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New Role for DCR-1/Dicer in *Caenorhabditis elegans* Innate Immunity against the Highly Virulent Bacterium *Bacillus thuringiensis* DB27

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Bacillus thuringiensis **produces toxins that target invertebrates, including** ***Caenorhabditis elegans*****. Virulence of** ***Bacillus*** **strains is often highly specific, such that** ***B. thuringiensis*** **strain DB27 is highly pathogenic to** ***C. elegans*** **but shows no virulence for another model nematode,** ***Pristionchus pacificus*****. To uncover the underlying mechanisms of the differential responses of the two nematodes to** ***B. thuringiensis*** **DB27 and to reveal the** ***C. elegans*** **defense mechanisms against this pathogen, we conducted a genetic screen for** ***C. elegans*** **mutants resistant to** ***B. thuringiensis*** **DB27. Here, we describe a** ***B. thuringiensis*** **DB27-resistant** ***C. elegans*** **mutant that is identical to** ***nasp-1*****, which encodes the** ***C. elegans*** **homolog of the nuclear-autoantigenic-sperm protein. Gene expression analysis indicated a substantial overlap between the genes downregulated in the** ***nasp-1*** **mutant and targets of** ***C. elegans dcr-1*****/Dicer, suggesting that** ***dcr-1*** **is repressed in** ***nasp-1*** **mutants, which was confirmed by quantitative PCR. Consistent with this, the** ***nasp-1*** **mutant exhibits RNA interference (RNAi) deficiency and reduced longevity similar to those of a** ***dcr-1*** **mutant. Building on these surprising findings, we further explored a potential role for** ***dcr-1*** **in** ***C. elegans*** **innate immunity. We show that** ***dcr-1*** **mutant alleles deficient in microRNA (miRNA) processing, but not those deficient only in RNAi, are resistant to** ***B. thuringiensis*** **DB27. Furthermore,** ***dcr-1*** **overexpression rescues the** ***nasp-1*** **mutant's resistance, suggesting that repression of** ***dcr-1*** **determines the** ***nasp-1*** **mutant's resistance. Additionally, we identified the collagen-encoding gene** ***col-92*** **as one of the downstream effectors of** ***nasp-1*** **that play an important role in resistance to DB27. Taken together, these results uncover a previously unknown role for DCR-1/Dicer in** ***C. elegans*** **antibacterial immunity that is largely associated with miRNA processing.**

Genetically tractable model organisms, including the nematode *Caenorhabditis elegans*, have provided detailed insights into the origin and fundamental principles of immunity. More than a decade of research on *C. elegans* has yielded a wealth of knowledge about its innate immune response to various pathogens, uncovering multiple signaling pathways critical to *C. elegans* survival after exposure to pathogens (for reviews, see references 1 to 7). Once activated, these pathways induce the expression of an array of antimicrobial effectors that differ, depending on the pathogen present (8–11). A variety of microbes, including bacteria (12), fungi (13), viruses (14), and microsporidia (15), have been used in *C. elegans* infection studies, with the main focus on etiological agents of human diseases. In some cases, work on *C. elegans* has led to the identification of conserved virulence factors of human pathogens (16, 17).

Among the natural pathogens of *C. elegans*, *Bacillus thuringiensis* has been extensively studied and *B. thuringiensis* likely coexists and coevolves with its host in the natural environment (18). *B. thuringiensis* produces a variety of pore-forming toxins (PFTs), called Cry toxins, that target the intestinal cells of insects (19) and nematodes (20). The ability of some Cry toxins to target *C. elegans* has been exploited to use this nematode as an *in vivo* system for studying PFTs (21), and several *C. elegans* defense mechanisms against PFTs have been discovered (22–27).

Although the *C. elegans* model has provided invaluable insight into the immune defense against pathogens, a comparative approach with other nematodes would enhance our understanding of the host response to pathogens. One nematode that has been used extensively for comparative studies with *C. elegans* is *Pristionchus pacificus*. In contrast to *C. elegans*, which is often found on compost heaps and rotten fruit (28), *P. pacificus* is found in tight association with scarab beetles (29). Not surprisingly, therefore, these two nematodes show different resistance patterns (30–32) and transcriptional responses to pathogens (11). For example, *C. elegans* dies when fed the human opportunistic bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus* and *B. thuringiensis* Cry5B toxin, whereas *P. pacificus* is resistant (20, 30). Moreover, we have previously shown (31) that out of 768 naturally isolated *Bacillus* strains, only 20 were pathogenic and show distinct patterns of virulence for *C. elegans* and *P. pacificus*. The most extreme difference was seen with regard to *B. thuringiensis* strain DB27. DB27 kills *C. elegans* in less than 24 h, representing one of the fastest killers of *C. elegans* known to date, whereas *P. pacificus* is resistant to this bacterial strain (31). Our previous study (31) also indicated that the determinants of *B. thuringiensis* DB27 virulence for *C. elegans* and also the mechanisms of *C. elegans* resistance to this pathogen are unknown. Specifically, we have shown that the *bre* and *daf-2* mutants, which are known to be resistant to *B. thuringiensis* PFT (22) and other bacterial pathogens (33), respectively, are as susceptible to *B. thuringiensis* DB27 as wild-type worms are (31). These findings suggest that potentially novel mechanisms are required to provide defense against this highly virulent *Bacillus* strain.

Here, we report a genetic screen for *B. thuringiensis* DB27-

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resistant mutants of *C. elegans* and show that a gene represented by two alleles is identical to the *nasp-1* gene C09H10.6. Further characterization revealed that the *nasp-1* mutant shows a reduced life span and reduced fecundity and is RNA interference (RNAi) deficient. Microarray analysis uncovered an unexpected enrichment of *dcr-1*-regulated genes in *nasp-1* mutants. We explored a potential role for *dcr-1* in *C. elegans* innate immunity and show that *dcr-1* allele mutants deficient in microRNA (miRNA) processing, but not those that are only deficient in RNAi, are resistant to *B. thuringiensis* DB27. Transgenic expression of *dcr-1* in *nasp-1* mutants can rescue the resistance phenotype of *nasp-1* mutants. We identified the collagen-encoding gene *col-92* as one of the downstream effectors of *nasp-1* and *dcr-1*. These findings describe NASP-1 as a novel *C. elegans* innate immunity regulator and identify a previously unknown role for DCR-1 in the *C. elegans* antibacterial immune response.

MATERIALS AND METHODS

Nematodes and bacterial strains. *C. elegans* strains were maintained on nematode growth medium (NGM) agar plates with *Escherichia coli* OP50 as a food source. The following strains were kindly provided by the *Caenorhabditis* Genetics Center (University of Minnesota): wild-type Bristol (N2), Hawaiian mapping strain (CB4856), DA453 *eat-2*(*ad453*), WM49 *rde-4*(*ne301*), WM27 *rde-1*(*ne219*), CB189 *unc-32*(*e189*), BB1 *dcr-1*(*ok247*)/*unc-32*(*e189*) *III*, YY11 *dcr-1*(*mg375*), VC1138 *drsh-1*(*ok369*), VC446 *alg-1*(*gk214*), BA1 *fer-1*(*hc1*), and BA15 *fer-15*(*hc15*). *btr-1*/*C09H10.9*(*tu439*) and *btr-1*(*tu440*) mutant worms were generated in this study and backcrossed to wild-type worms four times before analysis. Double mutants were generated by using standard procedures (34). *Serratia marcescens* C2 was isolated from Reunion Island, *S. aureus* Newman and *P. aeruginosa* PA14 were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, and *B. thuringiensis* DB27 was isolated from dung beetles (31).

Genetic screen for *B. thuringiensis* DB27-resistant nematodes. After ethyl methanesulfonate (EMS) mutagenesis (50 mM for 4 h at 20°C), single parental (P_0) mothers were separated onto NGM OP50 plates and allowed to lay 10 to 30 eggs. F_1 mothers were allowed to lay F_2 progeny that, upon reaching the L4 stage, were placed onto plates with pathogenic *B. thuringiensis* DB27. After 24 h of incubation at 25°C, single worms that survived pathogen exposure were transferred to NGM OP50 plates and allowed to reproduce. These candidates were retested several times in survival assay to confirm the resistance phenotype.

***C. elegans* killing assays.** Each bacterium was grown overnight in a shaking incubator at 30°C in Luria-Bertani (LB) broth, except *S. aureus* and *P. aeruginosa*, which were grown at 37°C. A 100-μl volume of the culture was spread to the edges of a 6-cm NGM plate to prevent worms from escaping and incubated overnight. Three to six independent replicates of 20 adult worms per plate were exposed to pathogens and monitored for survival. Survival assays were repeated multiple times and conducted at 25°C. Nematodes were transferred once a day to fresh plates and considered dead when they failed to respond to touch. We note that the exact nature of the killing assay (inoculum size etc.) might have an influence on the quantitative killing results reported in this study.

Complementation test, mapping, sequencing, and rescue of *tu439*. Complementation tests were performed by testing F_1 progeny from the crosses between *btr-2*(*tu438*) mutant males and *dpy-5*;*tu439* or *dpy-5*;*tu440* mutant hermaphrodites. To test if *tu439* and *tu440* are allelic, F_1 progeny from a cross between *tu439* mutant males and *dpy-5*;*tu440* mutant hermaphrodites were tested. For mapping, the *btr-1* mutant was crossed to Hawaiian strain CB4856 and approximately 400 F_2 cross progeny were isolated and screened for *B. thuringiensis* DB27 resistance. DNA was isolated from a total of 96 *tu439*-positive recombinant progeny and used for restriction fragment length polymorphism (RFLP)–single-nucleotide polymorphism (SNP) mapping (35). SNP data from the most informative recombinants identified a 160-kb region on chromosome II between the markers F38A3 and F37H8. Whole-genome sequencing of *btr-1* mutants revealed that both *btr-1* alleles contain two identical mutations in the mapping interval, which were found in the gene C09H10.6. For transformation rescue, a 2.3-kb PCR fragment, covering upstream and downstream regions of the C09H10.6 transcript, was amplified from wild-type genomic DNA and injected together with a *sur-5*::*gfp* plasmid as a coinjection marker into a *btr-1* mutant as described elsewhere (36).

For *dcr-1*, *dpy-5*, and *col-92* overexpression, respective transcripts with presumptive regulatory regions were PCR amplified from wild-type genomic DNA, purified, and used for injection together with the *sur-5*::*gfp* plasmid as a coinjection marker. For intestine-specific expression, a 3.5-kb *cpr-1* promoter fragment was amplified from wild-type DNA and fused with the *dcr-1* transcript by using Phusion High-Fidelity DNA polymerase (NEB) according to the manufacturer's instructions. The resulting construct was used for *btr-1* microinjection. Reporter gene constructs for gene expression analysis were created by the PCR fusion method as described previously (37). A *nasp-1*::GFP (green fluorescent protein) translational reporter was obtained by PCR amplification of the *nasp-1* transcript with the presumptive promoter region from wild-type genomic DNA, which in the next step was PCR fused to GFP, which was amplified from the pPD95.75 plasmid. A *col-92* transcriptional reporter was generated in the same way, with the exception that only a 1.4-kb *col-92* promoter region was fused to GFP. The resulting constructs were purified and used for injection together with the pRF4 plasmid (*rol-6*). Several independent lines were obtained and tested for all of the transgenic experiments described here.

Mutant identification by whole-genome sequencing. We sequenced a genomic library of the *btr-1*(*tu439*) mutant on the Illumina Genome Analyzer II platform and obtained 7,398,278 read pairs (2 × 100 bp). Raw reads were aligned with the *C. elegans* genome assembly (WS235) by using bwa (version 0.5.9-r16) (38). After the removal of duplicated reads to exclude false variant calls due to PCR amplification biases, 98.8% of the *C. elegans* assembly was covered by at least one read, with a genome-wide mean coverage of 15×. Variants were called by using SAMtools (version 0.1.18-r982:295) (39). In total, we identified 2,092 homozygous base substitutions with a SAMtools quality score of at least 20, of which six variants were located within the 160-kb mapping interval on chromosome II. Filtering for variants with a potential deleterious effect (nonsynonymous substitution, premature stop codons, and mutations near splice sites) could exclude three variants as intergenic and intronic, respectively. Two of the remaining three nonsynonymous substitutions were found to affect the *nasp-1* gene (amino acid changes, L53S and D307N), and one substitution at genomic position II:11069603, G to A (WS235), was found to cause an S-to-F amino acid exchange in various alternative transcripts of *unc-53*. A rescue experiment confirmed that mutations responsible for the phenotype occurred in the *nasp-1* gene.

Pumping rate measurement. Pharyngeal pumping was counted for 1 min starting on the first day of adulthood. Worms were placed on *B. thuringiensis* DB27 plates at 25°C and left undisturbed for 3 h before measurement. All worms remained on food during the period of observation. The *eat-2*(*ad453*) mutant, known to have low pumping, was used as a control.

Brood size measurement. Brood size was determined by picking L4 worms (one per plate) and transferring them to fresh plates every 24 h until egg laying ceased. The offspring were counted 2 days after the mothers were removed from the plates. Ten worms were used per strain per experiment, and experiments were repeated twice.

Life span assay. Life span assays were performed on NGM plates containing 0.1-mg/ml 5′-fluorodeoxyuridine (FUDR) to prevent progeny from hatching at 20°C. Plates were seeded with concentrated OP50 and allowed to dry overnight. A synchronized population of L4 worms grown on OP50 was placed onto the plates and scored every 48 h. For *col-92* RNAi life span assays, NGM plates containing 50 μg/ml carbenicillin, 1 mM isopropyl-β-D-thiogalactoside (IPTG), and 0.1 mg/ml FUDR were seeded

with *E. coli* HT115 expressing *col-92* double-stranded RNA (dsRNA) (experiment) or *E. coli* HT115 harboring the empty vector (control). A synchronized population of L4 worms grown on *col-92* RNAi bacteria or vector-containing bacteria were placed onto the RNAi plates with FUDR and scored every 48 h. Upon depletion of food, worms were transferred to fresh plates. Worms were considered dead when no response to touch was observed. Approximately 100 worms were used per strain per experiment, and experiments were repeated twice.

RNAi. To generate specific gene knockdowns, we used RNAi by feeding nematodes with *E. coli* expressing dsRNA that is homologous to a target gene. *E. coli* harboring the empty vector was used as a control. *E. coli* strain HT115 harboring the appropriate vector was grown in LB broth containing 100 μg/ml ampicillin at 37°C overnight. Bacteria were plated onto NGM plates containing 50 μg/ml carbenicillin and 1 mM IPTG and allowed to grow overnight at 37°C. Three adult hermaphrodites were placed on each RNAi plate and allowed to lay eggs. These eggs were grown on RNAi plates for 3 days at 22°C to the adult stage before being transferred to plates with *B. thuringiensis* DB27 for pathogenicity assay. All of the RNAi clones used in this study are part of the RNAi library obtained from Source BioScience UK Limited (Nottingham, United Kingdom). *cdc-25* RNAi plates were prepared as described above. However, worms were grown at 25°C to the adult stage. *cdc-25.1* encodes a CDC25 phosphatase homolog that affects embryonic viability and is necessary for cell proliferation in the germ line. In brief, gravid worms were laid on *cdc-25.1* RNAi plates for 4 h and then transferred to similar plates for an additional 4 h of egg laying. After that, gravid hermaphrodites were removed and eggs were left to hatch and grow in the presence of *cdc-25.1* RNAi to produce sterile worms. *cdc-25* RNAi by injection was performed as described below. For *mom-2* RNAi, three adult hermaphrodites were placed on *mom-2* RNAi plates and allowed to lay eggs. These eggs were grown on RNAi plates at 20°C to the adult stage. Single adults were subsequently placed onto freshly prepared *mom-2* RNAi plates and allowed to lay eggs for 24 h and then removed from the plates. After 16 h, the total amounts of eggs and hatched larvae were scored and viability was expressed as follows: (number of hatched larvae/total number of eggs) × 100. The *mom-2* coinjection experiments were conducted at 20°C as described previously (40).

dsRNA for injection experiments was prepared by *in vitro* transcription. DNA templates were PCR amplified from plasmids corresponding to the RNAi clones with T7 primers or PCR amplified from *C. elegans* genomic DNA with gene-specific primers with attached T7 promoters in case the gene is not present in the available RNAi library. PCR products were used for dsRNA synthesis in a single reaction with the TranscriptAid T7 high-yield transcription kit (Thermo Scientific). Concentrations of dsRNAs were determined with a spectrophotometer, and the quality and size of dsRNAs were assessed by gel electrophoresis. Young hermaphrodites were injected in the gonads with 300 to 600 ng/μl of dsRNA of the candidate gene. Given that *dpy-5* RNAi produces a clear morphological phenotype but has no effect on survival, *dpy-5* dsRNA was used as a positive control for injection and as a negative control in survival assays. Adult F_1 worms were used in the pathogen survival assay.

RNA collection for microarray experiments. Synchronized populations of wild-type and *tu439* mutant *C. elegans* worms were obtained by hypochlorite treatment and allowed to grow to the young adult stage on *E. coli* OP50 at 20°C. Worms were exposed to *B. thuringiensis* DB27 for 4 h and then picked into TRIzol reagent for RNA extraction. Four biological replicates were collected for each experimental condition and the control condition. For each biological replicate, about 200 young adult hermaphrodites were picked into 1 ml of TRIzol (Invitrogen) and total RNA was extracted according to the manufacturer's instructions and purified further by phenol-chloroform-isoamyl alcohol precipitation. The RNA pellet was suspended in RNase-free water and assessed on a NanoDrop spectrophotometer for quantity and RNA quality.

Microarray experiments and data analysis. Oligonucleotide microarrays for *C. elegans* containing ~43,000 unique probes for ~20,000 *C. elegans* genes were obtained from Agilent Technologies (NCBI GEO accession number GPL10094). All experiments are in a two-color format, where Cy5 and Cy3 dye-labeled cRNAs from experimental and control samples are cohybridized on the same microarray. The four biological replicates per experiment included two dye swap experiments to account for differences in dye labeling. Experimental procedures and data analysis were essentially the same as described before (41). Microarrays corresponding to three of the four biological replicates passed the quality criteria and were used in the final analysis. Genes with a false-discovery rate (FDR)-corrected *P* value of ≤0.05 and an absolute fold change greater than or equal to a cutoff of 1.5 on a $\log_2$ scale were called differentially expressed.

Expression cluster enrichment analysis. The list of microarray expression profiles in which a given *C. elegans* gene is known to be differentially expressed can be extracted from the Expression Cluster section of the WormBase (42) gene summary page for each gene. We retrieved all of the available expression clusters for *C. elegans* genes from the WormBase website (www.wormbase.org) and used them to identify expression clusters enriched in our expression profile, an approach that we have successfully used previously to analyze genome-wide trends in expression profiles (11, 41). *P* values for expression cluster enrichment in each expression profile were computed with a two-by-two Fisher exact test. An FDR-corrected *P* value cutoff of 0.05 was used as the significance threshold.

qRT-PCR. Synchronized *C. elegans* worms at the L4 stage were exposed to *B. thuringiensis* DB27 for 4 h before harvesting. Nematodes were collected by washing plates with M9 buffer, and RNA was extracted with TRIzol reagent. cDNA was synthesized with the SuperScript III kit (Invitrogen). Quantitative real-time PCR (qRT-PCR) was conducted with the LightCycler 480 SYBR green I master kit (Roche) on a LightCycler 480 real-time PCR instrument (Roche) in a 96-well format. Relative fold changes in transcripts were calculated by the comparative cycle threshold (C_T) method after normalization to *snb-1*, which has been used previously in qRT-PCR studies of *C. elegans* innate immunity (43–45). C_Ts were determined by the Roche LightCycler software. All samples were run in triplicate. The sequences of the primers used are available upon request.

Statistical analysis. Kaplan-Meier nonparametric comparisons and a log rank test were used for statistical analysis of *C. elegans* survival in the presence of pathogens and for life span analysis. In each case, curves represent the combined data from at least three independent experiments. Statistical analysis of the difference between two values was performed with Student's *t* test. Statistical significance was set at $P \leq 0.05$.

Microarray data accession number. Raw and processed data from all of the experiments described here are available under accession number GSE43905 in the NCBI Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/).

RESULTS

Forward genetic screen for *B. thuringiensis* DB27-resistant (*btr*) mutants. *B. thuringiensis* DB27 was isolated from a dung beetle (31) and was selected for detailed investigation because of its high virulence for *C. elegans*. This strain is 100% lethal in just 16 h under standard assay conditions (Fig. 1A). Our previous studies indicated that all of the *bre* and *daf-2* mutants tested were as susceptible to *B. thuringiensis* DB27 as wild-type worms are (31). In contrast to the observed susceptibility of *C. elegans*, the second model nematode organism, *P. pacificus*, is fully resistant to *B. thuringiensis* DB27 (31). To uncover a potential resistance mechanism and to better understand the specificity of *B. thuringiensis* virulence for certain nematode species, we conducted a forward genetic screen for mutants of *C. elegans* with strong resistance to *B. thuringiensis* DB27. Specifically, we screened for mutants that would extend *C. elegans* survival on monoxenic cultures of *B. thuringiensis* DB27 by at least a factor of 3, in order to select for major effectors. From 1,800,000 EMS-mutagenized gametes, we isolated

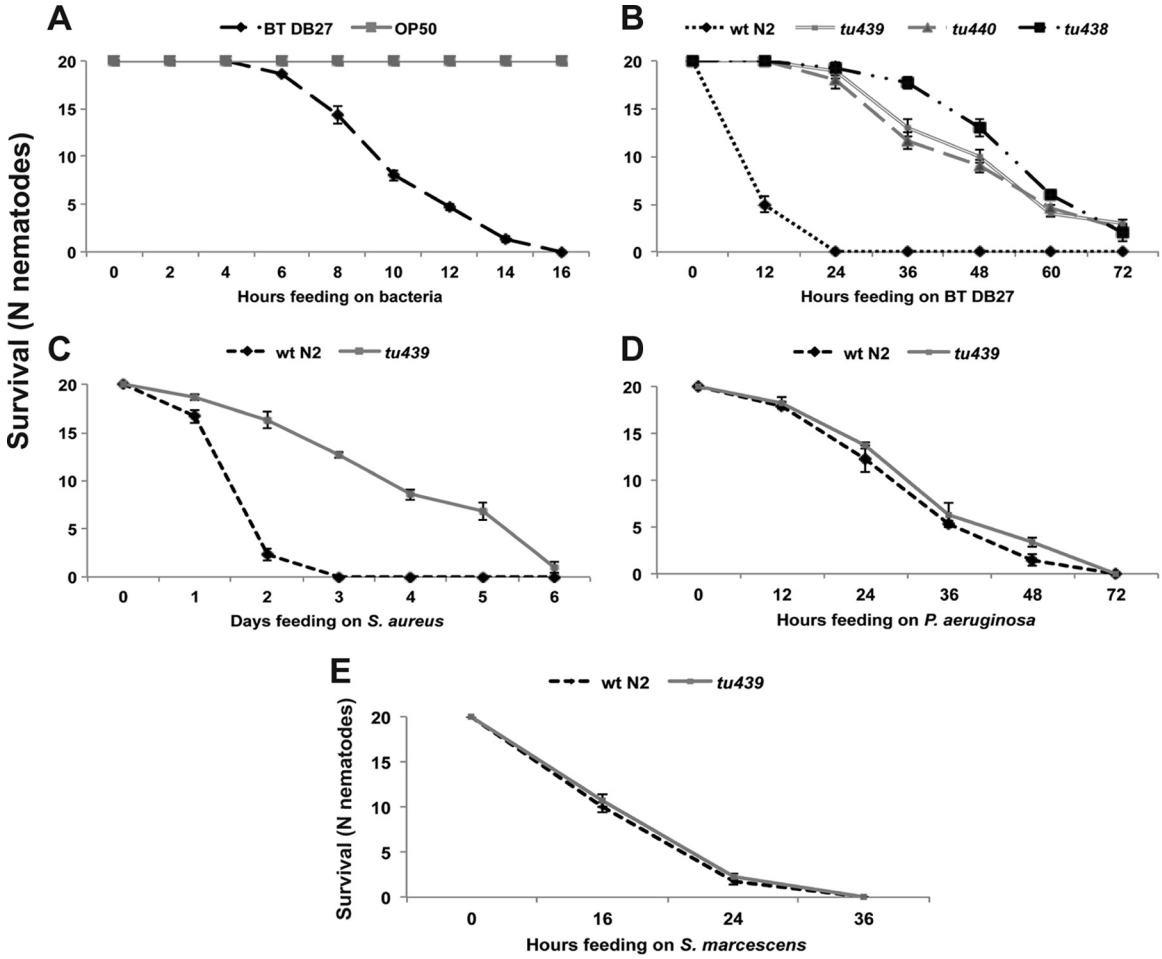

FIG 1 Differences in the survival of wild-type and *btr* mutant worms upon exposure to different pathogenic bacteria. (A) *C. elegans* wild-type (wt) N2 is highly susceptible to *B. thuringiensis* (BT) DB27, in contrast to standard *E. coli* OP50, which was used as a control ($P < 0.0001$). (B) Survival of *btr* mutants on monoxenic cultures of *B. thuringiensis* DB27. All alleles showed significantly ($P < 0.0001$) enhanced survival compared to that of the wild type. (C) *tu439* mutants show increased resistance to *S. aureus* compared to that of the wild type ($P < 0.0001$). (D, E) *tu439* mutants are as susceptible as wild-type worms to *P. aeruginosa* (D, $P > 0.05$) and to *S. marcescens* (E, $P > 0.05$). For survival curves, the numbers of live versus dead worms were scored over time. The number of worms alive (N nematodes) is plotted as a function of time. The data shown are means ± the standard errors of the means. For each condition in the survival assay, 60 to 80 worms (20 per plate) were used for each of multiple independent biological replicates.

three *btr* (*B. thuringiensis*-resistant) mutants that exhibited strong resistance to *B. thuringiensis*-mediated killing (Fig. 1B). Complementation tests revealed that these mutants fall into two complementation groups (data not shown) with two alleles (*tu439* and *tu440*) of a locus provisionally named *btr-1* and a single allele (*tu438*) of a second locus called *btr-2*. Here we further characterize *btr-1*.

***btr-1*(*tu439*) mutants are resistant to additional pathogens and have a reduced life span.** Considering that several pathogen-resistant *C. elegans* mutants are resistant to multiple pathogens (33), we exposed *btr-1*(*tu439*) mutant worms to additional pathogens to further characterize the specificity of the mutant. We found that *tu439* mutants exhibited stronger resistance to the Gram-positive pathogen *S. aureus* than wild-type worms did (Fig. 1C). In contrast, *tu439* mutants showed wild-type survival on the Gram-negative pathogens *P. aeruginosa* and *S. marcescens* (Fig. 1D and E). These results indicate that *tu439* confers resistance to two, but not all, of the pathogens tested.

To explore additional functions, we tested several physiological parameters of *tu439* mutant worms. First, we estimated the pumping rate as an indirect measurement of bacterial uptake to elucidate bacterial consumption. The pumping rate of the *tu439* mutant on DB27 is similar to that of the wild type, whereas the control *eat-2*(*ad453*) mutant shows the expected reduction of pumping in our assay system (Fig. 2A). Thus, pharyngeal pumping is unaffected, suggesting that pathogen consumption is not reduced in mutant worms. To rule out the possibility that bacteria might persist in the intestines of wild-type but not mutant worms, we fed worms with DB27 for 4 h, which is sufficient to observe accumulation of bacteria in the gut (see Fig. S1A in the supplemental material). We then shifted the worms to OP50 for 2 h and measured the DB27 CFU. Neither the wild-type nor the mutant worms showed a difference in their DB27 CFU counts after the shift to OP50, suggesting that the persistence of DB27 is not extended in wild-type worms (see Fig. S1B). Second, we observed that *tu439* mutants exhibit reduced fertility (see Fig. S1C). A similar phenotype was also annotated for other pathogen-resistant mu-

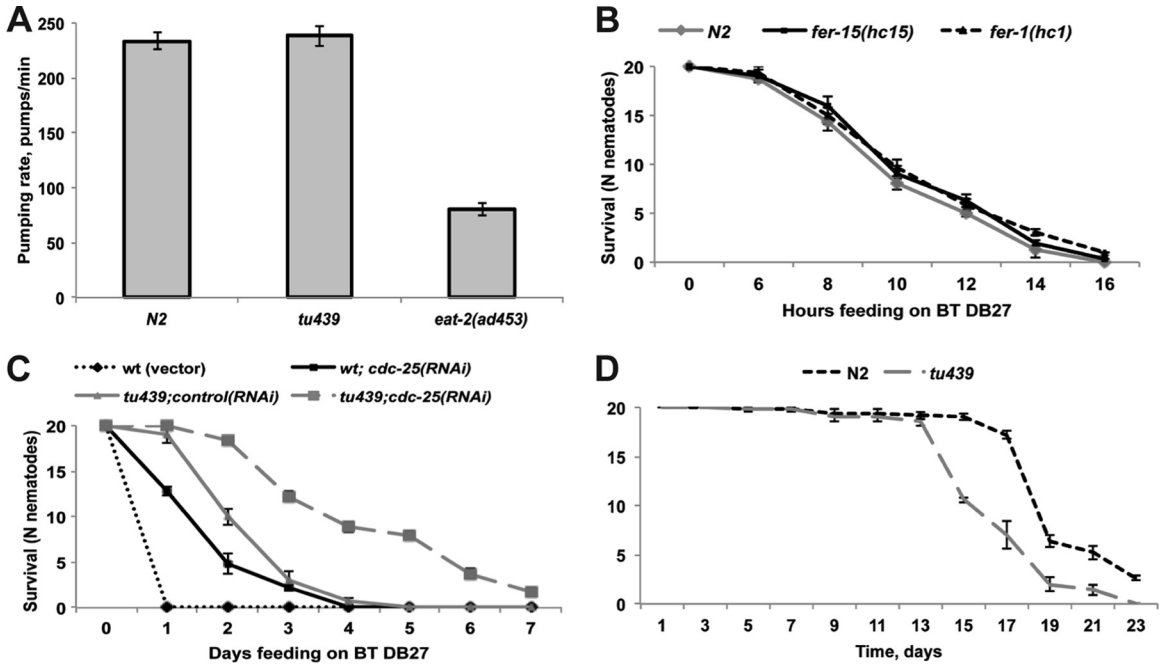

FIG 2 *tu439* mutants exhibit increased resistance to DB27 independently of reduced fertility and longevity. (A) The pumping rate of *tu439* mutants shows no difference from that of the wild type ($P > 0.05$). The control *eat-2* mutant strain shows the expected lower pumping rate than that of the wild type ($P < 0.0001$). At least 10 worms were scored per strain. The data shown are means ± the standard errors of the means. (B) *fer-1* and *fer-15* mutants show no difference ($P > 0.05$) from wild-type worms on a lawn of *B. thuringiensis* (BT) DB27. The data shown are means ± the standard errors of the means. For each condition in survival assays, 60 to 80 worms (20 per plate) were used for each of multiple independent biological replicates. (C) Wild-type and *tu439* nematodes were injected with *cdc-25* interfering dsRNA to obtain sterile worms or with control interfering dsRNA (see Materials and Methods) and exposed to DB27. *cdc-25* RNAi significantly increases the resistance of wild-type [$P < 0.0001$ for wt (vector) versus wt;*cdc-25* worms] and *tu439* mutant ($P < 0.0001$ for *tu439*;control versus *tu439*;*cdc-25* worms) worms. The survival of *tu439*;*cdc-25* mutant worms is significantly higher ($P < 0.0001$) than that of *tu439* mutant or wt;*cdc-25* worms alone. (D) The life span of *tu439* mutants is significantly ($P < 0.001$) lower than that of wild-type worms on standard food (*E. coli* OP50). At least 100 worms were used per strain per experiment, and each experiment was done twice. The data shown are means ± the standard errors of the means.

tants (46–48) and suggests that there is a tradeoff between reproduction and an elevated immune response. This raises the possibility that *tu439* confers increased resistance to *B. thuringiensis* DB27 because of diminished fecundity.

To elucidate this further, we assessed the survival of sterile mutants exposed to *B. thuringiensis* DB27. First, we investigated fecundity in combination with matricidal internal hatching of eggs by comparing the survival of wild-type worms to that of *fer-1* and *fer-15* mutants (47). *fer* mutants have a germ line but do not suffer from matricidal effects due to the lack of fertilization since their sperm production is affected (49). Both *fer* mutants are as susceptible to *B. thuringiensis* DB27 as wild-type worms are, suggesting that matricidal hatching of eggs does not contribute to *B. thuringiensis* DB27 killing and that increased resistance of *tu439* to DB27 is not due to a lack of matricide (Fig. 2B). This finding is further supported by the fact that males are susceptible to DB27 (not shown).

In a second set of experiments, we used *cdc-25* RNAi, which also causes sterility. *cdc-25* RNAi led to enhanced survival of wild-type worms exposed to *B. thuringiensis* DB27 (Fig. 2C). However, *cdc-25* RNAi in a *tu439* background resulted in a significant increase in resistance to DB27 that was stronger than the resistance of single *tu439* mutant or wild-type *cdc-25* RNAi worms (Fig. 2C). The cumulative effect of the two mutations suggests that the underlying molecular mechanisms are independent and that *tu439*-mediated resistance to *B. thuringiensis* DB27 is not due simply to reduced fecundity. Additionally, in contrast to sterile mutants, the *tu439* mutant is not resistant to *S. marcescens* and *P. aeruginosa* (Fig. 1D and E). Finally, we measured the life span of *tu439* mutants, given that life span extension often correlates with increased pathogen resistance in *C. elegans* (33, 47). Interestingly, we found that *tu439* mutants fed on *E. coli* OP50 show a slight but significant life span reduction in comparison to wild-type worms (Fig. 2D). These results show a novel relationship between life span and immunity and suggest that *tu439* mutant worm resistance to bacterial infection is not a simple consequence of extended longevity. Taken together, *tu439* mutant worms are resistant to several Gram-positive bacteria, have a reduced life span and fecundity, and show that immunity can be mechanistically uncoupled from aging.

***btr-1* is identical to the *C. elegans nasp-1* gene.** To clone *tu439*, we used RFLP-SNP mapping (35) and next-generation sequencing (50). In brief, we mapped *btr-1* to an approximately 160-kb region on chromosome II between the markers F38A3 and F37H8. Whole-genome sequencing of both alleles revealed mutations in the C09H10.6 gene, which has been described as the *C. elegans* homolog of the gene for human nuclear-autoantigenic-sperm protein (*nasp-1*) (Fig. 3A). *nasp-1* has previously been implicated in *C. elegans* female development (51).

Interestingly, the two alleles appear to contain two identical mutations, which might be due to the fact that they were derived from a single mother. The first mutation is a T-to-C substitution

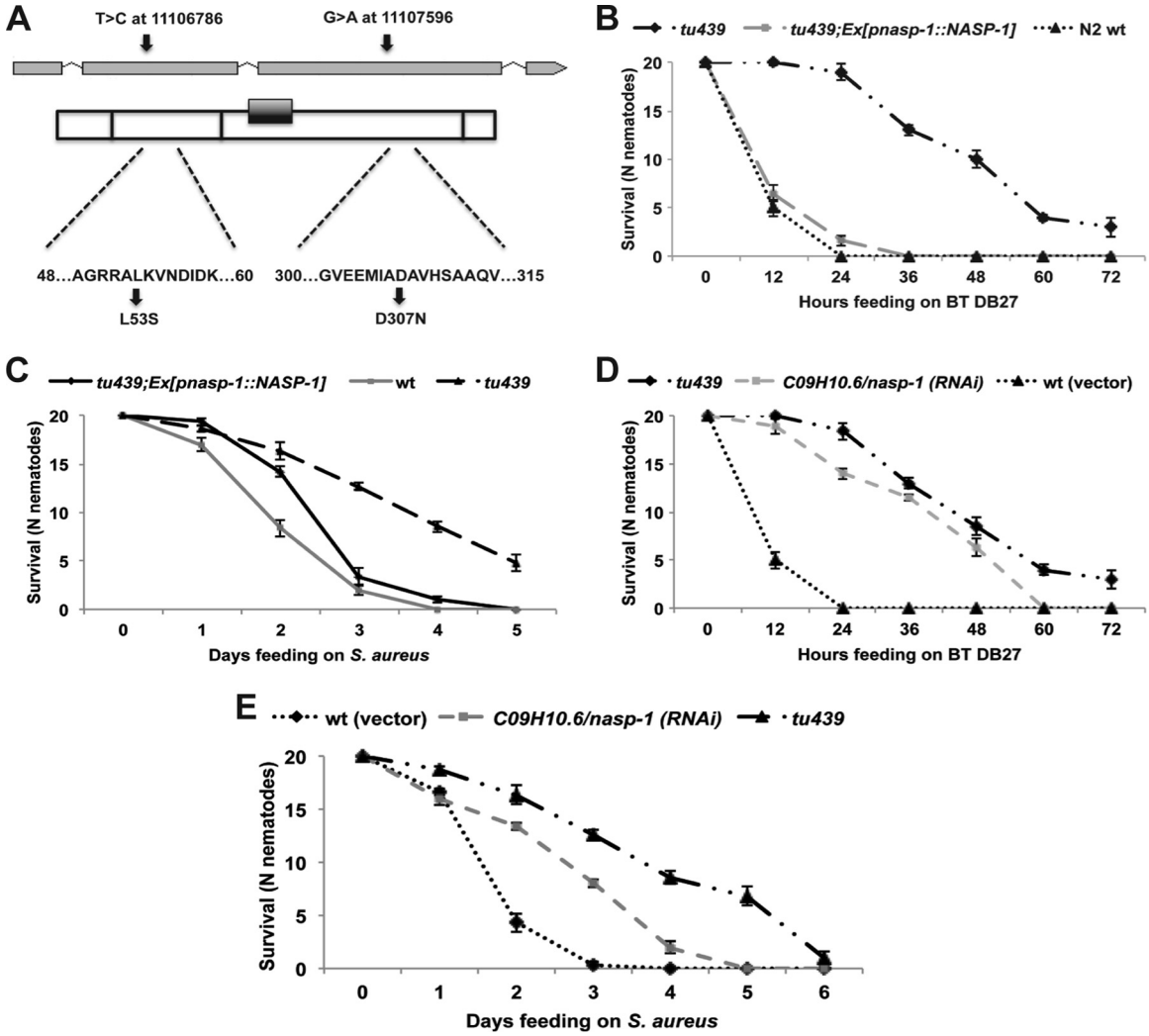

FIG 3 Identification of the *tu439* allele. (A, top) Exon-intron structure of the C09H10.9 gene, adapted from WormBase (WS235), with the positions of mutations indicated by arrows. Numbers indicate positions on chromosome II where single-nucleotide mutations occurred. (A, bottom) Schematic domain structure of the C09H10.9 protein. The black box shows the location of the TPR domain, which is not affected in either of the *btr-1* mutants. The dashed lines highlight the amino acid sequences flanking the mutation sites. The black arrows show the position and type of amino acid substitution. (B) Rescue experiment. Transgenic expression of *nasp-1* under the control of its endogenous promoter restores wild-type (wt) susceptibility to *B. thuringiensis* (BT) DB27 to the *tu439* mutant. (C) *tu439* mutant resistance to *S. aureus* is rescued by transgenic expression of *nasp-1* under the control of its endogenous promoter ($P > 0.05$ for the wild type versus the transgenic line). The data shown are means ± the standard errors of the means. (D) The survival on a lawn of *B. thuringiensis* DB27 of wild-type worms grown on dsRNA for C09H10.6, which corresponds to *tu439*, is significantly ($P < 0.0001$) greater than that of worms grown on dsRNA as a vector control. (E) The survival on a lawn of *S. aureus* of wild-type worms grown on dsRNA for C09H10.6, which corresponds to *tu439*, is significantly ($P < 0.001$) greater than that of worms grown on dsRNA as a vector control. The data shown are means ± the standard errors of the means. For each condition in survival assays, 60 to 80 worms (20 per plate) were used for each of multiple independent biological replicates.

in the second exon of *C. elegans nasp-1* resulting in a leucine-to-serine substitution at amino acid position 53, whereas the second mutation (a G-to-A change in exon 3) leads to a substitution from aspartic acid to asparagine at amino acid position 307 (Fig. 3A). To confirm that the resistance phenotype of *tu439* is due to these mutations in C09H10.6, we performed rescue and RNAi experiments. First, transgenic expression of a wild-type copy of C09H10.6 under the control of its endogenous promoter in the *tu439* mutant background resulted in almost complete restoration of wild-type susceptibility to *B. thuringiensis* DB27 (Fig. 3B) and *S. aureus* (Fig. 3C). Additionally, transgenic expression of C09H10.6 partially rescued the diminished reproduction of the *tu439* mutant (not shown). Second, C09H10.6 RNAi increases nematode resistance to *B. thuringiensis* DB27 (Fig. 3D) and *S. aureus* (Fig. 3E) in a way similar to that of *tu439*, providing additional evidence that the mutant is allelic to C09H10.6. Also, we noticed that *nasp-1* RNAi worms have slower growth and less fecundity at 25°C than vector-treated worms. The high conservation of the amino acids that were mutated (see Fig. S2 in the supplemental material), especially D307, suggests that our *nasp-1* mutant is a strong reduction-of-function allele. Also, the similarity between the *nasp-1* RNAi and the mutant phenotype suggests that the isolated alleles represent reduction-of-function rather than neomorphic mutations. In summary, we conclude that *btr-1*(*tu439*) is identical to the *nasp-1*-like gene C09H10.6. Following standard *C. elegans* nomenclature rules, we rename *btr-1* as *nasp-1*.

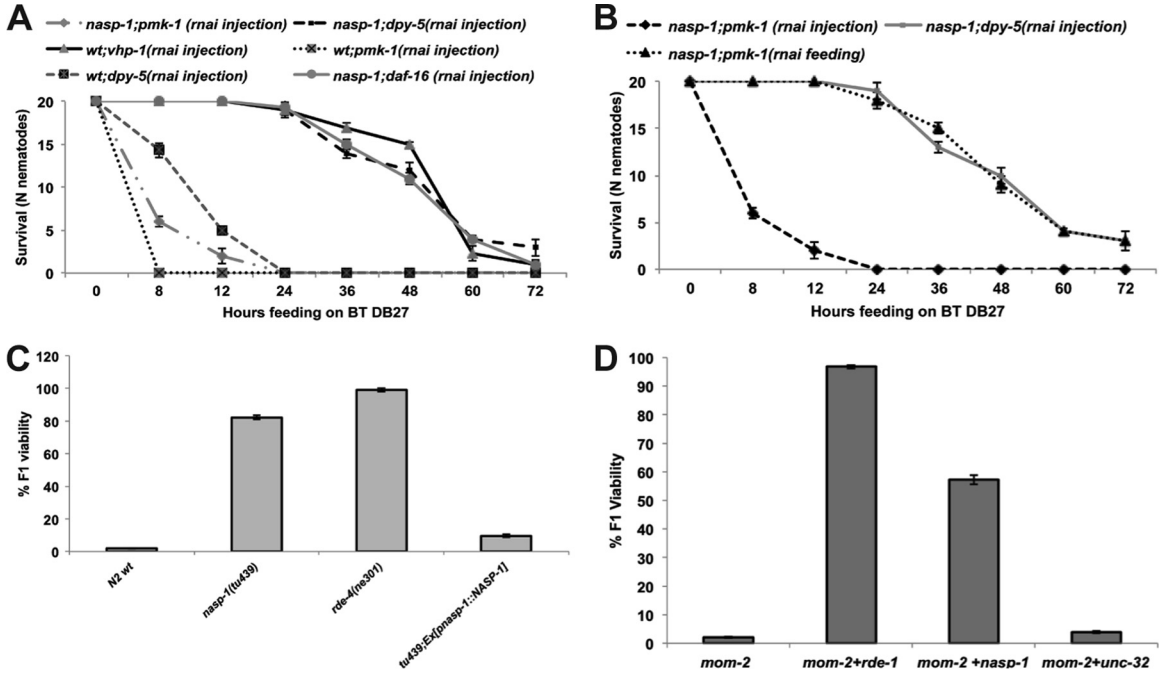

FIG 4 *nasp-1* mutant exhibits RNAi deficiency and requires PMK-1 but not DAF-16 for DB27 resistance. (A) *pmk-1* knockdown upon RNAi by injection leads to hypersusceptibility in wild-type (wt) worms ($P < 0.0001$ for wt;*dpy-5* versus wt;*pmk-1* worms) and abolishes the resistance of the *nasp-1* mutant ($P < 0.0001$ for *nasp-1*;*dpy-5* versus *nasp-1*;*pmk-1* mutant worms) to *B. thuringiensis* (BT) DB27 below the level of wild-type survival ($P < 0.05$ for *nasp-1*;*pmk-1* versus wt worms). *daf-16* knockdown upon RNAi by injection has no effect on *nasp-1* mutant ($P > 0.05$ for *nasp-1*;*dpy-5* versus *nasp-1*;*daf-16* mutant worms) resistance to *B. thuringiensis* DB27. *vhp-1* RNAi significantly ($P < 0.0001$) increases the survival of wild-type worms on a lawn of *B. thuringiensis* DB27 compared to control *dpy-5* RNAi, which was used as a positive control for injection and as a negative control in a survival assay (see Materials and Methods). (B) *pmk-1* knockdown upon RNAi by feeding has no effect on *nasp-1*-mediated resistance to *B. thuringiensis* DB27 ($P > 0.05$), while *pmk-1* knockdown upon RNAi by injection abolishes the resistance of the *nasp-1* mutant ($P < 0.0001$). The data shown are means ± the standard errors of the means. For each condition in survival assays, 60 to 80 worms (20 per plate) were used for each of multiple independent biological replicates. (C) *nasp-1* mutant is RNAi deficient. Wild-type and *nasp-1* and *rde-4* (positive control) mutant worms and *nasp-1* rescuing line worms were fed *E. coli* expressing dsRNA of *mom-2*, which is essential for viability. Viability was assessed as the proportion of hatched larvae relative to the total number of eggs laid. Both *rde-4* and *nasp-1* mutants show significantly ($P < 0.0001$) higher survival of progeny than the wild type, indicating that both mutants are resistant to RNAi-mediated *mom-2* lethality. *nasp-1* overexpression restores the *nasp-1*(*tu439*) mutant's susceptibility to *mom-2*-mediated lethality. The data shown are means ± the standard errors of the means. (D) Wild-type hermaphrodites were coinjected with each candidate dsRNA and *mom-2* dsRNA. *rde-1* RNAi was used as a positive control, while *unc-32* RNAi was used as a negative control. *nasp-1* RNAi significantly increases F_1 viability ($P < 0.0001$) relative to that of the negative control or *mom-2* RNAi alone. The data shown are means ± the standard errors of the means.

The p38 MAPK pathway is required for the *nasp-1* mutant's resistance. Next, we searched for potential interactions of *nasp-1* with known pathogen response regulators to further explore its biological role. First, we tested the p38 mitogen-activated protein kinase (MAPK) pathway (52) by performing injection of *pmk-1* dsRNA into a *nasp-1* mutant background. After *pmk-1* depletion, the resistance of the *nasp-1* mutant to *B. thuringiensis* DB27 is completely abolished, even below the wild-type level (Fig. 4A). Similarly, overactivation of PMK-1 by *vph-1* RNAi (53) strongly increased the survival of *C. elegans* on *B. thuringiensis* DB27 (Fig. 4A). In contrast, injection of *dpy-5* as a control dsRNA had no effect on the survival of wild-type and *nasp-1* mutant worms, although it did result in a dpy phenotype (Fig. 4A). Together, these results suggest that the effects of *pmk-1* and *vhp-1* on survival are gene specific and that *nasp-1* potentially may function in parallel with the p38 MAPK pathway. In contrast, RNAi of *daf-16*, another *C. elegans* innate immunity regulator (33), had no effect on the *nasp-1* mutant's resistance to *B. thuringiensis* DB27 (Fig. 4A), indicating that *nasp-1* does not confer resistance to *B. thuringiensis* DB27 by acting through *daf-16*/FOXO. This result also provides an additional argument that the *nasp-1* mutant's resistance is independent of reproduction, because increased pathogen resistance of most of the sterile mutants requires *daf-16* (47).

***nasp-1* mutants are deficient for RNAi by feeding.** When performing the RNAi experiments described above, we noticed that a knockdown of genes by RNAi in a *nasp-1* mutant background was only possible by injection of dsRNA, suggesting that the *nasp-1* mutant exhibits a sid phenotype (54). In contrast, RNAi by feeding in the *nasp-1* mutant background did not have the same effect (Fig. 4B). Specifically, *pmk-1* RNAi by feeding did not change the *nasp-1* mutant's resistance phenotype, whereas *pmk-1* RNAi by injection abolished the *nasp-1* mutant's resistance to *B. thuringiensis* DB27 completely (Fig. 4B). Similarly, we observed that *dpy-5* RNAi by injection resulted in a dpy phenotype in a *nasp-1* mutant background, whereas *dpy-5* RNAi by feeding did not (data not shown). These observations provide first evidence that *nasp-1* mutants might be defective in their RNAi response; however, they do not exhibit a complete rde phenotype but rather show similarity to sid mutants.

To further explore this phenotype, we fed *nasp-1* mutants with *E. coli* expressing dsRNA against *mom-2*, a gene essential for viability (40, 55). Almost no viable progeny were obtained from wild-

type worms, whereas nearly 80% of the *nasp-1* mutant progeny survived *mom-2* RNAi by feeding (Fig. 4C). Transgenic expression of *nasp-1* under the control of its endogenous promoter in the *tu439* mutant background resulted in almost complete restoration of the wild-type response to *mom-2*-mediated lethality. *rde-4* mutants, which are known to be RNAi deficient, were used as a control and showed nearly 100% viability (Fig. 4C). Additionally, we coinjected wild-type worms with dsRNA of *mom-2* and/or dsRNA of *nasp-1* or control dsRNA and scored the viability of the progeny. The survival of progeny indicates that RNAi against *nasp-1* enables worms to overcome the lethality of *mom-2* RNAi (Fig. 4D). Worms coinjected with dsRNA of a positive-control gene, *rde-1*, showed 97% survival, indicating strong protection from *mom-2* lethality by *rde-1* RNAi. Coinjection with dsRNA of *unc-32* or other genes dispensable for RNAi resulted in a very low survival rate. In contrast, coinjection of *nasp-1* dsRNA significantly rescued the lethality caused by *mom-2* dsRNA. Thus, in contrast to RNAi mutants, such as *rde-1* RNAi mutants, that are resistant to RNAi independently of the delivery method (56), *nasp-1* mutants are still able to perform RNAi when dsRNA is injected into the germ line. In this, they are similar to sid mutants (54), suggesting that they function primarily in the uptake or spreading of dsRNA rather than in modulation of the core RNAi machinery.

To provide further insight into this phenotype, we generated transgenic worms expressing a *nasp-1*-encoded translational protein fusion with GFP to study the site of action of *nasp-1*. Interestingly, expression was localized primarily in the pharynx (see Fig. S3 in the supplemental material). Expression was seen in the metacorpus and terminal bulb of the pharynx with a clear intracellular localization. Given that the pharynx is exposed to pathogens, this observation further supports the idea that *nasp-1* functions in innate immunity; however, the link to the sid phenotype remains unknown. Taken together, these results support the observation that *nasp-1* is required for a robust RNAi response.

The transcriptional response of *nasp-1* mutants to *B. thuringiensis* DB27 reveals a strong overlap with *dcr-1* mutants. To identify genes differentially regulated in *nasp-1* mutants when they are exposed to *B. thuringiensis* DB27, we used whole-genome microarrays on an Agilent platform. Microarray analysis revealed that differential expression in *nasp-1* mutants mostly resulted in downregulation (247 genes), whereas only 31 genes were more strongly expressed in *nasp-1* mutants than in wild-type worms (see Data Set S1 in the supplemental material, FDR-corrected *P* value of $\leq$0.05, fold change cutoff of 1.5 on a $\log_2$ scale). We validated these results by selecting a total of 12 down- and upregulated genes and confirmed their expression by qRT-PCR (Fig. 5A). Interestingly, 58 of the 247 downregulated genes were previously identified as genes that are induced by *B. thuringiensis* DB27, in comparison to OP50, in wild-type worms (11) (Fig. 5B). These results, together with the finding that most expression changes in *nasp-1* mutants result in transcriptional repression, suggest that NASP-1 normally acts as an activator of transcription.

Surprisingly, further characterization of downregulated genes in *nasp-1* mutants revealed a highly significant overlap with genes regulated by different components of the RNAi machinery (57), such as *rde-4*, *rde-3*, and *rrf-1* (see Fig. S4 in the supplemental material). The most significant enrichment was found for genes previously annotated as targets of the only *C. elegans* dicer gene, *dcr-1*. Specifically, 116 of the 247 *nasp-1*-repressed genes were previously identified as *dcr-1*-regulated genes (Fig. 5C). This strong overlap is further supported by quantitative PCR experiments showing that *dcr-1* expression is itself reduced in *nasp-1* mutants compared to that in the wild type (Fig. 5A). These findings are consistent with the reduced life span and RNAi deficiency of *nasp-1* mutants (see above). Thus, an intriguing possibility is that repression of DCR-1 function is responsible for the increased pathogen resistance observed in *nasp-1* mutants.

***dcr-1* is involved in innate immunity through its role in miRNA processing.** The unexpected correlation between downregulated genes in *nasp-1* and *dcr-1* mutants encouraged us to look for further interactions between these two loci. We hypothesized that if reduced DCR-1 function in *nasp-1* mutants were responsible for their increased resistance to *B. thuringiensis* DB27, *dcr-1* mutants should also be more resistant to the pathogen. Indeed, when we tested several *dcr-1* alleles, we found that the mutant for presumptive null allele *dcr-1*(*ok247*) showed greater survival on *B. thuringiensis* DB27 than the wild type (Fig. 6A). This is unlikely to be due to altered reproduction because the *dcr-1*(*ok247*) mutant is not resistant to *S. marcescens* (see Fig. S5A in the supplemental material) and *P. aeruginosa* (see Fig. S5B) and exhibits a reduced life span (58), in contrast to sterile mutants (59, 60). As the *dcr-1*(*ok247*) deletion allele was marked with a recessive allele of *unc-32* to facilitate the isolation of *dcr-1*(*ok247*) homozygous worms (57) and considering that the *unc-32* mutant was shown to have increased resistance to *Erwinia carotovora* and *Photorhabdus luminescens* (8), we also tested the survival of *unc-32* mutants on *B. thuringiensis* DB27 and found that they have a wild-type response to the pathogen (Fig. 6A). Furthermore, transgenic expression of *dcr-1* in a *dcr-1*(*ok247*) mutant restored wild-type susceptibility to *B. thuringiensis* DB27 (Fig. 6A). Together, these findings indicate a novel role for *dcr-1* in innate immunity to the pathogen *B. thuringiensis* DB27.

Interestingly, the effect of *dcr-1* on pathogen resistance is allele specific. We found that survival on *B. thuringiensis* DB27 was unchanged in the mutant with the activating *dcr-1*(*mg375*) allele (Fig. 6A). The *dcr-1*(*mg375*) allele has an impaired helicase domain and small interfering RNA (siRNA) production and, consequently, enhanced RNAi, whereas miRNA synthesis is intact (61). To further test the idea that resistance to *B. thuringiensis* DB27 is associated with altered miRNA processing, we investigated the effects of the *rde-4* and *rde-1* mutations on pathogen survival. *rde-1* and *rde-4* play a role in RNAi but not in miRNA processing (57), and they were shown to produce a reduced life span but normal survival on *S. marcescens*, although the immune response is upregulated (57). Indeed, *rde-1* and *rde-4* mutants did not show increased resistance to *B. thuringiensis* DB27 (Fig. 6B). In contrast, mutants with changes in the *drsh-1* and *alg-1* genes, which are involved in miRNA processing (62, 63), but not in RNAi, exhibited increased resistance to *B. thuringiensis* DB27 (Fig. 6C). To prove that the resistance of *drsh-1* and *alg-1* mutants is not due to impaired germ line development and fertility, we rendered both mutants sterile via *cdc-25* RNAi and compared their survival to that of control vector-treated worms. The survival of *alg-1* mutants was significantly increased after *cdc-25* RNAi compared to that of vector-treated *alg-1* mutants and compared to wild-type worms treated with either the empty vector or *cdc-25* RNAi (Fig. 6D), indicating that *alg-1* and *cdc-25* mutants exhibit resistance via distinct mechanisms and that *alg-1*-mediated resistance to *B. thuringiensis* DB27 is not simply a consequence of altered fertility. However, we did not observe any differences in survival between

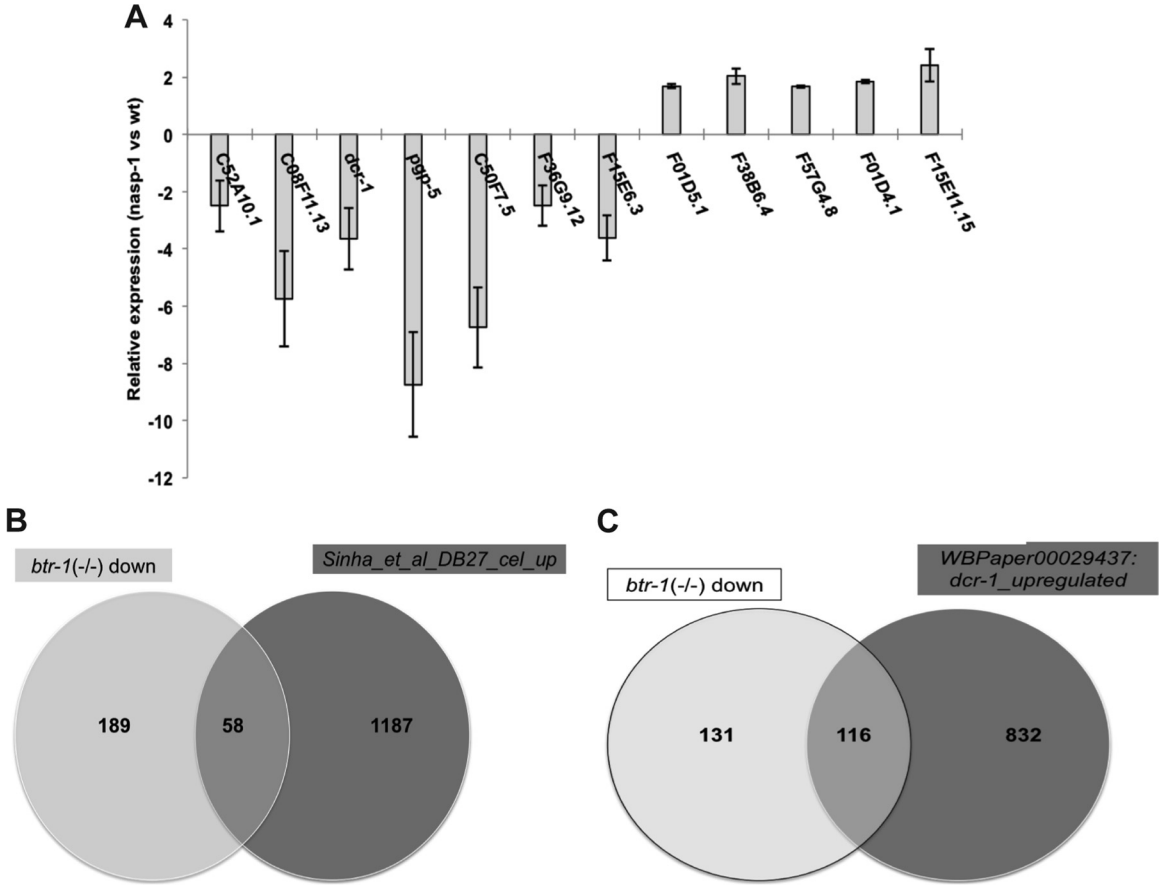

FIG 5 Transcriptional response of *nasp-1* mutant worms to *B. thuringiensis* DB27 infection. (A) qRT-PCR confirmation of microarray results. Wild-type (wt) and *nasp-1* mutant worms were exposed to *B. thuringiensis* DB27 for 4 h and used for RNA isolation. The data shown are the average of two independent RNA isolations. Each transcript was measured in triplicate and normalized to a control gene. Error bars show the standard errors of the means. (B) Venn diagram of the genes downregulated in the *nasp-1* mutant and upregulated in wild-type worms upon exposure to *B. thuringiensis* DB27 (11). The overlap is significant ($P = 1.11E-15$). (C) Venn diagram showing the overlap between genes downregulated in the *nasp-1* mutant and genes upregulated in the *dcr-1* mutant (47). The overlap is significant ($P = 3.70E-79$).

drsh-1 vector- and *drsh-1 cdc-25* RNAi-treated worms (Fig. 6E), suggesting that the increased resistance of *drsh-1* mutants to *B. thuringiensis* DB27 is indeed a secondary effect of sterility. Taken together, these results suggest that the impairment of DCR-1 function in miRNA processing confers resistance to *B. thuringiensis* DB27. Furthermore, these data, together with the downregulation of *dcr-1* expression in *nasp-1* mutants, support the conclusion that the resistance of *nasp-1* mutants is due to reduced DCR-1 activity.

***nasp-1* acts through DCR-1/Dicer.** The experiments described above are consistent with the hypothesis that *dcr-1* is genetically downstream of *nasp-1*. This conclusion is further supported by previous expression profiling of *dcr-1* mutants, which did not show any change in the expression of *nasp-1* in *dcr-1* mutants (57). To further test this hypothesis, we investigated whether overexpression of *dcr-1* would abolish the increased resistance of the *nasp-1* mutant to *B. thuringiensis* DB27. Indeed, *dcr-1* overexpression under the control of its endogenous promoter significantly reduced the survival of *nasp-1* mutant worms on *B. thuringiensis* DB27 (Fig. 6F). Additionally, *dcr-1* overexpression restored the reduced life span of *nasp-1* mutants (see Fig. S6 in the supplemental material). Given that *C. elegans dcr-1* is expressed at high levels in the intestine (58), which is also the major site of infection by ingested pathogens, we investigated whether upregulation of *dcr-1* specifically in the intestine could affect the *nasp-1* mutant's pathogen resistance. When we overexpressed *dcr-1* under the control of the intestine-specific *cpr-1* promoter, we noticed a significant decrease in the *nasp-1* mutant's survival on *B. thuringiensis* DB27 (Fig. 6F). Thus, the previously unknown role of DCR-1 in *C. elegans* innate immunity is genetically downstream of *nasp-1* and acts through the *C. elegans* intestine.

NASP-1 and Dicer resistance to *S. aureus* is due to impaired fecundity. Considering that the *nasp-1* mutant shows increased resistance to *S. aureus*, we investigated whether it is also mediated by the same mechanism that is employed in the defense against *B. thuringiensis* DB27. Given that the *nasp-1* mutant has reduced fecundity, a common feature of *S. aureus*-resistant mutants (47), we first tested if the *nasp-1* mutant's resistance to *S. aureus* depends on reproduction. As shown in Fig. 7A, *nasp-1* mutants injected with *cdc-25* interfering dsRNA survived *S. aureus* exposure longer than control-injected worms. However, their survival was similar to that of wild-type worms injected with *cdc-25* interfering dsRNA (Fig. 7A). Thus, *nasp-1* mutants do not have an additive effect with *cdc-25* RNAi for *S. aureus* resistance, as is the case for *B.*

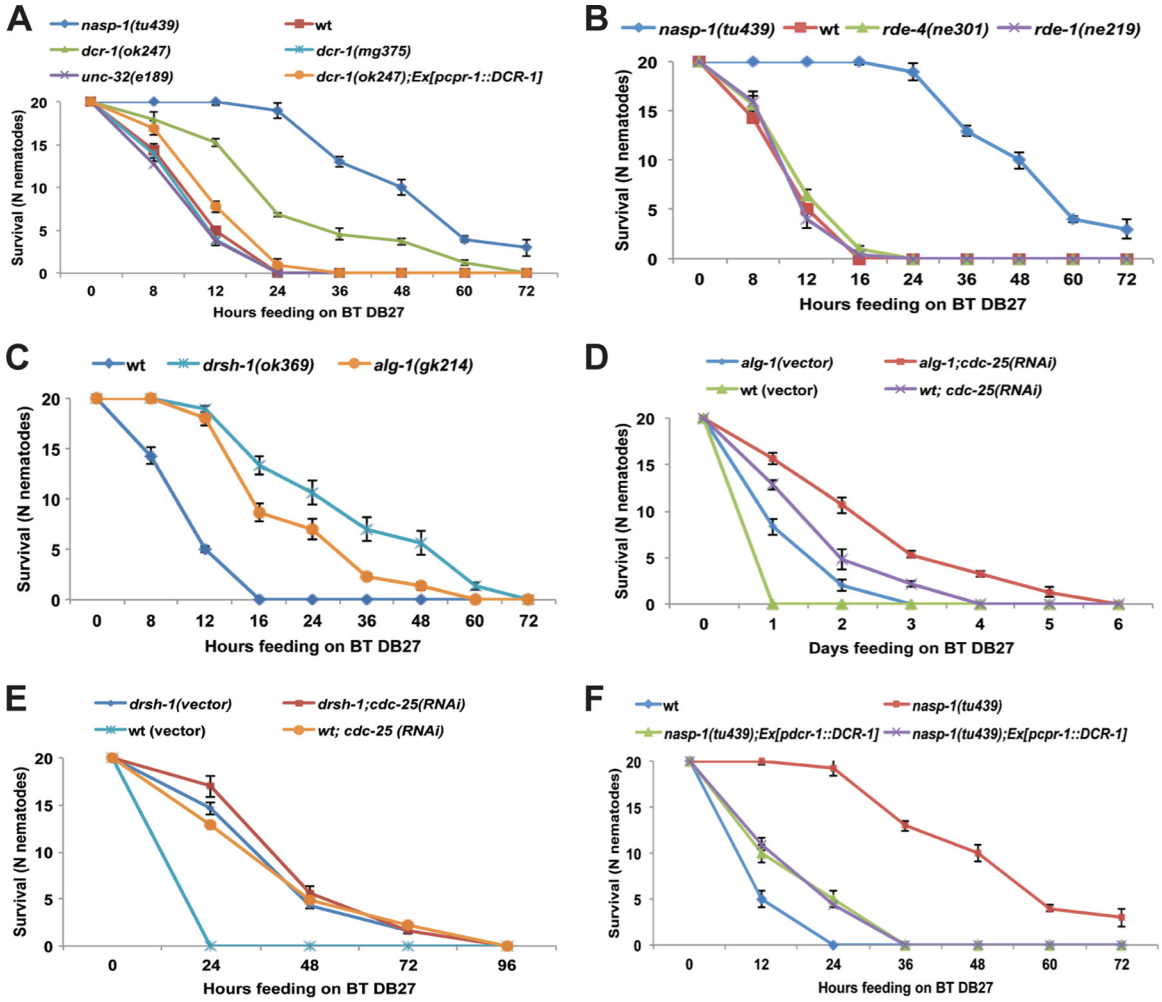

FIG 6 Repression of DCR-1 activity in miRNA processing but not in RNAi results in increased resistance to *B. thuringiensis* DB27. (A) The survival of different *dcr-1* alleles on a lawn of *B. thuringiensis* (BT) DB27 in comparison with wild-type (wt) and *nasp-1* mutant worms. *dcr-1*(*ok247*) mutant worms are significantly ($P < 0.0001$) more resistant to *B. thuringiensis* DB27 than wild-type worms are. *dcr-1*(*mg375*) mutant worms show wild-type susceptibility to the pathogen ($P > 0.05$). Transgenic expression of *dcr-1* rescues the increased resistance of the *dcr-1*(*ok247*) mutant to *B. thuringiensis* DB27. (B) RNAi-defective *rde-4*(*ne301*) and *rde-1*(*ne219*) mutants show survival comparable to that of wild-type worms ($P > 0.05$) upon exposure to *B. thuringiensis* DB27. (C) Survival of miRNA-deficient mutants upon exposure to *B. thuringiensis* DB27. *alg-1*(*gk214*) and *drsh-1*(*ok369*) mutants show resistance to DB27 greater than that of wild-type worms ($P < 0.0001$). (D, E) Wild-type, *alg-1* mutant (D), and *drsh-1* mutant (E) nematodes were fed with *cdc-25* RNAi vector to obtain sterile worms or with empty RNAi vector and exposed to DB27. *cdc-25* RNAi significantly increases the resistance of wild-type ($P < 0.0001$ for wt;vector versus wt;*cdc-25* worms) and *alg-1* (D) ($P = 0.002$ for *alg-1*;vector versus *alg-1*;*cdc-25*) worms but not *drsh-1* mutants (E) ($P > 0.05$ for *drsh-1*;vector versus *drsh-1*;*cdc-25* worms). The survival of *alg-1*;*cdc-25* mutant worms is significantly different ($P < 0.05$) from that of wt;*cdc-25* worms. The data shown are means ± the standard errors of the means. (F) Transgenic overexpression of *dcr-1* abolishes the resistance of the *nasp-1* mutant to *B. thuringiensis* DB27. The resistance of *nasp-1* mutants with transgenically expressed *dcr-1* under the control of its endogenous promoter (p*dcr-1*) or an intestine-specific promoter (p*cpr-1*) is significantly lower than that of *nasp-1* mutants ($P < 0.0001$). For each condition in survival assays, 60 to 80 worms (20 per plate) were used for each of multiple independent biological replicates. The data shown are means ± the standard errors of the means.

thuringiensis DB27 (Fig. 2C). These findings suggest that *nasp-1* and *cdc-25* RNAi increase resistance to *S. aureus* via the same mechanism and the *nasp-1* mutant's resistance to *S. aureus* is very likely due to partial sterility. In contrast, *nasp-1* and *cdc-25* RNAi function in independent pathways for *B. thuringiensis* DB27 resistance (Fig. 2).

This difference between *S. aureus* and *B. thuringiensis* DB27 was further explored by studying the allele specificity of Dicer mutants. There was no allele specificity in terms of Dicer resistance to *S. aureus* since both *dcr-1*(*ok247*) and *dcr-1*(*mg375*) mutant worms were more resistant to *S. aureus* than wild-type worms were (Fig. 7B). Also, *dcr-1* mutant resistance can be rescued by transgenic overexpression of *dcr-1* under the control of its endogenous promoter (Fig. 7B), indicating that Dicer might play a role in the *C. elegans* defense against *S. aureus*. Considering the reproductive defects of *dcr-1* mutants, which may contribute to increased resistance to *S. aureus*, we used *cdc-25* RNAi to sterilize *dcr-1*(*mg375*) mutant worms. The survival of *mg375* mutant worms was greater than that of vector-treated worms but was not significantly different from that of wild-type worms treated with *cdc-25* RNAi, indicating the absence of a cumulative effect of *dcr-1* and *cdc-25* (Fig. 7C). Thus, the *dcr-1* mutant's resistance to *S. aureus* is due to diminished reproduction, as in the case of the *nasp-1* mutant. We also assessed the survival of RNAi- and

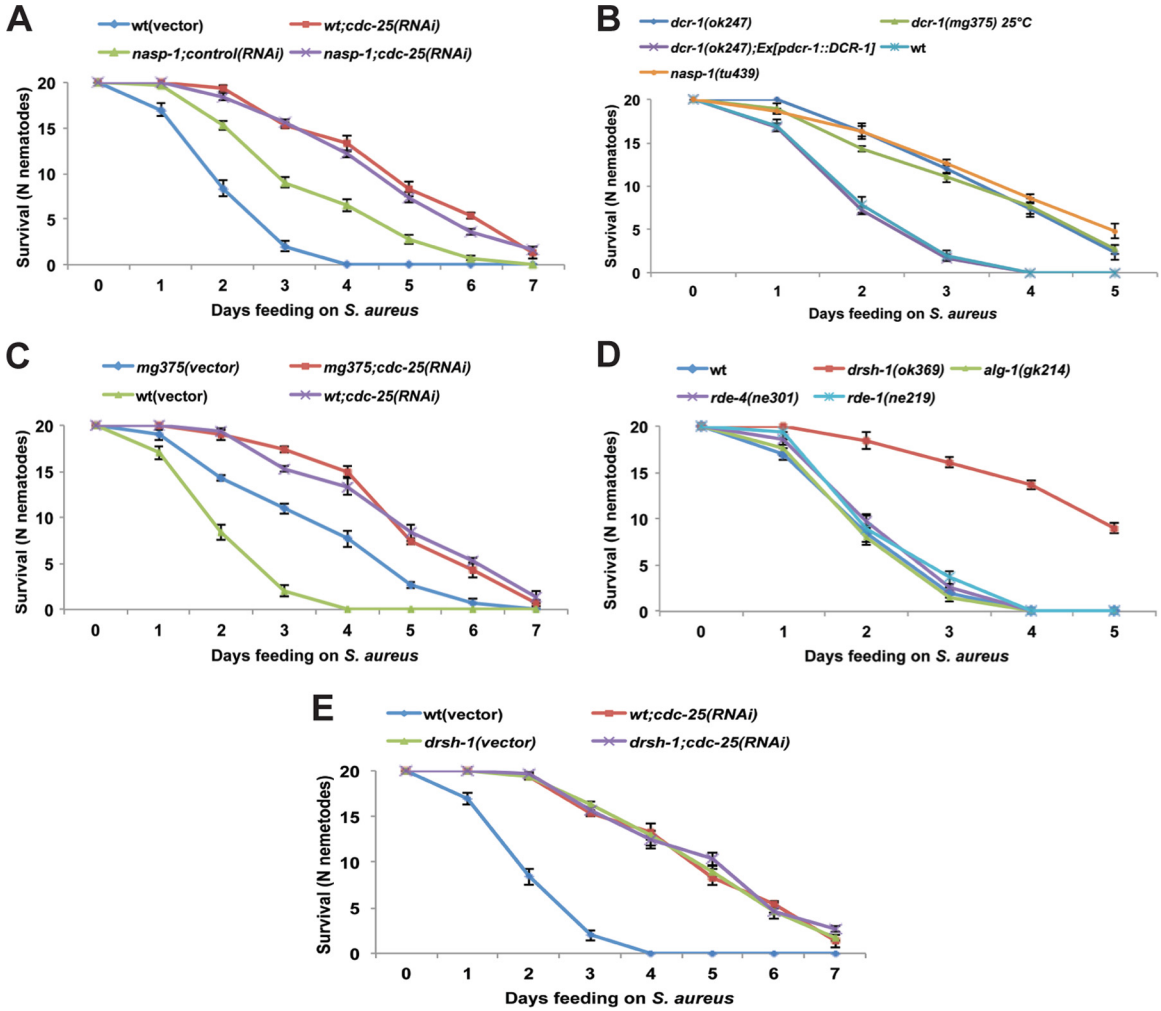

FIG 7 *nasp-1*- and *dcr-1*-mediated resistance to *S. aureus* is due to partial sterility. (A) Wild-type (wt) and *nasp-1* mutant nematodes were injected with *cdc-25* interfering dsRNA to obtain sterile worms or with control dsRNA (see Materials and Methods) and exposed to *S. aureus*. *cdc-25* RNAi significantly increases the resistance of wild-type ($P < 0.0001$ for wt;vector versus wt;*cdc-25* worms) and *nasp-1* mutant ($P < 0.0001$ for *nasp-1*;control versus *nasp-1*;*cdc-25* worms) worms. The survival of *nasp-1*;*cdc-25* mutant worms is not significantly different ($P > 0.05$) from wt;*cdc-25* worms. The data shown are means ± the standard errors of the means. (B) Both *dcr-1* mutants *ok247* and *mg375* grown at 25°C to induce sterility exhibit increased resistance to *S. aureus* ($P < 0.0001$ for *ok247* mutant versus wt worms; $P < 0.001$ for *mg375* mutant versus wt worms), which can be rescued by transgenic expression of *dcr-1*. The *ok247* and *mg375* mutants do not differ in the level of resistance ($P > 0.05$). The data shown are means ± the standard errors of the means. (C) Wild-type and *dcr-1*(*mg375*) mutant nematodes were fed with *cdc-25* RNAi vector to obtain sterile worms or with empty RNAi vector (see Materials and Methods) and exposed to *S. aureus*. *cdc-25* RNAi significantly increases the resistance of wild-type ($P < 0.0001$ for wt;vector versus wt;*cdc-25* worms) and *mg375* ($P = 0.018$ for *mg375*;vector versus *mg375*;*cdc-25* mutant worms) worms. The survival of *mg375*;*cdc-25* mutant worms is not significantly different ($P > 0.05$) from that of wt;*cdc-25* worms. The data shown are means ± the standard errors of the means. (D) Survival of wild-type worms and RNAi- and miRNA-deficient mutants upon exposure to *S. aureus*. Only *drsh-1*(*ok369*) mutant worms showed greater *S. aureus* resistance than wild-type worms ($P < 0.0001$). The data shown are means ± the standard errors of the means. (E) Wild-type and *drsh-1* mutant nematodes were fed with *cdc-25* RNAi vector to obtain sterile worms or with empty RNAi vector and exposed to *S. aureus*. *cdc-25* RNAi significantly increases the resistance of wild-type ($P < 0.0001$ wt;vector versus wt;*cdc-25* worms) but not *drsh-1* mutant ($P > 0.05$ *drsh-1*;vector versus *drsh-1*;*cdc-25* mutant worms) worms. The survival of *drsh-1*;*cdc-25* mutant worms is not significantly different ($P > 0.05$) from that of wt;*cdc-25* worms. For each condition in survival assays, 60 to 80 worms (20 per plate) were used for each of multiple independent biological replicates. The data shown are means ± the standard errors of the means.

miRNA-deficient mutants on *S. aureus*. Both *rde-1* and *rde-4* mutants showed a wild-type response to *S. aureus* (Fig. 7D). The *alg-1* mutant, which has increased survival on *B. thuringiensis* DB27, exhibited survival similar to that of the wild type on *S. aureus* (Fig. 7D), while only the *drsh-1* mutant was more resistant to *S. aureus* infection (Fig. 7D). However, as in the case of *B. thuringiensis* DB27, the increased resistance seen is the consequence of sterility since *cdc-25* RNAi did not further increase the survival of *drsh-1* mutants (Fig. 7E). Taken together, these results show that the *nasp-1* mutant's resistance to *S. aureus* is very likely due to impaired fecundity and at this stage it is impossible to disconnect immune and reproductive mechanisms that confer the *nasp-1* mutant's resistance to *S. aureus*.

The collagen gene *col-92* acts downstream of *nasp-1* and confers resistance to DB27. Finally, we wanted to uncover the molecular mechanisms of *nasp-1*- and *dcr-1*-mediated resistance to *B. thuringiensis* DB27. For this, we hypothesized that the repression of *dcr-1* activity in *nasp-1* mutants leads to overactivation of effec-

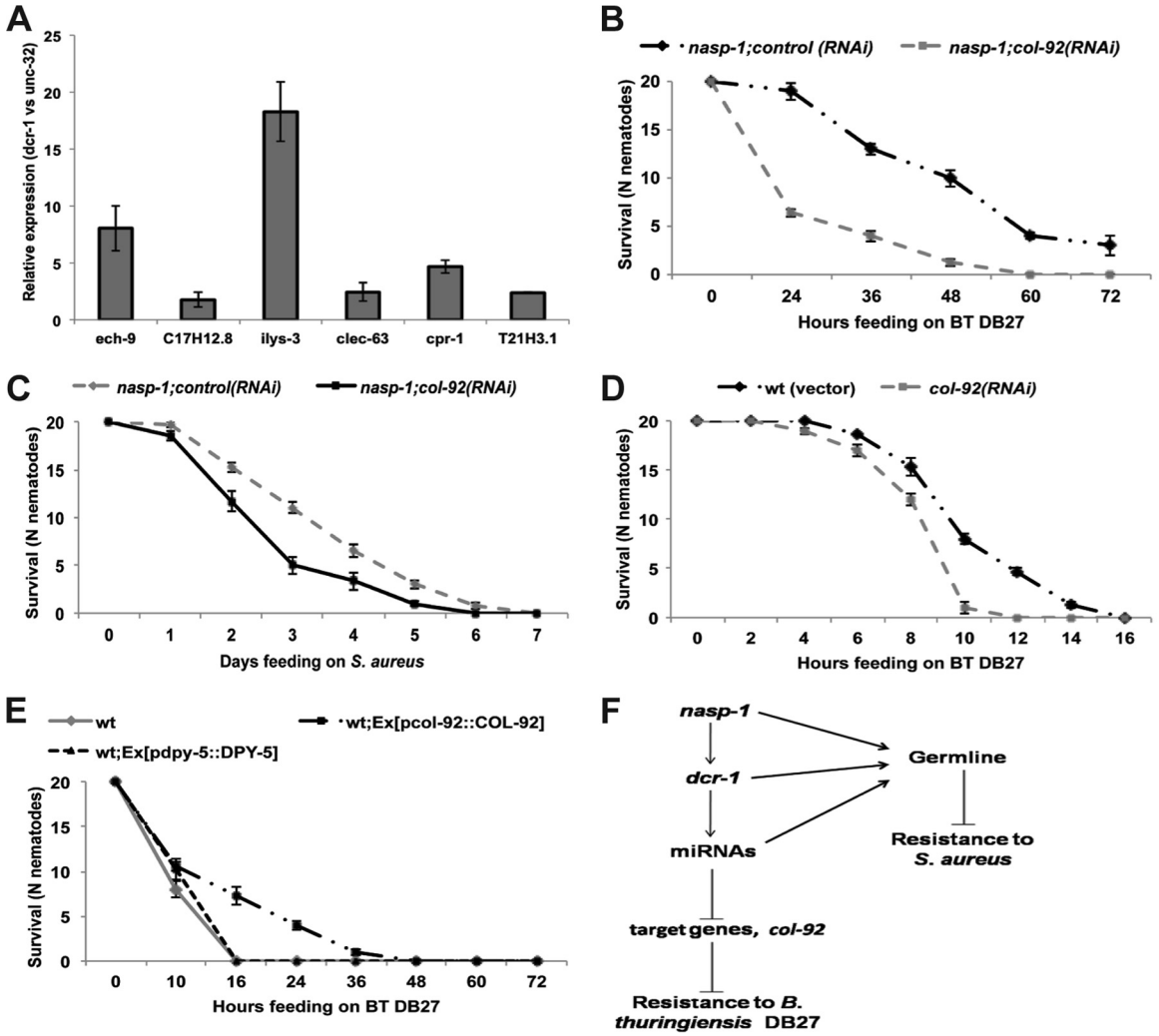

FIG 8 Collagen gene *col-92* acts downstream of *nasp-1* and is required for resistance to *B. thuringiensis* (BT) DB27. (A) Some of the DB27 response transcripts (selected from reference 11) show high expression in *dcr-1* mutant worms as determined by qRT-PCR. The data shown are averages of two independent RNA isolations. Each transcript was measured in triplicate and normalized to a control gene. Error bars show the standard errors of the means. (B) Injection of *col-92* interfering dsRNA significantly ($P < 0.001$) suppresses the *nasp-1* mutant's resistance to *B. thuringiensis* DB27 compared to the injection of a control gene (*dpy-5*). The data shown are means ± the standard errors of the means. (C) Injection of *col-92* interfering dsRNA significantly ($P = 0.021$) decreases the *nasp-1* mutant's resistance to *S. aureus* compared to the injection of a control gene (*dpy-5*). The data shown are means ± the standard errors of the means. (D) RNAi knockdown of *col-92* increases the susceptibility of wild-type (wt) worms to *B. thuringiensis* DB27 infection ($P < 0.01$). The data shown are means ± the standard errors of the means. (E) Transgenic expression of *col-92* but not of *dpy-5* significantly ($P < 0.02$) increases the survival of worms exposed to *B. thuringiensis* DB27. The data shown are means ± the standard errors of the means. (F) Mechanism by which *nasp-1* may regulate the innate immune response to *B. thuringiensis* DB27.

tor genes, which make *C. elegans* more resistant to infection. Consistent with this idea, we found that several *B. thuringiensis* DB27-responsive genes are more strongly expressed in *nasp-1* and *dcr-1* mutants than in the wild type (Fig. 8A). To test this hypothesis further, we used RNAi to knock down *nasp-1*-upregulated genes individually (see Data Set S1 in the supplemental material) in the *nasp-1* mutant background. While the knockdown of the majority of these genes resulted in no obvious effect on *nasp-1*-mediated resistance (see Fig. S7 in the supplemental material), *col-92* RNAi produced significant suppression of *nasp-1*-mediated resistance to DB27 (Fig. 8B; see Fig. S7) and *S. aureus* (Fig. 8C). Furthermore, knockdown of *col-92* in wild-type worms makes them hypersusceptible to *B. thuringiensis* DB27 (Fig. 8D), *S. aureus*, and *P. aeruginosa* (see Fig. S8A and B) but not to *S. marcescens* (see Fig. S8C). However, the life span of *col-92* RNAi worms was not affected (see Fig. S8D), suggesting that its hypersusceptibility to bacterial infection is not a result of impaired fitness. In contrast, *col-92* overexpression under the control of its endogenous promoter increases resistance to *B. thuringiensis* DB27 (Fig. 8E) and *S. aureus* (see Fig. S8E) infections. At the same time, overexpression of *dpy-5*, used as a control, resulted in no obvious effects on resistance to *B. thuringiensis* DB27 (Fig. 8E) and *S. aureus* (see Fig. S8E). Moreover, *dpy-5* RNAi and *col-93* RNAi had no effect on *C. elegans* survival upon exposure to *B. thuringiensis* DB27, indicating that the effect of *col-92* is specific and is unlikely to be due only to simple alteration of cuticle structure.

To shed more light on the immune function of *col-92*, we studied the expression of the gene. It should be noted that COL-92 is a

member of a nematode-specific family of collagens, characterized by a cuticle collagen N-terminal domain. Transgenic lines harboring a *col-92*–GFP transcriptional fusion construct showed that the gene is expressed predominantly in the hypodermis (see Fig. S9 in the supplemental material). Therefore, although *col-92* is a downstream target of NASP-1, its immune function awaits further characterization and might be associated with cuticle. Thus, the previously uncharacterized collagen gene *col-92* plays a pivotal role in the *C. elegans* defense against bacterial infection and represents a downstream target of NASP-1 and DCR-1.

DISCUSSION

In this study, we isolated a *B. thuringiensis* DB27-resistant mutant in *C. elegans* that we showed to be identical to *nasp-1*, a gene that has previously been implicated in female development (51). Both *nasp-1* alleles are reduction-of-function but not null alleles. Further characterization of *nasp-1* helped to discover a previously unknown function of *dcr-1*/Dicer in the immune response of *C. elegans*. Mammalian NASP1 is a tetratricopeptide (TPR) repeat domain-containing H1 linker histone binding protein that is part of a multichaperone complex implicated in nucleosome remodeling (64, 65). NASP1 is highly expressed in dividing cells and is required for normal cell cycle progression (65). A role for *C. elegans nasp-1* in chromatin remodeling is supported by its interactions with a variety of chromatin-remodeling proteins like linker H1 histones and histone deacetylase (51). We hypothesize that *nasp-1* might regulate the pathogen response of *C. elegans* via transcription and chromatin-remodeling mechanisms. Strikingly, it was shown that changes in NASP1 expression lead to the misregulation of a variety of genes in HeLa cells, including genes involved in the immune responses (66).

The RNAi deficiency of *C. elegans nasp-1* is similar to that of other chromatin-remodeling genes (40) and provided the entry point for the discovery of the function of *dcr-1* in *C. elegans* innate immunity, the most unexpected finding of this study. DCR-1 is an RNase III endoribonuclease whose primary function is the cleavage of dsRNA into smaller fragments that mediate RNAi (67). DCR-1 is required for miRNA processing and the synthesis of endo-siRNAs implicated in gene silencing and epigenetic regulation (67–69). Previous studies have shown a role for DCR-1 and the RNAi machinery in the *C. elegans* response to viral infections (14, 70). However, our results point toward a broader function of Dicer in innate immunity. To our knowledge, this study is the first to show a direct role for DCR-1 in antibacterial immunity. Results obtained by using different mutant alleles of *C. elegans dcr-1* support the conclusion that its function in pathogen defense is mediated primarily by impaired miRNA biogenesis. Further evidence for this conclusion comes from the analysis of other genes that allow discrimination between miRNA processing and RNAi. Consistent with our conclusion is a recent report showing that miRNA activity in the *C. elegans* intestine is largely dedicated to attenuating the activity of pathogen response genes (48). Specifically, Kudlow et al. (48) have shown that *ain-1* mutants, which have altered miRNA-induced silencing complex activity, are more resistant to *P. aeruginosa* than wild-type worms are. Additionally, those authors were able to identify specific miRNA families that are involved in damping of the immune response in the absence of infection. Together, these findings suggest that intestinal miRNAs act as negative regulators of infection response genes. However, we have been unable to identify a miRNA or siRNA that is likely to be a part of the mechanisms of *nasp-1*-mediated resistance to *B. thuringiensis* DB27. Such studies might be complicated by the fact that redundancy among small RNAs is a well-known phenomenon in *C. elegans*.

The increased resistance of *dcr-1* mutants to *B. thuringiensis* DB27 might be due to the upregulation of pathogen response genes. Indeed, our RT-PCR experiment showed induction of some of the *B. thuringiensis* DB27-responsive genes in *dcr-1* mutants (Fig. 8A), indicating that DCR-1 may function as a suppressor of the *C. elegans* immune response to *B. thuringiensis* DB27 via suppression of miRNA synthesis. By using a reverse genetic approach, we found that one of the *nasp-1*-upregulated genes, namely, *col-92*, is an important determinant of the *nasp-1* mutant's resistance that acts downstream of *nasp-1* and *dcr-1*. Collagens are differentially expressed in response to a variety of microbes (8, 71), suggesting that they may represent an essential part of the immune response. Nematodes, including *C. elegans*, are known for the unusually high number of collagens encoded in their genomes, and collagens have been implicated in life span and immunity (71–74). A potential role for collagens in the immune response might be in part responsible for the high number of nematode collagens, although direct proof of this assumption is hard to provide, even in the age of reverse genetics. One phenomenon that might hinder the identification of specific functions of collagens is redundancy, which is often seen among structurally related genes (73).

Our findings on the role of DCR-1 in *C. elegans* innate immunity strongly correlate with the identification of Dicer as a regulator of the mammalian immune response. Tang and coworkers found that the inhibition of Dicer in human cells upregulates major histocompatibility complex class I-related molecules A and B, which are innate immune system ligands for the NKG2D receptor expressed by natural killer cells (75). Those authors proposed further that a Dicer knockdown is indirectly linked to human innate immunity via the DNA damaging pathway. Whether a similar DNA-damaging pathway is involved in the upregulation of the *C. elegans* innate immune response awaits further analysis. Additionally, various miRNAs have been implicated in the regulation of diverse aspects of human innate immunity (76), including direct microbial killing. For example, *miR-223*-deficient neutrophils were shown to kill *Candida albicans* more effectively then wild-type cells do (77). A role for miRNAs in the human immune system is further supported by evidence that many immunological diseases are caused by alteration of miRNA function (76). Therefore, Dicer and miRNAs might play a conserved role as negative regulators in immunity.

Although alterations of *dcr-1* or miRNA functions are beneficial to *C. elegans* under pathogenic conditions because of immune response activation, this can be deleterious under normal growth conditions. Consistent with this, *nasp-1* mutants showed compromised reproduction, like many other pathogen-resistant mutants (47), indicating that the immune system might compete with the reproductive system for resources. Generally, chronic immune activation has been linked to several pathological disorders, like inflammatory bowel disease in humans (78). Therefore, it is crucial to any organism to have mechanisms of innate immune suppression in the absence of pathogens.

On the basis of our results, we propose the following mechanism for the function of *nasp-1* in the regulation of *C. elegans* innate immunity (Fig. 8F). First, *nasp-1* is a regulator of *dcr-1*. In

nasp-1 mutants, *dcr-1* function is repressed because of chromatin remodeling or related mechanisms, which leads to, besides RNAi deficiency, the inhibition of *dcr-1*-mediated miRNA processing. Consequently, certain miRNAs are not processed and, in turn, target genes, including *col-92*, are more strongly expressed than normal. As a consequence, mutant worms are more resistant to *B. thuringiensis* DB27 infection. Additionally, *nasp-1*, *dcr-1*, and *drsh-1* mutants exhibit increased resistance to *S. aureus* as a secondary consequence of altered reproduction likely because of germ line defects. In general, germ line defects and fecundity might play a role in the observed resistance phenotypes of *nasp-1* and *dcr-1* mutants. It is important to note, however, that *nasp-1 dcr-1* double mutants with *cdc-25* RNAi gave different results for resistance to *B. thuringiensis* DB27 and *S. aureus*, respectively. These findings indicate complex interactions between fecundity and nematode immunity.

Given the comparative framework of our work, what about the specificity of *nasp-1* and the resistance of *P. pacificus* to *B. thuringiensis* DB27? While *nasp-1* is clearly conserved in sequence, the amino acid positions that are changed in the two *C. elegans nasp-1* mutants are not. It is therefore possible that the exact molecular mechanisms of *nasp-1* and its role in nematode immunity did change during the course of evolution. More generally, our parallel work on innate immunity in *P. pacificus* has recently revealed that more general anatomical and physiological parameters are in part responsible for the observed differences between the two species. *P. pacificus* is not equipped with a grinder, as is typical of members of the family Diplogastridae, and so does not lyse its bacterial food in the pharynx. *P. pacificus* mutants hypersusceptible to bacterial pathogens are often Unc mutants with an abnormal defecation cycle, indicating that the regulation of the defecation cycle is crucial for innate immunity (32). Together, this comparative work on innate nematode immunity points toward the correlated action of anatomical, physiological, and molecular mechanisms that control nematode-bacterial interactions and regulate the evolution of innate nematode immunity.

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