A Spontaneous Translational Fusion of *Bacillus cereus* PlcR and PapR Activates Transcription of PlcR-Dependent Genes in *Bacillus anthracis* via Binding with a Specific Palindromic Sequence

Andrei P. Pomerantsev, Olga M. Pomerantseva, and Stephen H. Leppla*  
Microbial Pathogenesis Section, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, and Biological Defense Research Directorate, Naval Medical Research Center, Rockville, Maryland

Received 20 April 2004/Returned for modification 21 May 2004/Accepted 7 July 2004

Transformation of *Bacillus anthracis* with plasmid pUTE29-plcR-papR carrying the native *Bacillus cereus plcR-papR* gene cluster did not activate expression of *B. anthracis* hemolysin genes, even though these are expected to be responsive to activation by the global regulator PlcR. To further characterize the action of PlcR, we examined approximately 3,000 *B. anthracis* transformants containing pUTE29-plcR-papR and found a single hemolytic colony. The hemolytic strain contained a plasmid having a spontaneous *plcR-papR* intergenic region deletion. Transformation of the resulting plasmid pFP12, encoding a fused PlcR-PapR protein, into the nonhemolytic *B. anthracis* parental strain produced strong activation of *B. anthracis* hemolysins, including phosphatidylcholine-specific phospholipase C and sphingomyelinase. The fused PlcR-PapR protein present in a lysate of *B. anthracis* containing pFP12 bound strongly and specifically to the double-stranded palindrome 5'-TATGCATTATTTCATA-3' that matches the consensus PlcR-binding site. In contrast, native PlcR protein in a lysate from a *B. anthracis* strain expressing large amounts of this protein did not demonstrate binding with the palindromes. The results suggest that the activation of PlcR by binding of a PapR pentapeptide as normally occurs in *Bacillus thuringiensis* and *B. cereus* can be mimicked by tethering the peptide to PlcR in a translational fusion, thereby obviating the need for PapR secretion, extracellular processing, retrieval into the bacterium, and binding with PlcR.

The genomes of *Bacillus anthracis* (18) and *Bacillus cereus* (5) contain the nearly identical and structurally intact *plc* and *sph* genes encoding phosphatidylcholine phospholipase C (PC-PLC) and sphingomyelinase (SPH), respectively (16). These genes are weakly expressed in *B. anthracis* but are fully expressed in the strongly hemolytic *B. cereus*, where they are under the control of the global transcriptional regulator PlcR (7). At least 50 *B. anthracis* genes contain putative recognition sequences for binding of PlcR (18). It has been proposed that the inactivity of these genes in *B. anthracis* is due to a mutation that causes a C-terminal truncation of the *B. anthracis* PlcR protein, which renders it inactive (1). Activation of gene expression by PlcR in *Bacillus thuringiensis* and *B. cereus* is dependent on several additional genes. The small gene *papR* immediately downstream of *plcR* encodes a 48-amino-acid (aa) polypeptide, PapR, which appears to be secreted by the SecA pathway, processed extracellularly (20), and retrieved into the cell by oligopeptide permease (4). Inactivation of either *papR* (20) or some component of the oligopeptide permease complex (*oppB, oppD, and oppF*) (4) prevents activation of PlcR-dependent gene expression. Inside the cell, the resulting peptides, which may contain as few as 5 aa, act as cofactors to activate PlcR, allowing the protein to bind to its DNA target. It has been found that *papR* inactivation in *B. thuringiensis* can be physiologically complemented in vivo by adding the corresponding PapR C-terminal pentapeptide to growing bacterial cultures (20). This system has striking similarities to other peptide-responsive regulatory circuits in gram-positive bacteria, including those involving competence and sporulation (2, 15, 17).

We and others have sought to understand how components of the PlcR-PapR system might function in *B. anthracis*. Transformation of *B. anthracis* with a plasmid carrying the native *B. thuringiensis plcR* and *papR* genes was shown to cause modest transcriptional activation of several genes that are only very weakly expressed in the absence of active PlcR (11). The transcriptional activation was reflected in increased enzyme activity for several gene products, including hemolysins, but it was not clear whether the levels of their expression approached those observed in *B. thuringiensis* and *B. cereus*, where the PlcR regulon is fully active. However, in our recent studies, transformation of *B. anthracis* with the plasmid carrying the very similar *B. cereus plcR* and *papR* genes did not activate expression of *B. anthracis* hemolysins (16). To resolve this apparent discrepancy, we explored alternative methods to express PlcR and PapR in *B. anthracis*. In the course of these studies, we found a spontaneous fusion of the *B. cereus plcR* and *papR* genes, which we describe here. The translationally fused *B. cereus* PlcR-PapR protein, expressed from the fused gene under control of the *plcR* promoter in *B. anthracis*, gave strong activation of the endogenous *plc* and *sph* genes, leading to high levels of the corresponding PC-PLC and SPH activities. An antibody-based assay showed that the fused protein physically
bound to the PlcR palindrome sequence. These results are helpful in understanding how PlcR, PapR, and oligopeptide permease function to activate the PlcR regulon.

**MATERIALS AND METHODS**

**Growth conditions.** Escherichia coli strains were grown in Luria-Bertani (LB) broth and used as hosts for cloning. LB agar was used for selection of transformants. B. anthracis strains derived from Sterne 34F2 DeltaT (SdT) or Ames 33 (6) were grown in brain heart infusion medium (BHI) or in LB medium. Solid media were supplemented with 5% fresh sheep blood or 0.02% L-hemolysin (Sigma-Aldrich, St. Louis, Mo.), respectively, for the hemolytic and PC-PLC determination of DNA fragment length. All constructs were verified by DNA sequencing. The super-repressor (pURE29

**Plasmids**

<table>
<thead>
<tr>
<th>Plasmid or strain</th>
<th>Relevant characteristic(s)*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUTE29-plecR-papR</td>
<td>B. cereus 569 plecR-papR DNA fragment inserted into pUTE29</td>
<td>16</td>
</tr>
<tr>
<td>pUTE29-plecR</td>
<td>pUTE29-plecR-papR without papR gene</td>
<td>This work</td>
</tr>
<tr>
<td>pUTE29-plecR-papR</td>
<td>pUTE29-plecR-papR without plecR gene</td>
<td>This work</td>
</tr>
<tr>
<td>pFP12</td>
<td>pUTE29-plecR fusion to papR</td>
<td>This work</td>
</tr>
<tr>
<td>pAE5</td>
<td>Encodes B. cereus 569 plecR under control of B. anthracis protective antigen gene promoter</td>
<td>16</td>
</tr>
</tbody>
</table>

**Strains**

**B. anthracis**

Sterne 34F2 DeltaT (=SdT) Sterne 34F2 cured of pXO1; therefore, pXO1‘ pXO2‘

Ames 33 pXO1‘ pXO2‘ Ames derivative strain

SdT1 SdT electroporated with pUTE29-plecR-papR; Tc‘; does not produce intracellular PlcR; nonhemolytic strain

SdT2 SdT(pAE5); Km‘; produces intracellular B. cereus PlcR; weakly hemolytic

SdT5 SdT electroporated with pUTE29-plecR; Tc‘; does not produce intracellular PlcR; nonhemolytic

SdT6 SdT electroporated with pUTE29-papR; Tc‘; does not produce intracellular PlcR; nonhemolytic

SdT12 SdT electroporated with pFP12; Tc‘; produces intracellular PlcR-PapR fused protein; hemolytic

A12 Ames 33 electroporated with pFP12; Tc‘; produces intracellular PlcR-PapR fused protein; hemolytic

**E. coli**

XL2-Blue recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F‘ proAB lacIqZAM15 Tn10 (Tc‘) Amy Cm‘]

SCS110 rpsL (Smr) thr leu endA1 thi-1 lacY galK galT ana tonaA tsx dam ccm supE44D (lac-proAB)

* Km‘, kanamycin resistant; Tc‘, tetracycline resistant.

**DNA cloning and sequencing.** The QuikChange XL Site-Directed Mutagenesis kit (Stratagene, Inc., La Jolla, Calif.) was used for pUTE29-plecR creation. The entire pUTE29-plecR portion of pUTE29-plecR-papR (16) was amplified by PCR using the two primers Pr (TTTATATATCTATTATTATTATTATTTTTTC) and Pt (AAAACACCGTCTACTTFTAGCGGTGTGTGTTTTTTT) (Fig. 1). The resulting blunt-ended product was ligated and transformed into E. coli. Plasmid pUTE29-plecR was constructed from pUTE29-plecR-papR by deletion of the plecR gene with SmaI and SnaBI (Fig. 1). All junction regions of plasmids pUTE29-plecR-papR, pUTE29-plecR, and pUTE29-papR were sequenced by the dye-terminator technique with a Taq dye primer cycle sequencing kit. M13 reverse and forward primers were used initially; primers complementary to the determined sequences were subsequently used.

**DNA sequence alignments.** PlcR and PapR homology searches in the GenBank and SWISS Protein databases. Sprog (http://www.igb.uci.edu/tools/scratch/) protein secondary structure prediction software was used for helix-turn-helix (HTH) DNA binding motif search. The Clustal module of the GCG-Lite suite of sequence analysis programs (http://molbio.info.nih.gov/molbio/gglike/) was used for PapR amino acid sequences (20) were used for comparison.

**PC-PLC and SPH enzymatic assays.** Bacteria were grown in LB broth at 37°C. Aliquots of growing cultures were centrifuged 15 min at 8,500 x g. The supernatants and cell pellets were stored in dry ice. The proteins from selected supernatants were concentrated by Centriprep YM-10 units (Amicon, Inc., Beverly, Mass.) and stored at –20°C until used. Concentrations of the proteins in the samples were determined with the bicinchoninic acid (BCA) protein assay reagent (Pierce Biotechnology, Rockford, Ill.). PC-PLC and SPH enzymatic
FIG. 1. Plasmid pUTE29-pecR-papR contains the B. cereus pecR-papR region inserted into pUTE29. A derivative plasmid containing only papR was made by PCR amplification with primers PI and Pr (locations indicated by arrows). The regions responsible for the replication of pUTE29-pecR-papR are shown by arrows and designated as rep pBC16 (for replication in B. anthracis) and rep pUC18 (for replication in E. col).

activities of the samples were determined with, respectively, the Ampex Red PC-PLC assay kit and the Ampex Red SPH assay kit (Molecular Probes, Eugene, Oreg.). Fluorescence was measured with a Wallac 1420 VICTOR 96-well plate reader (Perkin-Elmer, Boston, Mass.) with excitation at 530 nm and emission at 590 nm. The activity of unknown enzymes was compared with that of the standard enzyme supplied with the kit to calculate milliunits per microgram of total protein.

Design of target DNA sequences for NoShift PlcR-binding assay. The NoShift transcriptional factor assay kit (Novagen, Inc., Madison, Wis.) was used to measure binding of PlcR proteins to double-stranded target DNAs as described by Bruggink and Hayes (Novagen Newsletter in Novations 18:13–16, 2003, Novagen, Inc.). The PlcR-binding site of the B. cereus 569 plcR gene (16) was synthesized with an additional 10 nucleotides flanking each end of the core palindrome sequence (shown in boldface in Table 2). This oligonucleotide was designated T1 (Table 2). The sequence of the putative 16-bp PlcR-binding site upstream of the B. anthracis plc start codon (16) was selected as an additional target. This T2 sequence contains one deviation from the PlcR consensus core sequence and from T1 (a T at the fourth position instead of G, shown in italic). Finally, an artificial sequence, T3, was designed to contain one additional mutation (C at the 11th position instead of T, also shown in italic). The deoxyoligonucleotides and their exact complements (Table 2) were synthesized by Sigma-Genosys (Woodlands, Tex.), with each strand containing biotin on the 3′ end. Duplexes were prepared from the complementary pairs of oligonucleotides according to the NoShift protocol. Thus, 10 μg each of sense and antisense oligonucleotide was added to 100 μl of a mixture of 0.5 M SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 75 mM NaCl, and 7.5 mM sodium citrate, pH 7.0, heated to 100°C for 10 min; slowly cooled to room temperature; and diluted with water to a concentration of 10 pmol/μl. Nonbiotinylated T1 duplex (T1 has the same sequence as T1, but synthesized without biotin) was diluted with water to a concentration of 50 pmol/μl and used as a specific competitor DNA. An oligonucleotide, NS, having no PlcR consensus sequences, was made in a nonbiotinylated form, diluted with water to 50 pmol/μl, and used as a nonspecific competitor.

Preparation of cell protein extract for NoShift PlcR-binding assay. Bacteria were grown in LB or BHI broth at 37°C up to A600 ~ 1.4. The cells were washed twice by ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 43 mM Na2HPO4, 27 mM KCl, 15 mM KH2PO4, pH 7.4), by centrifugation at 15,000 × g for 10 min at 4°C. Nuclease protein extraction kit (Novagen, Inc., Madison, Wis.) was used for the protein extraction. The cell pellet from a 25-ml culture was resuspended in 1,200 μl of prechilled NucBuster Extraction reagent 2 supplemented with 16 μl of 100× protease inhibitor cocktail and 16 μl of 100 mM dithiothreitol (DTT). The resuspended cells were added to prechilled FastPROTEIN BLUE tubes and homogenized with glass beads with a FastPrep FP120 instrument (Qiobiogene; BIO 101 Systems, Carlsbad, Calif.) at a speed of 6.0 for 2 to 30-s periods. After homogenization, the tubes were centrifuged for 15 min at 20,000 × g and the supernatants were transferred to new tubes for protein determination.

NoShift PlcR-binding assay. The binding affinity of SdT, SdT1, SdT2, and SdT12 protein extracts to different target DNA duplexes (T1, T2, and T3) was determined with the NoShift transcriptional factor assay kit (Novagen, Inc.). For measurement of binding efficiency, each reaction mixture contained 5 μl of 4× NoShift Bind buffer, 1 μl of pol(dI-dC)2-pol(dI-dC), 1 μl of salmon sperm DNA, 1 μl of biotinylated 50-pmol target DNA duplex, and various amounts of cell extract and competitive nonbiotinylated DNA complexes in a total reaction volume of 20 μl. After 30 min of incubation on ice, 80 μl of 1× NoShift Bind buffer was added to each reaction mixture, and the resulting 100 μl was dispensed into one well of a freshly prepared streptavidin plate and incubated for 1 h at 37°C. After the plates were washed with 1× NoShift Bind buffer, the binding of PlcR was detected by incubation for 1 h at 37°C with 100 μl of rabbit serum 1451 (directed to the C-terminal peptide of PlcR) (15) diluted 1:500 in NoShift antibody dilution buffer. After repeated washing of the plate, horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) (sc-2054; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) was added (1:1,000 dilution in NoShift antibody dilution buffer). After 0.5 h of incubation at 37°C, the wells were washed thoroughly. Finally, 100 μl of room temperature TMB (tetramethylbenzidine) substrate was added and the wells were incubated for 10 to 30 min at room temperature in the dark until a blue color developed. The reaction was stopped by adding 100 μl of 1 N HCl to each well, and A450 was measured with a Wallac 1420 VICTOR 96-well plate reader (Perkin-Elmer, Boston, Mass.).

Western immunoblotting of PlcR derivative proteins. Three different types of Western immunoblotting experiments were performed to (i) detect truncated B.

Western immunoblotting of PlcR derivative proteins. Three different types of Western immunoblotting experiments were performed to (i) detect truncated B.

TABLE 2. Oligonucleotide sequences used for the study of binding activities of PlcR proteins

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper strand</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>5′-TTATATAATTATAGCATATTTTATTATACATACAAAAATTTG-3′</td>
</tr>
<tr>
<td>T2</td>
<td>5′-GTATATAATTGATATTTAACATTTGATTTTTATTATTT-3′</td>
</tr>
<tr>
<td>T3</td>
<td>5′-GTATATAATTGATATTTAACATTTGATTAAAAATTTT-3′</td>
</tr>
<tr>
<td>NS</td>
<td>5′-CTCTTGTCTACCGCACCCGTTACTGTCAGC-3′</td>
</tr>
<tr>
<td>Lower strand</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>3′-TATATATAATTGTAGTAATTATTTTATTATACATAC-5′</td>
</tr>
<tr>
<td>T2</td>
<td>3′-CAATATTATAATTATTTATTTATTTATTTAATATAT-5′</td>
</tr>
<tr>
<td>T3</td>
<td>3′-CAATATTATAATTATTTATTTATTTAATATAT-5′</td>
</tr>
<tr>
<td>NS</td>
<td>3′-GGAGGACAGATGGGCGTGCCGTCATTATGAGAGG-5′</td>
</tr>
</tbody>
</table>

a Sequences related to the PlcR consensus 5′-TATG.A...T.CATA-3′ are indicated in boldface. Nucleotides within this consensus sequence that differ from T1 are shown in italic.
**antibiotics** PlcR, (ii) measure synthesis of _B. cereus_ PlcR in different strains of _B. anthracis_, and (iii) detect binding of fused PlcR-PapR to DNA in vivo.

For detection of truncated PlcR, the frozen bacterial cell pellets from the cultures from which supernatants were assayed for hemolytic enzymes were thawed, suspended in ice-cold PBS (0.15 M NaCl, 10 mM sodium phosphate, pH 7.5), added to prechilled FastPROTEIN BLUE tubes, and homogenized with glass beads with a FastPrep FP120 instrument (Qbiogene, BIO 101 Systems, Carlsbad, Calif.) at a speed of 6.0 for two 30-s periods. After homogenization, the tubes were centrifuged for 15 min at 20,000 _x_ g and the supernatants were transferred to new tubes for protein determination. Equal amounts of each sample (50 to 100 _µg_ of protein) were heated in 1× sample buffer (6× sample buffer contains 0.53 M Tris-HCl, pH 6.8, 10% sodium dodecyl sulfate [SDS], 36% glycerol, 0.6 M DTT, and 0.01% bromphenol blue) at 95°C for 5 min and separated on SDS-polyacrylamide (10 to 20%) gels (Novex precast gels; Invitrogen). The MultiMark multicolored standard (Invitrogen) was used for molecular weight markers. The separated proteins were transferred to nitrocellulose membrane (PROTRAN B85; Schleicher & Schuell) in the Novex transfer unit (Invitrogen). PlcR was detected with rabbit serum 1447, directed to the N-terminal peptide EIYNKVNELKKEEY (present in both _B. anthracis_ and _B. cereus_ PlcRs) as described previously (16). This serum was preadsorbed to _B. anthracis_ proteins to remove cross-reacting antibodies. This was accomplished by incubating a nitrocellulose membrane in an extract of _B. anthracis_ SDT (0.5 to 1 mg of protein/cm²) for 15 min at room temperature. The membrane was blocked with 3% (wt/vol) dry milk and then washed. A 1:200 dilution of the rabbit antiseraum was incubated with the washed membrane in the presence of 3% dry milk solution overnight 4°C. The antiseraum was removed and further diluted in 3% dry milk solution to a final dilution of 1:2,000, and this was used to probe the blots. Blots were developed with 0.2-µg/ml HRP-conjugated goat anti-rabbit IgG (sc-2054; Santa Cruz Biotechnology) and a chemiluminescent substrate (SuperSignal; Pierce Biotechnology). This method was used for the analysis presented in Fig. 6B.

In the second type of Western blot analysis, protein samples were prepared from the bacterial cell pellets as described above. Equal amounts of each sample (35 _µg_ of protein) were mixed with equal volumes of 2× Tris-glycine SDS sample buffer containing 126 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 0.005% bromphenol blue (Invitrogen); heated at 95°C for 5 min; and separated on SDS-polyacrylamide (10%) gels (Novex precast gels; Invitrogen). The BenchMark prestained protein ladder (Invitrogen) was used for molecular weight markers. The separated proteins were transferred to nitrocellulose membrane (PROTRAN B85; Schleicher & Schuell) in the Novex transfer unit (Invitrogen). PlcR was detected with a 1:2,000 dilution of rabbit serum 1451, directed to the N-terminal peptide LEKLGYDETESEEAY (16). The blot was developed with 1,000 HRP-conjugated goat anti-rabbit IgG (SKU 474-1506; KPL, Inc., Gaithersburg, Md.) and TMB 1-component membrane peroxidase substrate (KPL). This method was used for the analysis presented in Fig. 8.

In the third series of the Western blot experiments, duplicate cultures of _B. anthracis_ Sterne 34F2 DeltaT (SDT) with the plasmid pUTE29- plcR-papR (Fig. 1) did not reconstitute the PlcR regulon so as to activate hemolysins able to lyse sheep erythrocytes (16) (Fig. 2A). To examine whether the individual _B. cereus_ plcR or papR genes would do so, we eliminated either _plcR_ or _papR_ from pUTE29- plcR-papR and individually transformed the plasmids pUTE29- plcR and pUTE29- papR into _B. anthracis_ SDT. Again, neither the pUTE29-plcR nor the pUTE29-papR transforms induced hemolysis of fresh sheep blood (Fig. 2B and C). This suggests that the lack of PlcR-regulation in _B. anthracis_ cannot be attributed solely to the C-terminal truncation of the PlcR protein.

In a subsequent experiment, thousands of additional colonies were examined from the transformation of _B. anthracis_ SDT with plasmid pUTE29- plcR-papR, and a single strongly hemolytic colony was found. Restriction analysis of the plasmid pFP12 isolated from this colony revealed a single deletion in the region between the plcR and papR genes. No additional mutations were found by sequencing the entire pFP12 BamHI-HindIII fragment (Fig. 1). Consequent electrophoresis of pFP12 into SDT or a different _B. anthracis_ strain, Ames 33, also produced hemolytic colonies on blood agar (Fig. 2D) and halo-
forming colonies on plates with lecithin, indicating induction of PC-PLC and SPH activities. Ames 33 transformants with unmodified pUTE29-plcR-papR did not demonstrate hemolysis or hydrolysis of lecithin (data not shown).

Sequence analysis of plcR and papR genes in pUTE29-plcR-papR and pFP12. Sequence analysis of the B. cereus 569 plcR and papR genes from the plasmid pUTE29-plcR-papR (Fig. 1) showed that these sequences were identical to the previously reported sequences (GenBank accession no. AY195601). These genes encode the PlcR (285 aa) and PapR (48 aa) proteins. B. cereus 569 PlcR contains two sequential MKK amino acid motifs at the C terminus, whereas B. cereus 569 PapR contains the same 3-aa sequence at its N terminus. Apparently, recombination between the nucleotides encoding the MKK sequences at the end of PlcR and at the start of the PapR resulted in deletion of the 100-bp intergenic region to produce the translational fusion in the recombinant plasmid pFP12. The resulting 330-aa PlcR-PapR fusion protein has lost one MKK motif from PlcR and modified the remaining one to MKN (Fig. 3).

PlcR and PapR amino acid sequence analysis. BLAST searches for sequences matching B. cereus 569 PlcR showed that the N-terminal portion of the protein is homologous (E value, 4e-07) with transcriptional regulators belonging to the HTH XRE family of proteins (NCBI conserved domain database; http://www.ncbi.nlm.nih.gov/Structure/cdd/). PlcR secondary structure analysis showed that the DNA-binding domain of the protein comprised of residues 18 to 55 contains a Cro/C1 HTH motif (10), with its two alpha helices connected by a two-residue beta turn (Fig. 4).

In general, prokaryotic transcription factors with an HTH motif bind to palindromic DNA sequences as homodimers. Examples include the Cro protein of bacteriophage 434, which binds the palindrome AGTACAAACTTTCTTGTATTATA CAAGAAAGTTTGTACT (10), and bacteriophage λcl protein, which binds the palindrome ATTGTGGCACGCACAAC (9) (underlined sequences are inverted repeats). In structures such as the Cro protein, the HTH motif is part of the main body of the protein. In others, to which PlcR probably belongs, it is found in a small domain extending out of the main structure (10). Alignment of 18 PlcR sequences showed that the DNA-binding domain is located at the N-terminal portion of the protein, and it represents the most conservative segment of the molecule (data not shown).

In contrast, the most variable region of PlcR is the C-terminal portion. We found that sequences in this more variable domain fall into two groups (Fig. 5). In the more conservative first group (Fig. 5, top), containing eight B. thuringiensis sequences and one B. cereus PlcR sequence, the consensus sequence of the C-terminal octamer, ALVNKISR, exactly matches all but one actual sequence. The second group of C-terminal sequences containing six B. thuringiensis, one B. cereus, and two deduced (but not expressed) B. anthracis sequences.
quences (see figure legend) is somewhat more variable (Fig. 5, bottom). Each of these two sets of PlcR sequences has a matching set of PapR C-terminal pentapeptide sequences that fit a consensus. It has previously been shown with synthetic pentapeptides that the first amino acid in this C-terminal sequence of PapR controls the specificity of PlcR activation (20). Thus, peptides with N-terminal Leu (L) activated different Bacillus strains from peptides with Val (V) or Met (M). From the alignments in Fig. 5, it is now clear that this specificity is also reflected in the C-terminal PlcR sequences, suggesting these two regions may interact.

**Fused** *B. cereus* PlcR-PapR activates expression of the truncated *B. anthracis* PlcR and induces PC-PLC and SPH activities. We previously showed that the parental *B. anthracis* SdT strain produces no PlcR protein and the resulting PC-PLC or SPH activities are negligible. Although expression of the full-size *B. cereus* PlcR protein from the PA promoter of pAES in *B. anthracis* SdT2 took place from the beginning of the exponential phase of growth, the activities of the PlcR-regulated enzymes PC-PLC and SPH increased with time very slowly and did not reach values like those in *B. cereus* 569 (16). A similar analysis has now been performed with *B. anthracis* strains SdT1 and SdT12 (Fig. 6). Strain SdT12 containing pFP12 grew at rates similar to that of the SdT1 strain, which contains pUTE29-plcR-papR (Fig. 6A). Western blotting showed that the fused PlcR-PapR protein appeared during exponential growth in LB medium, and the intensity of the band increased up to 10 h (Fig. 6B), a time that corresponds to the stationary phase (8). However, expression of the truncated, chromosomally encoded *B. anthracis* PlcR was only evident at the onset of the stationary phase (8 h), and the level of expression was low in comparison with the fused protein. The discrepancy in expression can be explained by different copy number of the genes—high for the fused protein located on the plasmid pFP12 and low (probably, single) for the truncated protein located on the *B. anthracis* chromosome. Similarly, PC-PLC (Fig. 6C) and SPH (Fig. 6D) activities were detected in the pFP12 transformant strain SdT12 and again at the onset of the stationary phase. *B. anthracis* SdT1 strain with plasmid pUTE29-plcR-papR produced no PlcR protein (data not shown), and the PC-PLC and SPH activities were negligible (Fig. 6C and D).

**Fused** *B. cereus* PlcR-PapR protein binds specifically with the putative PlcR-binding palindrome. To demonstrate binding of PlcR proteins to their DNA targets, we employed a solid-phase antibody-based technique, available commercially as the NoShift assay. Various amounts of a SdT12 cell protein extract were incubated with three immobilized target DNA duplexes: T1, T2, and T3. Figure 7A shows linearity in the signal increasing between 2.5 and 12.5 μg of cell extract for all targets. However, the slope was much higher for the T1 duplex than for the T2 and T3 duplexes. Approximately 25 μg of SdT12 extract was enough to saturate 10 pmol of the T1 duplex, which contains the perfect putative PlcR-binding palindrome (TATTGnAnnnnTnCATA, where n is any of the four nucleotides, A, G, C, or T, (1). For the T2 duplex, having one mutation in the palindrome structure, and especially for T3 (two mutations), saturation did not occur even when 50 μg of SdT12 extract was added. These data unambiguously show that integrity of the palindrome is essential for the binding of the PlcR-PapR fused protein.

Figure 7B shows results of a competitive analysis using the same SdT12 cell protein extract. A signal/background ratio of >5:1 was achieved in the absence of competitor. Nonspecific, nonbiotinylated NS competitor did not effectively compete for
binding. However an equimolar amount of the nonbiotinylated, specific competitor T1C blocked most of the binding, decreasing the signal/background ratio to a value of 2. These data show that the binding of the PlcR-PapR fused protein to the palindrome is specific. The presence of functional PlcR proteins in extracts from three additional strains was also tested with the NoShift assay (Fig. 8, top). SdT and SdT1 extracts did not demonstrate any binding activity above that of controls (no cell extract added). Western blot analysis of the same extracts showed they contained no detectable PlcR (Fig. 8, bottom). The same analysis showed extensive production of full-length *B. cereus* PlcR protein in *B. anthracis* SdT2, exceeding the amounts of fused PlcR-PapR produced in *B. anthracis* SdT12 (Fig. 8, bottom). In spite of the presence of full-length PlcR protein in the SdT2 extract, its binding to the T1 duplex was barely detectable (Fig. 8, top). These data indicate that the PlcR-PapR fusion protein has strong binding to the target palindrome sequence compared to native PlcR.

**DNA digestion destroys DNA-PlcR-PapR complexes and induces aggregation of PlcR.** In the analysis of extracts for PlcR proteins described above (Fig. 8, bottom), we noted additional immunoreactive bands having decreased mobility than expected for monomeric PlcR proteins. Thus, the SdT2 sample contained the expected ~34-kDa band of PlcR and higher bands of >60 kDa. The SdT12 sample had a band of ~39 kDa, as expected for the PlcR-PapR fusion protein, together with bands of >100 kDa.

To examine the composition of these complexes, we pre-
pared two sets of the SdT2 and SdT12 extracts. In the first set, DNase activity was inhibited by addition of EDTA, whereas in the second, DNase was added along with magnesium. Extracts made with EDTA (Fig. 9A) produced rather discrete bands in comparison with the diffuse zones seen in Fig. 8. The SdT2 sample migrated mostly as a PlcR monomer (110 kDa) with small amounts of a presumed dimer and a trace of a larger species. The SdT12 samples prepared at different times of growth also demonstrated distinct bands. The 8-h sample contained a prominent band of 110 kDa, which decreased in amount at later times. The next band (100 kDa) changed little in intensity from 8 to 12 h but then decreased at 24 h. The last, most intense band, with a molecular mass close to 40 kDa, had a similar dependence on time (Fig. 9A). This band had a mobility consistent with it being the fused PlcR-PapR protein, having a calculated molecular mass of 38,855 Da.

Because the upper bands in the SdT2 sample (Fig. 9A) appeared to be larger than expected for a simple PlcR-PapR homodimer, we speculated that they contain two additional protein components with molecular masses around 42 and 22 kDa. Both of these components as well as PlcR-PapR homodimer bound to DNA because treatment of SdT2 samples with DNase I produced a band with the molecular mass expected for a PlcR-PapR dimer (Fig. 9B). DNase treatment also generated additional PlcR-PapR species having masses higher than the dimer. A similar conversion was obtained from the SdT2 sample, with the exception that the PlcR monomer seen in the EDTA extract remained present (Fig. 9B).

The formation of aggregated, slowly migrating material after DNase treatment suggested that PlcR proteins not able to bind DNA tended to stick to themselves or to other materials. To examine this, the DNase-treated samples were further treated with either Triton X-100 or 8 M urea (Fig. 10). Urea but not Triton X-100 disrupted the aggregates, especially in the SdT12 sample, where this treatment generated the PlcR-PapR monomer. These data confirm that PlcR-PapR monomer is a principal component of the aggregated complexes.

DISCUSSION

The studies reported here were begun to characterize the role of PlcR and PapR in transcriptional activation of PlcR-regulated genes in B. anthracis. In the initial study that identified PlcR as a global regulator in B. thuringiensis (7), it was shown that deletion of papR (originally orf2) along with its 3’ transcriptional terminator significantly reduced production of PlcR-regulated enzymes. The genomic structure (Fig. 2A) suggests that PapR can be encoded by transcripts starting at PlcR-regulated promoters upstream of both plcR and papR, with both putative transcripts terminating downstream of papR. Thus, the strong effect of deleting papR and its terminator could be due to a requirement for the orf2/papR product in the action of PlcR, or alternatively might result from destabiliza-
tion of the plcR transcript. To examine how expression of \textit{B. cereus} PlcR and PapR in \textit{B. anthracis} would affect expression of PlcR-regulated genes, we constructed two plasmids (Fig. 2). Both of the plasmids expressing single genes, pUTE29-plcR and pUTE29-papR, retained the proposed transcriptional terminator. We found that neither pUTE29-plcR nor pUTE29-papR, nor even the larger plasmid containing both genes, pUTE29-plcR-papR, converted \textit{B. anthracis} into a hemolytic strain (Fig. 2). This was surprising because insertion of the \textit{B. thuringiensis} plcR-papR operon into \textit{B. anthracis} was reported to confer hemolytic properties on the recipient \textit{B. anthracis} strain (11). A more recent report (20) defined the role of PapR in \textit{B. thuringiensis} by showing that it needs to be secreted from the bacterium, processed extracellularly to a pentapeptide, and then taken up by the cell through the oligopeptide permease. Inside the bacterium, the peptide interacts with PlcR to allow it to bind to the PlcR target sequence. The apparent difficulty of reconstituting this system in \textit{B. anthracis} raised the question of whether some components of the PlcR/PapR regulatory circuit are not functional in this species.

In the course of exploring this system in \textit{B. anthracis}, we found a spontaneous fusion of the \textit{B. cereus} PlcR and PapR proteins that strongly activates the PlcR regulon. This in-frame translational fusion produces a protein that retains the PlcR and PapR sequences except for 3 aa at the junction site. Its ability to strongly activate the expression of two PlcR-dependent genes (\textit{plc} and \textit{sph}) shows that the activities of both of the original proteins are retained in the fused protein. The functional properties of this fusion protein allow us to explain several aspects of the activation of the PlcR regulon in bacteria of the \textit{B. cereus} group.

As mentioned before, all known functional PlcR sequences contain a conservative DNA-binding domain with an HTH motif in the N-terminal portion of the molecule. This indicates that the DNA-binding properties of the PlcR protein will differ little between these bacteria (\textit{B. thuringiensis}, \textit{B. cereus}, and \textit{B. anthracis}). DNase footprinting analysis showed that even full-length PlcR binds very weakly to its recognition site in the absence of PapR (20). This suggests that additional interactions that involve the C-terminal region of PlcR are needed to gain sufficient DNA binding strength and specificity to initiate transcription. It is this C-terminal region that is truncated in \textit{B. anthracis} PlcR, thereby rendering it nonfunctional (1). A comparison of PlcR and PapR sequences showed that the C-terminal regions sort into two complementary sets (Fig. 5). These data are consistent with these sequences forming parts of the surfaces by which PapR interacts with PlcR.

A binding assay provided direct evidence for binding of PlcR to its DNA target, and for the influence of PapR on this binding. Only the fused PlcR-PapR protein effectively bound the specific PlcR-binding palindrome. Neither PlcR alone nor its truncated form gave a positive signal in these experiments. These data support the view that binding by the PlcR HTH motif to the DNA target is not strong enough to activate transcription and that PapR or peptides derived from it are needed to give the necessary binding affinity. Theoretically, PapR could interact with some additional motif of the palindrome or it could bind directly to PlcR. We favor the hypothesis that PapR stabilizes a PlcR homodimer. However, we cannot exclude that the stabilization occurs by interaction between the conserved part of C-terminal PapR pentapeptide, PFE(F/Y), and the AT-rich central part of the palindrome combining six A or T nucleotides (1, 12).

As noted above, studies of \textit{B. thuringiensis} showed that the 48-aa PapR peptide is processed, probably extracellularly, to release the C-terminal pentapeptide and that the latter is the active form which increases the affinity of PlcR for its DNA target, probably by inducing a change in the conformation of the PlcR (20). Thus, a defect in PapR can be complemented by the exogenous addition of a synthetic pentapeptide to \textit{B. cereus} or \textit{B. thuringiensis}. In the case of the fusion protein we found, it appears that the tethered PapR sequence can directly activate PlcR. This could occur by the active C-terminal region of PapR folding back to occupy the site normally occupied by the free pentapeptide. This could be a highly efficient process because of the entropy effect, even if the retention of sequences adjacent to the active pentapeptide significantly lowered its intrinsic affinity, as occurs in the analogous regulation of the Rap phosphatases by the Phr pentapeptides (14). In that case, a hexapeptide has 10-fold less activity than a pentapeptide. Alternatively, the PlcR-PapR fusion protein could be processed within the cell to produce short peptides that bind at the pentapeptide binding site. The PapR polypeptide sequence would be expected to remain within the bacterium and be available for intracellular processing because its original signal sequence is embedded in the middle of the fusion protein and will not be functional.

The normal mechanism of activation of PlcR by PapR-derived peptides has striking similarities to the \textit{Bacillus subtilis} Rap-Phr system (17). In both cases, extracellular processing of a pre-pro-peptide produces a pentapeptide that acts within the cell. The Phr pentapeptides have a consensus basic amino acid, lysine or arginine, at the second position. In contrast, the PapR pentapeptides are negatively charged (20), all containing a single conserved Glu (E). It is of interest that the genes encoding the Phr peptides overlap the ends of the cognate Rap phosphatase genes, and in addition, there is usually an internal $\sigma^H$ promoter sequence that may help to ensure adequate production of the peptide. It is not evident that there is a similar system that might enable PapR expression to exceed that of PlcR, as may be needed given the losses of peptide due to dilution outside the cell.

The work reported here also confirms our previous observation that the \textit{B. anthracis} plc and sph can be regulated by PlcR. These two genes were not listed among the 50 \textit{B. anthracis} genes that are putatively controlled by PlcR (18) because their PlcR recognition sequences differ slightly from the consensus. Apparently slight deviations from the consensus sequence can be tolerated, as shown by the fact that the \textit{B. thuringiensis} inhA2 gene is PlcR regulated, even though the PlcR-binding site has one nucleotide change from the consensus sequence (3). Also, our experiments on DNA-protein interaction showed that the single mutation in the palindrome structure (T2) corresponding to the \textit{B. anthracis} plc PlcR-binding site decreases but does not abolish binding.

Although the fused PlcR-PapR protein can activate PlcR-dependent genes in \textit{B. anthracis}, the kinetics of activation differs from the normal pattern in \textit{B. cereus}. Previously we showed that SPH activity appears during the exponential phase of growth of \textit{B. cereus} 569 and is rapidly lost at the onset of
stationary phase, at a time when the activity of PC-PLC reached its maximum (16). In contrast, both the PC-PLC and SPH activities appeared only at the onset of the stationary phase of growth in \textit{B. anthracis} containing the fused PlcR-PapR protein (Fig. 6). This pattern is characteristic of normal PlcR regulation in \textit{B. thuringiensis} (7). It is of interest that the fused PlcR-PapR protein was synthesized during the exponential phase of growth in \textit{B. thuringiensis} containing the fused PlcR-PapR protein (Fig. 6). This pattern is characteristic of normal PlcR-dependent gene expression. Consistent with this hypothesis, the PlcR-PapR protein formed higher-molecular-weight complexes of approximately 120 and 100 kDa (Fig. 9A). These could be complexes of the PlcR-PapR homodimer (78 kDa) with the exponential-phase $\sigma^A$ (molecular mass of $\sim$43 kDa; component 1) and the stationary-phase $\sigma^H$ (molecular mass of $\sim$25 kDa; component 2), respectively.

In addition to clarifying aspects of PlcR regulation through studies like those described here, the PlcR-PapR fusion protein we identified can be useful in mechanistic and structural studies of how PlcR interacts with its DNA target, in facilitating analysis of other components of the PlcR regulon, and in identifying additional genes regulated by PlcR.

REFERENCES


