Heat-shock transcription factor (HSF)-1 pathway required for *Caenorhabditis elegans* immunity

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Innate immunity comprises physical barriers, pattern-recognition receptors, antimicrobial substances, phagocytosis, and fever. Here we report that increased temperature results in the activation of a conserved pathway involving the heat-shock (HS) transcription factor (HSF)-1 that enhances immunity in the invertebrate *Caenorhabditis elegans*. The HSF-1 defense response is independent of the p38 MAPK/PMK-1 pathway and requires a system of chaperones including small and 90-kDa inducible HS proteins. In addition, HSF-1 is needed for the effects of the DAF-2 insulin-like pathway in defense to pathogens, indicating that interacting pathways control stress response, aging, and immunity. The results also show that HSF-1 is required for *C. elegans* immunity against *Pseudomonas aeruginosa*, *Salmonella enterica*, *Yersinia pestis*, and *Enterococcus faecalis*, indicating that HSF-1 is part of a multipathogen defense pathway. Considering that several coinducers of HSF-1 are currently in clinical trials, this work opens the possibility that activation of HSF-1 could be used to boost immunity to treat infectious diseases and immunodeficiencies.

heat-shock protein | innate immunity | MAPK | infection | pathogen

Increased temperature promotes expression of heat-shock (HS) proteins (HSPs) that are found in high levels in almost all inflammatory diseases (1). However, the precise mechanism by which increased temperature mediates innate immunity is not clear. The nematode *Caenorhabditis elegans*, which has evolved an immune system to recognize pathogens and respond accordingly (2–4), provides an excellent compromise between complexity and genetic tractability to dissect innate immunity pathways activated by heat stress.

A hallmark of *C. elegans* immunity is activation of defense responses through a conserved p38 MAPK pathway. As in mammalian innate immunity, the p38 MAPK signaling pathway is required for proper *C. elegans* defense against the human opportunistic pathogen *Pseudomonas aeruginosa*, which is the major cause of death in cystic fibrosis patients and immunocompromised individuals (5, 6). The pathway also is required to elicit an apoptotic response to *Salmonella enterica* in the *C. elegans* germline (7) and for defense to *Bacillus thuringiensis* toxin Cry5B (8). Based on the fact that *C. elegans* does not have NF-κB-like transcription factors and that the p38 MAPK pathway seems to be more ancient than NF-κB (9, 10), it has been postulated that the p38 MAPK pathway is the ancestral immune pathway of a common ancestor of insects, nematodes, and vertebrates (11, 12).

Although MAPKs have been involved in mammalian defense response and activation of HSPs (13–15), it was unknown whether HSPs and HS transcription factor (HSF)-1 function downstream of the MAPK-mediated immune responses. Here we report that activation of a conserved pathway involving HSF-1 triggers *C. elegans* immunity to bacterial pathogens. We demonstrate that both small and 90-kDa HSPs activated in an HSF-1-dependent manner are effectors responsible for the immune response. Our results show that the HSF-1 pathway regulates immunity independently of p38 MAPK and that it interacts with an insulin-like pathway that includes the insulin-like growth factor receptor DAF-2 and the forkhead transcription factor DAF-16. We provide direct evidence that HSF-1-regulated proteins are effectors of the DAF-2/DAF-16 pathway required for pathogen resistance.

Results and Discussion

HS Elicits *C. elegans* Immunity Independently of SEK-1/MAPKK. To study the role of HSPs in *C. elegans* defense response and their relationship with MAPKs, we first examined whether HS impacts *C. elegans* susceptibility to *P. aeruginosa*. After HS, wild-type N2 animals were exposed to *P. aeruginosa* by using slow-killing conditions (16) and death of animals on *P. aeruginosa*-containing plates was scored over the course of infection. The time required for 50% of the nematodes to die (TD_{50}) under these conditions was calculated in three independent experiments. HS-treated wild-type animals exhibited higher resistance ($TD_{50} = 53.5 \pm 3.35$ h) to *P. aeruginosa*-mediated killing than animals not exposed to HS ($TD_{50} = 42.5 \pm 2.87$ h) as shown in Fig. 1*A*. In mammals, the p38 MAPK signaling pathway is activated by various cytotoxic stresses (13–15), including HS. Therefore, we analyzed whether the NSY-1 MAPKKK/SEK-1 MAPKK/PMK-1 p38 MAPK pathway, which is crucial for *C. elegans* protection against *P. aeruginosa* (5, 6), is involved in the HS-mediated protection to the pathogen. Because PMK-1 activation requires SEK-1 (5), *sek-1*(*ag1*) mutants were exposed to mild HS or left untreated and were then exposed to the pathogen to address whether the kinase was involved in the HS-mediated protection to *P. aeruginosa* infection. As shown in Fig. 1*B*, HS-treated *sek-1*(*ag1*) mutants were more resistant to *P. aeruginosa* than untreated animals, indicating that HS-mediated protection is independent of SEK-1 activation. Although PMK-1 may also function independently of SEK-1 as a downstream target of MEK-1 in pathogen resistance (6), active PMK-1 was not observed in *sek-1*(*ag1*) mutants before or after HS (data not shown).

By using transgenic animals carrying additional *hsp-70* gene copies, it was found that HS increases the life span of *Drosophila melanogaster* (17) and *C. elegans* (18). In addition, animals overexpressing HSF exhibit an extended life span of ≈40% (19). Based on these results and on the short life span of *sek-1*(*ag1*) mutants on plates containing *Escherichia coli* ($TD_{50} = 36 \pm 7.2$h) (Fig. 1*C*), it is conceivable to postulate that HS may protect against bacterial infection by improving the general fitness of the animals rather than specifically boosting their immune system. However, under certain conditions, *E. coli* OP50 kills *C. elegans* (20), and proliferating *E. coli* OP50 is a cause of death (21). In addition, *sek-1*(*ag1*) animals are more rapidly killed by live *E. coli* than by killed *E. coli*, and HS treatment increases the protection of *sek-1*(*ag1*) animals against live *E. coli* ($TD_{50} = 126.2 \pm 8.2$ h vs. $TD_{50} = 36 \pm 7.2$ h) (Fig. 1*C*). These results, together with life-span experiments (5) using 5-fluoro-2′-

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Abbreviations: HS, heat shock; HSF-1, HS transcription factor-1; HSP, HS protein; NGM, nematode growth medium.

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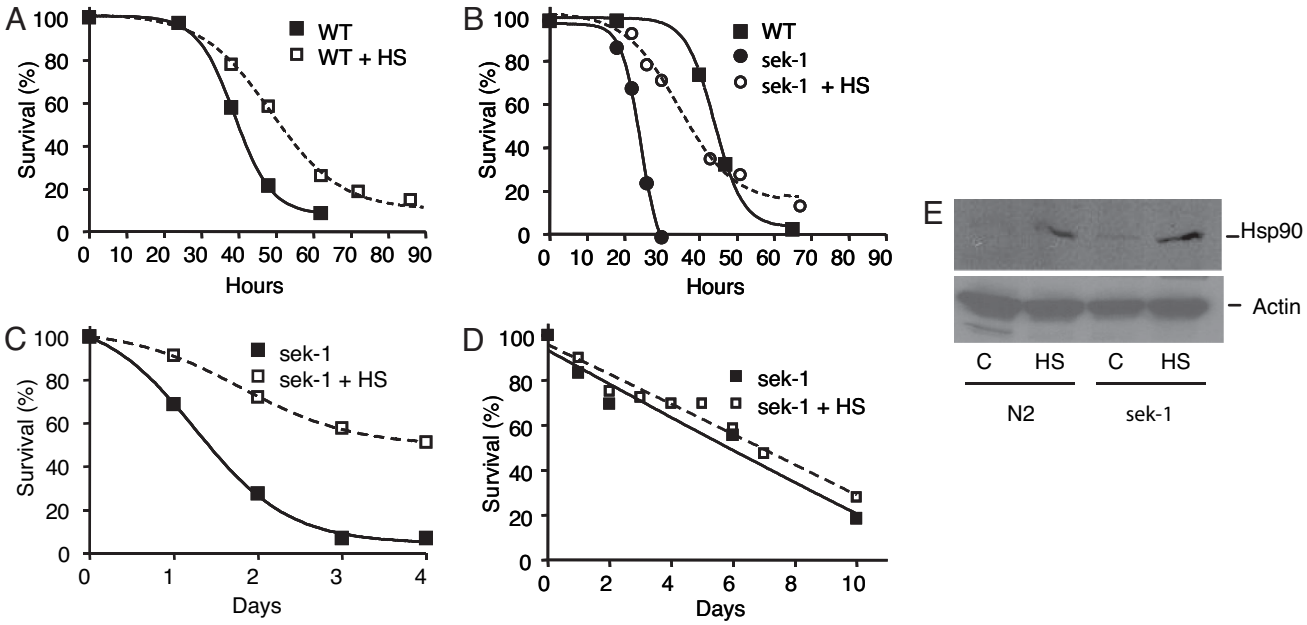

Fig. 1. HS enhances *C. elegans* immune response against *P. aeruginosa* in a SEK-1-independent manner. (*A*) Wild-type N2 animals untreated or HS-treated were exposed to *P. aeruginosa* PA14 ($P < 0.005$). (*B*) Wild-type, *sek-1*(*ag1*) ($P < 0.0001$), and *sek-1*(*ag1*) HS-treated animals ($P = 0.954$) were exposed to *P. aeruginosa*. (*C*) *sek-1*(*ag1*) animals untreated or HS-treated were exposed to live *E. coli* OP50 ($P < 0.002$). (*D*) *sek-1*(*ag1*) animals untreated or HS-treated also were exposed to heat-killed *E. coli* ($P > 0.1$). For each condition, 40–50 animals were used. (*E*) Immunological detection of Hsp90 in untreated control (C) and HS-treated wild-type or *sek-1*(*ag1*) animals. Detection of actin is used as a loading control.

deoxyuridine, which prevents transcription in both prokaryotes and eukaryotes, indicate that *sek-1*(*ag1*) nematodes are immunocompromised animals killed by live, replicating bacteria. Consistent with the idea that HS boosts defense response but not the overall fitness of the animals, the survival of wild-type animals grown on live or killed *E. coli* is not further enhanced by the HS treatment used in this study (Fig. 5, which is published as supporting information on the PNAS web site). Hsp90, which is up-regulated in mammalian systems upon HS and used as a marker of HSF activity, was found to be up-regulated by HS in a HSF-1-dependent manner (Fig. 6, which is published as supporting information on the PNAS web site) and independent of SEK-1 (Fig. 1*E*), raising the possibility that HSF-1 may be involved in a SEK-1-independent defense response against live bacteria.

HS-Mediated Immunity to *P. aeruginosa* Requires HSF-1. To test the hypothesis that HSF-1 is involved in a SEK-1-independent immunity to *P. aeruginosa*, we studied whether HSF-1 was involved in defense to the pathogen. The protection against *P. aeruginosa* by HS was not observed in *hsf-1* RNAi animals, which also appear to be more susceptible to the pathogen under control conditions (Fig. 2*A*), indicating that HSF-1 activity is required not only for HS-meditated protection but also for defense to *P. aeruginosa*-mediated killing under nonstressful conditions. The increased susceptibility to *P. aeruginosa* of animals lacking HSF-1 was confirmed by using the *hsf-1*(*sy441*) mutant, which carries a single G→A mutation that results in the truncation of the last 86 aa of HSF-1 and in the elimination of HS-induced expression of *hsp-16.2* (22) (Fig. 2*B*). As shown in Fig. 2*C*, *hsf-1* RNAi animals are more susceptible to *P. aeruginosa* than control animals when the infections are performed at 20°C, which is a more conducive temperature for animals lacking HSF-1.

The requirement of HSF-1 for HS-mediated immunity to *P. aeruginosa* (Fig. 2 *A–C*) suggests that animals exhibiting higher activity of HSF-1 would then be better protected from *P. aeruginosa*-mediated killing than wild-type animals. As shown in Fig. 2*D*, animals overexpressing *hsf-1* were significantly more resistant to *P. aeruginosa* infection than wild-type animals. Consistent with the idea that HSF-1 is involved in SEK-1-independent immunity to *P. aeruginosa*, Fig. 2 *E* and *F* shows that PMK-1 and HSF-1 function synergistically in the activation of immunity to *P. aeruginosa*. These results demonstrate that HSF-1 is involved in SEK-1-independent immunity to *P. aeruginosa* and suggest that HSPs may be required for protection.

A System of Chaperones Mediates HSF-1-Dependent Immunity. To address whether HSPs play a role in *C. elegans* defense to *P. aeruginosa*, we used RNAi to target HSP-encoding genes whose promoters contain HSF-1-binding elements and that are known to be heat-inducible (19, 23, 24). The results shown in Table 1 demonstrate that RNAi ablation of Hsp90/DAF-21 and members of the Hsp16 family increases susceptibility to *P. aeruginosa*, indicating that these HSPs are required for full immunity. Given the sequence similarity among members of the Hsp16 family, we cannot rule out the possibility of cross-RNAi. In addition, we cannot rule out the possibility of redundancy or incomplete RNAi when no RNAi phenotypes were detected. RNAi ablation of the HSPs required for *C. elegans* immunity diminished the increased resistance to *P. aeruginosa* of animals overexpressing *hsf-1* (Table 1) and did not enhance the increased susceptibility to *P. aeruginosa* of the *hsf-1*(*sy441*) mutant (Fig. 7, which is published as supporting information on the PNAS web site), suggesting that these HSPs may act downstream HSF-1. Taken together, these results indicate that HSF-1 regulates a system of chaperones that is required for proper innate immunity to *P. aeruginosa*. The statistically significant but modest effect that suppression of individual HSPs has on immunity (Table 1) is consistent with the idea that a network of chaperones is required for the HSF-1 effects on aging and immunity. RNAi inhibition of individual HSPs has no significant effects on the life span of wild-type animals and has modest effects on long-lived mutants (25). Furthermore, several HSPs appear to be required for proper antigen presentation (26).

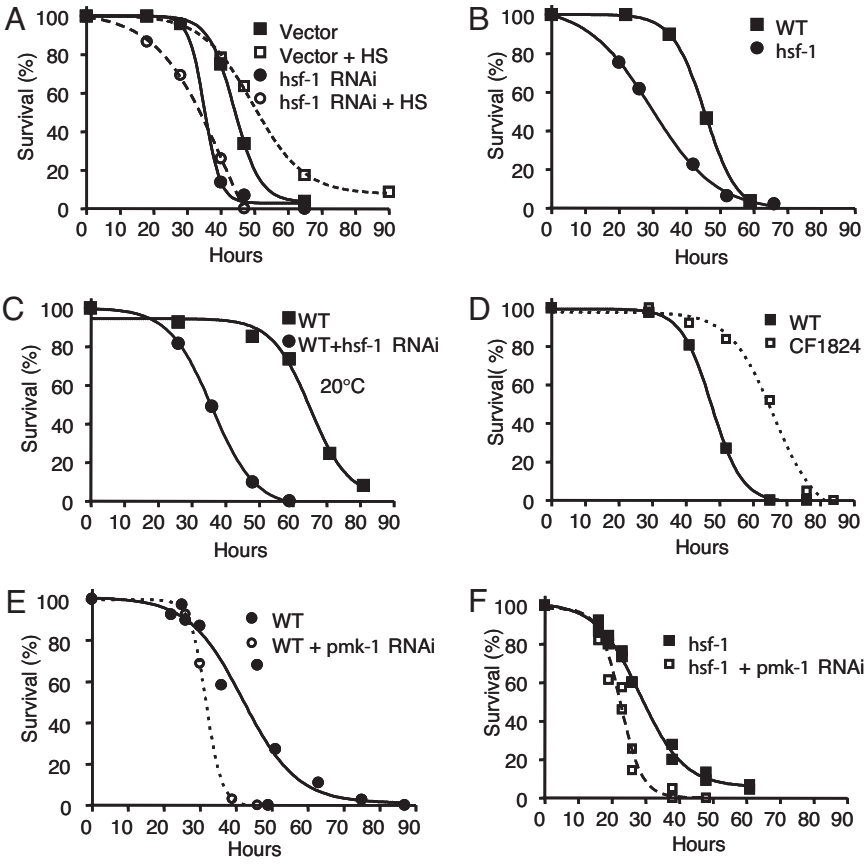

Fig. 2. HSF is required for *C. elegans* immunity to *P. aeruginosa*. (*A*) Wild-type worms grown on *E. coli* carrying a vector control were untreated or HS-treated and exposed to *P. aeruginosa* ($P < 0.03$). In addition, wild-type animals grown on *E. coli* expressing *hsf-1* double-stranded RNA were untreated or HS-treated and exposed to *P. aeruginosa* ($P = 0.2660$). *hsf-1* RNAi animals were more susceptible to *P. aeruginosa* compared with vector RNAi controls ($P < 0.005$). (*B*) Wild-type or *hsf-1*(*sy441*) animals were grown on *E. coli* carrying a vector control and exposed to *P. aeruginosa* ($P < 0.0001$). (*C*) Wild-type animals grown on vector control or *hsf-1* double-stranded RNA were exposed to *P. aeruginosa* at 20°C ($P < 0.0001$). (*D*) Wild-type or CF1824 (*hsf-1* overexpression) animals were exposed to *P. aeruginosa* ($P < 0.001$). (*E*) Wild-type animals grown on *E. coli* carrying a vector control or expressing *pmk-1* double-stranded RNA were exposed to *P. aeruginosa* ($P < 0.0001$). (*F*) *hsf-1*(sy441) animals grown on *E. coli* carrying a vector control or expressing *pmk-1* double-stranded RNA were exposed to *P. aeruginosa* ($P = 0.0023$). For each condition, 80–200 animals were used.

HSF-1 Is Required for the Immune Function of the DAF-2/DAF-16 Pathway. The DAF-2/DAF-16 pathway, whose role in life span has been very well established (27), has recently been shown to regulate aging and age-related diseases through small HSPs and other genes (19). DAF-16 is a forkhead transcription factor that regulates a wide variety of genes involved in longevity, stress response, metabolism, and development (28–30), is positively regulated by HS (19) and negatively regulated by DAF-2 (31). In addition, it has been shown that mutations in *daf-16* suppress the pathogen-resistant phenotype of *daf-2*(*e1370*) mutants (32). As previously shown, *daf-2*(*e1370*) mutants were resistant to *P. aeruginosa* (32) (Fig. 3*A*), and *daf-16*::*gfp* animals, which carry additional *daf-16* gene copies, also were found to be more resistant to the pathogen, as expected (Fig. 3*B*). Both *daf-2*(*e1370*) and *daf-16*::*gfp* nematodes showed higher levels of HSP90 than wild-type animals (Fig. 3*F*), suggesting that a higher activity of HSF-1 may be in part responsible for the increased resistance of these animals to *P. aeruginosa*. Enhanced resistance of *daf-2*(*e1370*) and *daf-16*::*gfp* animals to *P. aeruginosa* was reduced by the RNAi inhibition of *hsf-1* (Fig. 3 *C* and *D*). In addition, HS protection is not seen in *daf-16* RNAi animals (Fig. 3*E*). This reduction of the enhanced resistance phenotype of *daf-2*(*e1370*) and *daf-16*::*gfp* animals by RNAi ablation of *hsf-1* provides evidence that HSF-1-regulated proteins may be effectors of the DAF-2/DAF-16 pathway required for pathogen resistance.

Table 1. HSPs are required for *C. elegans* immunity to *P. aeruginosa*

Cosmid no.	Gene	TD_{50}, %	*n*	Significance*
N2				
C47E8.5	*hsp90*	87 ± 4.6	131	0.0012
F08H9.3	*hsp16*	85 ± 6.1	121	<0.0001
F08H9.4	*hsp16*	93 ± 1.2	170	<0.0001
T27E4.8	*hsp16.1*	82 ± 1.5	90	<0.0001
F43D9.4	*sip-1*	123 ± 4.2	81	>0.1
C12C8.1	*hsp70*	102 ± 3.4	130	>0.1
C30C11.4	*hsp70*	96 ± 9.4	132	>0.1
F44E5.4	*hsp70*	103 ± 3.6	133	>0.1
F44E5.5	*hsp70*	100 ± 14	130	>0.1
Vector		100 ± 11.3†	201	
CF1824				
C47E8.5	*hsp90*	81 ± 8.7	130	<0.0001
F08H9.3	*hsp16*	78 ± 9.2	120	<0.0001
F08H9.4	*hsp16*	82 ± 4.2	132	<0.0001
T27E4.8	*hsp16.1*	78 ± 5.4	105	<0.0001
Vector		100 ± 7.7‡	140	

*LogRank test.
†44 ± 5 h.
‡57 ± 4.4 h.

HSF-1 Is Part of a Multipathogen Defense Pathway. To study whether HSF-1 is part of an immune response specific to *P. aeruginosa* or whether it is required for immunity to pathogens in general, we infected *C. elegans* with *Yersinia pestis* (33), *S. enterica* (34, 35), and *Enterococcus faecalis* (20), as previously described. Fig. 4 *A–C* shows that *hsf-1* RNAi animals are more susceptible to the three pathogens studied. In addition, ablation of *hsf-1* expression by RNAi reduced the enhanced resistance to *Y. pestis*, *S. enterica*, and *En. faecalis* exhibited by *daf-2*(*e1370*) animals (Fig. 4 *A–C*), indicating that the HSF-1 is required for immunity to Gram-negative and Gram-positive bacteria.

Conclusions

Fever is an ancient immune mechanism used by metazoans in response to microbial infections. Although homeotherms are

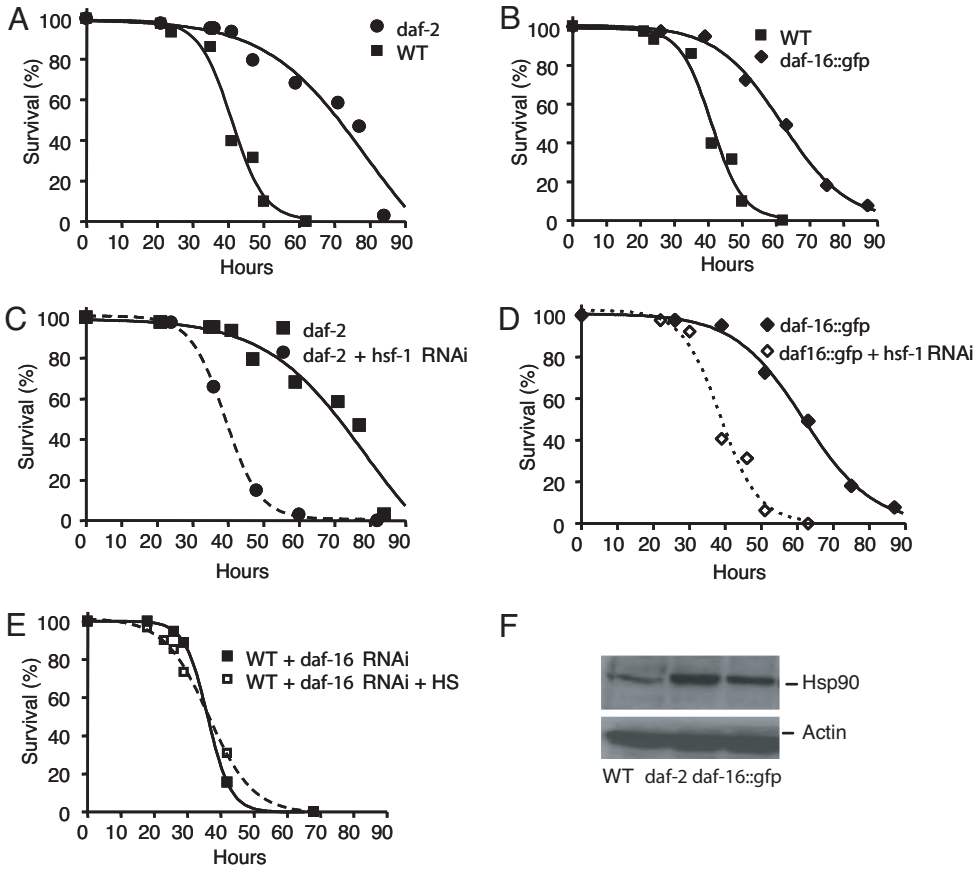

Fig. 3. The enhanced resistance phenotype of *daf-2*(*e1370*) and *daf-16*::*gfp* animals to *P. aeruginosa* requires HSF-1 activity. (*A* and *B*) Wild-type, *daf-2*(*e1370*) ($P < 0.0001$), and *daf-16*::*gfp* ($P < 0.0001$) animals were exposed to *P. aeruginosa*. (*C*) *daf-2*(*e1370*) grown on *E. coli* carrying a vector control or expressing *hsf-1* double-stranded RNA were exposed to *P. aeruginosa* ($P < 0.0001$). (*D*) *daf-16*::*gfp* grown on *E. coli* carrying a vector control or expressing *hsf-1* double-stranded RNA were exposed to *P. aeruginosa* ($P < 0.0001$). (*E*) Wild-type animals grown on *E. coli* expressing *daf-16* double-stranded RNA were untreated or HS-treated and exposed to *P. aeruginosa* ($P > 0.1$). For each condition, 80–100 animals were used. (*F*) Immunological detection of Hsp90 in WT, *daf-2*(*e1370*), and *daf-16*::*gfp* animals.

capable of internally increasing the body temperature, poikilotherms migrate toward warmer environments to increase their temperature in response to infections. The activation of the HSF-1 pathways by HS and its function in *C. elegans* immunity provides a molecular explanation for the beneficial role of behavioral fevers in poikilotherms. It will be interesting to study whether *C. elegans* responds to pathogen infections by migrating toward warmer areas to activate HSF-1-mediated immune responses.

Taken together, our results show that a conserved pathway involving HSF-1 contributes to *C. elegans* immunity to bacterial pathogens. HSF-1 acts downstream of DAF-2/DAF-16 in defense response and confers protection through a system of HSPs independently of the p38 MAPK pathway (Fig. 4*D*). At this point in our investigation, we cannot distinguish whether HSF-1 is part of a constitutive immune response or whether it is activated by pathogens. Initial attempts to address whether pathogens activate HSF-1 proved inconclusive, which is not surprising given the wide number of potential roles in the organism that could be played by HSF-1. Pathogens may lead to HSF-1 activation in only a subset of *C. elegans* cells that cannot be detected in whole-animal lysates. HSF-1 and the chaperone system may directly or indirectly regulate the expression of innate immunity genes. Another possibility is that the chaperone system may be required for recognition and degradation of bacterial virulence factors or for the proper folding of effector molecules of the immune system. The results presented here suggest that therapeutics aiming to boost the innate immune system through the activation of the HSF-1 pathway could be developed to treat bacterial infections.

Materials and Methods

Bacterial and Nematode Strains. The *E. coli* OP50 (36), *P. aeruginosa* PA14 (16), *S. enterica* SL1344 (37), *Y. pestis* KIM5 (38), *En. faecalis* OG1RF (39) strains were used. *C. elegans* strains used were wild-type N2, *sek-1*(*ag1*), *hsf-1*(*sy441*), *daf-2*(*e1370*), *daf-16*::*gfp* [*zIs356* (pDAF-16::DAF-16-GFP;*rol-6*)], and CF1824 [muEx265 (HSF-1p::HSF-1 cDNA + myo-3::GFP)].

***C. elegans* Killing Assay.** *C. elegans* wild-type N2 animals and mutants were maintained as hermaphrodites at 15°C, grown on modified nematode growth medium (NGM) agar plates, and fed with *E. coli* strain OP50 as described (36). *E. coli*, *P. aeruginosa*, and *S. enterica* cultures were grown in Luria–Bertani (LB) broth at 37°C. *Y. pestis* cultures were grown at 25°C. Bacterial lawns used for *C. elegans* killing assays were prepared by spreading 20 μl of an overnight culture of the bacterial strains on modified NGM agar (0.35% instead of 0.25% peptone) in plates 3.5 cm in diameter. Infections with *En. faecalis* OG1RF were performed as described in ref. 20. Briefly, *En. faecalis* liquid cultures were grown in brain–heart infusion broth at 37°C and plated on brain–heart infusion agar supplemented with 50 μg/ml gentamicin. Plates were incubated at 25°C for 12 h before seeding them with young adult nematodes grown at 15°C. The killing

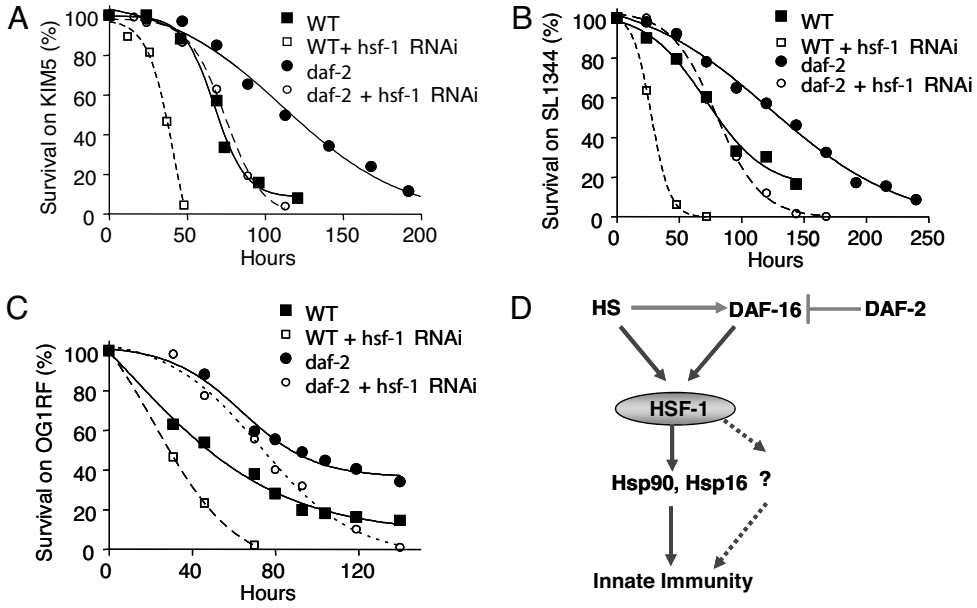

Fig. 4. HSF-1 is required for immunity to Gram-negative and Gram-positive pathogens. (*A*) Wild-type worms grown on *E. coli* carrying a vector control or on *E. coli* expressing *hsf-1* double-stranded RNA were exposed to *Y. pestis* KIM5 ($P < 0.0001$). *daf-2*(*e1370*) worms grown on *E. coli* carrying a vector control or on *E. coli* expressing *hsf-1* double-stranded RNA were exposed to *Y. pestis* KIM5 ($P < 0.001$). (*B*) Wild-type worms grown on *E. coli* carrying a vector control or on *E. coli* expressing *hsf-1* double-stranded RNA were exposed to *S. enterica* SL1344 ($P < 0.0001$). *daf-2*(*e1370*) worms grown on *E. coli* carrying a vector control or on *E. coli* expressing *hsf-1* double-stranded RNA were exposed to *S. enterica* SL1344 ($P < 0.0001$). (*C*) Wild-type worms grown on *E. coli* carrying a vector control or on *E. coli* expressing *hsf-1* double-stranded RNA were exposed to *En. faecalis* OG1RF ($P < 0.0001$). *daf-2*(*e1370*) worms grown on *E. coli* carrying a vector control or on *E. coli* expressing *hsf-1* double-stranded RNA also were exposed to *En. faecalis* OG1RF ($P < 0.0001$). (*D*) HSF-1 activated by HS and the DAF-2/DAF-16 pathway enhances *C. elegans* immunity. HSF-1 mediates protection via induction of Hsp90 and small HSPs in a PMK-1 independent manner.

assays were performed at 25°C unless otherwise indicated, and animals were scored and transferred twice a day to fresh plates. Animals were considered dead when they failed to respond to touch.

RNAi. We used the RNA interference technique to generate loss-of-function RNAi phenotypes by feeding worms with *E. coli* expressing double-stranded RNA that is homologous to a target gene (40, 41). Briefly, *E. coli* strain HT115(DE3) harboring the appropriate vectors were grown in LB broth containing 100 μg/ml ampicillin and 10 μg/ml tetracycline at 37°C overnight. Bacteria were plated onto NGM plates containing 100 μg/ml ampicillin and 10 mM isopropyl β-D-thiogalactoside and were allowed to grow overnight at 37°C.

Gravid adults were allowed to lay eggs on RNAi-expressing lawns of bacteria for 5 h. The eggs were allowed to develop into young adults on RNAi or vector control plates at 15°C. Under these conditions, *hsf-1* RNAi does not result in the developmental arrest observed when animals are grown at 25°C. Young adult animals grown under these conditions were HS-treated or untreated and subsequently transferred to *P. aeruginosa*-containing plates. Bacteria strains expressing double-stranded RNA to inactivate the *C. elegans* genes were obtained from Wellcome/Cancer Research (Cambridge, U.K.) and Open Biosystems (Huntsville, AL). The identity of the clones was confirmed by sequencing.

HS Treatment. Animals on NGM plates were given HS at 32°C in a water bath for 2 h and either used directly for protein preparation or allowed to recover at 25°C for 4 h before exposure to pathogen. As a control, animals on NGM plates were maintained at 15°C before exposure to pathogen. We found no significant difference between animals subjected to two insults at the same time (shift from 15°C to 25°C and exposure to the pathogen) and animals subjected to the two insults sequentially (shift from 15°C to 25°C and, after 4 h, exposure to the pathogen) (data not shown).

Statistical Analyses. Animal survival was plotted as a nonlinear regression curve with the PRISM 4.00 computer program. Survival curves are considered significantly different from the control when $P < 0.05$. Prism uses the product limit or Kaplan–Meier method to calculate survival fractions and the logrank test, which is equivalent to the Mantel–Heanszel test, to compare survival curves.

Immunological Detection of Proteins. Whole-worm lysates were prepared in the presence of protease inhibitors. Hsp90 was detected with a monoclonal antibody from Stressgen Biotechnology (San Diego, CA) and enhanced chemiluminesence approach. Actin was detected by using a polyclonal antibody from Sigma (St. Louis, MO). Multiple experiments were done, and the data presented are from representative experiments.

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