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Bacillus thuringiensis DB27 Produces Two Novel Protoxins, Cry21Fa1 and Cry21Ha1, Which Act Synergistically against Nematodes

Igor Iatsenko,[a] Iuliia Boichenko,[b] Ralf J. Sommer[a]

Max Planck Institute for Developmental Biology, Department of Evolutionary Biology,[a] and Department of Protein Evolution,[b] Tübingen, Germany

Bacillus thuringiensis **has been widely used as a biopesticide, primarily for the control of insect pests, but some *B. thuringiensis* strains specifically target nematodes. However, nematicidal virulence factors of *B. thuringiensis* are poorly investigated. Here, we describe virulence factors of nematicidal *B. thuringiensis* DB27 using *Caenorhabditis elegans* as a model. We show that *B. thuringiensis* DB27 kills a number of free-living and animal-parasitic nematodes via intestinal damage. Its virulence factors are plasmid-encoded Cry protoxins, since plasmid-cured derivatives do not produce Cry proteins and are not toxic to nematodes. Whole-genome sequencing of *B. thuringiensis* DB27 revealed multiple potential nematicidal factors, including several Cry-like proteins encoded by different plasmids. Two of these proteins appear to be novel and show high similarity to Cry21Ba1. Named Cry21Fa1 and Cry21Ha1, they were expressed in *Escherichia coli* and fed to *C. elegans*, resulting in intoxication, intestinal damage, and death of nematodes. Interestingly, the effects of the two protoxins on *C. elegans* are synergistic (synergism factor, 1.8 to 2.5). Using purified proteins, we determined the 50% lethal concentrations (LC_{50}s) for Cry21Fa1 and Cry21Ha1 to be 13.6 μg/ml and 23.9 μg/ml, respectively, which are comparable to the LC_{50} of nematicidal Cry5B. Finally, we found that signaling pathways which protect *C. elegans* against Cry5B toxin are also required for protection against Cry21Fa1. Thus, *B. thuringiensis* DB27 produces novel nematicidal protoxins Cry21Fa1 and Cry21Ha1 with synergistic action, which highlights the importance of naturally isolated strains as a source of novel toxins.**

Bacillus thuringiensis is a Gram-positive, spore-forming bacterium which is extensively used for biological control of insects and nematodes (1, 2). *B. thuringiensis* produces an array of virulence factors that contribute to its pathogenic effect. These virulence factors include exotoxins, extracellular proteases, enhancins, chitinase, and collagenase, which breach the epithelial cells of the insect gut (3). However, the major virulence factors of *B. thuringiensis* (4) are pesticidal proteins called Cry and Cyt produced during sporulation as a crystal inclusions.

These crystal proteins are pore-forming toxins lethal to insects and nematodes but nontoxic to vertebrates, which makes *B. thuringiensis* a safe and an effective pesticide that has been successfully used for many years (1, 4). While annotated Cry toxins are quite diverse in terms of sequence similarity (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/toxins2.html), they exhibit specific activity against insects of the orders Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Orthoptera, and Mallophaga (4), and they are also toxic to nematodes (5, 6). In contrast, Cyt toxins have shown mainly dipteran specificity, being able to kill mosquitoes and black flies (7). However, considering the diversity and amount of nematode species in soil, which is also ubiquitously inhabited by *B. thuringiensis*, nematodes also have to be considered the target for *B. thuringiensis* and its toxins (5). In support of this, several families of Cry proteins (Cry5, Cry6, Cry12, Cry13, Cry14, Cry21, and Cry55) were shown to be toxic to a number of free-living and parasitic nematodes (5, 8), but the full spectrum of nematicidal Cry toxins as well as their host targets is far from completion. Also, considering the growing problem of pest resistance to existing toxins, there is a high demand for new toxins (9).

Naturally isolated *Bacillus* strains have often been used as a source of new Cry toxins (see, for example, reference 10). Given that *B. thuringiensis* and nematodes coexist and coevolve in the natural environment, they undergo reciprocal changes, one of which is increased pathogen virulence (11). Therefore, naturally isolated *B. thuringiensis* strains may serve as a reservoir of novel Cry toxins, some of which may be used for the production of biological pesticides. In support of this, we have previously isolated several nematicidal *Bacillus* strains from environmental samples (12). One of the strains, *B. thuringiensis* DB27, was isolated from dung beetles and exhibits extreme toxicity to the model nematode *Caenorhabditis elegans* (12). Previously, we investigated *C. elegans* transcriptional response to *B. thuringiensis* DB27 infection (13) and elucidated the mechanisms of *C. elegans* resistance (14). However, the molecular mechanisms of the high virulence of *B. thuringiensis* DB27 for *C. elegans* are still unknown.

Here, we show that *B. thuringiensis* DB27 produces novel plasmid-encoded Cry protoxins (Cry21Fa1 and Cry21Ha1), which act synergistically to kill free-living and animal-parasitic nematodes via intestinal damage. We determined the 50% lethal concentrations (LC_{50}s) for Cry21Fa1 (13.6 μg/ml) and Cry21Ha1 (23.9 μg/ml) and found that they are comparable to the LC_{50} of nematicidal Cry5B. Additionally, we show that *C. elegans* conserved pathways provide protection against multiple pore-forming toxins.

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Address correspondence to Ralf J. Sommer, ralf.sommer@tuebingen.mpg.de.
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MATERIALS AND METHODS

Bacterial and nematode strains. The following strains were provided by the *Caenorhabditis* Genetics Center (University of Minnesota): *C. elegans* wild-type Bristol (N2), *Oscheius carolinensis*, *Pelodera strongyloides*, and *Panagrellus redivivus*. *C. elegans pmk-1*(*km25*), *jun-1*(*gk551*), *kgb-1*(*um3*), *xbp-1*(*zc12*), *bre-2*(*ye31*), and *bre-3*(*ye26*) mutants were also used in this study. Nematodes were maintained on nematode growing medium (NGM) agar plates with *Escherichia coli* OP50 as a food source and stored at 20°C. *Strongyloides papillosus* was maintained as described previously (15) and was kindly provided by Adrian Streit. *B. thuringiensis* DB27 was isolated by our group (12). Its plasmid-cured strain was generated in the current study. *E. coli* JM103 and protein expression vector pQE9 were provided by Raffi Aroian.

Nematode killing assays. (i) Vegetative cells. *B. thuringiensis* DB27 was grown overnight in a shaking incubator at 30°C in Luria-Bertani (LB) broth. A 40-μl volume of the culture was spread to the edges of 6-cm-diameter NGM plates, and plates were incubated for 12 to 14 h at 25°C before the assay. A total of 20 adult worms were placed into each plate in three to six independent replicates and were monitored for survival. Every 6 h (before bacteria on a plate started sporulation), worms were transferred to freshly prepared plates to ensure exposure to vegetative cells. Microscopy was used to monitor the state of bacteria on a plate.

(ii) Mixture of vegetative cells and spores. *B. thuringiensis* DB27 was grown overnight in a shaking incubator at 30°C in Luria-Bertani (LB) broth. An 80-μl volume of the culture was spread to the edges of 6-cm-diameter NGM plates, and plates were incubated for 24 h at 25°C before the assay. A total of 20 adult worms were placed into each plate in three to six independent replicates and were monitored for survival.

(iii) Spores. *B. thuringiensis* DB27 was grown overnight in a shaking incubator at 30°C in Luria-Bertani (LB) broth. An 80-μl volume of the culture was spread to the edges of 6-cm-diameter NGM plates, and plates were incubated for 24 h at 25°C before the assay. A total of 20 adult worms were placed into each plate in three to six independent replicates and were monitored for survival. Survival assays were repeated multiple times and conducted at 25°C.

Chemotaxis assays. Chemotaxis assays were modified from previous studies (16). Briefly, 25 μl of overnight *B. thuringiensis* DB27 suspension was placed 0.5 cm away from the edge of a 6-cm-diameter petri dish filled with NGM. The same amount of *E. coli* OP50 was placed on the opposing side and acted as the counterattractant. Approximately 50 to 200 J4/adult-stage *C. elegans* individuals were placed between the two bacterial spots. All nematodes used were previously fed on *E. coli* OP50. Plates were then sealed with Parafilm and stored at room temperature in the dark. After defined periods, the number of nematodes found in each bacterial spot was recorded. A chemotaxis index was used to score the response of the nematodes and was calculated as follows: number of nematodes in the test bacteria − number of nematodes in control bacteria/total number of nematodes counted (16). This gave a chemotaxis score ranging from −1.0 (total repulsion from test bacteria) to 1.0 (total attraction toward test bacteria). A score of around 0 means there were equal numbers of nematodes in all bacterial spots. Five plates were used per replicate, and the procedure was repeated five times.

Pulse-chase experiment. Pulse-chase experiments were conducted to find the minimum time required for *B. thuringiensis* DB27 to establish infection in *C. elegans*. Plates for pulse-chase experiments were prepared in the same way as for the nematode killing assay (mixture of vegetative cells and spores). Larval stage 4 (L4)-synchronized worms were exposed to *B. thuringiensis* DB27 for a defined period of time, washed five times with phosphate-buffered saline (PBS) buffer to remove surface bacteria, and then shifted to *E. coli* OP50 plates. Survival was scored after 24 h.

Plasmid isolation, gel electrophoresis, and plasmid curing. Plasmids of *B. thuringiensis* DB27 were extracted following the protocol of Reyes-Ramírez and Ibarra (17). Plasmids were resolved using 0.7% agarose gel, following a previously published protocol (18). Plasmid curing was performed by growing *B. thuringiensis* DB27 at 42°C with small amounts (0.0002%) of SDS in culture medium. Plasmid-cured derivatives were selected based on changes in colony morphology after plating on LB plates and verified by plasmid profiling.

Coomassie stain and EM of crystals. *B. thuringiensis* sporulation medium (the recipe can be found at http://www.bgsc.org) was used to produce large amounts of spore-crystal mixtures. *B. thuringiensis* DB27 spore-crystal mixtures were collected by centrifugation and washed three times with 1 M NaCl and ice-cold distilled water. The washed spore-crystal mixtures were resuspended in 1 ml of distilled water, and 10 μl of each sample was dropped onto a glass slide. Samples were fixed in 1% OsO_4, air-dried overnight, and then coated with gold. The scanning electron microscopy (SEM) observation was conducted on a Hitachi S-800 microscope (Hitachi, Japan), following the instructions for the device. For light microscopy, spore-crystal mixtures were spread on a glass slide, heat fixed, stained with Coomassie blue (0.133% Coomassie blue–50% acetic acid), and observed under ×100 magnification using immersion oil.

Solubilization and SDS-PAGE profiling of crystal proteins. Spore-crystal mixtures were collected and washed as described above. The spore-crystal pellet was resuspended in solubilization buffer (50 mM Na_2CO_3, 25 mM dithiothreitol [DTT], pH 10.5) and incubated at 37°C for 2 h. Insoluble remainings were removed by centrifugation, and the solubilized proteins from the supernatant were analyzed using SDS-PAGE.

Toxin cloning, protein expression, killing assay, and synergism assays. The Cry21 genes were PCR amplified from genomic DNA of *B. thuringiensis* DB27, digested with BamHI restriction enzyme, and ligated into the BamHI site of expression vector pQE9, generating pQE9(Cry21) plasmids for expression of His-tagged proteins. Recombinant plasmids were electroporated into *E. coli* JM103, which is used for Cry toxin expression (5). Bacteria were grown at 37°C to midlog phase (optical density at 600 nm [OD_{600}] = 0.6 to 0.8), and expression of Cry21 was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After induction, bacteria were grown at 30°C for 4 h and then 30 μl of bacterial culture was spread in the center of enriched nematode growth (ENG) plates (19) containing 1 mM IPTG (ENG-IC plates). Plates were incubated overnight at 25°C and then used for nematode toxicity assays. Synchronized nematodes at the adult stage (20 per plate) were transferred to toxicity plates and monitored for survival and intoxication. Nematodes were transferred to fresh plates every day and were considered dead when they failed to respond to touch. *E. coli* JM103 transformed with empty pQE9 vector was used as a control. The expression of Cry21 protoxin was verified by SDS-PAGE.

For synergism assays, *E. coli* protoxin-expressing cultures were grown and induced as described above. After induction, the OD_{600} of each culture, including the empty vector control, was measured and adjusted to 2.0. To determine synergism between two proteins, the respective protoxin-expressing *E. coli* cultures were mixed in equal amounts (100 μl plus 100 μl) and 30 μl of bacterial culture was spread in the center of ENG plates and used for toxicity assays. Given that the final amount of protoxin-expressing *E. coli* in synergism plates was 50%, empty-vector *E. coli* culture mixed with each of the protoxin-expressing *E. coli* cultures (Cry21 plus vector) was used for comparison and each was termed a single protoxin treatment. When all three protoxins were used, three *E. coli* cultures were mixed in equal amounts.

Protein purification. Bacteria were grown at 37°C to midlog phase (OD_{600} = 0.6 to 0.8), and then IPTG was added at 1 mM final concentration to induce the expression of Cry21Fa1. Since Cry21Fa1 yield is higher at lower temperatures, after IPTG was added, the temperature was reduced to 25°C. Expression was carried out during a 10-h time period, and then cells were harvested by centrifugation (6,328 × *g* for 20 min). Cry21Ha1 was expressed similarly with the exception that expression was carried out during a 6-h time period at 30°C. The bacterial pellet was resuspended in buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 0.5 mM β-mercaptoethanol, 4 mM $MgCl_2$, a protease inhibitor mix [Roche], phenylmethylsulfonyl fluoride [PMSF], DNase I), sonicated, and centrifuged at 35,000 rpm for 1 h at 4°C using an ultracentrifuge (Beckman).

The supernatant was applied onto a nickel-nitrilotriacetic acid (Ni-NTA) column (GE Healthcare). Bound protein was eluted from the column with a linear gradient of 0 to 0.5 M imidazole in a buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 0.5 mM β-mercaptoethanol). Fractions were analyzed by SDS-PAGE and Western blotting using anti-His antibodies. Cry21-containing fractions were pooled, dialyzed against buffer (20 mM Tris-HCl, 10 mM NaCl, 0.5 mM β-mercaptoethanol), and loaded on an anion exchange column (Mono Q; GE Healthcare). A linear gradient of 0 to 3 M KCl in the loading buffer was used to elute bound Cry21. The Cry21 fractions were identified by SDS-PAGE and Western blotting, pooled, and dialyzed against 20 mM Tris-HCl–150 mM NaCl–0.5 mM β-mercaptoethanol buffer. The final step was size exclusion chromatography (Superdex 200; GE Healthcare) performed using 20 mM Tris-HCl–150 mM NaCl–0.5 mM β-mercaptoethanol. Fractions were analyzed by SDS-PAGE and Western blotting, pooled, and concentrated. Western blotting was performed with standard procedures as described elsewhere (20). Briefly, protein samples were separated in 8% SDS-PAGE gels and then transferred to nitrocellulose membranes (GE Healthcare). The membrane was incubated overnight with a 1:1,000 dilution of anti-His antibodies. Horseradish peroxidase-conjugated secondary antibody was used at 1:5,000. The signal was visualized with enhanced chemiluminescence (Bio-Rad).

Liquid assay with purified Cry21 protoxins. Purified protoxins were used for *C. elegans* single-well toxicity assays as described in reference 19 to determine the effect of known concentrations of protoxin on single nematodes. L4 nematodes were individually placed into the wells of 96-well microtiter plates. Each well contained S medium (prepared as described in reference 19), 3 μl of a saturated OP50 culture as a standard food, the desired dose of purified Cry21 protoxin, and 2 μl of 8 mM FUDR (5-fluorodeoxyuridine). The final volume in each well was 120 μl. Wells containing buffer instead of protoxin were used as a control. To calculate LC_{50} values, L4 *C. elegans* hermaphrodites were subjected to a single-well assay and incubated in a humid chamber for 5 days at 25°C. Nematodes that did not show movement when touched were considered dead. Experiments were repeated at least three times per dose, and representative data were used to generate a semilog plot. The fraction of dead worms was plotted as a function of protoxin concentration using a semilog plot. Dead or intoxicated nematodes were not observed in buffer-containing control wells. The data were fitted to a line by the least-squares method, and LC_{50} was calculated from the line fit. Probit analysis (Minitab) was used to calculate 95% fiducial limits. The data (total number dead/number tested) are as follows: for Cry21Fa1, 22/24 at 58 μg/ml, 20/30 at 29 μg/ml, 20/48 at 14.5 μg/ml, 10/30 at 7.25 μg/ml, 6/36 at 3.1 μg/ml, and 3/36 at 1.6 μg/ml; and for Cry21Ha1, 22/24 at 80 μg/ml, 16/26 at 40 μg/ml, 10/28 at 20 μg/ml, 8/32 at 10 μg/ml, and 4/32 at 5 μg/ml.

To determine the potential synergism between Cry21Ha1 and Cry21Fa1 protoxins, nematodes were exposed to different protein ratios (1:1, 1:2, and 2:1) of Cry21Ha1/Cry21Fa1 mixtures in a single-well assay and observed LC_{50} values were calculated as described above. The theoretical (expected) LC_{50} values were calculated according to Tabashnik's equation (21), assuming a simple additive effect. The theoretical LC_{50} value is the harmonic mean of the intrinsic LC_{50} values of the components weighted by the ratio used in the mixture. The synergism factor (SF) was calculated by dividing the expected toxicity by the observed toxicity of the mixture. SF values greater than 1 indicate synergism.

Statistical analysis. Kaplan-Meier nonparametric comparison and a log-rank test (Minitab) were used for statistical analysis of survival curves based on the number of survivors at the sampled time points. In cases where multiple replicates were examined in one experiment, the average survival rate at each time point was determined. Bonferroni correction was applied when multiple comparisons were performed. Statistical significance was set at $P \leq 0.05$. Log-rank statistical analysis of survival curves is shown in Table S1 in the supplemental material.

Nucleotide sequence accession numbers. The nucleotide sequences for Cry21Fa1, Cry21Ga1, and Cry21Ha1 in strain DB27 have been deposited in the GenBank database (accession no. KF701307, KF771885, and KF771886, respectively) and designated Cry21Fa1, Cry21Ga1, and Cry21Ha1 by the *Bacillus thuringiensis* Toxin Nomenclature Committee.

RESULTS

***B. thuringiensis* DB27 kills *C. elegans* via intestinal damage.** The *B. thuringiensis* DB27 strain was isolated previously and potentially uses novel virulence factors, since *C. elegans bre* mutants resistant to *B. thuringiensis* Cry5B toxin are susceptible to *B. thuringiensis* DB27 (12). *B. thuringiensis* DB27 showed remarkable toxicity for *C. elegans*, killing 100% of worms in just 16 h (Fig. 1A) (12, 14). Microscopic examination of the bacterial culture used in this assay revealed the presence of a mixture of vegetative cells and spores (see Fig. S1A in the supplemental material). To elucidate which stages of *B. thuringiensis* DB27 are virulent to nematodes, the assay plates were prepared with pure vegetative cells or spores for verification by microscopy (see Fig. S1B and C). Interestingly, survival of the worms was not affected on pure vegetative cells (Fig. 1A) even after 36 h, while pure spores showed toxicity even higher than that seen with the mixture of spores and vegetative cells (Fig. 1A). These findings suggest that virulence factors are produced during sporulation but not by vegetative cells. Although the spores were the most virulent, we used in all subsequent assays mixed cultures of spores and vegetative cells to prevent starvation (22), which might affect the outcome of the assay.

Additionally, we noticed that *C. elegans* did not avoid *B. thuringiensis* DB27 in the chemotaxis assay, showing equal preferences for OP50 and *B. thuringiensis* DB27 (Fig. 1B).

To study whether *B. thuringiensis* DB27 is able to establish infection in *C. elegans*, we performed pulse-chase experiments, where worms were exposed to *B. thuringiensis* DB27 for a defined period of time and then shifted to *E. coli* OP50. We found that a 6-h pulse was sufficient to establish a lethal infection in nearly 100% of worms (Fig. 1C). After 3 h, nearly 40% of worms were already infected, suggesting that *B. thuringiensis* DB27 relies on an active infection process as part of its virulence mechanism. This is further supported by our microscopy observations. Compared to control worms on *E. coli* OP50 (Fig. 1D), *B. thuringiensis* DB27-infected worms exhibited strong intestinal changes, namely, intestinal shrinkage and dissociation from body walls (Fig. 1E). Furthermore, we could also show a dramatic accumulation of *B. thuringiensis* DB27 spores and cells in the *C. elegans* intestine (Fig. 1F), which is consistent with the pulse-chase experiments and confirms that *B. thuringiensis* DB27 relies on an active infection process. Similar levels of intestinal destruction and accumulation of spores in the intestine were reported previously for another nematicidal *B. thuringiensis* isolate (23). Together, these results show that *B. thuringiensis* DB27 infects the *C. elegans* intestine, leading to intestinal damage and subsequent death of the nematode.

***B. thuringiensis* DB27 is pathogenic to diverse nematodes.** To determine the specificity of *B. thuringiensis* DB27 virulence, we exposed several other nematodes to the pathogen. As shown in Fig. 1G and H, the nematodes *Oscheius carolinensis*, *Pelodera strongyloides*, and *Panagrellus redivivus* were also killed by *B. thuringiensis* DB27, although they showed different degrees of susceptibility. While *O. carolinensis* and *P. strongyloides* were more resistant to *B. thuringiensis* DB27 than *C. elegans* (Fig. 1G), *P. redivivus* was significantly more susceptible (Fig. 1H). In addition, we found that the free-living stage of the animal-parasitic nematode

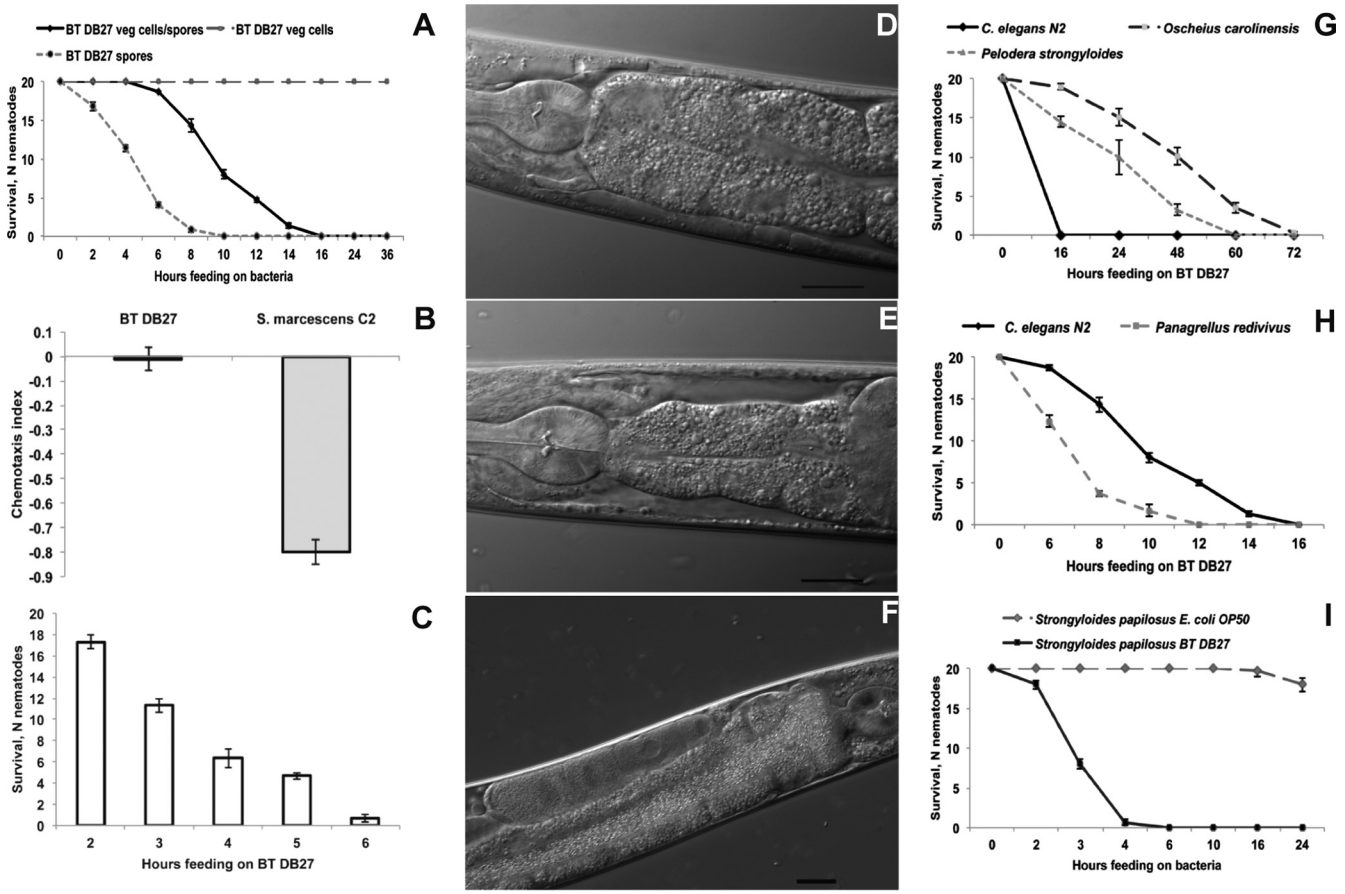

FIG 1 *B. thuringiensis* DB27 (BT DB27) kills diverse nematodes via intestinal infection. (A) *C. elegans* survival on monoxenic cultures of *B. thuringiensis* DB27. Pure spores of *B. thuringiensis* DB27 are significantly ($P < 0.0001$) more toxic to *C. elegans* than a mixture of spores and vegetative cells. Vegetative (veg) cells alone are not virulent. (B) *B. thuringiensis* DB27 did not repel *C. elegans* in a chemotaxis assay, in contrast to *Serratia marcescens*, used as a positive control. (C) *C. elegans* survival after a short exposure to *B. thuringiensis* DB27 (pulse-chase). The *x* axis shows the pulse (time of exposure to the pathogen). The *y* axis shows the number of nematodes (scored 24 h postinfection) that recovered after a given pulse. (D to F) *C. elegans* intestinal changes caused by *B. thuringiensis* DB27. Compared to control worms on OP50 (D), worms exposed to *B. thuringiensis* DB27 exhibit intestinal shrinkage (E) and accumulation of bacteria in the gut (F). Bar, 20 μm. (G to I) Other nematodes show levels of susceptibility that differ from that of *B. thuringiensis* DB27. (G) *O. carolinensis* and *P. strongyloides* are more resistant ($P < 0.0001$) to *B. thuringiensis* DB27-mediated killing than *C. elegans*. (H) *P. redivivus* is significantly ($P < 0.001$) more susceptible than *C. elegans*. (I) Animal-parasitic nematode *S. papillosus* is killed rapidly by *B. thuringiensis* DB27 compared to control *E. coli* OP50. For survival curves, the number of worms alive (N nematodes) is plotted as a function of time. The data shown are means ± standard errors of the means.

Strongiloides papillosus was also highly susceptible to *B. thuringiensis* DB27, being killed in as little as 4 h (Fig. 1I). Thus, *B. thuringiensis* DB27 is toxic to a number of free-living and also animal-parasitic nematodes, indicating its potential application as a nematicidal agent.

Role of plasmids in *B. thuringiensis* DB27 virulence. Given that *B. thuringiensis* major virulence factors are often encoded by plasmids (24), we next explored the role of *B. thuringiensis* DB27 plasmids in pathogenicity against nematodes. First, we extracted and gel-separated plasmids of *B. thuringiensis* DB27 and found that it harbored seven plasmids, ranging in size from 3 to above 16 kilobases (Fig. 2A). Next, we generated several plasmid-cured variants of *B. thuringiensis* DB27, some of which lost all seven plasmids (Fig. 2A). When we exposed *C. elegans* to those plasmid-cured variants, we found that they lost their virulence completely (Fig. 2B), suggesting that nematicidal factors are encoded by plasmids.

B. thuringiensis Cry toxins are almost exclusively encoded by plasmids (24) and are therefore potential nematicidal candidates produced by *B. thuringiensis* DB27. To explore that idea further, we tested whether *B. thuringiensis* DB27 produces Cry toxins. Light microscopy of Coomassie-stained spore-crystal mixtures indicated the presence of Cry protein crystals in *B. thuringiensis* DB27 (Fig. 2C) but not in a plasmid-cured derivative (Fig. 2D). Interestingly, crystals showed a strong association with spores, the phenotype previously described as spore-crystal association (SCA) (25). SEM images confirmed this phenotype (Fig. 2E) and showed striking similarity to the SCA of a rare filamentous *B. thuringiensis* strain (26). In contrast to wild-type *B. thuringiensis* DB27, the plasmid-cured variant did not produce any Cry protein crystals, as shown in light microscopy (Fig. 2D) and scanning EM images (Fig. 2F). To further confirm this phenotype, we solubilized proteins from spore-crystal mixtures of *B. thuringiensis* DB27 and its plasmid-cured variant and resolved them in an SDS-PAGE gel. While the *B. thuringiensis* DB27 profile revealed a dominant protein of around 130 kDa (see Fig. S2 in the supplemental material), which corresponds to the size of some Cry protoxins, the profile of the plasmid-cured variant showed no proteins at all

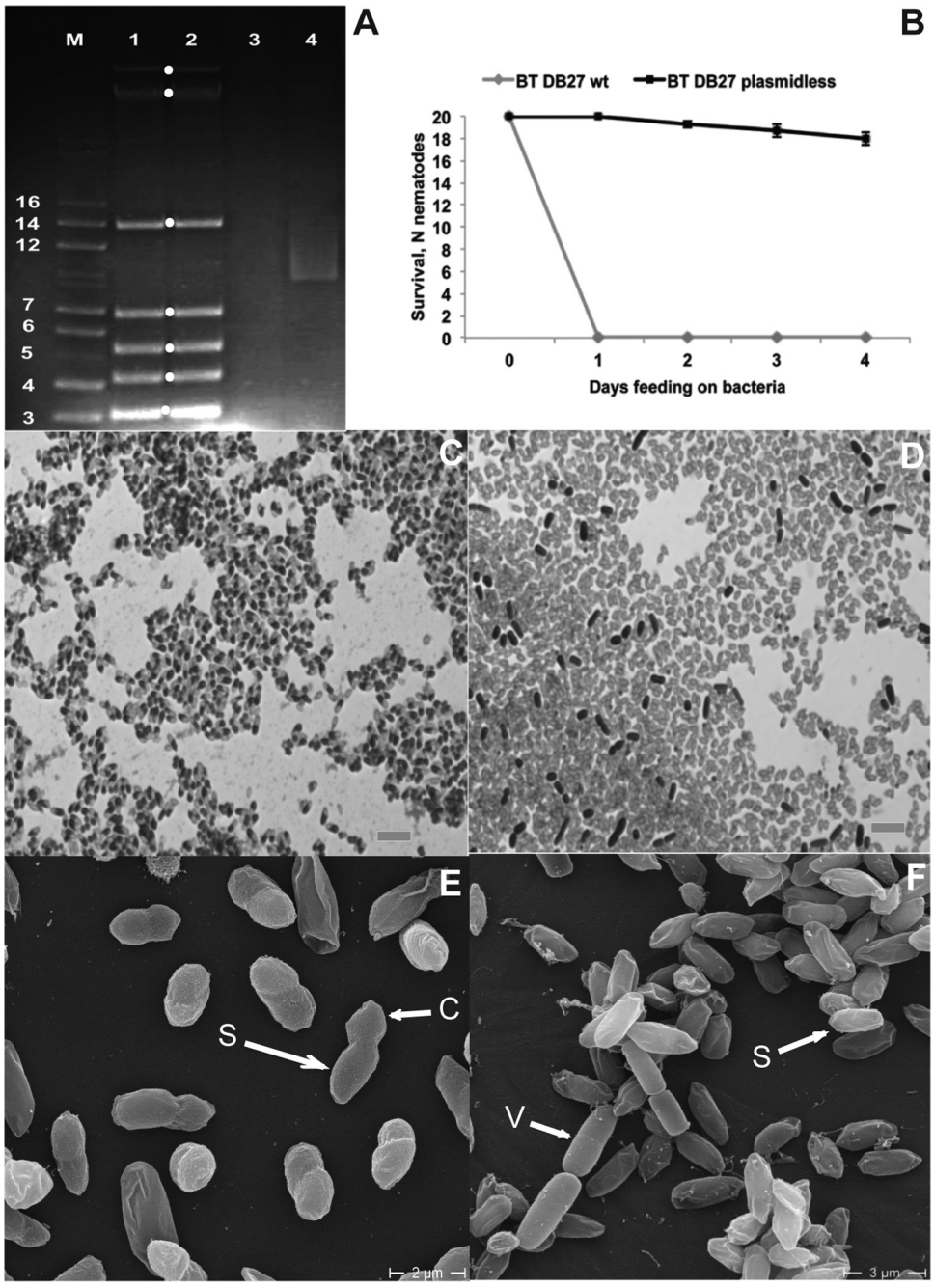

FIG 2 *B. thuringiensis* DB27 virulence factors are plasmid-associated Cry protoxins. (A) Plasmid profile of *B. thuringiensis* DB27 (lanes 1 and 2) and its plasmid-cured derivative (lanes 3 and 4). *B. thuringiensis* DB27 has seven plasmids (marked with dots), while the plasmid-cured variant lost all of them. M, marker (kb). Considering that the marker is represented by linear DNA fragments and the state of the plasmids (linear, circular, or supercoiled) is not known, it cannot be used as a determinant of the exact plasmid size. (B) *C. elegans* survival on the *B. thuringiensis* DB27 plasmid-cured variant is not affected ($P < 0.0001$) compared to that of the wild-type (wt) strain. (C and D) Light microscopy images (100×) of Coomassie-stained spore-crystal mixtures of wild-type *B. thuringiensis* DB27 (C) and its plasmidless derivative (D). Crystals (stained in black in panel C) show a strong association with the spore (SCA phenotype). No crystal inclusions are formed by plasmid-cured derivative. Rod-like black structures not attached to spores are vegetative cells. Bars, 100 μm. (E and F) Scanning electron microscopy images of spore-crystal mixtures of wild-type *B. thuringiensis* DB27 (E) and its plasmidless derivative (F). Arrows point to spores (S), crystals (C), and vegetative cells (V).

(see Fig. S2). These findings confirm our microscopy results showing that the plasmid-cured variant does not produce Cry protoxins. Together, these results indicate that *B. thuringiensis* DB27 virulence factors are plasmid-encoded Cry protoxins, which also agrees with the fact that sporulating cultures show high virulence activity (Fig. 1A). Consistent with this, vegetative cells that did not kill nematodes (Fig. 1A) did not produce any Cry proteins, as verified by SDS-PAGE (see Fig. S3); spores that showed the highest virulence also yielded the largest amount of Cry proteins (see Fig. S3); and spore/vegetative cell mixtures showed an intermediate

level of killing and an intermediate amount of Cry proteins (see Fig. S3). Thus, there is a correlation between the amounts of Cry protoxins produced by different stages and the levels of nematode lethality.

Candidate virulence factors identified by genome sequencing. To gain further insight into *B. thuringiensis* DB27 virulence factors, we sequenced the genome of this bacterium (27) and analyzed the genome sequence for the presence of potential virulence factors. Multiple proteases, enterotoxins, cytotoxins, collagenase, and chitinase were found (see Table S2 in the supplemental material). However, neither Cyt nor Vip (vegetative insecticidal protein) toxins implicated in *B. thuringiensis* insecticidal activity (7, 28) were found in the *B. thuringiensis* DB27 genome. Besides the circular chromosome, whole-genome sequencing revealed the presence of seven plasmids, ranging in size from 4 to 200 kb (See Table S3 in the supplemental material), which agrees with the observed plasmid profile (Fig. 2A). Given that our results suggest the nematicidal factors to be plasmid-encoded Cry protoxins, we concentrated specifically on plasmid-encoded factors. Indeed, we found several Cry-like toxins belonging to nematicidal families to be located on plasmids (See Table S3). Specifically, the 200-kb plasmid harbored a Cry-like toxin which showed similarity to the Cry21Ba1 toxin. In addition, the 8-kb and 6-kb plasmids also carried Cry-like toxins, both of which are similar to Cry21Ba1. Although a BLAST search identified sequence similarity of all three toxins to Cry21Ba1 toxin, more-detailed sequence comparisons revealed that all three proteins were different and that only the C-terminal part was conserved (see Fig. S4). Considering the low degree of sequence similarity to known Cry toxins, all three proteins were identified by the Cry toxin nomenclature committee as novel and were assigned new official names (See Table S3). Based on their sequence similarity, the newly identified Cry21 proteins are potentially nematicidal. However, they probably differ in the sensitivity spectrum of nematodes and the extent of toxicity.

Novel Cry21 protoxins show synergistic nematicidal activity. To test the potential role of these proteins in toxicity to *C. elegans*, we cloned them individually into the unique BamHI restriction site of the pQE9 *E. coli* protein expression vector. *E. coli* transformed with pQE9-Cry21 plasmids produced a Cry protein of above 130 kDa (not shown), which corresponds to the size of some Cry protoxins. This also suggests that the 130-kDa protein from the *B. thuringiensis* DB27 spore-crystal mixture corresponds to Cry21 protoxins, given the similarities in size.

When we fed *C. elegans* with *E. coli* expressing the three protoxins individually, we observed different degrees of intoxication (Fig. 3A). Cry21Fa1 was the most effective protoxin, killing worms in around 48 h. Cry21Ha1 showed moderate toxicity and caused 100% lethality in 5 days. In contrast, Cry21Ga1 did not show any obvious toxicity to *C. elegans* even after 5 days (Fig. 3A). Note that longer exposure times were not feasible in these experiments since even *E. coli* with empty vector is toxic to *C. elegans* on rich medium such as ENG. Worms fed with Cry21Fa1 or Cry21Ha1 protoxin exhibited classical intoxication phenotypes, such as slow movement, pale appearance, reduction in body size, and, finally, death of the worms. Additionally, in contrast to nematodes fed on *E. coli* with an empty vector (Fig. 3B), worms exposed to the *E. coli*-expressed protoxin exhibited intestinal shrinkage and damage (Fig. 3C), in similarity to worms fed with *B. thuringiensis* DB27 (Fig. 1E).

Given that Cry toxins often show synergistic action (29), we next fed *C. elegans* with mixtures of *E. coli* clones expressing different protoxins. As shown in Fig. 3D, combining Cry21Fa1 and Cry21Ha1 protoxins resulted in significantly higher *C. elegans* mortality compared to that seen with the single protoxins, suggesting that the two protoxins might act synergistically. A Cry21Ga1 combination with Cry21Fa1 and/or Cry21Ha1 did not increase *C. elegans* lethality compared to single protoxins (Fig. 3D), indicating that Cry21Ga1 (alone or in combination with other protoxins) is not involved in *C. elegans* killing. Interestingly, the *C. elegans nasp-1*(*tu439*) mutant, which is resistant to *B. thuringiensis* DB27-mediated killing, also exhibits increased resistance to the Cry21Fa1 and Cry21Ha1 protoxins (Fig. 3E). Thus, Cry21Fa1 and Cry21Ha1 are important nematicidal factors produced by *B. thuringiensis* DB27 that show potential synergistic action.

Quantitative effect of Cry21Fa1 and Cry21Ha1 on *C. elegans*. To quantify Cry21 protoxin actions, Cry21Fa1 and Cry21Ha1, which showed toxicity to *C. elegans* in a feeding experiment (Fig. 3A), were purified as a His-tagged proteins using affinity chromatography, verified by Western blotting (see Fig. S5 in the supplemental material), and used to intoxicate *C. elegans* in liquid assays. The purified protoxins showed clear dose-dependent action (Fig. 3F and G and Materials and Methods). While 58 μg/ml of Cry21Fa1 protoxin was sufficient to kill 100% of worms in 5 days, the estimated concentration of Cry21Fa1 that kills 50% of worms is 13.6 μg/ml (calculated LC_{50}). For Cry21Ha1, the LC_{50} is 23.9 μg/ml. Thus, Cry21Fa1 is almost twice as toxic as Cry21Ha1, which agrees with the results of the feeding experiment (Fig. 3A). While these values are very close to the LC_{50} of another nematicidal toxin, Cry5B (12.6 μg/ml) (30), direct comparison is not possible due to experimental differences in the toxin purification. When Cry21Ha1 and Cry21Fa1 protoxins were combined at different ratios (Table 1), all the observed LC_{50} values of protoxin mixtures were lower than the expected LC_{50} values and the mixtures exhibited clear synergistic activity. Specifically, the Cry21Fa1/Cry21Ha1 combination with a ratio of 2:1 exhibited the best synergistic activity (LC_{50} 6.1 μg/ml), representing a 2.6 reduction in the LC_{50} value compared to the expected LC_{50} (15.88 μg/ml) (Table 1).

***C. elegans* requires conserved defense pathways against pore-forming toxins.** Considering that multiple *C. elegans* pathways involved in the defense against nematicidal Cry5B toxin have been previously described (31–33), we wanted to test whether they are important for the defense against Cry21 protoxins. First, we tested *C. elegans bre* mutants that lack the receptor for Cry5B (32). Interestingly, those mutants showed slightly increased but not significantly different survival compared to wild-type animals when exposed to *E. coli*-expressed Cry21 protoxins (Fig. 4A). This result is consistent with previous studies and agrees with the fact that *bre* mutants do not show cross-resistance to multiple Cry toxins (32). This finding also indicates that Cry21Fa1 and Cry21Ha1 protoxins may require a different and/or additional receptor or epitope in comparison to the Cry5B toxin. The p38 mitogen-activated protein kinase (MAPK) pathway and the c-Jun N-terminal kinase (JNK) MAPK pathway were previously shown to play a central role in the *C. elegans* response to Cry5B pore-forming toxin (33). Consistent with this, we found that animals carrying mutations in the p38 MAPK pathway (*pmk-1*) or in a downstream transcription factor of JNK MAPK pathway *jun-1* were all hypersusceptible to Cry21Fa1 protoxin (Fig. 4B). JNK MAPK pathway mutant *kgb-1*

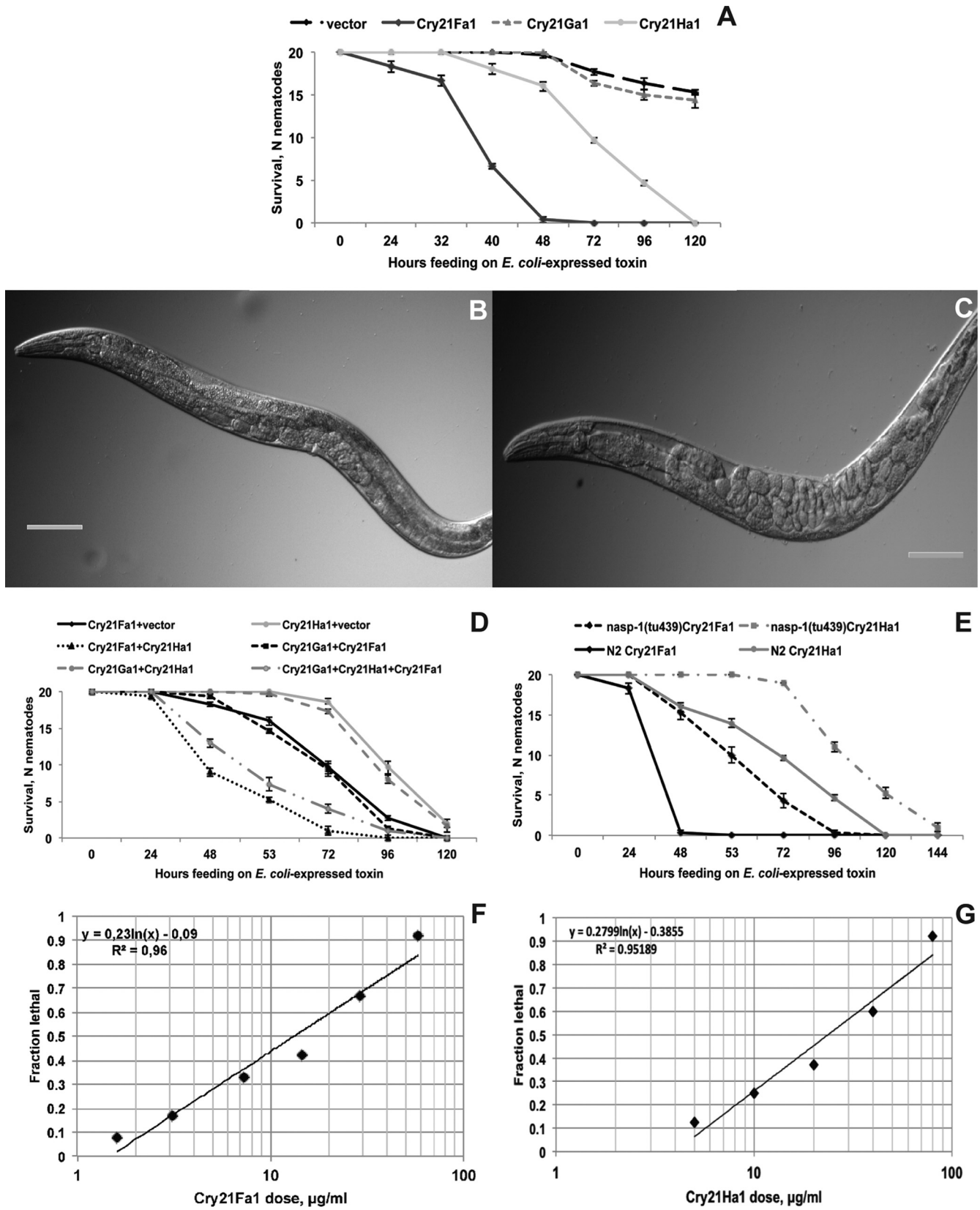

FIG 3 Cry21 protoxins are the nematicidal virulence factors of *B. thuringiensis* DB27. (A) *C. elegans* survival on *E. coli* which expresses individual Cry21 protoxins. Cry21Fa1 and Cry21Ha1 significantly ($P < 0.0001$) reduce *C. elegans* survival compared to the vector control. The Cry21Ga1 effect is similar ($P > 0.05$) to that of the vector control. (B and C) *C. elegans* fed with *E. coli*-expressed Cry21Fa1 protoxin exhibits dramatic intestinal shrinkage and destruction (C)

TABLE 1 Toxicity to *C. elegans* of Cry21Fa1 and Cry21Ha1 single protoxins and their mixtures

Cry21Fa1/ Cry21Ha1 ratio	LC_{50} (μg/ml)		Synergism factor[c]
	Observed[a]	Expected[b]	
1:0	13.6 (10.07–19.6)		
0:1	23.9 (18.07–33.25)		
1:1	8.7 (6.21–11.98)	17.33	1.99
1:2	7.98 (4.75–12.76)	19.08	2.39
2:1	6.1 (3.88–8.56)	15.88	2.6

[a] LC_{50}s were determined experimentally; 95% fiducial limits determined by Probit analysis are given in parentheses.
[b] Theoretical LC_{50}s were calculated by using Tabashnik's equation and assuming a simple additive effect.
[c] Synergism factors were calculated by dividing the expected LC_{50} by the observed LC_{50}.

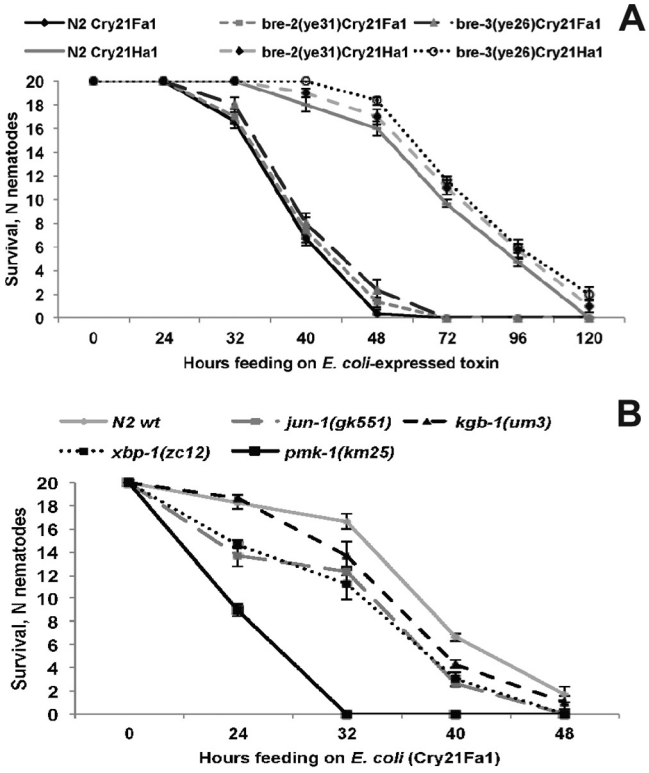

FIG 4 (A) *bre-2* and *bre-3* mutants are as susceptible to Cry21Fa1 and Cry21Ha1 protoxins as wild-type worms. (B) *C. elegans jun-1* ($P < 0.05$), *xbp-1* ($P < 0.05$), and *pmk-1* ($P < 0.0001$) mutants are hypersensitive to Cry21Fa1 protoxin compared to wild-type worms. Survival of the *kgb-1* mutant is not significantly ($P > 0.05$) different from wild-type survival. The data shown are means ± standard errors of the means.

was slightly, but not significantly, more susceptible to Cry21Fa1 protoxin (Fig. 4B). This surprising result suggests that the role of *kgb-1* in *C. elegans* defense against pore-forming toxins might be toxin dependent, and more-detailed investigation will be needed to elucidate the molecular mechanisms of the differentially protective role of *kgb-1*. Additionally, we found that *xbp-1* mutants, which are sensitive to Cry5B (31), are also more susceptible to Cry21Fa1 than wild-type worms (Fig. 4B), confirming the protective role of the *xbp-1* pathway against pore-forming toxins. Thus, *C. elegans* requires some conserved pathways for defenses against multiple Cry pore-forming toxins.

DISCUSSION

In the present study, we characterized novel virulence factors of the highly nematicidal *B. thuringiensis* DB27 strain. Combining plasmid curing, whole-genome sequencing, and a candidate gene approach, we identified three proteins related to Cry21Ba1 toxin as potential nematicidal factors. Previous methods of Cry toxin identification are clearly dominated by PCR-based techniques (34). While these methods proved to be useful, they have certain limitations. Therefore, whole-genome sequencing and genome mining become reasonable alternatives for the identification of novel Cry toxins (8, 35). Applying these techniques, we found that three different plasmids encode three Cry-like proteins. All three proteins appeared to be novel Cry protoxins and were assigned new official names.

In contrast to previous reports showing that the genes encoding Cry toxins are located on large plasmids (24), which is also true for Cry21Fa1 protoxin produced by *B. thuringiensis* DB27, we found that two other protoxins, Cry21Ga1 and Cry21Ha1, are encoded by small 8- and 6-kb plasmids, respectively. Interestingly, another nematicidal toxin, Cry55Aa1, was also encoded by a relatively small 17.7-kb plasmid (8). Whether this unusual location has any functional consequences is not clear yet and remains to be elucidated.

While all three novel proteins belong to the Cry21 family of nematicidal toxins, only two, Cry21Fa1 and Cry21Ha1, showed activity against *C. elegans*. Whereas they are potent as single protoxins, they also showed synergistic activity against *C. elegans*. Synergism between Cry toxins and Cyt toxins has been described previously (7, 29). Additionally, enzymes such as chitinase and collagenase and different proteases have been shown to have an enhancing effect on Cry toxin efficiency (3, 36). Given that whole-genome sequencing of *B. thuringiensis* DB27 revealed the presence of multiple enzymes with potential enhancing properties, we do not exclude their involvement in *B. thuringiensis* DB27 virulence, but their role awaits further investigation. At this stage, it is not known why Cry21Ga1 is not active against *C. elegans*. It is possible that Cry21Ga1 functions in combination with other toxins and/or enzymes. In addition to this, there are many other examples in which Cry toxins do not show pesticidal activity (37). Thus, the exact target host and molecular function of Cry21Ga1 in *B. thuringiensis* DB27 pathogenicity will require further investigation.

B. thuringiensis strains very often carry multiple plasmids with

compared to vector-fed control worms (B). Bars, 100 μm. (D) *C. elegans* survival upon exposure to combinations of *E. coli* clones that express different Cry21 protoxins. *C. elegans* survival is significantly ($P < 0.0001$) reduced when worms are exposed to the combination of Cry21Fa1 and Cry21Ha1 protoxins compared to exposure to each toxin individually. Combining Cry21Ga1 with either Cry21Fa1 or Cry21Ha1 does not significantly ($P > 0.05$) change the survival compared to that seen with the individual protoxins. The combination of all three proteins is almost as toxic as the combination of Cry21Fa1 and Cry21Ha1, suggesting that Cry21Ga1 has no synergistic effect. (E) The *nasp-1* mutant is significantly more resistant to Cry21Fa1 ($P < 0.0001$) and Cry21Ha1 ($P < 0.001$) protoxins than the wild-type strain. The data shown are means ± standard errors of the means. (F and G) *C. elegans* dose-dependent lethality to purified Cry21Fa1 (F) and Cry21Ha1 (G) protoxins in a liquid assay. A semilog plot of animals that died versus the concentration of toxin is shown. The data were fitted to a line by the least-squares method. The LC_{50}s for Cry21Fa1 (13.6 μg/ml) and for Cry21Ha1 (23.9 μg/ml) were calculated from the line fits. The data can be found in Materials and Methods.

different Cry toxins. *B. thuringiensis* DB27 carries seven plasmids, two of which harbor nematicidal Cry21 protoxins with synergistic activity. This trait may provide a strong selective advantage to the pathogen. First, loss of one of the toxins does not completely eliminate its virulence. Second, the synergistic action of two toxins facilitates faster host killing than is seen with strains with a single toxin. Third, the presence of multiple toxins drastically reduces the probability of the targeted host developing resistance (38). Consistent with this, several rounds of mutagenesis were needed in order to isolate a *C. elegans nasp-1* mutant resistant to *B. thuringiensis* DB27 (12), while multiple alleles of five *bre* mutants resistant to Cry5B toxins were isolated in a single mutagenesis screen (30).

Previous studies have shown that *C. elegans* exhibits avoidance behavior when challenged with different pathogens (39). *B. thuringiensis* was one of the pathogens that *C. elegans* strongly avoided (40). Other nematode-pathogenic *Bacillus* spp., such as *B. nematocida*, evolved strategies to attract nematodes (41). Interestingly, in the case of *B. thuringiensis* DB27, worms showed neither repulsion from nor attraction to the bacteria. The absence of host-aversive behavior very likely benefits the pathogen via increasing the chances of successful infection.

Interestingly, EM and light microscopy revealed that *B. thuringiensis* DB27 spores and crystals have strong associations. Crystals are normally located outside the exosporium and are separated from spores after lysis of the mother cell. However, in a few strains, such as *B. thuringiensis* subsp. *finitimus* strains (42) and *B. thuringiensis* subsp. *oyamensis* strain LBIT-113 (43), the parasporal crystals are located between the exosporium and the spore coat and continue to adhere to the spore after mother cell lysis. This phenotype has been previously described as spore-crystal association (SCA) (25). While many studies have concentrated on identification of genes responsible for this phenotype (25, 26, 44), the functional significance of SCA is not yet clear. Considering that Cry proteins are not stable in the environment, the exosporium may be used as a protective membrane. At the same time, SCA may be used as a secure strategy to deliver the spores together with toxins to the host gut, which is not guaranteed when crystals are separated from spores. Given that *B. thuringiensis* spores (45) and vegetative cells (46) have been shown to enhance the toxicity of Cry proteins, SCA thus ensures that the two components are always present together to achieve fast killing of the host. Therefore, *B. thuringiensis* DB27 SCA may be an important advantageous strategy combined with the production of several novel Cry21 protoxins acting synergistically to ensure efficient host killing.

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