

## Studies on the Function of Peroxidasins in Innate Immune System in *C. elegans*

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### Abstract

Peroxidasin is a unique member of peroxidase family in that it has extracellular matrix (ECM) motif as well as peroxidase activity. Peroxidasins are involved in consolidation the extracellular matrix during development and in innate immune defense. *C. elegans* has two functional peroxidasins, PXN-1 and PXN-2, and PXN-2 is known to contribute to innate immune system. However, it is not clear of PXN-1 function in innate immune system. Therefore, this study is focused on the function of PXN-1 and the relationship between PXN-1 and PXN-2 in innate defense system in *C. elegans*. When *pxn-1* was knocked down by RNAi, the worm turned to be more resistant to pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa* and the enhanced resistance was abolished in *pxn-1pxn-2* double knock down. By contrast, *pxn-2* knock down showed stronger susceptibility to the pathogens. These results suggest that *pxn-2* can contribute the pathogen resistance and *pxn-1* can suppress the pathogen resistance. To confirm the idea, overexpression experiments were performed. Overexpression of *pxn-1* showed more susceptible to pathogens compared to the control and double overexpression of *pxn-1pxn-2* overcame the susceptibility of *pxn-1* overexpression to the pathogens. On the other hand, *pxn-2* overexpression made the worm more resistant to the pathogens and the resistance was maintained in *pxn-1pxn-2* double overexpression. The comparison of the susceptibilities to the bacterial pathogens in above mentioned constructs suggests that PXN-1 suppress the function of PXN-2 in defense against bacterial pathogens in *Caenorhabditis elegans*.

**Keywords** : Peroxidasin, Innate Immune System, Pathogen, *C. elegans*

### 1. Introduction

Peroxidasin (PXN) is a member of peroxidase family and consists of two distinctive regions, secretory N-terminal and peroxidase C-terminal region<sup>[1,2]</sup>. It was first discovered in *Drosophila* in 1994 and the unique structure suggested functions of extracellular matrix (ECM) consolidation, phagocytosis, and pathogen defense<sup>[1]</sup>. Later, in *Caenorhabditis elegans* (*C. elegans*) *in vivo* study, peroxidasin-2 is involved in ECM consolidation to reinforce collagen network<sup>[2,3]</sup>.

Peroxidasins are animal heme peroxidases some of which are involved in immune defense. The best-studied one is myeloperoxidases (MPO), generating hypochlorous acid (HOCl) from H<sub>2</sub>O<sub>2</sub> and chloride to kill pathogens in mammalian system<sup>[4]</sup>. Moreover, SKPO-

1 (ShkT-containing peroxidase) peroxidase has a critical role in innate immunity against *Enterococcus faecalis* in *C. elegans*<sup>[5]</sup>. In addition, according to Shi *et al.*, peroxidasin is involved in murine lung host defense by recognizing gram-negative bacteria and by killing the bacteria<sup>[6]</sup>. The leucine repeats and immunoglobulin domains in N-terminal contribute the host-pathogen recognition and the peroxidase activity in C-terminal generates hypohalous acids to kill bacteria<sup>[6]</sup>.

There are two peroxidasin homologues, PXN-1 and PXN-2, in *Caenorhabditis elegans* (*C. elegans*). It is thought that *pxn-1* and *pxn-2* may have an antagonistic functional relationship in mechanosensory neurons in *C. elegans*<sup>[2]</sup>. A recent study suggests that PXN-1 regulate PXN-2 negatively in *C. elegans* neurodegeneration during aging<sup>[7]</sup>. However, it is yet to be studied how *pxn1* and *pxn2* interact each other in innate immunity. Therefore, this study focuses on how to affect *pxn-1* deficiency or overexpression on *pxn-2* function to pathogen resistance in *C. elegans*.

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## 2. Materials and Methods

### 2.1. Strains and Culture Conditions

Wild-type Bristol N2 strain and *pxn-1* (*ok785*) deletion mutant were obtained from the *Caenorhabditis elegans* Genetics Center (CGC) at the University of Minnesota. Breeding of *C. elegans* was carried out according to published protocols<sup>[8]</sup>.

*Bacillus subtilis* (KACC10854), *Staphylococcus aureus* (KACC10196) and *Pseudomonas aeruginosa* (KACC10259) were obtained from the Korean Agricultural Culture Collection (KACC), Suwon, Korea.

### 2.2. Constructs and Transformation

Fire feeding vector (L4440) was ligated with the green fluorescent protein gene (*gfp*), *pxn-1* genomic DNA (1.7kb, cut by BglII and SpeI) and *pxn-2* genomic DNA (1.4kb, cut by BglII and KpnI) to obtain *gfp* RNAi, *pxn-1* RNAi and *pxn-2* RNAi bacterial feeding constructs. The RNAi constructs were introduced to HT115 (DE3) bacteria. To knock down *pxn-1* and *pxn-2* gene expression, the experimental worms were fed on RNAi transformed HT115 at 48 hours and their eggs were used for survival assay. RNAi had been fed continuously until the hatched eggs were grown to young adults (56 hours post hatching) as described in Kamath *et al.*<sup>[9]</sup>.

To prepare the translational fusion constructs, *pxn-1* and *pxn-2* full length cDNA were ligated to heat shock promoters and fused with *gfp* to yield *Phs::pxn-1::gfp* and *Phs::pxn-2::gfp*. The 10 ng/ $\mu$ l construct was injected into wild type N2 or *pxn-1* (*ok785*) mutant. 80 ng/ $\mu$ l of plasmid pRF4 containing a dominant gene (*rol-6*) was co-injected for isolation by their rolling pattern. Microinjection was performed as previously described<sup>[10]</sup>. GFP expression was detected using a fluorescent microscope system (80i-DS-Fi1, Nikon). For ectopic expression, the transgenic worms were subjected to heat shock at 30°C for 1 hour every 12 hours and followed by pathogen survival assays.

### 2.3. Survival Assay

Gravid worms were treated with hypochlorite and then washed with M9 buffer several times to synchronize. The survived eggs were used for the experiments. To measure survival rates, approximately 1000 individual eggs of individual genotypes were grown to young

adults (56 h after hatching) at 20°C and the worms were transferred to a bacteria lawn plate either non-pathogenic *Bacillus subtilis* or pathogenic *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Subsequently, live worms in a specific plate were counted at every 6 hours.

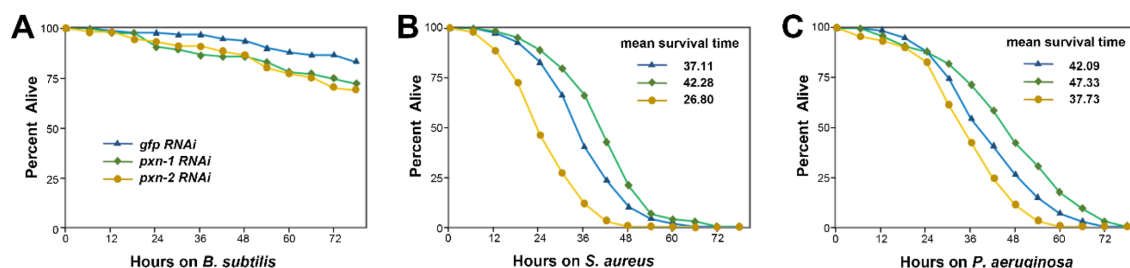
### 2.4. Statistical Analysis

All survival assays against pathogenic bacteria, *S. aureus* and *P. aeruginosa* including non-pathogenic *B. subtilis* were calculated and differentiated by using the log-rank test of OASIS 2<sup>[11]</sup>.

## 3. Results & discussion

### 3.1. *pxn-2* Gene Knock Down Caused Pathogen Susceptibility, but *pxn-1* Knock Down Gained the Resistance

To investigate functions of peroxidases in *C. elegans* innate immunity, we performed a worm survival assay against two pathogenic bacteria, *Staphylococcus aureus* (*S. aureus*), and *Pseudomonas aeruginosa* (*P. aeruginosa*). In addition, non-pathogenic bacteria, *Bacillus subtilis* (*B. subtilis*), were tested for pathogen sensitivity as a control. In *B. subtilis* treatments, knock-down of *pxn-1* (*pxn-1* KD) and *pxn-2* (*pxn-2* KD) by using RNAi showed similar survival rates and they were not significantly different from the *gfp* control rate (Fig. 1A). In contrast, pathogenic bacteria, *S. aureus* and *P. aeruginosa*, caused different responses in *pxn-1* KD and *pxn-2* KD worms. *pxn-2* KD was 28% more susceptible to *S. aureus* than the control in the mean survival time (Fig. 1B). In *P. aeruginosa*, *pxn-2* KD survival time was 10% less than the control in the mean survival time (Fig. 1C). On the other hands, *pxn-1* KD showed 13% enhanced resistance to the both pathogens compared to the control (Fig. 1B and C). Therefore, these results suggest that *pxn-2* KD causes susceptibility and *pxn-1* KD causes resistance to both pathogens. All three strains were more resistance to *P. aeruginosa* than to *S. aureus*. The different susceptibility can be the result of cell wall structure difference between *S. aureus*, a gram-positive and *P. aeruginosa*, a gram-negative. Recently, peroxidase can kill gram-negative bacteria by binding between immunoglobulin domain and lipopolysaccharide of bacteria and by generating hypohalous acids in murine lung defense<sup>[6]</sup>.



**Fig. 1.** Knock down of *pxn-1* gene in *C. elegans* resists to the pathogens and *pxn-2* knock down is sensitive to the pathogens. (A) Survival of *pxn-1* and *pxn-2* knock down worms to *B. subtilis*. (B) Survival of *pxn-1* and *pxn-2* knock down worms to *S. aureus*. (C) Survival of *pxn-1* and *pxn-2* knock down worms to *P. aeruginosa*. *gfp RNAi*, N2 treated with *gfp RNAi*; *pxn-1 RNAi*, treated with *pxn-1 RNAi*; *pxn-2 RNAi*, treated with *pxn-2 RNAi*. N > 600 individuals per each treatment. The survival rate is generated and differentiated by using OASIS 2.

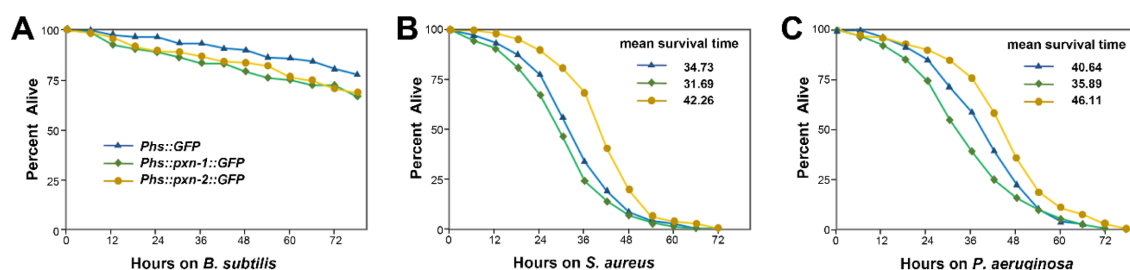
### 3.2. Overexpression of *pxn-2* Enhanced Resistance to the Pathogens

For further understanding of peroxidases' role in innate immune defense, we examined survival rates of *pxn-1* and *pxn-2* overexpressing worms fed on the pathogens. Heat shock promoter was used for ectopic expression during pathogen treatment. *pxn-1* and *pxn-2* overexpressing worms were slightly sensitive to *B. subtilis* compared to the *gfp* controls (Fig. 2). As expected by the RNAi knock down data (Fig. 1), *pxn-1* overexpression resulted in pathogen sensitivity; worms of *pxn-1* overexpression showed 9% decreased mean survival time compared to *gfp* control to *S. aureus* and 13% less to *P. aeruginosa* (Fig. 2B and C). The overexpression of *pxn-2* gained resistance against both pathogens, 22% and 14% longer survival time than the *gfp* control (Fig. 2B and C). These results conclude that *pxn-2* overexpression can strengthen the innate immune defense, but *pxn-1* overexpression restrict the defense. Therefore,

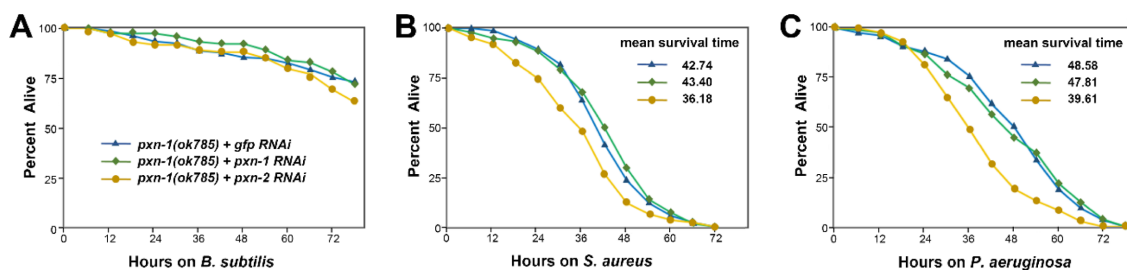
*pxn-2* may function on pathogen resistance and *pxn-1* may restrict the resistance via interacting with *pxn-2*.

### 3.3. Double Deficiency of *pxn-1* and *pxn-2* Compensate the Resistant Effect on *pxn-1* Knock Down to Pathogens

In addition to the above gene knock down and overexpression results, previous study showed that PXN-1 interacted with PXN-2 reversely in neurodegeneration of aged *C. elegans*. Therefore, *pxn-1* can function on *pxn-2* negatively in innate immune system. To test the relationship between *pxn-1* and *pxn-2*, *pxn-1* and *pxn-2* gene double knock down were constructed and the survival rate was examined. *pxn-1* mutant instead of *pxn-1 RNAi* was used in this experiment due to low efficiency of two different RNAi feeding<sup>[9]</sup>. For the control, *pxn-1* mutant fed on *gfp RNAi* was examined. There was no significant difference between the mean survival hours of control *pxn-1* (ok785) and *pxn-1* with



**Fig. 2.** Overexpression of *pxn-1* and *pxn-2* results in the opposite responses against the pathogens. (A) Survival of *pxn-1* and *pxn-2* overexpressing worms to *B. subtilis*. (B) Survival of *pxn-1* and *pxn-2* overexpressing worms to *S. aureus*. (C) Survival of *pxn-1* and *pxn-2* overexpressing worms to *P. aeruginosa*. *Phs::GFP*, heat shock promoter combined with *gfp*; *Phs::pxn-1::GFP*, heat shock promoter with *pxn-1* and *gfp*; *Phs::pxn-2::GFP*, heat shock promoter with *pxn-2* and *gfp*. N > 600 individuals per each treatment. The survival rate is generated and differentiated by using OASIS 2.



**Fig. 3.** Double knock down of *pxn-1pxn-2* reverse the resistance of *pxn-1* knock down to the pathogens. (A) Survival of *gfp*, *pxn-1* and *pxn-1pxn-2* knock down worms to *B. subtilis*. (B) Survival of *gfp*, *pxn-1* and *pxn-1pxn-2* knock down worms to *S. aureus*. (C) Survival of *gfp*, *pxn-1* and *pxn-1pxn-2* knock down worms to *P. aeruginosa*. *pxn-1 (ok785) + gfp RNAi*, *pxn-1 (ok785) mutant treated with gfp RNAi*; *pxn-1 (ok785) + pxn-1 RNAi*, *pxn-1 (ok785) treated with pxn-1 RNAi*; *pxn-1 (ok785) + pxn-2 RNAi*, *pxn-1 (ok785) treated with pxn-2 RNAi*. N > 600 individuals per each treatment. The survival rate is generated and differentiated by using OASIS 2.

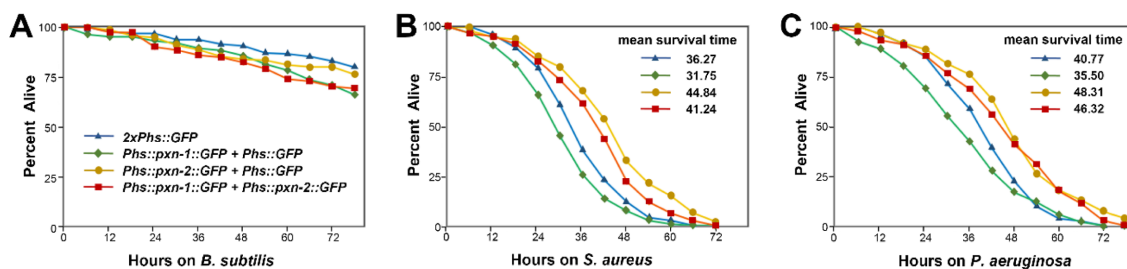
*pxn-1* RNAi, 42.74 and 43.3 hours respectively to *S. aureus* treatment and 48.58 and 47.81 hours to *P. aeruginosa* (Fig. 3B and C). However, the *pxn-1* mutant fed on *pxn-2* RNAi became sensitive to both pathogens, 36.18 mean survival hours to *S. aureus* and 39.61 hours to *P. aeruginosa* (Fig. 3B and C). These results suggest that additional *pxn-2* knock down in *pxn-1* mutant background eliminate the enhanced effect of *pxn-1* mutant survival in innate immune system (Fig. 1). In addition, the results suggest that *pxn-2* can affect *pxn-1* reversely in innate immune defense.

### 3.4. The Double Overexpression of *pxn-1pxn-2* Overcame Pathogen Susceptibility of *pxn-1* Overexpression

Double knock down experiment suggested a negative

interrelationship between *pxn-1* and *pxn-2* function in innate immunity. Thereafter, we confirm the idea by using double overexpressing worms against pathogens. At first, the amount of overexpression should be compensated. The used worms in this experiment were following: twice amount of *gfp* injection to N2 for the control ( $2x Phs::gfp$ ), *pxn-1* and *gfp* for *pxn-1* overexpression ( $Phs::pxn-1::gfp + Phs::gfp$ ), *pxn-2* and *gfp* for *pxn-2* overexpression ( $Phs::pxn-2::gfp + Phs::gfp$ ) and *pxn-1* and *pxn-2* for *pxn-1pxn-2* overexpression ( $Phs::pxn-1::gfp + Phs::pxn-2::gfp$ ).

Consistent with the previous result (Fig. 2), *pxn-1* overexpressing worms survived less than the control, 13% decreased mean survival time to both pathogens (Fig. 4B and C). *pxn-2* overexpression increase the mean survival hours, 24% and 19% increase to *S.*



**Fig. 4.** The pathogen resistance in double overexpression of *pxn-1pxn-2* recovers from the susceptibility in *pxn-1* overexpression. (A) Survival of *gfp*, *pxn-1*, *pxn-2* and *pxn-1pxn-2* overexpressing worms to *B. subtilis*. (B) Survival of *gfp*, *pxn-1*, *pxn-2* and *pxn-1pxn-2* overexpressing worms to *S. aureus*. (C) Survival of *gfp*, *pxn-1*, *pxn-2* and *pxn-1pxn-2* overexpressing worms to *P. aeruginosa*.  $2XPhs::GFP$ , twice amount of heat shock promoter with *gfp*;  $Phs::pxn-1::gfp + Phs::gfp$ , heat shock promoter with *pxn-1* and heat shock promoter with *gfp*;  $Phs::pxn-2::gfp + Phs::gfp$ , heat shock promoter with *pxn-2* and heat shock promoter with *gfp*;  $Phs::pxn-1::gfp + Phs::pxn-2::gfp$ , heat shock promoter with *pxn-1* and *gfp* and heat shock promoter with *pxn-2* and *gfp*. N > 600 individuals per each treatment. The survival rate is generated and differentiated by using OASIS 2.

*aureus* and to *P. aeruginosa* (Fig. 4B and C). *pxn-1pxn-2* double overexpression made the mean survival time longer than the control and *pxn-1* overexpression, but the survival time was no significantly different from *pxn-2* overexpression to *S. aureus* (Fig. 4B). In *P. aeruginosa*, *pxn-1pxn-2* double overexpressing worms survived longer than the control and the *pxn-1* overexpressing ones. However, the mean survival time of double overexpression was not significantly different from *pxn-2* overexpression (Fig. 4C). Double knock down and double overexpression results reinforce the idea of reverse interrelationship between *pxn-1* and *pxn-2*; *pxn-1* can suppress the resistant effect of *pxn-2* in innate immune defense in *C. elegans*. Peroxidase is considered as a peroxidase with a secretory motif, which previous studies focused on its antioxidant activity. Recently, the maintenance of extracellular matrix structure and their actions on innate immunity has focused to develop efficient bacteria killing agents<sup>[6,12]</sup>.

#### 4. Conclusion

In *Caenorhabditis elegans* innate immune system, *peroxidase 1* may suppress the resistant role of *peroxidase 2* against pathogenic *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Further research of peroxidase is necessary for developing a potential bactericidal agent and fast and massive screening can be done by using *C. elegans*.

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