Yersinia pseudotuberculosis mntH functions in intracellular manganese accumulation, which is essential for virulence and survival in cells expressing functional Nramp1

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Manganese has an important yet undefined role in the virulence of many bacterial pathogens. In this study we confirm that a null mutation in *Yersinia pseudotuberculosis mntH* reduces intracellular manganese accumulation. An *mntH* mutant was susceptible to killing by reactive oxygen species when grown under manganese-limited conditions. The *mntH* mutant was defective in survival and growth in macrophages expressing functional Nramp1, but in macrophages deficient in Nramp the bacteria were able to survive and replicate. In *Galleria mellonella*, the *mntH* mutant was attenuated. Taken together, these data suggest a role for manganese in *Y. pseudotuberculosis* during macrophage intracellular survival, protecting the bacteria from the antimicrobial products released during the respiratory burst.

INTRODUCTION

The mechanisms by which phagocytes eliminate pathogens are diverse and include low pH, antimicrobial enzymes and peptides, and oxidizing reagents such as superoxide, hydrogen peroxide and hypochlorous acid (Djaldetti *et al.*, 2002; Imlay, 2008). In addition, phagocytes are able to limit access to metal ions such as Fe^{2+} and Mn^{2+} which are essential for the viability and growth of bacteria. One of the key mechanisms by which phagocytes deprive bacteria of Fe^{2+} and Mn^{2+} involves the export of these ions across the phagosome membrane and into the host cell cytosol (Cellier *et al.*, 2007). The *Nramp1* gene in mice and other mammals encodes a symporter capable of restricting microbial access to divalent cations in the phagosome (Cellier *et al.*, 2007; Forbes & Gros, 2001). Functional Nramp1 has a profound influence on the ability of pathogens such as *Salmonella enterica* and *Mycobacterium bovis* to grow in phagocytes and to cause disease in mice. For example, the LD_{50} of *S. enterica* serovar Typhimurium is at least 10^4-fold higher in mice that produce functional Nramp1 compared with mice that do not (Plant & Glynn, 1976). However, it is also clear that Nramp1 is not a universally important factor in regulating pathogen growth in phagocytes. The growth of intracellular pathogens such as *Listeria monocytogenes* and *Legionella pneumophila* is unaffected by Nramp1 (Cellier *et al.*, 2007; Govoni & Gros, 1998).

Although Nramp1 is able to restrict the growth of some bacterial pathogens in phagocytes, it is clear that this growth restriction is not absolute and this reflects the ability of pathogens to access the limited supplies of Mn^{2+} ions using their own cation transport systems (Papp-Wallace & Maguire, 2006; Zaharik & Finlay, 2004). One of the key Mn^{2+} transporters in bacteria is MntH, which is often viewed as a functional orthologue of mammalian Nramp1. Consequently, this transporter has been shown to play a key role in permitting the intracellular growth of some pathogens in phagocytes (Anjem *et al.*, 2009; Johnston *et al.*, 2006; Perry, 1993). Although Mn^{2+} ions are essential for the growth of pathogens in phagocytes, the precise function of these ions is unclear. Some enzymes in intermediary metabolism and signal transduction systems require Mn^{2+} (Zaharik & Finlay, 2004). In addition, enzymes such as manganese-cofactored superoxide

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Abbreviations: ABC, ATP-binding cassette; ICPMS, inductively coupled plasma mass spectrometry; WT, wild-type.

dismutase (SodA) play direct roles in virulence of some pathogens, probably by detoxifying superoxide generated by phagocytes (Verneuil *et al.*, 2006). However, it has also been suggested that a key role for Mn^{2+} ions is to protect bacterial components from damage by reactive oxygen species (Forman *et al.*, 2010; Johnston *et al.*, 2006). The molecular mechanisms of protection are not clarified. There is some evidence that Mn^{2+} ions directly scavenge superoxide or peroxide (Imlay, 2008). Alternatively these ions may be able to compete with free iron for binding sites on proteins or nucleic acids and are thus able to minimize the damaging consequences of the Fenton reaction which occurs between peroxide and bound iron (Anjem *et al.*, 2009; Imlay, 2008).

There are three species of the genus *Yersinia* that are pathogenic for humans: *Yersinia pseudotuberculosis*, *Y. pestis* and *Y. enterocolitica*. *Y. pseudotuberculosis* and *Y. pestis* are closely related at a genetic level (Chain *et al.*, 2004; Thomson *et al.*, 2006) and pathogenic yersinia possess many common virulence factors (Brubaker, 1991; Carniel, 1999, 2002). Within the mammalian host there is evidence that the human pathogenic *Yersinia* species can survive both within and outside of host cells (Pujol & Bliska, 2005; Small *et al.*, 1987), and the ability to switch between these lifestyles is central to the pathogenesis of disease. The replication of yersinia in a range of cell types including macrophages and epithelial cells has been reported (Brubaker, 1991; Carniel, 1999; Horsburgh *et al.*, 2002) and the PhoP/Q system regulates survival in macrophages in both *Y. pestis* and *Y. pseudotuberculosis* (Grabenstein *et al.*, 2004). The PhoPQ regulon has been shown to sense changes in Mg^{2+} levels, which are reduced intracellularly. However, the sensing, transportation and role in virulence of other ions *in vivo*, such as Mn^{2+}, are unknown. In *Streptococcus pneumoniae*, the concentration of Mn^{2+} ions can influence virulence gene expression (Johnston *et al.*, 2006; Rosch *et al.*, 2009), and as metal ion homeostasis is essential for cellular physiology, transporters have been suggested as novel antimicrobial targets (Jakubovics & Valentine, 2009). In this study we investigate the role of the human pathogenic yersinia putative Mn^{2+} transporter, MntH, in survival within phagocytic vacuoles, providing new insight into the role(s) of Mn^{2+} ions in protecting bacteria from killing by phagocytes.

METHODS

Plasmids, bacterial strains and culture conditions. Plasmids and bacterial strains are listed in Table 1. Wild-type (WT) *Y. pseudotuberculosis* IP32953 was maintained in Luria–Bertani (LB) broth or on LB agar, incubated at 28 °C. LB broth or agar plates supplemented with either 50 μg kanamycin ml^{-1} or kanamycin and chloramphenicol (both 50 μg ml^{-1}) were used to culture the IP32953 Δ*mntH*::kan^r mutant or the complemented strain, respectively. *Y. pseudotuberculosis* was cultured at 28 °C on congo red magnesium oxalate (CRMOX) agar to confirm the presence of virulence plasmid pYV (Anderson *et al.*, 2009; Jakubovics & Valentine, 2009). Prior to stress assays, bacteria were grown in M9 minimal salts medium supplemented with 0.2 % glucose, 2 mM $MgSO_4$, 100 μM $CaCl_2$, 0.3 μM $MnCl_2$ (final concentration). *Escherichia coli* was cultured in LB broth or on LB agar at 37 °C.

Construction of a *Y. pseudotuberculosis mntH* mutant. Construction of an *mntH*-deficient mutant was carried out by using a previously published method (Datsenko & Wanner, 2000). All primers used are listed in Table 2. Briefly, primers were designed for *mntH* to be disrupted (Yptb2705_kan_for and Yptb2705_kan_rev) that included 20 bp complementary to the 5′ or 3′ kanamycin resistance cassette of the plasmid pUK4K followed by 50 bp of upstream or downstream sequence of the yersinia genome flanking the *mntH* gene. PCR products were generated by using the plasmid pUC4K as a template. Excess template was digested with *Dpn*I and the PCR product was purified by using the PCR cleanup kit (Qiagen). PCR products were transformed into *Y. pseudotuberculosis* IP32953/pAJD434 by electroporation. Following overnight incubation at 28 °C in LB supplemented with 0.8 % arabinose, transformants were selected on LB agar supplemented with kanamycin (50 μg ml^{-1}) and trimethoprim (100 μg ml^{-1}) for 48 h at 28 °C. Transformants were verified by PCR using screening primers Yptb2705_for and Yptb2705_rev. Mutant strains were cured of the pAJD434 plasmid by growth at 37 °C in LB medium supplemented with kanamycin (50 μg ml^{-1}). Cured mutant strains were screened for the virulence plasmid pYV by PCR with Yscp_for and Yscp_rev primers. The presence of the virulence plasmid was also confirmed by culture on CRMOX plates.

Complementation of the *mntH* mutation. The *mntH* gene of strain IP32953 was amplified by PCR (using primers Yptb2705_com_for and Yptb2705_com_rev) introducing *Xba*I and *Sph*I restriction sites to the amplicon, which was ligated into the pBAD33 vector. The plasmid was transformed into *E. coli* XL1 Blue MRF′ (Agilent Technologies) following the manufacturer's protocol. Transformants were selected on LB agar supplemented with 50 μg chloramphenicol ml^{-1} and confirmed by sequencing. The construct p2705-10Δsph was subsequently transformed into *Y. pseudotuberculosis mntH* by electroporation. Transformants were selected on LB agar supplemented with 50 μg kanamycin ml^{-1} and 50 μg chloramphenicol ml^{-1} and confirmed by PCR using primers Yptb2705_for and Yptb2705_rev (Table 2). The complemented strain produced two bands of 1.2 and 0.87 kb corresponding to mutant and WT alleles respectively, and was designated IP32953 *mntH*/*mntH*$^+$.

Inductively coupled plasma mass spectrometry (ICPMS). To determine the metal content of *Y. pseudotuberculosis*, *Y. pseudotuberculosis mntH* and the complemented *mntH*/*mntH*$^+$ strain, cultures were grown in 5 ml minimal salts medium with no added manganese overnight at 28 °C to deplete intracellular manganese. LB broth cultures (100 ml) were started from minimal salts cultures at OD_{590} 0.01 and grown overnight, shaking at 28 °C. These cultures were centrifuged at 4000 r.p.m. for 20 min. The wet cell pellet weight was measured and bacteria were chemically lysed using 5 ml Bugbuster (Novagen) (gram wet pellet cell paste)$^{-1}$ according to the manufacturer's instructions. Bacteria were resuspended in Bugbuster solution by pipetting and incubation on a rotating mixer at a slow setting for 20 min. Total protein for each sample was measured by using a Bio-Rad protein assay according to the manufacturer's instructions. Wet pellet weight and total protein for each sample were noted. Each sample was diluted 100-fold in 2 % molecular grade nitric acid and an internal control was added to a total volume of 25 ml (0.25 ml sample, 0.125 ml internal control, 24.625 ml 2 % nitric acid). Samples were analysed by ICPMS (Thermo X 1 series) for the presence of Mn^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+}, and the results were corrected using the appropriate buffers for reference and dilution factors using plasma lab software. Triplicate cultures of each strain were analysed during a single experiment, each sample was tested three times (technical replicates) and the experiment was repeated twice.

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Description	Reference
Strains		
Y. pseudotuberculosis		
IP32953	Wild-type	Chain *et al.* (2004)
IP32953 *mntH*	*mntH*::kan^r mutant of IP32953	This work
IP32953 *mntH*/*mntH*$^+$	*mntH*::kan^r mutant of IP32953, complemented	This work
E. coli		
XL1 Blue MRF′	Complemented with pBAD33*mntH*	Agilent Technologies
Plasmids		
pGEM-T-Easy	Cloning vector	Invitrogen
pAJD434	Encodes λ red recombinase genes under the control of an arabinose inducible promoter	Maxson & Darwin (2004)
pBAD33	Complementation vector, low copy number, chloramphenicol resistant	Guzman *et al.* (1995)
pBAD33mntH	Complementation construct (*mntH* and upstream native promoter cloned into pBAD33)	This work
pUC4K	Plasmid containing kanamycin resistance cassette	Taylor & Rose (1988)

Stress assays. Experiments were undertaken to determine the susceptibility of *Y. pseudotuberculosis* IP32953 or IP32953 *mntH* to hydrogen peroxide (H_2O_2). The spontaneous oxidation of pyrogallol (1,2,3-trihydroxybenzene) generates O_2^- and H_2O_2. Bacteria were cultured for 24 h in M9 minimal salts medium or LB broth and then diluted to an OD_{590} of 0.1. In three experimental replicates, aliquots of 100 µl *Y. pseudotuberculosis* IP32953 were spread on LB agar containing selective antibiotics. Aliquots of 100 µl IP32953 *mntH* and IP32953 *mntH*/*mntH*$^+$ were spread on LB agar (supplemented with kanamycin or kanamycin and chloramphenicol where appropriate) in triplicate. Filter paper discs (5 mm diameter) were placed in the centre of the plates to which 5 µl 1 M pyrogallol was added (Sigma-Aldrich). The cultures were incubated at 30 °C overnight for 24 h, and zones of inhibition were measured. Triplicate samples were included for each experiment, and the experiment was repeated three times.

Pyrogallol disc sensitivity assay. The spontaneous oxidation of pyrogallol (1,2,3-trihydroxybenzene) generates O_2^- and H_2O_2. To test susceptibility to exogenous superoxide anions, IP32953, IP32953 *mntH* and IP32953 *mntH*/*mntH*$^+$ were grown overnight in LB broth supplemented with 50 µg kanamycin ml^{-1} and diluted to OD_{590} 0.1. In three experimental replicates, aliquots of 100 µl *Y. pseudotuberculosis* IP32953 were spread on LB agar containing 1000 units catalase ml^{-1} (added to degrade H_2O_2). Aliquots of IP32953 *mntH* and IP32953 *mntH*/*mntH*$^+$ (100 µl) were spread on LB agar supplemented with kanamycin and 1000 units catalase ml^{-1}, in triplicate. Filter paper discs (5 mm diameter) were placed in the centre of the plates to which 5 µl 1 M pyrogallol was added (Sigma-Aldrich). The plates were incubated overnight at 30 °C and zones of inhibition surrounding the discs were measured.

Macrophage infection studies. Aliquots (1 ml) of 18 h cultures of *Y. pseudotuberculosis* IP32953, IP32953 *mntH* and IP32953 *mntH*/*mntH*$^+$ grown at 28 °C were centrifuged at 10 000 ***g*** for 5 min at 22 °C, resuspended in 100 µl 30 % mouse serum (Sigma-Aldrich) and incubated at 37 °C for 20 min. These opsonized bacteria were then adjusted to OD_{590} 0.01 in 50 ml of warmed L15 media (Sigma-Aldrich). Retrospective viable counts were determined by culture on LB agar.

The construction of Raw264.7 cells expressing wild-type (WT3) or mutant (MUT12) Nramp1 has been described previously (White *et al.*, 2004). Cells were routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal calf serum, 50 units penicillin ml^{-1}, 50 µg streptomycin ml^{-1} and 2 mM L-glutamine. Cells were seeded at a density of 2×10^5 ml^{-1} in Dulbecco's modified essential medium (Sigma-Aldrich) into 24-well tissue culture dishes and cultured for 24 h. The tissue culture medium was removed and monolayers were washed three times with PBS. One millilitre (10^6 cells) of the bacterial suspension in PBS was added, and the cells were incubated at 37 °C for 1 h. The suspension above the cell monolayer was removed, and the cells were washed three times with PBS. One millilitre of L15 medium containing 50 mg gentamicin ml^{-1} was

Table 2. Oligonucleotides used in this study

Name	Sequence (5′–3′)	Comments
Yptb2705_kan_for	GATACATCTCGTCGCCCATTAAGGAAGATTAAACTCTCCTTG-ATGGGGCCCACAGGAAACAGCTATGACC	Mutagenesis, forward
Yptb2705_kan_rev	CTGCAAGGTTATATAAGCCTCTTCGATTTCAGCAATGTTCTC-ATAACCATCAAGTCAGCGTAATGCTCTGC	Mutagenesis, reverse
Yptb2705_for	ATGCTTAATGGCCGTGCTGT	Screening, forward
Yptb2705_rev	AACAGTGGCCGCAGCATTAC	Screening, reverse
Yptb2705_com_for	ATGTGCTCTAGACAAATTTGAGGCAGGCGAGATTTTG	Complementation, forward
Yptb2705_com_rev	ATTAAGTGCATGCTAAAGCAGGCTAATCAGTAAATAGGC	Complementation, reverse
YscP_for	ATTAGAACCTGAGTATCAACC	Virulence plasmid pYV (*yscP* gene), forward
YscP_rev	AACAAATAACTCATCATGTCC	Virulence plasmid pYV (*yscP* gene), reverse

added, and the cells were incubated for 3 h at 37 °C. The cells were washed twice with PBS, and 1 ml L15 medium containing 10 mg gentamicin ml^{-1} was added to the cells. The cells were incubated at 37 °C. Twenty-four hours post-infection the growth medium was removed, the cells were washed with PBS, and 250 ml filter-sterilized water was added to the cells, which were lysed by aspiration. The lysate was diluted in PBS, and the number of viable cells was determined after growth at 28 °C for 48 h on LB agar. Triplicate samples were taken at all time points, and the assay was repeated three times.

Infection of *Galleria mellonella*. *G. mellonella* larvae (n=10) in three experimental replicates were infected with 10^6 c.f.u. IP32953, IP32953 *mntH* or IP32953 *mntH*/*mntH*$^+$ in 10 μl inocula by micro-injection (Hamilton syringe) in the right foremost leg. PBS injection and no injection controls were used (n=10, three experimental replicates). Survival at 72 h post-infection was recorded (Champion *et al.*, 2009).

Statistical analyses. Graphpad Prism software was used for all statistical analyses. Unpaired *t*-tests using Welch's correction were applied to pooled data from two or three experimental replicates for environmental stress, pyrogallol disc assays and *G. mellonella* bacterial load quantification.

RESULTS

Y. pseudotuberculosis mntH promotes the uptake of Mn^{2+} ions

We initially constructed a mutant of *Y. pseudotuberculosis* strain IP32953 in which the region corresponding to aa 8–281 of MntH was replaced with a kanamycin resistance cassette. This mutant was complemented by introducing a cloned DNA fragment incorporating a WT copy of the *mntH* gene along with an upstream region containing a putative promoter.

In our initial studies we tested whether MntH functions as a manganese-specific transporter in *Y. pseudotuberculosis*. ICPMS analysis of *Y. pseudotuberculosis* IP32953, IP32953 *mntH* and IP32953 *mntH*/*mntH*$^+$ showed a significant decrease (P=0.0027) in bioaccumulation of Mn^{2+} in the mutant compared with the WT (Fig. 1). Manganese bioaccumulation was partially restored in IP32953 *mntH*/*mntH*$^+$. However, no defect in bioaccumulation of Fe^{2+}, Cu^{2+} or Zn^{2+} was observed in the IP32953 *mntH* mutant (data not shown). These data suggest that MntH promotes specific uptake of Mn^{2+} cations.

Y. pseudotuberculosis mntH-defective mutant is susceptible to killing by H_2O_2 only when grown under manganese-limited conditions

To determine whether IP32953 *mntH* was susceptible to a range of environmental stresses, the survival of WT or MntH-deficient *Y. pseudotuberculosis* strains following exposure to a variety of stresses was compared. No significant difference in survival was detected between WT and MntH-deficient *Y. pseudotuberculosis* following exposure to low pH or temperature stress (data not shown).

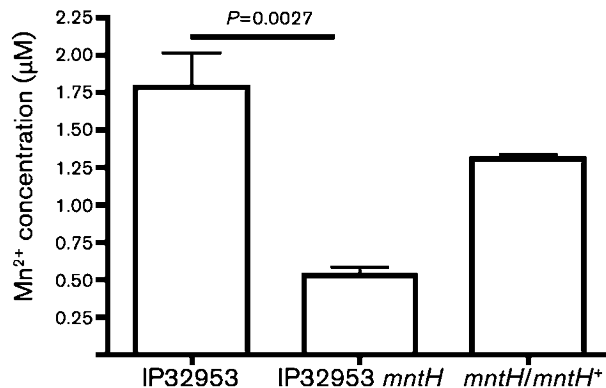

Fig. 1. Mn^{2+} accumulation in *Y. pseudotuberculosis* IP32953 (WT), IP32953 *mntH* mutant or complemented derivative IP32953 *mntH*/*mntH*$^+$ bacterial cells measured by ICPMS. Bacteria were cultured under low manganese conditions followed by the addition of LB broth. Error bars, SEM.

To investigate whether MntH plays a role in resistance to hydrogen peroxide, we grew bacteria under low manganese concentration conditions and then carried out pyrogallol disc diffusion assays on LB plates. The effect of peroxide on bacterial viability was assessed by measuring zones of inhibition. When grown under low manganese conditions in M9 minimal medium, IP32953 *mntH* showed a significantly increased (P=0.0195) sensitivity to peroxide (Fig. 2). The complemented strain showed a partial restoration of WT sensitivity. In contrast, when the bacteria were grown in rich medium (LB broth) there was no difference in the sensitivity of WT or mutant bacteria (data not shown).

Pyrogallol disc sensitivity assays on LB agar plates that had been impregnated with catalase were used to assess inhibition of the growth of WT, IP32953 *mntH* or IP32953 *mntH*/*mntH*$^+$ strains by exogenous superoxide anions. A significantly larger mean zone of inhibition

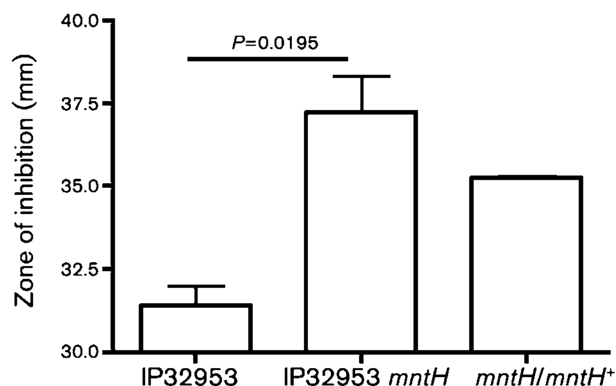

Fig. 2. Survival of *Y. pseudotuberculosis* IP32953 (WT), IP32953 *mntH* and complemented derivative IP32953 *mntH*/*mntH*$^+$. Bacteria were cultured under low manganese conditions after exposure to hydrogen peroxide. Error bars, SEM.

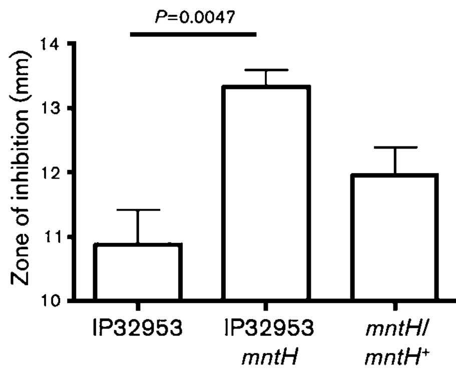

Fig. 3. Survival of *Y. pseudotuberculosis* IP32953 (WT), IP32953 *mntH* and complemented derivative IP32953 *mntH*/*mntH*$^+$ bacterial cells. Bacteria were cultured under low manganese concentrations after exposure to superoxide generated by pyrogallol treatment. Error bars, SEM.

(13.33 mm) was observed for IP32953 *mntH* than for WT bacteria (10.85 mm, $P=0.0047$) or the complemented mutant strain (11.95 mm, $P=0.0251$). There was no significant difference between the mean zone of inhibition between WT and the complemented strain, indicating that complementation had restored WT MntH activity (Fig. 3).

Differential ability of *Y. pseudotuberculosis* IP32953 *mntH* to survive in cells expressing functional Nramp1

It has previously been shown that a naturally occurring mutation in *Nramp1* (G169D) renders mice susceptible to pathogens such as *S. enterica* and *M. bovis*. These Nramp1 deficient mice are phenotypically indistinguishable from Nramp1$^{-/-}$ mutants (Vidal *et al.*, 1995).

To determine the influence of Nramp on the survival of WT or IP32953 *mntH Y. pseudotuberculosis* in macrophages, we infected RAW264.7 cells which expressed WT or G169D mutant (non-functional) Nramp1 (White *et al.*, 2004). No significant difference in the number of bacteria taken up by cells was observed based on enumerated bacteria recovered from lysed cells 1 h post-infection. Similarly, at 4 h post-infection there was no difference in the number of bacteria recovered from lysed cells (data not shown). However, 24 h after infection the cells were lysed and intracellular bacteria were enumerated. In cells expressing functional Nramp1, IP32953 *mntH* revealed a significant survival defect ($P=0.048$) compared with WT IP32953 (Fig. 4). Survival was restored to WT levels by complementation with an intact *mntH* gene. In contrast, in RAW264.7 cells which expressed G169D Nramp1 there was no difference in survival.

Contribution of MntH to virulence

G. mellonella larvae are used as an infection model for *Y. pseudotuberculosis* (Champion *et al.*, 2009). Larvae were

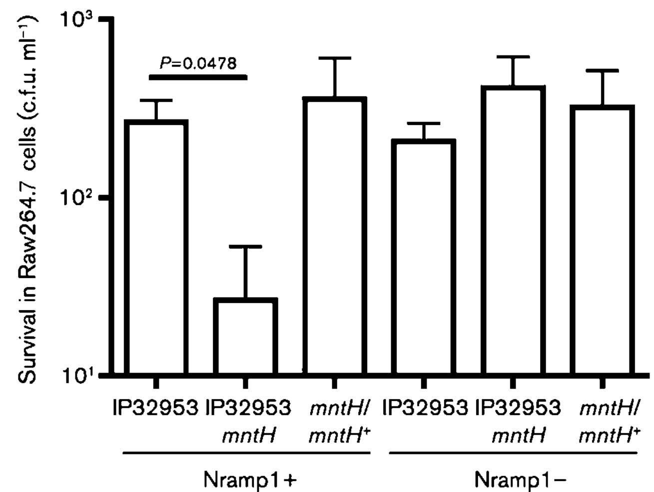

Fig. 4. Survival of *Y. pseudotuberculosis* IP32953 wild-type (WT), IP32953 *mntH* and complemented derivative IP32953 *mntH*/*mntH*$^+$ bacterial cells in Raw264.7 murine macrophages expressing Nramp WT (Nramp1+) or Nramp mutant (Nramp1−) proteins. $n=3$ per experiment and three experimental replicates were conducted; error bars, SEM. Bacteria were enumerated 24 h post-infection to measure bacterial survival.

infected with 10^6 c.f.u. IP32953, IP32953 *mntH* or IP32953 *mntH*/*mntH*$^+$. Survival at 72 h post-infection was observed. In all PBS-injected and uninfected controls, 100 % survival demonstrated that trauma from injection did not cause any larval mortality (data not shown). Larval survival following challenge with *Y. pseudotuberculosis* IP32953 *mntH* (mean 63 %) was significantly greater ($P=0.0442$) than survival following challenge with WT (mean 23 %). WT levels of larval killing were restored following infection with IP32953 *mntH*/*mntH*$^+$ (mean 30 %) (Fig. 5).

DISCUSSION

For many intracellular bacterial pathogens that have a lifestyle dominated by survival in the phagosome, the acquisition of manganese appears to be essential. Mn^{2+} transport via the natural resistance-associated macrophage protein (Nramp1) and ATP-binding cassette (ABC) family of transporters is widespread in both prokaryotes and eukaryotes (Papp-Wallace & Maguire, 2006). In eukaryotes, Nramp1 mediates Mn^{2+}, Fe^{2+} and Co^{2+} flux across the endosomal membrane, depleting the phagosome of cations (Jabado *et al.*, 2000). Eukaryotic expression of functional Nramp1 has been demonstrated to be a critical factor enabling macrophages to destroy a number of unrelated bacteria, all with an intracellular stage in their life cycle (Brown *et al.*, 1982; Glynn *et al.*, 1982; Plant *et al.*, 1982; Zwilling & Hilburger, 1994).

Bacterial orthologues of Nramp that have been characterized are selective Mn^{2+} transporters, named manganese

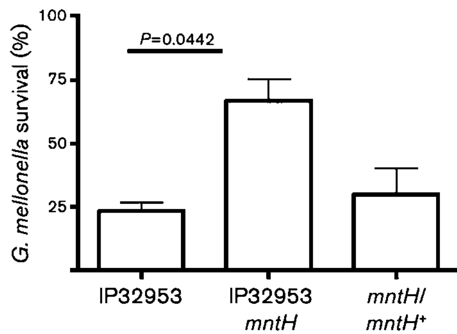

Fig. 5. Survival of *G. mellonella* at 72 h post-infection following challenge with *Y. pseudotuberculosis* IP32953, IP32953 *mntH* and complemented derivative IP32953 *mntH*/*mntH*$^+$ bacterial cells (10^6 c.f.u. per larvae) grown under low manganese conditions. $n=10$ per experiment and three experimental replicates were conducted; error bars, SEM. PBS-infected and uninfected controls were conducted for each experiment with 100 % survival. Pooled results are plotted.

transporter H (*mntH*), and are widespread in both Gram-negative and Gram-positive bacteria (Hao *et al.*, 1999; Horsburgh *et al.*, 2002; Kehres *et al.*, 2000; Que & Helmann, 2000; Reeve *et al.*, 2002). MntH mediates Mn^{2+} transport from the host phagosome into the bacterium resulting in competition between eukaryotic Nramp1 and prokaryotic MntH for phagosomal Mn^{2+}.

In *S. enterica*, the *mntH* gene encodes a manganese transporter that is required for resistance to H_2O_2-mediated killing (Govoni & Gros, 1998; Kehres *et al.*, 2002). In spite of the association of *mntH* with resistance to H_2O_2 killing, some of the previous studies on the role of *mntH* in virulence have failed to demonstrate a role in macrophage survival and virulence in mice. However, these studies have used macrophage cell lines such as J774 or RAW264.7 cells or BALB/c mice. These cells lines and mouse strains lack functional Nramp1 and therefore manganese may not have been limited in the phagosome. However, a role for MntH in virulence of *S. enterica* serovar Typhi has been revealed in mice expressing functional Nramp1 (Patzer & Hantke, 2001).

Nramp1 is expressed exclusively on professional phagocytic cells and is recruited to the membrane of phagosomes containing *Y. enterocolitica*, but the role of Nramp1 in controlling infection has not been reported (Djaldetti *et al.*, 2002). Our findings show that the survival of *Y. pseudotuberculosis* in macrophages is dependent on both host cell Nramp1 and bacterial MntH. The reduced ability of the *Y. pseudotuberculosis mntH* mutant to survive in macrophages was apparent only when cells expressing functional Nramp1 were infected. These data support the hypothesis that host Nramp1 and bacterial MntH compete for phagosomal manganese (Jabado *et al.*, 2000). Moreover, manganese accumulation in *Y. pseudotuberculosis* via MntH is critical for virulence in Nramp1-positive hosts. These data also strengthen the hypothesis that a phase of intracellular growth is required for *Y. pseudotuberculosis* infection.

The role of *mntH* in bacterial virulence might be obscured because of the redundancy provided by ABC transporters (Glynn *et al.*, 1982; Govoni & Gros, 1998; Kehres *et al.*, 2002). The *Y. pestis* YfeABCD transport system is involved in uptake of both iron and manganese (Bearden & Perry, 1999), and is required for full virulence of plague. Challenge studies using different mouse strains with either a functional or a non-functional *Nramp1* gene have not implicated a role for Nramp1 in resistance to the human pathogenic yersinia strains (Hancock *et al.*, 1986; Turner *et al.*, 2009). However, these studies were not conducted in Nramp1 isogenic mice and other genetic differences between the mouse strains used in these studies may have affected their resistance to infection by *Yersinia* species. In one report, a *yfeAB mntH* mutant of *Y. pestis* grew as well as the WT in J774 macrophages (Perry *et al.*, 2007). The use of J774 cells for this study may have obscured any phenotype. We have shown that the importance of MntH for *Y. pseudotuberculosis* survival is only apparent in macrophages expressing functional Nramp1. This suggests that it will be important to retest the *Y. pestis yfeAB mntH* mutant in cells expressing Nramp1.

Unlike *Y. pseudotuberculosis*, *Brucella abortus* possesses a single manganese transporter, MntH, inactivation of which results in attenuation of virulence. In *B. abortus*, *mntH* has an essential role in virulence in C57BL/6 mice that do not express a fully functional Nramp1 protein. Furthermore, *mntH* was critical for survival in primary macrophages from C57BL/6 mice (Anderson *et al.*, 2009). In *B. abortus*, pathogen bioaccumulation of manganese is solely dependent on MntH and so even in the absence of Nramp1, manganese accumulation will be severely limited. Taken together, these findings emphasize the critical nature of manganese in bacterial pathogenesis.

A role for manganese in bacterial pathogenesis has been clearly established yet the specific cellular role in which Mn^{2+} functions has not been clearly defined. In this study, we have shown that intracellular manganese accumulation is critical for *Y. pseudotuberculosis* survival during exposure to oxidative shock in low manganese conditions *in vitro*. Superoxide dismutase A (SodA) requires a manganese cofactor and functions in protecting the bacterium from cytotoxic superoxide during the respiratory burst in the host phagosome (Najdenski *et al.*, 2004). It is possible that the inability of the bacterium to accumulate Mn^{2+} results in decreased SodA formation and subsequent susceptibility to superoxide and attenuation of virulence.

Much work has established that, similar to many pathogens, acquisition of Fe^{2+} is essential for pathogenesis of *Yersinia* species (Perry, 1993). Bacterial manganese transport is regulated by MntR, an Mn^{2+}-specific transcription factor, as well as by Fur, PerR and OxyR. PerR and OxyR regulate Mn^{2+} transport in response to reactive oxygen. The mechanism by which Fur regulates manganese has not

been defined but probably relates to the relationship between cellular iron and manganese levels. The interplay of Fe^{2+} and Mn^{2+} homeostasis and the cross-regulation of the other regulons by both ions (Anjem *et al.*, 2009; Kehres *et al.*, 2002; Patzer & Hantke, 2001; Que & Helmann, 2000) suggest that manganese may substitute for iron in cells. However, internal levels of iron far exceed that of manganese (Anjem *et al.*, 2009; Outten & O'Halloran, 2001), indicating a specific role for manganese; otherwise it would be out-competed by iron. A role for manganese in iron homeostasis has been reported: severe limitation of manganese for *Bradyrhizobium* resulted in iron deficiency (Puri *et al.*, 2010). Analysis of the ion content of the *Y. pseudotuberculosis* IP32953 *mntH* revealed no difference in bacterial accumulation of Fe^{2+} relative to WT. However, in *Bradyrhizobium*, iron homeostasis was effected through haem, rather than Fe^{2+} (Puri *et al.*, 2010), which is in contrast with *E. coli* which relies on Fur/Fe^{2+} interactions (Escolar *et al.*, 1999). *Y. pseudotuberculosis* possesses both haemin and Fe^{2+} uptake systems, the majority of which are also possessed by *Y. pestis* (Forman *et al.*, 2010).

For most transport systems, the physiologically relevant cation transported by MntH has not been established. We have demonstrated that MntH is the dominant manganese transporter in *Y. pseudotuberculosis* based on ion accumulation. We have demonstrated that deletion of *mntH* in *Y. pseudotuberculosis* results in attenuation of virulence only in host cells where manganese is limited by Nramp1 expression. Mn^{2+} is associated with *Y. pseudotuberculosis* resistance to peroxide and superoxide through an unknown mechanism. To further understand the role that host Nramp1 status has in limiting intracellular bacterial access to manganese during infection and the subsequent effect on bacterial virulence requires *in vivo* isogenic Nramp1$^{+/+}$ and Nramp1$^{-/-}$ mouse studies. Moreover, the specific role(s) for manganese in the pathogenesis of disease caused by *Y. pseudotuberculosis* warrants further investigation.

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