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RESEARCH REPORT

Mitochondrial superoxide increase is essential for *Caenorhabditis elegans* against *Enterococcus faecalis* infection

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Abstract

Enterococcus faecalis infection can cause serious diseases including cancer development. Recently it has been reported that mitochondrial reactive oxygen species (mROS) are required for host immune defenses against bacteria and many mutations in mitochondrial electron transport chain (mETC) genes have an effect on mROS production. To identify the exact role of mROS during *E. faecalis* infection, we thus decide to knockdown the expression of *mev-1* and *isp-1* in *Caenorhabditis elegans* using RNAi. The knockdown of *mev-1* and *isp-1* causes increased susceptibility and increased resistance to *E. faecalis* infection, respectively. The *mev-1*(RNAi) can also down-regulate antimicrobial genes (C17H12.8, *mtl-1* and *bli-3*), whereas these antimicrobial genes are up-regulated in *isp-1*(RNAi) animals after bacterial infection. Further, significant increase of mitochondrial superoxide and mitochondrial *sod* expressions have been observed in *isp-1*(RNAi) animals. Conversely, the *mev-1*(RNAi) worms show a decrease of mitochondrial superoxide and mitochondrial *sod* expressions. Prooxidant paraquat, which is a mitochondrial superoxide generator, can increase survival rate of *mev-1*(RNAi) animals after *E. faecalis* infection. All together, the enhancement of mitochondrial superoxide contributes to anti-bacterial immunity and a better knowledge of them should open new avenues for preventive strategies against bacterial infection and also limiting the development of infection-associated cancer.

Key Words: *C. elegans*; *mev-1*; mitochondrial superoxide; *E. faecalis;* infection

Introduction

Although *Enterococcus* species were regarded as harmless commensals to humans for many years, they have emerged as major opportunistic pathogens in the nosocomial infections, notably by causing urinary tract infections, endocarditis and bacteraemia (Murray, 1990; Giannitsioti *et al.*, 2007; Arias and Murray, 2012). *Enterococcus faecalis* as the most abundant species recognized in enterococcal endocarditis (Murray, 1990; Tleyjeh *et al.*, 2005; Arias and Murray, 2012), has also been found in remarkably increased numbers in oral cancerous lesions and in human colon cancers (Balamurugan *et al.*, 2008; Boonanantanasarn *et al.*, 2012). Recently, increasing evidences suggest that *E. faecalis* infection is implicated in the development of gastric cancer by inducing genetic instability and mitochondrial dysfunction in gastric epithelial cells (Strickertsson *et al.*, 2013; Strickertsson *et al.*, 2014). However, treatment of *E. faecalis* infection has been clinically challenging due to the emergence of strains resistant to commonly used antibiotics (Singh *et al.*, 2015). Therefore, a better understanding of the pathogenesis of *E. faecalis* is urgent for limiting the development of infection-associated cancer and also promoting the further control of antibiotic resistance.

Reactive oxygen species (ROS) has been a target of study in host-pathogen interactions for decades. Recent studies suggest that *E. faecalis* can not only generate ROS in vitro, but also induce production of endogenous ROS in cancer cells (Boonanantanasarn *et al.*, 2012). Further, newly study reveals that ROS generating by *E. faecalis* infection down-regulates microRNA in Human Gastric Cancer Cells (Strickertsson *et al.*, 2013). All

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these results indicate the ROS homeostasis altering by the *E. faecalis* infection influences the host innate immunity. Since approximately 90 percentages of cellular ROS are derived from mitochondria (Balaban *et al.*, 2005), mitochondrial ROS (mROS) might mainly contribute to cellular redox status and affect host antibacterial responses. Therefore we hypothesize that mROS may be play an essential role for host defense against *E. faecalis* infection.

Previous studies suggest that *Caenorhabditis elegans* can be infected by *E. faecalis*, this feature makes *C. elegans* a simple model host to study the mechanism of *E. faecalis* infection (Garsin *et al.*, 2001). In this model, an intestinal infection triggers the release of ROS by Ce-Duox1/BLI-3 in what appears to be a protective response (Chavez *et al.*, 2009; Jain *et al.*, 2009). The ROS can actually diffuse back into cytosol through aquaporin and thereby activate redox signaling (Finkel, 2011). In addition, mitochondrial electron transport chain (mETC) genes have been identified to involve in ROS production. For example, *mev-1* mutants were known as shot-lifespan with high mROS production; while mutation in *isp-1* led to lifespan extension with low mROS level (Ishii *et al.*, 1998; Feng *et al.*, 2001; Yang and Hekimi, 2010). However, new evidence provides an argument that an enhancement of ROS increases the immunity and lifespan of *C. elegans* (Hwang *et al.*, 2014). Thus, the roles of mROS during the bacterial infections remain unclear (Murphy *et al.*, 2011).

In this work, we thus use the *E. faecalis* infected *C. elegans* as a model for understanding the role of mitochondrial superoxide in bacterial infection. The mETC knockdowns mediated changes in mitochondrial superoxide have been investigated in *C. elegans* during *E. faecalis* infection. The enhancement of immune response by increasing of mitochondrial superoxide has also been confirmed by prooxidant paraquat treatment. These results gave new insights to preventive strategies against bacterial infection and the further development of antibiotic resistance.

Materials and Methods

C. elegans *and bacteria strains*

The wild-type *C. elegans* strain N2 Bristol was provided by the *Caenorhabditis* Genetics Center (CGC). *C. elegans* were grown and maintained as previously described (Hope, 1999) at 20 °C. *E. coli* OP50, *E. coli* OP50:pFVP25.1and *E. coli* HT115 (DE3) were also obtained from CGC and cultured on Luria Bertani (LB) media supplemented appropriate antibiotics. *E. faecalis* (OG1RF) was a kindly gift from the First Affiliated Hospital of Lanzhou University (ATCC 47077), *E. faecalis* OG1RF:pMV158 was a kindly gift from Ludek Zurek (Doud and Zurek, 2012). Both of the *E. faecalis* strains were grown on Brain Heart Infusion (BHI) medium at 37 °C.

RNA interference

RNAi expressing clones were constructed as follows. Total RNA was extracted from wild type *C. elegans* L4 larvae using RNAiso Plus (Takara). cDNA was synthesized using Super-scriptII reverse transcriptase (Invitrogen) with oligo dT and random hexamer primers. Gene specific primers were used to amplify regions of target genes, then the target genes were cloned into the vector pL4440 (Addgene plasmid 1654) (Timmons *et al.*, 2001) and transformed into *E. coli* HT115 (DE3). All clones were verified by sequencing. Sequences of gene specific primers will be provided on request. Cultures of HT115(DE3) containing empty vector (pL4440) or target genes 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. 250 μl of bacteria cultures were spread onto 6 cm RNAi plates (NGM agar with 1 mM IPTG, 100 μg/ml ampicillin, and 5 μg/ml tetracycline), and the plates were incubated at 37 °C for 24 h and then equilibrated to room temperature before assay. RNAi was induced by feeding synchronized L1 worms through L4 stage with bacteria producing dsRNA to target relative genes (Rea *et al.*, 2007).

Drug plate preparation

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

Killing assays

E. faecalis OG1RF and *E. faecalis* OG1RF:pMV158 grown in BHI medium for 5 h was seeded on BHI plates and incubated at 37 °C for 24 h (Hoeven *et al.*, 2011). For mETC RNAi assay, synchronized worms fed on HT115 with mETC genes 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 60-75 worms were transferred to three replica plates. Worms were scored as live and dead at various points along the time course. For paraquart treatment assay, infected worms with mETC RNAi were transferred on drug plates after 16 h infection.

Quantitative real time PCR (qRT-PCR) analysis

Nematodes were treated as mentioned above in the killing assay. The worms exposed to *E. faecalis* and *E. coli* OP50 (non-infection control) for 16 h , followed by three times washing in M9 buffer. The total RNA was extracted using RNAiso Plus Reagent (TaKaRa). cDNA was generated using the Primescript RT reagent kit with gDNA Eraser (TaKaRa). The qRT-PCR was carried out with the SYBR Green methodology 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 three times, and each replicate was measured in triplicate. Fold change was calculated using the $2^{-\Delta\Delta Ct}$ method. Primers of C17H12.8 and *mtl-1* were graciously provided by Troemol (Troemel *et al.*, 2006). Primers of *sod-1* to *sod-5*, together with *act-1* control, were obtained from Yanase (Yanase *et al.*, 2009). Primers of *bli-3* were designed using Primer Premier 5.0, and the amplification specificity against the *C. elegans* genome was tested and the efficiency was identified with a dilution series of template. All the primers used in our research are listed in Table 1.

Table 1 List of primers used for qRT-PCR

Gene	Primer Name	Primer Sequence
C17H12.8	*C17H12.8-F*	5'TGTCATTTCAATGGAGGATATTGT3'
	C17H12.8-R	5'TGATGGAGTTGGAGGATATTGA3'
mtl-1	*mtl-1*-F	5'CTTGCAAGTGTGACTGCAAAAAC3'
	mtl-1-R	5'CTTGCAGTCTCCCTTACATCC3'
sod-1	*sod-1*-F	5'CGTAGGCGATCTAGGAAATGTG3'
	sod-1-R	5'AACAACCATAGATCGGCCAACG3'
sod-2	*sod-2-F*	5'AGCTTTCGGCATCAACTGTC3'
	sod-2-R	5'AAGTCCAGTTGTTGCCTCAAGT3'
sod-3	*sod-3*-F	5'TTCAAAGGAGCTGATGGACACT3'
	sod-3-R	5'AAGTGGGACCATTCCTTCCAA3'
sod-4	*sod-4*-F	5'GTTGTCTAAGTGCTGGTGG3'
	sod-4-R	5'TTCCACATGCAAGTCGGCT3'
sod-5	*sod-5*-F	5'GCAAAATGAATCATGGAGGAAG3'
	sod-5-R	5'AAGATCATCTCGATCGACGTGG3'
act-1	*act-1*-F	5'CCACGTCATCAAGGAGTCAT3'
	act-1-R	5'GGAAGCGTAGAGGGAGAGGA3'
bli-3	*bli-3*-F	5'GCTGCATAGAGATGCACGT3'
	bli-3-R	5'AGTGGACAGGATACTCCA3'

Staining and fluorescence imaging

MitoSOX™ Red (Invitrogen) was diluted in DMSO at high concentration 1 mM and frozen at -20 °C as a stock. Before staining stocks were diluted in M9 buffer at a 1:1000 dilution. Worms were collected as described in the qRT-PCR assays above and transferred into staining solution for 20 min. Worms were paralyzed by 20 mM sodium azide and mounted on microscopic glass slides, then 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.

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 Dancan's and Tukey's post-hoc test also using SPSS. Results were considered as significant with a p value of < 0.05.

Results and Discussion

Knockdown of mETC gene expressions preceded changes of resistance to E. faecalis *infection in* C. elegans.

Although previous work have been reported the effect of mETC gene expression on mROS production and lifespan of *C. elegans* (Ishii *et al.*, 1998; Feng *et al.*, 2001; Yang and Hekimi, 2010), recent studies revealed that *isp-1* mutants showed enhanced immunity against bacterial infection (Hwang *et al.*, 2014), while knockdown of *mev-1* led to susceptibility to *E. faecalis* (Feng *et al.*, 2015). All these data suggest a linkage between mROS level caused by mETC gene mutation and immune response to pathogenic bacteria. However, the precise role of mROS in host defense still need to elucidate. To investigate this question, we knocked down two mETC genes of *C. elegans*, *mev-1* and *isp-1*, by RNAi. The results of killing assay showed that *mev-1*(RNAi) caused increased susceptibility to *E. faecalis* infection (Fig. 1A), while *isp-1*(RNAi) endowed *C. elegans* more resistibility to *E. faecalis* infection (Fig. 1B). These results were consistent with the previous studies (Feng *et al.*, 2015; Hwang *et al.*, 2014).

Although good survival has been regarded as a primary phenotype for improved immunity, it is still hard to differentiate infection resistance from lifespan extension only by killing assay. Previous study suggested that *mev-1*(RNAi) caused high colonization of *E. faecalis* in the worm intestine (Feng *et al.*, 2015), which was a well-established pathogenic feature of infection. In this work, we also used a GFP-expressing *E. faecalis* OG1RF:pMV158 to test the accumulation of bacterium in the digestive tract of *isp-1*(RNAi) and wild-type *C. elegans*. After 16 h of bacterial exposure, *isp-1*(RNAi) worms accumulated less *E. faecalis* in the intestine lumen than control, whereas no difference in a gut colonization of *E. coli* OP50:pFVP25.1 was detected between both groups (Fig. 1C). This result demonstrated that *isp-1*(RNAi) decreased the colonization of *E. faecalis* in the worm intestine.

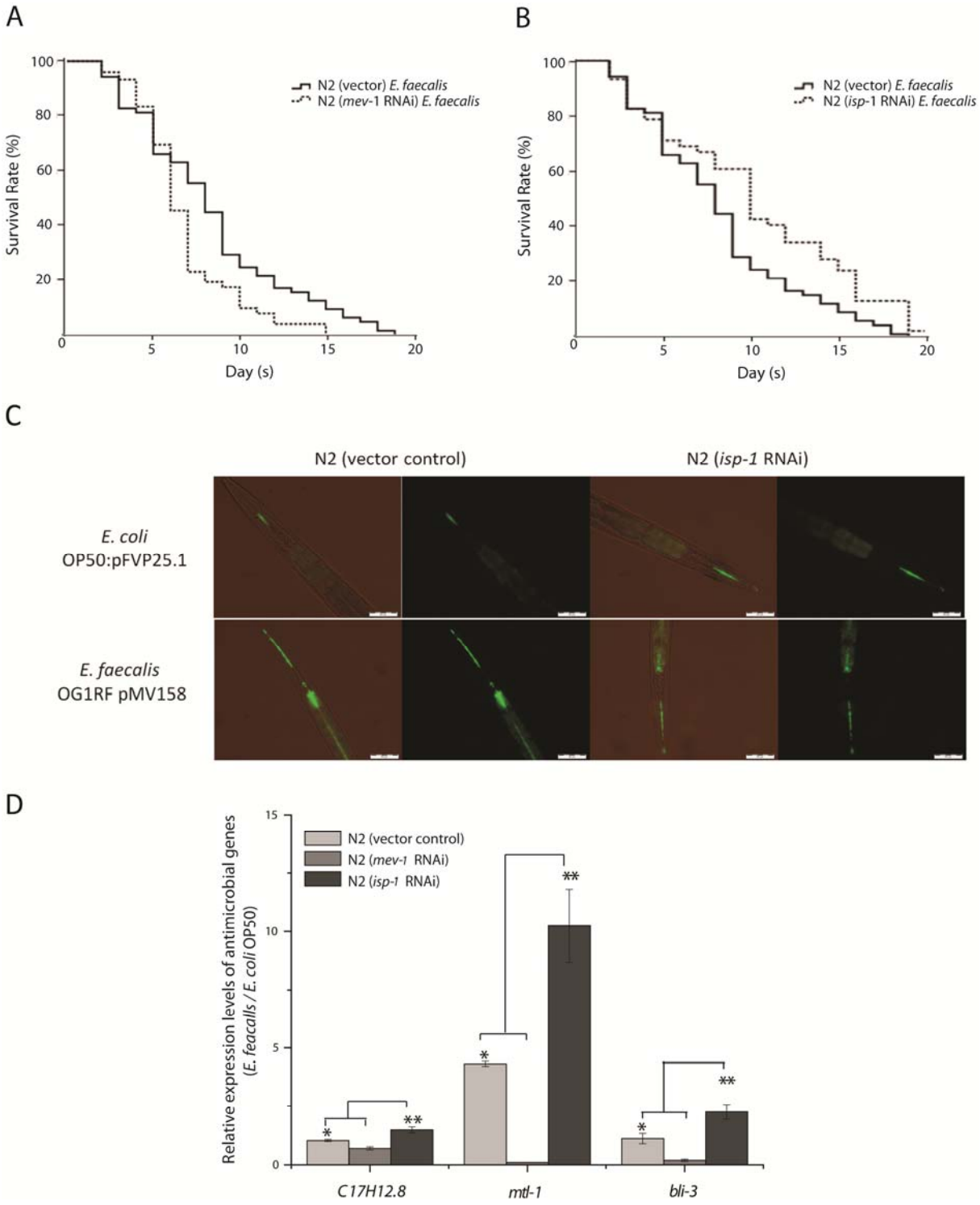

Fig. 1 Down-regulated mETC genes affect the resistance of *C. elegans* against *E. faecalis* infection. Survival curves of animals after *E. faecalis* infection. (A) N2 worms feeding *mev-1*(RNAi) and vector control through L4 and then exposed to *E. faecalis* for 16 h (B) N2 worms feeding *isp-1*(RNAi) and vector control through L4 and then exposed to *E. faecalis* for 16 h. Median survival and *p*-values of (A) and (B) are listed in Table 2. (C) Gut colonization of *E. coli* OP50:pFVP25.1 and *E. faecalis* OG1RF:pMV158 in vector control and *isp-1*RNAi worms. The data shown is representative of experiments repeated three times with 45 worms for each condition (D) The qRT-PCR analysis of antimicrobial genes (*C17H12.8*, *mtl-1*, *bli-3*) induced in *mev-1*(RNAi), *isp-1*(RNAi) and control worms when exposed to *E. faecalis* compared to *E. coli* OP50. Results are representative of three biological replicates, each replicate measured in triplicate and normalized to a internal control gene ± SD (*N = 3*). ***p < 0.001*, **p < 0.05*.

Table 2 The effect of *E. faecalis* and PQ on survival of *C. elegans*

Strains and genotype	Treatment	LT_{50} (in days)		*p*-value[a]
		Mean	SD	
N2	vector	7.51	0.55	
N2	*mev-1* RNAi	5.80	0.24	$p < 0.05$
N2	*isp-1* RNAi	9.60	0.75	$p < 0.0001$
N2	*mev-1* RNAi + 0.1 mM PQ	4.73	0.15	$p' = 0.601$
N2	*mev-1* RNAi + 0.5 mM PQ	8.69	0.58	$p = 0.591$
N2	*isp-1* RNAi + 0.1 mM PQ	13.60	2.31	$p'' = 0.985$
N2	*isp-1* RNAi + 0.5 mM PQ	4.13	0.96	$p < 0.0001$

The tabulated data shows the averaged results of experiments performed in triplicate in which about 60-75 N2 worms were assayed as previously described with specific treatment on *E. faecalis* OG1RF and PQ. Similar results were observed in other independent experiments; [a]*p-value* (log rank test) compared with vector control; *p'* compared with N2(*mev-1* RNAi); *p''* compared with N2(*isp-1* RNAi).

Furthermore, we tested the antimicrobial gene (C17H12.8, *mtl-1* and *bli-3*) expression changes after infection by *E. faecalis* (compared with *E. coli* OP50 normal food). All antimicrobial genes in *mev-1*(RNAi) worms were down-regulated (Fig. 1D), consistent with a susceptible phenotype as showed in Fig. 1A. In contrast, the expression of these genes was up-regulated in *isp-1*(RNAi) animals (Fig. 1D) causing resistance to *E. faecalis* (Fig. 1B). All these data above concluded that knockdown of *mev-1* and *isp-1* genes caused opposite host responses to *E. faecalis* infection, which provided us a great model to investigate the contributing factors for the susceptibility or resistance to bacterial infection.

mETC genes were closely associated with superoxide generation in E. faecalis *infected* C. elegans

Recently, it has been demonstrated that enhancement of superoxide dismutases (SODs) indicated significant increase in intracellular ROS levels (Yang *et al.*, 2007). Thus it is possible that the mETC, which is highly involved with ROS production, might also relate to *sod* gene expression levels. We evaluated five *sod* gene expression levels in mETC knockdown *C. elegans* after infection by *E. faecalis* (relative to OP50) and almost *sod* gene levels responded to *E. faecalis* were up-regulated in both mETC RNAis compared with normal food, except *sod-2* (Fig. 2). In *mev-1*(RNAi) animals, each induction fold of *sod* genes was lower than vector control. Conversely, *isp-1*(RNAi) worms showed higher induction fold of *sod* genes than control. These data supported for the relationship between superoxide related genes and mETC in *C. elegans* after *E. faecalis* infection.

To directly confirm the association between mETC and superoxide production, a MitoSOX™ Red was used to measure mitochondrial superoxide levels in mETC knockdown *C. elegans* after infection (Fig. 3). The results showed that mitochondrial superoxide significantly increased after *E. faecalis* infection in either vector control or *isp-1*(RNAi) worms, while the mitochondrial superoxide slightly decreased in *mev-1*(RNAi) animals (Figs 3A-G). Taken together, these results suggested that the component protein of mETC triggered immune response through an enhancement of mitochondrial superoxide generation during *E. faecalis* infection.

Mitochondrial superoxide influenced on both host defense and survival against E. faecalis *infection in* C. elegans

To emphasize an essential role of the mitochondrial superoxide in host defense, a mitochondrial superoxide generator paraquat (PQ) was used to treat our mETC RNAi worms after *E. faecalis* infection and the changes of mitochondrial superoxide levels were detected by MitoSOX™ Red dying assay. The PQ increased mitochondrial superoxide levels of both mETC RNAi worms in a dose-dependent manner (Figs 4A, B). In killing assay, no significant difference in survival rate was detected between all gene knockdown animals with and without low concentration PQ (0.1 mM) (Figs 4C, D). The high concentration PQ (0.5 mM) treatment restored infection survival of *mev-1* (RNAi) worms to vector control (Fig. 4C), whereas *isp-1* (RNAi) animals with high concentration PQ treatment showed severe susceptibility instead of resistance (Fig. 4D). Overall, the results are consistent with the assumption that mitochondrial superoxide is highly involved in the process of immune response against *E. faecalis* infection and the accurate increase of mitochondrial superoxide levels may serve as a determinant during the infection. Adequate mitochondrial superoxide enhancement can trigger host defense in *mev-1*(RNAi) *C. elegans* treated with high PQ. However, the homeostasis of mROS is also considered as an essential factor in host survival

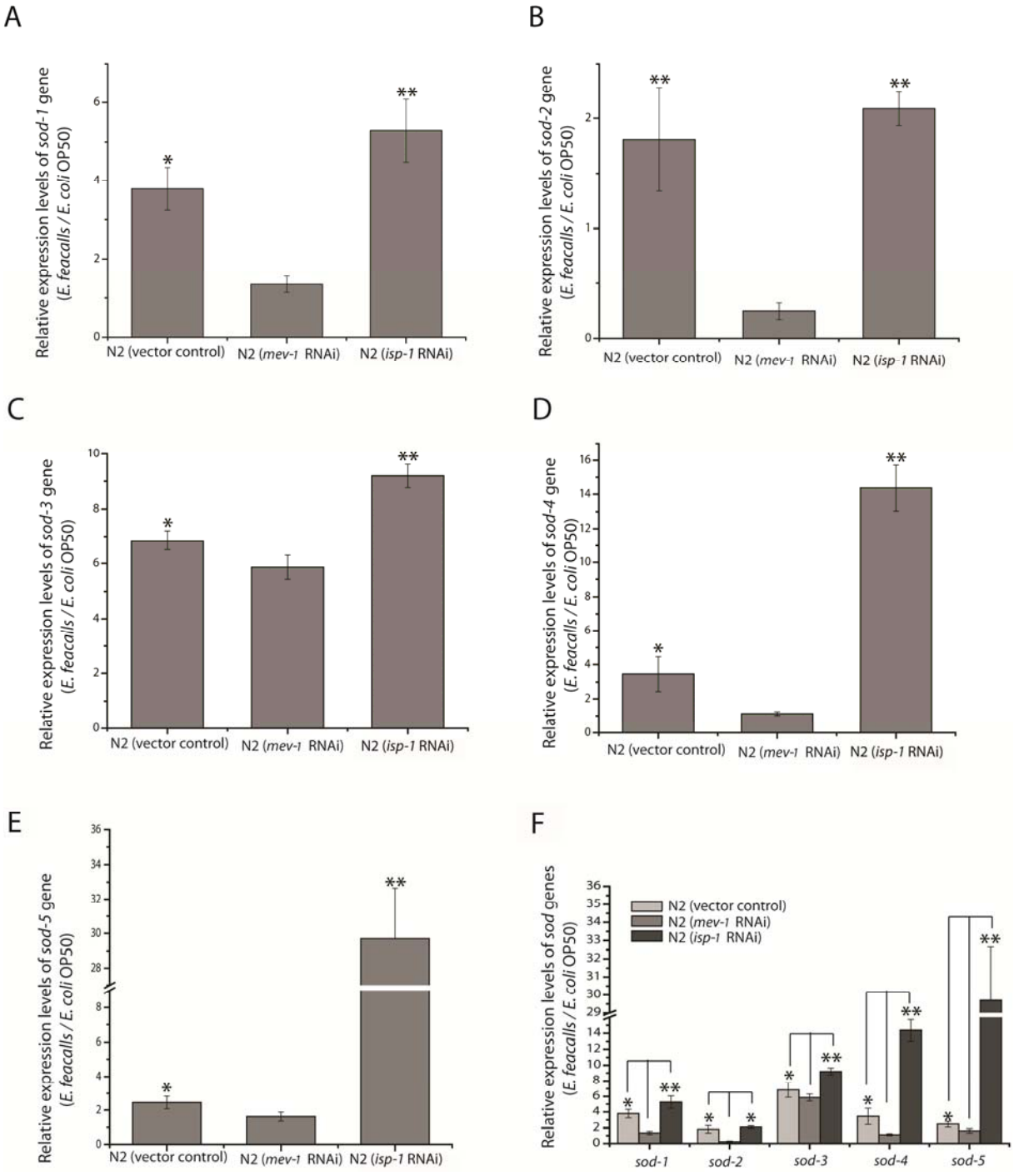

Fig. 2 sod gene expression levels in *C. elegans* are affected by mETC gene RNAi after *E. faecalis* infection. Changes in sod gene expressions after *E. faecalis* infection were assessed relative to normal food *E. coli* OP50 by qRT-PCR. (A) *sod-1* (B) *sod-2* (C) *sod-3* (D) *sod-4* (E) *sod-5* (F) *sod-1* to *sod-5*. Results are representative of three biological replicates, each replicate measured in triplicate and normalized to a internal control gene ± SD (*N* = *3*). ***p* < *0.001*, **p* < *0.05*.

(Sena and Chandel, 2012). Although the induction of mitochondrial superoxide resulted in host defense mechanisms, the over stimulation of mitochondrial superoxide can also cause death of host as shown in *isp-1* knockdown animal with high PQ treatment.

Although participation of mETC genes in aging and longevity has been reported in *C. elegans* (Ishii *et al.*, 1998; Feng *et al.*, 2001), their functions in immune response regulation have not been extensively studied. The longevity and pathogen resistance sharing the same signaling pathway has been demonstrated in *C. elegans* (Garsin *et al.*, 2003).

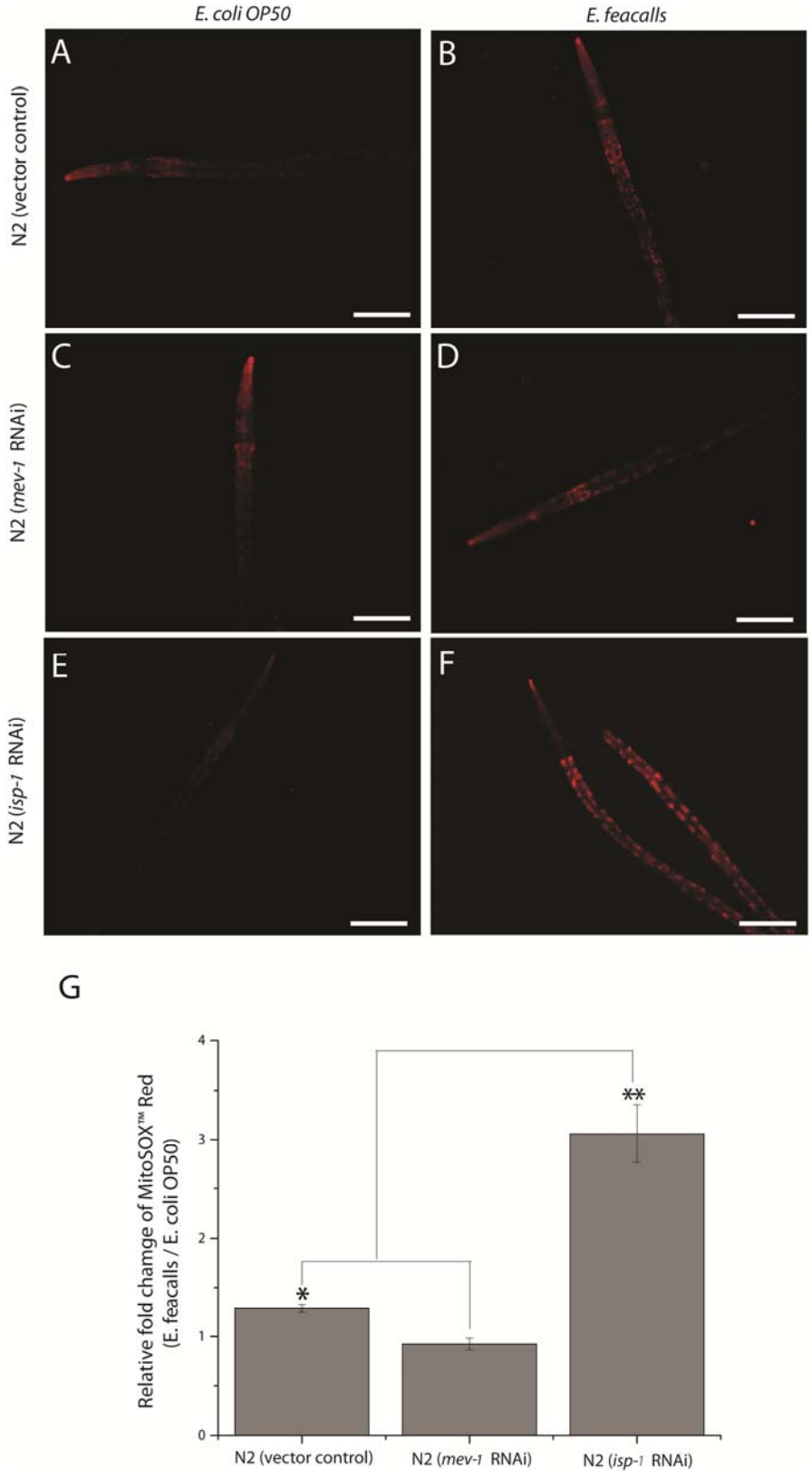

Fig. 3 Mitochondrial superoxide level in *C. elegans* by mETC RNAi after *E. faecalis* infection versus *E. coli* OP50. Mitochondria superoxide stained by MitoSOX™ Red (A) vector control worms fed on *E. coli* OP50 (B) vector control worms exposed to *E. faecalis* (C) *mev-1*(RNAi) worms fed on *E. coli* OP50 (D) *mev-1*(RNAi) worms exposed to *E. faecalis* (E) *isp-1*(RNAi) worms fed on *E. coli* OP50; (F) *isp-1*(RNAi) worms exposed to *E. faecalis* (G) Fluorescence fold changes after *E. faecalis* infection versus OP50. The fluorescence values of mitochondria superoxide stained by MitoSOX™ Red were calculated by Image J software. Results are representative of three biological replicates, each replicate measured in 45 worm individuals ± SD (*N = 45*). ***p < 0.001*, **p < 0.05*, scale bar = 100 μm.

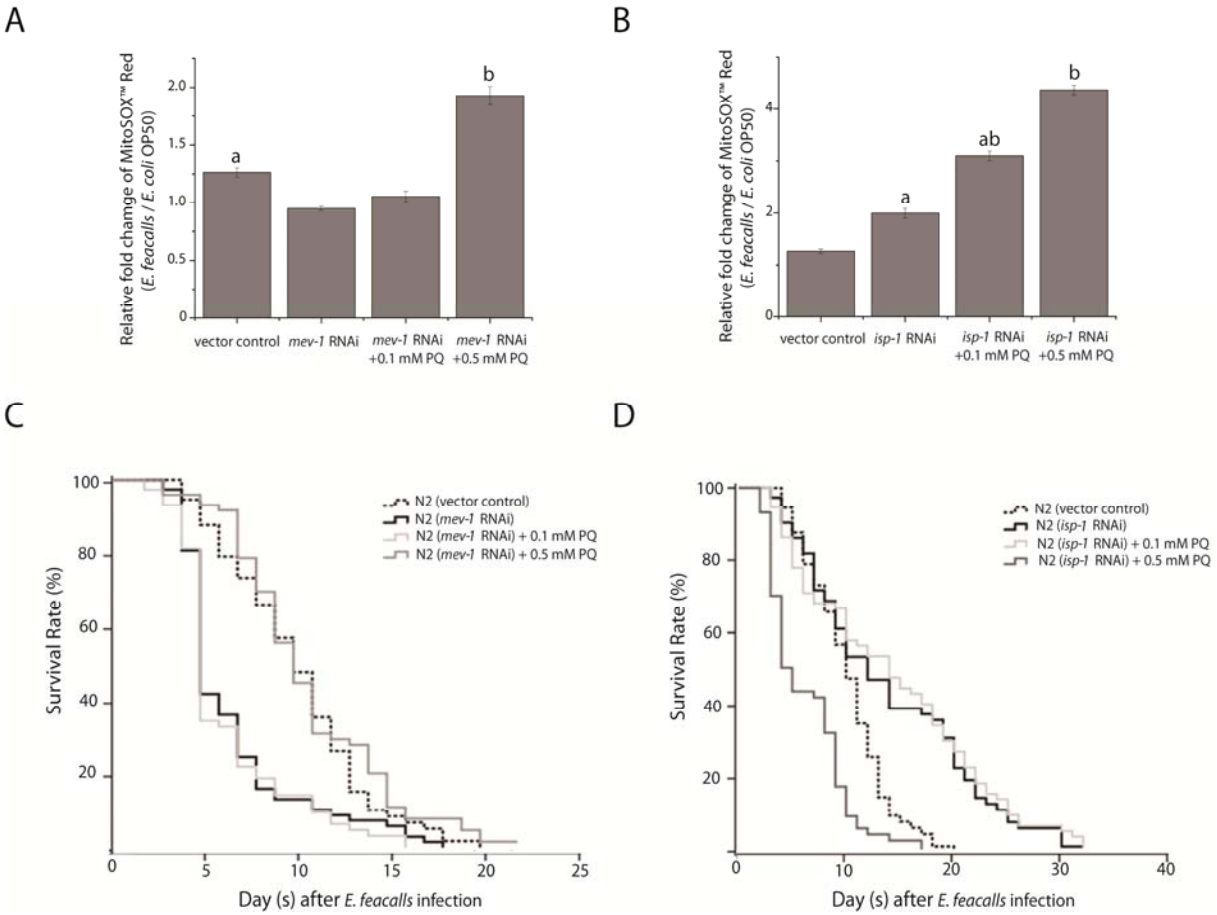

Fig. 4 Mitochondrial superoxide increase triggered by prooxidant PQ can benefit survival of mETC RNAi worms after *E. faecalis* infection. Fluorescence fold changes of (A) *mev-1*(RNAi) (B) *isp-1*(RNAi) worms pretreated with 0.1 mM and 0.5 mM PQ during *E. faecalis* infection. The fluorescence values were calculated by Image J software. Results are representative of three biological replicates, each replicate measured in 45 worm individuals ± SD ($N = 45$). Symbol "a" and "b" represent $p < 0.05$ and $p < 0.001$, while "ab" indicates $0.05 < p < 0.001$. Survival curves of (C) *mev-1*(RNAi) (D) *isp-1*(RNAi) worms pretreated with 0.1 mM and 0.5 mM PQ on *E. faecalis*. Median survival and *p*-values of (C) and (D) are listed in Table 2. The data shown is representative of experiments repeated three times with 60 - 75 worms for each condition.

Thus, it is not surprising that an enhancement of mitochondrial ROS increases both lifespan and immunity of *C. elegans* (Hwang *et al.*, 2014). However, the similar improved survival rate of *isp-1* mutants on *E. coli* and *E. faecalis* weakened the importance of mROS in pathogen resistance by attributing it to the impact of lifespan. In this work, *isp-1* (RNAi) animals showed decreased gut colonization and increased antimicrobial gene expression (Figs 1C, D). These results emphasized the resistance of *isp-1* to *E. faecalis* infection. The significant contribution of this study is using a well-established mROS concept in longevity to address the urgent problem in bacterial infection and infection-associated diseases.

Here, we clearly established the relationship between mitochondrial superoxide increase and the *E. faecalis* resistance. As shown in Figure 1, the *mev-1* and *isp-1* play opposite roles, as loss function of these gene results in totally opposite phenotypes in host defense. This mETC mediated resistance to *E. faecalis* can be explained by changes of mitochondrial superoxide production. We found that the *E. faecalis* infection induced the mitochondrial superoxide increase in *isp-1*(RNAi) and decrease in *mev-1*(RNAi). Prooxidant PQ were administrated to alter the mitochondrial superoxide levels in worms and the following killing assay results supported that mitochondrial superoxide increase was required in pathogen resistance (Fig. 4C). However, an optimal threshold of mitochondrial superoxide induction is necessary for host survival after bacterial infection. Over this threshold could cause damage to the host, while lower than the threshold showed little influence on survival after infection (Fig. 4D). All these inspire us a two-threshold model of mitochondrial superoxide induction: the increased level should surpass a lower threshold for pathogen resistance, but could not exceed the upper threshold for host survival (Fig. 5).

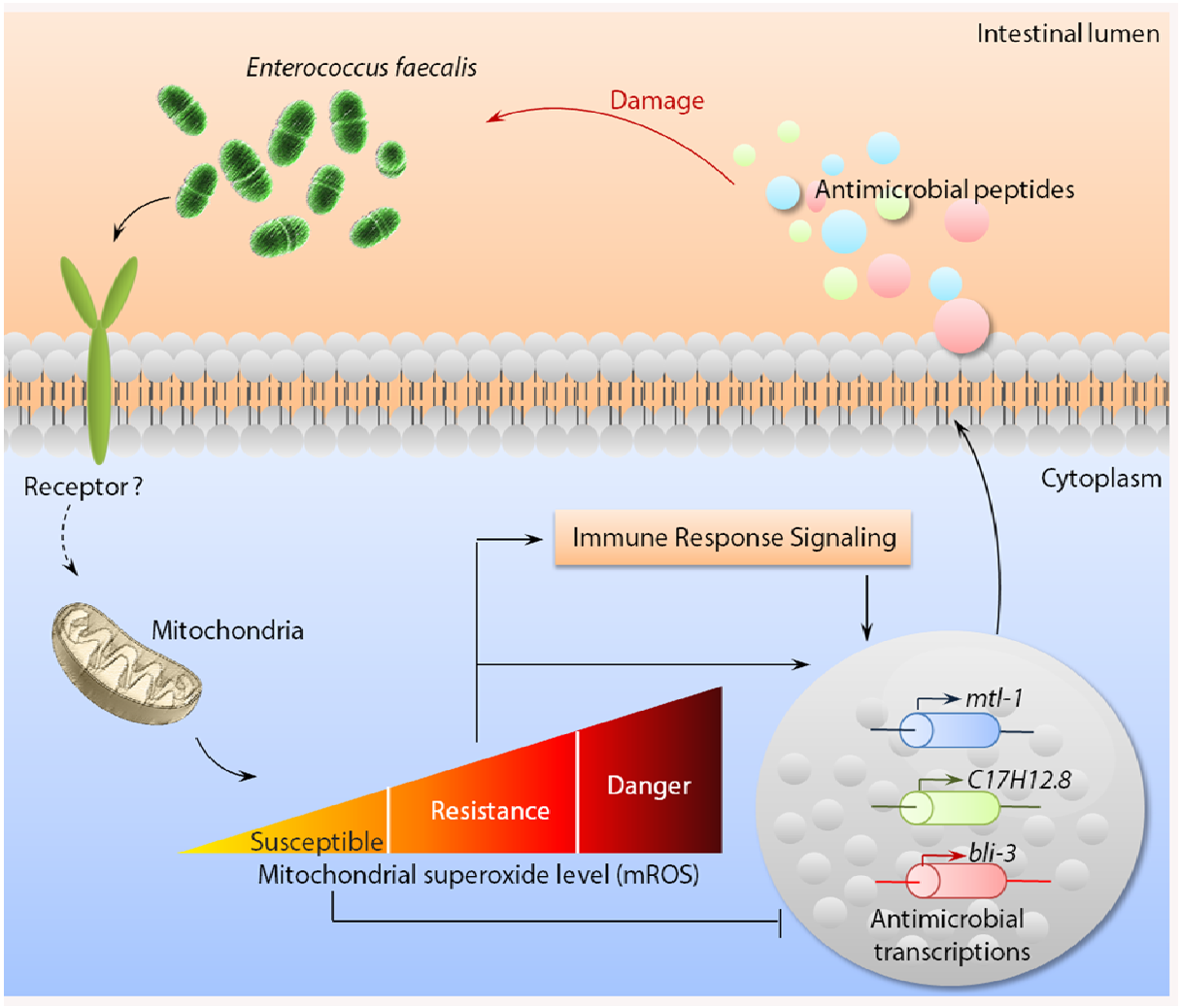

Fig. 5 Proposed model for mitochondrial superoxide induction determining resistance or susceptibility to infection (See the main text for a description).

In this work, we firstly report the enhancement of mitochondrial superoxide to regulate downstream antimicrobial molecules in bacterial resistance. Upon exposure to *E. faecalis*, mitochondria are activated by an unknown mechanism to produce changes of mitochondrial superoxide. Low mitochondrial superoxide induction in *mev-1*(RNAi) worms caused susceptibility to infection by down-regulated antimicrobial genes, whereas high mitochondrial superoxide up-regulated antimicrobial genes to resist infection (Fig. 5). As expression changes of *bli-3* showed similar tendency as antimicrobial genes, we predicted that *bli-3* served as an effecter molecule which was also regulated by mitochondrial superoxide level during infection (Fig. 5).

Previous study shows that reducing expression of a dual oxidase, Ce-Duox1/BLI-3, causes ROS production decrease and susceptibility to *E. faecalis* (Chavez *et al.*, 2009). Intriguingly, we found that mitochondrial superoxide fluctuation was closely linked to the expression of *bli-3* gene in present work. High mitochondrial superoxide induction was closely related to up-regulation of *bli-3* gene expression (Fig. 1D), which perfectly matched the survival phenotype during pathogen infection. Thus, we postulate that mitochondrial superoxide acts as an upstream signaling of BLI-3. Indeed, we observed that both cytoplasmic and extracellular SODs (*sod-1* and *sod-5* (Giglio *et al.*, 1994); *sod-4* (Fujii *et al.*, 1998), respectively) showed more significant upregulation than mitochondrial *sod-3* (Suzuki *et al.*, 1996) in *isp-1* (RNAi) worms after *E. faecalis* infection (Fig. 2). Superoxides were generated in mitochondria as signal molecules which can alternatively transfer to cytosol in form of H_2O_2 (Han *et al.*, 2003), which elicits intracellular signaling response to upregulate *bli-3* gene expression and activate BLI-3 to produce extracellular superoxide in protective response (Chavez *et al.*, 2009). Of course, insufficient superoxide increase cannot function and excessive mitochondrial superoxide increase may cause damage, resulting in more susceptible to *E. faecalis* infection. Enhancement of mitochondrial superoxide can also trigger intracellular signaling to protect host

cell from oxidative stress, such as cytosol *sod-1* and *sod-5*. On the other hand, extracellular *sod-4* was less unregulated to form a relative oxidative environment for bacterial defense. Concerning the concrete intracellular signaling response, a series of experiments will be performed in our future work.

Previous work indicates that insulin signaling contributes greatly to pathogen resistance in the model host *C. elegans* (Garsin *et al.*, 2003). The insulin signaling regulates downstream response via the transcription factor DAF-16. An association between MEV-1 and DAF-16 has already been reported in *C. elegans* (Feng *et al.*, 2015), which can explain susceptibility to *E. faecalis* infection. Moreover, it has also been reported that at least 4 others pathways have been shared in both pathogen defense and longevity of this nematode (Ewbank, 2006). For example, an oxidative stress-induced activation of DAF-16 requires the MAP2K SEK-1 (Kondo *et al.*, 2005). So it is reasonable to postulate that some other signaling pathway mediated by mitochondrial superoxide increase may trigger oxidative stress response through DAF-16.

Here, we use RNAi technology to investigate infection mechanism because recent research shows that ROS involved in infection process through regulating innate immune pathways (Hoeven *et al.*, 2011). RNAi technology is convenient to study which pathways mitochondrial superoxide relies on to respond to infection. Moreover, in this study, the *mev-1*(RNAi) animals showed sharply responsive ability to exogenous agent PQ in a dose-dependent manner. In this context, PQ can also be recognized as an antibacterial agents, which indicates the possibility that *mev-1* animals can be used as a sensitive model to evaluate efficiency of new anti-bacterial drugs targeting mitochondrial superoxide level. Further investigation on this target is important to develop more effective preventive strategies and promising antibiotic against bacterial infection.

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