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A mycobacterial virulence gene cluster extending RD1 is required for cytolysis, bacterial spreading and ESAT-6 secretion

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Summary

Initiation and maintenance of infection by mycobacteria in susceptible hosts are not well understood. A screen of *Mycobacterium marinum* transposon mutant library led to isolation of eight mutants that failed to cause haemolysis, all of which had transposon insertions in genes homologous to a region between *Rv3866* and *Rv3881c* in *Mycobacterium tuberculosis*, which encompasses RD1 (*Rv3871–Rv3879c*), a known virulence gene cluster. The *M. marinum* mutants showed decreased virulence *in vivo* and failed to secrete ESAT-6, like *M. tuberculosis* RD1 mutants. *M. marinum* mutants in genes homologous to *Rv3866-Rv3868* also failed to accumulate intracellular ESAT-6, suggesting a possible role for those genes in synthesis or stability of the protein. These transposon mutants and an *ESAT-6*/*CFP-10* deletion mutant all showed reduced cytolysis and cytotoxicity to macrophages and significantly decreased intracellular growth at late stages of the infection only when the cells were infected at low multiplicity of infection, suggesting a defect in spreading. Direct evidence for cell-to-cell spread by wild-type *M. marinum* was obtained by microscopic detection in macrophage and epithelial monolayers, but the mutants all were defective in this assay. Expression of *M. tuberculosis* homologues complemented the corresponding *M. marinum* mutants, emphasizing the functional similarities between *M. tuberculosis* and *M. marinum* genes in this region that we designate extRD1 (extended RD1). We suggest that diminished membranolytic activity and defective spreading is a mechanism for the attenuation of the extRD1 mutants. These results extend recent findings on the genomic boundaries and functions of *M. tuberculosis* RD1 and establish a molecular cellular basis for the role that extRD1 plays in mycobacterial virulence. Disruption of the *M. marinum* homologue of *Rv3881c*, not previously implicated in virulence, led to a much more attenuated phenotype in macrophages and *in vivo*, suggesting that this gene plays additional roles in *M. marinum* survival in the host.

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Introduction

Despite more than a century of research on prevention and treatment, tuberculosis remains one of the world's most significant infectious diseases, with more than 2 million deaths each year (Dolin *et al*., 1994). The availability of the complete genome sequence of *Mycobacterium tuberculosis* (Cole *et al*., 1998) and of methods for forward genetics (Bardarov *et al*., 1997; Pelicic *et al*., 1997; Camacho *et al*., 1999; Cox *et al*., 1999; Rubin *et al*., 1999) has led to important advances in understanding the pathogenesis of tuberculosis. Comparative genomics of *M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium leprae*, the vaccine strain *M. bovis BCG* (BCG) and *Mycobacterium microti* has revealed several chromosomal regions that are absent from the genomes of the less virulent organisms (Mahairas *et al*., 1996; Behr *et al*., 1999; Gordon *et al*., 1999; Brodin *et al*., 2002). This analysis has focused particular attention on one region, termed region of difference 1 (RD1), which encompasses nine genes (from *Rv3871–Rv3879c*), because it is missing from the less virulent BCG (Mahairas *et al*., 1996; Behr *et al*., 1999; Gordon *et al*., 1999) (see Fig. 1D). In addition, an overlapping deletion has been found in *M. microti* that involves 13 ORFs (*Rv3864–Rv3876*) when compared to the *M. tuberculosis* genome (Brodin *et al*., 2002) (see Fig. 1D). The overlap between RD1 and the *M. microti* deletion includes *CFP-10* (*Rv3874*) and *ESAT-6* (*Rv3875*), whose secreted protein products are prominent T and B cell antigens in patients infected with *M.*

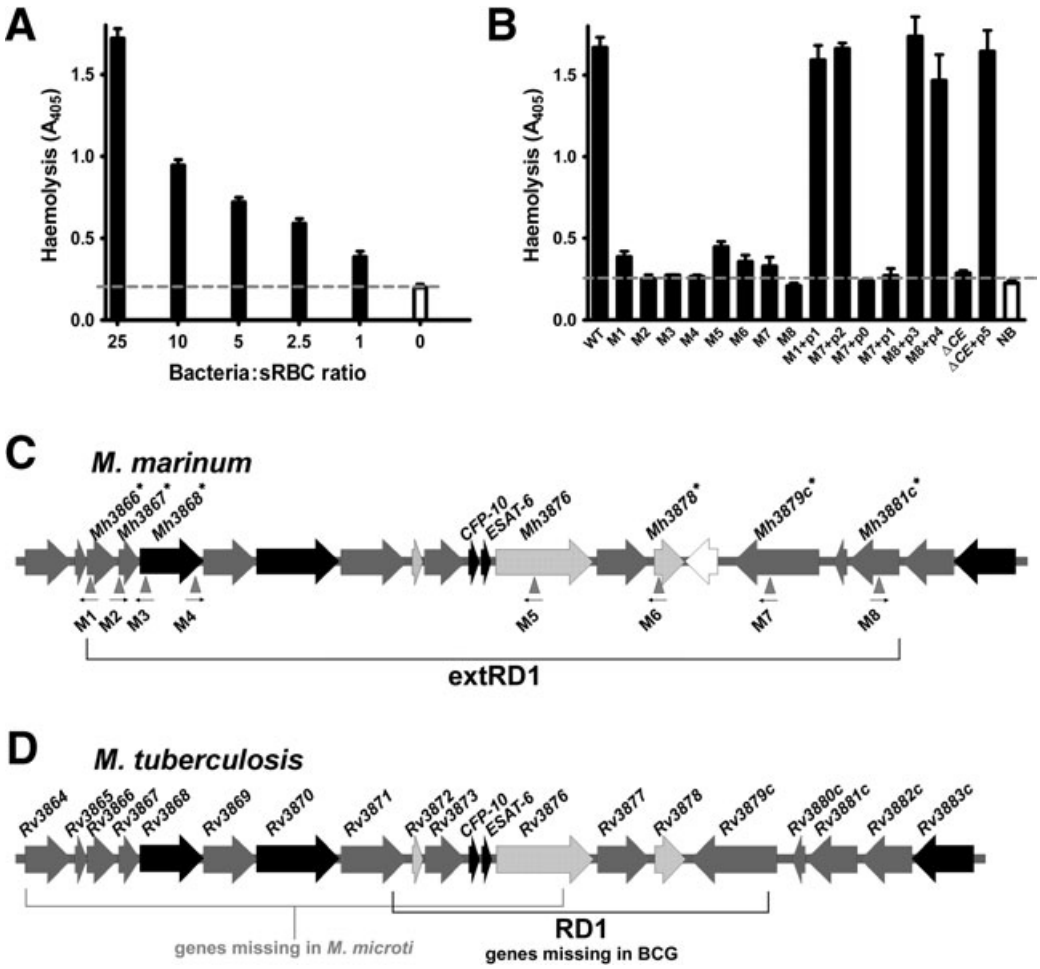

Fig. 1. Identification and sequence analysis of *M. marinum* genes involved in haemolysis of sRBC. Haemolysis was determined by measuring the release of haemoglobin at a wavelength of 405 nm (A_{405}) for *M. marinum* WT at varying bacterium:sRBC ratios (A), or for WT, the extRD1 mutants (M1–M8), and the complemented strains at a bacterium:sRBC ratio 25:1 (B). NB, no bacteria; grey dotted line, background of measurement; p0 indicates the empty complementation vector pLYG206.*Zeo*; p1, p2, p3, p4 and p5 are pLYG206.*Zeo* expressing *Rv3866*, *Rv3879c*, *Mh3880c-Mh3881c*, *Rv3881c* and *M. tuberculosis CFP-10/ESAT-6* genes respectively; Δ*CE*, *M. marinum* mutant with deletion of both *CFP-10* and *ESAT-6* genes. Error bars indicate standard deviation of data from three experiments with each performed in duplicate. The extRD1 regions in *M. marinum* and *M. tuberculosis* are diagrammed in C and D respectively. Filled areas represent extRD1 genes with directions of transcription, and filling colours indicate sequence similarities: black, >90%; dark grey, 70–89%; light grey, 55–69%; and open, <54%. RD1 indicates *M. tuberculosis* genes deleted in BCG. *Mh* denotes *M. marinum* genes corresponding to *M. tuberculosis* homologues. The extRD1 region covering *Mh3866–Mh3881c* is indicated in C, in which *Mh* designations are shown only for *M. marinum* genes mutated in this study, and the new genes that are described in this study are indicated by asters (*). Triangles indicate sites of transposon insertions for M1–M8, and the black arrows beneath show directions for transcription of *kan*^{r} in the transposon.

tuberculosis. An important role for RD1 in mycobacterial pathogenesis has been demonstrated experimentally in several ways. Incorporation of *M. tuberculosis* genes extending through the *M. microti* and BCG deletions into the BCG or *M. microti* chromosome enhances virulence of these organisms (Pym *et al*., 2002); conversely, a deletion of RD1 genes from *M. tuberculosis* greatly decreases its capacity to cause disease (Lewis *et al*., 2003). Recently, by using signature-tagged mutagenesis (Stanley *et al*., 2003) and transposon site hybridization (Sassetti and Rubin, 2003) screens and targeted gene deletions (Hsu *et al*., 2003; Guinn *et al*., 2004), several individual RD1 genes of *M. tuberculosis* have been shown to be essential for virulence in mice. One major clue about the role of RD1 genes has come from recent discoveries that demonstrate these genes may constitute an alternative secretion system required for ESAT-6 and CFP-10 secretion (Hsu *et al*., 2003; Pym *et al*., 2003; Stanley *et al*., 2003; Guinn *et al*., 2004). ESAT-6 itself enhances permeability of artificial membranes, suggesting a possible direct role in cytolysis/cytotoxicity (Hsu *et al*., 2003). These data have led to the hypothesis that ESAT-6 and CFP-10, which can form a 1:1 complex (Renshaw *et al*., 2002), may be effectors of the enhanced pathogenicity of

the mycobacteria that contain RD1. However, how exactly ESAT-6 and CFP-10 potentiate mycobacterial virulence at the molecular and cellular level is not fully understood. Moreover, the functions of *M. tuberculosis* genes missing in *M. microti* that do not overlap with RD1 (Fig. 1D) are not understood. As a result, the virulence function of the genetic region containing RD1 remains an area of intense investigation.

Mycobacterium marinum causes a systemic tuberculosis-like disease in fish and frogs, its natural hosts, and a localized disease in immunocompetent humans. Both the human and animal infections are marked by the presence of a granulomatous host response, a hallmark of diseases caused by *M. tuberculosis* (Ramakrishnan *et al*., 1997; Talaat *et al*., 1998; Prouty *et al*., 2003). *M. marinum* is genetically closely related to *M. tuberculosis* (Tonjum *et al*., 1998) and has been used increasingly as a model for understanding the pathogenesis of tuberculosis (Ramakrishnan *et al*., 1997; 2000; Talaat *et al*., 1998; Davis *et al*., 2002; Gao *et al*., 2003a,b; Prouty *et al*., 2003). Like *M. tuberculosis*, *M. marinum* grows and persists in host macrophages *in vivo* and *in vitro*. Infection by either leads to lysis of host cells, suggesting the existence of a membranolytic activity in both species that may be important in dissemination of infection (Ramakrishnan and Falkow, 1994; Valdivia *et al*., 1996; Bouley *et al*., 2001; Gao *et al*., 2003a). Although *M. tuberculosis* has been shown convincingly to reside exclusively in macrophage phagosome (Russell, 2001; Clemens *et al*., 2002), *M. marinum* can escape from the phagosome into the cytosol and polymerize actin (Stamm *et al*., 2003), suggesting another potential role for membranolytic activity in this organism. In the present study we have used transposon mutagenesis (Gao *et al*., 2003a) to understand the haemolytic activity that is characteristic of pathogenic mycobacteria including *M. tuberculosis* and *M. marinum* and that has been associated with virulence (Rudnicka *et al*., 1999). Haemolysis may also be related to mycobacteria-mediated cytolysis of nucleated cells, which also has been suggested to have a role in pathogenesis of tuberculosis (Maslow *et al*., 1999). By screening an *M. marinum* transposon mutant library, we identified eight mutants with abolished haemolytic activity and found that all of the mutants had transposon insertions in *M. marinum* genes homologous to *M. tuberculosis* RD1 and surrounding genes, a gene cluster that we refer to as extended RD1 (extRD1). These mutants and others we created by homologous recombination demonstrate that haemolytic, cytolytic and cytotoxic activities of *M. marinum* are tightly linked, require multiple individual genes of extRD1 and correlate with secretion of ESAT-6. Like BCG, *M. microti* and *M. tuberculosis* strains missing genes in RD1, *M. marinum* mutants with mutations in individual extRD1 genes had significantly decreased virulence *in vivo*. Furthermore, these mutants showed diminished spreading in macrophage and epithelial cell monolayers and markedly reduced dissemination of infection *in vivo*. Because bacterial spreading is a characteristic linked to pathogenicity for several intracellular pathogens including *Listeria* and *Shigella* (Goldberg, 2001), we propose that this defect may account for the decreased virulence of the *M. marinum* extRD1 mutants. Thus, this work demonstrates that genes in the extRD1 region are required for mycobacterial expression of a membranolytic activity necessary for efficient bacterial spreading among host cells. These results extend the recent knowledge of the genomic boundaries and functions of RD1 and establish a cellular basis for the role that extRD1 plays in mycobacterial virulence.

Results

A screen for M. marinum *haemolysis mutants identifies RD1 and surrounding genes required for haemolysis*

We screened mutants from an *M. marinum* transposon mutant library for diminished contact-dependent haemolysis of sheep red blood cells (sRBC). Wild type (WT) *M. marinum* was haemolytic to sRBC, which could be detected after 1 h coincubation of the bacteria with sRBC at a ratio as low as 1:1, and haemolysis was augmented at increasing ratios of bacteria to sRBC (Fig. 1A). Human erythrocytes also were lysed efficiently by *M. marinum* (data not shown). We identified eight mutants (M1–M8) that reproducibly showed markedly reduced haemolytic activity, four of which (M2, M3, M4 and M8) were completely non-haemolytic (Fig. 1B). We sequenced chromosomal DNA flanking individual transposon insertions in those mutants and found that all of the insertions were clustered in a genomic region containing ORFs homologous to *M. tuberculosis* RD1 genes (Fig. 1C and D). Alignment of an *M. marinum* assembled contig sequence (mar478c10.p1k, http://www.sanger.ac.uk/Projects/M_marinum/) to this region of the *M. tuberculosis* chromosome revealed that both the ORF sequences and their genomic organization are highly conserved between the two mycobacterial species (Fig. 1C and D). For this reason, we have used the prefix *Mh* to denote the *M. marinum* homologue of each *M. tuberculosis* gene, based on the sequence of H37Rv (Cole *et al*., 1998). Four of the transposon insertions were located in three individual *M. marinum* ORFs (*Mh3866–Mh3868*) in a region homologous to *M. tuberculosis* genes missing in *M. microti*; another three insertions were in *M. marinum* ORFs (*Mh3876*, *Mh3878* and *Mh3879c*) homologous to *M. tuberculosis* RD1. One insertion was in a nearby ORF (*Mh3881c*) homologous to *Rv3881c*. The insertions in *Mh3866*, *Mh3867*, *Mh3868* and *Mh3881c* were all beyond

the so-far-defined boundaries of *M. tuberculosis* RD1 but produced similar abolition of the haemolytic phenotype. These results indicate that production of haemolytic activity requires multiple genes on the mycobacterial chromosome that include RD1 and surrounding genes, and we refer to this entire gene cluster (*Mh3866–Mh3881c*) as the extended RD1 (extRD1, Fig. 1C and D). BCG, which lacks RD1, lacks haemolytic activity (King *et al*., 1993), suggesting that RD1 is important in haemolysis by *M. bovis* and probably other mycobacteria as well.

M. marinum *extRD1 mutants are complemented by expression of homologous genes from* M. tuberculosis

We expressed *M. tuberculosis* homologues in four of the *M. marinum* extRD1 mutants to determine whether these could complement the mutants' defect in haemolysis. As shown in Fig. 1B, expression of *Rv3866*, *Rv3879c* and *Rv3881c* genes, respectively, in M1 (*Mh3866::Tn*), M7 (*Mh3879c::Tn*) and M8 (*Mh3881c::Tn*) resulted in a complete recovery of haemolysis, and there was no significant difference in complementation of M8 by *Rv3881c* compared to the *Mh3880c–Mh3881c* operon from *M. marinum*. Complementation was specific to expression of the appropriate *M. tuberculosis* gene, for neither an empty plasmid nor a plasmid expressing a non-corresponding gene (*Rv3866*) restored haemolysis by *Mh3879c::Tn* (Fig. 1B). Therefore, extRD1 genes are conserved between *M. marinum* and *M. tuberculosis* not only for structure but also for function, further evidence for involvement of this region in the known haemolytic activity of other pathogenic mycobacteria (King *et al*., 1993).

extRD1 genes are required for rapid cytolysis to macrophages

To explore potential biological functions for the haemolytic activity during mycobacterial infection, we first examined whether haemolytic activity of *M. marinum* was related to cytolysis to macrophages. J774 murine macrophage-like cells were incubated with *M. marinum* strains, and cytolysis was detected by permeabilization of the plasma membrane to ethidium homodimer-1. As shown in Fig. 2A and E, WT *M. marinum* at a bacterium:cell ratio of 50:1 produced massive cytolysis to J774 cells within 1 h of incubation. Cytolysis to J774 cells was detected at a bacterium:cell ratio as low as 3 and augmented at increasing ratios. In contrast, none of the extRD1 mutants exhibited significant cytolysis to J774 cells (Fig. 2B, C and E), even at a bacterium:cell ratio up to 200. Cytolysis by M1, M7 and M8 was fully complemented by expression of *Rv3866*, *Rv3879c* and *Rv3881c* (or *Mh3880c–Mh3881c*) genes respectively (Fig. 2C–E). Thus, the extRD1 genes required for haemolysis are also necessary for rapid cytolysis of macrophages, suggesting a tight genetic linkage between the two effects.

extRD1 genes are required for cytotoxicity to macrophages and epithelial cells following intracellular infection

To determine whether the RD1-dependent haemolytic/cytolytic activity is related to cytotoxicity that occurs during mycobacterial infection, we infected J774 cells with *M. marinum* WT and extRD1 mutants at a low multiplicity of infection (moi) of 0.1 ($\approx 10^4$ bacteria internalized by 10^5 cells). Cytotoxicity to the cells was examined by use of ALAMAR BLUE to measure the cell metabolic activity. As shown in Fig. 2F, WT bacteria produced massive cytotoxicity to J774 cells by 5 days after infection, with a loss of 92% of the metabolic activity of the cell monolayer. In contrast, all extRD1 mutants showed markedly reduced cytotoxicity to J774 cells. The cytotoxic defects of M1 and M7 were fully restored by expression of corresponding *M. tuberculosis* homologues (Fig. 2F). Diminished cytotoxicity by the *M. marinum* extRD1 mutants and complementation by expression of *M. tuberculosis* homologues were also observed during infections of murine bone marrow-derived macrophages (BMDMs) and human A549 epithelial-like cells (data not shown). These results demonstrate that extRD1 genes are required for cytotoxicity to macrophages and epithelial cells following intracellular infection that is initiated at a low inoculum. In addition, the close correlation between cytotoxicity and haemolysis/cytolysis argues that cytotoxicity resulting from intracellular growth of mycobacteria is probably related in mechanism to the rapid haemolytic and cytolytic activities that occur after addition of extracellular bacteria.

extRD1 genes are required for secretion of ESAT-6 and CFP-10

Genes in *M. tuberculosis* RD1 are required for secretion of ESAT-6 and CFP-10 (Hsu *et al*., 2003; Pym *et al*., 2003; Stanley *et al*., 2003; Guinn *et al*., 2004). To assess whether this was true for *M. marinum* as well, we examined secretion of these molecules in the mutants we had identified. As shown in Fig. 3A, WT *M. marinum* secreted a significant amount of ESAT-6 into the culture medium ($\approx$ 5 ng protein/3×10^8 bacteria), which was approximately two times more than in the bacterial cell lysate. GroEL1, a cytosolic protein that has been used as a marker for bacteriolysis when present in the culture medium (Sonnenberg and Belisle, 1997; Stanley *et al*., 2003), was detected solely in the cell lysates of both WT and extRD1 mutants (Fig. 3A), demonstrating that ESAT-6 in the supernatant was secreted from WT *M. marinum* and not simply a result of cell lysis. In contrast to WT *M. marinum*,

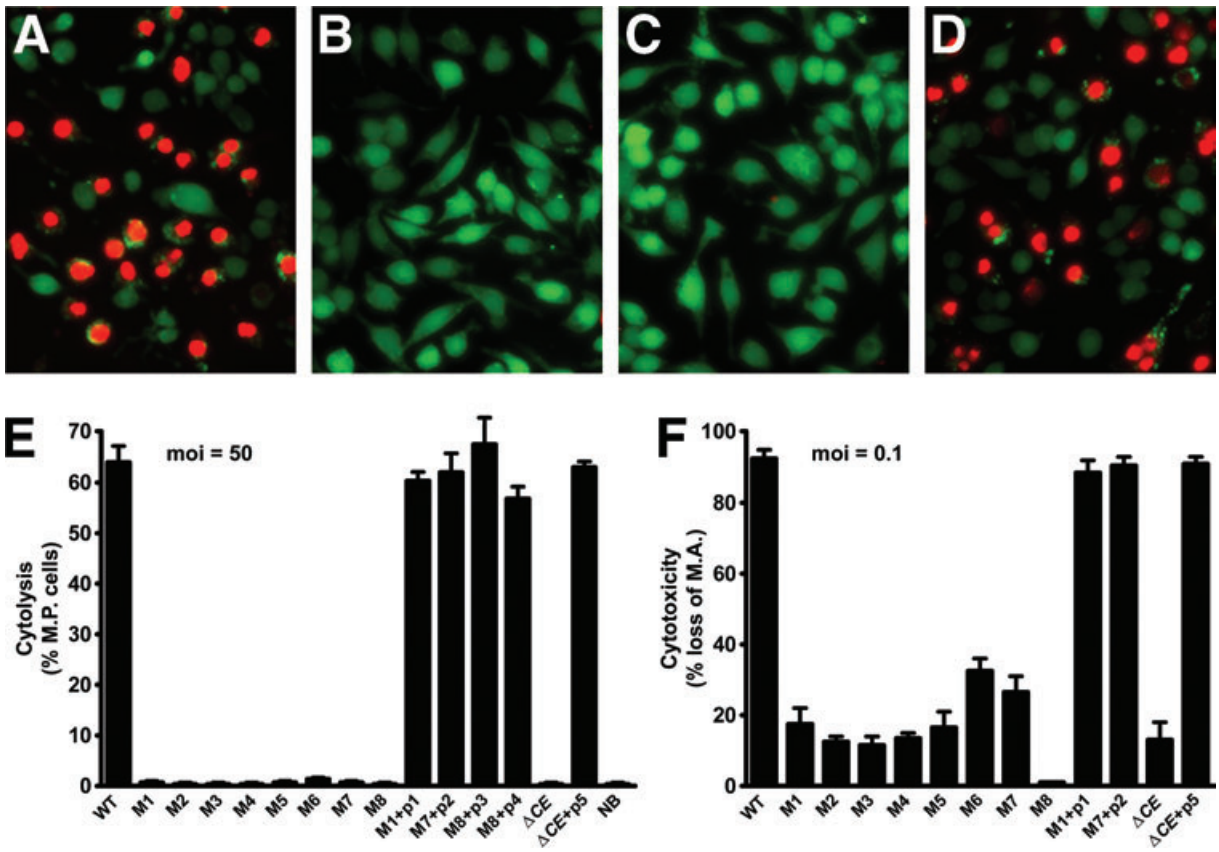

Fig. 2. Cytolysis and cytotoxicity of *M. marinum* WT and extRD1 mutants to J774 cells. J774 cells were incubated with *M. marinum* WT (A), M3 (*Mh3868::Tn*) (B) or M1 (*Mh3866::Tn*) (C), or M1 expressing *Rv3866* (D) at moi 50 for 1 h, and cytolysis was detected by staining the cells with calcein AM (green) for live cells and ethidium homodimer-1 (red) for membrane-permeabilized cells. (E) Quantification of the percentage of membrane-permeabilized (M.P.) cells following incubation with *M. marinum* strains under the same conditions as shown above in A–D. (F) Cytotoxicity of *M. marinum* to J774 cells infected at moi 0.1 (10^4 CFU internalized by 10^5 cells), as determined by measuring cell monolayer metabolic activity (M.A.) using ALAMAR BLUE and expressed as percentage loss of M.A., which is calculated as [1 – (activity in infected cells/activity in uninfected cells)] × 100. The graph is the summation of three experiments, each performed in duplicate, and error bars indicate standard deviation. M1–M8, Δ*CE* and p1–p5 are described in the legend of Fig. 1.

none of the extRD1 mutants secreted a detectable amount of ESAT-6 (Fig. 3A). Lack of ESAT-6 in the culture filtrate was a specific defect in secretion for several of the mutants (M5–M8), because the protein was detected in cell lysates in an amount similar to WT (Fig. 3A). Interestingly, mutants M1–M4 (*Mh3866::Tn*, *Mh3867::Tn* and *Mh3868::Tn*) had markedly reduced intracellular accumulation of ESAT-6, suggesting a role for these genes in either protein synthesis or protein stability (Fig. 3A).

Similar to ESAT-6, CFP-10 was secreted into the culture medium by WT *M. marinum*, which was approximately two to three times more than in the cell lysate (Fig. 3A). The secretion of CFP-10 was markedly reduced or abolished for several of the mutants (M1–M4 and M8), but it was only modestly decreased for the other mutants (M5–M7) (Fig. 3A). Therefore, secretion of CPF-10 and ESAT-6 are not tightly coupled, suggesting that they may not be secreted in a fixed 1:1 ratio. All extRD1 mutants produced CFP-10 in the cell extracts (Fig. 3A), although its production in M2–M4 was substantially reduced. These results suggest that failure of accumulation of CFP-10 in the culture filtrates from the non-haemolytic mutants probably resulted from failure of secretion, and that association with ESAT-6 is not necessary for stable production of the protein in the bacterial cells. The defects in secretion of ESAT-6 and CFP-10 were fully restored by expression of *M. tuberculosis* homologues in M1, M7 (Fig. 3A) and M8 (Fig. 3B).

To determine the specificity of the secretion defect in extRD1 mutants, we examined secretion of fibronectin attachment protein (FAP) and the antigen-85 complex (Ag85), both of which are well-characterized secreted proteins with signal sequences. There was no significant difference between *M. marinum* WT and extRD1 mutants in either the production or secretion of these proteins (Fig. 3A). Furthermore, there were no detectable differences between WT and the mutants in cell wall lipids, including mycolic acids, polar and apolar lipids (data not

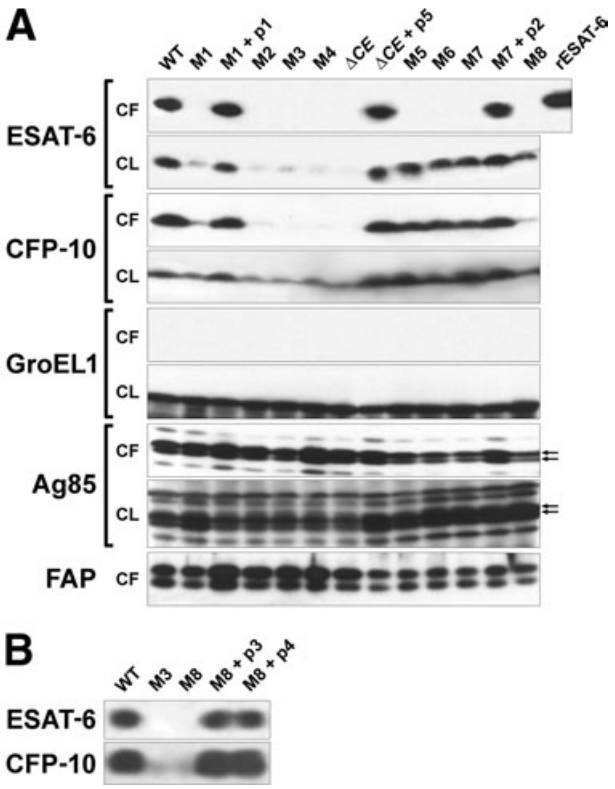

Fig. 3. Production and secretion of ESAT-6 and CFP-10 by *M. marinum* WT, extRD1 mutants and complemented strains. Proteins in the culture filtrates (CF) or cell lysates (CL) equivalent to 3×10^8 bacteria were separated by SDS-PAGE and detected by immunoblotting. M1, *Mh3866::Tn*; M2, *Mh3867::Tn*; M3, *Mh3868::Tn*; M4, *Mh3868::Tn*; Δ*CE*, *M. marinum* mutant deleted for *CFP-10* and *ESAT-6*; M5, *Mh3876::Tn*; M6, *Mh3878::Tn*; M7, *Mh3879c::Tn*; M8, *Mh3881c::Tn*. p1–p5 are complementation plasmids as described in the legend of Fig. 1. rESAT-6 indicates recombinant ESAT-6 protein (5 ng). Antigen-85 complex (Ag85), fibronectin attachment protein (FAP) and GroEL1 were used as controls for general secretion and for cell lysis, as described in the text. Proteins specifically recognized by the antibodies are indicated by black arrows on the right.

shown). Together, these results demonstrate that multiple individual genes in extRD1 are required for secretion of ESAT-6 and CFP-10. These data are consistent with the hypothesis (Gey Van Pittius *et al*., 2001; Pallen, 2002) and recent reports on *M. tuberculosis* (Hsu *et al*., 2003; Pym *et al*., 2003; Stanley *et al*., 2003; Guinn *et al*., 2004) that RD1 genes may encode a multicomponent secretion system, with at least ESAT-6 and CFP-10, both lacking signal peptides, as targets for secretion.

Secretion of ESAT-6 is essential for haemolysis, cytolysis and cytotoxicity

ESAT-6 and CFP-10 are targets of the immune response in tuberculosis and have been implicated in the pathophysiology of infection (Wards *et al*., 2000; Pym *et al*., 2002; 2003). Because both are secreted proteins that may act as effectors of RD1, we examined their role in haemolysis, cytolysis and cytotoxicity, which we have shown to require extRD1 genes. We created an *M. marinum* mutant deleted for both *ESAT-6* and *CFP-10* genes (Δ*CE*) by homologous recombination and confirmed that the mutant produced no ESAT-6 protein in either the bacterial cell or the culture medium (Fig. 3A). Compared to WT *M. marinum*, Δ*CE* was extremely defective in haemolysis (Fig. 1B), cytolysis (Fig. 2E) and cytotoxicity (Fig. 2F), similar to other extRD1 mutants. Expression of *M. tuberculosis ESAT-6*/*CFP-10* genes completely restored the ability for Δ*CE* to produce and secrete both proteins (Fig. 3A) and to produce haemolysis, cytolysis and cytotoxicity (Figs 1B, 2E and F). Because ESAT-6 was not secreted by any of the extRD1 mutants, but CFP-10 was secreted at least to some extent by several of the mutants, ESAT-6 secretion is more closely associated with the effector functions of extRD1 genes.

extRD1 genes are required for virulence to zebrafish

Mycobacterium tuberculosis RD1 has been shown to be required for full virulence in the mouse infection model (Pym *et al*., 2002; Hsu *et al*., 2003; Lewis *et al*., 2003; Stanley *et al*., 2003; Guinn *et al*., 2004). A number of fish species are natural hosts for *M. marinum*, and zebrafish has recently been established as a relevant laboratory model that manifests both acute disseminated disease and chronic persistent infection (Davis *et al*., 2002; Prouty *et al*., 2003). To determine whether RD1 is required for *M. marinum* virulence *in vivo*, we infected zebrafish intraperitoneally (i.p.) with *M. marinum* WT and three of the extRD1 mutants and examined severity of infection by monitoring fish survival, mycobacterial growth *in vivo* and histopathology. At 10^5 CFU per fish, WT-infected fish all died 4–5 weeks after infection (Fig. 4A). In contrast, even after 8 weeks, none of the fish infected by this dose of M8 (*Mh3881c::Tn*) died, and only one of four fish infected by M3 (*Mh3868::Tn*) died (Fig. 4A). At a lower inoculum (10^3 CFU per fish), none of the fish died following infection by either WT or the mutants for the entire 9 weeks of the experiment. With respect to *M. marinum* growth *in vivo*, at 10^5 CFU per fish, WT CFU increased about 1000-fold in fish livers during the first 3 weeks of infection, after which the fish began to die (Fig. 4B). In contrast, CFUs for M3 and Δ*CE* showed a much slower increase during the first 3 weeks (≈ 1% of WT) and simply sustained or declined slightly thereafter (Fig. 4B). The extent of this growth defect is similar to that reported for RD1-deleted *M. tuberculosis* in mice (Lewis *et al*., 2003; Guinn *et al*., 2004); thus the functional homology between *M. tuberculosis* and *M. marinum* extRD1 includes effects on *in vivo* virulence as well as secretion of ESAT-6 and CFP-10. Mutation of the *Mh3881c* gene, not a part of RD1, caused the most

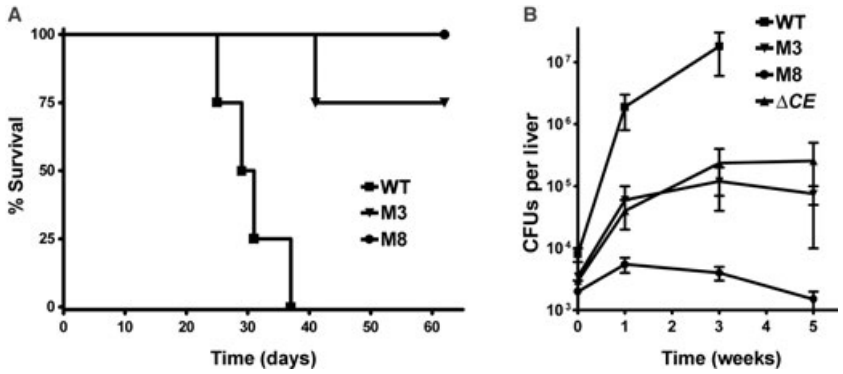

Fig. 4. Virulence of *M. marinum* WT and extRD1 mutants to zebrafish.
A. Survival of zebrafish following intraperitoneal (i.p.) infection by 10^5 *M. marinum* WT, M3 (*Mh3868::Tn*) or M8 (*Mh3881c::Tn*) during 8 weeks after infection. Four fish were infected by each *M. marinum* strain. Injection of the same volume of PBS was used as a negative control.
B. Enumeration of *M. marinum* CFUs in infected fish livers at 1 day or 1, 3 or 5 weeks after infection. Eight fish were infected by each *M. marinum* strain, two of which were examined for CFUs at each time point. Error bars indicate standard deviation.

severe attenuation *in vivo*, with minimal growth during the first week and a decrease in CFU thereafter (Fig. 4B).

Histopathological studies of infected fish supported attenuation of the mutants. At 3 weeks after infection, fish infected by 10^5 WT *M. marinum* developed massive necrotic damage of the livers; large numbers of mycobacteria were observed in damaged tissues, and granulomas were rarely detected (Fig. 5A and B). In contrast, no significant tissue damage was observed in fish infected by M3, M8 or Δ*CE*; instead, granulomas containing few mycobacteria were observed (data not shown). After inoculation with 10^3 CFU per fish, fish infected by both WT and the mutants developed granulomas, but significantly fewer granulomas and fewer mycobacteria per granuloma were detected in the livers of fish infected by M3 (Fig. 5E and F) compared to WT (Fig. 5C and D). No granulomas were detected in the livers of fish infected by M8; a few granulomas were seen in tissues of the peritoneal space, which was the original site of the infection (Fig. 5G and H). Together, these results indicate that extRD1 genes affect dissemination of infection by *M. marinum* in zebrafish and suggest that *Mh3881c* may be particularly important for growth *in vivo*.

extRD1 genes potentiate M. marinum *growth in macrophage culture*

Macrophages are primary sites for intracellular growth of mycobacteria (Russell, 2001). Because extRD1 genes are required for full virulence of *M. marinum* to fish, we examined whether they are also required for *M. marinum* growth in macrophages. When BMDMs were infected by WT *M. marinum* at moi 0.01 (≈ 10^3 CFU internalized by 10^5 cells), the bacteria replicated extensively in these cells and the CFU increased by ≈ 500-fold over the 6 days of intracellular infection (Fig. 6A). In contrast, although most of the extRD1 mutants showed initial growth similar or close to WT, they had markedly reduced growth at later time points, and by 6 days after infection their CFUs were ≈ 2-logs less than WT (Fig. 6A). M8 was the most attenuated, with a considerable loss of viability soon after infection in BMDMs and complete sterilization by 6 days (Fig. 6A). The growth defects of M1, M7 and M8 were restored by complementation with the appropriate *M. tuberculosis* genes (Fig. 6B). There were no significant differences between *M. marinum* WT and extRD1 mutants for growth in the bacterial culture medium, except that Δ*CE* showed slightly delayed exit from the initial lag phase of culture (Fig. S1).

extRD1 genes are required for M. marinum *spreading in cell culture*

Because (i) the majority of *M. marinum* extRD1 mutants showed an intracellular growth defect that was most significant at late time points of infection and (ii) these mutants lacked cytolysis and cytotoxicity, we hypothesized that they might exhibit defective spreading in cell culture. To test this possibility, we directly observed *M. marinum* spreading in confluent BMDM monolayers infected with *M. marinum* strains expressing green fluorescent protein (GFP) at exceedingly low moi 0.001 (10^3 CFU internalized by 10^6 cells). As illustrated in Fig. 7A–C, WT *M. marinum* developed fluorescent foci of infection (FFI) that increased in size with the time-course of the infection. FFI were detectable by 48 h as individual macrophages filled with replicating GFP-expressing bacteria. *M. marinum* spreading was apparent by 72 h (Fig. 7A), when FFI contained 4–10 cells filled with the bacteria, indicating *M. marinum* spreading into neighbouring cells. At 120 h, each FFI contained 20–40 cells (Fig. 7B and C), demonstrating further *M. marinum* spreading. When observed at a higher magnification, single WT bacteria were detected in cells surrounding the heavily infected cells that formed the FFI (Fig. 7G and H). In contrast, all extRD1 mutants lacked the ability to spread from the initially infected cells, although they (except M8) achieved a high bacterial density in these cells, as shown by the intense GFP fluorescence throughout the cytoplasm of individual infected cells (Fig. 7D–F, I and J). M8 was unable to form even the single cell-based FFI (Fig. S2). The spreading defects of M1, M7, M8 and Δ*CE* were fully complemented by

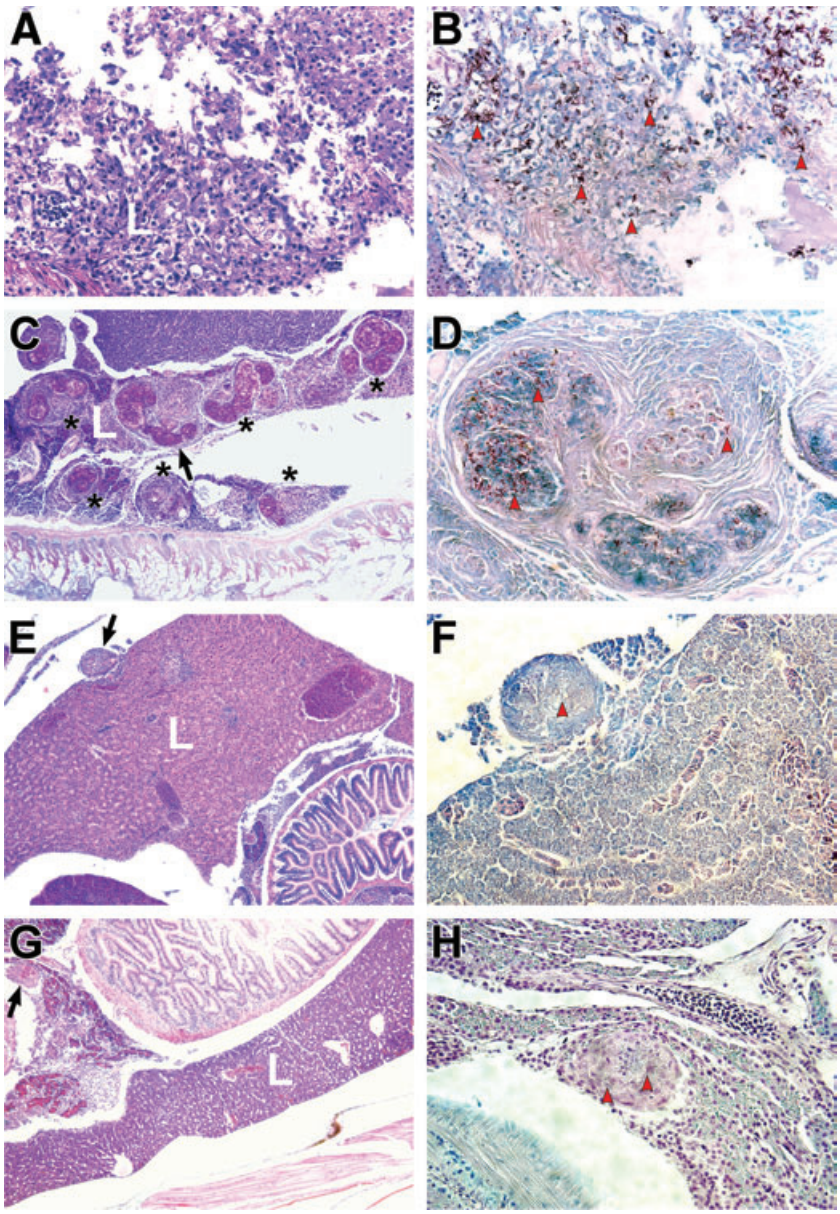

Fig. 5. Histopathology of zebrafish infected by *M. marinum* WT and extRD1 mutants. Fish were infected i.p. by 10^3 *M. marinum*: WT (A and B), M3 (*Mh3868::Tn*) (C and D), and M8 (*Mh3881c::Tn*) (E and F) and processed for histopathological studies at 8 week after infection. A, C and E are HandE stain sections observed at low magnification using a 10× objective to show the frequency of granulomas (the granulomas indicated by black arrows in A, C and E are shown at high magnification (using a 40× objective) in B, D and F respectively; additional granulomas in C are indicated by (*). B, D and F are acid-fast stain sections observed at high magnification to show *M. marinum* bacteria (bacteria are indicated by arrowheads) in granulomas. L indicates the liver.

expressing corresponding *M. tuberculosis* or *M. marinum* genes (Fig. S2).

Mycobacterium marinum spreading was also observed for WT, but not the extRD1 mutants, in A549 lung epithelial cell monolayers, even in the presence of 20 μg ml^{-1} amikacin, a concentration sufficient to kill >95% of extracellular WT *M. marinum* in 4 h (Fig. 8). Compared to cultures without amikacin, the presence of 20 μg ml^{-1} of the antibiotic considerably reduced but did not eliminate formation of FFI by WT *M. marinum* (Fig. 8A, B, D and E). Formation of multicellular FFI with no apparent cell lysis and in presence of amikacin (Fig. 8B and E) suggests that *M. marinum* is capable of direct cell-to-cell spread without significant exposure to the extracellular milieu. Because the absence of amikacin led to more robust spreading accompanied by significant cell lysis (Fig. 8A and D), it is possible that spreading of bacteria to uninfected cells through lysis of primarily infected cells with subsequent uptake of bacteria by neighbouring cells also occurred. None of the *M. marinum* extRD1 mutants were able to spread in A549 cell monolayer even in the absence of the antibiotic, and the defects of M1, M7, M8 and Δ*CE* were abolished after expression of the corresponding *M. tuberculosis* or *M. marinum* genes (Figs 8 and S3). The defects of the mutant strains for spreading in A549 cells was confirmed in a plaque assay, in which the mutants' defects were again complemented by expression of the corresponding *M. tuberculosis* or *M. marinum* genes (Fig. 9).

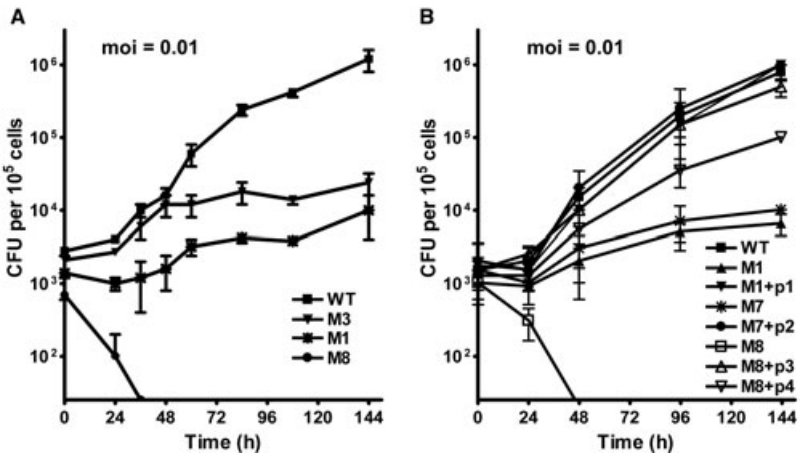

Fig. 6. Growth of *M. marinum* WT, extRD1 mutants and complemented strains in BMDM cell cultures following infection at moi 0.01 (≈ 10^3 CFU internalized by 10^5 cells).
A. Growth of WT and three extRD1 mutants in BMDM cell cultures. M1, *Mh3866::Tn*; M3, *Mh3868::Tn*; M8, *Mh3881c::Tn*.
B. Complementation of three extRD1 mutants by expression of *M. tuberculosis* homologues. p1–p4 are the same as described in the legend of Fig. 1. Error bars indicate standard deviation of data from duplicate samples.

Together, these data demonstrate that extRD1 genes are required for mycobacteria cell-to-cell spread during infection of macrophages and lung epithelial cells.

Discussion

The possibility that genes within the RD1 region in *M. tuberculosis* and *M. bovis* contribute to virulence first received attention when it was realized that all strains of BCG, the attenuated *M. bovis* used for vaccination against tuberculosis, have a deletion in this region of the chromosome (Mahairas *et al*., 1996; Behr *et al*., 1999; Gordon *et al*., 1999). An important role for RD1 in pathogenesis was demonstrated directly by enhancement of the virulence of BCG and *M. microti* with expression of this region of the *M. tuberculosis* chromosome and by reduction of the virulence of *M. tuberculosis* with its deletion. Very recently, several groups have reported that *M. tuberculosis* RD1 genes other than *ESAT-6* and *CFP-10* are required for secretion of these two immunodominant antigens and for virulence in mice (Hsu *et al*., 2003; Pym *et al*., 2003; Stanley *et al*., 2003; Guinn *et al*., 2004). Numerous questions remain about how this region affects virulence: for example, which of the genes in this region are required for, and how do they participate in, mycobacterial infection of host cells; which of the genes are involved in secretion of ESAT-6 and CFP-10; and whether and how the secretion of ESAT-6 and CFP-10 potentiates the virulence of mycobacteria. This study demonstrates that multiple genes in the *M. marinum* chromosomal region considerably larger than RD1 (extRD1) spanning *Mh3866–Mh3881c* are required for virulence both *in vivo* and *in vitro* and for secretion of ESAT-6 and CFP-10. The extRD1 genes are required for disseminated *M. marinum* infection *in vivo* and cell-to-cell spread *in vitro* in cultured macrophages and epithelial cells, both of which are genetically associated with the secretion of ESAT-6. We propose that extRD1 genes potentiate virulence by contributing to secretion of a membranolytic activity that involves ESAT-6 and that this activity is required for mycobacteria cell-to-cell spread in the host. The ability of mycobacteria to spread from cell-to-cell probably facilitates dissemination of infection among macrophages and dendritic cells and may be involved in early events in infection when inhaled or ingested bacilli come in contact with both epithelial and immune cells.

Spreading of mycobacteria in cultured cell monolayers has been observed previously for *M. tuberculosis*, in which two potentially different modes of cell-to-cell spread are described (Byrd *et al*., 1998; Castro-Garza *et al*., 2002). In one case, addition of extracellular antibiotic did not affect spreading, suggesting that the spreading bacteria were not exposed to the extracellular milieu sufficient for antibiotic effect, and in the other, addition of antibiotic abolished spread, suggesting a requirement for release into the extracellular medium before uptake by uninfected host cells for spreading. In our study, continuous incubation with amikacin at 20 times the MIC for *M. marinum* only moderately reduced the size of FFI compared to the absence of antibiotic (Fig. 8). This suggests that the bacteria are not exposed to extracellular milieu long enough to be killed by amikacin before rapid uptake into uninfected cells and may suggest that spreading in the presence of amikacin is predominantly a result of direct cell-to-cell spread similar to *Listeria* or *Shigella*. Our recent demonstration that *M. marinum* can escape the phagosome and polymerize actin (Stamm *et al*., 2003) suggests a cellular mechanism for this direct cell-to-cell spread. While this may not be directly relevant to cell-to-cell spread by *M. tuberculosis*, which does not escape the phagosome, it certainly is intriguing that *M. tuberculosis* RD1 genes are thought to control host cell cytolysis (Hsu *et al*., 2003) and cell-to-cell spread (Guinn *et al*., 2004). The otherwise very close parallels between *M. tuberculosis* and *M. marinum* in organization, structure and function of the genes in extRD1 raise the intriguing question of why

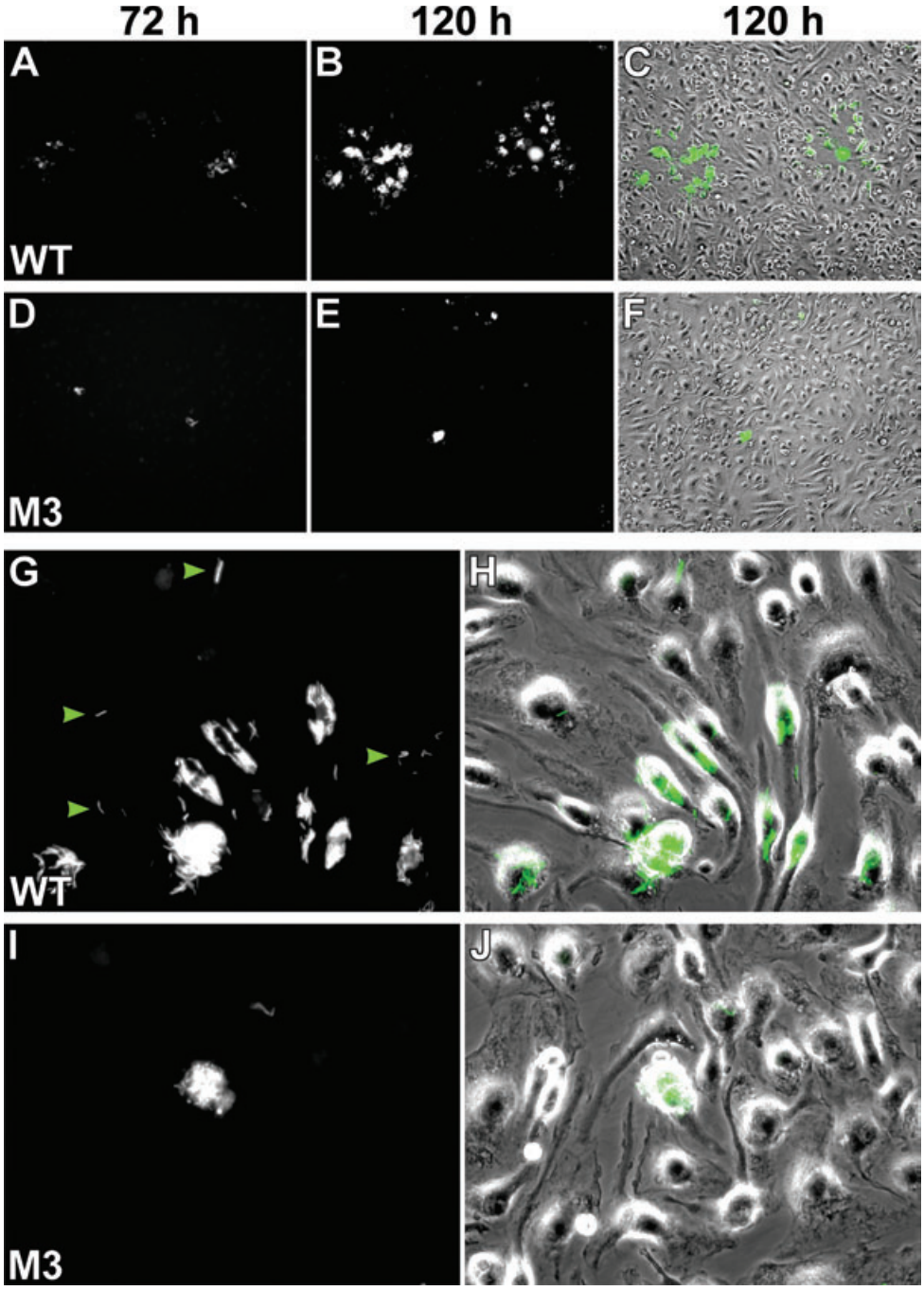

Fig. 7. Detection of cell-to-cell spread in BMDMs after infection by *M. marinum* WT and M3 (*Mh3868::Tn*) expressing GFP. BMDMs were infected by *M. marinum* strains at moi of 0.001 (10^3 CFP internalized by 10^6 cells), followed by an overlay with 0.8% agarose and incubation for 120 h.

A–F. Examination of fluorescent foci of infection (FFI) at low magnification using a 10 × objective. (C) and (F) are the phase images of (B) and (E) respectively.

G and H. Examination of cell-to-cell spread at 120 h after infection for WT *M. marinum* at high magnification using a 40× objective. Cell-to-cell spread was evident by observing single bacteria (arrow heads) in cells surrounding heavily infected cells forming FFI.

I and J. Lack of cell-to-cell spread for M3, although it is competent for growth in the primarily infected cell, as indicated by detection of a large number of bacteria inside a cell without apparent spreading into adjacent cells. H and J are the phase images of G and I respectively.

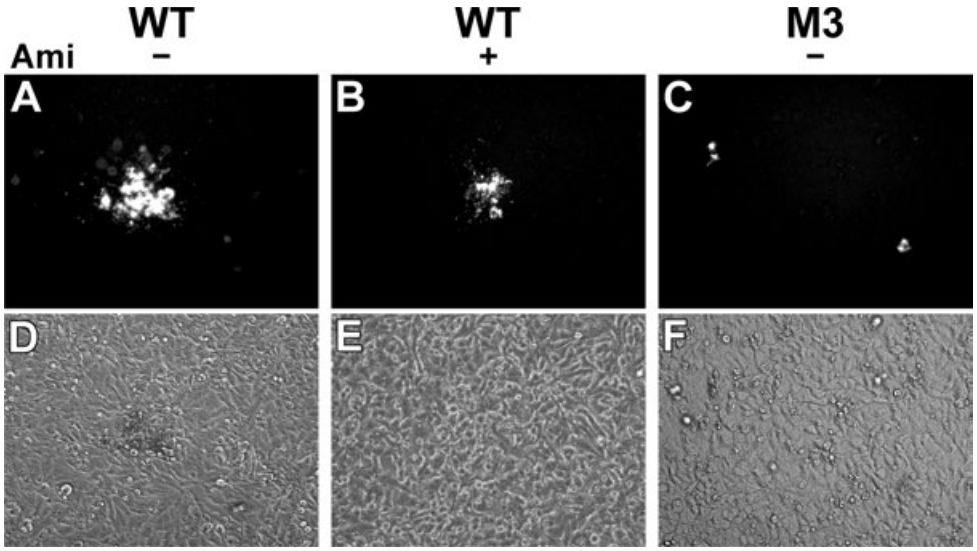

Fig. 8. Detection of cell-to-cell spread in A549 lung epithelial cells by *M. marinum* WT and M3 (*Mh3868::Tn*) expressing GFP. A549 cells were infected by *M. marinum* strains at moi 0.001 (10^3 CFU internalized by 10^6 cells) and overlaid with 0.8% agarose in the presence (+) or absence (–) of 20 μg ml^{-1} amikacin (Ami). FFI were observed at 120 h after infection. D, E and F are the phase images of A, B and C respectively.

M. tuberculosis does not lyse phagosome membranes. The answer to this question will probably require a more detailed molecular understanding of the mechanisms of membranolysis for the two closely related organisms.

Our study confirms recent work in *M. tuberculosis* that has demonstrated that individual genes near those encoding ESAT-6 and CFP-10 are required for their secretion and that these genes and products are required for full virulence in macrophages and in the mouse model of infection (Hsu *et al*., 2003; Sassetti and Rubin, 2003; Stanley *et al*., 2003; Guinn *et al*., 2004). Our work extends these data in several important ways. First, we show that the chromosomal region (*Mh3866–Mh3881c*) required for secretion of ESAT-6 and CFP-10 extends beyond the known boundaries of *M. tuberculosis* RD1 (*Rv3871–Rv3879c*). Mutations in *M. marinum* genes on both sides of the *M. tuberculosis* RD1 homologues led to loss of ESAT-6 and CFP-10 secretion. Thus, the putative secretion system is probably more complex than previously considered (Pallen, 2002; Hsu *et al*., 2003; Stanley *et al*., 2003; Guinn *et al*., 2004). Moreover, we demonstrate that in addition to the other recently characterized RD1 genes (Hsu *et al*., 2003; Stanley *et al*., 2003; Guinn *et al*., 2004), another two genes in RD1, *Mh3878* and *Mh3879c*, also are required for secretion of ESAT-6. Second, we found discordance between the secretion of ESAT-6 and CFP-10 upon mutation of several extRD1 genes, including *Mh3876*, *Mh3878* and *Mh3879c*. This demonstrates that the complex between these two molecules is not required for secretion of CFP-10. However, this does not exclude the possibility that the complex may facilitate ESAT-6 secretion. Our data show a very close linkage between

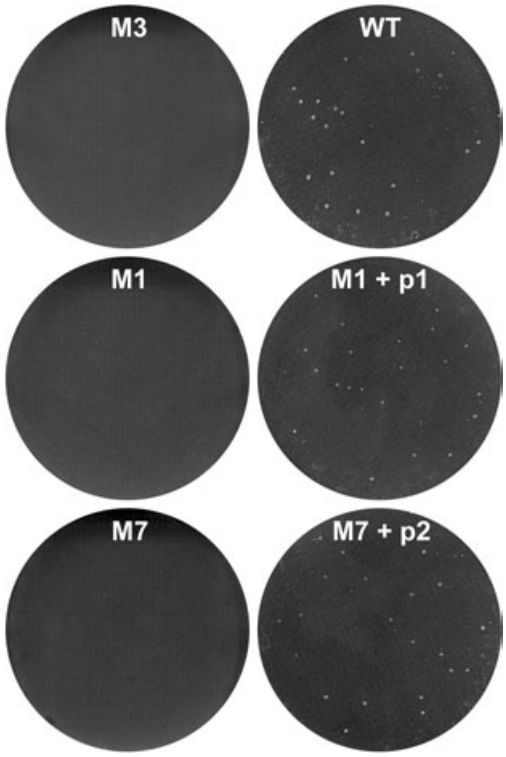

Fig. 9. Plaque formation by *M. marinum* strains in A549 cell monolayers. The cell monolayers were infected by *M. marinum* strains at moi 0.001 (10^3 CFU internalized by 10^6 cells), overlaid with 0.8% agarose, and incubated in culture medium for 7 days before visualization of plaques. M1, *Mh3866::Tn*; M3, *Mh3868::Tn*; M7, *Mh3879c::Tn*. p1 and p3 are complementation plasmid as described in the legend of Fig. 1.

failure to secrete ESAT-6 (but not CFP-10) and loss of virulence and are compatible with the suggestion that ESAT-6 is required for cytolysis (Hsu *et al*., 2003) and that this requirement is reflected in the haemolysis and cytotoxicity assays and in cell-to-cell spread of infection. Our data do not rule out the possibility that CFP-10 is necessary but not sufficient to produce these phenotypes nor do they exclude the possibility that as yet unidentified effectors of RD-1 are required for host membrane lysis and/or virulence. Third, we demonstrate that the extRD1 genes are differentially involved in the intracellular accumulation and secretion of ESAT-6. extRD1 mutants M1–M4, disrupted for genes *Mh3866–Mh3868* upstream of RD1, failed to accumulate ESAT-6 in the bacterial cell (Fig. 3A), which cannot be a simple secondary consequence of loss of secretion, because intracellular ESAT-6 did accumulate in other mutants that failed to secrete the protein. This is not a result of a polar effect of the transposon insertions on the ESAT-6 gene, because ESAT-6 secretion was restored to the *Mh3866* mutant by expression of *Rv3866* alone. These genes therefore may be involved in stabilization of ESAT-6 in the bacteria before secretion and may be chaperones for this secretion system. Alternatively, these genes may be involved in transcription or translation of *ESAT-6*. Fourth, we have genetically linked the phenotypes of haemolysis, nucleated cell cytolysis and cytotoxicity to each other, to secretion of ESAT-6, to mycobacteria cell-to-cell spread and to virulence. We believe that the previously reported defects for BCG in haemolysis, cytolysis and cell-to-cell spread (King *et al*., 1993; Dobos *et al*., 2000; Castro-Garza *et al*., 2002) result from the deletion of RD1 genes. Fifth, we have provided direct evidence for mycobacteria cell-to-cell spread that involves the function of extRD1 genes, by microscopic detection of FFI and examination of plaque formation. The recent studies by Guinn *et al*. (2004) and Hsu *et al*. (2003) have suggested a role for *M. tuberculosis* RD1 genes in cell-to-cell spread by evaluation of the percentage of cells infected and the average CFU per cell, at different times after infection. While these conclusions were drawn based on statistical analysis of the entire culture monolayer, our visualization of these events adds considerable support to the inferences of these previous studies. Our assay for FFI is particularly useful, for it allows dynamic observation of individual spreading events at multiple times after infection. Finally, we demonstrate that *M. marinum* extRD1 genes are functionally conserved with their *M. tuberculosis* homologues both for biochemical and virulence functions in the bacterium's natural host, zebrafish.

Some of our newly identified extRD1 genes are predicted to encode proteins with possible roles in protein synthesis or secretion. For example, Mh3868 or Rv3868 is predicted to be a member of the AAA-ATPase protein family that carries out diverse cellular functions (Lupas and Martin, 2002). A common cheperone-like activity underlies this functional diversity, which involves structural remodelling, unfolding and disassembly of proteins and protein complexes (Lupas and Martin, 2002). Because there is no transmembrane segment predicted by the sequence of Mh3868 or Rv3868, the encoded protein may serve as a cytosolic chaperone to facilitate protein translocation across the cytoplasmic membrane, perhaps functionally interacting with the other two AAA-ATPases encoded in RD1 that have been shown to be required for secretion of ESAT-6 and CFP-10 (Stanley *et al*., 2003). Consistent with this hypothesis, *Mh3866* (interrupted in M1) and its homologue *Rv3866* are predicted to encode a periplasmic substrate-binding protein for high affinity transport of bacterial proteins across the cytoplasmic membrane. Alternatively, Mh3868 or Rv3868 could be involved in protein synthesis and/or stability, because its absence resulted in decreased intracellular accumulation as well as secretion of ESAT-6. Consistent with a possible role for these genes in protein synthesis at some step, Mh3867 is predicted to contain a RNA-binding domain. Clearly more detailed biochemical analysis of the products of these genes will be required to determine precisely how they are involved in the putative multicomponent secretion system that also requires multiple genes within RD1.

Finally, we demonstrate that M8 (*Mh3881c::Tn*) is far more attenuated *in vivo* and in macrophages than any other extRD1 mutant, including Δ*CE*. This does not result from a polar mutation of downstream genes, because M8 is complemented by expression of either *Rv3881c* or *Mh3881c*. This suggests that the *Mh3881c*/*Mh3880c* operon gene products perform additional function(s) required for mycobacterial survival during infection in addition to the secretion of ESAT-6 and CFP-10. However, we have been unable to find structural motifs in the predicted protein that suggest the nature of these additional roles or why Mh3881c is required for ESAT-6 and CFP-10 secretion. Further study of this operon to understand function and potential importance as a drug or vaccine target is clearly warranted.

Experimental procedures

Bacterial strains and media

Mycobacterium marinum strain M and the DH5α strain of *E. coli* were cultured and genetically manipulated as described (Gao *et al*., 2003a,b). Unless specified, *M. marinum* strains were cultured in 7H9 liquid with Tween 80 and ADC supplement or 7H10 agar with OADC supplement.

Isolation of M. marinum *mutants defective in contact-dependent haemolysis*

An *M. marinum* transposon mutant library was generated by

the M^4 (*mariner* transposon mutagenesis in *M. marinum*) procedure as previously described (Gao *et al*., 2003a). Briefly, the mutagenesis vector harbouring the *mariner* transposon was transformed into *M. marinum* WT and the mutants selected by a two-step procedure: first for single cross-over by kanamycin resistance, and second for double cross-over by resistance to sucrose and kanamycin. Approximately 1000 individual mutants were screened for haemolysis to sRBC. Contact-dependent haemolysis was assayed as previously described (King *et al*., 1993; Gao *et al*., 1999; Alli *et al*., 2000) with modifications. Briefly, *M. marinum* grown in 7H9 medium to mid- to late-log phase were washed twice with phosphate buffered saline (PBS) and mixed with sRBC (Remel). For the initial mutant screen, 120 µl of *M. marinum* suspension (containing 5×10^8 bacteria) was mixed with 120 µl of sRBC (containing 2×10^7 cells) in each well of the round-bottomed 96-well plates and centrifuged at 2600 *g* for 10 min. After incubation at 32°C, 5% CO_2 for 1–2 h, the pellets were resuspended, centrifuged, and absorbance of the supernatant was measured at 405 nm. Defibrinated human RBCs (Jain, 1989) were similarly tested for haemolysis by *M. marinum* strains.

Analysis of rapid cytolytic activity of M. marinum *to macrophages*

The murine J774 macrophage-like cells (ATCC TIB67) were maintained as described (Gao *et al*., 2003a). *M. marinum* strains grown to mid- to late-log phase and washed twice with DME medium were added to the cell monolayers, centrifuged at 1500 *g* for 10 min and incubated at 32°C, 5% CO_2 for 1 h. Cytolysis was examined at the end of the incubation by fluorescence microscopy after staining the cells for 40 min with 2 µM calcein AM and 4 µM ethidium homodimer-1 (Molecular Probes). Calcein AM stains live cells with green fluorescence after being metabolized by the cells, while ethidium homodimer-1 penetrates through permeabilized plasmic membrane of dead cells to stain the nuclei with red fluorescence.

Analysis of M. marinum *intracellular growth and cytotoxicity*

Mouse BMDMs and A549 human alveolar epithelial-like cells were cultured as previously described (Gao and Abu Kwaik, 1999; Gao *et al*., 2003b). Infection of BMDMs, J774 and A549 cells and enumeration of intracellular M. marinum CFUs were also as described (Gao *et al*., 2003a,b). For examination of intracellular growth, the cells were infected by *M. marinum* strains at moi 0.01 (10^3 CFU internalized by 10^5 cells) and incubated at 32°C, 5% CO_2 for 6 days. For detection of cytotoxicity, J774 cells were infected by *M. marinum* strains at moi 0.1 (10^4 CFU internalized by 10^5 cells) and incubated at 32°C, 5% CO_2 for 4 days. Cytotoxicity was determined by replacing the culture medium with fresh medium containing 10% ALAMAR BLUE (Trek Dignosis) that measures the metabolic activity of only the live cells (Gao *et al*., 1997). In addition, microscopic examination of live and dead cells in the monolayers was performed by staining with calcein AM and ethidium homodimer-1, as described above. Statistical analysis of the results was based on the ALAMAR BLUE assay, because a portion of the cells in the monolayers detached, particularly in WT-infected monolayer, and cytolysis could not be accurately determined by microscopic scoring.

Analysis of M. marinum *cell-to-cell spread*

BMDMs or A549 cells were infected by *M. marinum* strains at very low moi 0.001 (10^3 CFU internalized by 10^6 cells). Extracellular bacteria were killed by incubation of the infected monolayers with 200 µg ml^{-1} amikacin for 2 h. Then, the monolayers were overlaid with 0.8% agarose and incubated in culture medium with or without 20 µg ml^{-1} amikacin (≈ 20 × MIC). *M. marinum* strains expressing GFP were used to detect cell-to-cell spread, in which FFI were detected by fluorescence microscopy. The GFP-expressing *M. marinum* strains were generated by transformation with plasmid pmsp12.GFP.Apr (Chan *et al*., 2002) (generously provided by Lalita Ramakrishnan, University of Washington) and selection on 7H10 plates containing 30 µg ml^{-1} apramycin. In addition, a plaque assay, in which cells were fixed with 10% formalin and stained with crystal violet, was used to detect cell-to-cell spreading, exactly as previously described (Fernandez *et al*., 1989).

Analysis of production and secretion of ESAT-6 and CFP-10

Mycobacterium marinum strains grown to mid-log phase in 7H9 medium were washed and then grown in Sauton's medium (Bryan, 1998) for 3–5 days at 30°C. Culture filtrates were prepared by pelleting the bacteria at 2600 *g* and concentrating the supernatants 100-fold using centriplus concentrators (Amicon) with a 3 kDa cut-off. Pellets were washed twice with Sauton's and weighed, following which a portion were plated for quantification of CFUs and the rest subjected to bead-beating to obtain cell lysates. Cell lysates were obtained from disrupted bacteria by centrifugation for 5 min at 2300 *g*. Culture filtrates and cell lysates were normalized to the weight of the bacterial pellets. The samples were separated by SDS-PAGE on a 4–20% gradient gel, and the proteins were detected by Western blotting, developed by enhanced chemiluminescence and exposed to films. Anti-ESAT-6 (Mab HYB 76-8) and anti-CFP-10 (K8493) antibodies were generous gifts from Peter Andersen (Statens Serum Institut, Denmark); the anti-Ag85 serum was from Marcus Horwitz (University of California, Los Angeles); anti-GroEL1 (CS-44) and purified recombinant ESAT-6 protein were from John Belisle (Colorado State University, NIH contract NO1-AI-75320). Protein bands on films are quantified by densitometry that determines relative protein amount.

Extraction and analysis of lipids

Mycobacterium marinum cell wall lipids were metabolically labelled by incubating with [^{14}C]-acetic acid (1 µCi ml^{-1}) for 2 h. Extraction and separation of *M. marinum* cell wall lipids to polar lipids, apolar lipids and mycolic acid methyl esters were performed as described (Besra, 1998). Polar and apolar

lipids were resolved in one or two dimensions on high performance thin layer chromatography (HPTLC) plates (EM Science) on either silica or reverse phase surface. Mycolic acids were first separated to α-, methoxy- and keto-mycolates on silica HPTLC plates by hexanes:ethyl acetate (95:5, v/v), and the purified fractions from preparative plates were further resolved on reverse phase HPTLC plates by chloroform:methanol (40:60, v/v). [^{14}C]-labelled lipids on HPTLC plates were visualized by exposure to a Cyclone phosphor screen, and analysed by the Cyclone Phosphor System software (Packard Instrument Company).

Analysis of transposon insertion junction sequences and similarity searches

Recovery and sequencing of the transposon insertion sites were performed as described (Gao *et al*., 2003a). Similarity searches were performed using BLASTp and BLASTn at the NCBI site (http://www.ncbi.nlm.nih.gov/BLAST/) and the incomplete *M. marinum* genome database at the Sanger Center site (http://www.sanger.ac.uk/Projects/M_marinum/). Domain homology searches for predicted protein sequences were also performed at the SMART site (http://smart.embl-heidelberg.de/). The complete genome sequences for *M. tuberculosis*, *M. leprae* and *M. bovis* were obtained from the NCBI site using Accession No. NC_000962, NC_002677 and NC_002945 respectively.

Generation of M. marinum *CFP-10/ESAT-6 deletion mutant*

The vector for allelic exchange was constructed by cloning the ZEOCIN-resistant gene under the mycobacterial groEL promoter (Gao *et al*., 2003a) into the blunt-ended *Bam*HI site of pPR27 (Pelicic *et al*., 1997) to create pLYG304.*Zeo*. To generate *M. marinum CFP-10*/*ESAT-6* deletion mutant (Δ*CE*), a left arm fragment was polymerase chain reaction (PCR) amplified from *M. marinum* genomic DNA using primers DeltaCE-LF (5′-GCCCTCTAGAAGTCAGCGACGGCGCAG CCAG-3′) and DeltaCE-LR (5′-GGCGGAATTCGTGTTGACG ACGCTCACCAGTCGTC-3′), and a right arm fragment using primers DeltaCE-RF (5′-CGGGAATTCGCGTAGAATACCGA AGCACGAGATCGGG-3′) and DeltaCE-RR (5′-CCGCAAG CTTCTAGATTCATGCCGGTTTGGCGTGGC-3′). The left and right arms and the kanamycin-resistant gene (*kan*^{r}) (derived from pUC4K, Pharmacia) were ligated into the *Xba*I and *Hin*dIII sites of pBluescript (Stratagene) to generate pBS.*CE*/*kan*. The *Xba*I fragment of pBS.*CE*/*kan* containing the *kan*^{r} gene was cloned into the *Xba*I site of pLYG304.*Zeo* to generate the plasmid for homologous recombination. Correct construction of the plasmid was confirmed by sequencing the junctions between the arms and the *kan*^{r} gene and between the arms and the plasmid backbone. The plasmid was introduced into *M. marinum* WT by electroporation and the transformants were sequentially selected first for *kan*^{r}, then for resistance to sucrose and kanamycin, and finally for sensitivity to ZEOCIN. Deletion of *CFP-10* and *ESAT-6* from the *M. marinum* chromosome was confirmed by PCR that amplifies chromosomal sequence encompassing the junctions of the arms using primers DeltaCE.check-F1 (GGCG GCTCAGCGGGAGATCCACCGAG) and DeltaCE.check-R1 (CACCTTCTTCACGAGGCAGACCTCAGCGCC). The PCR products were sequenced to confirm chromosomal disruption of the target genes.

Complementation of M. marinum *extRD1 mutants with* M. tuberculosis *or* M. marinum *genes*

Complementation was performed for four of the *M. marinum* extRD1 mutants: M1 (*Mh3866::Tn*), M7 (*Mh3879c::Tn*), M8 (*Mh3881c::Tn*) and Δ*CE*. The coding sequences of *Rv3866*, *Rv3879c*, *Mh3880c–Mh3881c*, *Rv3881c*, and *M. tuberculosis CFP-10* and *ESAT-6* genes (Cole *et al*., 1998; http://www.sanger.ac.uk/Projects/M_marinum/) were PCR amplified using primers pair one (5′-CGGAATTCGCCAGCAG GTCTGTGCCATAGCGAGTCG-3′ and 5′-CGGTCGACGGA GATGTGAGGGGTCAGCCTCGG-3′), pair two (5′-GGGTAC GTAGTATTACCAGGCCGACGGGCAGC-3′ and 5′-GGGAAT TCACGCAGCGCCTGTTGCTGTCCTGGC-3′), pair three (5′-GGCGCGGAGGCCAAGGTGGTGT-3′ and 5′-TCAGACC ATCCCGTTGTTCATTG-3′), pair four (5′-GTAAAGGTGGCG TGGCCGAATGCGA-3′ and 5′-TCACTTCGACTCCTTACTG TCCTGGC-3′) and pair five (5′-GCGAATTCCCGTAATGAC AACAGACTTCCCGGCCACCC3′ and 5′-GGAAGCTTGCC CTATGCGAACATCCCAGTGACGTTGCC-3′), and cloned into pLYG206.*Zeo* (Gao *et al*., 2003b) to generate p1, p2, p3, p4 and p5. *M. marinum* mutant strains were transformed by electroporation with corresponding complementing plasmids and selected for resistance to ZEOCIN.

Infection of zebrafish and histopathological studies

Zebrafish AB strain was maintained as described (Lee *et al*., 2003). For infection, fish were anaesthetized in 0.02% tricaine (ethyl 3-aminobenzoate methanesulphonate) (Sigma-Aldrich) for 3–5 min and injected i.p. with 5–10 μl of *M. marinum* suspension in PBS containing a known number of bacteria. The same volume of PBS alone was injected into control fish. For survival studies, four fish were infected with each *M. marinum* strain. To evaluate CFUs in fish organs, eight fish were infected by each *M. marinum* strain and two fish were sacrificed at 1 day and 1, 3 and 5 weeks after infection. Fish were anaesthetized and sacrificed at a higher concentration of tricaine (0.05%) for a longer period of time (>20 min) and sterilized for 2 min in 70% ethanol before dissection. Organs were homogenized in 0.1% Triton X-100, and serially diluted samples were plated onto 7H10 plates for enumeration of CFUs. Incubation in 0.1% Triton X-100 for 30 min did not affect the viability of both *M. marinum* WT and the extRD1 mutants. For histopathology, fish were fixed in 10% formalin for 24 h before embedding in paraffin and sectioning.

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Supplementary material

The following material is available from
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Fig. S1. Growth curves for *M. marinum* WT and extRD1 mutants *in vitro* in 7H9 broth.

Fig. S2. Detection of cell-to-cell spread in BMDMs after infection by *M. marinum* WT and M1 (*Mh3866::Tn*), M7 (*Mh3879c::Tn*), M8 (*Mh3881c::Tn*) and ΔCE expressing GFP.

Fig. S3. Detection of cell-to-cell spread in A549 lung epithelial cells by *M. marinum* WT and M1 (*Mh3866::Tn*), M7 (*Mh3879c::Tn*), M8 (*Mh3881c::Tn*) and ΔCE expressing GFP.

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