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DAF-16-Dependent Suppression of Immunity During Reproduction in *Caenorhabditis elegans*

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ABSTRACT

To further understand how the nematode *Caenorhabditis elegans* defends itself against pathogen attack, we analyzed *e*nhanced *p*athogen *r*esistance (*epr*) mutants obtained from a forward genetic screen. We also examined several well-characterized sterile mutants that exhibit an Epr phenotype. We found that sterility and pathogen resistance are highly correlated and that resistance in both *epr* and sterile mutants is dependent on DAF-16 activity. Our data indicate that a DAF-16-dependent signaling pathway distinct from previously described pathways is involved in the activation of genes that confer resistance to bacterial pathogens. The timing of DAF-16-dependent gene activation in sterile mutants coincides with the onset of embryonic development in wild-type animals, suggesting that signals from developing embryos normally downregulate the immune response.

ONE of the most important traits for natural selection is the maximal production of progeny. However, reproduction comes at the expense of decreased survival of the parent. A negative relationship between reproduction and longevity has been reported across a diverse array of organisms including plants, insects, and mammals (Fowler and Partridge 1989; Westendorp and Kirkwood 1998; Hautekeete *et al.* 2001). Several studies have linked successful reproduction and immunocompetence (McKean and Nunney 2001; Fedorka *et al.* 2004) and reviewed by Klein and Nelson (1999). Because reproduction utilizes resources necessary for somatic cell maintenance, growth, and survival, it appears likely that expression of immunity and reproduction must compete for energy allocation. This trade-off between increased reproduction and decreased survival rate may maximize survival of offspring depending on the availability of food and the presence of pathogens.

Both vertebrates and invertebrates rely on innate immunity as the first line of defense against pathogen attack. In plants, insects, and vertebrates, a wealth of data show that this ancient mechanism relies in part on the recognition of pathogen-associated molecular patterns (PAMPs) by defined genomically encoded receptors and subsequent expression of antimicrobial effector molecules (Tosi 2005; Akira *et al.* 2006; Ryan *et al.* 2007). Invertebrates and plants appear to depend solely on innate immunity because they lack an adaptive immune system that somatically generates an array of immune receptors that are selected and amplified in immune cells. Work carried out during the past 15 years shows that innate immune signaling pathways are at least partially conserved between vertebrate and invertebrates, including the innate immune response pathways of the model nematode *Caenorhabditis elegans* (reviewed by Ausubel 2005). Mounting evidence suggests that *C. elegans* is a facile model to study both the evolutionary origins of innate immunity as well as the resistance mechanisms by which hosts combat pathogen attack (reviewed by Kurz and Ewbank 2003; Millet and Ewbank 2004; Schulenburg *et al.* 2004; Gravato-Nobre and Hodgkin 2005; Kim and Ausubel 2005).

A wide variety of human pathogens, including the Gram-negative bacterial pathogen *Pseudomonas aeruginosa* and the Gram-positive bacterial pathogen *Staphylococcus aureus*, infect and kill *C. elegans* (Tan *et al.* 1999a; Sifri *et al.* 2003). Because *C. elegans* is normally propagated on bacterial lawns in the laboratory, the nematodes can be easily infected by simply transferring them from their normal laboratory food, *Escherichia coli* strain OP50, to a lawn of the pathogen of choice. Infection with live but not heat-killed pathogens leads to premature death of the worms and in many cases it has been demonstrated that many of the same bacterial virulence factors are required for nematode killing and for maximum virulence in mammalian hosts (Mahajan-Miklos *et al.* 1999; Tan *et al.* 1999b; Aballay *et al.* 2000; Sifri *et al.* 2003).

Both forward and reverse genetic approaches have been used to identify signaling pathways involved in activating innate immune responses. For example, a

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forward genetic screen for immunocompromised mutants identified a p38 MAPK signaling cascade (Kim *et al.* 2002) that functions to promote immunity in *C. elegans*. A candidate gene approach revealed that derepression of the FOXO/forkhead-like transcription factor DAF-16, by loss-of-function mutations in the upstream components of the insulin/IGF-1 pathway, confers resistance to a variety of pathogens (Garsin *et al.* 2003). When an insulin-like ligand binds to DAF-2, the only insulin/IGF-1 receptor in *C. elegans*, it activates a conserved phosphorelay cascade via AGE-1, a phosphatidylinositol 3-kinase [PI(3)K] (Morris *et al.* 1996), leading to phosphorylation of DAF-16, which blocks its translocation to the nucleus (reviewed by Nelson and Padgett 2003; Mukhopadhyay *et al.* 2006). Disruption of the DAF-2 signaling cascade by mutation of *daf-2* or *age-1*, for example, promotes DAF-16 accumulation in the nucleus (Henderson and Johnson 2001; Lee *et al.* 2001; Lin *et al.* 2001), concomitant entry into the dauer developmental pathway (Gottlieb and Ruvkun 1994), and activation of a variety of stress-related responses leading to increased longevity (Murakami and Johnson 1996; Henderson and Johnson 2001). Temperature-sensitive loss-of-function mutations in *daf-2* or *age-1* increase longevity up to twofold when feeding on nonpathogenic *E. coli*, which is completely suppressed by loss-of-function mutations in *daf-16* (Kenyon *et al.* 1993; Lin *et al.* 1997).

Since pathogen resistance is DAF-16-dependent in *daf-2* and *age-1* mutant animals, it appears likely that the genes regulated downstream of DAF-16 confer the pathogen resistance phenotype. Genomewide transcriptional analyses have shown that DAF-16 is predicted to transcriptionally target >500 genes whose functions include antioxidant, metabolic, antimicrobial, and stress responses in animals with reduced expression of DAF-2 (McKean and Nunney 2001; McElwee *et al.* 2003). Murphy *et al.* (2003) further demonstrated that the oxidative stress genes *mtl-1* and *sod-3* and the antimicrobial genes *dod-6* and *lys-7* play an important functional role in the increased life span of *daf-2* animals. It appears that a variety of DAF-16-dependent genes play an important role in conferring enhanced immunity. SOD-3 is not only expressed in intestinal tissue, a site of infection, after exposure to the Gram-positive bacterial pathogen *Enterococcus faecalis*, but is also required for resistance to *E. faecalis* (Chavez *et al.* 2007). Similarly, *lys-7* is induced by *Microbacterium nematophilum*, a nematode-specific Gram-positive bacterial pathogen, as well as the Gram-negative bacterial pathogen *Serratia marcescens* and plays a functional role in conferring resistance to *M. nematophilum* (Mallo *et al.* 2002; O'Rourke *et al.* 2006). Additionally, *dod-6* encodes a ShK-toxin-like domain that is implicated in immunity (O'Rourke *et al.* 2006; Shapira *et al.* 2006; Troemel *et al.* 2006).

Several lines of evidence suggest that DAF-16 can be activated (dephosphorylated) independently of the DAF-2 signaling pathway (Hamilton *et al.* 2005; Hansen *et al.* 2005). Elimination of germ line stem cells by laser ablation or by mutation of *glp-1* or *mes-1* results in DAF-16 translocation to the nucleus (Lin *et al.* 2001) and DAF-16-dependent increased longevity (Hsin and Kenyon 1999; Arantes-Oliveira *et al.* 2002; Libina *et al.* 2003). Moreover, removal of the germ line precursor cells in *daf-2* mutants further increased their longevity (Hsin and Kenyon 1999; Arantes-Oliveira *et al.* 2003). The ankyrin-repeat-containing protein KRI-1, acting upstream of DAF-16, is required for increased longevity in *glp-1* animals (Berman and Kenyon 2006). In contrast, *kri-1* does not suppress the enhanced longevity phenotype of *daf-2* mutants, suggesting that DAF-2 and KRI-1 act in parallel to activate DAF-16 (Berman and Kenyon 2006). Whether these different signaling inputs regulate distinctive sets of DAF-16-dependent genes is not known.

Do all *C. elegans* mutants that exhibit an enhanced pathogen resistance phenotype depend on DAF-16 activity? Male *C. elegans* exhibit pathogen resistance toward the fungal pathogen *Cryptococcus neoformans*, and resistance is partly suppressed by a *daf-16* mutation (van den Berg *et al.* 2006). The sterile mutant *fer-1* is at least partially resistant to *P. aeruginosa* (Tan *et al.* 1999a) and *Salmonella enterica* (Aballay *et al.* 2000); however, it has not been demonstrated whether pathogen resistance depends on DAF-16 activity, requires other cellular-based defense mechanisms, or is simply explained by the lack of pathogen-induced matricide caused by internal hatching of embryos. It is thought that the resistance of sterile mutants to pathogens may be related to the observation that pathogen-mediated killing often coincides with retention of eggs in *C. elegans* hermaphrodites, resulting in internal hatching of the eggs and matricidal death (Aballay *et al.* 2000; O'Quinn *et al.* 2001; Sifri *et al.* 2003). Internal hatching of retained embryos results in a "bagging" phenotype because the hermaphrodite corpses fill up with hatchlings. Importantly, however, because sterile and male animals are still killed by pathogens (Tan *et al.* 1999a; Aballay *et al.* 2000; Kim *et al.* 2002), it appears that killing is not due solely to bagging.

In this report, we investigate DAF-16-dependent defenses against bacterial infection. A forward genetic screen for *S. aureus*-resistant mutants resulted in the isolation of six mutants, all of which exhibited DAF-16-dependent resistance. Two of the genes corresponding to these mutants were mapped and cloned; one corresponded to AGE-1, a component of the insulin signaling pathway, and the other to INX-14, a gap junction protein involved in oocyte maturation that is not a component of the insulin signaling pathway. All six mutants exhibited a small brood size, suggesting a correlation between sterility and pathogen resistance. Reverse genetic analysis showed that sterile mutants in general were resistant to *P. aeruginosa* in a DAF-16-dependent manner. Our results suggest that activation of DAF-16 in sterile mutants is distinct from DAF-2 and the germ

line/stem cell-based regulation of DAF-16. In addition, the timing of the DAF-16-dependent defense response coincides with the onset of embryonic development. These results suggest the existence of a previously unknown pathway that modulates DAF-16 activity and can lead to pathogen resistance in sterile mutants.

MATERIALS AND METHODS

Bacterial strains: Bacterial strains used in this study include *E. coli* strain OP50 and *P. aeruginosa* strain PA14 cultured at 37° in Luria–Bertani (LB) broth and *S. aureus* strain NCTC8325 cultured in tryptic soy (TS) broth containing 5 μg/ml nalidixic acid (Sigma, St. Louis). *E. coli* strain HT115 carrying the RNAi vector L4440 or L4440-derived plasmids engineered to express double-stranded RNA (dsRNA) targeting the *C. elegans* genes were obtained courtesy of the Ahringer laboratory (Kamath *et al.* 2003) and maintained in LB broth containing 50 μg/ml ampicillin and 15 μg/ml tetracycline.

***C. elegans* strains:** All strains were cultured on nematode growth media (NGM) supplemented with *E. coli* OP50 as a food source as described by Brenner (1974) and maintained at 15°, unless otherwise noted. Strains used in this study include DR1572 [*daf-2(e1368)*III], GR1329 [*daf-16(mgDf47)*I], GR1309 [*daf-2(e1368);daf-16(mgDf47)*], CB61 [*dpy-5(e61)*I], CB4037 [*glp-1(e2141)*III], CB4108 [*fog-2(q71)*V], CB4856 (wild-type Hawaiian isolate), CF512 [*fer-15(b26)*II;*fem-1(hc17)*IV], JK816 [*fem-3(q20)*IV], TJ1052 [*age-1(hx546)*II], TJ356: N2; *zIs356* [*pG30 (DAF-16::GFP)*IV], VC832 [*tag-296(ok1189)*I/*hT2*[*bli-4(e937) let-?(q782) qIs48*](I;III)], and N2 (wild-type Bristol isolate). FX02864 [*inx-14(tm2864/+)*I] and FX02593 [*inx-14(tm2593/+)*I] were generated by the National Bioresource Project (http://www.nbrp.jp/index.jsp) and subsequently balanced with *hT2*[*bli-4(e937) let-?(q782) qIs48*](I;III)] to create AU0210 [*tm2864/hT2*] and AU0211 [*tm2593/hT2*], respectively.

The following double mutant strains were constructed for this study using standard genetic techniques: AU0144 [*daf-2 (e1368);glp-1(e2141)*III], AU0145 [*daf-2(e1368)*III;*fem-3(q20)*IV], AU0146[*daf-2(e1368)*III;*fog-2(q71)*V], AU0147 [*daf-16(mgDF47)*I; *glp-1(e2141)*III], AU0148 [*daf-16(mgDF47)*I;*fem-3(q20)*IV], and AU0166 [*daf-16(mgDF47)*I;*fog-2(q71)*V].

Genetic screen for *C. elegans* mutants with enhanced resistance to pathogens: N2 hermaphrodites in the L4 stage (P_0 generation) were mutagenized by a 6-hr exposure to 25 mm ethyl methanesulfonate (EMS) (Sigma). Progeny (the F_1 generation) were collected, allowed to mature to gravid adults, and their embryos harvested using hypochlorite treatment (Epstein 1995). Embryos were allowed to hatch in M9 buffer overnight, resulting in a synchronized culture of F_2 animals arrested at the L1 larval stage, which were then transferred to 10 cm NGM plates spread with *E. coli* OP50, and incubated for ~55 hr at 15° until the animals reached the L4 larval stage. Worms were then eluted from the growth plates in M9 and washed three times in M9 containing ampicillin (100 μg/ml) to kill the *E. coli.* Washed worms were transferred to 10 cm TS agar plates spread with *S. aureus* and incubated at 25°. Control wild-type N2 animals were treated identically (with the exception of EMS treatment) in parallel to mutagenized animals. After 48 hr, a time at which all animals on the control plates were dead, surviving nematodes were manually transferred from the killing plates to NGM plates containing *E. coli* OP50 to recover putative mutants. Animals that produced offspring were retested in the *S. aureus* killing assay to confirm their pathogen resistance phenotype. Selected mutants were backcrossed at least three times (*ag17* was backcrossed five times) to the parental wild-type N2 worms. For each round of backcrossing, ~20 F_2 animals were singled and the progeny (F_3 generation) tested for resistance using the *S. aureus* killing assay. Lines exhibiting a strong resistance phenotype were selected for at least three additional rounds of backcrossing.

SNP-based mapping of *ag12*: To identify the chromosomal location of the genetic lesions responsible for the resistance phenotype in the *ag12* mutant, a single nucleotide polymorphism (SNP)-based mapping strategy similar to the method described by Davis *et al.* (2005) was employed using the *C. elegans* Hawaiian strain CB4856. *C. elegans* Hawaiian males were mated to *ag12* hermaphrodites and the resulting F_2 population was tested for their resistance against *S. aureus.* Resistant animals were then genotyped using three defined SNPs per chromosome for all six chromosomes and the linkage patterns analyzed. *ag12* showed strong linkage to the central region of chromosome II, where *age-1* is located.

Mapping of *ag17*: Using a SNP-based mapping strategy described above, the F_2 population generated from a cross between *ag17* and CB4856 was tested for its resistance against *P. aeruginosa* and both wild-type and enhanced resistance animals were genotyped with SNPs. *P. aeruginosa* was used for mapping because CB4856 showed a slightly enhanced resistance toward *S. aureus* compared to wild-type N2. A large number of F_2 worms were generated by multiple rounds of crosses and were examined individually for both their genotypes and phenotypes. *ag17* showed strong linkage to the central region of chromosome I within a 0.25-MU interval bounded by SNP markers F21C3 and H15M21. Of 61 genes that were found within this region according to WormBase (Harris *et al.* 2003), 50 RNAi clones were available in the Ahringer library (Kamath *et al.* 2003) and were analyzed for their role in pathogen resistance. Nine RNAi clones (F21C3.5, F52A8.5, F07A5.1, T28F4.1, C26C6.1, C26C6.2, C26C6.5, T25G3.3, and D2030.3) caused pathogen resistance phenotype in wild-type animals; however, four of the nine RNAi clones also exhibited obvious anatomical defects and were not analyzed further. The five candidate genes were sequenced as described below.

Complementation analysis: Male *age-1(hx546)* nematodes were mated with *ag12* or wild-type hermaphrodites, and male *ag12* or wild-type nematodes were mated with *age-1(hx546)* hermaphrodites. The F_1 progeny resulting from each cross were tested for their resistance phenotype using the *S. aureus* killing assay.

Similarly, complementation analyses were used to confirm that the mutation in *ag17* was in *inx-14* by mating male animals of *ag17* with AU0210 or AU0211. The F_1 progeny resulting from the crosses were tested for their resistance to *P. aeruginosa.*

To complement the *inx-14* mutation in *ag17*, the cosmid F07A5, obtained from Sanger Institute (Cambridge, UK), or a PCR product containing a full-length copy of *inx-14* (5–10 ng/μl) was injected into *ag17* or wild-type animals as described by Mello and Fire (1995). The primers *inx-14_1A* (5′-GTCT GTCACTCTCTAACTATCTACAC-3′) and *inx-14_4R* (5′-GAA GACGACATCTCCGAGTTG-3′) were used to amplify a 9.9-kb PCR product containing *inx-14* from *C. elegans* N2 genomic DNA. The co-injection marker pTG96 encoding *sur-5::GFP* (Yochem *et al.* 1998) (10 ng/μl) was used as a control.

RNA interference: All the RNAi clones used in this study were from the Ahringer library (Kamath *et al.* 2003), except for RNAi clones corresponding to *fem-3* and *fog-2* (Rual *et al.* 2004) and *daf-2* (Dillin *et al.* 2002). All of the RNAi constructs that contributed to data presented in figures were verified by DNA sequence analysis. Each RNAi clone in *E. coli* strain HT115 was grown overnight at 37° in LB with 50 μg/ml ampicillin and 15 μg/ml tetracycline and seeded onto RNAi agar plates containing 10 μg/ml carbenicillin and 5 mm isopropylthiogalactosidase. RNAi clones were incubated overnight at room temperature to induce dsRNA expression. Two

gravid adult *epr* mutants were transferred to each plate in at least triplicate and incubated at 15° to allow them to lay eggs. When sterile mutants were fed with RNAi clones, they were raised at the permissive temperature and then allowed to lay eggs at 15° for ~48 hr or at 20° for ~16 hr then moved to 25°. After feeding on RNAi plates, L4-stage animals were transferred for pathogen infection assays. Worms grown under the same conditions on *E. coli* HT115 expressing dsRNA targeting *unc-22* were included as a positive control to confirm the efficacy of RNAi. Animals grown on *E. coli* harboring the empty L4440 vector were used as a negative control (Fire *et al.* 1998).

Sequencing of the *age-1* and *inx-14* genes: From the genomic DNA isolated from adult wild-type *C. elegans* or *ag12* animals, the *age-1* gene was amplified using the primers *age-1F* (5′-ATGCATGTTAACATTTTACATC-3′) and *age-1R* (5′-TCA GTAGTGTTTGACTGC-3′). From the resulting PCR product, each exon of the *age-1* PCR product in the wild-type and mutant genomic DNA was sequenced in both the forward and reverse direction and analyzed using the Vector NTI Suite 7 software package (InforMax, Bethesda, MD). The sequences obtained from wild-type and *ag12* DNA were compared to the published sequence in WormBase (Harris *et al.* 2003). Sequence alignments of homologous Ras-binding domains (RBDs) from other PI3 kinases were obtained from the Conserved Domain Database (Marchler-Bauer *et al.* 2005).

A similar method was used to sequence the 5 candidate genes (C26C6.1, C26C6.2, F07A5.1, F52A8.5, and T28F4.1) corresponding to *ag17* that had been identified by RNAi analysis in both wild type and *ag17*. The primers used to amplify the region where the mutation was found were *inx-14_4F* (5′-GAT ATAAATTGAATGACACTGAT-3′) and *INX-14_8R* (5′-CTTTG TGAAATTATGGTGTACTG-3′).

C. *elegans* growth conditions for pathogen infection assays and for quantitative RT–PCR: For pathogenicity assays, *epr* mutants were maintained at 15°, unless otherwise noted, until the L4 larval stage. Temperature-sensitive sterile mutants were allowed to lay eggs and grow at 20° for ~18 hr and were then shifted to 25° until the L4 larval stage. *daf-2* animals were allowed to grow until the L3 larval stage at 20° and were then shifted to 25°. For qRT–PCR, the growth stage of animals was synchronized by hypochlorite treatment (Epstein 1995). About 3000 animals arrested at the L1 larval stage were plated on 10-cm NGM plates seeded with OP50 and grown at 15° for 48 hr then moved to 25° for 24 hr to the young adult stage before harvesting for RNA extraction. For RNA extraction from adults, animals were prepared and grown until the L4 larval stage as described above, then transferred onto fresh 10-cm NGM plates seeded with OP50 for another 26 hr at 25° before harvesting. For RNA extraction from 4-day-old adults, animals were transferred onto fresh 10-cm NGM plates seeded with OP50 twice a day for four consecutive days after the L4 larval stage at 25° before harvesting. These transfers were done to separate adults from their progeny. Temperature-sensitive sterile mutants were monitored to ensure complete sterility when used for either infection assays or qRT–PCR.

Pathogen infection assays: *C. elegans* killing assays were performed as previously described for *S. aureus* strain NCTC8325 (Sifri *et al.* 2003) and *P. aeruginosa* strain PA14 (Tan *et al.* 1999a). Briefly, a saturated culture of *S. aureus* was diluted 1:5 in TS broth containing 5 μg/ml nalidixic acid. Ten microliters of diluted culture was plated on TS agar plates containing 5 μg/ml nalidixic acid, and incubated for 3 hr at 37°. Five microliters of saturated cultures of *P. aeruginosa* PA14 in LB was plated on modified NGM plates (Tan *et al.* 1999a) and incubated for 24 hr at 37° followed by another incubation at room temperature for ~24 hr. For the pathogen assays in the presence of 5-fluorodeoxyuridine (FUDR), FUDR was added to the edge of the lawn to give a final concentration of 0.1 mg/ml and dried before the animals were placed on the plates. Killing assays were carried out in triplicate and performed by manually transferring ~30 L4-staged animals from *E. coli* OP50 plates to pathogen plates ($t = 0$). Worm mortality was monitored over time, and nematodes were considered dead when they failed to respond to head tapping with a platinum wire. For pathogen assays involving FUDR pretreatment, animals were transferred at the L4 larval stage to plates containing OP50 with 0.1 mg/ml FUDR for 24 hr at 25°. Subsequently, pathogen infections were performed as described above without FUDR. For the pathogen assays that involved mated *fer-15;fem-1* animals, *fer-15;fem-1* was grown at the nonpermissive temperature until the L4 larval stage and then mated with wild-type male animals at 25° for 24 hr on OP50. Subsequently, mated *fer-15;fem-1* animals were transferred onto plates containing *P. aeruginosa* and tested for survival.

Longevity assays: Longevity assays were performed as previously described (Wolkow *et al.* 2000). About 30 L4 hermaphrodites that had been raised at 15° were transferred to each of three NGM plates containing 0.1 mg/ml FUDR, seeded with *E. coli* OP50, and incubated at 25°. The life span was defined as the length of time from when animals were put down on the plates at the L4 larval stage ($t = 0$) until they were scored as dead.

Brood size measurements: Animals were grown at 15° until the L4 larval stage then singled onto plates seeded with OP50 and incubated at 25° to allow them to lay eggs. Adult animals were transferred once a day for 3 consecutive days to fresh plates. The total number of progeny that grew up from a single animal was counted. At least eight animals were used for the analysis for each strain.

DAF-16 nuclear localization assays: A synchronous culture of TJ356 animals expressing *DAF-16::GFP* was seeded onto relevant RNAi plates at 20° for one or two generations as necessary to induce sterility. *inx-8* RNAi caused complete sterility in animals in the first generation. *fem-3*, *glp-1*, *inx-9*, and *inx-14* RNAi caused complete sterility in the majority of animals in the second generation. *fem-1*, *fer-1*, and *fog-2* RNAi caused complete sterility only in a small number of animals. On day 1 of adulthood, DAF-16 nuclear translocation in intestinal cells was analyzed using an Axioplan 2 fluorescent microscope and processed using Openlab 4.0.3 (Improvision, Coventry, UK) and Photoshop CS2 9.0.2 (Adobe, San Jose, CA) software. Except for the animals treated with control RNAi, only completely sterile animals were analyzed as shown in Figure 7 and supplemental Figure S3 at http://www.genetics.org/supplemental/.

RNA extraction and qRT–PCR: Both RNA extraction and qRT–PCR were performed as described (Troemel *et al.* 2006). Briefly, animals were harvested from OP50 plates by washing once with M9 buffer and then total RNA was extracted using TRI Reagent (Molecular Research Center, http://mrcgene.com) according to the manufacturer's protocol. The RNA was reverse transcribed to make cDNA using the Retroscript kit (Ambion, Austin, TX), and the resulting cDNA was subjected to qRT–PCR analysis using SYBR green detection on an iCycler machine (Bio-Rad, http://bio-rad.com). Primers for qRT–PCR for *sod-3*, *mtl-1*, and *lys-7* have been described (Troemel *et al.* 2006). Other primers were manually designed, and their specificity was tested against the cDNA and their efficiency was calculated using a serially diluted cDNA template. *snb-1* was used as a control gene because expression of *snb-1* was very similar among wild-type and different mutant backgrounds used in this study. All the values were normalized to *snb-1*, and then each value was expressed in fold change compared to that of wild type. Fold change was calculated according to the Pfaffl method (Pfaffl 2001). Primer sequences are listed in supplemental Table 2 at http://www.genetics.org/supplemental/).

Statistical analysis: Each survival and longevity curve is based on data from 60 to 100 animals. Curves were analyzed by calculating the TD_{50} (the time for 50% of the nematodes to die) using a nonlinear regression analysis (GraphPad Prism, version 4.0). The significance of the differences between different survival assays was assessed using a log-rank test (GraphPad Prism, version 4.0).

RESULTS

A forward genetic screen for mutants with enhanced resistance to pathogens: Mutations in the *daf-2* or *age-1* genes in the *C. elegans* insulin-like signaling pathway confer DAF-16-dependent resistance to both Gram-negative and Gram-positive bacterial pathogens (Garsin *et al.* 2003), demonstrating that the immune response of *C. elegans* can be genetically enhanced. To identify additional genes that when mutated result in enhanced resistance to killing by bacterial pathogens, we undertook a forward genetic screen for mutants with an *e*nhanced *p*athogen *r*esistance (Epr) phenotype.

EMS-mutagenized L4 larval stage N2 animals were transferred from their normal laboratory food *E. coli* strain OP50 to the Gram-positive human pathogen, *S. aureus* strain NCTC8325. This strain of *S. aureus* kills essentially 100% of *C. elegans* wild-type animals within 48 hr and prevents progeny from maturing past the L1 larval stage (Sifri *et al.* 2003), thereby facilitating the identification of pathogen-resistant mutants without developing progeny obscuring the assay. From 33,000 genomes screened, 319 putative *epr* mutants were identified in the primary screen on the basis that they appeared healthy after 48 hr of exposure to *S. aureus*. Of these 319 putative mutants, 110 produced broods when transferred to *E. coli*. After retesting, 39 of the 110 fertile putative *epr* mutants reproducibly exhibited enhanced resistance to *S. aureus*. Among these 39 mutants, 6 mutants with the strongest resistance to *S. aureus* were further characterized. When these 6 mutants (*ag12*, *ag13*, *ag14*, *ag15*, *ag17*, and *ag19*) were mated with each other in all combinations, the pathogen resistance phenotype was complemented in all cases (data not shown), indicating that the 6 mutations fell into six different complementation groups. These 6 mutants exhibited various degrees of resistance to *S. aureus* after being backcrossed at least three times to wild-type animals. The resistance phenotypes of *ag12*, *ag15*, *ag17*, and *ag19* are shown in Figure 1A and supplemental Table 1 at http://www.genetics.org/supplemental/). The resistance phenotypes of *ag13* and *ag14* are shown in supplemental Figure S1A.

All six mutants also exhibited enhanced resistance to the Gram-negative bacterial pathogen *P. aeruginosa* (Figure 1B; supplemental Figure S1C and supplemental Table 1 at http://www.genetics.org/supplemental/). Unless otherwise noted, further characterization of the *epr* mutants was carried out using *P. aeruginosa* because it elicits less matricidal death (bagging) than *S. aureus*

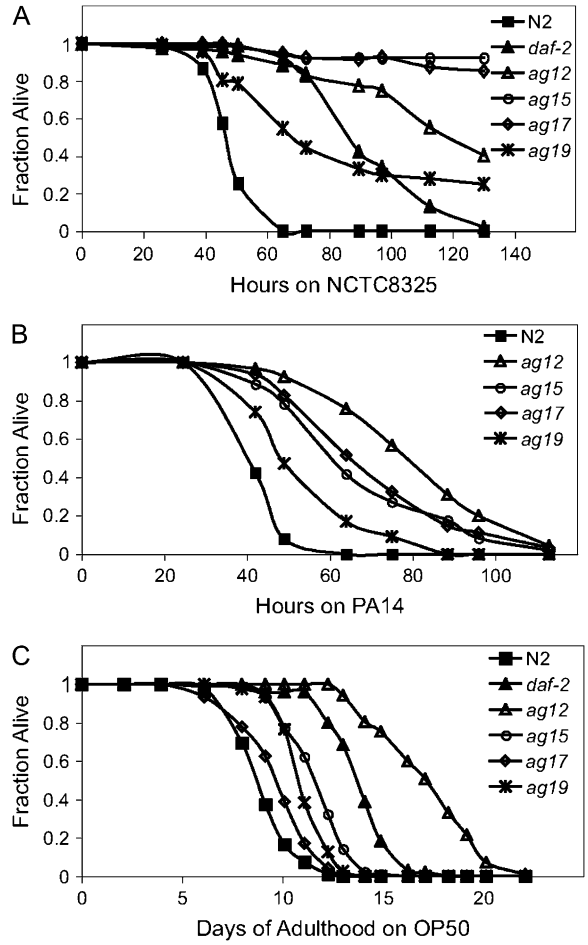

Figure 1.—*C. elegans epr* mutants exhibit enhanced resistance to both *S. aureus* and *P. aeruginosa* and various degrees of enhanced longevity on *E. coli*. L4 larval-stage *epr* mutants, *ag12*, *ag15*, *ag17*, and *ag19*, were transferred from *E. coli* OP50 plates onto plates containing *S. aureus* NCTC8325 (A), *P. aeruginosa* PA14 (B), and OP50 (C) and monitored for their survival over time at 25°. The time at which 50% of the worms had died (TD_{50}) for this assay for each *epr* mutant is shown in supplemental Table 1. *daf-2(e1368)* was included as positive controls (A and C). The experiment was repeated at least three times with similar results.

and because *P. aeruginosa* is still infectious in the presence of FUDR, unlike *S. aureus* (J. Irazoqui, personal communication).

Mutants in the *daf-2* insulin signaling pathway exhibit both enhanced longevity and enhanced pathogen resistance, both of which can be completely suppressed by a mutation in *daf-16* (Garsin *et al.* 2003; Kenyon *et al.* 1993). To determine whether any of the six pathogen-resistant mutants carried defects in the insulin signaling pathway components, we examined their longevity on *E. coli* OP50 (Figure 1C; supplemental Figure S1B and supplemental Table 1) and determined whether DAF-16 was required for their enhanced resistance to *P. aeruginosa* (Figure 2, A and B, and supplemental Figure S1C). One mutant, *ag12*, exhibited significantly increased longevity on OP50 (Figure 1C and supplemental Table 1).

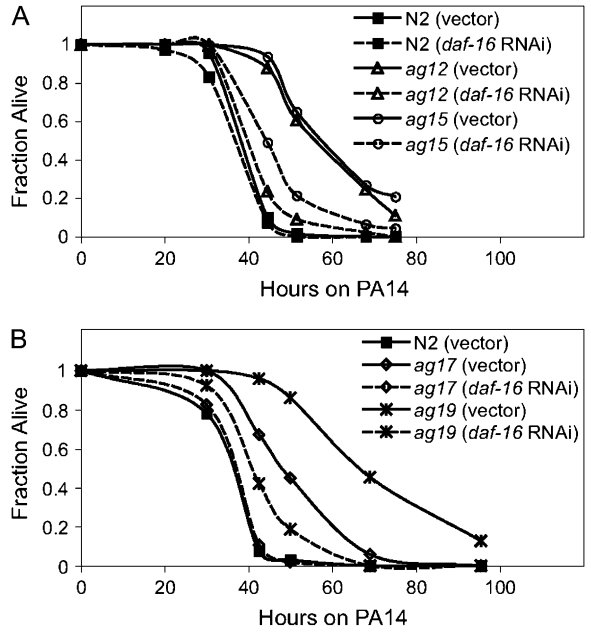

FIGURE 2.—*epr* mutants require DAF-16 for pathogen resistance. *epr* mutants, *ag12* and *ag15* (A), *ag17* and *ag19* (B), were fed with either a vector control or *daf-16* RNAi and then transferred to *P. aeruginosa* PA14 plates. The differences in survival kinetics between wild-type N2 and *epr* mutants (*ag12*, *ag15*, *ag17*, and *ag19*) treated with the control RNAi were significant ($P < 0.0001$). The differences in survival kinetics between the *epr* mutants treated with control RNAi and *daf-16* RNAi were also significant ($P < 0.0001$). Experiments with *ag12* and *ag17* were repeated three times and experiments with *ag15* and *ag19* were repeated twice with similar results.

daf-16 RNAi completely suppressed the enhanced pathogen resistance phenotype of *ag12* (Figure 2A). *ag12* also exhibited a dauer-constitutive phenotype at 25° (data not shown). All three of these phenotypes are characteristic of mutations in the *daf-2* signaling pathway, suggesting that *ag12* might encode a component of the *daf-2* signaling pathway. There was a modest increase in longevity of two other *epr* mutants, *ag15* and *ag19*, on OP50. In contrast, there was no increase in longevity of *ag14* (supplemental Figure S1B). The longevity on OP50 of the remaining two mutants, *ag13* and *ag17*, while statistically greater than wild type, was only marginally increased (Figure 1C and supplemental Figure S1B). Furthermore, none of these latter five mutants became dauer at 25° (data not shown), suggesting that they did not encode components of the *daf-2* signaling pathway. Unexpectedly however, the enhanced resistance to *P. aeruginosa* in all of these latter five mutants was suppressed by *daf-16* RNAi (Figure 2, A and B, and supplemental Figure S1C).

***ag12* has a mutation in *age-1*:** Using a SNP-based mapping strategy (DAVIS *et al.* 2005), we mapped *ag12* to the central region of chromosome II (see MATERIALS AND METHODS for details). The insulin signaling pathway gene, *age-1*, is located in this region (MORRIS *et al.* 1996) and the *age-1(hx546)* mutation failed to complement *ag12* for *S. aureus* susceptibility and longevity on OP50 (data not shown). Sequencing of PCR products corresponding to the exons of the *age-1* gene in *ag12* genomic DNA revealed a single point mutation in exon 3 resulting in a cytosine to thymine transition (see MATERIALS AND METHODS for details). This mutation results in the substitution of a highly conserved leucine residue with phenylalanine in the RBD of the AGE-1 protein (Figure 3). Thus, consistent with its dauer and longevity phenotypes, *ag12* appears to be defective in a component of the *daf-2* signaling pathway, namely the PI3 kinase, *age-1*.

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                  *    *    :.   .   :  ..   .
P3C2B_HUMAN  V-LKPCGLEEFLQ-NKHALGSHEYIQYCR
P3C2G_HUMAN  I-LSVCGSEEFLQ-NDHCLGSHKMFQ---
PK3CG_PIG    V-LRVCGRDEYLV-GETPIKNFQWVRQCL
PK3CG_MOUSE  V-LRVCGRDEYLV-GETPLKNFQWVRQCL
PK3CB_HUMAN  V-LQVSGRVEYVF-GDHPLIQFQYIRNCV
PK3CB_RAT    V-LQVSGRVEYVF-GDHPLIQFQYIRNCV
PK3CD_HUMAN  T-LQVNGRHEYLY-GSYPLCQFQYICSCL
PK3CA_BOVIN  I-LKVCGCDEYFL-EKYPLSQYKYIRSCI
P91634_DROME I-LKVSGRDEYLL-GDYPLIQFLYIQEML
Q620C1_CAEBR I-LQLAGRTSFVTRPEISLVSYDGIRSEL
AGE1_CAEEL   F-LQLAGRTTFVTNPDVKLTSYDGVRSEL
                                 ↑
```

FIGURE 3.—*ag12* harbors a point mutation in the RBD of the PI(3)K AGE-1. Protein alignment of the RBDs of PI(3)Ks among different species including *C. elegans*, which is shown at bottom. The conserved leucine residue, which is mutated to phenylalanine in *ag12*, is indicated by an arrow.

***ag17* has a mutation in *inx-14*:** Because five of the *epr* mutants exhibited substantially shorter longevity on OP50 than *daf-2* or *age-1* mutants (Figure 1C) and because they did not become dauer at elevated temperatures, it did not seem likely that they carried mutations in components of the DAF-2 insulin signaling pathway. However, similar to *daf-2* and *age-1* animals, these five *epr* mutants exhibited enhanced resistance to *P. aeruginosa* in a DAF-16-dependent manner (Figure 2, A and B, and supplemental Figure S1C), suggesting additional mechanisms to activate DAF-16 to exert a pathogen resistance phenotype in addition to the canonical DAF-2 insulin signaling pathway. To further investigate this possibility, we chose *ag17* for additional analysis because its longevity on OP50 was only marginally increased compared to that of wild type (Figure 1C) and because it exhibited a very high level of resistance to *S. aureus*-mediated killing (Figure 1A).

As described in detail in MATERIALS AND METHODS, SNP-based mapping showed strong linkage of *ag17* to the central region of chromosome I within a 0.25-MU interval bounded by SNP markers F21C3 and H15M21 (data not shown). This map position was confirmed by showing that *ag17* is tightly linked to *dpy-5*, which also maps to the central region of chromosome I [in a cross between *ag17* and the semidominant *dpy-5(e61)* mutant, 100% of F_2 Dpy animals had progeny with wild-type resistance, while 90% of F_2 non-Dpy had progeny with enhanced resistance to pathogens; data not shown].

Perusal of WormBase (http://www.wormbase.org/release WS176, June 19, 2007) showed 61 predicted genes in the 0.25-MU interval on chromosome I where *ag17* maps. A combination of RNAi feeding analysis and sequencing of five candidate genes identified a single point mutation in exon 5 of the *inx-14* gene in *ag17* resulting in a guanine-to-adenine transition. INX-14 is a predicted member of the innexin family of proteins, which have been shown to form gap junctions that are important for cell–cell communication (reviewed by Phelan (2005). INX-14 in particular is thought to form sheath/oocyte gap junctions involved in oocyte maturation and fertilization (Whitten and Miller 2007). The identified mutation in *ag17* causes an arginine-to-histidine substitution in *inx-14* near the end of the predicted third transmembrane domain (Bairoch and Apweiler 2000).

To confirm that the arginine-to-histidine mutation in *inx-14* was responsible for the enhanced pathogen phenotype of *ag17*, we obtained two additional alleles of *inx-14*, *tm2864* and *tm2593*, from the National Bioresource Project (http://www.shigen.nig.ac.jp) for complementation analysis. Because both the *tm2864* and *tm2593* alleles cause sterility when homozygous, the mutations were first balanced with hT2 marked with GFP and then crossed into *ag17*. F_1 cross progeny that inherited the hT2 balancer chromosome did not show the pathogen resistance phenotype of *ag17* (data not shown). In contrast, F_1 progeny lacking the balancer and therefore heterozygous for *ag17* and an *inx-14* deletion were resistant to pathogens (data not shown), indicating that *inx-14* failed to complement *ag17* and suggesting that the mutation in *ag17* is an allele of *inx-14*. These *trans*-heterozygotes exhibited complete sterility and a stronger Epr phenotype relative to *ag17* homozygotes (data not shown), suggesting that the mutation in *ag17* is most likely not null.

We also attempted to rescue the phenotype of *ag17* with a wild-type genomic copy of *inx-14*. Extrachromosomal expression of *inx-14* in transgenic animals carrying either the cosmid *F07A5* or a PCR product corresponding to the full-length *inx-14* gene resulted in partial complementation of the pathogen resistance phenotype of *ag17* (supplemental Figure S2 and data not shown). The fact that the pathogen resistance of *ag17* was only partially complemented in these experiments is most likely due to the fact that INX-14 is expressed in the germ line (Govindan *et al.* 2006) and transgenes are generally expressed at low levels in the germ line (Dernburg *et al.* 2000). Nevertheless, the data presented in this section support the conclusion that the pathogen-resistant phenotype of *ag17* is due to a mutation in *inx-14*. From here on, *ag17* is referred to as *inx-14(ag17)*.

Twenty-five genes make up the innexin family of proteins in *C. elegans* (Starich *et al.* 2001). To determine whether any other innexin proteins are also involved in pathogen resistance, we carried out RNAi feeding analysis of all of the innexin genes except for *inx-2*, *inx-21*, and *inx-22* (for which RNAi constructs were not available in the Ahringer RNAi library). Among the 22 genes examined, *inx-8*, *inx-9*, and *inx-14* RNAi clones caused resistance to *P. aeruginosa* (data not shown). We also observed that these three RNAi clones resulted in sterility (data not shown). In this study we use the term "sterility" to refer to the phenotype of animals that do not produce viable progeny, without implying a molecular mechanism leading to no brood or a small brood size.

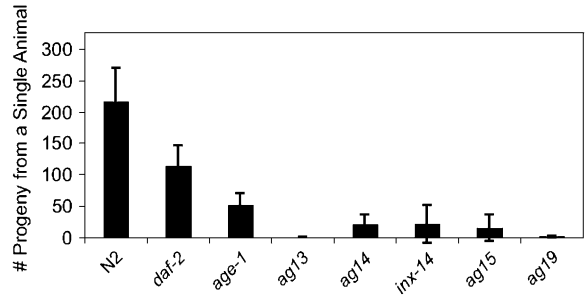

Figure 4.—*epr* mutants have a small brood size at 25°. N2, *daf-2(e1368)*, *age-1(ag12)*, *ag13*, *ag14*, *inx-14(ag17)*, *ag15*, and *ag19* were cultured at 15° and then transferred at the L4 larval stage to 25°. The total number of progeny that hatched from a single animal was counted. The average of more than eight animals for each genotype is shown. Error bars represent standard deviation.

Sterility is a common phenotype of pathogen resistance mutants: The screen for *epr* mutants identified *inx-14(ag17)*, which is not a component of the canonical insulin signaling pathway, although its pathogen resistance depends on DAF-16 (Figure 2B). Importantly, *inx-14(ag17)* exhibited a partially penetrant sterility phenotype leading to a smaller brood size at 25°, which is the temperature at which the pathogen infection assays were performed. Because INX-14 is required to regulate oocyte maturation and sperm recruitment to the spermatheca, the site of fertilization (Whitten and Miller 2007), fertilization may be disrupted in *inx-14* mutants. Moreover, in the process of mapping *inx-14 (ag17)*, an unexpectedly high number of RNAi clones were found to cause pathogen resistance (9 of 50) and these mostly coincided with sterility (8 of 9). Finally, as reported above, RNAi against *inx-8* and *inx-9* in addition to *inx-14* resulted in both resistance to *P. aeruginosa* and in sterility. To examine whether the other *epr* mutants also have a temperature-sensitive sterility phenotype, we compared the brood size of each mutant to that of wild-type animals at 25°. Similar to *inx-14(ag17)*, the other five *epr* mutants also exhibited a much smaller brood size than wild-type animals (Figure 4).

To test the hypothesis that partially sterile mutants are pathogen resistant because of a lower frequency of internal progeny hatching, we examined *inx-14(ag17)*

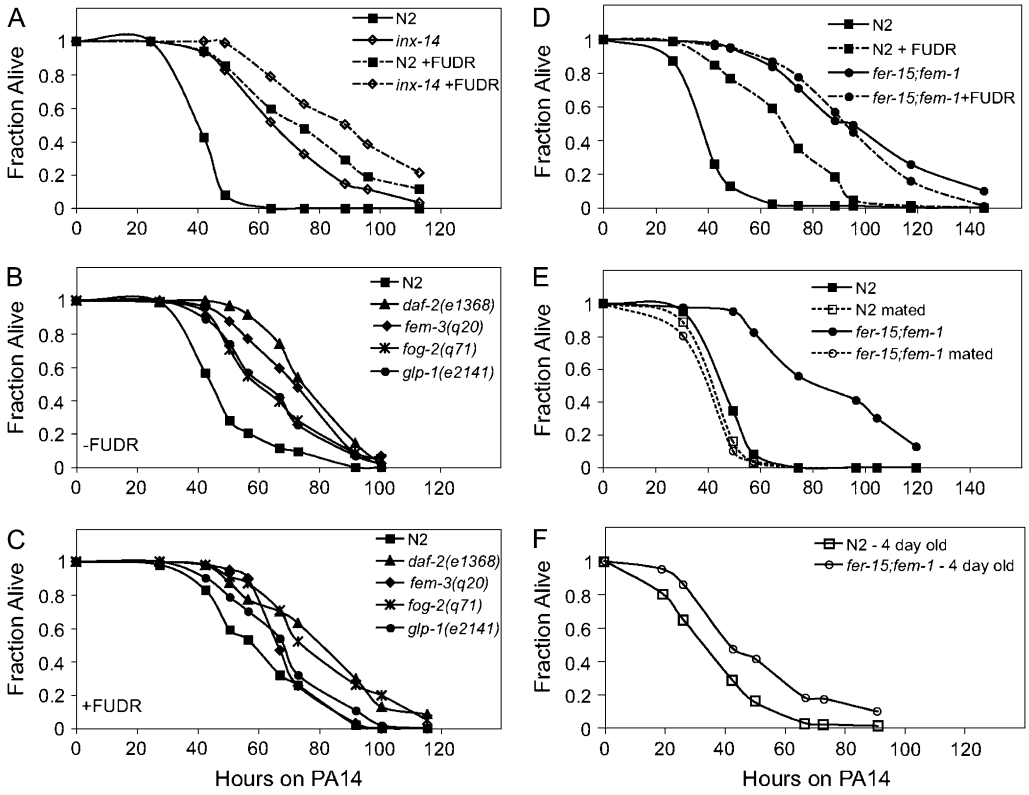

FIGURE 5.—*inx-14(ag17)* mutants and sterile mutants are more resistant than wild-type animals in the presence and absence of FUDR (A–D). N2 and *inx-14(ag17)* animals were transferred at the L4 larval stage onto plates preseeded with *P. aeruginosa* PA14 with and without FUDR (A). *inx-14(ag17)* exhibited enhanced pathogen resistance compared to wild type in the absence of FUDR ($P < 0.0001$) and in the presence of FUDR ($P < 0.005$). *glp-1(e2141)*, *fog-2(q71)*, and *fem-3(q20)* were grown at the nonpermissive temperature of 25° and then transferred onto plates preseeded with PA14 in the absence (B) or presence (C) of FUDR. Compared to wild-type N2, *daf-2(e1368)*, *glp-1(e2141)*, *fog-2(q71)*, and *fem-3(q20)* exhibited significantly enhanced pathogen resistance in the absence ($P < 0.0001$) or presence of FUDR ($P < 0.05$). N2 and *fer-15;fem-1* were pretreated or untreated with FUDR prior to infection with PA14 in the absence of FUDR (D). FUDR-pretreated *fer-15;fem-1* exhibited enhanced pathogen resistance ($P < 0.0001$) compared to FUDR-pretreated wild-type N2. N2 and *fer-15;fem-1* were mated with wild-type males prior to infection with PA14 (E). Mated *fer-15;fem-1* animals no longer exhibited enhanced pathogen resistance ($P = 0.2961$) compared to mated N2. Four-day-old adults of N2 and *fer-15;fem-1* were infected with PA14 (F). The difference in pathogen resistance phenotype between N2 and *fer-15;fem-1* was much smaller in 4-day-old adults than in 1-day-old adults (E). Similar results were observed in two independent experiments for *inx-14(ag17)* and three independent experiments for the other sterile mutants.

pathogen resistance in the presence of FUDR, which prevents the production of viable eggs. Overall, both wild-type and *inx-14(ag17)* animals died more slowly when feeding on *P. aeruginosa* in the presence of FUDR; however, *inx-14(ag17)* animals were still more resistant to *P. aeruginosa* than wild-type animals on FUDR (Figure 5A). Similarly, when *daf-2(e1368)* was compared to wild type in the presence of FUDR, *daf-2(e1368)* exhibited enhanced resistance to *P. aeruginosa* (Figure 5C). These data suggest that the DAF-16-dependent enhanced pathogen resistance phenotype of *daf-2(e1368)* and *inx-14(ag17)* is not simply a consequence of a reduced level of matricidal hatching of progeny.

Sterility activates a signaling pathway(s) to enhance resistance to *P. aeruginosa* infection through DAF-16 activity: All of the *epr* mutants that were examined in detail exhibited partially sterile phenotypes as well as DAF-16-dependent pathogen resistance. Therefore, we examined a variety of previously characterized sterile mutants to determine whether they also exhibit Epr phenotypes that could be suppressed by a *daf-16* mutation.

glp-1 is a sterile mutant that lacks mitotic germ-line stem cells (and thus the entire germ line) and exhibits DAF-16-dependent increased longevity on OP50 (ARANTES-OLIVEIRA *et al.* 2002). In comparison, *fog-2*, *fer-15;fem-1*, and *fem-3* are sterile mutants that have mitotic germ cells, but they differentiate into only oocytes (*fog-2*), oocytes with dysfunctional sperm (*fer-15;fem-1*), or only sperm (*fem-3gf*) (and thus produce unfertilized embryos) (BARTON *et al.* 1987; SCHEDL and KIMBLE 1988; MCCARROLL *et al.* 2004). *fem-3*, *fog-2*, and *glp-1* animals all showed enhanced pathogen resistance either in the absence (Figure 5B) or presence (Figure 5C) of FUDR compared to wild-type animals.

One problem in interpreting the results of pathogen-mediated killing assays carried out in the presence of FUDR is that FUDR could be affecting the virulence of the pathogen as well as the fertility of *C. elegans*. To circumvent this problem, we exposed animals to FUDR at the L4 larval stage for 24 hr and then transferred the animals to lawns of *P. aeruginosa* PA14. This FUDR pretreatment was sufficient to maintain the sterility of wild-type animals throughout the course of the infection assay (data not shown). Wild-type animals pretreated with FUDR survived longer on *P. aeruginosa* compared to wild-type animals without the FUDR pretreatment (Figure 5D), exhibiting similar killing kinetics as animals treated continuously with FUDR (Figure

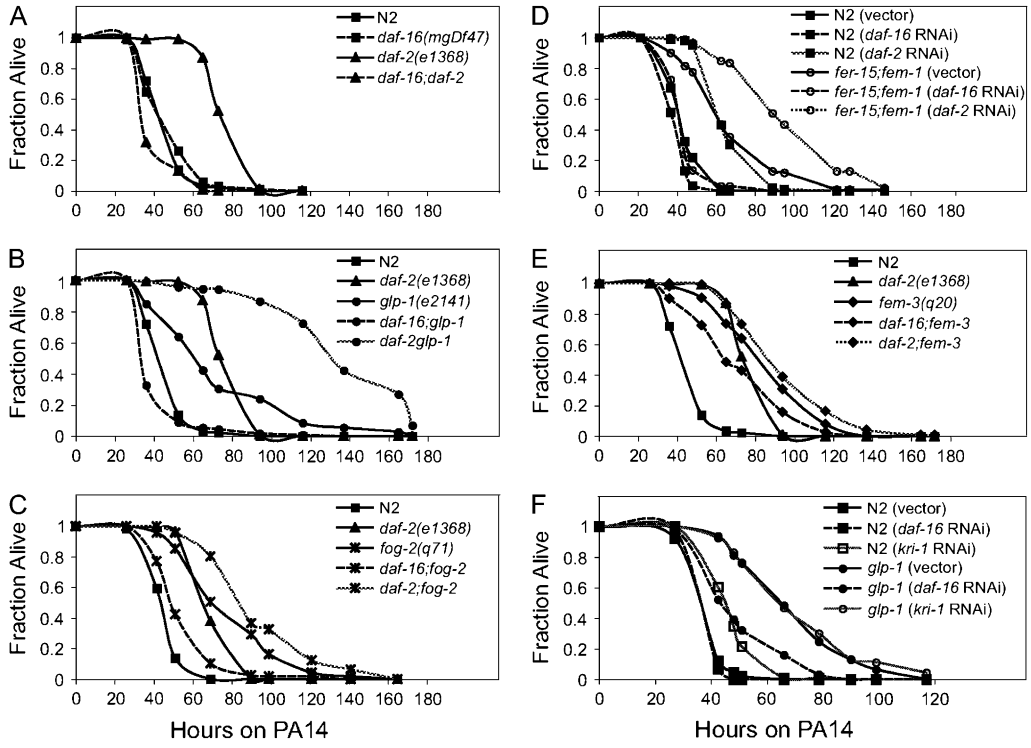

FIGURE 6.—*daf-16* is required for the pathogen resistance of sterile mutants. The survival kinetics of N2 were compared to those of *daf-16* and *daf-2* upon *P. aeruginosa* PA14 infection (A). Similarly, the survival kinetics of the following sterile mutants were compared to their respective *daf-16* and *daf-2* mutants; *glp-1* to *daf-16; glp-1* and *daf-2 glp-1* (B), *fog-2* to *daf-16;fog-2* and *daf-2; fog-2* (C), *fer-15;fem-1* with control RNAi to *fer-15;fem-1* with *daf-16* RNAi and with *daf-2* RNAi (D), and *fem-3* to *daf-16;fem-3* and *daf-2; fem-3* (E). In wild-type N2, a *daf-16(mgDf47)* mutation caused no significant change in pathogen resistance ($P = 0.6281$), whereas a *daf-2(e1368)* mutation enhanced the pathogen resistance significantly ($P < 0.0001$). In *glp-1(e2141)*, *fog-2(q71)*, *fer-15;fem-1*, and *fem-3(q20)* animals, either the *daf-16(mgDf47)* mutation or *daf-16* RNAi suppressed the pathogen resistance phenotype of the respective sterile mutants ($P < 0.0001$). *daf-2(e1368)* or *daf-2* RNAi significantly increased the pathogen resistance phenotype of the respective sterile mutants ($P < 0.05$). Similar results were observed in three independent experiments with *glp-1(e2141)*, *fer-15; fem-1*, and *fem-3(q20)* and two independent experiments with *fog-2(q71)*. N2 and *glp-1(e2141)* animals were fed either with vector control RNAi, *daf-16* RNAi, or *kri-1* RNAi and then tested for PA14 resistance (F). The differences between *glp-1(e2141)* treated with control RNAi and with *daf-16* RNAi were significant ($P < 0.0001$). The differences between *glp-1(e2141)* treated with control RNAi and with *kri-1* RNAi were not significant ($P = 0.3990$). *kri-1* RNAi in *glp-1* animals appeared to be effective because 100% of *glp-1* animals ($n = 94$) treated with *kri-1* RNAi appeared paler and smaller compared to the *glp-1* animals treated with the control RNAi, as previously reported (BERMAN and KENYON 2006). This experiment was repeated three times with similar results.

5, A and C). This result indicated that FUDR affected the sterility of *C. elegans* rather than the virulence of *P. aeruginosa*. Moreover, *fer-15;fem-1* animals pretreated with FUDR were significantly more resistant to *P. aeruginosa* than wild-type animals pretreated with FUDR (Figure 5D). This result demonstrates that *fer-15;fem-1* animals, like the other sterile mutants examined in Figure 5, B and C, are more resistant to killing than wild-type animals even in the absence of bagging.

Finally, as shown in Figure 6, removal of DAF-16 in sterile mutants, either with RNAi or by mutation, resulted in a striking suppression of the Epr phenotype. This suppression was complete for *glp-1*, *fer-15;fem-1*, and *fog-2* mutants (Figure 6, B–D) and partial for *fem-3gf* (Figure 6E). The effects of DAF-16 appeared to be specific to the Epr phenotype, as elimination of DAF-16 function did not suppress the sterility of any of the mutants tested (data not shown).

The results in this section show that the pathogen resistance of sterile mutants appears to rely on DAF-16 and is not simply a consequence of reduced internal hatching of progeny. These results are similar to the effects of the germ line on life span; removal of the germ line increases life span in a DAF-16-dependent manner. However, unlike the effects on life span, which are only observed in animals lacking germ-line stem cells, the effects on pathogen resistance are also observed in sterile animals that have stem cells, such as *fog-2* mutants. The main difference between these sterile animals and wild-type animals is the presence of developing embryos.

Sterility causes DAF-16 nuclear translocation: The results described in the previous section demonstrated that a variety of sterile mutants exhibit a DAF-16-dependent Epr phenotype. These findings prompted us to determine whether DAF-16 is translocated into the nucleus in these sterile mutants. Previously, ablation of germ cells (LIN *et al.* 2001) or mutation of *glp-1* (BERMAN and KENYON 2006) had been shown to cause DAF-16 nuclear translocation. We used RNAi to examine DAF-16 nuclear translocation in a variety of sterile mutants, including those that contain germ cells. We performed feeding RNAi targeting *fer-1*, *fem-1*, *fem-3*, *fog-2*, *glp-1*, *inx-8*, *inx-9*, or *inx-14* and then examined DAF-16 nuclear translocation in strain TJ356 (carrying a *DAF-16::GFP*). As shown in Figure 7, all RNAi constructs tested caused

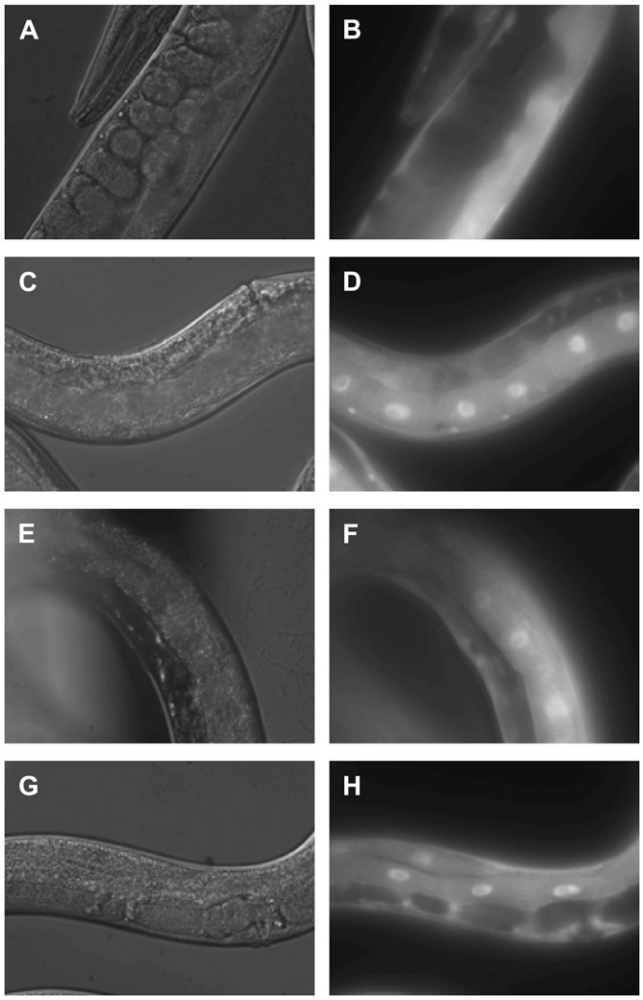

Figure 7.—*DAF-16::GFP* is localized to the nucleus in animals treated with RNAi clones that cause sterility. Shown are representative images of *DAF-16::GFP* protein in intestinal cells of TJ356 animals fed with various RNAi clones including L4440 vector control (A and B), *glp-1* (C and D), *inx-14* (E and F), or *fog-2* (G and H). Differential interference contrast images are in A, C, E, and G and GFP images are in B, D, F, and H. Only fully sterile animals were analyzed for *DAF-16::GFP*.

DAF-16 nuclear translocation in sterile adult animals (Figure 7, supplemental Figure S3 at http://www.genetics.org/supplemental/), and data not shown).

KRI-1 is not required for pathogen resistance of *glp-1* mutants: Animals lacking germ-line stem cells such as *glp-1* mutants exhibit DAF-16-dependent life-span extension when feeding on *E. coli* OP50 (Arantes-Oliveira *et al.* 2002). It is thought that germ-line cells signal through the KRI-1 protein to mediate DAF-16 nuclear translocation in intestinal cells (Berman and Kenyon 2006). The role of KRI-1 is specific to germ-line-mediated enhanced longevity since a mutation in *kri-1* only suppresses the longevity phenotype of *glp-1* but not of *daf-2* mutants (Berman and Kenyon 2006). Our analysis of the pathogen resistance of sterile mutants suggested that KRI-1 is not involved in conferring resistance in *glp-1* animals since all sterile mutants tested were pathogen resistant, not just those lacking germ-line stem cells. Consistent with this reasoning, we found that KRI-1 was not required for the enhanced resistance of *glp-1* (Figure 6F) or *fer-15;fem-1* (data not shown).

A *daf-2* mutation can enhance the pathogen resistance of sterile animals: To determine whether the enhanced pathogen resistance of *glp-1*, *fog-2*, *fem-3*, and *fer-15;fem-1* mutants was additive with *daf-2* pathogen resistance, we examined the pathogen resistance of double mutants that were both sterile and defective in *daf-2*. As shown in Figure 6, B–E, all double mutants exhibited more enhanced pathogen resistance than single mutants alone. These data are consistent with a model in which sterility and *daf-2* independently upregulate pathogen resistance. However, since *daf-2(e1368)* is not a null mutation, it is formally possible that *daf-2* could act in the same pathway as the sterile mutations to confer pathogen resistance. But the fact that the *daf-2(e1368)* mutation can enhance the resistance of fully sterile mutants confirms that *daf-2* pathogen resistance is not just a consequence of its slightly smaller brood size (Figure 4).

DAF-16-dependent genes are upregulated in sterile adult animals: Previous transcriptional profiling studies identified a variety of genes that are expressed in a DAF-16-dependent manner in animals treated with *daf-2* RNAi (Murphy *et al.* 2003) or in a *daf-2* mutant (McElwee *et al.* 2003). We reasoned that if the pathogen resistance phenotype of most of the sterile mutants was a consequence of activated DAF-16, as suggested by the nuclear translocation of DAF-16 in the sterile mutants (Figure 7), then these sterile mutants should exhibit a transcriptional profile similar to that of *daf-2* animals. To test this hypothesis, we examined the expression of four DAF-16-dependent genes by quantitative RT–PCR that had been identified as DAF-2/DAF-16 dependent in three published transcriptional profiling studies (McElwee *et al.* 2003; Murphy *et al.* 2003; Troemel *et al.* 2006). We selected these four DAF-16-dependent genes because of their robust regulation by DAF-16 in other studies (*mtl-1* and *sod-3*) (Hsu *et al.* 2003; Wolff *et al.* 2006), and because they have been implicated in immunity (*lys-7* and *dod-6*) (Mallo *et al.* 2002; Murphy *et al.* 2003; O'Rourke *et al.* 2006). The expression levels of these genes were examined in four different sterile mutant backgrounds: *glp-1*, *fer-15;fem-1*, *fem-3*, and *inx-14(ag17)*, as well as *daf-2* and *daf-16* mutants.

We first compared expression of DAF-16-dependent genes in 1-day-old adults, in which wild-type animals contain many developing embryos. As expected, all the DAF-16-dependent genes tested were more highly expressed in *daf-2* mutants (Figure 8A). These four genes were either expressed at lower levels or at not substantially different levels in *daf-16* animals compared to wild-type animals (presumably because DAF-16 is not very active in well-fed wild-type animals) (Figure 8A).

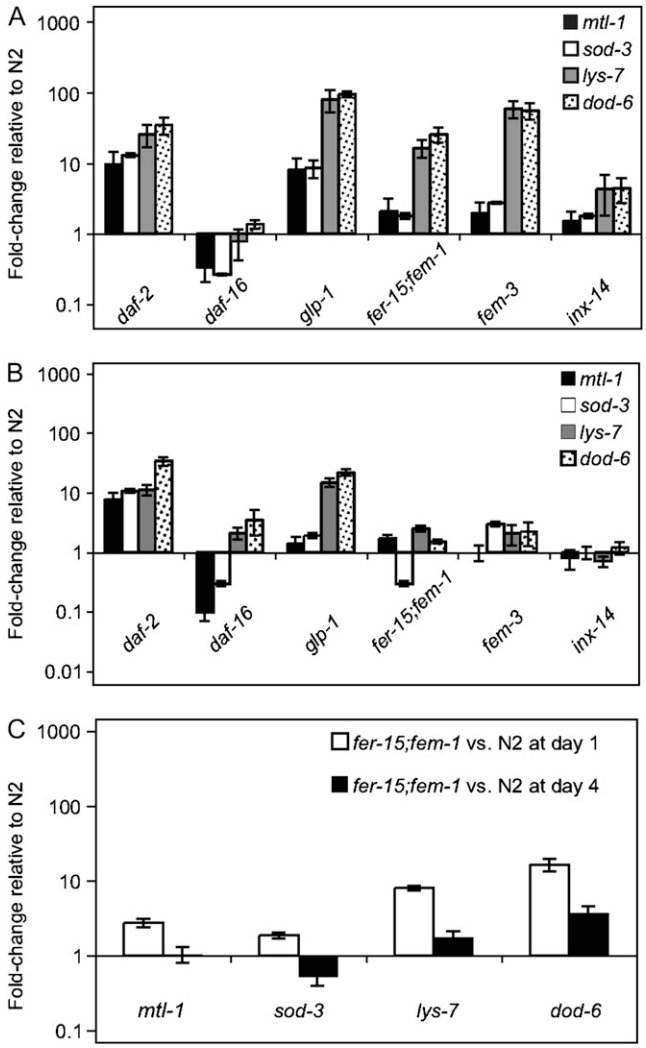

FIGURE 8.—DAF-16-dependent genes are upregulated in adult sterile mutants. The expression of four DAF-16-dependent genes, *mtl-1*, *sod-3*, *lys-7*, and *dod-6*, in gravid adult animals (A) or young adult animals without embryos (B) was determined by qRT–PCR and compared to that of wild-type N2. Mutants analyzed were *daf-2(e1368)*, *daf-16(mgDf47)*, *glp-1 (e2141)*, *fer-15;fem-1*, *fem-3(q20)*, and *inx-14(ag17)*. DAF-16-dependent gene expression in 4-day-old *fer-15;fem-1* adults was compared to 4-day-old wild-type N2 (C). Fold changes represent the average of at least three different biological samples. Error bars represent standard error of the mean.

Similar to *daf-2* mutants, all four DAF-16-dependent genes, *mtl-1*, *sod-3*, *dod-6*, and *lys-7*, were expressed more highly in *glp-1* mutants compared to wild-type animals (Figure 8A). Furthermore, *lys-7* and *dod-6* were also expressed more highly in *fer-15;fem-1* and *fem-3* mutants compared to wild type (Figure 8A). *lys-7* and *dod-6* were also more highly expressed in *inx-14(ag17)* adult animals; however, the levels of expression were slightly lower compared to the other sterile mutants perhaps due to the observation that the *inx-14(ag17)* mutation causes variable sterility (Figure 8A). In contrast, the expression of two other DAF-16-dependent genes, *mtl-1* and *sod-3*, was not substantially different in *fer-15;fem-1*, *fem-3*, or *inx-14(ag17)* mutants compared to wild-type animals (Figure 8A).

To test whether the difference in DAF-16-dependent gene expression observed between sterile and fertile animals was due to the presence of developing embryos, we compared DAF-16-dependent gene expression in animals at an earlier stage. We analyzed animals slightly older than the L4 larval stage, when embryos have not yet developed in wild-type animals. Consistent with the hypothesis that developing embryos suppress the expression of DAF-16-dependent genes, none of the four DAF-16-dependent genes, *mtl-1*, *sod-3*, *lys-7*, or *dod-6* were differentially expressed in the sterile mutants *fer-15;fem-1*, *fem-3*, or *inx-14(ag17)* compared to wild-type animals (Figure 8B). In *glp-1* mutants, *lys-7* and *dod-6* were more highly expressed than wild-type animals (Figure 8B). All four genes were more highly expressed in *daf-2* mutants and were either expressed less or not substantially differently in *daf-16* mutants compared to wild type (Figure 8B). We also examined expression in postreproductive adults in which egg laying had ceased. In these older animals, there was less difference in DAF-16-dependent gene expression between the sterile mutant *fer-15;fem-1* and wild-type animals compared to what was seen in 1-day-old adults (Figure 8C).

To further test the hypothesis that the presence of embryos might reduce the pathogen resistance of sterile animals, we forced *fer-15;fem-1* to produce progeny by mating them with wild-type males prior to infection. *fer-15;fem-1* mutants produce defective sperm at 25° but do produce functional oocytes (McCarroll *et al.* 2004). Thus *fer-15;fem-1* is capable of producing progeny if functional sperm is provided. When *fer-15;fem-1* "females" were mated with wild-type males, viable progeny were produced (data not shown), and the mated *fer-15;fem-1* animals became as susceptible to *P. aeruginosa* as wild-type animals (Figure 5E). This result supports the hypothesis that developing embryos suppress immunity.

As a final test of whether the presence of embryos suppresses immunity, we analyzed the Epr phenotypes of wild-type and sterile animals in the postreproductive period. When 4-day-old adults were infected with *P. aeruginosa*, both wild-type and *fer-15;fem-1* animals died faster than L4 larval-stage animals (Figure 5F). Moreover, the difference in Epr phenotype between *fer-15;fem-1* and wild type was markedly less in the older animals compared to the 1-day-old adult animals (compare Figure 5, D and F).

DISCUSSION

Reproduction and immunity: Here we provide genetic and phenotypic evidence that suggests that embryonic development causes immune suppression in *C. elegans*. The following observations support a model of

embryo-mediated immunosupppression. First, animals without embryos exhibit DAF-16-dependent pathogen resistance that is not simply a consequence of matricidal internal embryo hatching in wild-type animals. Second, the enhanced pathogen resistance phenotype of sterile *fer-15;fem-1* mutants is completely lost when *fer-15;fem-1* is induced to make embryos by mating with wild-type animals. Third, DAF-16 is located in the nucleus in sterile animals, and DAF-16-dependent immune-related genes are expressed at higher levels in sterile animals relative to wild-type animals carrying embryos. Fourth, the enhanced pathogen resistance (and DAF-16-dependent gene activation) seen in sterile *fer-15;fem-1* mutants compared to fertile wild-type animals is greatly reduced when comparing these strains during their postreproductive period. It is important to note that our data do not provide any information concerning the stage(s) of embryogenesis at which the putative immunoregulatory signals are generated. Indeed, it is possible that the signal is not generated by the embryo *per se*, but rather by adult tissues that are affected by the presence of embryos. Analysis of mutants defective in different stages of embryonic development may confirm the existence of a signal that is produced during embryogenesis to downregulate a DAF-16-dependent immune pathway.

The results reported here are most consistent with a model in which embryo-mediated immunosuppression involves the inhibition of DAF-16 activity. However, we cannot rule out the possibility that a DAF-16-independent mechanism targeting the same effector genes as DAF-16 could be involved in mediating pathogen resistance of sterile mutants. For example, removing DAF-16 left substantial resistance in the gain-of-function *fem-3* mutant that was characterized. Alternatively, the pathogen resistance phenotype of sterile mutants could potentially be explained by a radical disruption of metabolic homeostasis as a consequence of sterility in which enhanced metabolic resources are recruited to immunity.

If pathogen resistance in sterile mutants is due solely to a lack of embryo-mediated immunosuppression, why are sterile animals more resistant than wild-type animals when progeny production is blocked by FUDR (Figure 5C)? In the absence of FUDR, eggs are fertilized and reach about the 100-cell stage of development, at which point they are laid (Bany *et al.* 2003). In contrast, FUDR blocks in the mid-proliferation stage of embryonic development (Stroeher *et al.* 1994). Under our FUDR assay conditions, eggs are fertilized, go through multiple rounds of cell division, and are laid; however, the cellular organization is disturbed inside the eggs, and they fail to hatch (data not shown). Thus, it is likely that FUDR does not completely block the generation of the putative signal during embryogenesis in wild-type animals to downregulate DAF-16 activation of defense response pathways and that any stage of embryo development from fertilization to egg laying could generate the signal leading to immune deficiency. Thus, instead of blocking the embryo-derived signal completely, FUDR may affect the level of the signal at least to some degree because wild-type animals survive longer on pathogens with FUDR than without FUDR. This difference in pathogen resistance between the wild-type animals in the presence and absence of FUDR cannot be explained solely as an effect of FUDR on the virulence of the pathogens (Figure 5D). Nevertheless, the results of experiments using FUDR highlight the requirement of normal embryonic development to downregulate immunity in adult animals.

Why does embryonic development decrease immunity in adult animals? To successfully generate offspring, an adult animal must store enough energy for both its own cellular maintenance and to foster embryonic development. The expression of defense-related antimicrobials is presumably energy intensive and competes with embryos for limited nutrients. Although it may be beneficial to turn down defense mechanisms to save energy for successful reproduction in the absence of pathogen attack, our data suggest that wild-type animals prioritize reproduction over immunity even in the presence of a lethal pathogen.

Screen for *epr* mutants highlights sterility and DAF-16-dependent pathogen resistance: When we carried out a forward genetic screen to identify factors important for pathogen resistance, the 6 Epr mutants that were characterized in depth had fertility defects. Importantly, however, our characterization of sterile mutants indicates that lack of internal progeny hatching is not the sole mechanism by which animals become resistant to bacterial pathogens like *P. aeruginosa*. This is in agreement with previous findings that showed that the inability to bag does not fully account for the resistance of *C. elegans* male animals to the fungal pathogen *C. neoformans* (van den Berg *et al.* 2006). In fact, we found that most sterile mutants had pathogen sensitivity similar to wild-type animals when DAF-16 was removed, despite the fact that they were still completely sterile and did not die from internal progeny hatching.

In addition to being partially sterile, all six *epr* mutants we characterized exhibited DAF-16-dependent resistance, suggesting that a major resistance mechanism in *C. elegans* is likely to be governed by DAF-16. The *epr* mutant screen identified a new allele of *age-1*, a component of the DAF-2 insulin-like signaling pathway. AGE-1 is a class IA phosphoinositide-3-OH kinase (PI3K) most similar to the mammalian PI3K p110 form (Morris *et al.* 1996). Mutations in the *C. elegans* AGE-1 RBD have not been previously reported. Our data suggest that the RBD in AGE-1 has a role in both longevity and pathogen resistance. Our screen also identified a new allele of *inx-14*, which is not directly related to DAF-2 signaling. The *inx-14(ag17)* allele that we identified causes a very modest increase in longevity and incomplete sterility, but in agreement with previous results (Hamilton *et al.*

2005), our data show that INX-14 functions upstream of DAF-16.

A new mode of DAF-16 activation: DAF-16 was translocated to the nucleus in the animals induced to become sterile by RNAi knockdown of eight of eight genes examined. This observation is consistent with the observed DAF-16-dependent pathogen resistance of the sterile mutants we examined. Furthermore, our observations that a *daf-2* mutation or *daf-2* RNAi enhanced the resistance of *glp-1*, *fog-2*, *fer-15;fem-1*, and *fem-3* animals (Figure 6, B–E) suggest that the DAF-16-dependent signaling pathway leading to pathogen resistance in these sterile mutants works in parallel with the DAF-2 pathway. Finally, whether the germ-line stem cells were absent (*glp-1*) or not (*fer-15;fem-1*), these sterile mutants exhibited enhanced pathogen resistance that was DAF-16 dependent and KRI-1 independent. Even though our epistasis experiments were not carried out with null alleles of *daf-2* or *kri-1*, these data suggest the existence of a signaling pathway that is distinct from the KRI-1 and DAF-2 pathways that functions to promote resistance in a DAF-16-dependent manner. Hereafter, the proposed third pathway is referred as the "defense response pathway."

If the three pathways, DAF-2, KRI-1, and the defense response pathway, act independently upstream of DAF-16 to exert pathogen resistance, why are *glp-1* animals, which have both KRI-1 and the defense response pathways activated, not more pathogen resistant than the sterile mutants that only have the defense response pathway activated (Figure 5B)? One possibility is that the KRI-1 pathway may be responsible for regulating genes that function in longevity and the defense response pathway is dedicated to regulating genes to enhance pathogen resistance.

DAF-16-dependent pathogen response: Previous transcriptional profiling analyses of *P. aeruginosa*-infected worms indicated that DAF-16 is not required for the induction of certain classes of antimicrobial genes, including those regulated by the p38 MAPK pathway (Troemel *et al.* 2006). In addition, there is almost no overlap between *P. aeruginosa*-elicited gene expression and genes that are activated by DAF-16, even though in both cases the activated genes appear to function as immune effectors (Shapira *et al.* 2006; Troemel *et al.* 2006). For example, *lys-7* or *dod-6* were not differentially regulated in worms feeding on *P. aeruginosa* compared to worms feeding on *E. coli* (Shapira *et al.* 2006; Troemel *et al.* 2006), even though both *lys-7* and *dod-6* have been implicated in a role in immunity (Mallo *et al.* 2002; O'Rourke *et al.* 2006; Shapira *et al.* 2006; Troemel *et al.* 2006).

DAF-16 could still play a very important role in immunity in the wild, even if it is not required for the immune response to infection under the particular conditions we use to carry out killing assays in the laboratory. Indeed, DAF-16-regulated genes likely do play a role in immunity, as illustrated by the fact that upregulation of DAF-16, either through transgenic overexpression (Singh and Aballay 2006) or through a *daf-2* mutation (Garsin *et al.* 2003), causes marked enhanced pathogen resistance. However, as discussed above, we cannot rule out the possibility that a DAF-16-independent mechanism that targets the same effector genes as DAF-16 could be involved in mediating pathogen resistance in sterile mutants.

Immune effectors downstream of DAF-16: Our analysis of several DAF-16-dependent genes revealed that *lys-7* and *dod-6*, but not *sod-3* or *mtl-1*, were highly expressed in 1-day-old adult sterile mutants compared to wild type (Figure 8A). Previously, it was shown that two other pathogens, *M. nematophilum* and *S. marcescens*, also induce *lys-7* (Mallo *et al.* 2002; O'Rourke *et al.* 2006), suggesting that it plays an important role as an immune effector. However, inactivation of neither *lys-7* nor *dod-6* by RNAi had a significant effect on *P. aeruginosa* (data not shown). A likely explanation for these negative results is functional redundancy at the level of downstream effectors.

Our results also suggest that DAF-16 activity must be differentially modified to orchestrate expression of different subsets of DAF-16-activated genes in these sterile mutants and in a *daf-2* mutant. Our analyses of DAF-16-dependent gene expression also highlighted that *glp-1* exhibited higher levels of expression of the DAF-16-dependent genes tested in young adult animals unlike the other sterile mutants (Figure 8). This may be due to the fact that *glp-1* mutants are anatomically different than wild-type animals virtually their entire lives, whereas mutants that are sterile because of a lack of oocytes or sperm are not different from wild-type animals until adulthood. Indeed, *glp-1* mutants have already been shown to upregulate DAF-16 via the KRI-1 signaling pathway, which is thought to be specific to mutants like *glp-1* that are deficient for germ-line stem cells from hatching onward (Hsin and Kenyon 1999; Berman and Kenyon 2006).

DAF-16-independent resistance of *fem-3*: Unlike the other sterile mutants examined in this study, the pathogen-resistant phenotype of *fem-3* was only partially suppressed by *daf-16*. Previously, it has been shown that male *C. elegans* exhibit enhanced resistance to the fungal pathogen *C. neoformans* and that this resistance is only partially dependent on DAF-16 (van den Berg 2006). The *fem-3gf* strain, which was used in this study, is a gain-of-function mutation that results in production of excess sperm but not oocytes (Barton *et al.* 1987). One interesting possibility is that the *fem-3gf* strain behaves similarly to true males and its pathogen resistance relies on both DAF-16-dependent and DAF-16-independent factors.

Pathogen resistance and longevity: Determining whether there are mechanistic differences underlying increased longevity and enhanced resistance in *C.*

elegans is complicated because the readout for both pathogen resistance and longevity is survival. Thus, if a particular mutant has both a long-lived and a pathogen-resistant phenotype, is pathogen resistance simply a reflection of increased longevity, or vice versa? One argument that longevity and pathogen resistance are distinct phenomena is the poor correlation between the degree of pathogen resistance and the degree of increased longevity. For example, *inx-14(ag17)* is one of the most pathogen-resistant mutants that we examined, but it only exhibits a modest, although statistically significant, increase in longevity (Figure 1). [Previously, a genomewide RNAi screen in *C. elegans* revealed that RNAi inactivation of *inx-14* extends longevity in a DAF-16-dependent manner (Hamilton *et al.* 2005)]. Likewise, as previously reported (Garsin *et al.* 2003), although *daf-2* mutants exhibit both increased longevity and pathogen resistance, *daf-2* mutations have different quantitative effects on longevity and resistance. The *daf-2 (e1370)* allele extends life span when feeding on *E. coli* about twofold but confers a fivefold increase in life span when feeding on *S. aureus* (Garsin *et al.* 2003). Similarly, even though all of the previously characterized sterile mutants analyzed in this study exhibited pathogen resistance, some of them are reported to have essentially wild-type longevity (*fog-2* and *fer-15;fem-1*) (Arantes-Oliveira *et al.* 2002; McCarroll *et al.* 2004). There are also examples of long-lived mitochondrial mutants that do not exhibit pathogen resistance (D. Kim, personal communication). Finally, the gene expression data shown in Figure 8 suggest that particular DAF-16-regulated genes can be differentially activated in long-lived *daf-2* mutants and pathogen-resistant sterile mutants. Taken together, these results suggest distinct molecular mechanisms underlying life-span extension and pathogen resistance. On the other hand, it is certainly possible that increased longevity *per se* can contribute to pathogen resistance as measured by worm survival in the presence of a pathogen. Because of this, additional mechanistic understanding, defining independent measures of innate immunity, will be key to distinguishing longevity and immune-related phenomena.

Conclusion: We found that certain sterile mutants have enhanced pathogen resistance due to upregulation of the transcription factor DAF-16. Unlike the signaling pathway between the germ line and DAF-16, which involves germ cells that suppress life span, this proposed pathway seems to involve a signal generated during embryonic development that suppresses immunity in adult animals. This proposed new signaling pathway functions in adults with developing embryos, but not in younger or older adults that lack embryos. Identifying the pathway by which embryonic development suppresses the pathogen resistance of *C. elegans* may shed light on the interconnections between reproduction and immunity and the general mechanism by which animals maintain a systemwide energy balance.

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