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Functional assessment of Nramp-like metal transporters and manganese in *Caenorhabditis elegans*

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ABSTRACT

Nramp1 (natural resistance-associated macrophage protein-1) is a functionally conserved iron–manganese transporter in macrophages. Manganese (Mn), a superoxide scavenger, is required in trace amounts and functions as a cofactor for most antioxidants. Three Nramp homologs, *smf-1*, *smf-2*, and *smf-3*, have been identified thus far in the nematode *Caenorhabditis elegans*. A GFP promoter assay revealed largely intestinal expression of the *smf* genes from early embryonic through adult stages. In addition, *smf* deletion mutants showed increased sensitivity to excess Mn and mild sensitivity to EDTA. Interestingly, these *smf* deletion mutants demonstrated hypersensitivity to the pathogen *Staphylococcus aureus*, an effect that was rescued by Mn feeding or knockdown of the Golgi calcium/manganese ATPase, *pmr-1*, indicating that Mn uptake is essential for the innate immune system. This reversal of pathogen sensitivity by Mn feeding suggests a protective and therapeutic role of Mn in pathogen evasion systems. We propose that the *C. elegans* intestinal lumen may mimic the mammalian macrophage phagosome and thus could be a simple model for studying Mn-mediated innate immunity.

Introduction

Nramps (natural resistance-associated macrophage proteins) are a family of membrane proteins that facilitate the transport of heavy metal ions. Members of the Nramp family of protein transporters are evolutionarily conserved and are found in almost all organisms, from bacteria to man. In *Saccharomyces cerevisiae*, the three Nramp homologs Smf1p, Smf2p, and Sm3p, encoded by three distinct genes, are quite closely related [1]. Among the various divalent metals, these transporters are best known for manganese trafficking [2]. Increased levels or deprivation of intracellular manganese ions allows Smf1ps to shuttle between vacuoles and the plasma membrane for necessary degradation of the transporter or allows for manganese uptake from the environment, respectively [1].

The Nramps, in general, are well known to possess a significant conserved role in eukaryotic host defense. Besides being associated with innate resistance to certain bacterial infections, including several other infectious diseases in several vertebrate species, the Nramps in humans are known to play a crucial role in innate immunity [3]; therefore, they may be ascribed as the first line of intracellular defense against infection in humans. Likewise, mouse Nramp1 plays an important role in controlling infection by intracellular parasites and is exclusively expressed in monocytes/macrophages and polymorphonuclear leukocytes [4]. Nramp2, a more ubiquitously expressed transporter, acts as a divalent metal transporter capable of transporting iron, manganese, copper, zinc, cadmium, and lead [5].

Transition metals, including iron (Fe), zinc (Zn), and manganese (Mn), are essential cofactors for numerous proteins involved in vital functions, such as respiration, defense against oxidative stress, and cell division. Moreover, these metals are critical to both bacterial metabolism and virulence [6]. Depending on the microbe physiology, precise functions in survival, growth, and virulence may be affected by the amount of Mn ions available [7]. Transport of these essential metals out of macrophage phagosomes could exert pleiotropic effects on microbial metabolism and the capacity to perturb phagosome maturation.

In the nematode *Caenorhabditis elegans*, three Nramp type transporters, *smf-1*, *smf-2*, and *smf-3*, have been identified to date. *C. elegans*, a genetically tractable animal, is a facile and inexpensive model organism that has been used as a means for host–pathogen

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interaction studies, specifically to identify the host genes that interact with invading pathogens. In the present study we identified and characterized the Nramp homologs in *C. elegans*, namely, the three *smf* genes. The presence of these metal transporters in this model organism provides several advantages in determining their molecular identities. Mutants with deleted *smfs* were hypersensitive to excess Mn and displayed pathogen susceptibility, but subsequent Mn feeding facilitated the reversal of this phenotype. Our results describe the role of nematode *smfs* in Mn homeostasis and illustrate the function of Mn in the host–pathogen interaction. Recovery from pathogen sensitivity by Mn feeding suggests the protective and therapeutic potential of this metal in a pathogen evasion system. The *C. elegans* intestine may physiologically mimic the mammalian macrophage phagosome, functioning as a simple model for innate immune response studies.

Materials and methods

Caenorhabditis elegans strains, bacterial strains, and cosmid clones. *C. elegans* strains wild-type N2, *daf-16* (m26), *daf-2* (e1370), *smf-1* (ok1748), *smf-2* (gk133), and *smf-3* (ok1035) were obtained from the *Caenorhabditis elegans* Genetics Center (CGC) at the University of Minnesota, USA (Supplementary Material; Figure 1). Bacterial strains *Staphylococcus aureus* (KACC10196) and *Bacillus subtilis* (KACC10854) were obtained from the Korean Agricultural Culture Collection (KACC), Suwon, Korea. Cosmids K11G12 and Y69A2AR were obtained from A. Coulson at the Sanger Center, UK.

Construction of double mutant strains. Double mutants of *smf-1* (ok1748); *smf-3* (ok1035) and *smf-2* (gk133); and *smf-3* (ok1035) were constructed by standard genetic methods.

DNA constructs and microinjection. Approximately 1.5 kb of the 5′ upstream regions of *smf-1*, *smf-2*, and *smf-3* were amplified by PCR using cosmid K11G12 as a template for *smf-1* and *smf-2* and cosmid Y69A2AR as a template *for smf-3*. These PCR products were cloned into the promoterless *gfp* vector pPD95.77 to obtain *smf-1 pro::gfp*, *smf-2 pro::gfp*, and *smf-3 pro::gfp* (Supplementary Material; Figure 1). Microinjection was carried out as described [8].

Worm survival assays. For EDTA and manganese sensitivity experiments, L4 stage worms were transferred from normal NGM plates onto two different plates containing either 20 mM EDTA or 20 mM $MnCl_2$. Worms were incubated at 20 °C, and the number of dead animals was scored as described [9].

To examine pathogen susceptibility, *S. aureus* and *B. subtilis* strains were grown overnight at 37 °C in BHI liquid media. Bacterial lawns were prepared by spreading 10 μl of an original saturated bacterial culture diluted 10-fold onto 60-mm tissue culture plates containing solid BHI media (Difco). The plates were then incubated at 37 °C for 6 h, followed by 25 °C for 6 h. Worms were transferred to the plates and further incubated at 25 °C. The survival rate of each strain was scored after 3 days. Heat-killed *S. aureus* plates were prepared by heat treatment of the seeded plates at 65 °C for 2 h.

In the manganese (Mn) feeding experiment, L2 stage larvae were preincubated on NGM plates containing 1 mM $MnCl_2$ for 24 h and then washed on normal NGM plates for 24 h before being transferred to test plates.

In vitro transcription and RNA-mediated interference. Approximately 1.2 kb of *pmr-1* double stranded RNA was generated from yk218a11 cDNA clone as described earlier [9].

Statistical analysis. Data pertaining to survival rates of worms are presented as means ± SD. The survival rates of each animal strain were counted after 3 days following treatment. Forty animals were tested for each data point for a single experiment set, and each experiment was repeated six times. Data were evaluated by one-way analysis of variance (ANOVA) using the software Microcal Origin 6.0 (Microcal Software Inc., MA, USA). A level of $P < 0.05$ was considered the threshold for statistical significance between the control and the various experimental groups.

Results and discussion

The C. elegans smf genes

Thus far, three Nramp (natural resistance-associated macrophage protein)-like transporters, the *smf* genes, have been identified in the *C. elegans* database (see Wormbase; http://www.wormbase.org). The *smf* genes (*smf-1*, *smf-2*, and *smf-3*) have been physically mapped to Chromosome X (LGX) on cosmid K11G12 (*smf-1* and *smf-2*) and to Chromosome IV (LGIV) on cosmid Y69A2AR (*smf-3*). Both *smf-1* and *smf-2* have been identified as immediate neighboring genes on LGX (Supplementary Material; Figure 1). Alignment studies have shown that the worm Smf proteins show ~17–21% identity to yeast Smf proteins, ~41–48% identity to human Nramp1 or Nramp2, and ~42–59% identity to each other at the amino acid level (Supplementary Material; Figure 2).

Expression pattern of C. elegans smfs

In eukaryotes, the trafficking of manganese to antioxidant enzymes for enzyme activity is in part dependent on metal transporters like Nramps. In a step towards characterizing the physiological roles of the Nramp homologs in *C. elegans*, we first examined the temporal and spatial expression patterns of the *smf* genes. Transgenic worms containing the *smf-1 pro::gfp* or *smf-3 pro::gfp* construct demonstrated strong GFP expression at all stages of development, beginning as early as the comma stage embryo and continuing through larval and adult stages (Fig. 1A–J). Expression was spatially confined to intestinal cells, excretory cells, vulval epithelial cells, and neuronal cells. Fluorescence was largely observed at the apical ends of the adult and larval intestines (Fig. 1A, B, D, and G); in neuronal cells, particularly in the head neurons and hypodermis (Fig. 1E); in H-shaped excretory cells (Fig. 1F); and in vulval epithelial cells (Fig. 1H). Worms expressing the *smf-3 pro::gfp* construct exhibited distinct intestinal expression, which encompassed most of the intestine (Fig. 1I and J). In mammals, Nramp1s are abundantly expressed in the endosomal–lysosomal compartment of macrophages and are recruited to phagosomal membranes following phagocytosis [10]. In the present study, although *smf-1* and *smf-3* were expressed in various tissues and cell types, they were dominantly expressed in the intestines. Thus, they may have roles in the transport of metal ions in the intestine.

Under the control of the *smf-2* promoter, no expression of GFP was detected in the worms, indicating that the fragment selected as the putative promoter for *smf-2* may have lacked promoter activity. Besides being functionally related proteins, both *smf-1* and *smf*-2 physically mapped close together on Chromosome X (Supplementary Material; Figure 1) and therefore were found together in operons. *C. elegans* operons appear to be a means of coregulating functionally related proteins, similar to bacterial operons, and thus related genes do occur in operons [11].

SMF knockdown animals are sensitive to high concentrations of EDTA and Manganese

In order to investigate the *in vivo* function of the worm Smfs and to further study the transport activity of these metal transporters in response to metals, we first performed worm survival assays under high concentrations of EDTA (a metal ion chelator). Since SMFs are metal transporters, we reasoned that depletion of metals by a chelating agent could make the worms hypersensitive and have

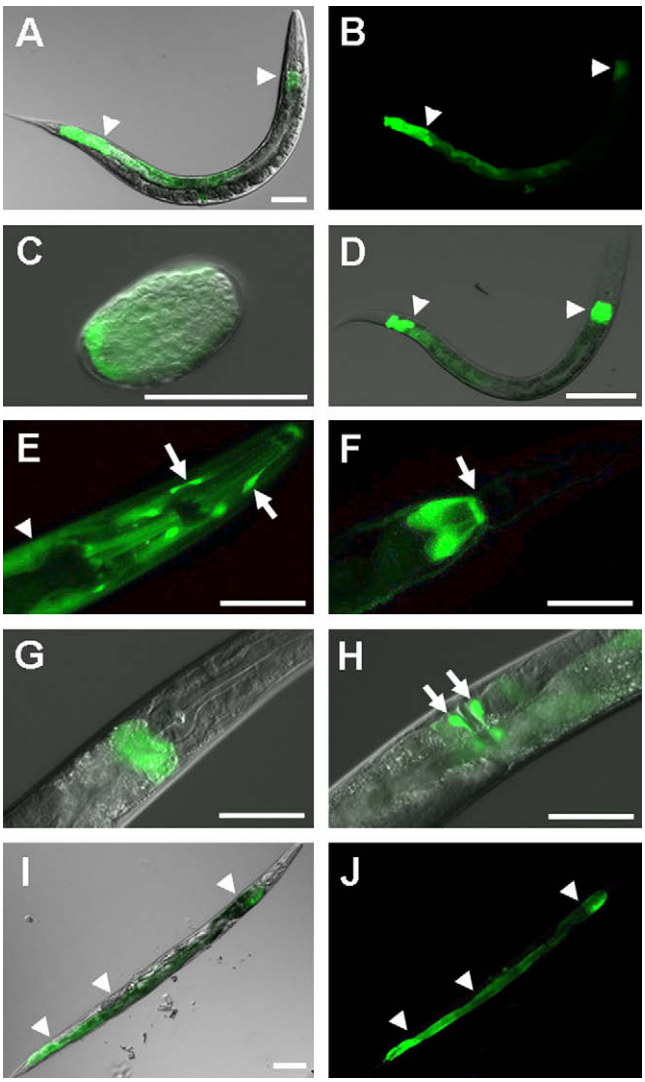

Fig. 1. GFP expression under the control of *smf* promoters. Transgenic animals expressing *smf-1::gfp* or *smf-3::gfp* were observed under a fluorescence microscope and photographed. (A,B) High GFP expression (from *smf-1 pro::gfp*) in an adult worm in the apical regions of the intestine (arrow heads); (C) in an embryo; (D) in apical regions of intestine in an L2 larva (arrowheads), (E) in head neurons (arrows) and the hypodermis (arrowhead), (F) in H-shaped excretory cells (arrow), (G) in the anterior intestine, and (H) in vulval epithelial cells (arrows). (I,J) Under the control of *smf-3* promoter, adult worms display strong GFP expression along the intestine (arrowheads). Each scale bar indicates 50 μm.

an effect on the overall survival of the worms. We grew the worms on plates containing 20 mM EDTA and scored the survival rate. As expected, the survival rates of the *smf* mutant worms in presence of 20 mM EDTA decreased in comparison to the control untreated mutant animals (Fig. 2; Supplementary Material, Table 1; $P < 0.01$). This overall trend of reduced survival rate was comparable to the wild-type N2 animals treated with 20 mM EDTA ($P < 0.05$). We further tested the effect of the metal ion chelator on *smf* double mutants and observed a similar trend in worm survival rates (Fig. 2 and Supplementary Material; Table 1).

We also studied the role of these worm Smfs in metal homeostasis by performing survival assays under different Mn^{2+} stresses. Although Mn is nutritionally essential for the growth and survival of all living organisms, given that it functions as a redox cofactor for some enzymes and as an activator at metal binding sites of other enzymes [12], an excess of Mn could be a growth limiting constraint. When the plates were supplemented with high concentrations of Mn^{2+}, the survival rates of *smf* mutants dramatically decreased (Fig. 2 and Supplementary Material, Table 1; $P < 0.01$ or $P < 0.001$). We concurrently examined the survival rates of insulin/IGF-1 signaling pathway-related mutants, e.g., *daf-16* and *daf-2* mutants [13]. *daf-2* mutants are known to be resistant to different stressors, such as pathogens or oxidative stress, while *daf-16* mutants are stress-sensitive. Therefore, we used these strains as negative and positive controls, respectively, for subsequent experiments [14].

Among the three Nramp transporters in yeast (Smf1p, Smf2p, and Smf3p), the Smf3p transporter affects iron trafficking [15] whereas Smf1p and Smf2p appear to function in manganese homeostasis. Moreover, *smf1Δ* mutants exhibit greater sensitivity to metal chelators than *smf2Δ* mutants [16]. These observations are to some extent contradictory to ours, however, in that the mutants in our study responded differently when exposed to high concentrations of the metal ion chelator EDTA versus high Mn stress. In fact, the *smf-2* mutants demonstrated a greater response to high Mn stress ($P < 0.001$) compared to the *smf-1* or *smf-3* mutants. This could be explained by the fact that the response to marked perturbation of Mn^{2+} ion homeostasis in a deleted *smf-2* was more severe than in the other mutants, which may indicate that *smf-2* plays a more significant role in metal trafficking in the nematode.

SMF mutant animals are sensitive to pathogens

Caenorhabditis elegans has been used as a model host for the identification of virulence genes in pathogens, and has also been used to identify the host genes that interact with invading pathogens. Pathogenic *S. aureus* causes a wide array of diseases and is one of the fastest killers that infect the worm [17]. In order to test the effect of invading pathogens on worms with defective metal transporters, such as SMFs, we allowed wild-type N2 worms and *smf* mutant worms to grow on plates seeded with the pathogenic *S. aureus* or with the non-pathogenic *B. subtilis* and subsequently scored the survival rate. We observed an overall reduced survival rate for all three of the *smf* mutants in comparison to the N2 wild-type worms (Fig. 3 and Supplementary Material, Table 1; $P < 0.001$). A similar trend was also observed for the double mutants. The worms grew normally, however, and displayed normal survival rates when grown on heat-killed *S. aureus* or on non-pathogenic *B. subtilis* (Fig. 3A).

It is well known that both infection and injury cause cellular stress. To address the question of whether pathogen stress can provoke *smf* expression, we used *smf-1 pro::GFP* and *smf-3 pro::GFP* transgenic lines and allowed them to grow on plates seeded with *S. aureus*. We found that GFP expression, which was originally localized to the intestinal ends, displayed greater GFP expression throughout the intestine upon pathogen stress (Fig. 3B). This suggests that *smf-1*, *smf-3*, or both are transcriptionally induced upon pathogen stress. It further implies that metal transporters such as Smfs may have important roles in the innate immunity of the worm by acting predominantly in the intestines.

Manganese uptake in worms is essential for recovery from pathogen susceptibility and for the activity of the innate immune system

Nramp1 may weaken a key defense mechanism of many microbes by depleting the phagosomal lumen of Mn^{2+} [18]. Manganese not only plays an important role as a cofactor for the majority of antioxidant enzymes, but also functions as a scavenger of superoxide radicals [15]. Therefore, we were interested in assessing whether the hypersensitivity to *S. aureus* could be alleviated by manganese feeding. We allowed the pathogen-fed animals (both wild-type and *smf* single and double mutants) to feed on 1 mM $MnCl_2$ and then scored their survival rate. Interestingly, the animals that otherwise showed reduced survival rates when

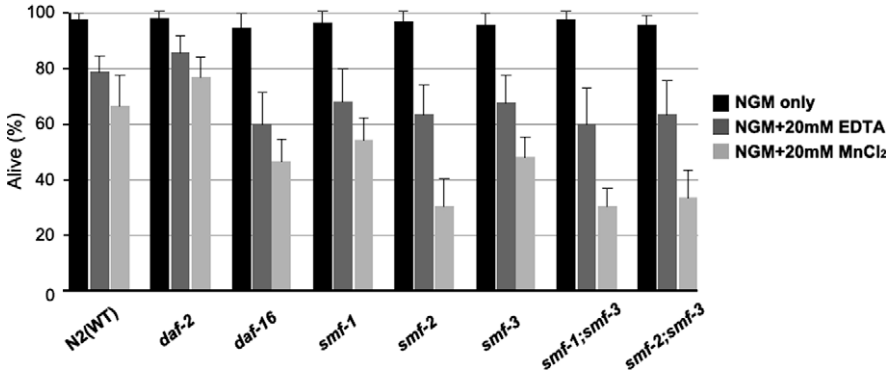

Fig. 2. SMF deletion mutants are sensitive to high concentrations of EDTA and excess manganese. Young adult worms were laid on NGM plates and on NGM plates with 20 mM EDTA or 20 mM $MnCl_2$. The survival rates of each strain were counted after 2 days. We tested 40 animals for each data point of a single experimental set, and each experiment was repeated six times. *daf-2* and *daf-16* stains were used as controls. Statistical analysis was performed using one-way ANOVA.

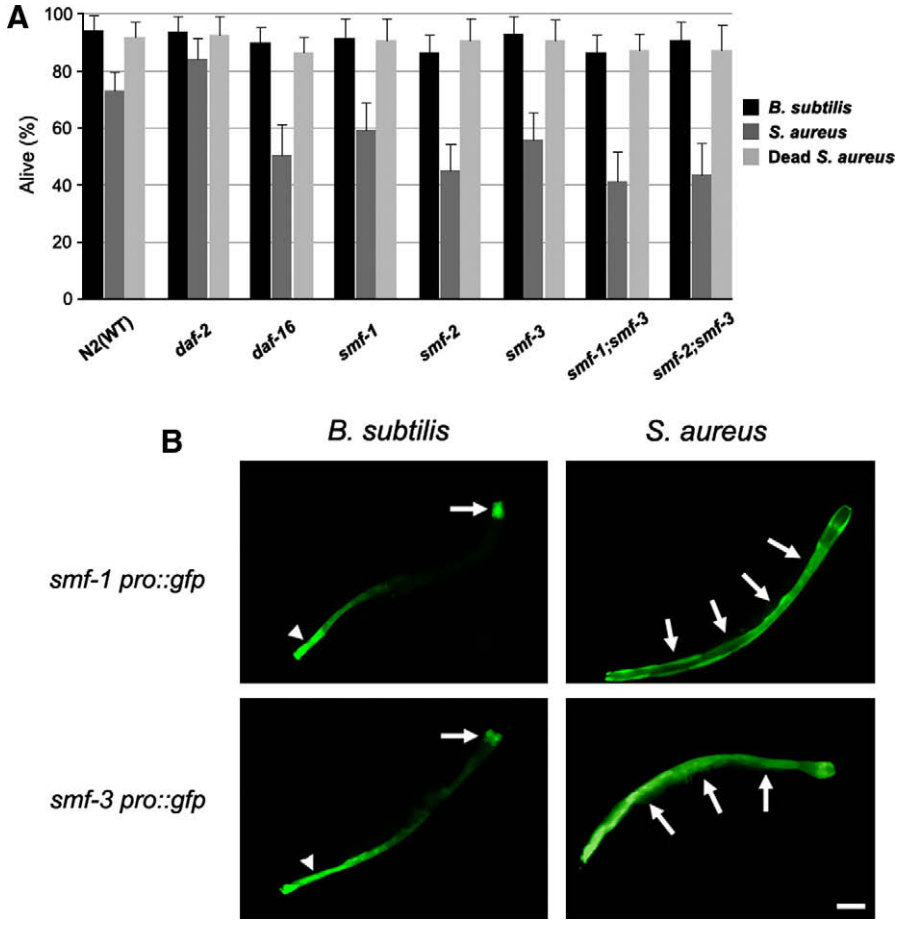

Fig. 3. SMF knockdown animals are sensitive to pathogens. (A) Young adult worms were laid on non-pathogenic *B. subtilis*, pathogenic *S. aureus*, or heat-killed *S. aureus* seeded on BHI plates. The survival rates of each strain were counted after 3 days. We tested 40 animals for each data point of a single experimental set, and each experiment was repeated six times. *daf-2* and *daf-16* strains were used as controls. Statistical analysis was performed using one-way ANOVA. (B) *smf-1*, *smf-3*, or both were transcriptionally induced upon pathogen stress. The *smf-1 pro::gfp* line and *smf-3 pro::gfp* line exposed to non-pathogenic *B. subtilis* showed strong intestinal GFP expression at the anterior (arrow) and posterior (arrowhead) ends. Adult *smf-1 pro::gfp* line and *smf-3 pro::gfp* lines exhibited even stronger GFP expression throughout the whole intestinal (arrows) under pathogenic *S. aureus* stress. Each scale bar indicates 50 μm.

fed on *S. aureus* displayed a significant reduction in pathogen-induced hypersensitivity upon 1 mM $MnCl_2$ feeding, particularly the *smf* mutants ($P < 0.001$). This signifies that Mn uptake is obligatory to build up the innate immune system (Fig. 4).

The *C. elegans* PMR1 (CePMR-1) protein, a Golgi calcium/manganese ATPase, is known to transport Ca^{2+} and Mn^{2+} into the Golgi apparatus when expressed in COS-1 mammalian cells [9]. Moreover, PMR1 in *C. elegans* is important for the regulation of Ca^{2+} and Mn^{2+} ions. We previously reported that PMR-1 knockdown can render animals resistant to stresses, such as oxidative stress [9]. Concurrent with these findings, we were interested in examining whether hypersensitivity towards pathogen stress could also be reduced by knockdown of the *pmr-1* gene. In order to test this, we performed *pmr-1* RNAi on *smf* single and double mutants following *S. aureus*

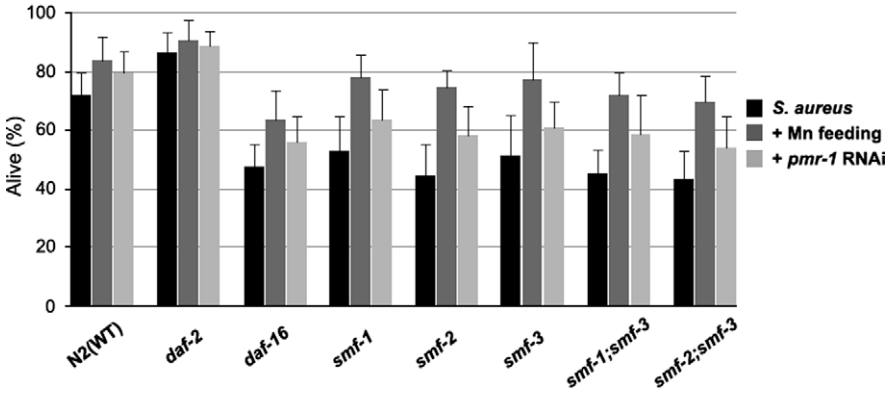

Fig. 4. Manganese uptake is essential for recovery from pathogen stress. Young adults grown on *S. aureus* BHI plates for 1 day were supplemented with 1 mM $MnCl_2$ or *pmr-1* RNAi. The survival rates of each strain were counted after 3 days. We tested 40 animals for each data point of a single experimental set, and each experiment was repeated six times. Statistical analysis was performed using one-way ANOVA.

pathogen stress. Because PMR-1 is a Ca^{2+}/Mn^{2+} ATPase pump, we reasoned that knockdown of *pmr-1* would make *smf* mutant animals resistant to pathogenic stresses in terms of survival. As anticipated, the RNAi worms responded differently to the pathogen stress. Interestingly, *pmr-1* RNAi animals pre-exposed to *S. aureus* displayed a reasonably reduced sensitivity towards the pathogenic stress (Fig. 4; $P < 0.05$). In other words, the absence of *pmr-1* resulted in the recovery of pathogen susceptible worms or rendered the worms resistant to the pathogen (Fig. 4). This could be due to the fact that Mn in the cytosol was partly elevated by *pmr-1* knockdown, which probably augmented the host's Mn-dependent antioxidant machinery ([9] and this study).

Although Nramp1 orthologs are typically recognized for their functions in metal ion trafficking, the most significant role that has been established for these metal transporters is their conserved role in eukaryotic host defense systems. Because metal cofactors like iron and manganese are obligatory in various physiological functions, including cellular replication and protection against oxidative stress, their cellular levels influence the survival, growth, and virulence of microbes [7]. Systems lacking specific transporter(s) have been shown to exhibit defects in antioxidant functions due to defective metal trafficking. Maintaining a delicate homeostatic balance between oxidant generation, antioxidant protection, and repair of ROS-induced damage appears to be intimately connected to the regulation of various physiological parameters. In the nematode, it is clear that the immune system is assisted by the generation of ROS to fight invading pathogens. Metals like Mn, however, would be required by the antioxidant machinery to counteract this oxidative stress. Thus, enhanced generation of ROS in the *smf* mutants could be a plausible explanation for their pathogen susceptibility. However, in *smf* mutants under these prescribed conditions, pathogens demonstrated a better survival rate in the host. Thus, ROS function as a double-edged sword in combating pathogens and carrying out essential cellular functions on one hand and causing cellular damage via increased oxidative injury on the other hand.

The *C. elegans* intestine presents many advantages because this system can mimic the host–pathogen interactions that occur during phagocytosis. It remains the best approach to advance our knowledge of macrophage phagosome biology, particularly when used in conjunction with functional approaches. Macrophages play a pivotal role in the resolution of microbial infections via the process of phagocytosis. Many microorganisms, however, have evolved efficient strategies to obstruct the weaponry of macrophages. Just as a better understanding of the components involved in phagosome formation and maturation is necessary to devise novel approaches aimed at counteracting these microbial strategies, the roles of the Nramp homologs of the Smf family can be similarly dissected, particularly with regard to connecting the worm intestinal lumen with the macrophage phagosome, which may contribute substantially to Mn-mediated innate immunity. Furthermore, recovery from pathogen sensitivity in response to Mn feeding suggests a protective and therapeutic role of the metal in pathogen evasion systems. Further experiments are necessary to develop the worm as a model for manganese toxicology studies and to gain insight into the biology of pathogen–host invasion, with special attention to survival rates according to functional metal transporters.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.09.082.

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