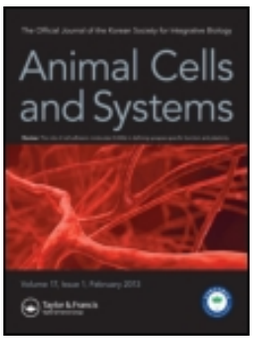

Animal Cells and Systems

ISSN: 1976-8354 (Print) 2151-2485 (Online) Journal homepage: https://www.tandfonline.com/loi/tacs20

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To cite this article: Raymond Febus, Sun-Kyung Lee & Joohong Ahnn (2014) Calcineurin differentially functions in innate immune response of *Caenorhabditis elegans* fed with gram-positive or gram-negative bacteria, Animal Cells and Systems, 18:6, 394-398, DOI: 10.1080/19768354.2014.972981

To link to this article: https://doi.org/10.1080/19768354.2014.972981

Published online: 11 Nov 2014.

Animal Cells and Systems, 2014
Vol. 18, No. 6, 394–398, http://dx.doi.org/10.1080/19768354.2014.972981

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Calcineurin differentially functions in innate immune response of *Caenorhabditis elegans* fed with gram-positive or gram-negative bacteria

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(*Received 8 August 2014; received in revised form 27 September 2014; accepted 30 September 2014*)

Innate immunity in *Caenorhabditis elegans* involves multiple processes including growth factor pathways, neuroendocrine signaling mediating various kinase activities. In this study, we report that calcineurin, a Ca^{2+}/calmodulin-dependent serine/threonine phosphatase, regulates the nematode's vulnerability to bacterial pathogens. Interestingly, calcineurin seems to differently function in host–pathogen interaction of gram-positive and gram-negative bacteria. In addition, loss of calnexin, a Ca^{2+}-binding chaperone in endoplasmic reticulum, compensates immune response of the nematode to gram-negative bacteria, while that of calreticulin has no effect. The results suggest that *C. elegans* has evolved different immune response strategy to gram-positive or gram-negative bacteria, utilizing the same immune machinery in different modes.

Keywords: calcineurin; calreticulin; calnexin; innate immunity

Introduction

Calcineurin is a Ca^{2+}/calmodulin-dependent serine/threonine phosphatase, which is evolutionarily highly conserved from yeast to mammals (Crabtree 2001). Mammalian calcineurin activates nuclear factor of activated T-cell transcription factor, which is a regulator of interleukin-2, a key cytokine required for T-cell growth (Crabtree 1999). Catalytic subunit A (CnA) and a regulatory subunit B (CnB) form a heterodimer of calcineurin, which is not active until dissociated by binding of calmodulin loaded with Ca^{2+} to CnB (Hashimoto et al. 1990).

The innate immune system, also known as a non-specific immune system and the first line of defense, is comprised of cells and mechanisms that defend the host from infection by other organisms in a non-specific manner (Garsin et al. 2001). This means that the cells of the innate immune system recognize and respond to pathogens in a generic way. It does not confer long-lasting or protective immunity to the host yet provide immediate defense against possible infection. Therefore, the innate immunity provides fundamental self-defense systems in almost all classes of organisms. The vertebrate innate immune system includes cytokine-driven activation of immune cells, the activation of the complement cascade to identify bacteria, the clearance of foreign substances or dead cells by white blood cells, and physical and chemical barrier against infectious agents (Gravato-Nobre & Hodgkin 2005).

Caenorhabditis elegans is a nematode that lives in the soil where it feeds on bacteria. *C. elegans* possesses an inducible innate immune system which involves the *daf-2* insulin-like pathway, the p38 MAPK pathway, and the TGF-β-like pathway (Kurz & Tan 2004). These pathways are all well conserved in vertebrates, where they play various roles in metabolism, cell proliferation, growth, etc. The induction of an immune response is triggered by the recognition of pathogen-associated molecular patterns (PAMP) found only in micro-organisms with pattern recognition receptors (PRR) encoded in host genome (Medzhitov & Janeway 1997). No obvious PRR has been described for *C. elegans*, while *tol-1* has been reported as the unique worm homologue of the Drosophila Toll protein that is critical for the insect defense against microbes (Hoffmann 2003). However, the role of *tol-1* in immune response activation in the nematode seems to be very limited, and Rel homology domain protein which is required in fly Toll pathway is absent in *C. elegans.*

Pseudomonas aeruginosa is an opportunistic gram-negative human pathogen. Normally, nonpathogenic *P. aeruginosa* is able to infect human in immune-compromised condition. The flagellum of *P. aeruginosa* is an important factor in the infection process, playing a role of motility and attachment apparatus, and also recognized as a PAMP inducing innate immunity. *P. aeruginosa* can infect *C. elegans* causing intestinal inflammation, and eventual accelerated death, by suppressing intestinal immune defense through virulence factor mediated activation of *daf-2* (Evans et al. 2008).

Staphylococcus aureus is a facultative anaerobic gram-positive coccal bacterium. *S. aureus* appears as grape-like clusters when viewed through a microscope and has large, round, golden-yellow colonies, often with hemolysis, when grown on blood agar plates. *S. aureus* is able to infect a wide range of hosts and very versatile in its

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infection areas ranging from superficial skin infections to toxic shock syndrome. *S. aureus* treatment itself has become difficult due to the emergence of antibiotic resistant strains (Irazoqui, Troemel, et al. 2010).

In this study, using *C. elegans* to study, the innate immune system has led to several discoveries about the intricacies of the innate immune system. *tax-6 (jh107)*, the calcineurin gain of function mutants, was resistant to the gram-negative bacteria *P. aeruginosa* (PA14), compared to wild type and loss of function mutants, *tax-6 (ok2065)* or *cnb-1(jh101)*. Interestingly, when the same mutants were grown on *S. aureus* (SA), a gram-positive pathogen, the reverse results were observed. These results suggest that calcineurin's role in gram-negative infections and gram-positive infections could be associated with different signaling pathways that are specific to gram-negative and gram-positive bacterial infections.

Methods and materials

C. elegans *strains and maintenance*

N2, *tax-6 (ok2065)*, and *cnx-1(nr2009)* were obtained from *C. elegans* Genetics Center (CGC) and *rcan-1 (tm1925)* and *rcan-1 (tm2021)* from National BioResource Project (NBRP). *crt-1 (jh101)*, *cnb-1 (jh103)*, and *tax-6 (jh107)* were described previously (Park et al. 2001; Bandyopadhyay et al. 2002). Nematodes were grown at 20°C according to standard protocol and were synchronized by plating 2–6 gravid adults' mutants on regular OP50 nematode growth media (NGM) plates. After 12 hours, each adult worm was transferred to new OP50 NGM plates. Each mutant strain was allowed to grow for 72 hours or until L4 larval stage or young adult. These worms were plated onto SA, PA14, or OP50 NGM plates.

Bacterial strains

The following bacterial strains were obtained from Frederick M. Ausubel: *P. aeruginosa* (PA14), PA14-GFP (green fluorescent protein [GFP] tag to visualize via microscopy), *S. aureus* (NCTC 8325 and Newman).

Killing assays

Killing assays were performed by using stock bacterial strain and incubated them on selective media plates overnight at 37°C. With the growth on the selective media plate, (i.e. NGM plates with rifampin 50 µg/ml), a single colony was picked, inoculated into selective LB broth, and incubated overnight at 37°C. When the LB broth was turbid (OD600 ≈ 0.5), 20 µl was plated onto non-seeded modified NGM (nematode growth medium) agar plates (50 mM NaCl, and 0.35% peptone) with 3.5-cm diameter and spread to cover the entire plate, "big lawn," and then incubated overnight at 37°C. Each newly seeded NGM plate was taken out and allowed to cool at RT for 12–24 hours before nematodes were added. Each plate was seeded with 25–60 nematodes and maintained at 25°C. Wild type nematodes, N2, were added to an OP50 NGM plate as a control. Each plate was counted for survival every 8–16 hours. Worms lost, burrowed or climbed onto the plate wall, were censored and removed from statistical analysis. A worm was considered dead when there was no response to a tap on the head or tail after three taps with a worm picker. Each plate was transferred to a new plate every 24 hours until eggs are no longer present, then every 48 hours depending on bacterial lawn and food availability. New bacterial stock was produced every two days to maintain healthy bacteria for seeding onto NGM plates.

Statistical analyses

Student's *t*-test was used to determine significance of single comparisons. One-way analyses of variance (ANOVAs) with Fisher's test were performed with online application for the survival analysis at a significance level of 0.05 for all multiple comparisons (Yang et al. 2011).

Results

Loss of function calcineurin mutants tax-6(ok2065) *and gain of function mutant* tax-6(jh107) *showed different resistance to gram-positive and gram-negative bacteria*

Calcineurin is critical in mammalian immune system function. To see whether calcineurin functions in innate immunity of nematodes, we fed *C. elegans* with bacteria, which have been known to be pathogenic to worms. First, calcineurin mutants were fed with gram-positive *S. aureus* (SA). The survival rate for *N2* worms on OP50 was 272 ± 17 hours in average (Table 1). The survival rate for *N2* when grown on *S. aureus* (SA) came out to be around 93 ±

Table 1. Average survivals.

Bacterial strain	Worm strain	Survival (hours)
OP 50	*N2*	272 ± 2
S. aureus	*N2*	93 ± 9
	tax-6 (jh107)	90 ± 6
	tax-6(ok2065)	55 ± 7*
	cnb-1(jh103)	53 ± 4*
P. aeruginosa	*N2*	72 ± 3
	tax-6 (ok2065)	50 ± 3*
	tax-6 (jh107)	87 ± 13*
	cnb-1(jh103)	66 ± 11*
	crt-1(jh101)	76 ± 28
	cnx-1(nr2009)	74 ± 6
	rcan-1(tm1925)	68 ± 6
	rcan-1(tm2021)	72 ± 5
	cnx-1(nr2009);crt-1(jh101)	60 ± 16*

Note: $n > 300$.
*$P < 0.01$ relative to N2.

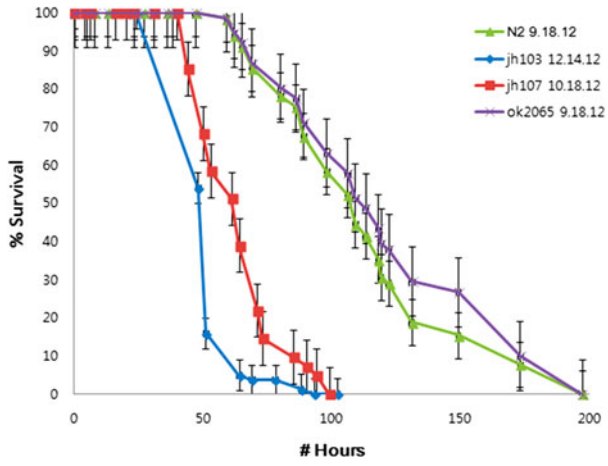

Figure 1. Calcineurin gain-of-function strain *tax-6(jh107)* and loss-of-function mutants *tax-6(ok2065)* fed with SA. The survival curves for these two mutant strains are presented with N2 as a control. The *tax-6(ok2065)* mutant survives slightly longer than the *tax-6(jh107)* mutants (n > 100 per strain, done in triplicate).

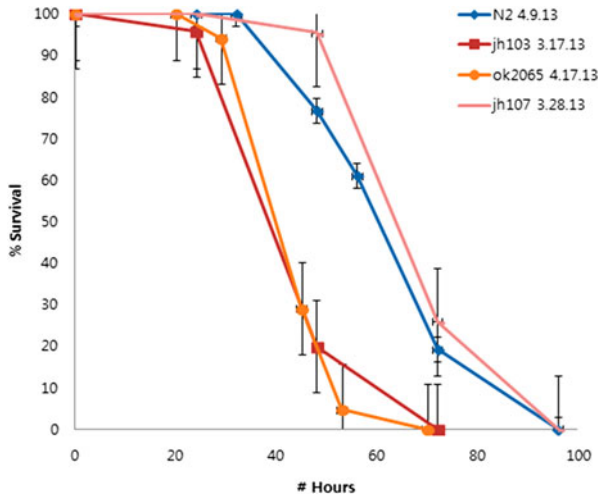

Figure 2. Calcineurin mutants fed with PA14. While *tax-6 (jh107)* lived slightly longer than N2, calcineurin subunit B mutant *cnb-1(jh103)* showed a similar reduced resistance as *tax-6(ok2065)* (n > 100 per strain, done in triplicates).

9 hours, indicating that SA is pathogenic to worms as previously reported (Figure. 1). The overall survival rate for *tax-6(ok2065)* mutants was 90 ± 6 hours, which was comparable to wild-type. However, the gain of function *tax-6(jh107)* mutants survived for only 55 ± 7 hours. We also found that *cnb-1(jh103)*, a null calcineurin B subunit mutant, showed a survival rate at 53±4 hours. The different resistances of loss-of-function and gain-of-function calcineurin mutants suggest that there might be a specific function of calcineurin in vulnerability of the worms to pathogenic gram-positive bacteria.

To see whether calcineurin functions in the response to pathogenic gram-negative bacteria, we set out to feed calcineurin mutants with *P. aeruginosa*. The survival for the N2 control on PA14, a pathogenic gram-negative bacteria *P. aeruginosa,* was 72 ± 3 hours which was similar with previously published data (Irazoqui, Urbach, et al. 2010). The survival rates of the calcineurin loss-of-function mutants *tax-6*(*ok2065)* when grown on PA14 was 63 ± 11 hours and the calcineurin gain-of-function mutants *tax-6(jh107)* survival rates were similar to N2 at 87 ± 13 hours (Figure. 2).

rcan-1 *(regulator of calcineurin-1) mutants showed no vulnerability to PA14*

RCAN (regulator of calcineurin) is a calcineurin binding protein which regulates the function of calcineurin. Dysregulation of RCAN is involved in many human diseases, such as Down syndrome, Alzheimer's disease, and cardiac hypertrophy (Klee et al. 1998). To see whether RCAN plays a role in innate immune response, we fed *C. elegans* RCAN deletion mutants *rcan-1(tm1925)* and *rcan-1(tm2021)* with PA14. Both mutants showed similar survival to PA14, and there was no statistical significance between the two mutants and wild type (Figure. 3).

Knock-out of calnexin increase immune protection of C. elegans *to PA14*

Calreticulin and calnexin are endoplasmic reticulum (ER)-resident lectin-like chaperones (Park et al. 2001). They bind and buffer Ca^{2+} in ER to regulate Ca^{2+} homeostasis (Lee & Michalak 2012). *crt-1(jh101)* and *cnx-1(nr2009)*, *C. elegans* calreticulin and calnexin null mutants, have been reported that they showed reduced brood size and embryonic lethality. When fed with PA14, *crt-1(jh101)*

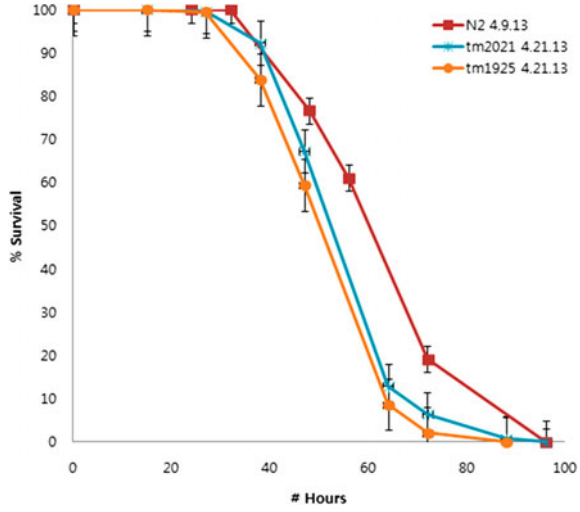

Figure 3. RCAN mutants *rcan-1(tm1915)* and *rcan-1(tm2021)* fed on PA14 showed a slight, but not significant decreased survival compared to N2 (n > 100 and each strain was run in triplicates).

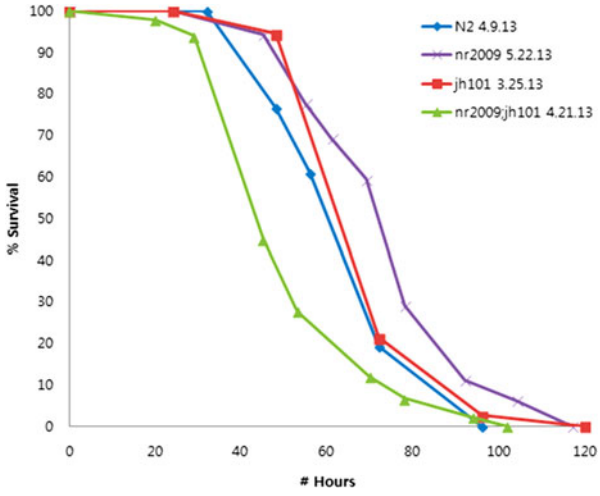

Figure 4. Calreticulin *crt-1(jh101)* and calnexin mutants *cnx-1 (nr2009)* fed with PA14. *cnx-1(nr2009)* has a slight resistance to the PA14 gram-pathogen compared to wild type whereas calreticulin mutants *crt-1(jh101)* fed on PA14 shows a similar survival as N2 ($n > 100$ per strain, each strain ran in triplicate). Calreticulin and calnexin double mutants, *cnx-1(nr2009);crt-1 (jh101)*, showed a decreased life span compared to the single mutants *crt-1(jh101)* and *cnx-1(nr2009)*.

showed a survival similar to that of N2. Interestingly, however, the survival of *cnx-1(nr2009)* was slightly extended, compared to wild-type. Finally, *cnx-1(nr2009); crt-1(jh101)* fed on PA14 drastically lowered the survival, when compared to N2 (Figure. 4). These data indicate that calreticulin and calnexin are required for proper immune response in *C. elegans* and may have different roles to mediate innate immunity to bacteria.

Discussion

Calcineurin is a well-conserved protein that is present in *C. elegans*, which does not have an adaptive immune system but an apparent innate immune system, and mammals which have both an innate immune system and adaptive immune system, while calcineurin plays a vital role in the adaptive immune system's ability to develop and differentiate T cells, as well as proliferation of cytokines, it would appear that calcineurin could play a vital role in the innate immune system of *C. elegans*.

Gram-negative and gram-positive bacteria, SA and PA14, are pathogenic to *C. elegans*, and an inducible innate immune system, which involves the *daf-2* insulin-like pathway, the p38 MAPK pathway, and the TGF-β-like pathway, is required. Here, we found that calcineurin functions in innate immune response to the bacteria and may play in different mode in response to gram-negative or gram-positive bacteria. It would appear that *tax-6 (jh107)* gain of function mutant interferes with *C. elegans* immune response when grown on SA, whereas tax-6 (*ok2065)* loss of function mutant does not (Figure 1). This suggests that the overactive dephosphorylation by calcineurin may suppress immune response while exposed to a gram-positive pathogen. Interestingly, *cnb-1(jh103)*, calcineurin loss of function in the B subunit, also seems to have a suppressed immune system, possibly due to the overall pleiotropic effect of the mutation (Bandyopadhyay et al. 2002). Surprisingly, when the gain of function mutant *tax-6(jh107)* was fed on gram-negative PA14, it showed a slight resistance compared to the loss of function mutant, *tax-6(ok2065)* which showed an increased susceptibility. Altogether, these results suggest that there might be separate pathways that involve calcineurin in the response of gram-positive and gram-negative bacteria.

Calnexin is an integral ER membrane protein whose luminal domain is structurally and functionally similar to calreticulin. Despite the similarity between calnexin and calreticulin, it has been reported that their expression patterns and knock-out phenotypes in both mammalian and *C. elegans* are different (Lee et al. 2005; Kraus et al. 2010). *cnx-1(nr2009)* appeared to be more resistant to pathogenic bacteria, while *crt-1(jh101)* did not. *cnx-1 (nr2009)* is a null mutant that has reproductive defects when grown at 25°C, as well as some other reproductive defects in general. These reproductive defects of *cnx-1 (nr2009)* may be compensatory for the increased resistance to PA14.

It is unclear what the direct correlation or pathway that calcium-binding proteins are involved. However, the evidence shown here seems to point to the fact that calcineurin does play a role in the innate immunity of *C. elegans* response to both gram-negative and gram-positive pathogens.

Further studies of both biochemical and genetic approaches would be beneficial to the direction of identifying different pathways involved in *C. elegans* immune responses involving calcineurin. Being able to identify the different proteins that are up-regulated and down-regulated in calcineurin mutants during a pathogenic bacterial infection would provide valuable information regarding specific immune pathways in *C. elegans*.

A continued study of the innate immune system of *C. elegans* using a pathogenic feeding system along with mutating the different *C. elegans* lines may reveal an evolutionary link in the innate immune system that might stem from *C. elegans*.

Acknowledgments

Some strains were obtained from CGC and NBRP as described above. Frederick M. Ausubel provides PA and SA pathogenic strains used in the study. Hyun Jin Kim, Joon Kyung Sung, and Young-Ho Jung are appreciated for their technical supports.

Funding

This work was supported by Korean Ministry of Education (Basic Science Research Program no. 2013R1A1A2005836) and Women Scientist program (no. 2013R1A1A3A04006010) in the Basic Science Research Program through the National Research Foundation of Korea grant funded by Korean Ministry of Science, Information and Communication Technology & Future Planning.

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