VirB10, VirB11) (Vogel *et al*., 1998). *L. pneumophila* also harbours the *lvh* genes, which encode a type IV secretion system distinct from Icm/Dot (Segal *et al*., 1999). The Lvh system is dispensable for multiplication within macrophages and *A. castellanii* but partially required for conjugation. Interestingly, *lvh–dotB* or *lvh–icmE* double mutants are completely defective for conjugation.

Legionella pneumophila is phagocytosed by macrophages and *A. castellanii* by coiling or conventional phagocytosis (Horwitz, 1984; Bozue and Johnson, 1996). Phagocytosis of the bacteria by human monocytes involves complement receptor (CR) 1 and CR3 and is enhanced under different conditions, such as opsonization with fresh serum (Payne and Horwitz, 1987; Marra *et al*., 1990) or immunoglobulins (Horwitz and Silverstein, 1981; Nash *et al*., 1984) or previous growth in *A. castellanii* (Cirillo *et al*., 1999). However, the mechanism and receptors mediating phagocytosis do not seem to influence the intracellular fate of *L. pneumophila* fundamentally.

Legionella pneumophila wild-type but not *icm*/*dot* mutant bacteria block maturation of the phagosome within minutes after internalization (Roy *et al*., 1998; Wiater *et al*., 1998). This finding prompted us to investigate whether phagocytosis was also affected by the pathogen. In this study, we compared the uptake of *L. pneumophila* wild-type with *icm*/*dot* mutant strains using a gentamicin protection assay and fluorescence microscopy. We found that the *icm*/*dot* mutants are defective for efficient phagocytosis by macrophages and *A. castellanii*. The defect in efficient phagocytosis of the *icm*/*dot* mutants was not the result of a defect in adherence. However, efficient uptake of an *icmT* mutant could be partially restored in a co-infection with wild-type bacteria, indicating that secreted effector(s) are involved. These results suggest that the *L. pneumophila icm*/*dot* genes function to stimulate uptake of the bacteria upon initial contact of the pathogen with its host cell.

Results

Legionella pneumophila icm/dot *mutants are defective for phagocytosis by HL-60 cells*

Within minutes after internalization, wild-type *L. pneumophila* but not *icm*/*dot* mutant bacteria are found in a compartment that neither fuses with lysosomes nor acidifies (Roy *et al*., 1998; Wiater *et al*., 1998). In addition, expression of *dotA* before macrophage uptake but not continuous signalling by the Icm/Dot transporter is required to establish a replication-permissive phagosome (Roy *et al*., 1998; Coers *et al*., 1999). The rapid kinetics of the endocytic block prompted us to analyse phagocytosis of *L. pneumophila* in detail. As a host cell, we initially chose HL-60 macrophages, a well-established model of *L. pneumophila* uptake, intracellular multiplication and host cell killing (Marra *et al*., 1990).

Differentiated human HL-60 cells were infected with *L. pneumophila* wild-type strain JR32 or *icm*/*dot* mutants and, after killing extracellular bacteria with gentamicin, the cells were lysed, and the number of viable intracellular bacteria was determined. Using this assay, we recovered about 10 times more viable wild-type *L. pneumophila* than *icm*/*dot* mutants from the HL-60 macrophages (Fig. 1A). This phenotype was statistically significant ($P < 0.04$, Student's *t*-test) and was observed for *icm*/*dot* mutants in chromosomal region II (*icmT*, *-S*, *-R*, *-Q*, *-P*, *-O*, *-N*, *-M*, *-L*, *-K*, *-E*, *-G*, *-C*, *-D*, *-J*, *-B*, *-F*) as well as region I (*dotB*, *-A*, *icmV*, *-X*). The *L. pneumophila* strains carried the mobilizable plasmid pMMB207αb (pMMB), the non-mobilizable derivative pMMB207αb-Km-14 (pMMB-MobA) or plasmids encoding *icmM*, *-K*, *-E* (pMKE) or *icmM*, *-L*, *-E* (pMLE). The latter two plasmids were introduced into the *icmL* or *icmK* mutants, respectively, to rule out polar effects on downstream genes in the putative operon formed by *icmMLKE* (Segal *et al*., 1998). Disruption of *tphA*, a gene in region II, did not impair phagocytosis. This result corresponds with the finding that *tphA* is also not required for intracellular multiplication within macrophages (Purcell and Shuman, 1998). In addition, the phenotype of the *icmF* mutant was somewhat weaker than that of other *icm*/*dot* mutants, correlating with its only partial requirement for intracellular multiplication (Purcell and Shuman, 1998).

Treatment of the HL-60 macrophages with cytochalasin B, an inhibitor of actin polymerization, led to the recovery of about 10–20 times less wild-type bacteria ($P < 0.01$), indicating that, under these conditions, phagocytosis was largely abolished, and gentamicin eradicated extracellular *L. pneumophila*. Cytochalasin might reduce the adherence of differentiated HL-60 cells (data not shown), thus decreasing the number of gentamicin-resistant intracellular cfu observed. However, cytochalasin-dependent decrease in phagocytosis was confirmed in independent experiments using green fluorescent protein (GFP)-expressing *L. pneumophila* and immunofluorescence microscopy as a more direct assay to enumerate intracellular bacteria (see below).

The defect in efficient phagocytosis of the *icm*/*dot* mutant strains was complemented by supplying the corresponding *icm*/*dot* genes on plasmids (Fig. 1B; $P < 0.05$). In general, complementation was more efficient if the complementing genes were encoded by

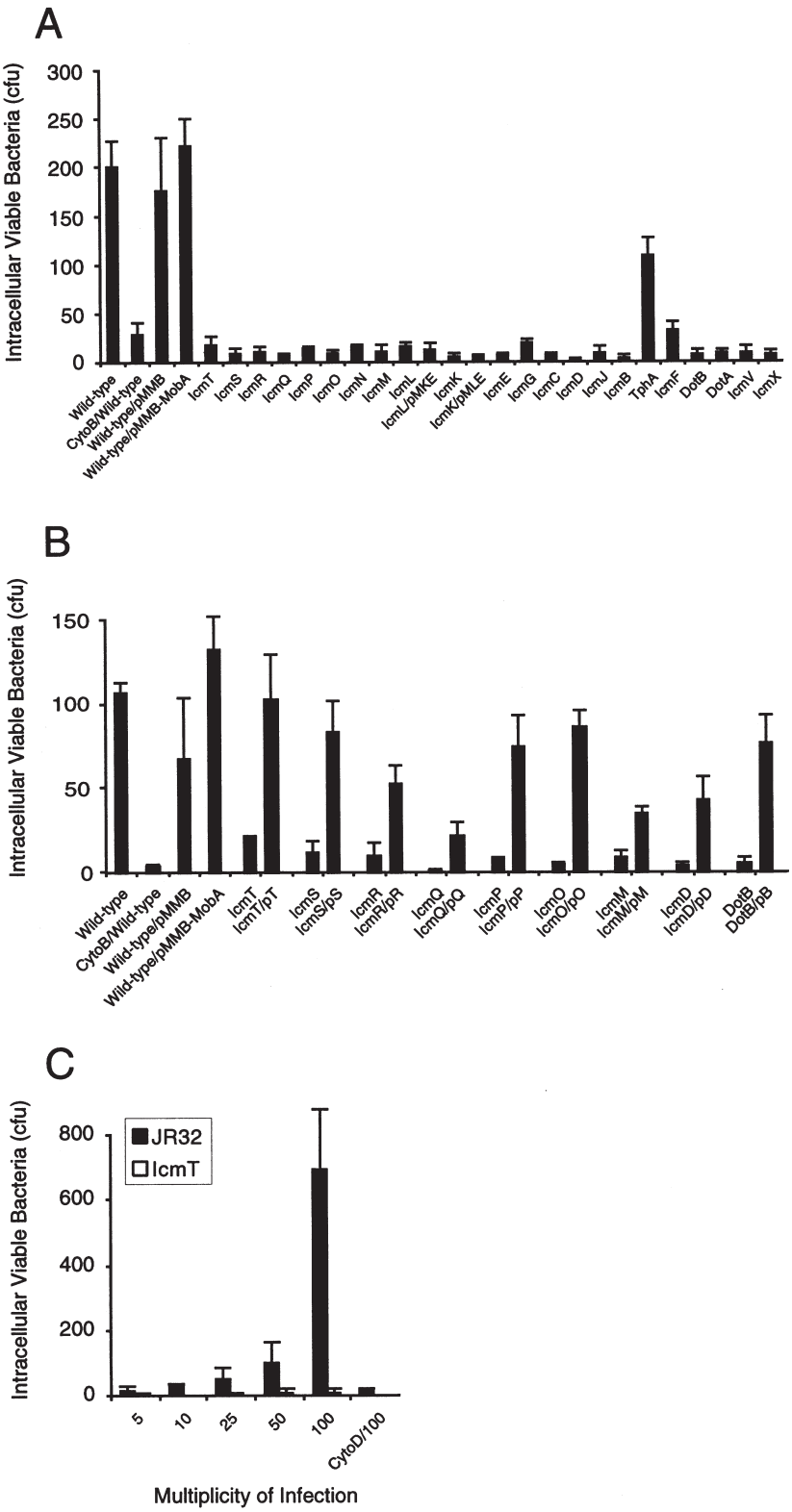

Fig. 1. *Legionella pneumophila icm*/*dot* mutants are defective for phagocytosis by HL-60 macrophages as determined by gentamicin protection assay. Differentiated HL-60 macrophages were infected at an MOI of 100 (A and B) or the MOI indicated (C) with *L. pneumophila* wild-type (JR32) or *icm*/*dot* mutant strains (Table 1) harbouring empty or corresponding complementing plasmids (Table 2). Before infection, macrophages were treated with cytochalasin-B or -D where indicated (CytoB, -D). The infected macrophages were washed with gentamicin-containing buffer, lysed 60 min after infection, and lysates were plated to count cfu. Mean and standard deviation of experiments performed in triplicate are shown. Similar results were obtained in at least three independent experiments.
A. Ten times more wild-type bacteria (JR32) than *icm*/*dot* mutants were phagocytosed by HL-60 cells. The strains harboured the mobilizable plasmids pMMB207 (*icmD*, *-J*, *-B*) or pMMB207αb (pMMB; *icmR*, *-Q*, *-M*, *-L*, *-K*, *-E*, *-V*, *-X*, *dotA*) or the non-mobilizable plasmid pMMB207αb-Km-14 (pMMB-MobA; *icmT*, *-S*, *-P*, *-O*, *-N*, *-G*, *-C*, *-F*, *tphA*, *dotB*).
B. The defect in phagocytosis of the *icm*/*dot* mutant strains was complemented by supplying the corresponding *icm*/*dot* genes on plasmid pMMB207 (*icmD*), pMMB (*icmR*, *-Q*, *-M*) or pMMB-MobA (*icmT*, *-S*, *-P*, *-O*, *dotB*).
C. Wild-type *L. pneumophila* (JR32, closed bars) was phagocytosed more efficiently than an *icmT* mutant strain (GS3011, open bars) at different MOIs.

the non-mobilizable plasmid pMMB207αb-Km-14 rather than by the mobilizable plasmids pMMB207 or pMMB207αb. Moreover, the wild-type strain JR32 transformed with pMMB207αb was phagocytosed less efficiently than JR32 transformed with pMMB207αb-Km-14 ($P = 0.02$). These findings are consistent with the idea that conjugal nucleoprotein complexes compete with effector proteins as substrates of the Icm/Dot transport

machinery during phagocytosis as well as in later steps of intracellular multiplication (Segal and Shuman, 1998b).

Differentiated HL-60 cells are not very efficient phagocytes (see below; data not shown) and, therefore, the cells were routinely infected with *L. pneumophila* at a high multiplicity of infection (MOI) of 100. However, wild-type *L. pneumophila* were also more efficiently phagocytosed than *icmT* mutant bacteria at lower MOIs of 5–50 bacteria per macrophage (Fig. 1C).

The gentamicin sensitivity of *L. pneumophila* wild-type strain JR32 and the *icmT* mutant GS3011 was compared by incubating the strains with different concentrations of gentamicin for 1 h. No significant differences in the susceptibility towards gentamicin were found, ruling out the possibility that the apparent difference in phagocytic efficiency between wild-type and *icm*/*dot* mutant strains was caused by increased drug sensitivity of the mutant strains (data not shown).

We considered the possibility that the decreased number of viable *icm*/*dot* mutant bacteria resulted from more rapid killing of the latter by the HL-60 cells within the standard 1 h infection time. To test this idea, gentamicin-treated infected cells were lysed as early as 15 min after infection. At this time point, about fives times fewer *icmT* mutant bacteria were recovered from the cells compared with wild-type strain JR32 ($P = 0.001$), and the defect for phagocytosis was complemented by supplying plasmid-encoded *icmT* (data not shown). If phagocytosis was blocked with cytochalasin B, about 15 times more bacteria were killed within 15 min ($P = 0.002$), thus validating the short incubation time with gentamicin. From these results, it seems unlikely that the differences in viable wild-type and *icm*/*dot* mutant bacteria result from more efficient killing of the mutants.

The apparent efficiency of phagocytosis varied to some degree between independent experiments (Fig. 1). After differentiation, the HL-60 cells did not always adhere similarly to the wells and, consequently, different numbers of infected cells were left after the washing steps (see *Experimental procedures*). Importantly, however, in 10 independent experiments performed in triplicate, about 10 times more intracellular viable wild-type *L. pneumophila* were recovered compared with *icm*/*dot* mutant bacteria (data not shown).

In an independent approach, we used fluorescence microscopy to quantify phagocytosis of GFP-expressing wild-type or *icm*/*dot* mutant *L. pneumophila* directly. Infected HL-60 cells were washed and fixed 20 min after infection, and extracellular bacteria were immunolabelled with a rhodamine-conjugated, serotype-specific anti-*L. pneumophila* antibody. Thus, intracellular (green) bacteria could easily be distinguished from extracellular (yellow; superimposition of green and red) bacteria (Fig. 2A). Wild-type and *icm*/*dot* mutant strains grown to stationary phase were examined by fluorescence microscopy for GFP expression before infections. Although $> 95\%$ of wild-type bacteria and *icm*/*dot* mutants expressed GFP, individual bacteria showed some differences in fluorescence intensity. Thus, more faintly green fluorescing bacteria might appear red in the overlay fluorescence image. In addition, the green and red fluorescence of some unattached bacteria did not co-localize in the overlay image, as these bacteria moved during the time required to acquire two image series of 40 sections with different fluorescence filters.

Compared with wild-type strain JR32, 10–20 times fewer *icm*/*dot* mutants (*icmT*, *-S*, *-R*, *-Q*, *-P*, *-O*, *dotA*, *-B*, *icmW*) were scored within differentiated HL-60 cells (Fig. 2B; $P < 0.03$). The wild-type strain was phagocytosed about 10 times more efficiently than an *icmT* mutant strain, regardless of whether the bacteria had been grown to stationary phase on agar plates or in liquid culture (data not shown). The *icmN* mutant was phagocytosed about two times less efficiently than wild-type bacteria. As expected, the *tphA* mutant was phagocytosed as efficiently as wild type, and cytochalasin D severely diminished the uptake of wild-type bacteria.

Interestingly, individual macrophages infected with JR32 phagocytosed large numbers of bacteria (up to 60 bacteria per macrophage), resulting in a population of highly infected cells that was not observed in infections with the *icmT* mutant (Fig. 2C) or the *icmS*, *-R*, *-Q*, *-P*, *-O*, *-W* and *dotB* and *-A* mutants (data not shown). Furthermore, JR32 infected more HL-60 macrophages ($31 \pm 6\%$) than the *icmT* mutant ($10 \pm 7\%$) or the other *icm*/*dot* mutants.

Similar results were obtained using flow cytometry to analyse HL-60 cells infected with GFP-expressing *L. pneumophila* strains. Compared with HL-60 cells infected with the wild-type strain JR32, two to three times fewer cells infected with *icm*/*dot* mutant strains (*icmT*, *-S*, *-R*, *-P*, *dotB*) showed a fluorescence intensity higher than the intrinsic fluorescence of uninfected cells (data not shown).

We also compared phagocytosis of GFP-expressing JR32 or *icmT* mutant bacteria by murine J774A.1 macrophages. Using fluorescence microscopy, these cells were found to phagocytose approximately three times more wild-type *L. pneumophila* than *icmT* mutants (data not shown).

In summary, the above experiments demonstrate that *L. pneumophila icm*/*dot* mutants are taken up less efficiently by human and mouse macrophages, indicating that the secretion system encoded by these genes governs the initial interaction of the bacterium with its host cell.

Human monocyte-derived macrophages phagocytose icm/dot *mutants less efficiently than wild-type* L. pneumophila

We next addressed the question whether *L. pneumophila*

icm/*dot* mutants are defective for uptake not only by macrophage cell lines but also by primary macrophages. Human monocytes were isolated from buffy coats and matured into macrophages to yield human monocyte-derived macrophages (HMMΦ). Within these host cells, the wild-type strain JR32 replicated, and *icm* mutants (*icmT*, *-S*) were killed (data not shown). To test whether the phagocytic efficiency of HMMΦ is affected by the *L. pneumophila icm*/*dot* genes, the macrophages were infected with either the GFP-expressing wild-type strain JR32 or *icm*/*dot* mutants, and intracellular bacteria were counted by fluorescence microscopy (Fig. 3A) as described above. At 20 min after infection, approximately eight times (*icmT*, *-S*, *-R*), four times (*icmQ*), 15 times (*icmP*, *-O*) or 35 times (*icmW*) fewer intracellular *icm* mutants were phagocytosed compared with wild-type bacteria (Fig. 3B; $P < 0.013$). The *dotB* and *icmN* mutant strains were phagocytosed by HMMΦ about two times less efficiently than wild-type, and the *tphA* mutant was not impaired for phagocytic uptake. Cytochalasin D-treated

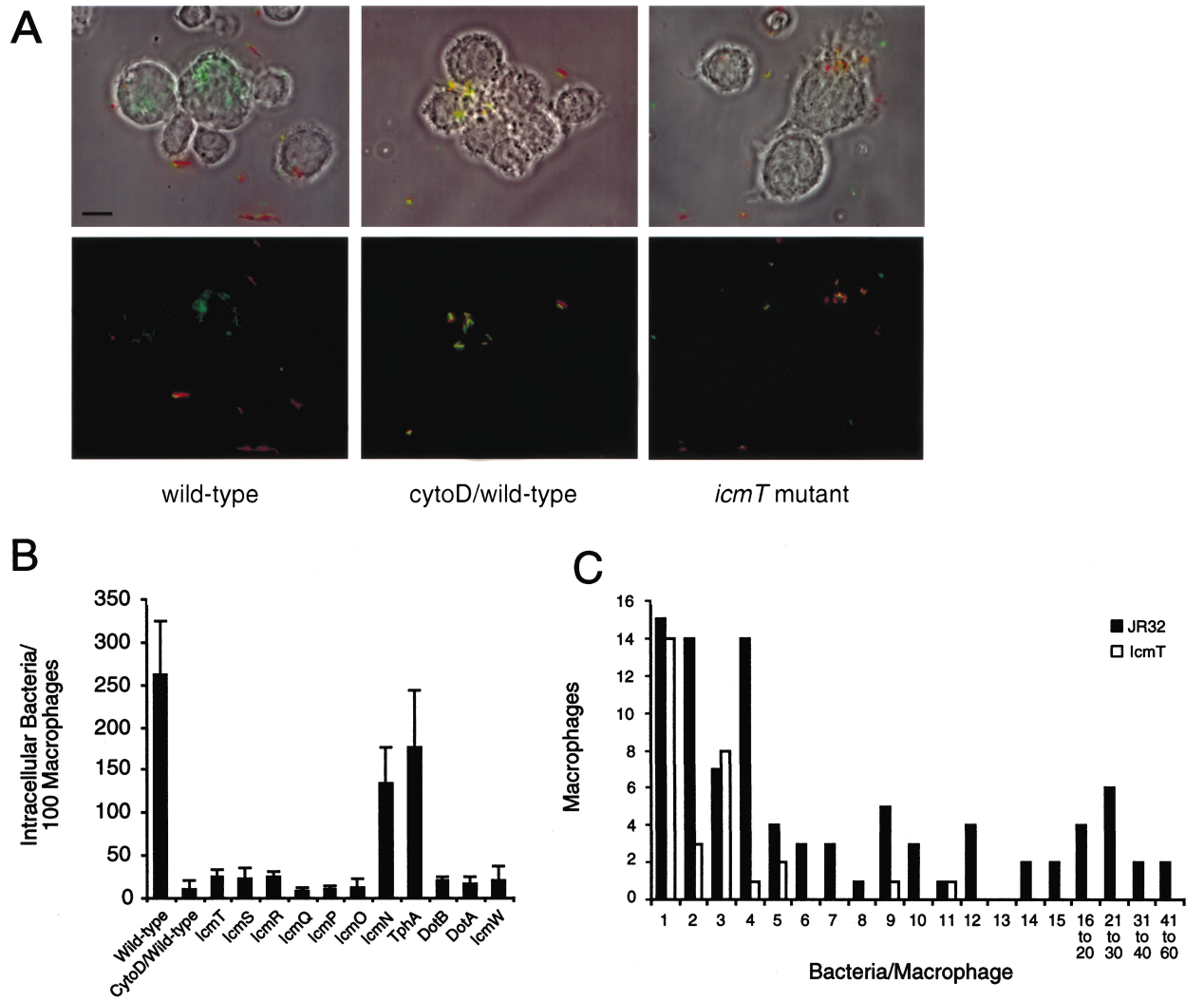

Fig. 2. Fluorescence microscopy of HL-60 macrophages infected with wild-type and *icm*/*dot* mutant *L. pneumophila.*
A. Fluorescence micrographs of differentiated HL-60 cells infected for 20 min at an MOI of 150 with GFP-expressing wild-type bacteria (JR32) in the absence and presence of cytochalasin D or with an *icmT* mutant (LELA4086). Extracellular bacteria were labelled with a rhodamine-conjugated antibody. Z-section images of infected cells were merged into one layer, and either red and green fluorescence images (bottom) or fluorescence images and a brightfield picture (top) were overlain. Thus, intracellular bacteria are labelled green, extracellular bacteria are labelled yellow (red and green superimposed). Bar represents 10 μm.
B. *icm*/*dot* mutants are defective for efficient phagocytosis by HL-60 cells. Intracellular wild-type bacteria (JR32), *icm*/*dot* mutants (LELA4086, GS3001, GS3012, LELA3463, GS3002, GS3003, GS3007, LELA2883, LELA3118, LM1445) or a *tphA* mutant strain (MW627) were counted within 100 randomly selected macrophages. Mean and standard deviation of three independent experiments are shown.
C. Wild-type and *icmT* mutant bacteria are distributed differently within HL-60 cells, yielding a population of highly infected cells (12–60 bacteria per macrophage) specific for the wild-type strain. Individual wild-type and *icmT* mutant bacteria were counted within 100 HL-60 cells and plotted against the number of macrophages containing the same number of intracellular bacteria. The sum of three independent experiments is shown.

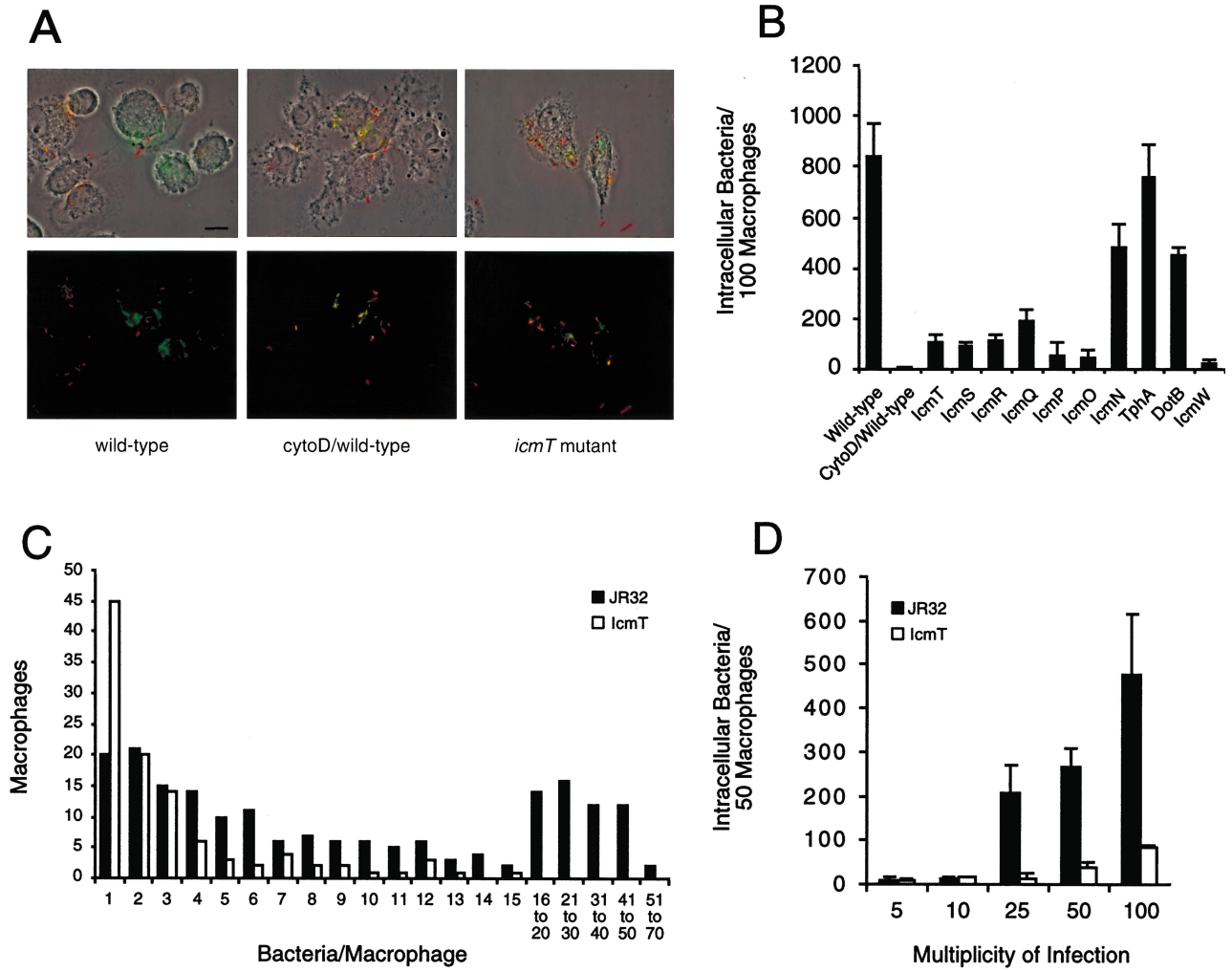

Fig. 3. Wild-type *L. pneumophila* promotes its uptake by human monocyte-derived macrophages.
A. Fluorescence micrographs of human monocyte-derived macrophages (HMMΦ) infected for 20 min at an MOI of 100 (A–C) or at the MOI indicated (D) with GFP-expressing wild-type bacteria (JR32) in the absence and presence of cytochalasin D or with an *icmT* mutant (LELA4086). Extracellular bacteria were labelled with a rhodamine-conjugated antibody. Z-section images of infected HMMΦ were merged into one layer, and either the red and green fluorescence images (bottom) or the fluorescence images and a brightfield picture (top) were overlain. Thus, intracellular bacteria are labelled green, extracellular bacteria are labelled yellow (red and green superimposed). Bar represents 10 μm.
B. *icm*/*dot* mutants are defective for efficient phagocytosis by HMMΦ. Intracellular wild-type bacteria (JR32), *icm*/*dot* mutants (LELA4086, GS3001, GS3012, LELA3463, GS3002, GS3003, GS3007, LELA2883, LM1445) or a *tphA* mutant strain (MW627) were counted within 100 randomly selected macrophages. Mean and standard deviation of three independent experiments are shown. Similar results were obtained with HMMΦ cultured for 6–13 days.
C. Wild-type and *icmT* mutant bacteria are distributed differently within HMMΦ, resulting in a population of highly infected cells (16–70 bacteria per macrophage) specific for the wild-type strain. Individual wild-type and *icmT* mutant bacteria were counted within 100 HMMΦ and plotted against the number of macrophages containing the same number of intracellular bacteria. The sum of three independent experiments is shown.
D. HMMΦ were infected at the MOI indicated with a GFP-expressing wild-type (JR32, closed bars) or *icmT* mutant strain (GS3011, open bars), and intracellular bacteria were counted within 50 randomly selected macrophages. At a threshold around an MOI of 25, promotion of phagocytosis by the wild-type strain became evident. Mean and standard deviation of three independent experiments are shown.

HMMΦ phagocytosed $< 1\%$ of wild-type *L. pneumophila* compared with untreated cells ($P = 0.008$).

Strikingly, the distribution of wild-type and *icmT* mutant bacteria within HMMΦ was different (Fig. 3C). Most HMMΦ infected with the *icmT* mutant strain contained only one or a few bacteria per macrophage. In contrast, many HMMΦ infected with wild-type *L. pneumophila* contained several bacteria per macrophage, and a population of heavily infected macrophages (16–70 bacteria per macrophage) was only observed for HMMΦ infected with wild-type bacteria. The population of highly infected macrophages was also absent from infections with the *icmS*, *-R*, *-Q*, *-P*, *-O* and *-W* mutant bacteria (data not shown). Significantly more HMMΦ were infected by wild-type *L. pneumophila* than by the *icmT* mutant ($64 \pm 12\%$ or $35 \pm 3\%$ respectively; $P = 0.03$) or other *icm*

mutants. As expected, HMMΦ were more efficient phagocytes than HL-60 cells and phagocytosed approximately three to four times more wild-type as well as *icm*/*dot* mutant bacteria (Figs 2B and 3B).

HMMΦ were infected with GFP-expressing wild-type or *icmT* mutant bacteria at different MOIs (Fig. 3D). At an MOI of 5 or 10, wild-type as well as *icmT* mutant bacteria were phagocytosed with similar efficiency, and the overall uptake of bacteria was very low (10–15 bacteria per 50 macrophages). At an MOI of 25, however, a threshold was observed, at which the difference in phagocytosis between wild type and the *icmT* mutant became significant ($P = 0.02$). At MOIs from 25 to 100, the number of intracellular bacteria increased (up to 600 bacteria per 50 macrophages), whereas the ratio of wild-type to *icmT* mutant bacteria remained about 6–15.

The lvh *genes complement a* dotB *mutant for efficient phagocytosis*

The *dotB* and *icmE* genes show sequence homology to two genes within the *L. pneumophila lvh* locus, encoding a type IV secretion system distinct from the *icm*/*dot*-encoded apparatus (Segal *et al*., 1999). The Lvh system is dispensable for intracellular growth of *L. pneumophila* in HL-60 cells and *A. castellanii*. Furthermore, deletion of *lvh*, *dotB* or *icmE* individually has only a minor effect on conjugation. However, the *lvh–dotB* or *lvh–icmE* double mutants conjugate five orders of magnitude less efficiently (Segal *et al*., 1999). In order to test whether the *lvh* locus is involved in phagocytosis, we infected HMMΦ with GFP-expressing *lvh*, *lvh–dotB* or *lvh–icmE* mutant strains (Fig. 4). Whereas the *lvh* mutant was phagocytosed similarly to the wild-type and *dotB* mutant strains, phagocytosis of the *lvh–dotB* mutant was severely diminished ($P = 0.01$). The *icmE* mutant and, consequently, the *lvh–icmE* mutant was also impaired for phagocytosis. As both the *lvhB11* and *dotB* genes share homology with the large family of *virB11* ATPases, these results suggest that *lvhB11* can substitute for the *dotB* gene not only for conjugation but also for a virulence-related trait such as phagocytic efficiency.

Wild-type and icmT *mutant* L. pneumophila *adhere similarly and compete for binding to HMMΦ, and uptake is triggered* in trans

The differences in phagocytic efficiency between *L. pneumophila* wild type and *icm*/*dot* mutants might result from either decreased adherence of the mutants to the host cell or a defect in bacterial signalling. We chose HMMΦ to analyse binding of *L. pneumophila* to macrophages, as *L. pneumophila* was found to adhere well to these cells (Fig. 3A). The HMMΦ were treated with cytochalasin D to block phagocytosis and infected with either GFP-expressing wild-type or *icmT* mutant strains. Extracellular bacteria were labelled with a rhodamine-conjugated anti-*L. pneumophila* antibody. Similar numbers of extracellular bacteria were counted on HMMΦ infected with either wild-type or *icmT* mutant bacteria (Fig. 5A, 'single strains'). Therefore, the differences in phagocytic efficiency between wild-type and *icmT* mutant bacteria cannot be accounted for by differences in adherence. Equal numbers of bacteria were also counted in co-infection experiments with unlabelled wild-type and GFP-expressing *icmT* bacteria (Fig. 5A, 'competition'). Furthermore, compared with infections with one bacterial strain, 50% fewer wild-type and *icmT* mutant bacteria adhered to the macrophages in the co-infections ($P = 0.04$). These results indicate that wild-type and *icmT* mutant bacteria adhering to macrophages compete for the same receptor(s) on HMMΦ.

In additional experiments, adherence of wild-type or *icm*/*dot* mutant strains to HL-60 cells or HMMΦ was compared by determining colony-forming units (cfu). Cytochalasin D-treated macrophages were infected with bacteria and, after removing non-adherent bacteria by washing, the cells were lysed, and cfu were determined. Similar numbers of *icm*/*dot* mutants (*icmT*, *-S*, *-R*, *-Q*, *-P*, *-O*, *-N*, *-M*, *-E*, *-G*, *-C*, *-D*, *-J*, *-B*, *tphA*, *-F*, *dotB*, *-A*, *icmV*, *-W*, *-X*) and wild-type *L. pneumophila* were recovered in this assay, indicating that similar numbers of wild-type and *icm*/*dot* mutant bacteria adhered to cytochalasin D-treated macrophages (data not shown).

To test whether stimulation of phagocytosis by wild-type *L. pneumophila* would promote phagocytosis of an *icmT* mutant strain in a co-infection, GFP-expressing *icmT* mutant bacteria were used to infect HMMΦ simultaneously with either unlabelled wild-type or unlabelled *icmT* mutant bacteria. The MOI of the unlabelled strains was kept constant at 100, and GFP-expressing *icmT* was used at two different MOIs (Fig. 5B). At an MOI of 50 ($P = 0.007$) or 125 ($P = 0.032$), about two times more GFP-expressing *icmT* bacteria were phagocytosed in the presence of unlabelled JR32 compared with co-infections with the unlabelled *icmT* mutant. Therefore, wild-type *L. pneumophila* promotes the phagocytosis of *icmT* mutant bacteria, suggesting that secreted effector molecules are involved. Given that wild-type and *icmT* mutant bacteria adhere similarly to macrophages and compete for the same receptor(s), this result argues that, independently of adherence, wild-type *L. pneumophila* promotes phagocytosis by secreting (an) effector(s).

Acanthamoeba castellanii phagocytoses icm/dot *mutants less efficiently than wild-type L. pneumophila*

Growth of *L. pneumophila* in its natural host *A. castellanii*

requires the *icm*/*dot* genes (Segal and Shuman, 1999a). To analyse whether these genes are also involved in phagocytosis of *L. pneumophila* by *A. castellanii*, the amoebae were infected with either the GFP-expressing wild-type strain JR32 or *icm*/*dot* mutants. Extracellular bacteria were labelled with a rhodamine-conjugated anti-*L. pneumophila* antibody, and intracellular bacteria were counted by fluorescence microscopy as described above for macrophages. At 20 min after infection, about three to four times fewer *icm* mutants (*icmT*, *-S*, *-P*, *-O*) were phagocytosed compared with wild-type bacteria (Fig. 6A, $P < 0.05$). Wild-type *L. pneumophila* was more efficiently phagocytosed than *icmT* mutant bacteria at MOIs as low as 1 or 5 (Fig. 6B). These results indicate that the *icm*/*dot* genes are involved in the initial steps of the interaction between *L. pneumophila* and amoebae. Moreover, phagocytosis of *L. pneumophila* by amoebae or macrophages seems to be mechanistically similar.

Discussion

Intracellular multiplication of *L. pneumophila* depends on several properties of the organism. The ability of the bacteria to survive the antimicrobial defences of phagocytic host cells is the first challenge that must be met. Phagocytosis of most other organisms results in the formation of a phagosome that rapidly fuses with lysosomes, and the internalized organism is either killed or can tolerate the inhospitable environment of the phagolysosome. *Legionella* and some other intracellular pathogens have the ability to prevent phagosome–lysosome fusion (Sinai and Joiner, 1997; Garcia-del Portillo, 1999). Genetic analysis has revealed that the *icm/dot* gene products are required for preventing phagosome–lysosome fusion, intracellular multiplication and organelle trafficking (Kirby and Isberg, 1998; Segal and Shuman, 1998a; Vogel and Isberg, 1999). Very rapidly after internalization, it is possible to distinguish whether *Legionella*-containing phagosomes have either fused or not fused with lysosomes. Phagosomes that have not fused provide a hospitable environment in which the bacteria multiply within a vacuole and eventually kill the host cell. *Legionella* contained in fused phagolysosomes, however, are unable to grow and are destroyed. Approximately 85% of the phagosomes containing viable wild-type bacteria do not fuse and are called 'specialized' or '*Legionella*-specific' phagosomes. In contrast, > 90% of the phagosomes containing either dead bacteria or viable *icm*/*dot* mutant bacteria fuse and form phagolysosomes (Horwitz, 1987; Roy *et al*., 1998; Wiater *et al*., 1998). Thus, the ability of the bacteria to influence the fusion properties of the phagosome is key to the ultimate fate of the bacteria.

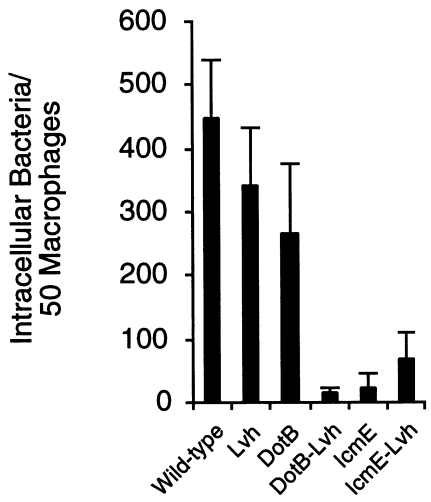

Fig. 4. The *lvh* genes complement a *dotB* mutant for efficient phagocytosis. HMMΦ were infected at an MOI of 100 with GFP-expressing wild-type bacteria (JR32) or *icm*/*dot* (LELA2883, LELA4432), *lvh* (GS28G) or *lvh*–*icm*/*dot* (LELA2883-28, LELA4432-28) mutant strains. Extracellular bacteria were labelled with a rhodamine-conjugated antibody, and intracellular bacteria were counted within 50 randomly selected macrophages. The *lvh* genes, encoding a type IV secretion system distinct from the *icm*/*dot*-encoded apparatus, functionally complemented a *dotB* mutant for efficient phagocytosis. Mean and standard deviation of triplicates performed in two independent experiments are shown.

It has been proposed that the *icm*/*dot* complex functions to deliver effectors to the host cell (Kirby and Isberg, 1998; Segal and Shuman, 1998a). One presumed function of the effectors is to modify the fusion properties of the phagosome. An alternative possibility is that the bacteria use an effector to modify the process of phagocytosis. The experiments described here tested the idea that *L. pneumophila* influences the process of phagocytosis itself. We found that wild-type *L. pneumophila* are taken up by phagocytic host cells more efficiently than *icm*/*dot* mutants (Figs 1A, 2B, 3B and 6A). Not only was the net number of bacteria taken up higher, but wild-type bacteria were found in very high numbers inside individual phagocytes (Figs 2C and 3C). These heavily infected cells were not detected in infections with *icm*/*dot* mutants. The defect in phagocytosis of an *icmT* mutant was not the result of a defect in adherence (Fig. 5A) and, because similar results were obtained using mammalian or protozoan host cells, these effects appear to be independent of the receptor(s) used by the bacteria to adhere to host cells. The adherence receptor used by *L. pneumophila* does not seem to affect its intracellular survival profoundly, as the bacterium multiplies within phagocytes after being taken up under different opsonizing or non-opsonizing conditions (Horwitz and Silverstein, 1981; Nash *et al*., 1984; Payne and Horwitz, 1987; Marra *et al*., 1990; Cirillo *et al*., 1999). Interestingly, in co-infection experiments, the presence of wild-type *L. pneumophila* could promote increased uptake of an *icmT* mutant *in trans* (Fig. 5B). This finding provides evidence that the Icm/Dot transport system indeed

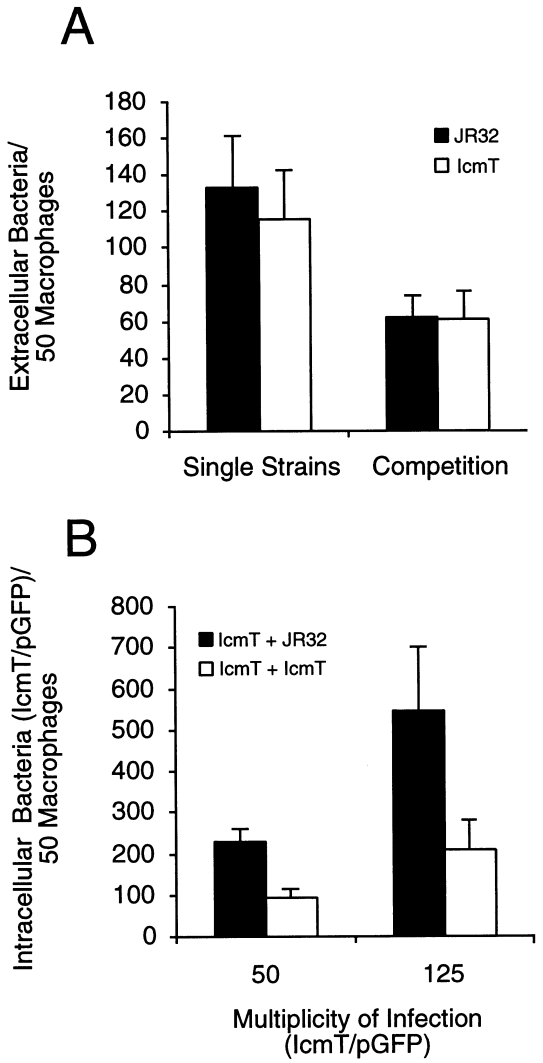

Fig. 5. Wild-type and *icmT* mutant bacteria adhere similarly and compete for binding to HMMΦ, and uptake is triggered *in trans*.
A. HMMΦ were treated with cytochalasin D and infected at an MOI of 100 with either a GFP-expressing wild-type strain (JR32, closed bars) or a GFP-expressing *icmT* mutant strain (GS3011, open bars) alone ('single strains') or synchronously with unlabelled wild-type and GFP-expressing *icmT* mutants at an MOI of 100 each ('competition'). Extracellular bacteria were labelled with a rhodamine-conjugated antibody and counted on 50 randomly selected macrophages. Similar numbers of wild-type and *icmT* mutant bacteria adhered to HMMΦ and, in co-infections, the strains competed for the same adherence receptor(s). Mean and standard deviation of quintuplets (single strains) or quadruplets (competition) performed in three or two independent experiments, respectively, are shown.
B. HMMΦ were co-infected with a GFP-expressing *icmT* mutant strain (GS3011, MOI 50 or 125) and either unlabelled wild-type (JR32, closed bars) or unlabelled *icmT* mutant bacteria (GS3011, open bars) at an MOI of 100 each. After labelling extracellular bacteria with a rhodamine-conjugated antibody, intracellular bacteria were scored within 50 randomly selected macrophages. Twice as many GFP-expressing *icmT* bacteria were phagocytosed in the presence of JR32 compared with co-infections with the *icmT* mutant. Results are the mean and standard deviation of three independent experiments.

secretes (an) effector(s). In accordance with this result, wild-type *L. pneumophila* was recently found to rescue a *dotA* mutant if the two strains were fed to the macrophages simultaneously but not sequentially, and the wild-type strain supported intracellular multiplication of *dotA* mutant bacteria residing in the same phagosome but not in distinct phagosomes (Coers *et al*., 1999).

Upregulation of phagocytosis by Icm/Dot-secreted effectors might immediately lead to either a *Legionella*-specific or a non-specific phagosome that is subsequently modified by wild-type but not *icm*/*dot* mutant *Legionella*. In a 'concerted'-type model, a *Legionella* effector triggers increased uptake into a specialized type of phagosome that does not fuse with lysosomes (Fig. 7, pathway A). In the absence of the effector, normal phagocytosis results in the formation of a phagosome that does fuse with lysosomes, eventually leading to an acidic phagolysosome, in which *icm*/*dot* mutants are degraded (Sadosky *et al*., 1993; Purcell and Shuman, 1998; Solomon *et al*., 2000). Alternatively, in a 'sequential'-type model, *L. pneumophila* could upregulate phagocytosis by altering aspects of cell physiology related to phagocytosis (pathway B). This might occur by increasing the rate of membrane recycling or stimulating the turnover of actin filaments. In this case, although the quantity of phagosome formation is altered, the quality of wild-type and mutant phagosomes is the same. The effector(s) would then modify the newly formed phagosome. Although the concerted model makes an attractive functional connection between phagosome formation and phagosome fate, we cannot distinguish these models at the present time.

Many of the experiments reported here were carried out by infecting host cells with MOIs of up to 100 bacteria per cell. The fact that the differences in uptake between wild-type and mutant *Legionella* were more apparent at the higher MOI raised several interesting issues. First, do the high MOIs used kill the host cells by the immediate cytotoxicity phenomenon (Husmann and Johnson, 1994; Kirby *et al*., 1998)? Our results indicate that, not only are the cells alive enough to carry out phagocytosis, but also the permeability barrier of the infected cells seems to be intact, as the anti-*Legionella* antibodies used to distinguish intracellular from extracellular bacteria are excluded from the cells (Figs 2A and 3A). Secondly, is the high MOI infection 'physiologically relevant'? There are several possible answers because the exact conditions of infection of protozoa in the wild and alveolar macrophages in human lungs are not known. After a successful infection, large numbers of newly replicated bacteria are released from lysed host cells. If new host cells are in the same vicinity, a high MOI would indeed occur. In the case of Legionnaires' disease, whether or not the bacteria alone or bacteria within infected protozoa (e.g. *Hartmanella vermiformis*) are the infectious agent has been debated

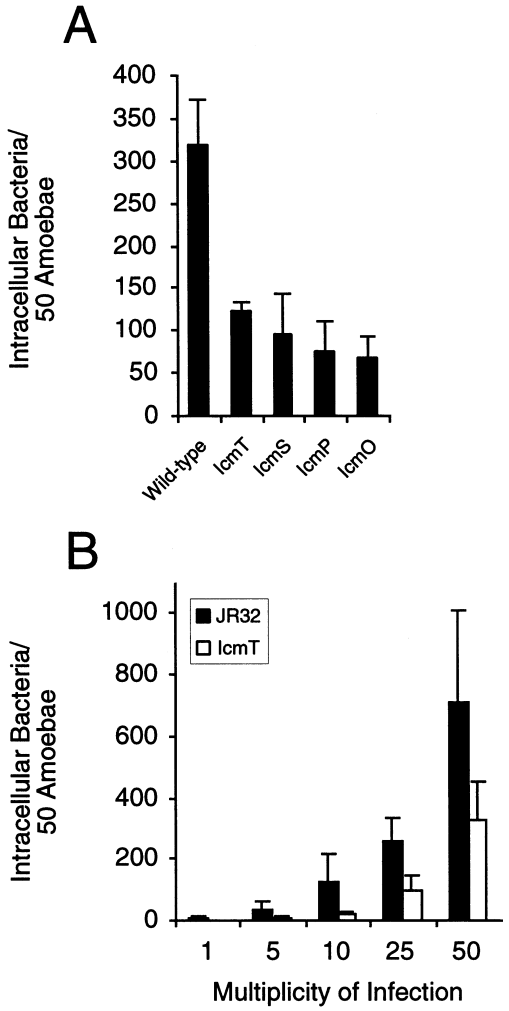

Fig. 6. *L. pneumophila* promotes internalization by *A. castellanii*. *A. castellanii* was infected for 20 min at an MOI of 25 (A) or the MOIs indicated (B) with GFP-expressing wild-type (JR32) or *icmT* (GS3011), *icmS* (GS3001), *icmP* (GS3002) or *icmO* (GS3003) mutant strains. Extracellular bacteria were labelled with a rhodamine-conjugated antibody, and intracellular bacteria were counted within 50 randomly selected amoebae. The *icm* mutant strains are defective for efficient phagocytosis by *A. castellanii*. Mean and standard deviation of three independent experiments are shown.

(Brieland *et al*., 1997; reviewed by Harb *et al*., 2000). Infected protozoa harbour many hundreds of organisms and could release them inside alveoli. During experimental aerosol infection of animals, suspensions of 10^8-10^9 organisms ml^{-1} are used to nebulize the air in a closed compartment (Marra *et al*., 1992), and the exact MOI with respect to alveolar macrophages in the animal lungs is difficult to quantify. Finally, are the experiments described here comparable with the cell biology experiments performed by others? Several studies in which phagocytosis of *Legionella* or the properties of the *Legionella*-containing phagosome were characterized by microscopy used MOIs ranging from 20 to 100 or even higher (Horwitz, 1983a; 1984; Payne and Horwitz, 1987; Clemens and Horwitz, 1992; Cirillo *et al*., 1994; Swanson and Isberg, 1995; Brieland *et al*., 1997; Venkataraman *et al*., 1997; Coers *et al*., 1999). Therefore, those observations should be directly comparable with the data presented here.

Our results presented here represent the first report of a bacterial secretion system (Icm/Dot) promoting phagocytosis by professional phagocytes. In contrast, inhibition of phagocytosis by pathogenic bacteria using a type III secretion system is a well-known phenomenon. Pathogens avoiding phagocytosis by this means include *Yersinia* spp. (Lian *et al*., 1987), enteropathogenic *Escherichia coli* (Goosney *et al*., 1999) and *Pseudomonas aeruginosa* (reviewed by Ernst, 2000). Only recently, *Helicobacter pylori* has been reported to resist phagocytosis by macrophages and polymorphonuclear neutrophils using a type IV secretion system (Allen *et al*., 2000; Ramarao *et al*., 2000). Interestingly, within 2 h after internalization, virulent *H. pylori* type I (harbouring the *cag* pathogenicity island), but not type II strains, induced the formation of homotypic fusions of phagosomes, yielding 'megasomes' with multiple viable bacteria (Allen *et al*., 2000). The *H. pylori* effector(s) affect phagocytosis *in trans* as, after preinfection of macrophages, the pathogen prevented the internalization of latex beads as well as opsonin-independent phagocytosis of *Neisseria gonorrhoeae* (Ramarao *et al*., 2000). Similarly, extracellular wild-type *L. pneumophila* triggered the uptake of *icmT* mutant bacteria in a co-infection experiment *in trans* (Fig. 5B). However, once internalized, the *L. pneumophila* effector(s) act only *in cis* (within a discrete phagosome) rather than perturbing endocytosis in general (Coers *et al*., 1999). The different effects of *L. pneumophila* and *H. pylori* on phagocytosis represents yet another example of the notion that the effectors of conserved secretion systems may perform opposite functions and are tailored for the unique needs of specific pathogens. Other plant and animal pathogens harbouring type IV secretion systems include *A. tumefaciens*, *Bordetella pertussis*, *Rickettsia prowazekii* and *Brucella* spp. (Christie and Vogel, 2000). However, for *L. pneumophila* and most other bacteria, the effector(s) exerting virulence functions have not yet been identified.

Experimental procedures

Reagents

Bacto agar, proteose peptone and yeast extract were purchased from Difco Laboratories. Phosphate-buffered saline (PBS) was from BioWhittaker. RPMI-1640 medium (RPMI), L-glutamine (Gln) and Dulbecco's phosphate-buffered salt solution (DPBS) were from Mediatech. Fetal calf serum (FCS) was purchased from Sigma-Aldrich, and normal human AB serum (NHS) was from Gemini Bio-Products. Ficoll-Paque Plus and Percoll were obtained from Amersham Pharmacia.

The rabbit anti-*L. pneumophila* Philadelphia 1 rhodamine-conjugated antibody was purchased from m-Tech, and the mounting media Citifluor no. 1 was from Ted Pella. All other reagents were from Sigma-Aldrich.

Bacteria and plasmids

The bacterial strains used in this study are listed in Table 1. *L. pneumophila* was grown on CYE agar plates (Feeley *et al*., 1979) or AYE broth (Horwitz and Silverstein, 1983). Antibiotics were used at the following concentrations: chloramphenicol (5 μg ml^{-1}); gentamicin (10 μg ml^{-1}); and kanamycin (50 μg ml^{-1}).

Strain GS3012 was constructed by replacing the *icmR* gene with a kanamycin resistance cassette by allelic exchange with plasmid pGS-Le-38-Km-1 as described before (Segal and Shuman, 1997). Plasmid pGS-Lp-32 (Segal and Shuman, 1997) was digested with *Eco*RV, and a 1721 bp fragment (236–1957 in the sequence) containing the *icmTSR* genes was cloned into pUC-18 digested with *Hin*cII to generate pGS-Lp-38. Subsequently, the kanamycin resistance cassette was cloned into the *Dra*III and *Sac*I sites (located in the *icmR* gene) of pGS-Lp-38 to generate pGS-Lp-38-Km-1. This plasmid was digested with *Pvu*II, and the fragment containing the disrupted *icmR* gene was cloned into the *Eco*RV site of the allelic exchange vector pLAW-344 (Wiater *et al*., 1994) to generate pGS-Le-38-Km-1.

Strain LM1445 was constructed by replacing the *icmW* gene with a gentamicin resistance cassette by allelic exchange with plasmid pLM804 as described above for strain GS3012. The *icmW* gene including 0.2 kb upstream and 0.45 kb downstream sequence (1117 bp) was amplified by polymerase chain reaction (PCR) using the primers 5′-TCTAGAGCCATTCACCAAATACAAGG-3′ (*Xba*I site) and 5′-GCGGCCGCTTGATTGATCTATTAAGCC-3′ (*Not*I site) and blunt end ligated into the plasmid pZErO-2.1, yielding plasmid pLM785. Subsequently, the gentamicin resistance cassette was cut with *Hin*cII and cloned into the *Sma*I site of the *icmW* gene, yielding plasmid pLM788. The fragment containing the disrupted *icmW* gene was released from pLM788 by digestion with *Xba*I and *Not*I, filled in with Klenow polymerase and cloned into the *Eco*RV site of the allelic exchange vector pLAW-344 to generate pLM804.

Plasmids were introduced into the *L. pneumophila* strains by electroporation or conjugation as described previously (Segal and Shuman, 1998b). The plasmids used in this study are listed in Table 2. Plasmid pGS-GFP-04 was constructed by digesting plasmid pGFP-mut-2 (Cormack *et al*., 1996) with *Eco*RI and *Hin*dIII and cloning the released fragment into the same sites of pMMB207. In this vector, the *gfp* gene is under the control of the P*tac* promoter. Plasmid pGS-BCD-05 was constructed by screening the *L. pneumophila* pLAFR1 library (Szeto and Shuman, 1990) using a fragment from the plasmid pAB-11 as a probe. Plasmid pAB-11 harbours the group V *Eco*RI fragment from strain LELA2883 that contains an insertion in the *dotB* gene (Sadosky *et al*., 1993). Several positive

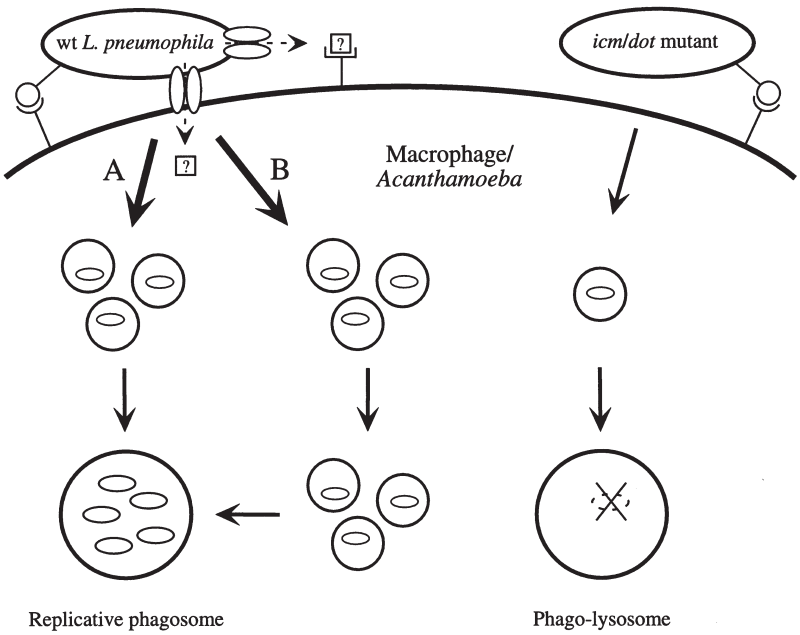

Fig. 7. Model of *L. pneumophila*-triggered efficient phagocytosis. Wild-type and *icm*/*dot* mutant bacteria adhere to macrophages by binding to and competing for the same receptor(s), which include complement receptor (CR)1 and CR3. Upon binding, wild-type *L. pneumophila* upregulates phagocytosis by secreting unknown effectors via the Icm/Dot transport system. The effectors might either be injected directly into the host cell or bind extracellularly to host cell receptors. In a 'concerted'-type model, upregulation of phagocytosis by wild-type *L. pneumophila* leads directly to a specialized type of phagosome that does not fuse with lysosomes and, ultimately, yields a replication-permissive phagosome (pathway A). Alternatively, in a 'sequential'-type model, increased phagocytosis would initially lead to a non-specialized phagosome that is subsequently altered by wild *L. pneumophila* to prevent fusion with lysosomes (pathway B). In the absence of secreted effectors, normal phagocytosis results in the formation of a fusogenic phagosome eventually leading to acidic phagolysosomes, in which *icm*/*dot* mutants are degraded. *L. pneumophila* also promotes phagocytosis by *A. castellanii*. Therefore, the mechanism of efficient phagocytosis seems to be similar for macrophages and protozoan hosts.

Table 1. *L. pneumophila* strains.

Strain	Genotype and features	Reference
GS28G	JR32 *lvh*::Gm	Segal *et al*. (1999)
GS3001	JR32 *icmS3001*::Km	Segal and Shuman (1997)
GS3002	JR32 *icmP3002*::Km	Segal and Shuman (1997)
GS3003	JR32 *icmO3003*::Km	Segal and Shuman (1997)
GS3007	JR32 *icmN3007*::Km	Segal *et al*. (1998)
GS3008	JR32 *icmM3008*::Km	Segal *et al*. (1998)
GS3009	JR32 *icmL3009*::Km	Segal *et al*. (1998)
GS3010	JR32 *icmK3010*::Km	Segal *et al*. (1998)
GS3011	JR32 *icmT3011*::Km	Segal and Shuman (1998b)
GS3012	JR32 *icmR3012*::Km	This study
JR32	Salt-sensitive isolate of AM511	Sadosky *et al*. (1993)
LELA921	JR32 *icmX921*::Tn*903*dII*lacZ*	Sadosky *et al*. (1993)
LELA1205	JR32 *icmD1205*::Tn*903*dII*lacZ*	Sadosky *et al*. (1993)
LELA1275	JR32 *icmF1275*::Tn*903*dII*lacZ*	Sadosky *et al*. (1993)
LELA1747	JR32 *icmV1747*::Tn*903*dII*lacZ*	Sadosky *et al*. (1993)
LELA2883	JR32 *dotB2883*::Tn*903*dII*lacZ*	Sadosky *et al*. (1993)
LELA2883-28	LELA2883 *lvh*::Gm	Segal *et al*. (1999)
LELA3118	JR32 *dotA3118*::Tn*903*dII*lacZ*	Sadosky *et al*. (1993)
LELA3463	JR32 *icmQ3463*::Tn*903*dII*lacZ*	Sadosky *et al*. (1993)
LELA3473	JR32 *icmR3473*::Tn*903*dII*lacZ*	Sadosky *et al*. (1993)
LELA3896	JR32 *icmB3896*::Tn*903*dII*lacZ*	Sadosky *et al*. (1993)
LELA4086	JR32 *icmT4086*::Tn*903*dII*lacZ*	Sadosky *et al*. (1993)
LELA4432	JR32 *icmE4432*::Tn*903*dII*lacZ*	Sadosky *et al*. (1993)
LELA4432-28	LELA4432 *lvh*::Gm	Segal *et al*. (1999)
LM1445	JR32 *icmW*::Gm	This study
MW627	JR32 *tphA*::Km	Purcell and Shuman (1998)
MW635	JR32 *icmG*::Km	Purcell and Shuman (1998)
MW645	JR32 *icmC*::Km	Purcell and Shuman (1998)
MW656	JR32 *icmJ*::Km	Purcell and Shuman (1998)

cosmids were identified. One of them (pGS-cos-4) was digested with *Bam*HI, and a 12 kb fragment containing the *dotBCD* genes was subcloned into pUC-18 to generate pGS-BCD-01. The insert from this plasmid was cloned using *Bam*HI sites into pMMB207αb-Km-14 to generate pGS-BCD-05.

Cell culture of macrophages and A. castellanii

The human leukaemia cell line HL-60 (ATCC CCL-240), murine J774A.1 macrophages (ATCC TIB-67) and human monocyte-derived macrophages (HMMΦ) were grown in a humidified atmosphere of 5% CO_2 at 37°C in RPMI supplemented with 2 mM Gln and 10% (v/v) heat-inactivated (56°C, 30 min) FCS (HL-60, J774A.1) or 10% (v/v) heat-inactivated NHS (HMMΦ) respectively.

Peripheral blood monocytes (PBMs) were isolated from buffy coats obtained through the New York Blood Center. The PBMs were isolated by Ficoll-Paque Plus and Percoll gradient centrifugations as described previously (Colotta *et al*., 1984; Hilbi *et al*., 1997). Briefly, 38 ml of buffy coat diluted 1:5 with PBS was placed on 11.4 ml of Ficoll-Paque Plus and centrifuged at 900 *g* for 30 min at room temperature. To remove platelets, 5 ml of washed leucocytes was placed on 15 ml of heat-inactivated FCS and centrifuged at 200 *g* for 15 min. The leucocytes were resuspended in 14 ml of RPMI containing 2 mM Gln and 10% (v/v) heat-inactivated FCS, placed on the same volume of 46% Percoll and centrifuged at 600 *g* for 30 min. Washed PBMs were resuspended at a density of 10^6 cells ml^{-1} in RPMI containing 2 mM Gln and 10% (v/v) heat-inactivated NHS and matured in Teflon beakers for at least 5 days to yield HMMΦ. In some experiments, the PBMs were matured directly on sterile coverslips in 24-well plates.

Acanthamoeba castellanii (ATCC 30234) was grown in 30 ml of proteose peptone–yeast extract–glucose medium (PYG) in a 75 cm^2 tissue culture flask at 28°C (Moffat and Tompkins, 1992). The amoebae were split once a week, fed with PYG 24 h before use and prepared for infections as described previously (Segal and Shuman, 1999a). Briefly, non-adherent amoebae were removed by gently shaking the flask and replacing the medium with fresh PYG. Adherent amoebae were detached by tapping the flask vigorously. The resulting suspension was centrifuged for 10 min at 220 *g*, resuspended at a concentration of 4×10^5 amoebae ml^{-1} and used to plate on sterile coverslips (see below) or to inoculate 30 ml of fresh PYG (50–100 μl 30 ml^{-1}).

Gentamicin protection assay

HL-60 cells (1×10^5 per well, 96-well plate) were differentiated into macrophages by incubation for 48 h with 100 ng ml^{-1} phorbol 12-myristate 13-acetate (PMA) in RPMI supplemented with 2 mM Gln and 10% (v/v) fresh NHS (RGN medium). Before the infection, the HL-60 cells were placed for 1 h in RGN medium containing cytochalasin D (10 μM) where indicated. The bacterial strains used for the infections were grown on CYE agar plates for 2–3 days and harboured the plasmid pMMB207 (*icmD*, *-J*, *-B*), pMMB207αb (*icmR*, *-Q*, *-M*, *-L*, *-K*, *-E*, *-X*, *dotA*), pMMB207αb-Km-14 (*icmT*, *-S*, *-P*, *-O*, *-N*, *-G*, *-C*, *-F*, *tphA*, *dotB*) or the corresponding complementing plasmids (Table 2). Bacteria suspended in RGN medium (OD_{600} of $0.3 = 5 \times 10^8$

Table 2. Plasmids used in this study.

Plasmid	Features	Reference
pGS-BCD-05	*dotBCD* in pMMB207αb-Km-14	This study
pGS-GFP-04	*gfp* in pMMB207	This study
pGS-Lc-34-14	*icmPO* in pMMB207αb-Km-14	Segal and Shuman (1998b)
pGS-Lc-35	*icmQ* in pMMB207αb	Segal and Shuman (1997)
pGS-Lc-36b	*icmR* in pMMB207αb	Segal and Shuman (1997)
pGS-Lc-37-14	*icmTS* in pMMB207αb-Km-14	Segal and Shuman (1998b)
pGS-Lc-47[a]	*icmNMLKEG* in pMMB207αb	Segal *et al.* (1998)
pGS-Lc-47-D3	*icmNMKEG* in pMMB207αb	Segal *et al.* (1998)
pGS-Lc-47-D4	*icmNMLEG* in pMMB207αb	Segal *et al.* (1998)
pMMB207	RSF1010 derivative, *IncQ*, *oriT*, *lacI*^{q}, P*tac*, Cm^r	Morales *et al.* (1991)
pMMB207αb	pMMB207, *lacZ*α, MCS	Segal and Shuman (1997)
pMMB207αb-Km-14	pMMB207αb with *mobA*::Km	Segal and Shuman (1998b)
pMW100[b]	*icmGCDJB-tphA-icmF* in pMMB207	Purcell and Shuman (1998)
pUC-18	*oriR*(colE1), MCS, Ap^r	Yanisch-Perron *et al.* (1985)
pZErO-2.1	*oriR*(colE1), *lacZ*α, *ccdB*, P*lac*, MCS, Km^r	Invitrogen

a. Used for complementation of the *icmM* mutant strain GS3008.
b. Used for complementation of the *icmD* mutant strain LELA 1205.

bacteria ml^{-1}) were centrifuged onto the HL-60 cells (700 *g*, 10 min) at an MOI of 100, and the infected cells were incubated for another 10 min at 37°C. To kill extracellular bacteria, the infected macrophages were washed twice with PBS containing 0.1 mg ml^{-1} gentamicin and incubated for another 40 min in RGN containing 0.1 mg ml^{-1} gentamicin. At 60 min after infection, the infected macrophages were lysed with 0.2 ml H_2O for 10 min, and aliquots of the lysates were plated on CYE agar plates. Intracellular viable bacteria were counted as cfu. As an input control, dilutions of the bacterial suspensions in RGN medium were also plated.

To compare the sensitivity towards gentamicin of *L. pneumophila* wild-type strain JR32 and the *icmT* mutant strain GS3011, 1×10^5 agar-grown bacteria per 0.2 ml of RGN were incubated with 0.025 or 0.1 mg ml^{-1} gentamicin for 1 h, and cfu were determined as described above.

Immunofluorescence staining and fluorescence microscopy

For immunofluorescence, the phagocytes were seeded on sterile coverslips in 24-well plates at the following densities: HL-60 cells (1.5×10^6 per well, differentiated for 48 h with PMA), HMMΦ (0.25×10^6 per well, overnight) or *A. castellanii* (0.25×10^6 per well, overnight). Before infection, the phagocytes were washed two or three times with RPMI containing 2 mM Gln (HL-60, HMMΦ) or Ac buffer (*A. castellanii*) (Moffat and Tompkins, 1992), respectively, and placed in 0.5 ml of RGN (HL-60, HMMΦ) or Ac buffer (*A. castellanii*), containing cytochalasin D (10 μM, 1 h) where indicated.

The *L. pneumophila* strains used for the infections were grown to stationary phase either on CYE plates for 2–3 days (HL-60 cells) or in AYE broth for about 24 h (HMMΦ, *A. castellanii*). Before infection, the bacteria were suspended or diluted, respectively, in RGN (HL-60, HMMΦ) or Ac buffer (*A. castellanii*), and the phagocytes were infected with bacteria at the MOIs indicated. In all experiments, the bacteria were centrifuged onto the phagocytes (700 *g*, 10 min) to synchronize infection and incubated for another 10 min at 37°C (macrophages) or 28°C (*A. castellanii*). Infected macrophages were washed five times with DPBS and fixed for 15 min with DPBS containing 3.7% formaldehyde. Infected *A. castellanii* were washed five times with 20% DPBS (Kaiser *et al.*, 1999) and fixed for 15 min with 20% DPBS containing 3.7% formaldehyde. The fixed cells were washed five times, blocked for 30 min with 5% non-fat milk in DPBS or 20% DPBS (blocking buffer), respectively, and incubated for 1 h with a rhodamine-conjugated rabbit anti-*L. pneumophila* Philadelphia 1 antibody, diluted 1:100 in blocking buffer. After washing five times, the coverslips were mounted onto microscopy slides using the mounting media Citifluor no. 1.

The samples were viewed with an inverted fluorescence microscope (Nikon Eclipse TE200) using a 100× oil immersion phase-contrast objective (numerical aperture 1.4). The microscope was equipped with fluorescein isothiocyanate and Texas red filter sets, a digital camera and a computer-controlled Z-axis drive. METAMORPH software (Universal Imaging) was used to acquire and process digital images. Z-sections were acquired every 0.5 μm (spanning a total of 20 μm), merged into one layer by optimizing for fluorescence, and either the green and red fluorescence images or both fluorescence images and a brightfield picture were overlain. Thus, intracellular (green) and extracellular (yellow = superimposition of green and red) bacteria could easily be distinguished. Bacterial internalization or adhesion was scored by randomly selecting 50–100 phagocytes.

Statistical analysis

Statistical analysis was performed by two-tailed Student's *t*-test for paired samples, taking $P < 0.05$ as significant.

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