The Bacillus thuringiensis PlcR-Regulated Gene \( \text{inhA}2 \) Is Necessary, but Not Sufficient, for Virulence

Sinda Fedhila,1* Michel Gohar,1,2 Leyla Slamti,3 Patricia Nel,1 and Didier Lereclus1,3

Unite´ G´en´etique Microbienne et Environnement, Institut National de la Recherche Agronomique, La Mini`ere, 78285 Guyancourt Cedex,1 and Unite´ de Biochimie Microbienne, Centre National de la Recherche Scientifique (URA2172), Institut Pasteur, 75724 Paris Cedex 15,3 France, and Bayer CropSciences, Ghent B-9000, Belgium2

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We previously reported that Bacillus thuringiensis strain 407 Cry 32\(^{-}\) secretes a zinc-requiring metalloprotease, \( \text{InhA}2 \), that is essential for virulence in orally infected insects. Analysis of the \( \text{inhA}2 \)-lacZ transcriptional fusion showed that \( \text{inhA}2 \) expression is repressed in a PlcR\(^{-}\) background. Using DNase I footprinting experiments, we demonstrated that PlcR activates \( \text{inhA}2 \) 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 \( \text{InhA}2 \). The results indicated that overexpression of \( \text{inhA}2 \) in the \( \Delta \text{plcR} \) strain did not restore the wild-type phenotype. However, virulence was fully restored in the \( \Delta \text{inhA}2 \) complemented mutant. Thus, \( \text{inhA}2 \) 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 \( \text{InhA}2 \) 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 \( \delta \)-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 septicaemia (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 \( \text{plcR} \) 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 (TATGNN\( \varepsilon \)TNCATA), 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 (\( \text{papR} \)) that lies immediately downstream of \( \text{plcR} \) (36). \( \text{papR} \) 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 \( \text{papR} \) considerably reduced the amounts of up to 56 exported proteins (15). Moreover, the inactivation of \( \text{papR} \) 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, \( \text{InhA}2 \) (13). \( \text{InhA}2 \) is a zinc metalloprotease that is highly homologous to the \( B. \) thuringiensis \( \text{InhA} \), 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 \( \text{InhA}2 \) plays a major role in potentiating the toxicity of Cry proteins in orally infected insects (13). Recent studies have shown that \( \text{InhA}2 \) is synthesized by \( B. \) cereus strain ATCC 14579 and is

* Corresponding author. Mailing address: Unite´ G´en´etique Microbienne et Environnement, Institut National de la Recherche Agronomique, La Mini`ere, 78285 Guyancourt Cedex, France. Phone: 33-1-30-83-36-36. Fax: 33-1-30-43-80-97. E-mail: sindah@jouy.inra.fr.

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of one of the B. cereus proteases strongly downregulated in ΔpCR 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 ΔpCR 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 Cry1C belonging to serotype 1 (27) and the 407 Cry1C ΔpCR strain carrying a pCRR disrupted gene (33) were used throughout the present study. The 407 Cry1C inhA2 strain carrying an inhA2 disrupted gene has been described previously (13). Escherichia coli K-12 strain TG1 [Δlac-proAB] supE thi lsd25 (F’ traD36 proA+ proB+ lacIq lacZΔM15) (14) was used as a host for the construction of plasmids and cloning experiments. E. coli strains SCS 110 (pPlcR), the leu en36 thi-1 lacgal gpt1 as lamps t-x ecx dam cpl44 Δ(lac-proAB) (F’ traD36 proA+ proB+ lacIq lacZΔM15; Stratagene, La Jolla, Calif.) and ET12557 (F’ dam-13:Teq dam-6: hald Mut4 recF143 spc-202: Tn10 gptK2 gptT22 uma14 pac Y1 S-l5 leu B 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, and tetracycline at 5 μg/ml in LB medium with vigorous agitation at 37°C and 30°C, respectively. The following antibiotic concentrations were used for bacterial selection: ampicillin at 100 μg/ml and tetracycline at 5 μg/ml in LB medium with vigorous agitation at 37°C and 30°C, respectively.

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 (5) transformed with pH10C (34) as described by Gommet 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 lysozyme (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). Amplicified 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 using a centrifugal filter device (Ultrafree-DA; Amicon Labortories).

Trans-complementation of ΔpCR and the ΔinhA2 mutant strains with inhA2. A 473-bp HindIII/BamHI fragment containing the promoter region of the kana-mycin resistance gene aaph3 was amplified by PCR using the primers Km1 (5'-GGCGGTAGTGGAGTAAAG-3') and Km2 (5'-CCGCAAGACGGTACTTATTAGAC-3') and pDG783 DNA carrying the aaph3 gene from Enterococcus faecalis (38) as a template. PCR products 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 HindIII/BamHI sites of the gram-positive-gram-negative shuttle plasmid, pHT315 (3). The resulting plasmid was designated pHT315Aaph3. A 2.571-bp BamHI/EcoRI 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 Cry1C chromosomal DNA as a template and oligonucleotides inhA2-1 (5'-GGCGGGA TCCACCGTTATTTAAGCTT-3') and inhA2-2 (5'-CCGGAATTCCTTCCCTCCATATTGT-3'). The DNA fragment harboring the inhA2 coding sequence was digested and ligated downstream of the promoter region of pHT315Aaph3. The ligation mixture was used to transform B. thuringiensis strain 407 Cry1C ΔpCR and strain 407 Cry1C inhA2. The transformant clones harboring the recombinant pHT315Aaph3/pinhA2 were designated 407 Cry1C ΔpCR (pinhA2) and 407 Cry1C inhA2 (pinhA2).

Construction of the inhA2-lacZ transcriptional fusion. The inhA2-lacZ transcriptional fusion was cloned by constructing a 593-bp BamHI/PstI DNA fragment harboring the inhA2 promoter between the BamHI and PstI sites of pHT304-18Z (2). The DNA fragment was generated by PCR amplification performed on 407 Cry1C chromosomal DNA with the primers H9252 (5’-AAAA CTGCAAGCCACCAAGCTAATTATTCAATTTG-3') and H989 (5’-CCGCAGATTCCCTTCCCTCCATATTGT-3'). The DNA fragment harboring the inhA2 coding sequence was digested and ligated downstream of the promoter region of pHT315Aaph3. The ligation mixture was used to transform B. thuringiensis strain 407 Cry1C ΔpCR and strain 407 Cry1C inhA2. The transformant clones harboring the recombinant pHT315Aaph3/pinhA2 were designated 407 Cry1C ΔpCR (pinhA2).

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). Aα-G Maxam and Gilbert reactions (28) were carried out on the appropriate 32P-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 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 Vh. The strips were then equilibrated first in urea-sodium dodecyl sulfate-Tris-dithiothreitol, followed by a second equilibration in urea-sodium dodecyl sulfate-Tris-acetamid. 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 by N-terminal sequencing by mass spectrometry.
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 (10^7/l/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 pHIT304-18. The recombinant plasmid (pHT304 inhA2-Z) was introduced into B. thuringiensis wild-type and ΔplcR 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 ΔplcR 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 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′-TATGNAN4TNCATA-3′) (1).
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 ΔplcR 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 ΔplcR spores might be due to the absence of the InhA2 protein. To test this hypothesis, we measured the virulence of a ΔplcR strain expressing the inhA2 gene. A ΔplcR 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− ΔinhA2 mutant strain to verify that InhA2 complements the avirulent InhA2− phenotype. *B. thuringiensis* ΔplcR and ΔinhA2 mutant strains, carrying the inhA2 gene under the control of the aphA3 promoter, were referred to as 407 Cry− [ΔplcR (pinhA2)] and 407 Cry− [ΔinhA2 (pinhA2)], 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 ΔinhA2 strain (data not shown). In the ΔplcR 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 ΔplcR 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− [ΔplcR (pinhA2)] and 407 Cry− [ΔinhA2 (pinhA2)] 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− ΔplcR, 407 Cry− [ΔplcR (pinhA2)], and 407 Cry− [ΔinhA2 (pinhA2)] 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 ΔinhA2 mutant were not synergistic. However, the 407 Cry− [ΔinhA2 (pinhA2)] spores completely recovered the wild-type virulent phenotype, thus demonstrating that the complemented strain was functional. In contrast, the 407 Cry− [ΔplcR (pinhA2)] spores were as ineffective as the parental ΔplcR 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-
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 (TATGNAN₄TCNATA) 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’-CATGNAN₄TNCATA-3’) located just upstream of the −35 consensus promoter box of inhA2. This sequence differs from the previously defined PlcR box by one residue (C₁ versus T₁) (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 is 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 nonfunctional in B. thuringiensis strain 407 Cry⁻ [Δ plcR (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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