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The *Bacillus thuringiensis* PlcR-Regulated Gene *inhA2* Is Necessary, but Not Sufficient, for Virulence

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We previously reported that *Bacillus thuringiensis* strain 407 Cry 32⁻ secretes a zinc-requiring metalloprotease, InhA2, that is essential for virulence in orally infected insects. Analysis of the *inhA2-lacZ* transcriptional fusion showed that *inhA2* expression is repressed in a PlcR⁻ background. Using DNase I footprinting experiments, we demonstrated that PlcR activates *inhA2* transcription directly by binding to a DNA sequence showing a one-residue mismatch with the previously reported PlcR box. It was previously reported that PlcR is essential for *B. thuringiensis* virulence in oral infection by contributing to the synergistic properties of the spores on the insecticidal activity of the Cry1C protein. We used complementation experiments to investigate whether the PlcR⁻ phenotype was due to the absence of InhA2. The results indicated that overexpression of *inhA2* in the Δ*plcR* strain did not restore the wild-type phenotype. However, virulence was fully restored in the Δ*inhA2* complemented mutant. Thus, *inhA2* is the first example of a PlcR-regulated gene found to be directly involved in virulence. However, it is not sufficient for pathogenicity when the other members of the PlcR regulon are lacking. This suggests that InhA2 may act in concert with other PlcR-regulated gene products to provide virulence.

Bacillus thuringiensis and *Bacillus cereus* are two gram-positive spore-forming bacteria belonging to the *cereus* group, which also includes the human pathogen *Bacillus anthracis* and the nonpathogenic *Bacillus mycoides*. Molecular analysis showed that *B. thuringiensis* and *B. cereus* share the same genetic background (7, 8, 21). *B. thuringiensis* is well known for its entomopathogenic properties, partly due to the cytoplasmic crystallized δ-endotoxins (also termed Cry proteins) that are specifically active against insect larvae (35). *B. cereus* does not produce crystallized proteins and is an opportunistic human pathogen, causing food-borne gastroenteritis (23). In some rare cases, *B. cereus* is responsible for systemic and local infections such as endophthalmitis, periodontitis, meningitis, or pneumonia (4, 6).

Although these bacteria infect distinct hosts (insects versus mammals), they share some common pathogenic features. Indeed, the intrahemocoelic administration of low inocula of *B. cereus* or acrystalliferous *B. thuringiensis* strains to susceptible insect larvae leads to lethal septicemia (20, 32, 37, 39). Furthermore, in some insects, the presence of spores from both of these species, but not from other bacterial species, strongly increases the killing activity of *B. thuringiensis* crystals administrated via the oral route (10, 33). Finally, the opportunistic properties of *B. thuringiensis* have been demonstrated in mice by infection via nasal instillation of spores (22, 23). *B. thuringiensis* and *B. cereus* produce common potential virulence factors, which are thought to facilitate their development within the host. These factors include degradative enzymes and toxins (18, 19). A large number of *B. thuringiensis* genes, encoding potential virulence factors, are regulated by a pleiotropic transcriptional activator named PlcR (1, 25). The transcription of the *plcR* gene is both autoregulated (25) and under the control of the sporulation key factor Spo0A (26). Alignment of the promoter regions of about 15 PlcR-regulated genes from *B. thuringiensis* and *B. cereus* revealed the presence of a highly conserved palindromic sequence ($TATGNAN_4TNCATA$), named the PlcR box (1, 30). This sequence is located in various positions upstream from the transcription start site and is essential for transcription (1). PlcR acts by binding to the PlcR box. This binding requires the product of a small gene (*papR*) that lies immediately downstream of *plcR* (36). *papR* is regulated by PlcR and encodes a quorum-sensing effector that controls the expression of the PlcR regulon in members of the *B. cereus* group. Analysis of the extracellular proteome in the *B. cereus* strain ATCC 14579 revealed that the disruption of *plcR* considerably reduced the amounts of up to 56 exported proteins (15). Moreover, the inactivation of *plcR* decreased the pathogenicity of *B. cereus* and *B. thuringiensis* in both insects and mice, suggesting that one or several PlcR-regulated genes are involved in pathogenicity (33).

We recently characterized a new *B. thuringiensis* virulence factor, InhA2 (13). InhA2 is a zinc metalloprotease that is highly homologous to the *B. thuringiensis* InhA, which was originally identified as an extracellular protease that specifically hydrolyzes the antibacterial peptides cecropins and attacins from the insect *Hyalophora cecropia* (9, 12). We showed that InhA2 plays a major role in potentiating the toxicity of Cry proteins in orally infected insects (13). The expression of *inhA2* is induced at the onset of the stationary phase and is negatively regulated by Spo0A (13). Recent studies have shown that InhA2 is synthesized by *B. cereus* strain ATCC 14579 and is

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one of the *B. cereus* proteases strongly downregulated in Δ*plcR* mutants (15). However, the *B. cereus inhA2* gene does not display the previously reported PlcR box consensus target upstream of its coding sequence and the mechanism by which PlcR affects InhA2 production has not been determined.

In the present study we investigated the involvement of the pleiotropic regulator PlcR in the control of *inhA2* expression. Experiments with *inhA2′-lacZ* transcriptional fusions and DNase I footprinting demonstrated that *B. thuringiensis inhA2* belongs to the PlcR regulon. We also assessed the virulence of the Δ*plcR* mutant after transcomplementation with *inhA2* in orally infected *Galleria mellonella*. Our findings indicate that *B. thuringiensis* InhA2 is not sufficient by itself for providing virulence in the absence of PlcR-regulated genes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The sporogenic acrystalliferous *B. thuringiensis* strain 407 Cry$^-$ belonging to serotype 1 (27) and the 407 Cry$^-$ Δ*plcR* strain carrying a *plcR* disrupted gene (33) were used throughout the present study. The 407 Cry$^-$ Δ*inhA2* strain carrying an *inhA2* disrupted gene has been described previously (13). *Escherichia coli* K-12 strain TG1 [Δ(*lac-proAB*) *supE thi hsd*Δ5 (*F′ traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*ΔM15)] (14) was used as a host for the construction of plasmids and cloning experiments. *E. coli* strains SCS 110 [*rpsL* (Str^r) *thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB*) (*F′ traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} lacZΔM15; Stratagene, La Jolla, Calif.] and ET12567 (*F*$^-$ *dam-13*::Tn*9 dcm-6 hsdM hsdR recF143 zjj-202*::Tn*10 galK2 galT22 ara14 pacY1 xyl-5 leuB6 thi-1*) were used to generate unmethylated plasmid DNA prior to *B. thuringiensis* transformation. Electroporation was used to transform *E. coli* (11) and *B. thuringiensis* (27).

E. coli and *B. thuringiensis* cells were routinely grown in Luria broth (LB) medium with vigorous agitation at 37 and 30°C, respectively. The following antibiotic concentrations were used for bacterial selection: ampicillin at 100 μg ml^{-1} (for *E. coli*) and kanamycin at 200 μg ml^{-1} and erythromycin at 10 μg ml^{-1} (for *B. thuringiensis*). Bacteria with the Lac$^+$ phenotype were identified on LB plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at 40 μg ml^{-1}.

Spores of *B. thuringiensis* strains were obtained by culturing cells in 40 ml of sporulation-specific (HCT) medium (24) at 30°C for 3 days. Spores were harvested by centrifugation (5,000 × *g* for 10 min), washed with distilled water (twice, each time with 40 ml), and finally resuspended in 6 ml of sterile distilled water. The concentrations of the spore preparations were estimated by plating dilutions onto LB agar plates containing appropriate antibiotics.

The Cry1C toxin was prepared from the asporogenic strain 407 Δ*sigK* (5) transformed with pHT1C (34) as described by Gominet et al. (16).

DNA manipulation. Plasmid DNA was extracted from *E. coli* and *B. thuringiensis* by a standard alkaline lysis procedure by using QIAprep spin columns (Qiagen), with the following modification in the first step of the lysis procedure for *B. thuringiensis*: incubation at 37°C for 1 h with 5 mg of chicken egg white lyzosyme (14,300 U/mg). Chromosomal DNA was extracted from *B. thuringiensis* cells harvested in mid-log phase as described previously (29). Restriction enzymes and T4 DNA ligase were used as recommended by the manufacturer (New England Biolabs). Oligonucleotide primers were synthesized by Genset (Paris, France). PCRs were performed in a GeneAmp PCR system 2400 thermal cycler (Perkin-Elmer). Amplified DNA fragments were purified by using the QIAquick PCR purification Kit (Qiagen) and separated on 0.7% agarose gels after digestion. Digested DNA fragments were extracted from agarose electrophoresis gels by using a centrifugal filter device (Ultrafree-DA; Amicon Laboratories).

Trans-complementation of Δ*plcR* and the Δ*inhA2* mutant strains with *inhA2*. A 473-bp *Hin*dIII/*Bam*HI fragment containing the promoter region of the kanamycin resistance gene *aphA3* was amplified by PCR by using the primers Km1 (5′-GAGGTGATAGGTAAG-3′) and Km2 (5′-CCCAAGAAGCTAATTATA AC-3′) and pDG783 DNA carrying the *aphA3* gene from *Enterococcus faecalis* (38) as a template. PCRs were carried out in a volume of 100 μl containing a 200 μM concentration of deoxynucleoside triphosphates, 1.5 mM MgSO4, 50 pmol of each primer, 0.5 μg of DNA template, and 0.5 U of *Pwo* DNA polymerase (Roche Boheringer) in a 1× reaction buffer. The amplified DNA fragment was digested with the appropriate restriction enzymes and inserted between the *Hin*dIII/*Bam*HI sites of the gram-positive–gram-negative shuttle plasmid, pHT315 (3). The resulting plasmid was designated pHT315Ω*paphA3*. A 2,571-bp *Bam*HI/*Eco*RI DNA fragment carrying a promoterless *inhA2* gene (positions −46 and +126 with respect to the ATG and TAA codons of the *inhA2* coding sequence, respectively) was obtained by PCR amplification by using the 407 Cry$^-$ chromosomal DNA as a template and oligonucleotides *inhA2*.1 (5′-CGCGGA TCCCACCGATTTATCTG-3′) and *inhA2*.2 (5′-CCGGAATTCCTTTCCCCA CATAATTTG-3′). The DNA fragment harboring the *inhA2* coding sequence was digested and ligated downstream of the promoter region of *aphA3* gene between the *Eco*RI/*Bam*HI sites of pHT315Ω*paphA3*. The ligation mixture was used to transform *B. thuringiensis* strain 407 Cry$^-$ Δ*plcR* and strain 407 Cry$^-$ Δ*inhA2*. The transformant clones harboring the recombinant pHT315Ω*paphA3-inhA2* were designated 407 Cry$^-$ [Δ*plcR* (p*inhA2*)] and 407 Cry$^-$ [Δ*inhA2* (p*inhA2*)].

Construction of the *inhA2′-lacZ* transcriptional fusion. The *inhA2′-lacZ* transcriptional fusion was constructed by cloning a 593-bp *Bam*HI/*Pst*I DNA fragment harboring the *inhA2* promoter between the *Bam*HI and *Pst*I sites of pHT304-18′Z (2). The DNA fragment was generated by PCR amplification performed on 407 Cry$^-$ chromosomal DNA with the primers B6sqBt (5′-AAA CTGCAGCCCAGCAAACGTAATTGCTTC-3′) and InB9 (5′-CGCGGATCC CTCTTTTGCTGGCGTTTCTGC −3′). The recombinant plasmid, designated pHT304Ω*inhA2′-Z*, was introduced into *B. thuringiensis* wild-type and Δ*plcR* mutant strains by electroporation. The transformants were named 407 Cry$^-$ (pHT304Ω*inhA2′-Z*) and 407 Cry$^-$ Δ*plcR* (pHT304Ω*inhA2′-Z*), respectively, and were resistant to erythromycin (10 μg ml^{-1}).

β-Galactosidase assay. Cells of *B. thuringiensis* strains harboring plasmid *lacZ* transcriptional fusions were cultured in LB medium in the absence of antibiotics at 30°C with vigorous shaking. β-Galactosidase specific activities were measured as described previously (29). Specific activities are expressed in units of β-galactosidase per milligram of protein (Miller units).

DNase I footprinting. DNase I footprinting assays were performed by using purified PlcR as previously described (36). A+G Maxam and Gilbert reactions (28) were carried out on the appropriate ^{32}P-labeled DNA fragments and loaded alongside the DNase I footprinting reactions. Gels were dried and analyzed by autoradiography.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was conducted as described previously (15). The culture supernatant was collected 2 h after the onset of stationary phase, centrifuged, and filtered. Proteins were precipitated by using the deoxycholic acid-trichloroacetic acid method (31), washed with ethanol ether (1:1), and dissolved in a urea-thiourea-CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}-ampholine mixture. A total of 20 μg of proteins were loaded onto each IPG strip for the first dimension and isoelectrofocusing was performed for 35,000 V·h. The strips were then equilibrated first in urea-sodium dodecyl sulfate-Tris-dithiothreitol, followed by a second equilibration in urea-sodium dodecyl sulfate-Tris-acetamide. The second dimension was done on a 10 to 12.5% gradient acrylamide gel. Gels were then silver stained and scanned for image analysis. Proteins were identified by mass spectrometry after trypsin digestion or by N-terminal sequencing by

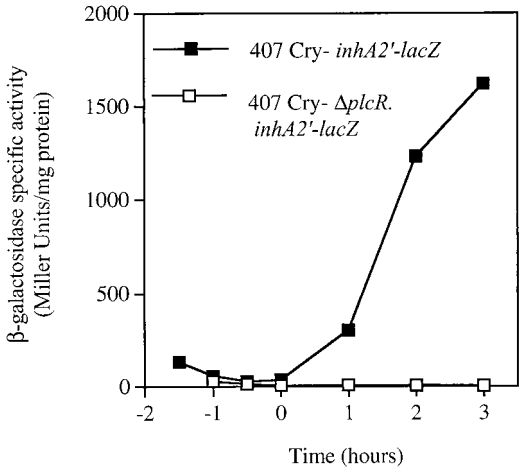


FIG. 1. PlcR is a positive regulator of *B. thuringiensis inhA2* expression. Specific β-galactosidase activity (Miller units) of strain 407 Cry$^-$ (■) and strain 407 Cry$^-$ Δ*plcR* (□) harboring the transcriptional *inhA2′-lacZ* fusion. The cells were grown in LB medium at 30°C.

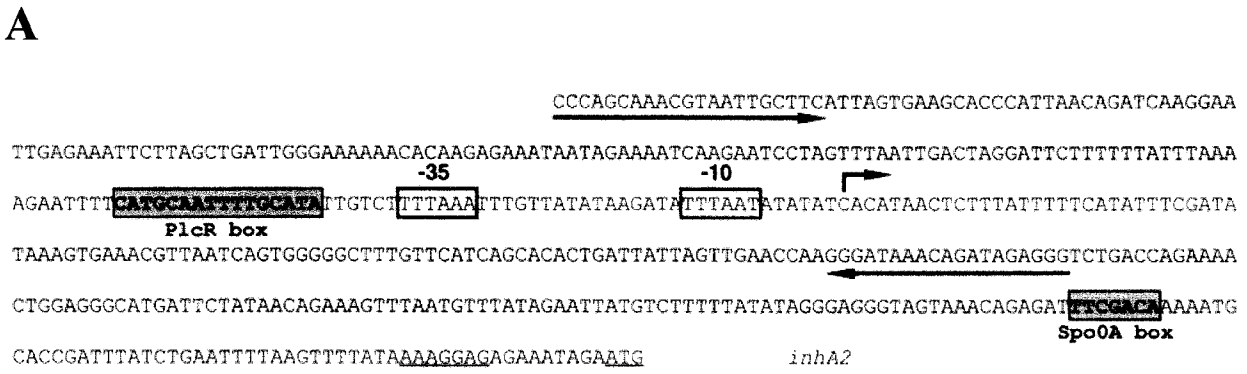
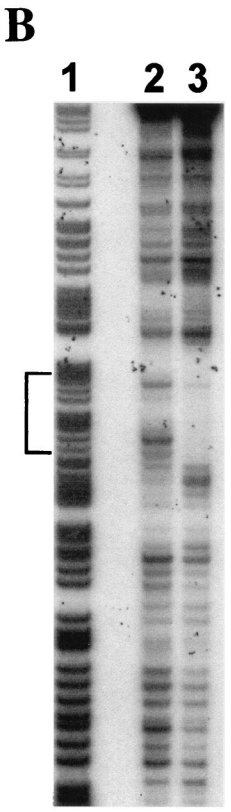


FIG. 2. PlcR directly activates *inhA2* transcription. Sequence of the *inhA2* promoter region (A). The putative −10 and −35 boxes of the *inhA2* promoter are boxed. The potential ribosome-binding site and the start codon (ATG) are underlined. The potential reverse Spo0A box and the PlcR box are shaded. The broken arrow indicates the transcription start site. Arrows indicate the positions of primers used in the DNase I footprinting experiments. DNase I footprinting analysis of PlcR binding to the *inhA2* promoter region (B). Lane 1 indicates the A+G Maxam and Gilbert reactions of the labeled template strand of *inhA2* promoter region. Lane 2, no PlcR; lane 3, 100 pmol of purified PlcR. Brackets indicate the region protected by PlcR.



using the Edman method. Mass spectrometry and N-terminal sequencing were performed by the Unité de Recherches en Biochimie et Structure des Protéines at INRA (Jouy-en-Josas, France).

Insects and force-feeding assays. *G. mellonella* eggs were hatched at 30°C, and the larvae were reared on beeswax and pollen (Naturalim). Groups of 20 last-instar *G. mellonella* larvae, weighing ca. 200 mg, were force-fed with spore-crystal suspensions in sterile water (10 μl/larva) by using a 0.5-by-25-mm needle (Burckard Manufacturing) and a microinjector (Burckard). The larvae were kept in individual boxes containing beeswax and pollen at 25°C. A control group was fed with sterile water. Experiments were repeated three times, and mortality was recorded daily over a 3-day period.

Statistical analysis. Means and standard errors of the means were calculated and plotted, and the statistical significance of the difference between groups was estimated by using the log linear model.

RESULTS

Effect of the *plcR*-null mutation on *inhA2* expression. In *B. thuringiensis* strain 407 Cry$^-$ grown in LB medium, *inhA2* transcription is induced at the onset of stationary phase (13). Recent data showed that the closely related species *B. cereus* strain ATCC 14579 produces InhA2 and that the amount of this protease is significantly decreased in the isogenic strain carrying a disrupted *plcR* gene (15). These findings led us to check whether InhA2 was controlled by PlcR at the transcriptional level. The present study was carried out in *B. thuringiensis* strain 407 Cry$^-$ as the predicted amino acid sequence of *B. cereus* strain ATCC 14579 InhA2 (http://www.integratedgenomics.com/genomereleases.html#list0) is 99% identical to that of *B. thuringiensis* InhA2 (accession no. AF421888). Moreover, the nucleotide sequences of both gene promoter regions are identical (result not shown). We thus investigated the effect of the *plcR*-null mutation on *B. thuringiensis inhA2* expression by analyzing a plasmid transcriptional fusion between the 480-bp DNA region extending upstream from the *inhA2* start codon and the *lacZ* gene in pHT304-18′Z. The recombinant plasmid (pHT304Ω*inhA2′-Z*) was introduced into *B. thuringiensis* wild-type and Δ*plcR* mutant strains. Cells were cultured at 30°C in LB medium, and β-galactosidase production was monitored at different stages of bacterial growth between t_{-1} and t_{+3} (t_n indicates the number of hours before [−] or after [+] the onset of the stationary phase). The *inhA2′-lacZ* fusion was not expressed when introduced into the Δ*plcR* mutant strain (β-galactosidase level of <10 Miller units), whereas its expression increased during the stationary phase when introduced into the wild-type strain (Fig. 1). These results indicate that PlcR positively controls *inhA2* expression during bacterial growth.

PlcR directly activates *inhA2* expression. Analysis of the *inhA2* promoter sequence revealed an atypical PlcR box (5′-CATGCAATTTTGCATA-3′) located 5 bp upstream of the previously identified −35 promoter sequence (Fig. 2A). This box displayed one mismatch compared to the reported PlcR binding consensus sequence (5′-TATGNAN$_4$TNCATA-3′) (1).

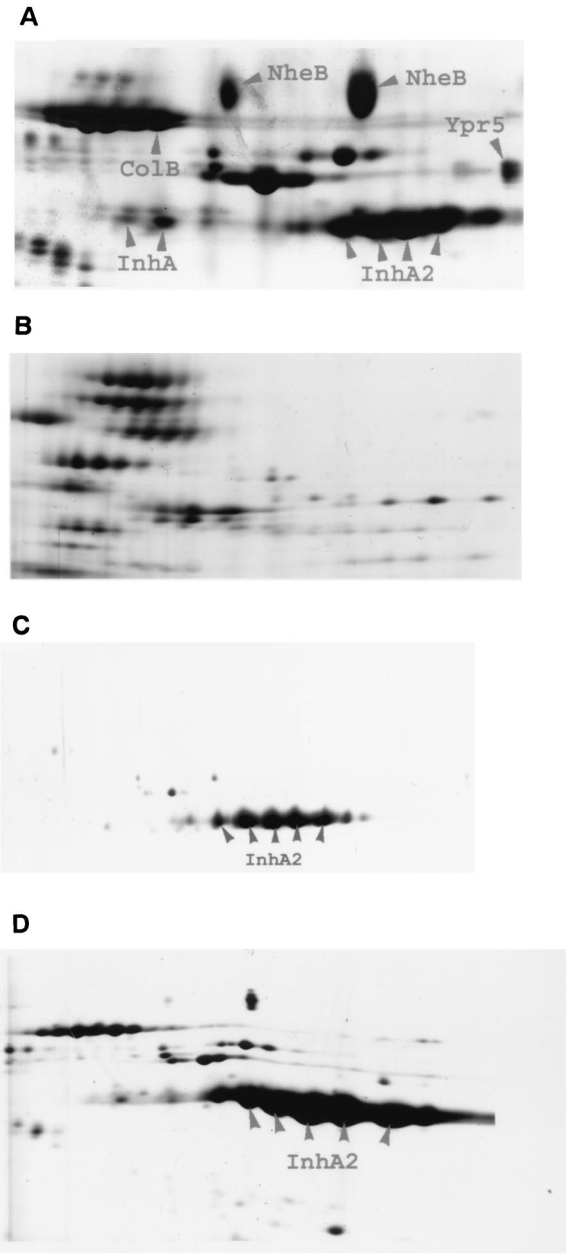


FIG. 3. Two-dimensional gel electrophoresis of *B. thuringiensis* culture supernatants collected 2 h after the onset of the stationary phase. The gel area shown is the InhA/InhA2 zone, between isoelectric points 5.1 (left) and 5.9 (right) and in the molecular mass range from 70 to 90 kDa. (A) Strain 407 Cry$^-$; (B) strain 407 Cry$^-$ Δ*plcR*; (C) strain 407 Cry$^-$ [Δ*plcR* (p*inhA2*)]; (D) strain 407 Cry$^-$ [Δ*inhA2* (p*inhA2*)]. ColB, collagenase B; Ypr5, enhancin; NheB, component B of enterotoxin Nhe.

This mismatch concerned the first nucleotide of the PlcR box 5′, a thymine, which was substituted with a cytosine in the *inhA2* gene. To investigate whether PlcR directly activates *inhA2* gene expression by binding this putative PlcR box, we performed a DNase I footprinting experiment with purified PlcR and the *inhA2* DNA promoter region extending 213 bp upstream and 112 bp downstream of the transcription start site (Fig. 2B). The footprinting assay showed that PlcR binds to the promoter region and that the area protected by this pleiotropic regulator exactly overlaps the putative PlcR box.

Effect of InhA2 production on the pathogenicity of *B. thuringiensis* Δ*plcR* spores against orally infected *G. mellonella* larvae. PlcR and InhA2 play a major role in the pathogenicity of *B. thuringiensis* against *G. mellonella* infected via the oral route (13, 33). We evaluated the synergistic effect of the spores on the insecticidal activity of the crystal protein Cry1C. Since the expression of *inhA2* depends on PlcR, the loss of synergy with Δ*plcR* spores might be due to the absence of the InhA2 protein. To test this hypothesis, we measured the virulence of a Δ*plcR* strain expressing the *inhA2* gene. A Δ*plcR* strain was complemented by introducing a plasmid harboring the *inhA2* coding sequence cloned downstream of the constitutive *aphA3* gene promoter. This recombinant plasmid was also introduced into the 407 Cry$^-$ Δ*inhA2* mutant strain to verify that InhA2 complements the avirulent InhA2$^-$ phenotype. *B. thuringiensis* Δ*plcR* and Δ*inhA2* mutant strains, carrying the *inhA2* gene under the control of the *aphA3* promoter, were referred to as 407 Cry$^-$ [Δ*plcR* (p*inhA2*)] and 407 Cry$^-$ [Δ*inhA2* (p*inhA2*)], respectively. Two-dimensional electrophoresis was performed on extracellular proteins as described previously (15). Dense spots corresponding to InhA2 were observed in the wild-type strain (Fig. 3A). These spots were abolished in the Δ*inhA2* strain (data not shown). In the Δ*plcR* strain, faint spots, migrating to the same pI and MW as InhA2, were detected (Fig. 3B). Mass spectrometry failed to identify these faint spots, which might be unrelated to InhA2. However, the presence of small amounts of InhA2 in the Δ*plcR* culture supernatant, even though the plasmid transcriptional fusion showed that *inhA2* is not expressed in this mutant, may reflect a weak transcriptional activity of *inhA2* in a chromosomal context. The mutant strains 407 Cry$^-$ [Δ*plcR* (p*inhA2*)] and 407 Cry$^-$ [Δ*inhA2* (p*inhA2*)] secreted the InhA2 protease and the InhA2 spots for these strains were as dense as for the wild-type strain (Fig. 3C and D).

We next assessed the synergistic activity of *B. thuringiensis* 407 Cry$^-$, 407 Cry$^-$ Δ*plcR*, 407 Cry$^-$ [Δ*plcR* (p*inhA2*)], and 407 Cry$^-$ [Δ*inhA2* (p*inhA2*)] spores on the killing effect of the Cry1C toxin (Fig. 4). The ingestion of Cry1C-containing crystals alone (3.3 μg of protein/larva) or of spores alone (2×10^6 spores/larva) resulted in very low levels of mortality (<13% for the crystals and 0% for the spores from all *B. thuringiensis* strains). The coingestion, of the same concentrations of Cry1C-containing crystals and spores from the parental 407 Cry$^-$ strain resulted in a significant increase in mortality, demonstrating synergy. As reported previously (13), spores from the Δ*inhA2* mutant were not synergistic. However, the 407 Cry$^-$ [Δ*inhA2* (p*inhA2*)] spores completely recovered the wild-type virulent phenotype, thus demonstrating that the complemented strain was functional. In contrast, the 407 Cry$^-$ [Δ*plcR* (p*inhA2*)] spores were as ineffective as the parental Δ*plcR* mutant strain to provide a synergistic or even a cumulative effect on Cry1C insecticidal activity. These results indicate that the effect of *plcR* inactivation on synergy is not reversed by the presence of the InhA2 metalloprotease in bacterial cells.

DISCUSSION

We previously reported that *B. thuringiensis* strain 407 Cry$^-$ possesses two related genes, *inhA* and *inhA2*, encoding puta-

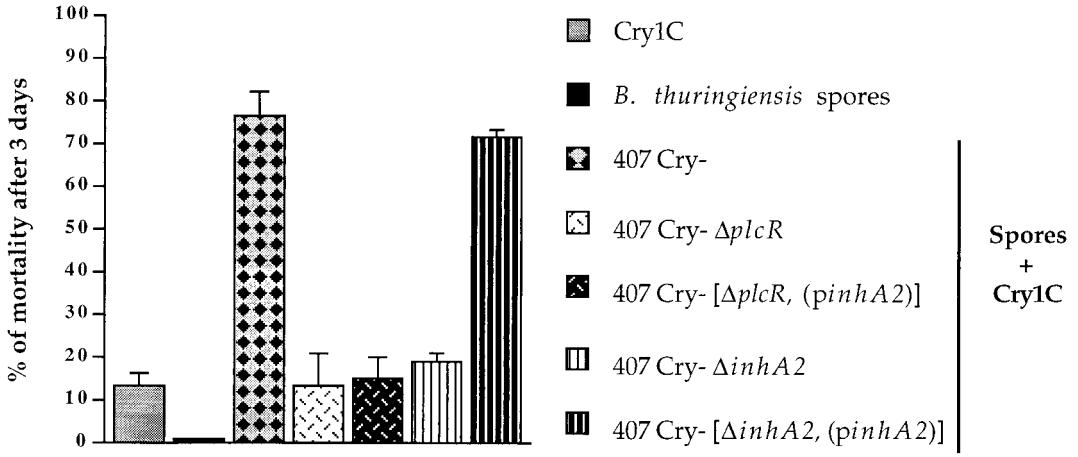


FIG. 4. Contribution of InhA2 to the virulence of *B. thuringiensis* against insects. Last-instar *G. mellonella* larvae were force-fed with spores alone (2×10^6 spores/larva), crystals alone (3.3 μg/larva) or spore-crystal mixtures. For all of the strains, spores alone caused no mortality. Vertical bars indicate the standard error of the mean. The results were obtained from three pooled independent experiments.

tive zinc-requiring metalloproteases (13). *inhA* and *inhA2* are inversely regulated by the sporulation factor Spo0A. *inhA* appears to be positively regulated by Spo0A and thus to be overproduced in sporulation-specific medium (17), whereas *inhA2* is repressed by Spo0A and needs a relatively rich medium (such as LB) to be expressed (13). In the present study, we showed that the expression of *inhA2* depends on the pleiotropic regulator PlcR. Although a reverse Spo0A box has been identified downstream of the *inhA2* promoter (13) (Fig. 2A), there is no evidence that Spo0A directly represses *inhA2* expression, and *inhA2* repression might be due to the inhibitory effect of Spo0A on *plcR*. Indeed, the transcription of *plcR* is repressed by Spo0A, resulting in the complete loss of *plcR* expression when the cells are grown in a sporulation-specific medium (26).

PlcR activates the expression of several potential virulence genes in *B. cereus* and *B. thuringiensis* at the end of the exponential growth phase. These genes encode secreted proteins, including phospholipases C, hemolysins, enterotoxins, and proteases (1, 25, 30). PlcR acts by binding to a highly conserved palindromic sequence ($TATGNAN_4TNCATA$) located in the promoter region of PlcR-regulated genes (1, 36). Our footprinting experiments show that PlcR activates *inhA2* transcription directly by binding to the DNA sequence (5′-$CATGNAN_4$ TNCATA-3′) located just upstream of the −35 consensus promoter box of *inhA2*. This sequence differs from the previously defined PlcR box by one residue (C_1 versus T_1) (1), but this substitution does not result in a loss of function. Thus, this DNA sequence is a PlcR recognition target for *inhA2* activation. This is the first example of a functional PlcR box that differs from that previously published. A similar DNA motif is present upstream of the *inhA2* coding sequence in *B. cereus* strain ATCC 14579. The observation that InhA2 production was significantly diminished in this *B. cereus* strain carrying a disrupted *plcR* gene (15) is consistent with the expression of *inhA2* being dependent on PlcR in *B. cereus*.

B. thuringiensis spores significantly synergize the insecticidal activity of Cry proteins when coingested by susceptible larvae, and this synergy is abolished by the disruption of the *plcR* and *inhA2* genes (13, 33). PlcR-regulated toxins and degradative enzymes may facilitate the spread of the bacterium through host tissues, thus allowing bacterial cells to gain access to alternative sources of nutrients and to cause septicemia. Since phospholipases C, enterotoxins, or hemolysins were not found to be essential on their own in providing synergism (D. Lereclus, unpublished data), *inhA2* is the first example of PlcR-regulated gene shown to be essential for virulence. This raised the question as to whether the absence of InhA2 alone could explain the avirulent phenotype of the Δ*plcR* mutant. To answer this question, we tested the virulence of a Δ*plcR* strain complemented with the *inhA2* gene. The results showed that spores from this strain were as inefficient as the Δ*plcR* spores in potentiating the toxic effect of crystals. InhA2 was, however, able to complement the Δ*inhA2* virulence defect. There are two hypotheses to explain why InhA2 was unable to compensate for the absence of PlcR. First, the correct maturation of InhA2 requires the product of a PlcR-regulated gene, and thus InhA2 would be not functional in *B. thuringiensis* strain 407 Cry^- [Δ*plcR* (p*inhA2*)]. Alternatively, InhA2 might have to cooperate with one or several unidentified PlcR-regulated factors. The possible cooperative properties of members of the PlcR regulon highlighted the multifactorial characteristic of *B. thuringiensis* virulence against orally infected insects. The identification of these factors may improve our understanding of the genetic and biochemical bases of the interaction between *B. thuringiensis* and its host after oral infection.

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