Biochemical and Biophysical Research Communications 459 (2015) 481–487

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Molecular mechanisms of resistance to human pathogenic bacteria in *Caenorhabditis elegans* by MEV-1 mediated oxidative stress

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ARTICLE INFO

Article history:
Received 17 February 2015
Available online 5 March 2015

Keywords:
Mitochondrial superoxide
mev-1
Oxidative stress
Infection
DAF-16

ABSTRACT

Both mutation and knockdown in *mev-1* gene render *Caenorhabditis elegans* susceptibility to *Enterococcus faecalis* infection. However, the mechanisms by which of MEV-1 defects pathogen resistance remain unclear. Here we show that *mev-1*RNAi causes a dramatic decrease in oxidative stress and antioxidant enzyme expressions, thereby leading to increased *E. faecalis* accumulation in nematode intestine. Mitochondrial superoxide change after infection induced these oxidative responses through DAF-16 activity. All together, this highlights MEV-1 as a key regulatory component for determining genetic responsiveness to oxidant/antioxidant imbalance that is associated with innate immunity.

1. Introduction

A number of human pathogens can infect *Caenorhabditis elegans* including the gram-positive bacterium *Enterococcus faecalis* [1–3], and trigger conserved host defense mechanisms [4]. These features make *C. elegans* a favorable model to study the innate immunity of humans and other animal hosts [5,6]. In contrast to other model animals, *C. elegans* lacks professional immune cells and no PRRs have yet been identified [7]. This raises the hypothesis of a more ancient mechanism as a major resistance strategy for *C. elegans*. It is therefore not surprising that reactive oxygen species (ROS) has been widely studied in the nematode defense mechanism against bacteria [8–10]. In particular, the essential roles of mitochondrial ROS (mROS) in immunity have clearly been established in many species [10–12]. Recently, there is increasing that the mROS homeostasis is crucial for immune systems against pathogenic bacteria [13]. The molecular mechanism by which ROS homeostasis influences the *C. elegans* innate immune remains unclear.

Defects in mitochondrial machineries can affect or distort their functions in a large number of physiological processes [14]. For example, *mev-1* mutations in complex II of mitochondrial electron transport chain (mETC) causes increased susceptibility to *E. faecalis* infection (Feng, under review). The *mev-1* phenotypes also result in overproduction of mROS and short life span, which is similar to the observed in *daf-16 C. elegans* [15,16]. This suggests a possible link between MEV-1 and DAF-16. The DAF-16 is an important regulator of genes that rapidly respond to and neutralize the effects of oxidative stress, such as catalase and superoxide dismutase (SOD) [17,18]. Actually, the DAF-16 resides in the cytoplasm and only becomes translocated to nuclei upon activating stimuli [10]. In addition, the DAF-16 protein highly resides in the nuclei of *mev-1* mutants even under normal growth conditions [19].

In this study, we investigated the regulatory mechanisms underlying the effect of *mev-1* mediated oxidative stress in *C. elegans* when *E. faecalis* infection. Influences of mitochondrial superoxide on the expression of the antioxidant enzymes superoxide dismutase (SOD) and glutathione S-transferase (GST) were also examined. Moreover, we dissected the genetic requirements that are attributable to the increased oxidative stress response in *C. elegans*.

2. Materials and methods

2.1. Nematode and bacterial strains and growth conditions

The *C. elegans* strains used in this study were: wild-type N2 Bristol, CF1038 (*daf-16(mu86)I*), TJ356 (*daf-16::GFP (zIs356 IV)*),

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http://dx.doi.org/10.1016/j.bbrc.2015.02.132
0006-291X/

CF1553 (*sod-3p::GFP (muIs84)*) and CL2166 (*gst-4p::GFP(dvIs19 III)*). All worm strains were obtained from the *Caenorhabditis* Genetics Center (CGC). Growth and manipulation of *C. elegans* were performed as previously described [20] at 20 °C. *E. faecalis* OG1RF was a kindly gift from the First Affiliated Hospital of Lanzhou University (ATCC 47077). *E. faecalis* OGRF1:pMV158 was a kindly gift from Ludek Zurek [21]. Both of the *E. faecalis* strains were grown on Brain Heart Infusion (BHI) medium at 37 °C. *Escherichia coli* OP50, *E. coli* OP50:pFVP25.1and *E. coli* HT115 (DE3) were obtained from CGC and cultured on Luria Bertani (LB) media supplemented appropriate antibiotics.

2.2. RNA interference

RNAi expressing clone were constructed as follows. Briefly, total RNA was extracted from wild type *C. elegans* L4 larvae using RNAiso Plus (Takara) and synthesized cDNA using Super-scriptII reverse transcriptase (Invitrogen). Target genes were amplified and cloned into the vector pL4440 (Addgene plasmid 1654) [22] and then transformed into *E. coli* HT115 (DE3). The clone was verified by sequencing. The sequences of primers can be provided on request. Cultures of HT115(DE3) containing empty vector (pL4440) or target gene were prepared in LB broth containing 100 μg/ml ampicillin and 5 μg/ml tetracycline and grown overnight at 37 °C. Optical density values at 590 nm (OD 590) were adjusted to 0.9. Plates for the RNAi assay were prepared as follows. Bacteria cultures were spread onto plates (NGM agar + 1 mM IPTG, 100 μg/ml ampicillin, and 5 μg/ml tetracycline) and incubated at 37 °C for 24 h. For double RNAi, cultures containing two genes prepared above were mixed to ratio of 1:1. RNAi was induced by feeding synchronized L1 worms through L4 stage with bacteria prepared above to target relative genes [23].

2.3. Killing assays

E. faecalis OG1RF and *E. faecalis* OGRF1:pMV158 grown in BHI medium for 5 h were seeded on BHI plates and incubated at 37 °C for 24 h [8]. Synchronized worms were feeding on HT115 with *mev-1* gene or empty vector until the L4 stage larvae. Then the worms were washed three times in M9 buffer and transferred to *E. faecalis* plates. After 16 h of infection, a total of 80 worms were transferred to three replica plates. Worms were scored as live and dead at various points along the time course. For colonization analysis of *E. faecalis*, after 16 h of infection, worms were washed three times in M9 buffer and mounted on microscopic glass slides and observed under fluorescence microscopy OLYMPUS BX53 (Olympus). For paraquart treatment assay, synchronized worms were feeding on HT115 with *mev-1* gene or empty vector until the L4 stage larvae on drug plates before infection.

2.4. Drug plates preparation

Paraquat (Sigma) was added into NGM media from a stock solution (50 mM) before pouring the plates. Plates were made freshly each week.

2.5. Quantitative real time PCR (qRT-PCR) analysis

Nematodes were treated as mentioned above in the killing assays. The worms exposed to *E. faecalis* and *E. coli* OP50 for 16 h, followed by three times washing in M9 buffer. The total RNA was extracted and cDNA was generated using the Primescript RT reagent kit with gDNA Eraser (TaKaRa). The qRT-PCR was carried out using SYBR Premix Ex Taq II (TaKaRa) and performed on a BIO-RAD S1000 Thermal Cycler. Cycle threshold (Ct) values were normalized against the control gene *act-1*. All the tests were repeated at least three times, and each replicate was measured in triplicate. Fold change was calculated using the $2^{-\Delta\Delta Ct}$ method. The sequences of primers can be provided on request.

2.6. Fluorescence imaging

Animals were collected as described in the qRT-PCR assays above and paralyzed by 20 mM sodiumazide. Then worms were mounted on microscopic glass slides and observed under fluorescence microscopy OLYMPUS BX53 (Olympus). The fluorescence pictures of worms were taken by DP72 CCD (Olympus). The fluorescence signal intensity was quantified using Image J software. Results are the average of three biological replicates, each replicate measured in 15 worm individuals ± SD (N = 15).

2.7. Statistics

Results are all presented as mean ± SD. Animal survival was plotted by SPSS version17.0 software (SPSS Inc.), and Kaplan–Meier method was employed to calculate survival fractions. For other analysis, statistical significance was determined by ANOVA with Tukey's post-hoc test also using SPSS. Results were considered as significant with a P value of <0.05.

3. Results

3.1. mev-1 RNAi Enhanced susceptibility to E. faecalis infection relates to oxidative stress

It has recently been showed that increased ROS levels promoted longevity by increasing immunity [24]. The *mev-1* mutants also enhance the production of mROS in non-infected conditions [25], whereas the knockdown of *mev-1* reduced ROS production during *E. faecalis* infection (Fig. 1A). Interestingly, we showed that *mev-1*RNAi *C. elegans* were due to enhanced susceptibility to *E. faecalis* infection rather than enhanced innate immunity (Fig. 1B). To further understand the mechanisms of *mev-1* in host defense, a GFP-expressing *E. faecalis* OG1RF:pMV158 was used to detect a bacterial fade in the digestive tract of *C. elegans*. After 16 h of bacterial treatment, *mev-1*RNAi worms accumulated more *E. faecalis* in the intestine lumen than wild-type, whereas no difference in a gut colonization of *E. coli* OP50:pFVP25.1 was detected between both groups (Fig. 1C). This result demonstrated that *mev-1*RNAi causing high colonization of *E. faecalis* in the worm intestine.

Next, we chose to focus on *mev-1* mediated oxidative stress in resistance against *E. faecalis*. We hypothesized that the *mev-1* might lack resistance because of a low oxidative stress response in a worm intestine. We thus used an accumulation of lipofuscin that represented an oxidative stress at the host–pathogen interface to test this hypothesis [9]. After 16 h exposure to *E. faecalis*, both empty vector and *mev-1*RNAi worms showed increased lipofuscin accumulation along the intestine compared with *E. coli* OP50 (Fig. 1D). However, the enhancement of lipofuscin accumulation in *mev-1*RNAi worms was less than vector control (Fig. 1D). These results are consistent with our hypothesis. Taken together, we demonstrated that *C. elegans* lacking *mev-1* is sensitive to infection by *E. faecalis* due to a low intestinal oxidative stress.

3.2. Knockdown of mev-1 defects in DAF-16-dependent oxidative stress against E. faecalis infection

In 2007, Chávez and colleague proposed a model whereby *C. elegans* responds to pathogens by producing ROS in the intestine while simultaneously inducing oxidative stress response enzymes

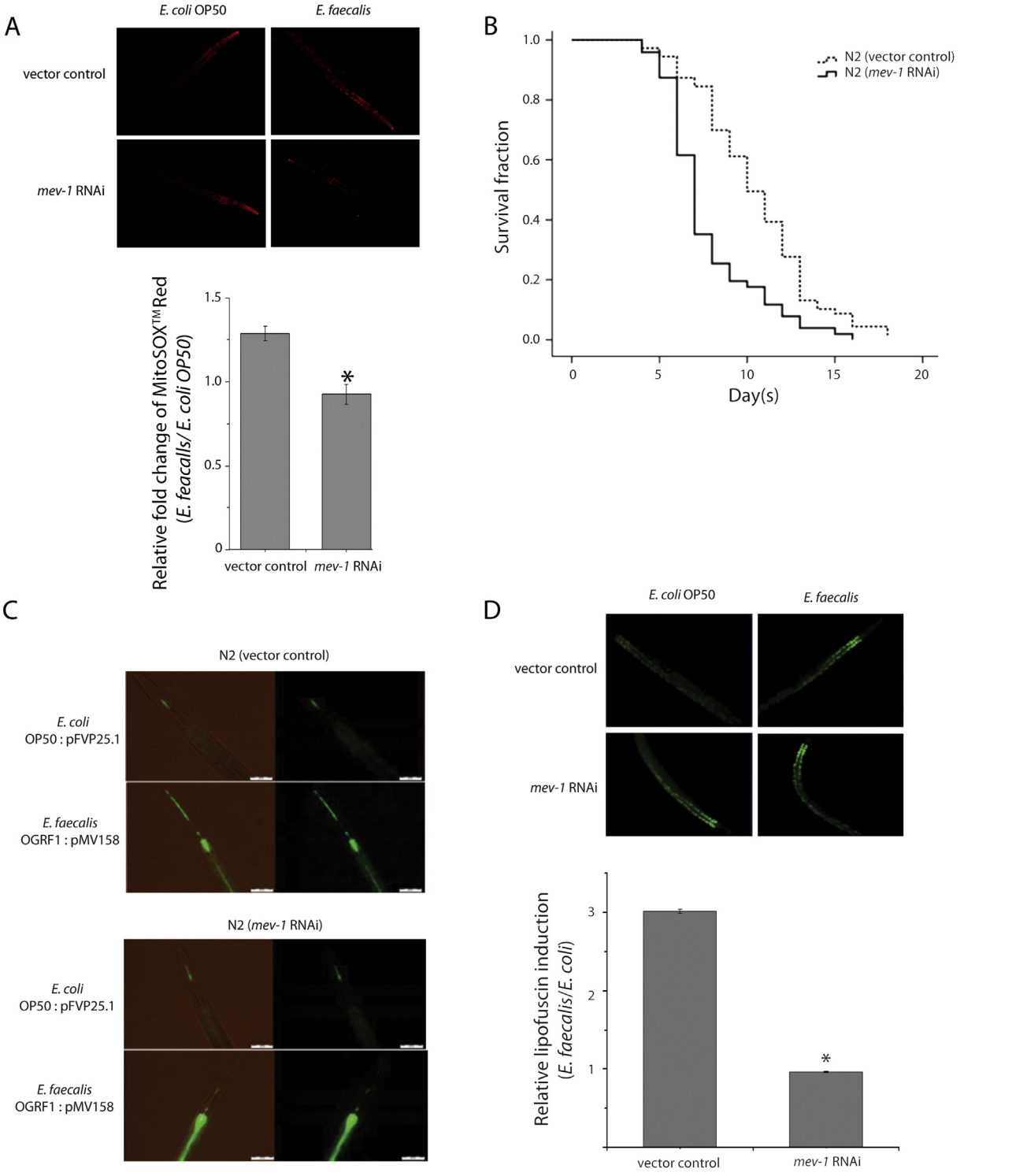

Fig. 1. *mev-1*RNAi causes increased susceptibility to *E. faecalis* infection and attenuated lipofuscin accumulation. (A) The fluorescence pictures (top panel) and density quantification (lower panel) of MitoSOX™ Red staining in vector control and *mev-1*RNAi worms on *E. coli OP50* and *E. faecalis* exposure (n ≥ 80 animals per each treatment, $^{*}P \leq 0.05$, detailed method can be found in the supplementary material). (B) Survival rate of *mev-1*RNAi and vector control on *E. faecalis* OGRF1 in wild type N2 (n = 80/group). (C) Gut colonization of *E. coli* OP50:pFVP25.1 and *E. faecalis* OGRF1:pMV158 in vector control and *mev-1*RNAi worms (n ≥ 60 animals for each condition). (D) The fluorescence pictures (top panel) and density quantification (lower panel) of lipofuscin accumulation in vector control and *mev-1*RNAi worms on *E. coli* OP50 and *E. faecalis* exposure (n ≥ 75 animals/group, $^{*}P \leq 0.05$). These all experiments were repeated three times independently with at least 180 worms in each treatment group.

to protect adjacent tissues [10]. We next tested if anti-oxidative gene expression is *mev-1* regulated. Expression of *sod-3* and *gst-4* genes in response to *E. faecalis* infection is the best characterized immune response of *C. elegans*. The SOD-3 is a major enzyme against oxidative stress by catalyzing the removal of superoxide [26], while the GST-4 enzyme is involved in the phase II detoxification process during oxidative stress [23]. We treated the transgenic *C. elegans* (CF1553 and CL2166) expressing GFP as a

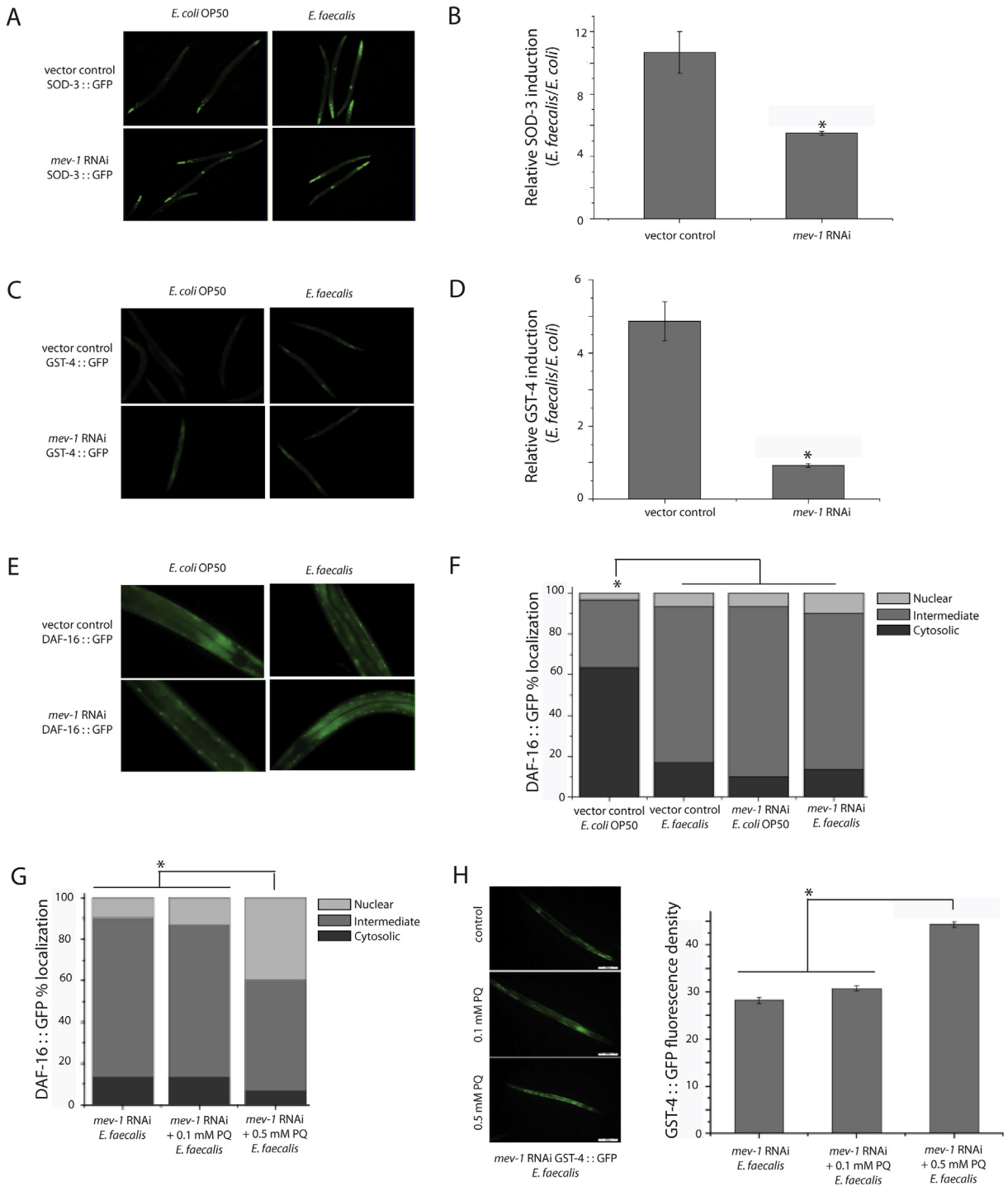

Fig. 2. *mev-1* RNAi down regulates DAF-16-dependent oxidative stress against *E. faecalis* infection. The expression pattern of SOD-3::GFP (A) and GST-4::GFP (C) in *mev-1*RNAi and control worms exposed to *E. coli* OP50 and *E. faecalis* for 16 h. The data shown is representative of experiments repeated three times independently, with at least 80 worms for each condition. The qRT-PCR analysis of oxidative stress genes *sod-3* (B) and *gst-4* (D) induced in *mev-1*RNAi and control worms exposed to *E. faecalis* compared with *E. coli* OP50. Abundance changes relative to an internal control gene as represented as mean (three biological replicates, n = 45/group) ± standard deviation, $^*P < 0.05$. DAF-16 nuclear localization image (E) and analysis (F) in TJ356 worms exposed to *mev-1*RNAi and vector control prior to *E. coli* OP50 and *E. faecalis* exposure. (G) TJ356 pretreated with 0.1 mM and 0.5 mM PQ in *mev-1*RNAi worms were exposed to *E. faecalis* for 16 h. DAF-16 is present in cytosolic, intermediate, or nuclear fractions. Quantification of DAF-16 translocation in F and G is representative of three biological replicates ±SD, with each replicated being the mean of 15 worms ($^*P < 0.05$). (H) The expression pattern and quantification of GST-4::GFP in *mev-1*RNAi with 0.1 mM and 0.5 mM PQ exposed to *E. faecalis* for 16 h. The data shown is representative of three biological replicates ±SD, with each replicated being the mean of 15 worms ($^*P < 0.05$).

reporter transgene for inducible *sod-3* and *gst-4* expression. Strains CF1553 and CL2166 feeding with empty vector and *mev-1*RNAi were then exposed to *E. faecalis* for 16 h. The results showed that the expression levels of SOD-3::GFP and GST-4::GFP were significantly increased after pathogenic infection compared to regular food *E. coli* in empty vector (Fig. 2A), whereas it dramatic decreased in *mev-1*RNAi animals (Fig. 2C). The results were confirmed expression of *sod-3* and *gst-4* by qRT-PCR (Fig. 2B and D), suggesting that oxidative stress response during infection has been blocked in *mev-1*RNAi background.

It is well-known that DAF-16 regulates many genes encoding oxidative stress response enzymes, including *sod-3* and *gst-4* [17,27–29]. Although high DAF-16 is reported in the nuclei of *mev-1* RNAi *C. elegans* under the normal atmospheric conditions [19], no data has been reported in this mutant after bacterial infection. Here we used a DAF-16::GFP transgenic line TJ356 to examine DAF-16 activity and localization. As shown in Fig. 2E, exposure to *E. faecalis* causes increase nuclear localization of DAF-16::GFP in vector control compared to regular food *E. coli* OP50. On the other hand, the *mev-1*RNAi animals do not display significant change in DAF-16::GFP nuclear localization versus OP50 (Fig. 2F). The oxidative stress in *mev-1*RNAi worms may not reach the minimum threshold to induce DAF-16 translocation when bacterial infection, causing decreased expression of oxidative stress response genes.

Thus it is possible that DAF-16 translocation against pathogens will present under *mev-1*RNAi background when oxidative stress above the minimum threshold. The paraquat (PQ) was demonstrated to increase mitochondrial superoxide levels in *C. elegans* [15]. To test this, the *mev-1*RNAi animals were pretreated with 0.1 mM and 0.5 mM PQ, and then exposed to *E. faecalis* for 16 h. The nuclear localization of DAF-16::GFP showed no significant difference in animals with and without low concentration PQ (0.1 mM) (Fig. 2G). The high concentration PQ (0.5 mM) treatment could enhance the DAF-16 nuclear localization in *mev-1*RNAi worms (Fig. 2G). In addition, a high concentration of PQ could also trigger expression of oxidative stress gene in *mev-1*RNAi *C. elegans* (Figs. 2H and S1). These observations were consistent with our assumption.

3.3. DAF-16 is required for mev-1 mediated transcriptional activation of oxidative stress genes

To confirm whether *mev-1* mediated induction of oxidative stress gene requires *daf-16* activity, we preformed *daf-16;mev-1*double RNAi in CL2166. The expression level of GST-4::GFP showed no significant difference between *daf-16;mev-1*double RNAi and single *daf-16* RNAi to resist *E. faecalis* challenges (Fig. 3A). These data were supported by a similar survival fraction in CF1038 with *mev-1*RNAi and empty vector after 16 h *E. faecalis* infection (Fig. 3B). These results suggest that MEV-1 triggers transcriptional activation of oxidative stress gene through DAF-16 activity that lead more tolerant to *E. faecalis* infection.

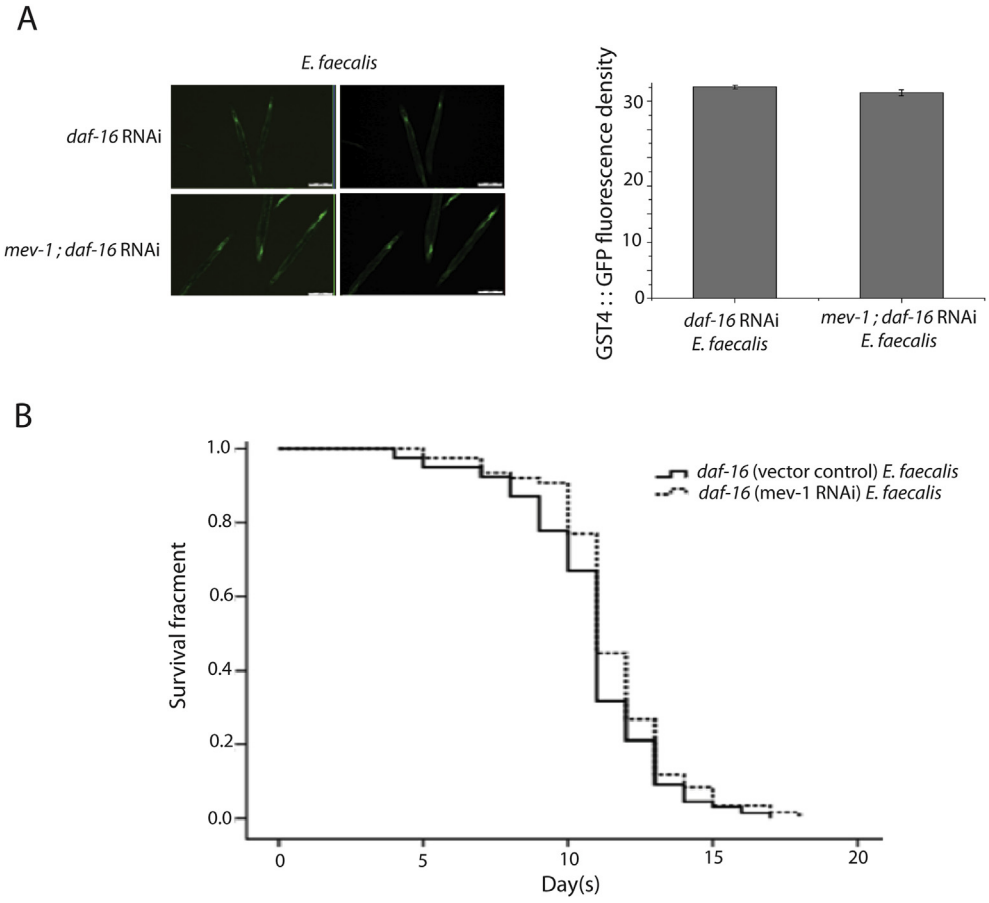

Fig. 3. DAF-16 is required for *mev-1* mediated transcriptional activation of oxidative stress genes. (A) The expression pattern and quantification of GST-4::GFP in *daf-16*RNAi and *mev-1;daf-16* RNAi exposed to *E. faecalis* for 16 h. The data shown is representative of three biological replicates ±SD, with each replicated being the mean of 15 worms, $P = 0.325$. (B) Survival rate of *mev-1*RNAi and vector control on *E. faecalis* OGRF1 in *daf-16* mutant, $P = 0.065$. The data shown is representative of experiments repeated three times independently with at least 80 worms for each condition.

4. Discussion

Increased mitochondrial ROS has recently been reported to enhance immunity against pathogenic bacteria [24]. It has been unclear how the level of reactive oxygen species regulates and promotes oxidative stress as a protective immune mechanism in *C. elegans*. Here we demonstrate that an optimal ROS appears to be crucial for animal immune protection in *E. faecalis* infection. The *mev-1* encoded a subunit of enzyme succinate dehydrogenase cytochrome b, which governs the rate of ROS production and recruitment to foreign microorganisms by modulating the cellular response to oxidative stress. The lack of *mev-1* causes susceptibility in *C. elegans* during *E. faecalis* infection rather than enhanced immune system. This defect likely explains the failure of *mev-1* to reach a minimum threshold of oxidative stress to resist bacterial infection. As can be seen in Fig. 1A, the high intestinal accumulation of *E. faecalis* has been detected under *mev-1*RNAi background. This bacterial accumulation is a common reason in worm susceptibility because pathogens can produce toxic substances to kill the host [30].

There are several studies in which protective actions of ROS in *C. elegans* are mainly attributed to their antioxidative activity [9,10]. An important feature of the oxidative stress response is to protect worms from the cytotoxicity of elevated ROS generation in response to bacterial infection [10]. Although *mev-1* mutants display higher ROS levels than wild type under regular condition [16,31], the *mev-1* RNAi worms produce lower amount of superoxide production than WT against *E. faecalis* (Fig. 1A). The ROS productions in our *mev-1*RNAi animals fail not only to promote bacterial resistance, but also to stimulate antioxidant response element during pathogenic infection (Figs. 2 and 3). However, treatment with high doses of ROS-generating chemical paraquat can restore the defective processing of *mev-1* knockdown (Fig. 2).

Oxidative damage always occurs when an imbalance in oxidant–antioxidant equilibrium [32]. The increased prevalence of lipofuscin with aging and immune function is also due to an accumulation of oxidative damage [33,34]. It seems likely that *C. elegans* resistance to an *E. faecalis* lost by an oxidant/antioxidant imbalance because of a low oxidative stress enzymes (Fig. 2). Reduction of antioxidant genes (*ctl-1*, *ctl-2*, and *sod-3*) by RNAi decreases the resistance of bacterial infected *C. elegans* [9], which is consistent with our study. Therefore, the oxidative damage might injure surrounding tissue in *mev-1*RNAi background that causes increased susceptibility to *E. faecalis*. It is well known that most of antioxidant enzyme levels are clearly under genetic control, but it still need to elucidate additional insight into the molecular mechanisms of their expression [35]. In rats exposed to hyperoxia, interleukin (IL)-1 and tumor necrosis factor (TNF)-α can induce activities of various antioxidant enzymes, including MnSOD (*sod-3*) and catalase [36]. In *C. elegans* model, the pathogen resistance caused by insulin signaling requires activity of the transcription factor DAF-16 [37–40]. Here, we suggest that *mev-1* regulates ROS-mediated expression of oxidative stress genes through DAF-16 in *E. faecalis* resistance (Figs. 2 and 3). The molecular genetics of *mev-1* suggest a defect in electron transport and mitochondrial respiration, causing a superoxide overproduction and a short life span of *C. elegans* [16]. One argument for this assumption is that mild inhibition of mitochondrial respiration extends longevity of many organisms [41,42]. Moreover, new evidence has emphasized that

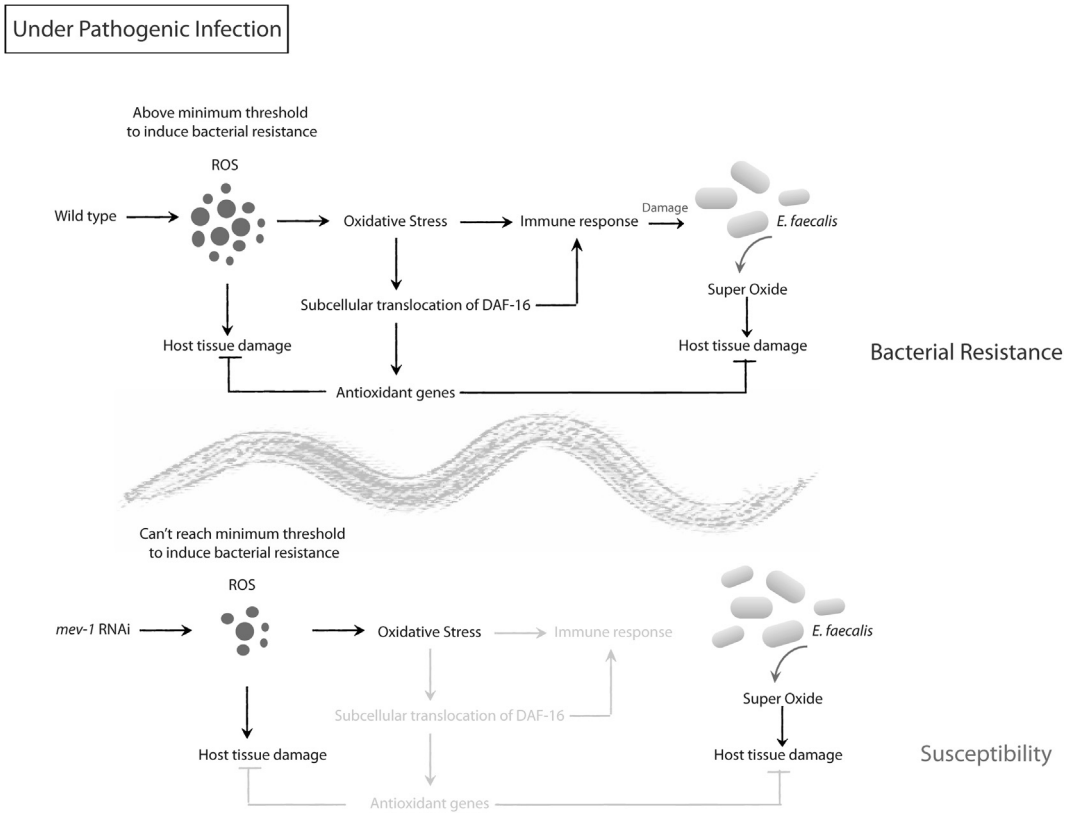

Fig. 4. Schematic diagram of proposed roles for MEV-1 in ROS-dependent bacterial resistance. In wild type, mitochondrial MEV-1 promotes ROS productions in response to *E. faecalis*. A ROS threshold requires for bacterial resistance. Certain amounts of ROS can stimulate DAF-16 dependent oxidative stress response, and thereby protects issues against oxidative damage. It implies that the MEV-1 plays an essential role in regulation of ROS or oxidant/antioxidant homeostasis. In addition, MEV-1 mediated oxidative stress is also crucial in the regulation of immune system activity to battle with invaders. Lack of MEV-1 conversely results in decrease ROS productions during bacterial infection, causing susceptibility in this nematode. Because *mev-1*RNAi animals loss the activation in anti-oxidative response and immune system, during *E. faecalis* infection.

mitochondrial ROS promotes long lifespan and immunity by reducing mitochondrial respiration [24] and there is reasonable agreement with our results. One of the most interesting findings from this study is a novel function of mitochondrial superoxide as the endogenous stress signal against pathogen. Based on these data, we propose a model for oxidative stress regulation by *mev-1*, which enhances immunity (Fig. 4). This model provides a new insight into the mechanism of ROS against pathogenic infection by *mev-1*.

Conflict of interest

None.

Acknowledgments

This work was supported by the special international cooperation project of MOST, 2012DFA30480; the key project of Gansu Province science and technology, 1002WKDE55; Fundamental Research Funds for the Central Universities of China (lzujbky-2013-75) and Gansu Province Scientific Research Project Fund (No. 2GS064-A43-019-02). Nematode strains used in this work were provided by CGC. *E. faecalis* used in this work was obtained from the First Affiliated Hospital of Lanzhou University.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.132.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.132.

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