tions at critical checkpoints during both the induction and effector phases of two different induced autoimmune diseases.

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A Conserved p38 MAP Kinase Pathway in *Caenorhabditis elegans* Innate Immunity

Dennis H. Kim,[1]* Rhonda Feinbaum,[1]* Geneviève Alloing,[1]† Fred E. Emerson,[1] Danielle A. Garsin,[1] Hideki Inoue,[2] Miho Tanaka-Hino,[2] Naoki Hisamoto,[2] Kunihiro Matsumoto,[2] Man-Wah Tan,[1]‡ Frederick M. Ausubel[1]§

A genetic screen for *Caenorhabditis elegans* mutants with enhanced susceptibility to killing by *Pseudomonas aeruginosa* led to the identification of two genes required for pathogen resistance: *sek-1*, which encodes a mitogen-activated protein (MAP) kinase kinase, and *nsy-1*, which encodes a MAP kinase kinase kinase. RNA interference assays and biochemical analysis established that a p38 ortholog, *pmk-1*, functions as the downstream MAP kinase required for pathogen defense. These data suggest that this MAP kinase signaling cassette represents an ancient feature of innate immune responses in evolutionarily diverse species.

The evolutionary conservation in the mechanisms of innate immunity between *Drosophila melanogaster* and mammals suggests that the study of immune function in diverse species may yield key insights into the evolutionary origins and molecular mechanisms of the mammalian innate immune system (*1*). The development of experimental host-pathogen systems that use invertebrate hosts greatly facilitates the genetic analysis of immune function of a host organism. Here, we report on our initial efforts toward the comprehensive forward genetic analysis of immune function in the nematode, *Caenorhabditis elegans*.

To screen for mutants with enhanced susceptibility to pathogens (Esp), we exposed mutagenized F_2 generation L4 larval stage nematodes to *Pseudomonas aeruginosa* strain PA14 under slow killing assay conditions, which involve an infection-like process requiring live pathogenic bacteria (*2*). The population of worms was then screened after a period of 16 to 30 hours for dead animals (wild-type animals typically began to die at approximately 34 hours). Under these conditions, hermaphrodite animals died as gravid adults; thus, putative mutants were recovered by transferring individual dead worms containing their brood to plates seeded with the standard laboratory nematode food source, *Escherichia coli* OP50, whereupon the progeny were recovered. Potential mutants were rescreened by a comparative evaluation of survival on OP50 and pathogen PA14.

We screened an estimated 14,000 haploid genomes and selected for further study two of an initially isolated 10 mutants, *esp-2(ag1)* and *esp-8(ag3)*, that had similar and the most penetrant *Esp* phenotypes. When animals were exposed to PA14 under standard assay conditions, 100% of *esp-2* animals, 90% of *esp-8*, and 0% of wild-type animals were killed by 31 hours (Fig. 1A). To monitor the course of infection in the *esp-2* and *esp-8* mutant animals, worms were fed green fluorescent protein (GFP)–labeled PA14 (*2*). Accumulation of PA14 in the intestine (detected as early as 6 hours in *esp-2* and *esp-8* animals after exposure to GFP-labeled PA14) was correlated with the kinetics of killing. After 20 hours on GFP-labeled PA14, *esp-2* and *esp-8* animals had an overwhelming accumulation of PA14 in the anterior portion of their intestines, whereas wild-type animals showed minimal accumulation of GFP-labeled PA14 (Fig. 1D). Of note, the enhanced susceptibility of *esp-2* and *esp-8* mutant animals was not specific to the Gram-negative pathogen, *P. aeruginosa*, because the mutants were also hypersusceptible to the Gram-positive pathogen *Enterococcus faecalis* (Fig. 1C), which also kills *C. elegans* (*3*).

An implicit concern with the isolation of *C. elegans* mutants with enhanced susceptibility to pathogens is that many loss-of-function mutations might compromise the overall health of the worm, resulting in a secondary increased sensitivity to pathogens. In fact,

[1]Department of Genetics, Harvard Medical School, and Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114, USA. [2]Department of Molecular Biology, Graduate School of Science, Nagoya University and CREST, Japan Science and Technology Corporation, Chikusa-ku, Nagoya 464-8602, Japan.

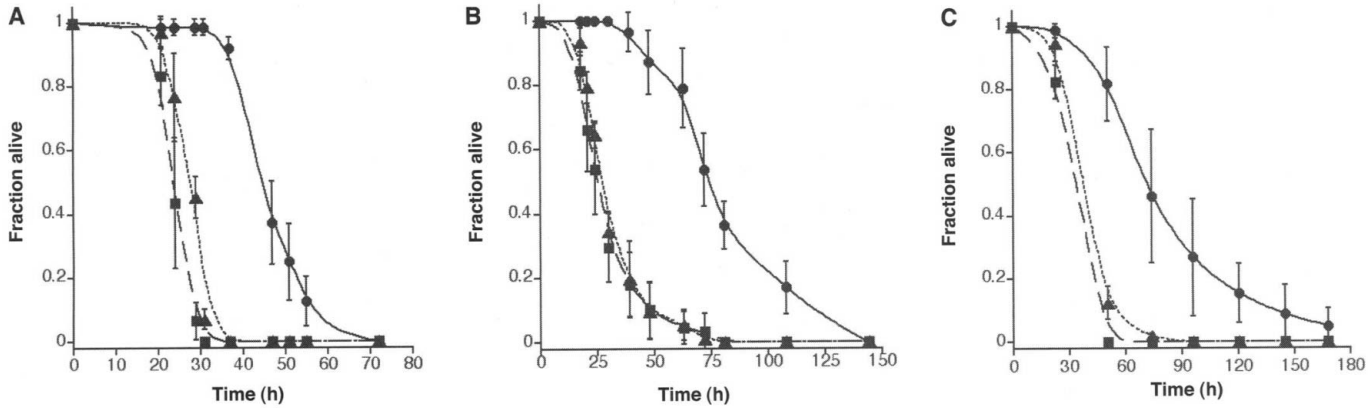
*These authors contributed equally to this work.
†Present address: Laboratoire de Biologie Vegetale et Microbiologie, Universite de Nice-Sophia Antipolis, Parc Valrose 06108 Nice Cedex 2, France.
‡Present address: Department of Genetics, Stanford University School of Medicine, 300 Pasteur Drive, Room M337, Stanford, CA 94305–5120, USA.
§To whom correspondence should be addressed. E-mail: ausubel@molbio.mgh.harvard.edu

another mutant isolated in an Esp screen, *esp-1(aj3)*, exhibits pharyngeal grinder dysfunction due to a mutation in a troponin T gene expressed predominantly in the pharyngeal muscle; the Esp phenotype in this case is likely due to accelerated accumulation of pathogenic bacteria that pass through the defective grinder into the intestine (*4*). Importantly, *esp-2* and *esp-8* were shown to have normal feeding behavior as well as defecation, and furthermore, on *E. coli* OP50, *esp-2* and *esp-8* mutant animals were observed to develop at a normal rate and reach adulthood concomitant with N2 wild-type worms.

To assess the relative fitness of *esp-2* and *esp-8* mutants, we determined the life-span of *esp-2* and *esp-8* animals and showed that their longevity was equivalent to wild-type worms (Fig. 2). The only pleiotropic phenotype that is readily apparent in the *esp-2* and *esp-8* mutants is an egg-laying defect. To determine whether a matricidal egg-laying defect contributes to the Esp phenotype, we constructed double mutants of *esp-2* and *esp-8* with the temperature-sensitive sterile *glp-4(bn2)* mutant, resulting in animals that do not generate a brood at the restrictive temperature (*5*). In the *glp-4* mutant background, the *esp-2* and *esp-8* mutations retain the ability to confer sensitivity to PA14 (Fig. 1B). Thus, the *esp-2* and *esp-8* mutations confer enhanced susceptibility to both Gram-negative and Gram-positive pathogens, and this phenotype cannot be attributed to diminished fitness, impaired feeding or defecation, or defective egg-laying.

To identify the genes corresponding to *esp-2* and *esp-8* mutations, we used high-resolution single-nucleotide polymorphism mapping (*6*), sequencing of candidate genes, and transformation rescue (*7*). *esp-2* was mapped to a 110-kilobase (kb) region of chromosome X and rescued by microinjection of a 9.2-kb fragment that contained the R03G5.2 coding sequence and 5 kb of upstream sequence. R03G5.2 encodes SEK-1 (*8*), a *C. elegans* MAP kinase kinase (MAPKK) homologous to mammalian MKK3/MKK6 (50% sequence identity) and MKK4 (43% sequence identity). The *esp-2/sek-1(ag1)* allele is a C-to-T substitution that results in the mutation of highly conserved Gly^{212} to Arg^{212}. The *sek-1(km4)* allele (*8*), which carries a deletion of the kinase domain of *sek-1*, also exhibited the Esp phenotype to a degree comparable to *esp-2/sek-1(ag1)* (Fig. 3A).

esp-8 was mapped to a 400-kb region of chromosome II and rescued with a 13-kb fragment containing the F59A6.1 coding sequence and 3.8 kb of upstream promoter sequence. F59A6.1 encodes the *C. elegans* ortholog of the mammalian MAPKKK ASK1 that has recently been found to correspond to the *nsy-1* locus (*9*). The *esp-8/nsy-1(ag3)* mutation is a C-to-T change that converts the codon for Gln^{1013} to a premature stop codon just after the kinase domain. Another putative null allele, *nsy-1(ky397)* (*9, 10*), also showed sensitivity to PA14 comparable to *esp-8/nsy-1(ag3)* (Fig. 3A). Genetic and biochemical data suggest that NSY-1 is a direct activator



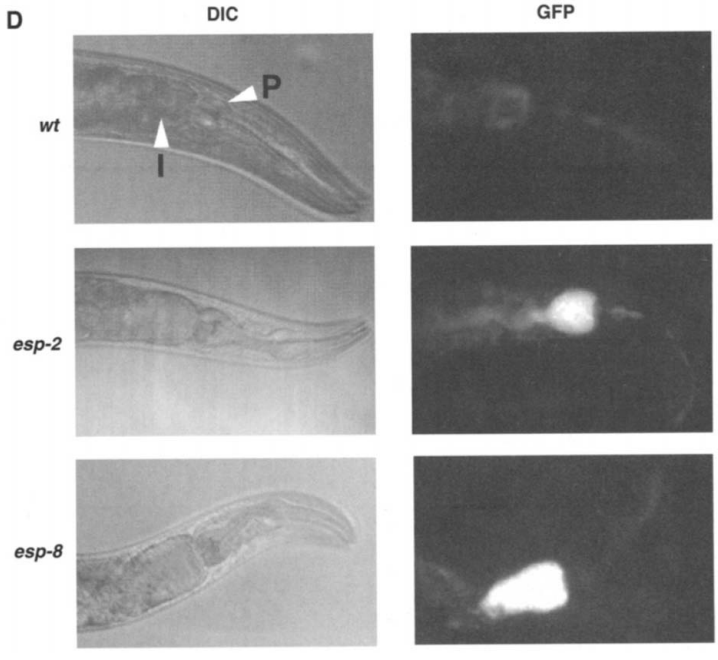


Fig. 1. *Caenorhabditis elegans* mutants with enhanced susceptibility to pathogens. (**A**) Survival curve of wild-type N2 *C. elegans* (circles) and *esp-2(ag1)* (squares) and *esp-8(ag3)* (triangles) mutants feeding on *P. aeruginosa* PA14. Approximately 25 L4-stage wild-type N2, *esp-2*, or *esp-8* worms were placed on each of three agar plates with PA14 lawns under standard slow killing (SK) assay conditions (*2*). The fraction of live animals was determined at each time point; worms were considered dead when they did not respond to gentle touch. Error bars represent the standard deviation between the three plates at each time point. Multiple trials of the experiment were done, and the data presented are from a representative experiment. (**B**) Survival curve of *glp-4(bn2)* (circles), *glp-4(bn2);esp-2(ag1)* (squares), and *glp-4(bn2);esp-8(ag3)* (triangles) worms feeding on PA14. Worms were grown at the nonpermissive temperature so that no germ-line proliferation occurred. (**C**) Survival curve of wild-type N2 (circles) and *esp-2* (squares) and *esp-8* (triangles) mutants feeding on *E. faecalis*. Wild-type N2, *esp-2*, and *esp-8* worms at the L4 larval stage were exposed to *E. faecalis* (strain OG1RF) under standard assay conditions (*3*). (**D**) Differential interference contrast (DIC) and GFP fluorescence microscopy of wild-type N2, *esp-2*, and *esp-8* worms fed GFP-labeled PA14. L4-stage worms were exposed to GFP-labeled PA14 under standard SK conditions, and live worms were mounted for microscopy in phosphate-buffered saline with sodium azide (5 mM). Worms are shown after 20 hours of feeding on GFP-labeled PA14. The arrows in the DIC microscopy image of the wild-type worm refer to the intestine (I) and pharynx (P). Weak fluorescence discernible in the wild-type animal is autofluorescence visible in worms not exposed to GFP-labeled bacteria.

of SEK-1 in the signaling pathway mediating asymmetric neuronal cell fate in AWC sensory neurons (*8*).

Although the Nsy phenotype corresponds to *nsy-1* and *sek-1* function in the AWC neurons, *nsy-1* and *sek-1* are expressed in a number of tissues types, including the intestine (*8, 9*). Whereas the signal transduction pathways that are involved in the Nsy and Esp phenotypes apparently share MAPKKK (ESP-8/NSY-1) and MAPKK (ESP-2/SEK-1) components, these pathways likely respond to different inputs. NSY-1 and SEK-1 have been shown to act downstream of UNC-43, a Ca^{2+}/calmodulin-dependent kinase, in the Nsy pathway (*8–10*). Two *unc-43* null mutants, *n1186* and *e755* (*11*), that have a null Nsy phenotype were found to have little (*e755*) or no (*n1186*) Esp phenotype (Fig. 3B).

Because mammalian MKK3/MKK6 MAP kinase kinases specifically activate the p38 family of MAP kinases and MKK4 MAP kinase kinase can activate p38 MAP kinase as well as JNK MAP kinases, we tested the role of p38 and JNK MAP kinases in the defense response of *C. elegans*. Two p38 MAP kinase orthologs, *pmk-1* and *pmk-2*, organized in an operon, have been identified in *C. elegans* (*12*). A mutant carrying a deletion of *pmk-2* has been reported to be L1 larval lethal (*12*). No *pmk-1* mutants are available, but RNA interference (RNAi) of *pmk-1* has been reported to give no apparent phenotype (*12*). We found that inhibition of PMK-1 activity (Fig. 4A), but not PMK-2 activity, by feeding RNAi (*13*) resulted in a strong Esp phenotype (*14*). We confirmed the efficacy of *pmk-1* RNAi by immunoblotting with an antibody prepared against PMK-1 (*14*), which showed a marked decrease in PMK-1 protein levels in animals subjected to *pmk-1* feeding RNAi (Fig. 4B). Furthermore, *esp-2/sek-1(ag1)* and *esp-8/nsy-1(ag3)* mutants had markedly diminished levels of p38 MAP kinase activation compared to wild-type N2 worms in the presence of PA14 (Fig. 4C). We have not ruled out the possibility that JNK also plays a role in the defense response in *C. elegans*, although the single available JNK mutant (*jnk-1*) (*15*) exhibited no enhanced susceptibility to PA14 (*16*).

The downstream targets of this p38 MAP kinase pathway involved in mediating the nematode defense to pathogen remain to be elucidated. Previous work in our laboratory has shown that infection of *C. elegans* with *Salmonella* induces programmed cell death, which appears to be associated with a protective response (*17*). Underscoring the importance of the p38 MAP kinase pathway in mediating the response to pathogens, recent data from our laboratory have shown that *Salmonella*-induced programmed cell death

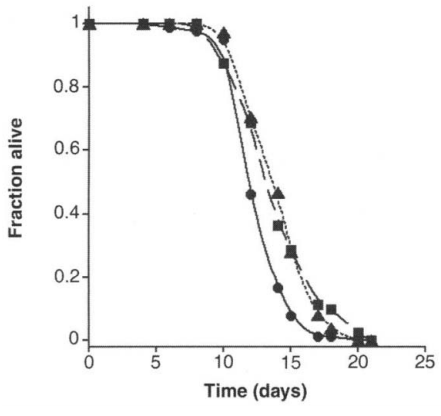


Fig. 2. Characterization of the life-span of *esp-2* and *esp-8* mutants. Life-span curve of wild-type N2 *C. elegans* (circles), and *esp-2* (squares) and *esp-8* (triangles) mutants. Life-span assays were performed as described (*25*). For each strain, 25 L4 animals were placed on each of three plates that contained 5-fluoro-2′-deoxyuridine and had been seeded with *E. coli* OP50.

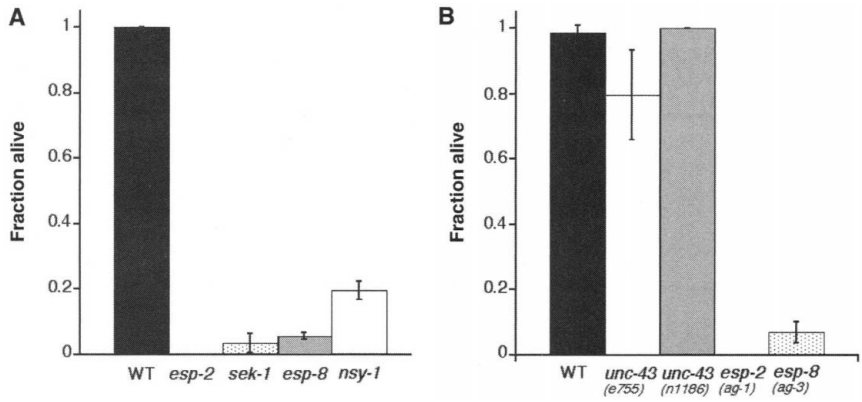


Fig. 3. Susceptibility of Nsy mutants to PA14. (**A**) Survival of wild-type N2, *esp-2(ag1)*, *sek-1(km4)*, *esp-8(ag3)*, and *nsy-1(ky397)* worms after 30 hours on PA14. The *sek-1(km4)* allele is a deletion of kinase domains II through XI of SEK-1 and exhibits no detectable kinase activity (*8*). The *nsy-1(ky397)* allele is a point mutation that creates a stop at codon 1129 (*9*). Experimental conditions were as in Fig. 1A. The *esp-2/sek-1* alleles and *esp-8/nsy-1* alleles were tested in separate experiments, and therefore the data for *esp-2/sek-1* alleles cannot be directly compared to that of the *esp-8/nsy-1* alleles. (**B**) Survival of *unc-43* Ca^{2+}/calmodulin-dependent protein kinase II mutants after 30 hours on PA14. *esp-2(ag1)* and *esp-8(ag3)* were tested in the same experiment with the *unc-43* alleles, thus enabling a direct comparison of the data between *unc-43* and the *esp-2(ag1)* and *esp-8(ag3)* mutants.

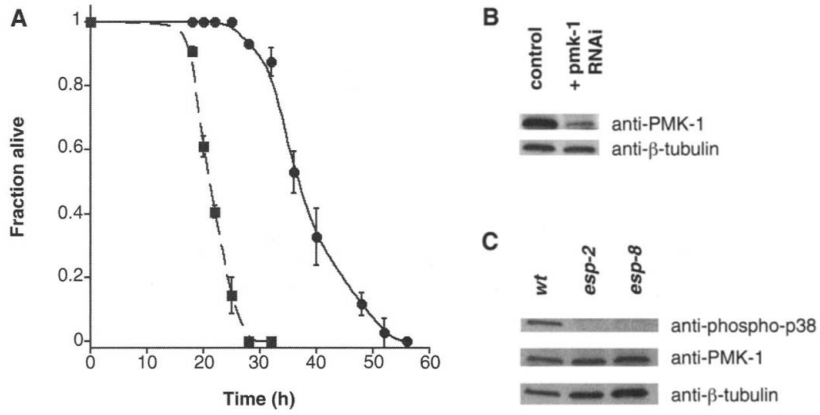


Fig. 4. Susceptibility of animals with reduced PMK-1 activity. (**A**) Survival curve of L4-stage N2 animals exposed to PA14 after being prefed on *E. coli* HT115 carrying the L4440 expression vector only (circles) or HT115 carrying plasmid pDK177 (squares), which contains sequence specific to *pmk-1* (*14*). Worms at the L4 stage were transferred to plates containing PA14, and SK assays were performed as in Fig. 1A. (**B**) Immunoblot analysis of lysates from wild-type N2 animals growing on a lawn of HT115 carrying vector only (pL4440) or HT115 producing double-stranded RNA corresponding to *pmk-1* (pDK177) by using antibodies against PMK-1 or β-tubulin (loading control) (*14*). Exposure to HT115 was performed as in (A), and the synchronized worms were harvested as young adults. (**C**) Immunoblot analysis of lysates from *esp-2(ag1)* and *esp-8(ag3)* mutants compared with wild-type N2 lysates by using an antibody recognizing the doubly phosphorylated activated form of p38 MAP kinase and antibodies against PMK-1, and β-tubulin (loading control) (*14*). Synchronized L4 populations of N2 wild type, *esp-2/sek-1(ag1)*, and *esp-8/nsy-1(ag3)* worms were transferred to PA14 plates in parallel, and lysates were prepared for immunoblotting after 6 hours when the worms were predominantly young adults.

in the *C. elegans* host is dependent on the p38 MAP kinase pathway (*18*).

We have used a direct forward genetic approach in *C. elegans* to identify a requirement for a p38 MAP kinase pathway in the immune response of a whole animal. Extensive biochemical and cell biological studies in mammalian systems have implicated a critical role for p38 MAP kinase signaling in the cellular immune response to bacterial lipopolysaccharide and the proinflammatory cytokines, interleukin-1 and tumor necrosis factor (*19*). However, the genetic analysis of mammalian MAP kinase pathways, including the p38 MAP kinase pathway, has been complicated by embryonic lethality and presumed redundancy (*20*). Evidence implicating p38 and JNK MAP kinases in the modulation of the insect immune response has also been based primarily on cell biological studies (*21, 22*). Recently, a MAPKKK, dTAK1, was identified in a direct screen for *Drosophila* mutants with increased susceptibility to bacterial challenge (*23*). dTAK1 appears to function in the Imd pathway upstream of the IKK complex in the *Drosophila* immune response. The genetic analysis of *C. elegans* defense against pathogens, combined with recent data from plant pathogen resistance (*24*), underscores the conservation of a MAP kinase signaling cassette in innate immunity of phylogenetically diverse organisms. Furthermore, our results here suggest that the p38 MAP kinase pathway in particular represents an ancient, evolutionarily conserved component of the metazoan defense against pathogen attack.

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