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Selection of a *Bacillus pumilus* Strain Highly Active against *Ceratitis capitata* (Wiedemann) Larvae[▽]

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***Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), the Mediterranean fruit fly (medfly), is one of the most important fruit pests worldwide. The medfly is a polyphagous species that causes losses in many crops, which leads to huge economic losses. Entomopathogenic bacteria belonging to the genus *Bacillus* have been proven to be safe, environmentally friendly, and cost-effective tools to control pest populations. As no control method for *C. capitata* based on these bacteria has been developed, isolation of novel strains is needed. Here, we report the isolation of 115 bacterial strains and the results of toxicity screening with adults and larvae of *C. capitata*. As a result of this analysis, we obtained a novel *Bacillus pumilus* strain, strain 15.1, that is highly toxic to *C. capitata* larvae. The toxicity of this strain for *C. capitata* was related to the sporulation process and was observed only when cultures were incubated at low temperatures before they were used in a bioassay. The mortality rate for *C. capitata* larvae ranged from 68 to 94% depending on the conditions under which the culture was kept before the bioassay. Toxicity was proven to be a special characteristic of the newly isolated strain, since other *B. pumilus* strains did not have a toxic effect on *C. capitata* larvae. The results of the present study suggest that *B. pumilus* 15.1 could be considered a strong candidate for developing strategies for biological control of *C. capitata*.**

The Mediterranean fruit fly (medfly), *Ceratitis capitata*, is considered a highly invasive agricultural and economically important pest throughout the world. In less than 200 years the range of this species has expanded from its native habitat in sub-Saharan Africa, and it has become a cosmopolitan species (26) that is present on five continents (14, 46). The wide distribution of the medfly is attributed, among other things, to its remarkably polyphagous behavior (more than 300 host plants have been reported) (43), to its resistance to cold climates (65), and to successful establishment after multiple introductions (30, 49) as a result of the increasing frequency of global trade (46).

Medfly infestations cause serious economic losses and sometimes result in complete loss of crops (76). Numerous methods have been tried to control medfly populations, including chemical products, such as malathion and other organophosphate insecticides (4, 8), classic biological control programs based on the release of some of parasitoids and predators (38, 41, 44), toxic baits (2, 13, 31, 32, 35, 56), mass trapping systems (24, 51), the sterile insect technique (7, 34, 61, 63, 72, 73), and development of integrated strategies of management (71). In spite of all of these attempts, control of Mediterranean fruit fly populations has been ineffective, and losses associated with this pest worldwide are constantly increasing (21, 46).

Insecticides based on microbial agents (bacteria, fungi, and viruses) are a promising alternative that has received a great deal of attention for control of *C. capitata* (5, 13, 18, 40, 55), but so far no such insecticide has reached a commercial stage. Among the microbial insecticides, bacteria are very successful agents in biological control programs (17, 29). The entomopathogenic bacteria belonging to the genus *Bacillus* are natural agents used for biological control of invertebrate pests and are the basis of many commercial insecticides. Three species of the genus *Bacillus* have been mass produced and commercialized: *Bacillus sphaericus*, *Bacillus thuringiensis*, and *Paenibacillus popilliae* (formerly *Bacillus popilliae*) (29, 54). These organisms have different spectra and levels of activity that are correlated with the nature of the toxins, which are very frequently produced during sporulation (16, 17). *B. thuringiensis* was the first *Bacillus* species used in biological control programs for pests and human vector disease insects (17, 62). During its stationary phase, this Gram-positive, aerobic, ubiquitous, endospore-forming bacterium produces parasporal crystalline inclusions composed mainly of two types of insecticidal proteins (Cry and Cyt toxins) (62) that are toxic to a variety of insects, in some cases at the species level.

There have been some reports of *B. thuringiensis* strains active against other fruit flies (3, 37, 58, 59, 67), but there has been no report of any *Bacillus* strain with activity against *C. capitata*.

The aim of this study was to search for novel bacteria belonging to the genus *Bacillus*, specifically *B. thuringiensis*, with activity against adults and larvae of *C. capitata* that could be used as biological control agents. Isolation of 115 bacterial strains, evaluation of the insecticidal activities of these strains, and identification of a novel strain of *Bacillus pumilus* that is highly toxic to *C. capitata* larvae are reported here. In addition, we found that toxicity was observed only when cultures of *B. pumilus* strain 15.1 were exposed to low temperatures. The isolation of this novel pathogenic strain could be important for future development of biotechnological strategies aimed at reducing the economic losses caused by *C. capitata*.

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MATERIALS AND METHODS

Insects and rearing conditions. Our colony of *C. capitata* was established from a laboratory colony maintained at the Centro de Ecología Química Agrícola (CEQA) of the Universidad Politécnica de Valencia (Spain). The laboratory conditions used for rearing and bioassays were 25 ± 2°C, 65% ± 5% relative humidity, and a photoperiod consisting of 16 h of light and 8 h of darkness. Eggs laid through a net placed on one side of an insect-rearing cage were collected in a plastic container filled with distilled water. Larvae from 0.5 ml of eggs were raised on an artificial diet containing 200 g wheat bran, 50 g sucrose, 25 g brewer's yeast, 2 g Nipagin (methyl 4-hydroxybenzoate; Sigma), 2 g Nipasol (propyl 4-hydroxybenzoate; Sigma), 7.5 ml HCl 37%, and 500 ml distilled water (27). Third-instar larvae were transferred into a pupation chamber with sand and were maintained there for at least 1 week before they were collected using a sieve. Adult flies were fed an artificial diet comprised of 20% (wt/wt) yeast autolysate and 80% (wt/wt) sucrose. Water was provided to the flies via a damp yellow sponge.

Bacterial isolation and culture conditions. Bacterial strains were isolated from field samples, such as soil, decomposed and fresh fruits and leaves, dust, stagnant water, dead insects, soil mollusks, vegetable waste, ashes, dried mud, beach sand, and seawater, collected from an agricultural area in Almuñecar, which is in the province of Granada (south coast of Spain). This area has a well-established population of medfly throughout the year, which is considered the most damaging pest for local agriculture. Bacterial isolation was performed using the method described by Travers et al. (68), with minor modifications. In brief, 0.5 g of each field sample was placed in a 250-ml flask containing 10 ml LB (10 g tryptone, 5 g yeast extract, and 5 g NaCl in 1 liter of distilled water) supplemented with 0.25 M sodium acetate, which inhibited *B. thuringiensis* spore germination selectively. The mixture was incubated with aeration for 4 h at 250 rpm and 30°C to allow germination of any spores other than *B. thuringiensis* spores. All nonsporulating bacteria and all vegetative cells resulting from sporulating bacteria were removed by heat. Instead of using the tube-in-tube flowthrough pasteurization device used by Travers et al. (68), 1 ml of each mixture was placed in a 1.5-ml Eppendorf tube and heated in a water bath at 80°C for 10 min. Next, 100 μl of each sample was plated on LB plates and incubated overnight at 30°C. Typical *Bacillus* sp. colonies were selected for experiments.

At the same time, several bacteria were isolated from dead larvae of *C. capitata* found in Japanese plums (*Prunus salicina*) and custard apple (*Annona cherimolla*) picked in Almuñecar. Each larva was surface sterilized by immersing it three times in 1 ml of 70% ethanol for 5 min and rinsed three times with 1 ml of sterile water. Larvae were individually homogenized under aseptic conditions in 1 ml of LB. Tenfold serial dilutions were plated on LB plates and incubated at 30°C for 24 h. All of the bacteria isolated were kept in 40% (vol/vol) glycerol at −80°C.

Bacteria were routinely grown in LB and in T3 medium (3 g tryptone, 2 g tryptose, 1.5 g yeast extract, 0.05 M sodium phosphate [pH 6.8], and 0.005 g $MnCl_2$ in 1 liter of distilled water) until sporulation was desired. Sporulation of bacteria in T3 medium was monitored using an Olympus BH2-RFCA phase-contrast microscope. The total number of cells in a culture was determined by plating 10-fold serial dilutions on LB plates. The number of spores was determined by plating 10-fold dilutions of a 1-ml aliquot that had been heated at 80°C in a water bath for 10 min. The number of vegetative cells was calculated by subtracting the number of spores from the total number of cells.

Biotin requirement assays were performed by comparing bacterial growth in 50 ml of Spizizen minimal medium (66) supplemented with 0.05 μg/ml of D-biotin with bacterial growth in 50 ml of Spizizen minimal medium not supplemented with D-biotin.

Toxicity assays for screening. (i) Bioassays with *C. capitata* adults. The susceptibility of *C. capitata* adults to each bacterial isolate was tested under laboratory conditions. Each bacterial isolate was cultured in 7 ml of T3 medium at 30°C and 240 rpm for 144 h, until sporulation was complete (determined by microscopic inspection). Cultures were poured into 13-ml sterile glass bottles and offered to flies through a sponge. The adult diet was also supplied in each plastic bioassay chamber (12.5 by 10 by 5 cm). Each bioassay was performed with 10 newly emerged flies (five males and five females). In each set of bioassays a culture of a strain negative for expression of Cry toxins, *B. thuringiensis* IPS 78/11 (74), *Escherichia coli* XL1Blue/pSV10-wt (unpublished data) expressing a high level the Cry1Ac1 toxin active against Lepidoptera, T3 medium (no bacteria), and distilled water were included as negative controls. Mortality was recorded daily for 20 days. Under the bioassay conditions used the life span of a *C. capitata* adult was 28 to 34 days.

(ii) Bioassays with *C. capitata* larvae. The susceptibility of *C. capitata* first-instar larvae to each bacterial isolate was tested under laboratory conditions. For screening, each bacterial isolate was cultured in 3 ml of T3 medium at 30°C and 240 rpm for 72 h until sporulation was complete. The bioassays were performed in 48-well sterile Cellstar microplates (Greiner Bio-One) at 25°C. The artificial diet used was composed of two autoclave-sterilized mixtures (mixtures A and B) that were mixed just before use. Mixture A contained 52 g fine-grain wheat bran, 20 g sucrose, 10 g brewer's yeast, 1.5 ml HCl 37%, and 100 ml distilled water, while mixture B contained 1.6 g agar in 100 ml of distilled water. Five hundred microliters of artificial diet and 100 μl of a bacterial culture were dispensed sequentially in each well of a microplate, mixed thoroughly using a sterile toothpick, and left to settle at room temperature. One first-instar larva was placed on the surface of the diet, and once preparation of the plate was complete, a transparent film (Saran Wrap) was used to seal the wells. A hole was punctured over each well to provide sufficient aeration for the larvae. Each bacterial culture was tested with 24 larvae, and each bacterial isolate was bioassayed at least twice. In each set of bioassays, a culture of the acrystalliferous strain *B. thuringiensis* IPS 78/11, *E. coli*/pSV10-wt, and T3 medium were used as negative controls. Mortality was recorded 4, 10, and 15 days after initiation of the bioassay.

The mortality results of the screening bioassays were corrected using Abbott's formula (1), transformed (arcsine of the square root scale to normalize the variance), and analyzed using one-way analysis of variance (ANOVA). Treatment means were separated using Tukey's studentized range honestly significant difference (HSD) test, and treatments were compared with controls using Dunnett's test. All analyses were carried out using SPSS (version 16.0).

DNA isolation, PCR amplification, and 16S rRNA sequencing. Total DNA was obtained from 1.5 ml of an overnight bacterial culture in LB using a DNAeasy tissue kit (Qiagen) by following the supplier's instructions for Gram-positive bacteria. Two fragments (approximately 800 and 1,000 bp) of the 16S rRNA gene were amplified by PCR using two sets of universal primers, primers 533F (5′-GTGCCAGC[M]GCCGCGGTA-3′) (12) and 16SB1 (5′-TACGG[Y]TACCTTGTTACGACTT-3′) (19) and primers fD1 (5′-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3′) and rD2 (5′-GACTACCAGGGTATCTAATCC-3′) (75). The PCRs were carried out using approximately 500 ng of total bacterial DNA, 10 μl of 10× PCR buffer, 8 μl of $MgCl_2$ (25 mM), 10 μl of deoxynucleoside triphosphates (dNTPs) (2 mM each), 3.3 μl of each primer (20 μM), 0.5 μl of *Taq* polymerase (5 U/μl), and enough Milli Q water so that the final volume of the mixture was 100 μl. The PCR mixtures were denatured at 94°C for 5 min, which was followed by 45 cycles of 94°C for 45 s, 52°C for 45 s, and 72°C for 45 s and then a final extension at 72°C for 5 min. Amplification was checked by electrophoresis on a 0.8% (wt/vol) agarose gel. The bands of interest were excised from the gel, and the DNA was extracted using a QIAquick gel extraction kit (Qiagen). The PCR-amplified DNA fragments were sequenced using the same sets of primers that were used for amplification. Sequencing reactions were carried out using a final volume of 20 μl and a BigDye Terminator cycle sequencing kit (Applied Biosystems). The mixture contained 3 μl of PREMIX, 2 μl of 10× buffer, 140 ng of template DNA, 3 μl of primer (1 μM), and 8 μl of Milli Q H_2O. Sequencing PCRs were performed under the following conditions: 94°C for 3 min and then 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The sequencing products were analyzed with an automatic sequencer with four capillaries (model 3100 Avant genetic analyzer; Applied Biosystem/Hitachi) at the sequencing service of the University of Granada. Chromatograms were visualized with Chromas (version 1.45), and sequences were analyzed with the NCBI tool BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Bioassays with strain 15.1 and *C. capitata* larvae. After the first screening, the bioassays were modified slightly in order to determine and optimize the toxicity of strain 15.1. In order to accelerate the effect of the entomopathogenic activity, 50-ml portions of the sporulated cultures in T3 medium were frozen for at least 6 h, lyophilized, and resuspended in 5 ml of sterile distilled water. One hundred microliters of a concentrated culture was dispensed into each well and mixed with 500 μl of the larva diet. The bioassay plates were kept at 4°C for 96 h before a *C. capitata* larva was placed in each well. All bioassays were performed at least twice using 48 larvae for each replicate and using *B. pumilus* M1, *B. pumilus* M2, and T3 medium as negative controls. All results were subjected to ANOVA followed by means separation using the least-significant difference (LSD) procedure ($P > 0.05$).

To evaluate vegetative cell toxicity, LB cultures that were incubated at 30°C and 240 rpm for 10 h were used in bioassays.

Bioassays with culture fractions were performed using the supernatant and pellet fractions of sporulated cultures obtained by centrifugation at 35,000 × *g* for 20 min at 4°C (Beckman J2-21 M, JA-20 rotor). Supernatants were filtered using cellulose acetate syringe filters with a pore size of 0.20 μm (Sartorius, Goettingen, Germany), frozen, lyophilized, and resuspended in 0.1 volume of sterile distilled water before they were used in the bioassays. Pellets were resus-

pended in 5 ml of distilled water and kept at 4°C before they were used in the bioassays.

The dependence of toxicity on a low temperature and the incubation time was proven using bacterial cultures kept at 4°C or −20°C for 0, 96, and 168 h before they were lyophilized and included in a bioassay plate.

Fifty percent lethal concentrations (LC_{50}) and LC_{90} were determined using 2-fold dilutions ranging from 20× to 0.625× of the original culture (4.65×10^8 to 1.45×10^7 CFU/ml). Cultures were kept at −20°C for 168 h before lyophilization to activate toxicity. The average larval mortality data were subjected to Probit analysis (Minitab 15.1.30.0) to calculate the LC_{50}, LC_{90}, and confidence limits.

Nucleotide sequence accession number. The sequence of the 16S rRNA gene of *B. pumilus* strain 15.1 determined in the present study has been deposited in the GenBank database under accession no. EU978469.

RESULTS

Bacterial isolation. Thirty-seven diverse field samples were collected at an agricultural locality in the south of Spain. In this area, *C. capitata* is well established and is the most damaging fruit pest. It was assumed that some of the environmental samples collected would be good sources of natural enemies for this insect. Environmental samples (soil, dust, stagnant water, dead insects, etc.) were used as sources for isolation of bacteria belonging to the genus *Bacillus*. The total number of heterotrophs able to grow on LB was determined for each sample and ranged from 10^5 to 10^7 bacteria for soil, soil snails, and partially decomposed vegetable samples and from 0 to 10^3 bacteria for fresh fruit and leaves collected directly from trees. Using the selective method described by Travers et al. (68) for *Bacillus* spp. (and frequently used for *B. thuringiensis* isolation), we isolated approximately 10^4 bacteria from the 37 field samples. A total of 110 *Bacillus*-like colonies were randomly picked, streaked on LB plates, and obtained as pure cultures. After checking the ability of these organisms to grow in liquid T3 medium, as well as spore formation using a phase-contrast microscope, we selected 104 strains for further study. In addition, 11 bacterial strains were randomly isolated from *C. capitata* dead larvae (DLs strains) found inside fruits picked in fields. The activities of the 115 bacterial strains isolated were tested using both medfly adults and larvae.

Bioassays with adults and larvae of *C. capitata*. The biological activities of the 115 bacterial isolates were evaluated using adults and first-instar larvae of *C. capitata*. In each set of bioassays a strain negative for expression of Cry toxins (*B. thuringiensis* IPS 78/11), an *E. coli* strain expressing at high level the Cry1Ac1 toxin active against Lepidoptera (*E. coli* XL1Blue/pSVC10-wt), and T3 medium (no bacteria) were included as negative controls for toxicity. None of the 115 bacterial strains caused significant mortality of *C. capitata* adults compared with the negative controls. The corrected mortality rates with the 115 bacterial isolates at the end of the experiment ranged from 0% to 40% (data not shown), while the average mortality rates with the negative controls ranged from 5% to 30%. Similar results were obtained in bioassays with larvae (data not shown). The maximal corrected mortality rates with the 115 bacterial isolates ranged from 0% to 36%, while the average mortality rates with the negative controls ranged from 1% to 12% after 15 days, at the end of the experiment. There were no significant differences in the cumulative mortality rates for *C. capitata* larvae fed with whole cultures of the 115 bacterial isolates after 4 days ($P > 0.05$) and after 10 days ($P > 0.05$). However, there was a significant effect 15 days after the bioassays were initiated ($P = 0.048$). Nevertheless, the mean mortality rates for larvae with the 115 bacterial isolates were not significantly different as determined by Tukey's studentized range test; all treatments were in the same group after 15 days of treatment. Moreover, Dunnett's test showed that the percentages of mortality caused by the negative controls were not significantly different ($P > 0.05$) from the percentages of mortality caused by the 115 bacterial isolates.

Although none of the strains tested was toxic for *C. capitata* larvae or adults in the experiment described above, when we replicated the larva bioassays, due to circumstances beyond our control we introduced a slight modification of the screening methodology when a subset of bacterial isolates was assayed. Instead of starting the bioassay just after the diet was mixed with the bacterial culture, we added the larvae 96 h later than usual. During this time, the bioassay microplates, in which the bacterial culture was mixed with the larva diet, were stored at 4°C. After this subtle modification, the toxicity of the 15.1 strain changed noticeably, and the noncorrected mortality rates were 78% and 100% at 10 and 15 days, respectively, after the bioassay was initiated (while the mortality rates with the *B. thuringiensis* IPS 78/11 and T3 medium controls were 29.1% and 30.4%, respectively, at 15 days after the bioassay was started). Additionally, it was observed that most of the larvae in the microplates had necrosed bodies.

To double check this surprising result, a bioassay was performed with the 15.1 strain using only two microplates. After the bacterial culture and the diet were mixed, *C. capitata* neonates were immediately placed on one of the microplates, while the other plate was kept at 4°C for 96 h before newly obtained neonates were added. The results were consistent with our previous observation; the mortality rate for *C. capitata* larvae was higher after 15 days for the bioassay plate kept for 96 h at the low temperature before the bioassay (94.44% ± 7.86%) than for the bioassay plate used immediately for the bioassay (6.25% ± 4.66%). Given the unexpected behavior of the 15.1 strain together with its potent effect on *C. capitata* larvae, we selected this strain for further characterization.

Characterization and identification of the 15.1 strain. Strain 15.1 was isolated from a partially decomposed common reed plant (*Phragmites australis*). The 15.1 strain formed pearlescent rough colonies with concentric rings of growth on LB and T3 medium plates.

Identification of the 15.1 strain was carried out by PCR amplification of two overlapping fragments of the 16S rRNA gene (*rrsE*) whose amplicon sizes were around 780 and 1,023 bp. The amplicons spanned from position 1 to position 780 and from position 515 to position 1,538 of the *E. coli* 16S rRNA gene sequence (GenBank accession no. AE005174). Both strands of the two PCR products were sequenced, and the resulting 16S rRNA sequence was compared to the sequences deposited in the GenBank database. The 16S rRNA gene sequence of strain 15.1 was 100% identical to the 16S rRNA gene sequences of several strains of *B. pumilus*.

To verify that the 15.1 strain is a *B. pumilus* strain, a biochemical test was performed. It has been reported that *B. pumilus* strains are biotin dependent when they are grown in minimal medium with glucose (45). To determine that the 15.1 strain is dependent on the presence of biotin, growth curves for

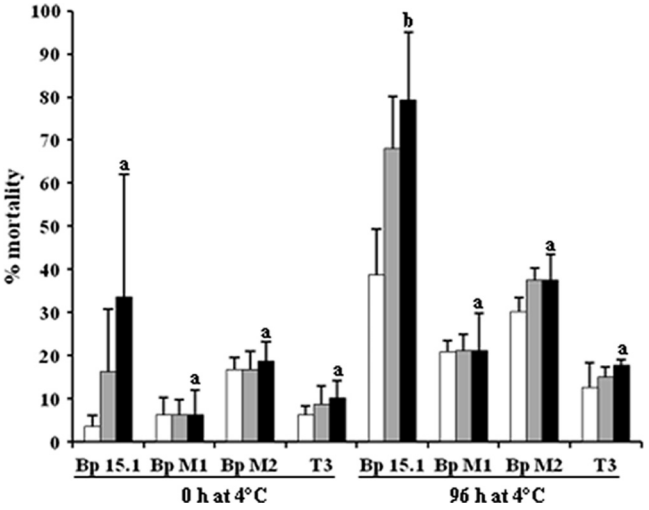

FIG. 1. Mortality of first-instar larvae of *C. capitata* caused by cultures of *B. pumilus* 15.1, *B. pumilus* M1, and *B. pumilus* M2 and T3 medium (no bacteria) used immediately after growth (0 h at 4°C) or kept for 96 h at 4°C. Open bars, mortality 4 days after initiation of the bioassay; gray bars, mortality 10 days after initiation of the bioassay; black bars, mortality 15 days after initiation of the bioassay. Statistical differences between *B. pumilus* 15.1 and controls were evaluated by ANOVA for larval mortality at 15 days ($P < 0.01$). The same letter above bars indicates that there was not a significant difference. Bp, *B. pumilus*.

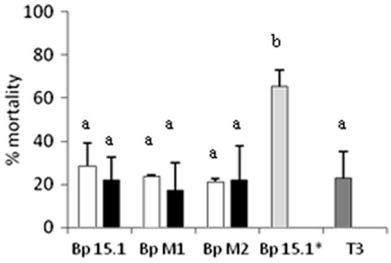

FIG. 2. Cumulative mortality of first-instar larvae of *C. capitata* 15 days after initiation of the experiment caused by separate fractions of *B. pumilus* 15.1, *B. pumilus* M1, and *B. pumilus* M2 cultures, a reconstituted 15.1 culture (mixed supernatant and pellet fractions) (Bp 15.1*), and T3 medium (no bacteria) kept for 96 h at 4°C. Open bars, supernatants; black bars, pellets; light gray bar, reconstituted culture; dark gray bar, T3 medium. The reconstituted culture exhibited significant toxicity ($P < 0.05$) compared to the supernatant and pellet fractions, control strains, and T3 medium. The same letter above bars indicates that there was not a significant difference. Bp, *B. pumilus*.

this strain in a minimal medium (66) without biotin and in the minimal medium with biotin for 80 h were examined. The results (data not shown) showed that the 15.1 strain was not able to grow in the minimal medium when biotin was not present. In contrast, the biotin-supplemented minimal medium supported bacterial growth (doubling time, 112 min).

Characterization of the toxic effect of *B. pumilus* strain 15.1. To speed up the toxic effect of *B. pumilus* strain 15.1 on *C. capitata* larvae, the cultures were concentrated 10-fold by lyophilization. A direct consequence of this modification was that a mortality rate of approximately 50% for *C. capitata* larvae was observed 4 days after initiation of the bioassay (instead of 10 days when the culture was not concentrated). After identification of the 15.1 strain, we compared its activity against *C. capitata* with the activities of other *B. pumilus* strains. To this end, two *B. pumilus* strains, strains M1 and M2, which were kindly provided by C. Calvo (69), were included in our bioassays. The mortality rates with both of these strains were determined and compared to the mortality rate with the T3 medium control, and there were no significant differences ($P > 0.05$). The mortality rate with *B. pumilus* 15.1 was also compared with the mortality rates with the three negative controls (strains M1 and M2 and T3 medium), and this analysis showed that there were significant differences ($P < 0.01$) (Fig. 1). These results indicate that the toxicity of *B. pumilus* 15.1 is a characteristic of this strain and not a characteristic of the species *B. pumilus*. *B. pumilus* strains M1 and M2 were used as negative controls in all further experiments.

In order to characterize the toxicity of *B. pumilus* 15.1 for larvae of *C. capitata*, a series of experiments were carried out. First, we checked if the toxicity of *B. pumilus* 15.1 for *C. capitata* larvae is a characteristic of sporulated cultures or if vegetative cells are also toxic. To this end, a lyophilized culture of vegetative cells containing 8×10^5 CFU/ml viable cells was treated using the conditions required to obtain maximal mortality with a sporulated culture (10× concentration and cold treatment) and used in a bioassay. The cumulative mortality rates obtained with a lyophilized vegetative culture of *B. pumilus* 15.1 that had been maintained at 4°C for 96 h after 4 days (11.49% ± 6.64%), 10 days (17.01% ± 8.11%), and 15 days (19.10% ± 7.16%) were low and not significantly different ($P > 0.05$) than the values for the negative controls (LB and other *Bacillus* strains incubated under the same conditions). These results showed that vegetative cells are not active against *C. capitata* larvae, suggesting that toxicity is correlated with the sporulation process and that incubation of vegetative cells at a low temperature does not result in a toxic culture.

Furthermore, we tried to determine which fraction of a 15.1 sporulated culture is toxic. Hence, three bioassays, one using the supernatant fraction, one using the pellet fraction (spores), and one using a mixture containing the supernatant and pellet fractions, were performed. The results showed that neither of the fractions tested exhibited significant toxicity ($P > 0.05$) (Fig. 2) compared to the controls. The reconstituted culture (mixture of the supernatant and pellet fractions) showed significant toxicity compared to both of the fractions and the control strains. These results suggested that both fractions are involved in toxicity.

The fact that the sporulated culture of *B. pumilus* 15.1 requires incubation for at least 96 h at 4°C in the larva diet to show toxicity against *C. capitata* (Fig. 1) led us to consider the possibility that spore germination could take place in the larva diet and that germination could cause toxicity. Since the *C. capitata* larva diet is not a suitable environment for bacterial germination due to its low pH (pH 3) and high concentration of solutes (242 mM sucrose), we checked this hypothesis to rule out the possibility that toxicity could be linked to spore germination. For this analysis, a liquid version of the larva diet used in the bioassays (without wheat bran and agar) was prepared. A lyophilized culture of *B. pumilus* 15.1 was resuspended in the diet at the same culture/diet ratio that was used in standard bioassays, and the bacterial suspension was divided

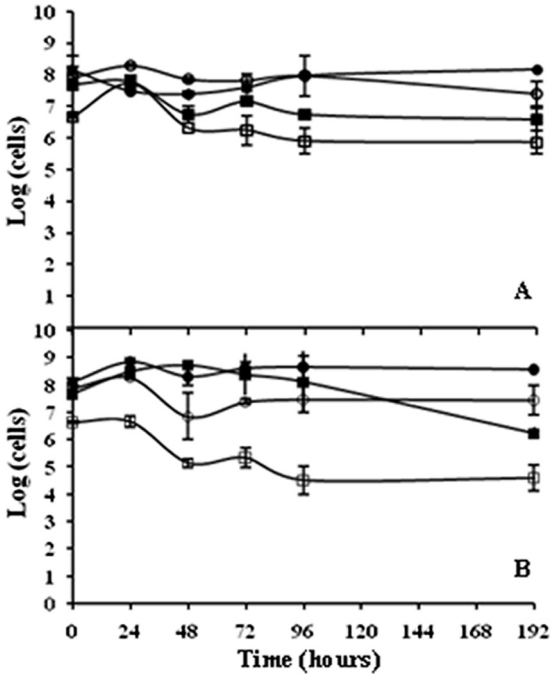

FIG. 3. Numbers of total viable cells (filled symbols) and spores (open symbols) of *B. pumilus* 15.1 (circles) and *B. pumilus* M1 (squares) over time in a liquid version of the larva diet. Cell counts were determined at two different temperatures, 4°C (A) and 25°C (B).

into two aliquots; then one of the aliquots was incubated at 4°C, and the other was incubated at 25°C to simulate the bioassay conditions. The numbers of total viable cells and spore cells were determined at different time points, and the number of vegetative cells was calculated for each time point.

The number of *B. pumilus* 15.1 spores was constant at either 4°C and 25°C during the experiment (Fig. 3), indicating that no spore germination occurred in the diet. Unexpectedly, the behavior of the control strain *B. pumilus* M1 was different, and the spore and total cell counts decreased with time when this strain was incubated at 25°C. This decrease in the number of cells could have been due to germination and death of vegetative cells because of the low pH of the medium.

Evaluating the effect of temperature on toxicity. Spore germination was not observed when spores were exposed to the larva diet, suggesting that exposure of the culture to a low temperature was the cause of the high toxicity. To test this hypothesis, a slight variation of our standard bioassays was used. Instead of leaving the sporulated cultures in the bioassay microplates mixed with the diet, we incubated the cultures for 4 days in the growth flasks. After the sporulated cultures were exposed to two temperatures (4°C and −20°C), they were used in the bioassay along with a culture that was not subjected to a temperature treatment and was used as a control. The results showed that the 15.1 strain was toxic only when cultures were kept at low temperatures (Table 1) and that activation of toxicity was independent of contact with the larva diet.

The LC_{50} and LC_{90} for *B. pumilus* 15.1 sporulated cultures kept for 96 h at −20°C were determined to be 4.18×10^7 CFU/ml (95% confidence interval, 3.45×10^7 to 4.9×10^7 CFU/ml) and 1.56×10^8 CFU/ml (95% confidence interval, 1.31×10^8 to 1.93×10^8 CFU/ml), respectively. The LC_{50} and LC_{90} for cultures used directly in the bioassays without incubation at a low temperature were estimated to be 4.66×10^{10} CFU/ml and 3.7×10^{13} CFU/ml, respectively.

DISCUSSION

Isolation of 115 bacterial strains, evaluation of the insecticidal activity of these strains, and identification of a novel strain of *B. pumilus* that is highly toxic to first-instar larvae of *C. capitata* are described here. To our knowledge, the entomopathogenic activity of *B. pumilus*, the fact that the toxicity increases when sporulated cultures of the strain are exposed to low temperatures, and the isolation of a *Bacillus* strain pathogenic for *C. capitata* larvae are reported here for the first time. These are important findings for the development of pest control strategies that can help reduce economic losses in fruit crops.

Chemical treatment is the most common method used to reduce the economic impact that *C. capitata* has on crops, but the chemical tools available are increasingly restricted by global policies. For example, in the summer of 2007, malathion was banned for agricultural application in the European Economic Community (70).

Biological control of *C. capitata* using microorganisms could overcome the disadvantages of chemical control since microorganisms are safer and there are fewer resistance problems. Unfortunately, very few microorganisms that exhibit activity against *C. capitata* have been described; the organisms that have been described include the fungi *Mucor hiemalis* (40), *Metarhizium anisopliae*, and *Beauveria bassiana* (5, 18, 22, 23, 39, 52, 55), the bacteria *Serratia marcescens* (11) and *Saccharopolyspora spinosa* (50), the microsporidian parasite *Octosporea muscaedomesticae* (53), and the entomopathogenic nematodes *Heterorhabditis bacteriophora*, *Heterorhabditis zealandica*, and *Steinernema khoisanae* (47). Notwithstanding these reports, no efficient product that can be applied in the field is commercially available yet. Therefore, a search for new microorganisms is necessary.

The *Bacillus*-based insecticides that have been described are specific for their targets, harmless to humans and higher ver-

TABLE 1. Mortality of *C. capitata* larvae after 10 days caused by two *Bacillus* strain cultures kept under different conditions

Bacterial strain or medium	Exposure time (h)	% Mortality[a]		
		Culture		Culture + diet (4°C)
		4°C	−20°C	
B. pumilus 15.1	0	22.92 ± 0.00 a	22.92 ± 0.00 a	16.37 ± 14.45 a
	96	93.61 ± 1.94 b	84.10 ± 2.85 b	67.92 ± 12.19 b
	168	66.67 ± 10.42 b	60.42 ± 18.75 b	
B. pumilus M1	0	16.67 ± 2.08 a	16.67 ± 2.08 a	6.25 ± 0.00 c
	96	37.50 ± 4.17 c	30.21 ± 3.12 c	21.28 ± 0.00 a
	168	17.71 ± 1.04 c	16.67 ± 6.25 a	
T3 medium	0	11.11 ± 0.00 c	16.57 ± 6.16 a	8.61 ± 4.44 c
	96	9.25 ± 2.98 c	9.25 ± 2.98 c	15.09 ± 2.35 c

[a] Cultures were either mixed (Culture + diet) or not mixed (Culture) with the larva diet. The values are means ± standard deviations. Values followed by the same letter are not significantly different ($P < 0.05$).

tebrates (60, 64), and easy to store and apply. Hence, we focused on a search for natural occurring bacteria belonging to the genus *Bacillus* (in particular *B. thuringiensis*), using the selective method proposed by Travers et al. (68). A wide range of field samples were taken from the natural habitat of *C. capitata* and used as a source of microorganisms that could be a natural enemy of this fly. Of the 115 bacterial strains screened, only one, a strain isolated from a partially decomposed common reed plant, was highly pathogenic for first-instar larvae of *C. capitata*. The 15.1 strain seems to be specific for the larval stage, since no toxicity for *C. capitata* adults was observed when the same conditions that resulted in maximum toxicity for larvae were used (unpublished results). Activity against only one of the possible insect stages has been observed previously for *B. thuringiensis* isolates that exhibit activity against larvae of other fruit flies (3, 59).

The 16S rRNA of the 15.1 strain was 100% identical to the 16S rRNA of several *B. pumilus* strains whose sequences have been deposited in the GenBank database. Based on the colony appearance, including concentric rings of growth, and the requirement for biotin when the strain is grown in minimal medium, we concluded that the 15.1 strain is a *B. pumilus* strain (10, 45).

B. pumilus 15.1 did not exhibit toxicity under standard bioassay conditions. Toxicity was observed only when a bacterial culture (on a bioassay plate or in a growth flask) was incubated at a low temperature (4°C or −20°C) for at least 96 h before initiation of the bioassay. Activation of toxicity seems to be a time-dependent effect of low temperature, because the toxicity increased up to the maximal value at 96 h and decreased slightly after the cultures were incubated for 168 h. A narrower low-temperature incubation time course is being tested in order to further examine this characteristic. This finding opens the door for exploration of novel entomopathogenic activities under conditions that have not been tested to date.

B. pumilus is a ubiquitous bacterium with a wide range of activities that are important from a biotechnological point of view. Some *B. pumilus* strains show fungicidal activity and have been used as biological control agents against phytopathogenic fungi (9, 42). Other *B. pumilus* strains have been reported to be plant growth-promoting rhizobacteria (25, 36, 57, 77), while some others showed potent antibacterial activity (6). Still others have been used as probiotics (15, 20, 28). However, *B. pumilus* is not considered an insect pathogen like other members of the genus *Bacillus*, such as *B. thuringiensis* or *B. sphaericus*. As far as we know, there has been only one previous report of a patent of a *B. pumilus* strain active against insects, and this strain is active against the corn rootworm (*Diabrotica undecimpunctata*) and the armyworm (*Spodoptera exigua*) (33). The special conditions required to obtain toxicity against *C. capitata* could explain why this bacterium is not considered a traditional entomopathogen, since incubation of cultures at low temperatures is not part of any standard bioassay. The effect of low temperature on toxicity is surprising and has also been observed for *B. thuringiensis* strain Ormilia isolated from Greece (37). This strain shows significant toxicity against *Dacus oleae* in standard bioassays, but its toxicity increases dramatically when a culture is kept for 11 days at 4°C before it is used in a bioassay. So far, the mechanism of this increased toxicity has not been studied.

There have been previous reports of increases in the activities of some enzymes when they are exposed to low temperatures; e.g., nitrite reductase from *Alcaligenes* sp. was studied by Masuko et al. (48), who found that the enzymatic activity increased 2.5- to 4.5-fold when the enzyme was incubated at a low temperature (−20°C), and it was demonstrated that the increased activity was related to minor conformational changes in the molecule, particularly in the hydrophobic region of the protein, which could also be true for *B. pumilus* 15.1. A conformational change in the virulence factor induced by low temperature might have occurred, although the possibility that there are other mechanisms (e.g., protease activation) cannot be ruled out.

Based on our results, it is likely that the virulence factor responsible for the toxicity of *B. pumilus* 15.1 is synthesized during sporulation (cultures with vegetative cells are not toxic, even after incubation at low temperatures) and is further activated or processed when the organism is incubated at a low temperature (4°C or −20°C). Toxicity was not related to germination of the spores in the insect diet, since (i) the number of spores in the diet was constant during the bioassay (no germination was observed) and (ii) the virulence factor could be activated when sporulated cultures were kept at low temperatures in the growth flasks. It would be interesting to investigate why both the supernatant and bacterial fractions are needed for full activation and toxicity and to identify the virulence factor and the mechanisms by which this factor is activated. A clear understanding of the mechanism of toxicity should help optimize the effect on *C. capitata* larvae. Work to determine the activity of the 15.1 strain against other insects and to characterize this bacterium biochemically and molecularly is under way.

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