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Distinct Mutations in PlcR Explain Why Some Strains of the *Bacillus cereus* Group Are Nonhemolytic

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***Bacillus thuringiensis*, *Bacillus cereus*, and *Bacillus anthracis* are closely related species belonging to the *Bacillus cereus* group. *B. thuringiensis* and *B. cereus* generally produce extracellular proteins, including phospholipases and hemolysins. Transcription of the genes encoding these factors is controlled by the pleiotropic regulator PlcR. Disruption of *plcR* in *B. cereus* and *B. thuringiensis* drastically reduces the hemolytic, lecithinase, and cytotoxic properties of these organisms. *B. anthracis* does not produce these proteins due to a nonsense mutation in the *plcR* gene. We screened 400 *B. thuringiensis* and *B. cereus* strains for their hemolytic and lecithinase properties. Eight Hly$^-$ Lec$^-$ strains were selected and analyzed to determine whether this unusual phenotype was due to a mutation similar to that found in *B. anthracis*. Sequence analysis of the DNA region including the *plcR* and *papR* genes of these strains and genetic complementation of the strains with functional copies of *plcR* and *papR* indicated that different types of mutations were responsible for these phenotypes. We also found that the *plcR* genes of three *B. anthracis* strains belonging to different phylogenetic groups contained the same nonsense mutation, suggesting that this mutation is a distinctive trait of this species.**

Bacillus thuringiensis, *Bacillus cereus*, and *Bacillus anthracis* are three closely related species belonging to the *B. cereus* group (20, 25, 48). These bacteria are gram-positive spore-forming microorganisms, the spores of which can be isolated from many sources, including insects, soils, dust, and foods (10, 11, 22). *B. anthracis*, the etiologic agent of anthrax, produces a capsule and toxins encoded by genes carried by the pXO1 and pXO2 plasmids (for a review see reference 35). The expression of these genes is under the control of the key regulator AtxA and the minor regulators AcpA and PagR (16, 24, 36). *B. thuringiensis* is an entomopathogenic bacterium that produces specific insecticidal toxins encoded by plasmid genes (45). These toxins, the Cry and Cyt proteins, are synthesized when the bacterium enters the stationary phase or the sporulation phase (2). *B. cereus* is an opportunistic pathogen that is responsible for food poisoning, pneumonia, and endophthalmitis, possibly due to production of an emetic toxin, enterotoxins, and degradative enzymes (3, 8, 15, 26, 34). *B. cereus* and *B. thuringiensis* generally produce extracellular proteins that are potentially involved in virulence. Transcription of the genes encoding these proteins is under control of the pleiotropic activator PlcR (1, 30, 38). These putative virulence factors include phospholipases (phosphatidylinositol-specific phospholipase C [PI-PLC], phosphatidylcholine-specific phospholipase C [PC-PLC], sphingomyelinase), cholesterol-dependent hemolysins (cereolysin Clo in *B. cereus* and thuringiolysin Tlo in *B. thuringiensis*), proteases (ColB, InhA, Sfp, NprB, NprP2), a cytotoxin (CytK), and hemolytic (Hbl) and nonhemolytic (Nhe) enterotoxins. Disruption of the *plcR* gene in *B. cereus* strain ATCC 14579 and *B. thuringiensis* strain 407 Cry$^-$ drastically reduces the hemolytic (Hly$^-$) and cytotoxic properties (43) of these strains, decreases their motility, and attenuates the symptoms of endophthalmitis (9). Two-dimensional (2D) gel electrophoresis has shown that most of the proteins secreted by wild-type *B. cereus* ATTC 14579 (including phospholipases, hemolysins, enterotoxins, and proteases) are not secreted by the Δ*plcR* mutant strain (13). Opp, an oligopeptide permease, is necessary for *plcR* expression, suggesting that uptake of a small peptide is required to activate PlcR-regulated gene expression (14). PlcR activation requires a small peptide, designated PapR, which acts as a cell-cell signaling peptide. Agaisse and collaborators (1) showed that *papR* is a PlcR-regulated gene located 70 bp downstream from *plcR* and encoding a 48-amino-acid peptide. This peptide is secreted by the cell and then reimported via Opp, presumably as a pentapeptide. Intracellular PapR is required for binding of PlcR to its DNA targets and thus is required to trigger expression of the PlcR regulon (46).

The importance of the PlcR regulon in the *B. cereus* group was recently emphasized by the publication of the genome sequences of *B. cereus* ATCC 14579 and *B. anthracis* Ames (21, 42). More than 100 genes putatively belong to the PlcR regulon on the basis of the presence of a PlcR DNA-binding sequence upstream from these genes in *B. cereus* ATCC 14579. Most of these genes are also present in *B. anthracis*. However, *B. anthracis* does not produce the PlcR-regulated extracellular proteins even though the corresponding genes are present. Most *B. cereus* and *B. thuringiensis* strains are hemolytic on

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sheep blood agar plates, whereas *B. anthracis* is not hemolytic. This characteristic is commonly used to distinguish between *B. anthracis* and *B. cereus* or *B. thuringiensis*, although it is not considered to be a decisive criterion (29). *B. anthracis* Sterne does not produce PlcR-regulated proteins due to a nonsense mutation in the *plcR* gene (1). Mignot and collaborators (33) showed that this mutation in the *plcR* gene of *B. anthracis* might result from incompatibility between the PlcR and AtxA regulons. Indeed, *B. anthracis* is unable to sporulate when it is complemented with a functional *plcR* gene that restores expression of the PlcR regulon. Point mutations in regulators or in major virulence genes could be one of the mechanisms responsible for the genetic shaping and divergence of closely related populations (5). For example, there is genetic variability among different *Clostridium difficile* clinical isolates due to deletions in the *tcdC* gene, which is the putative transcriptional repressor of the A and B toxins and thus affects the toxicity of strains (47). Thus, a point mutation in a key regulator gene, in association with acquisition of mobile genetic elements harboring virulence factors (transposons, plasmids, bacteriophages), may result in distinctive differences between closely related species that may lead to a specialization, like multiplication in mammals for *B. anthracis* (for a review see reference 18, 19).

In this study, we first aimed to determine whether, because of their monomorphism, distinct *B. anthracis* strains had the same *plcR* mutation. We then sought *B. thuringiensis* and *B. cereus* strains that had lost phenotypic characteristics like hemolysis and lecithinase activity. We wanted to find out whether inactivation of a pleiotropic regulator, such as PlcR, was restricted to *B. anthracis* or if it was widespread in other species belonging to the *B. cereus* group.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The acrystalliferous strain *B. thuringiensis* 407 Cry$^-$, which belongs to serotype 1 (31), and the *B. cereus* ATCC 14579 strain were used as reference strains throughout this study. The hemolysis assays were performed with *B. cereus* and *B. thuringiensis* strains originating from the Institut National de la Recherche Agronomique and Institut Pasteur collections. The type strain of each *B. thuringiensis* serotype was tested (28). Strains 05, 17, 26, and 45 are *B. thuringiensis* strains; the numbers indicate the serotypes. Bt1, Bt13, and Bt32 were identified as *B. thuringiensis* strains and LM112.3 was identified as a *B. cereus* strain on the basis of the presence or absence of crystal inclusions. These four strains, which have not been serotyped yet, were isolated from the environment; the *B. thuringiensis* strains were isolated from forest soils near Versailles (France), and the *B. cereus* strain was isolated from the coleopteran *Otiorhynchus* (Curculionidae). *B. anthracis* RA3R is a field strain cured for pXO2 (39). *Escherichia coli* strain ET 12567 (*dam13*::Tn*9 dcm-6 hsdM hsdR recF143 zjj201*::Tn*10 galK2 galT22 ara-14 lacY1 xyl-5 leuB6 thi-1 tonA31 rpsL136 hisG4 tsx-78 mtlI glnV44* F$^-$) (32) was used as a host for plasmid construction and for preparation of plasmid DNA for *B. thuringiensis* and *B. cereus* transformation. *E. coli* and *B. thuringiensis* cells were transformed by electroporation, as previously described (12, 31). *E. coli* strains were grown at 37°C in Luria-Bertani broth (LB) (1% tryptone, 0.5% yeast extract, 0.5% NaCl). *B. thuringiensis* and *B. cereus* strains were generally grown at 30 or 37°C in LB or in brain heart infusion (BHI) (Difco) broth. Specific media were used for the enzyme activity and motility assays (see below).

The antibiotic concentrations used for bacterial selection were as follows: 100 μg of ampicillin ml^{-1} for *E. coli*; and 10 μg of erythromycin ml^{-1} and 200 μg of kanamycin ml^{-1} for *B. thuringiensis*. Bacteria with the Lac$^+$ phenotype were identified on LB plates containing X-Gal (5-bromo-4-chloro-3-indolyl-D-galactoside) (80 μg ml^{-1}).

Plasmid construction. pHT304Ω*plcR-papR* was constructed by inserting a fragment containing the *plcR* and *papR* genes under control of their own promoter between the XbaI and HindIII sites of pHT304 (4). This fragment was amplified from chromosomal DNA of the *B. thuringiensis* 407 Cry$^-$ strain with primers S1X and S2H (Table 1).

TABLE 1. Sequences of the primers used in this study

Primer	Sequence (5′ −3′)[a]
26C200Y.2	CTTTTGCATGATTATATCTTACCTTC
26C200Y.3	GAAGGTAAGATATAATCATGCAAAAG
phind26	CCCAAGCTTATATTTATCTACTGATTTTATTTACGAG
S1	CTATTATTATATGTGAGATGAATTGTATG
S1X	GCTCTAGACTATTATTATATGTGAGATGAATTGTATG
S2	GTAAAGACGTTTGGATGTTACTCC
S2H	CCCAAGCTTGTAAAGACGTTTGGATGTTACTCC
S3	CGCAATTGCAAACATTTATGCTGA
S3Bwei	TGCGATTGCAAATATTTATGCTGAAAAT
S4	CATTATCATGCAATGCCTCTAATTGT
S4Bt1	CTCTATATAAAGATTTTGGTATACATC
S4Bt13	TATTTTCAGGTAATGCCTCTAATTGC
S4LM112.3	GTTTTCGCATAATTATGTCTTACCTTCAC

[a] Restriction sites are underlined.

The *plcR* gene from strain 26 was amplified from chromosomal DNA by using primers S1X and phind26 (Table 1). The fragment was then inserted between the XbaI and HindIII sites of pHT304 to generate pHT304Ω*plcR*26wt.

The plasmid carrying the modified *plcR* gene from strain 26 (pHT304Ω*plcR*26mt) was constructed as follows. Chromosomal DNA from strain 26 was used as a template for PCR amplification with the S1X-26C200Y.2 and 26C200Y.3-phind26 primer pairs (Table 1). The two resulting fragments were mixed and PCR amplified with primers S1X and phind26. The fragment generated was inserted between the XbaI and HindIII sites of pHT304. In all the cases, the nucleotide sequences of the cloned DNA fragments were verified by sequencing.

DNA manipulations. Plasmid DNA was extracted from *E. coli* by a standard alkaline lysis procedure by using Qiagen kits. Chromosomal DNA was extracted from *B. thuringiensis* and *B. cereus* cells grown to the mid-exponential phase in LB or BHI medium and was purified as previously described (37). Restriction enzymes and T4 DNA ligase were used as recommended by the manufacturers. The oligonucleotide primers used for PCR amplification were synthesized by Proligo (Paris, France). Primers S1 and S2 were used to amplify the *plcR-papR* region, and this region was sequenced by using the primers listed in Table 2. Primer sequences are shown in Table 1. PCR were carried out in 100-μl reaction mixtures containing each deoxynucleoside triphosphate at a concentration of 200 μM, 3.5 mM $MgSO_4$, 50 pmol of each primer, 0.5 μg of chromosomal DNA, and 0.5 U of Pwo DNA polymerase (Roche Boehringer) in 1× reaction buffer. The PCR were performed with a GeneAmp PCR system 2400 thermal cycler (Perkin-Elmer). The reaction conditions were as follows: incubation for 5 min at 94°C,

TABLE 2. Primers used for sequencing the *plcR-papR* regions of various *B. anthracis*, *B. cereus*, and *B. thuringiensis* strains

Strain	Primers used for sequencing
B. anthracis RA3R	S1, S2, S3, S4
B. thuringiensis strains	
407 Cry$^-$	S1, S2, S3, S4
05	S1, S2, S3, S4
17	S1, S2, S3, S4
26	S1, S2, S3, S4
45	S1, S2, S3, S4
Bt1	S1, S2, S3, S4Bt1
Bt13	S1, S2, S3, S4Bt13
Bt32	S1, S2, S3Bwei, S4Bt13
B. cereus LM112.3	S1, S2, S3, S4LM112.3

followed by 30 cycles of 30 s at 48 or 50°C for annealing, 1 min at 72°C for extension, and 30 s at 94°C for denaturation and then incubation at 72°C for 10 min. Nucleotide sequences were determined by Genome Express (Montreuil, France).

Enzyme activity assays. Columbia agar plates containing 5% sheep blood (BioMérieux) were used to assay the hemolytic activity of the *B. thuringiensis* and *B. cereus* strains. The hemolytic activity was also evaluated on agar plates containing washed human erythrocytes (5%). Erythrocytes from defibrinated human blood were washed twice in phosphate-buffered saline (44) before they were added to the medium.

The lecithinase activity was assayed on BHI agar plates containing 5% egg yolk (Difco). The plates were checked after 24 and 48 h, and the assays were repeated at least twice.

2D-PAGE. For preparation of samples for 2D polyacrylamide gel electrophoresis (2D-PAGE) cells were grown in 50 ml of LB at 30°C in 500-ml flasks with rotation at 175 rpm, harvested 2 h after they entered the stationary phase, and centrifuged at 5,000 × g for 10 min at 4°C. The supernatant was rapidly filtered through a membrane (pore size, 0.2 μm). Proteins were precipitated twice by using the deoxycholate-tetrachloroacetic acid method (41). The pellet was washed twice with ethanol-ether (1:1), dried, and stored at −80°C until it was used. The protein content of the pellet was quantified by the Bradford method (7).

2D-PAGE was performed as described previously (13). For gel analysis gels were scanned at 300 dpi and 8-bit depth by using a SHARP JX-330 scanner equipped with a film-scanning unit and were analyzed with the ImageMaster 2D program from Amersham BioScience. The spots were identified on the basis of mobility and by comparison with a reference gel as described previously (13).

Nucleotide sequence accession numbers. *plcR* nucleotide sequences have been deposited in the EMBL database under the following accession numbers: serotypes 1 to 14, AJ582632 to AJ582638 and AJ582669 to AJ582675; serotypes 17 and 26, AJ583460 and AJ583461; isolates Bt13, Bt32, and LM112.3, serotype 45, and isolate Bt1, AJ583463 to AJ583467; and *B. anthracis* RA3R, AJ585425.

The accession numbers of the *papR* sequences of the nonhemolytic strains are AJ586123 to AJ586131. The accession numbers of the *papR* sequences of *B. cereus* ATCC 14579 and *B. thuringiensis* 407 Cry$^-$ (serotype 1) have been given previously (46).

RESULTS

Comparison and analysis of the PlcR sequences from various *B. anthracis*, *B. cereus*, and *B. thuringiensis* strains. We analyzed the hemolytic properties of 400 *B. thuringiensis* and *B. cereus* strains (from the Institut Pasteur and the Institut National de la Recherche Agronomique collections) on sheep blood agar plates. The Hly$^-$ strains were subsequently screened for lecithinase activity on egg yolk agar plates. Eight Hly$^-$ Lec$^-$ strains were found and selected. The *plcR-papR* regions of these strains and of the *B. anthracis* RA3R strain were sequenced and aligned with those of *B. cereus* ATCC 14579, *B. thuringiensis* 407 Cry$^-$, *B. anthracis* Sterne, and *B. anthracis* Ames (Fig. 1A). The *B. anthracis* RA3R strain was chosen because phylogenetically it is distantly related to the *B. anthracis* Ames and Sterne strains. The latter strains both belong to the A3b group, whereas *B. anthracis* RA3R is in the B2 group (23). Although the three *B. anthracis* strains belong to different phylogenetic groups, they all have the same point mutation leading to the same truncated PlcR.

The PlcR sequences of the *B. cereus* and *B. thuringiensis* strains differ essentially in the carboxy-terminal part of the protein. Strain LM112.3 (*B. cereus*) has a truncated PlcR, but the mutation is located at a different place in the sequence than the mutation in *B. anthracis*. The Bt32 protein lacks two residues in the middle of the protein. PlcR from strain Bt1 is 15 residues longer than PlcR from strain 407 Cry$^-$. Mutations that could potentially affect PlcR activity were less obvious in the other Hly$^-$ Lec$^-$ strains. PlcR from strain 45 has two more residues than PlcR from strain 407 Cry$^-$, but it is also divergent throughout the sequence. We compared the PlcR sequences of strains 05, 17, 26, and Bt13 to those of hemolytic strains belonging to serotypes 1 to 14 (data not shown) (see Materials and Methods for the accession numbers of the nucleotide sequences). The PlcR sequence of strain 17 differed at five positions, positions 8, 15, 154, 207, and 244 (Fig. 1A). PlcR from strain 26 differed at two sites, E186D and H/Y200C; the latter difference presumably had a drastic effect. No mutations were detected in strains 05 and Bt13.

Alignment of the predicted PapR polypeptide sequence of each nonhemolytic strain with the sequences of the hemolytic strains revealed no obvious mutations, except in *B. thuringiensis* serotype 17 (Fig. 1B). Indeed, the PapR pentapeptide, which is located at the carboxy terminus of the PapR protein and is required for PlcR activation, contains an isoleucine at position 1. When the first residue of the pentapeptide is an isoleucine, PlcR is not activated in *B. thuringiensis* 407 Cry$^-$ (46). However, addition of synthetic pentapeptides (with a leucine, a valine, or an isoleucine at position 1 of the pentapeptide) on sheep blood agar plates did not restore the hemolytic activity in strain 17 (data not shown). The absence of hemolysis and lecithinase activity might be due to the absence of the genes conferring these phenotypes or to the absence of a functional PlcR protein. This is discussed below.

Together, our results indicate that the possible causes for PlcR inactivity can be summarized as follows: (i) a nonsense mutation leading to a truncated protein (*B. cereus* LM112.3 and *B. anthracis*), (ii) deletions (Bt32) or additions (Bt1), (iii) divergence throughout the sequence (*B. thuringiensis* serotype 45), (iv) point mutations (*B. thuringiensis* serotype 17 and serotype 26), and (v) mutations in genes required for PlcR expression or activity (e.g., *papR*, *opp*, gene coding for a protease responsible for PapR maturation).

Point mutation in PlcR restores activity in *B. thuringiensis* serotype 26. We next tested the hypothesis that a point mutation could be sufficient to explain the loss of PlcR activity in nonhemolytic strains. This hypothesis was examined by using strain 26; we hypothesized that a cysteine at position 200 may have a drastic effect on PlcR activity in this strain. We constructed pHT304Ω*plcR*26mt, in which the cysteine codon was replaced by a tyrosine codon. This plasmid and pHT304Ω*plcR*26wt (carrying the wild-type *plcR* gene from strain 26) were introduced into strain 26, which had two potential mutations (Fig. 1A). We then assayed the enzymatic activities of the transformants (Fig. 2). Both the lecithinase and hemolytic activities were fully restored when strain 26 was complemented with pHT304Ω*plcR*26mt, whereas no lecithinase activity and only weak hemolysis were detected when it was complemented with pHT304Ω*plcR*26wt. Similarly, the protease activity of strain 26 on BHI milk agar plates was also restored when the strain was complemented with pHT304Ω*plcR*26mt (data not shown).

Complementation of the Hly$^-$ Lec$^-$ strains with functional *plcR* and *papR* genes. pHT304 and pHT304Ω*plcR-papR* were introduced into each Hly$^-$ Lec$^-$ strain except the *B. anthracis* strain. The transformants were then assayed on sheep blood agar plates, human blood agar plates, and egg yolk agar plates. In parallel, the extracellular proteomes of each wild-type strain and the corresponding complemented strain were analyzed by

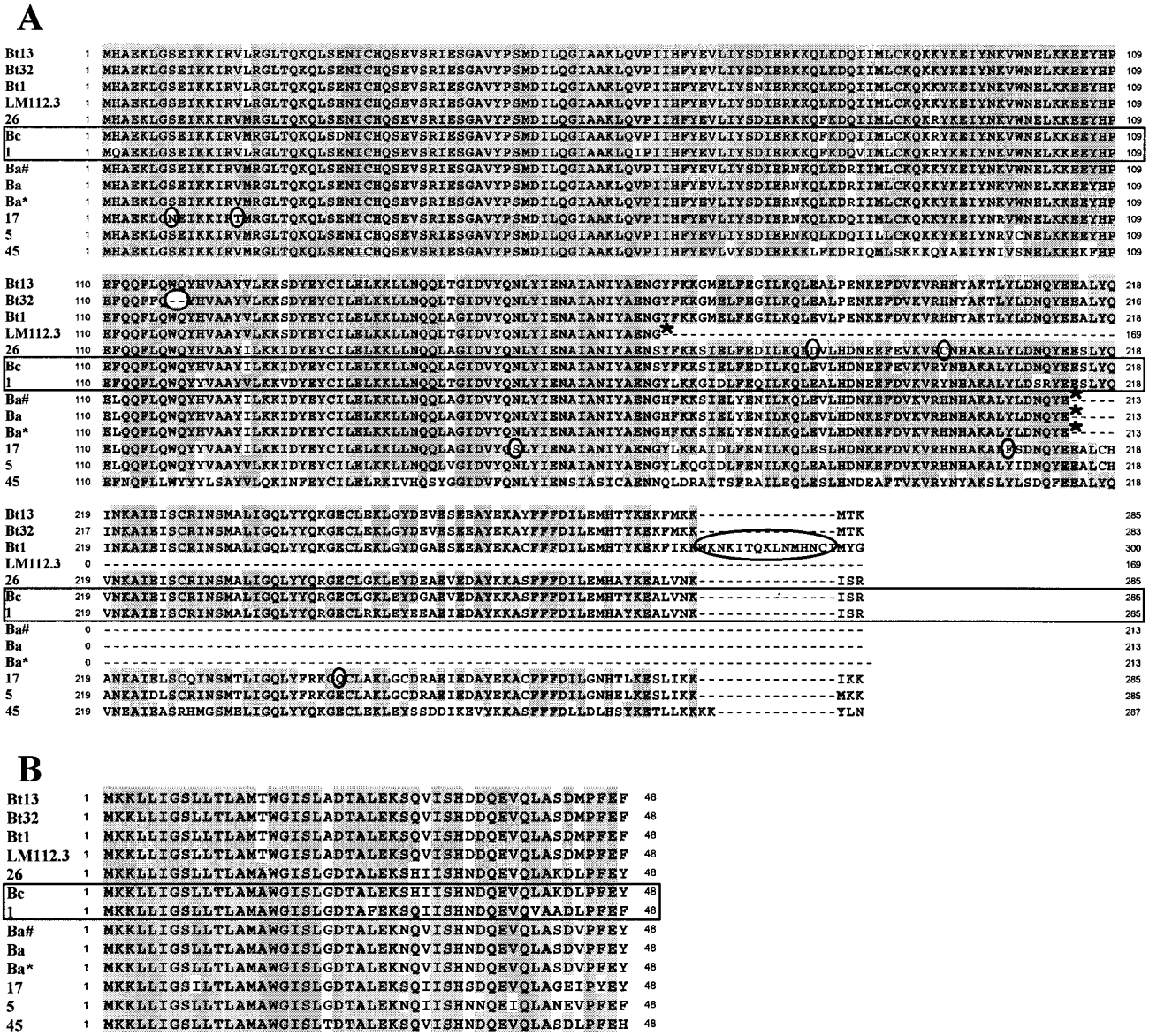

FIG. 1. Comparison of the PlcR (A) and PapR (B) sequences of various *B. anthracis*, *B. cereus*, and *B. thuringiensis* strains. Ba, *B. anthracis* (a superscript asterisk, no superscript, and a superscript number sign indicate the Sterne, Ames, and RA3R strains, respectively); Bc, *B. cereus* ATCC 14579; LM112.3, *B. cereus* LM112.3; Bt1, Bt13, and Bt32, *B. thuringiensis* Bt1, Bt13, and Bt32, respectively; 26, 17, 5, and 45, *B. thuringiensis* strains 26, 17, 5, and 45, respectively; 1, *B. thuringiensis* 407 Cry$^-$ strain. Bt1, Bt13, and Bt32 were isolated from the environment for this study and have not been serotyped yet. Sequences corresponding to the sequences of the hemolytic strains are enclosed in boxes. The sequences were aligned by using Megalign (DNASTAR). In panel A, the nonsense mutations in PlcR are indicated by stars. The other mutations thought to be responsible for the loss of hemolytic and lecithinase activities are circled. The nucleotide sequences of *plcR* of *B. thuringiensis* 407 Cry$^-$, *B. cereus* ATCC 14579, *B. anthracis* Sterne, and *B. anthracis* Ames have been described previously (1, 30, 42).

2D-PAGE, and the presence of the potential virulence factors belonging to the PlcR regulon was determined for each strain (Table 3). An example of each assay for strain LM112.3 is shown in Fig. 3, and an example of 2D-PAGE of the extracellular proteome of strain LM112.3 is shown in Fig. 4. All the results are summarized in Table 3. Hemolysis, both of sheep blood and of human blood, and lecithinase activity were restored when strains 17, 26, Bt32, and LM112.3 were complemented with pHT304Ω*plcR-papR*. Bt13(pHT304Ω*plcR-papR*) exhibited hemolytic activity with human blood and lecithinase activity, whereas only the latter was observed in the case of Bt1(pHT304Ω*plcR-papR*). The phenotypes of strains 05 and 45, which were hemolytic on human blood agar plates, did not change on blood agar and egg yolk agar plates when the strains were complemented with *papR* and *plcR*. Similarly, 2D-PAGE analysis of these two strains revealed no difference between the extracellular proteomes of the wild-type strains and those of the corresponding complemented strains. The extracellular proteome of strain 05 with or without *plcR* and *papR* (data not shown) was considerably different from that of *B. cereus* ATCC 14579 described previously (13). The former lacked almost all the major proteins of the PlcR regulon except CytK, sphingo-

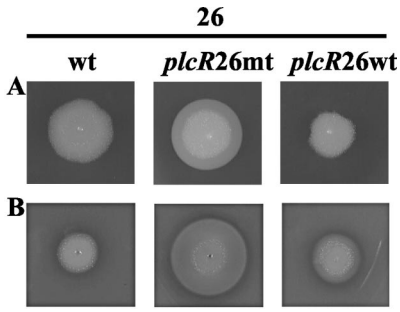

FIG. 2. Complementation of *B. thuringiensis* serotype 26 with mutated *plcR* restored both hemolytic activity on sheep blood plates (A) and lecithinase activity on egg yolk plates (B). wt, *B. thuringiensis* serotype 26 wild type; *plcR*26mt, *B. thuringiensis* serotype 26(pHT304Ω*plcR*26mt); *plcR*26wt, *B. thuringiensis* serotype 26(pHT304Ω*plcR*26wt).

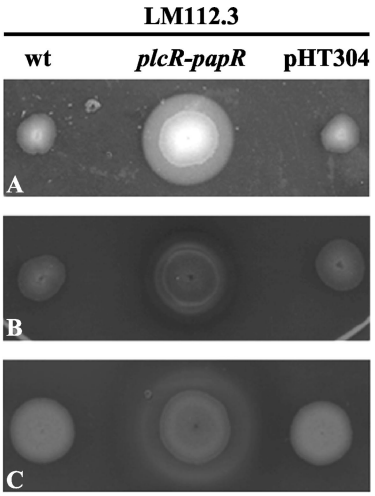

FIG. 3. Complementation with *plcR* and *papR* restored PlcR-regulated enzyme activities of the LM112.3 strain. wt, *B. cereus* wild-type strain LM112.3; *plcR-papR*, *B. cereus* LM112.3(pHT304Ω*plcR-papR*); pHT304, *B. cereus* LM112.3(pHT304). The plates were incubated at 30°C for 24 h. (A) Lecithinase activity on egg yolk agar plates was restored when LM112.3 was complemented with *plcR* and *papR*. (B and C) Hemolytic activity on sheep blood agar plates (B) and on human blood agar plates (C). Hemolysis was observed only with the LM112.3(pHT304Ω*plcR-papR*) strain.

myelinase, and Tlo. Both wild-type and complemented strain 45 produced Sfp, NheA, NheB, NprP2, Tlo, HblL2, PI-PLC, and sphingomyelinase. Wild-type strain Bt1 produced Sfp, NheA, NprP2, NprB, and sphingomyelinase, and it produced ColB, NheB, Tlo, HblL2, and PC-PLC when it was complemented with *plcR* and *papR*. CytK, NheA, NheB, NprP2, NprB, and HblL2 were produced by both wild-type strain Bt13 and the complemented Bt13 strain, but Tlo was only weakly produced by the wild-type strain. The amount of Tlo was greater in Bt13(pHT304Ω*plcR-papR*) than in Bt13. ColB, Sfp, sphingomyelinase, and PC-PLC were found only in the extracellular proteome of Bt13(pHT304Ω*plcR-papR*). Wild-type Bt32 and Bt32(pHT304Ω*plcR-papR*) both expressed Sfp, but NprB, Tlo, HblB, L1, L2, PI-PLC, and PC-PLC were found only in Bt32(pHT304Ω*plcR-papR*). None of these proteins were present in wild-type strains 17, 26, and LM112.3, but all were present when these strains were complemented with *plcR* and *papR*.

DISCUSSION

Eight of the 400 *B. thuringiensis* and *B. cereus* strains screened in this study displayed a nonhemolytic phenotype on sheep blood agar plates and were also lecithinase negative on egg yolk agar plates, like *B. anthracis*. These two characteristics

TABLE 3. Complementation results

Strain[a]	2D-PAGE analysis[b]														Agar plates assays[c]		
	ColB	Sfp	CytK	NheB	NheA	NprP2	NprB	Tlo; Clo	HbIL2	HbIL1	HBlB	PI-PLC	Smase	PC-PLC	Sheep blood	Human blood	Egg yolk
05 wt	−[d]	−	+	−	−	−	−	+	−	−	−	−	+	−	−	+	−
05 *plcR-papR*	−	−	+	−	−	−	−	+	−	−	−	−	+	−	−	+	−
17 wt	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
17 *plcR-papR*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
26 wt	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
26 *plcR-papR*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
45 wt	−	+	−	+	+	+	−	+	+	−	−	+	+	−	−	+	−
45 *plcR-papR*	−	+	−	+	+	+	−	+	+	−	−	+	+	−	−	+	−
Bt1 wt	−	+	−	−	+	+	+	−	−	−	−	−	+	−	−	−	−
Bt1 *plcR-papR*	+	+	−	+	+	+	+	+	+	−	−	−	+	+	−	−	+
Bt13 wt	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
Bt13 *plcR-papR*	+	+	+	+	+	+	+	—[e]	+	−	−	−	+	+	−	+	+
Bt32 wt	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Bt32 *plcR-papR*	−	+	−	−	−	−	+	+	+	+	+	+	−	+	+	+	+
LM112.3 wt	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
LM112.3 *plcR-papR*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] wt, wild type; *plcR-papR*, PHT304Ω*plcR-papR*.

[b] A 2D-PAGE analysis of the extracellular proteomes of the wild-type and the *plcR-papR*-complemented strains was performed. Co1B, collagenase; Sfp, subtilase family protein; CytK, hemolysin cytolysin K; NheB, enterotoxin Nhe component B; NheA, enterotoxin Nhe component A; NprP2, neutral protease; NprB, neutral protease; Tlo/Clo, hemolysin thuringiolysin O/cereolysin O; HbIL2, enterotoxin Hbl lytic component L2; HbIL1, enterotoxin Hbl lytic component L1; HblB, enterotoxin Hbl binding component B; PI-PLC, phosphatidylinositol-specific phospholipase C; Smase, sphingomyelinase; PC-PLC, phosphatidylcholine-specific phospholipase C.

[c] Enzymatic activities were determined on sheep blood agar plates, human blood agar plates, and egg yolk agar plates.

[d] −, negative (protein or activity absent); +, positive (protein or activity present). A spot was considered positive if its volume was equal to or greater than 0.1% of the total spot volume (pixel density × area).

[e] —, There was an increase in the amount of protein.

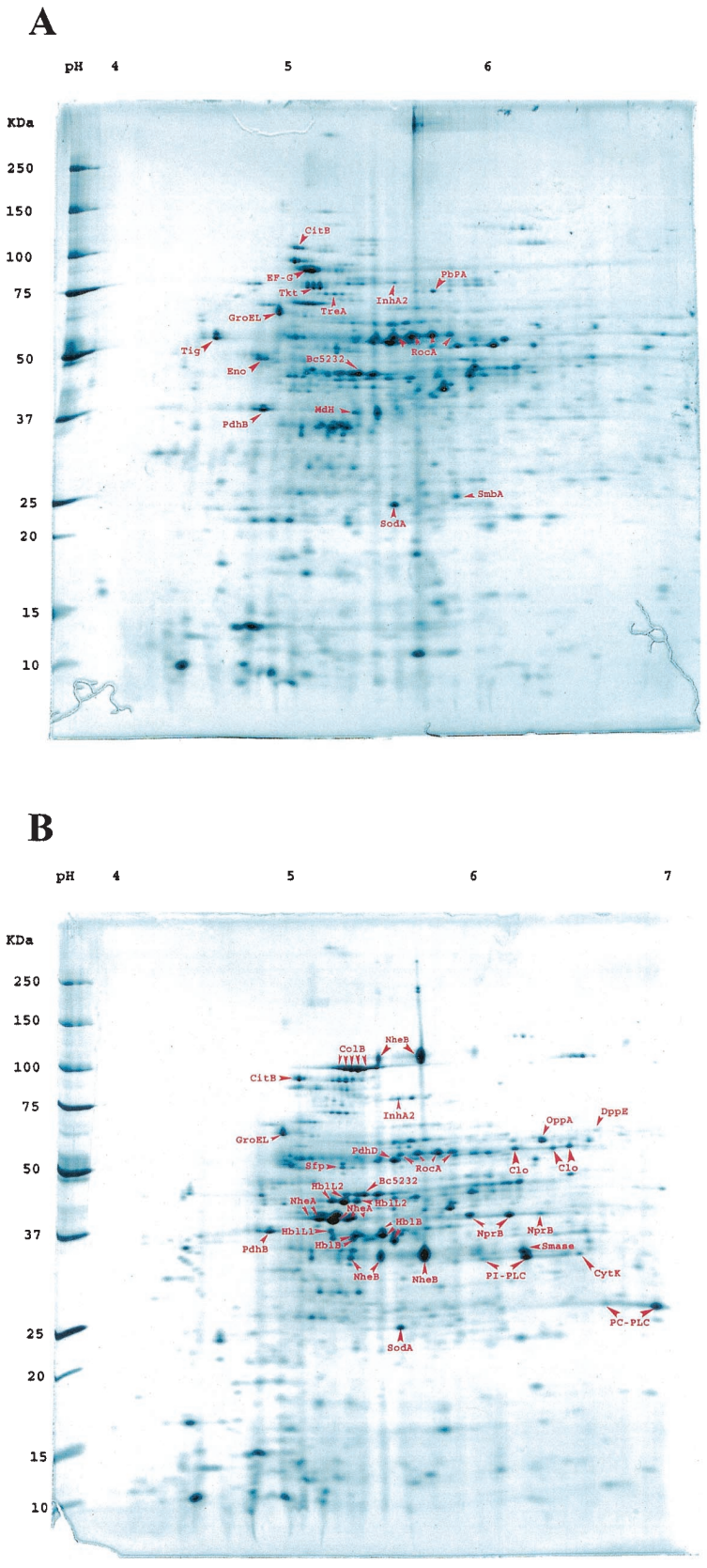

FIG. 4. 2D-PAGE of the extracellular proteomes of two wild-type strains and the corresponding *plcR-papR*-complemented strains. Proteins of interest are indicated by arrowheads and were identified by comparison with 2D-PAGE results for the *B. cereus* ATCC 14579 strain. (A) *B. cereus* wild-type strain LM112.3. (B) *B. cereus* LM112.3(pHT304Ω*plcR-papR*). For abbreviations of the proteins see Table 3, footnote *b*.

are known to depend on the presence of PlcR-regulated proteins (43). Sequence analysis of three *B. anthracis* strains belonging to the A3b and B2 phylogenetic groups (23) indicated that these strains have identical *plcR* sequences and harbor the same nonsense mutation in the *plcR* gene. This is in agreement with the monomorphism of *B. anthracis* and strongly suggests that the nonsense mutation is a distinctive feature of this species. This is emphasized by the fact that all of the *B. cereus* and *B. thuringiensis* mutations that led to an inactive PlcR polypeptide differed from the mutation found in *B. anthracis*. Thus, the point mutation in the *plcR* gene of *B. anthracis* might be used to design specific primers to detect *B. anthracis* strains.

Analysis of the PlcR sequences of the eight Hly$^-$ Lec$^-$ strains revealed that the amino-terminal part of the protein, which is the putative helix-turn-helix DNA-binding domain, is very well conserved. In contrast, the region located downstream of the 75th residue, which contains the tetratricopeptide repeat domain region (40), is highly variable. These domains are involved in protein-protein interactions or protein-peptide interactions (6, 27). Thus, the loss of PlcR activity in these strains might be due to a failure to form multimers or to the lack of an interaction with the activating peptide PapR rather than to an inability to bind DNA.

B. cereus LM112.3 had a truncated, presumably inactive PlcR. In this strain, PlcR is 169 residues long, whereas the PlcR proteins of hemolytic strains are 285 residues long. Analysis of the extracellular proteome of strain LM112.3 showed that the PlcR-regulated proteins examined in this study were not produced in the wild-type strain and that production of these proteins was restored in the *plcR-papR*-complemented strain. Furthermore, the enzymatic activities tested were restored in the complemented strain. *B. thuringiensis* serotypes 17 and 26 responded in a similar manner to complementation. For these three strains, complementation with *plcR* and *papR* completely restored the enzymatic activities tested. This was not the case with strains Bt1, Bt13, and Bt32. The enzymatic activities of Bt32 were restored, but about one-half of the PlcR-regulated proteins were absent from the complemented secretome and Sfp seemed to be regulated independently of PlcR. The genes encoding the proteins that were not induced after PlcR complementation might be absent from the Bt32 strain, as is the case for a large number of *B. cereus* strains (17). However, mutations in the coding sequences of these genes or in the PlcR boxes upstream of these genes might also explain the absence of these proteins in the secretome. One-half of the PlcR-controlled proteins examined were present in wild-type strains Bt13 and Bt1, suggesting either that they are not under control of PlcR or that PlcR is not totally inactive in these strains. The latter hypothesis might be in agreement with the mutation found in the PlcR sequence of Bt1 (addition of 15 residues at the carboxy-terminal end) and with the apparent absence of mutations in the PlcR sequence of Bt13. Tlo, which confers the ability to lyse human blood (33), was present in wild-type strain Bt13. However, probably because of the small amount of Tlo, this strain was nonhemolytic on human blood agar plates. Strain Bt1(pHT304Ω*plcR-papR*) was also nonhemolytic on human erythrocytes even though expression of Tlo was induced, suggesting that Tlo was inactive. In this strain, only lecithinase activity, corresponding to PC-PLC activity, was restored. In *B. cereus* ATCC 14579 and *B. thuringiensis* 407 Cry$^-$, NheA, NheB, HblL1, hb1L2, and HblB are expressed from two operons (1, 38). These proteins were produced in strain Bt1(pHT304Ω*plcR-papR*) as they are independently regulated. The fact that the complemented strain displayed no hemolytic activity with sheep erythrocytes is compatible with the absence of two of the Hbl components as determined by 2D-PAGE. Neither the enzymatic activities nor the secretome profiles of *B. thuringiensis* strains 05 and 45 were changed by complementation (both strains were hemolytic on human blood). Various PlcR-regulated proteins were present in both *B. thuringiensis* serotype 05 and 45 wild-type strains and in these strains transformed with pHT304Ω*plcR-papR*. This strongly suggests that the *plcR* and *papR* genes are functional in these two strains. To verify this hypothesis, we transformed the 05 and 45 wild-type strains with the pHT304Ω*plcA′-lacZ* plasmid. This plasmid carries a transcriptional fusion between the promoter of the *plcA* gene (belonging to the PlcR regulon) and the reporter gene *lacZ* (30). This fusion has been shown to reflect *plcR* expression or PlcR activity. The *plcA′-lacZ* fusion was expressed when it was introduced into strains 05 and 45 (results not shown), confirming that PlcR is produced and functional in these two strains. The Hly$^-$ phenotype on sheep blood agar plates and the Lec$^-$ phenotype are likely due to the absence or inactivity of the genes encoding the Hbl components and PC-PLC.

In conclusion, the work described here showed that inactivation of the *plcR* gene is not restricted to *B. anthracis*, but none of the *B. cereus* and *B. thuringiensis* strains contained the nonsense mutation found in *B. anthracis*. Three strains contained mutations that could be predicted to affect PlcR activity (truncations, deletions, and insertions). The other strains either contained no mutations in PlcR or had sequence variations that may or may not have affected their activity. However, one point mutation was confirmed to be responsible for the loss of PlcR activity. Thus, about 1% (4 of 400) of the strains belonging to the *B. cereus* group were deficient for expression of the PlcR regulon due to mutations in PlcR. The causes of these mutations are unknown. It has been shown that in *B. anthracis* incompatibility with the AtxA regulon results in a drastic reduction in sporulation, and it was suggested previously that the mutation in *plcR* occurred after the acquisition of plasmid pXO1 carrying *atxA* (33). In the four strains with an inactive PlcR regulon which we identified, complementation with a functional *plcR* gene did not affect growth and sporulation (results not shown), suggesting that in these strains counterselection of PlcR did not result from an incompatibility event. The latter hypothesis might be true for *B. anthracis*, but we cannot exclude the possibility that the *plcR* mutation occurred prior to acquisition of the AtxA regulon. Inactivation of PlcR might have been caused by the biological cost of the useless PlcR regulon in some ecosystems and the resulting reduction in fitness of the bacterium.

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