This study shows that the *Bacillus anthracis* pXO1 virulence plasmid carries a Rap-Phr system, BXA0205, which regulates sporulation initiation in this organism. The BXA0205Rap protein was shown to dephosphorylate the Spo0F response regulator intermediate of the phosphorelay signal transduction system that regulates the initiation of the developmental pathway in response to environmental, metabolic, and cell cycle signals. The activity of the Rap protein was shown to be inhibited by the carboxy-terminal pentapeptide generated through an export-import processing pathway from the associated BXA0205Phr protein. Deregulation of the Rap activity by either overexpression or lack of the Phr pentapeptide resulted in severe inhibition of sporulation. Five additional Rap-Phr encoding systems were identified on the chromosome of *B. anthracis*, one of which, BA3790-3791, also affected sporulation initiation. The results suggest that the plasmid-borne Rap-Phr system may provide a selective advantage to the virulence of *B. anthracis*.

*Bacillus anthracis*, the etiological agent of anthrax, is a gram-positive spore-forming organism that primarily infects ruminants but can also be highly pathogenic to other mammals, including humans. The intrinsic spore resistance to extreme stresses such as desiccation, solvents, extreme pH, temperature, UV, and ionizing radiation plays a major role in anthrax pathogenesis. Sporulation is essential for survival in the environment, and it evidently contributes to anthrax diffusion, because spores are usually present when the infection is initiated (27).

The process of sporulation has been extensively studied in *Bacillus subtilis* and shown to be the result of a complex differentiation pathway that has its onset in a signal transduction system called phosphorelay. The phosphorelay is a more complex version of the two-component signal transduction systems, because it is composed of multiple central elements and it is modulated by a variety of ancillary factors (40, 41).

In *B. subtilis*, five histidine sensor kinases (KinA, -B, -C, -D, and -E) can respond to a multiplicity of inducing signals and activate the pathway by autophosphorylating and transferring the activating phosphoryl group to an intermediate response regulator acceptor called Spo0F. From Spo0F, the phosphoryl group is then transferred to the Spo0A response regulator through the Spo0B phosphotransferase. Spo0A is the critical transcription regulator for sporulation initiation. Accumulation of its activated form, Spo0A~P, during growth progressively results in repression of genes not required for sporulation and the activation of genes necessary for spore formation (7, 19, 28, 53).

Negative inputs into the phosphorelay are brought about mainly by aspartyl phosphate phosphatases that specifically dephosphorylate the response regulator components of the system. The three members of the Spo0E family of phosphatases dephosphorylate Spo0A~P, while three members of the Rap family of phosphatases dephosphorylate the Spo0F~P intermediate (18, 36, 38). Rap proteins are structurally organized in tetratricopeptide repeats (TPR) which provide the basis for protein-protein or protein-ligand regulatory mechanisms (10, 37). Rap proteins in fact are often inhibited by specific pentapeptide ligands, which results from an export-import pathway of the pre-pro precursor product of the rap-associated phr genes (37).

The Spo0A and Spo0F response regulator proteins are very conserved in amino acid sequence between *B. subtilis* and *B. anthracis* (49). The response regulator domain of the Spo0A proteins is highly conserved (79% identical residues) and all of the residues involved in the interaction with the Spo0E phosphatase are identical (50). In the DNA-binding domain, all the residues known to make contact with the target DNA promoters are also conserved, suggesting that the sequence of the “OA box” is invariant in these two organisms (60). The Spo0F proteins (77% identity) also are invariant in all the residues known to affect the interaction with the Rap phosphatase proteins (54). Amino acid conservation is also significantly high in the Spo0B proteins, in particular in the residues known to make interaction with the response regulator, thus maintaining specificity in protein-protein interaction through an identical molecular mechanism in the two organisms (58). Amino acid conservation of the histidine domain has also allowed the identification of nine sensor histidine kinases presumably involved in *B. anthracis* sporulation initiation (6).

In this communication we report the identification and characterization of Rap proteins of *B. anthracis* and investigate their role in the sporulation process of this organism.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The *B. subtilis* strains used in this study are all derivatives of the parental strain JH642 and thus carry the *trpC2* and

---

*Rap Phosphatase of Virulence Plasmid pXO1 Inhibits Bacillus anthracis Sporulation†*

Cristina Bongiorni, Ricarda Stoessel, Dorinda Shoemaker, and Marta Perego∗

The Scripps Research Institute, Department of Molecular and Experimental Medicine, Division of Cellular Biology, 10550 North Torrey Pines Road, La Jolla, California 92037

Received 19 September 2005/Accepted 24 October 2005

Copyright © 2006, American Society for Microbiology. All Rights Reserved.

JOURNAL OF BACTERIOLOGY, Jan. 2006, p. 487–498 Vol. 188, No. 2

0021-9193/06/$08.00 doi:10.1128/JB.188.2.487–498.2006
The fragment used to test the presence of a promoter in front of the BA3791 gene was obtained by PCR amplification using the oligonucleotides RappXO15 and RappXO13 located upstream of the BA3791 gene. The fragment cloned in pHT315-BXA0205phr containing the BA0205 gene was subcloned from the pHT315-1582, pHT315-3016, pHT315-3760, and pHT315-4060. The promoters of each phr gene were subcloned by direct PCR amplification with primers RappXO15 Kpn and RappXO13 Bam at the 5′ end and an internal BamHI site at the 3′ end (see Table S1 in the supplemental material). Each fragment was cloned in pHT315-BXA0205phr by using a 6-codon deletion at the 3′ end of the phr gene. The fragment cloned in pHT315-BXAm was used as a template for the construction of the transcriptional fusion to the E. coli lacZ gene (42).

The primers used in this work are listed in Table S1 in the supplemental material. The promoter and coding sequence of the phr genes were amplified by PCR of B. anthracis 34F2 chromosomal DNA, using oligonucleotide primers that introduced appropriate restriction sites. Fragments were cloned into pHT315 to generate the derived plasmids, named pHT315-BXA0205, pHT315-3790, pHTo315-1582, pHTo315-1580, and pHTo315-4080.

In vivo analysis of the active pentapeptide inhibitor of the BA3790 and BA3791 genes were amplified by PCR using the oligonucleotides that introduced an NdeI site at the 5′ end and a BamHI site at the 3′ end using oligonucleotides pHT315-3790phr and pHT315-3790phr containing the 6-codon deletion at the 3′ end of the phr gene. The fragment cloned in pHT315-BXAm was used as a template for the construction of the transcriptional fusion to the E. coli lacZ gene (42).

The primers used in this work are listed in Table S1 in the supplemental material. The promoter and coding sequence of the phr genes were amplified by PCR of B. anthracis 34F2 chromosomal DNA, using oligonucleotide primers that introduced appropriate restriction sites. Fragments were cloned into pHT315 to generate the derived plasmids, named pHT315-BXA0205, pHT315-3790, pHTo315-1582, pHTo315-1580, and pHTo315-4080.

In vivo analysis of the active pentapeptide inhibitor of the BA3790 and BA3791 genes were amplified by PCR using the oligonucleotides that introduced an NdeI site at the 5′ end and a BamHI site at the 3′ end using oligonucleotides pHT315-3790phr and pHT315-3790phr containing the 6-codon deletion at the 3′ end of the phr gene. The fragment cloned in pHT315-BXAm was used as a template for the construction of the transcriptional fusion to the E. coli lacZ gene (42).

The primers used in this work are listed in Table S1 in the supplemental material. The promoter and coding sequence of the phr genes were amplified by PCR of B. anthracis 34F2 chromosomal DNA, using oligonucleotide primers that introduced appropriate restriction sites. Fragments were cloned into pHT315 to generate the derived plasmids, named pHT315-BXA0205, pHT315-3790, pHTo315-1582, pHTo315-1580, and pHTo315-4080.

In vivo analysis of the active pentapeptide inhibitor of the BA3790 and BA3791 genes were amplified by PCR using the oligonucleotides that introduced an NdeI site at the 5′ end and a BamHI site at the 3′ end using oligonucleotides pHT315-3790phr and pHT315-3790phr containing the 6-codon deletion at the 3′ end of the phr gene. The fragment cloned in pHT315-BXAm was used as a template for the construction of the transcriptional fusion to the E. coli lacZ gene (42).

The primers used in this work are listed in Table S1 in the supplemental material. The promoter and coding sequence of the phr genes were amplified by PCR of B. anthracis 34F2 chromosomal DNA, using oligonucleotide primers that introduced appropriate restriction sites. Fragments were cloned into pHT315 to generate the derived plasmids, named pHT315-BXA0205, pHT315-3790, pHTo315-1582, pHTo315-1580, and pHTo315-4080.

In vivo analysis of the active pentapeptide inhibitor of the BA3790 and BA3791 genes were amplified by PCR using the oligonucleotides that introduced an NdeI site at the 5′ end and a BamHI site at the 3′ end using oligonucleotides pHT315-3790phr and pHT315-3790phr containing the 6-codon deletion at the 3′ end of the phr gene. The fragment cloned in pHT315-BXAm was used as a template for the construction of the transcriptional fusion to the E. coli lacZ gene (42).

The primers used in this work are listed in Table S1 in the supplemental material. The promoter and coding sequence of the phr genes were amplified by PCR of B. anthracis 34F2 chromosomal DNA, using oligonucleotide primers that introduced appropriate restriction sites. Fragments were cloned into pHT315 to generate the derived plasmids, named pHT315-BXA0205, pHT315-3790, pHTo315-1582, pHTo315-1580, and pHTo315-4080.

In vivo analysis of the active pentapeptide inhibitor of the BA3790 and BA3791 genes were amplified by PCR using the oligonucleotides that introduced an NdeI site at the 5′ end and a BamHI site at the 3′ end using oligonucleotides pHT315-3790phr and pHT315-3790phr containing the 6-codon deletion at the 3′ end of the phr gene. The fragment cloned in pHT315-BXAm was used as a template for the construction of the transcriptional fusion to the E. coli lacZ gene (42).

The primers used in this work are listed in Table S1 in the supplemental material. The promoter and coding sequence of the phr genes were amplified by PCR of B. anthracis 34F2 chromosomal DNA, using oligonucleotide primers that introduced appropriate restriction sites. Fragments were cloned into pHT315 to generate the derived plasmids, named pHT315-BXA0205, pHT315-3790, pHTo315-1582, pHTo315-1580, and pHTo315-4080.

In vivo analysis of the active pentapeptide inhibitor of the BA3790 and BA3791 genes were amplified by PCR using the oligonucleotides that introduced an NdeI site at the 5′ end and a BamHI site at the 3′ end using oligonucleotides pHT315-3790phr and pHT315-3790phr containing the 6-codon deletion at the 3′ end of the phr gene. The fragment cloned in pHT315-BXAm was used as a template for the construction of the transcriptional fusion to the E. coli lacZ gene (42).
RESULTS

Identification of Rap-encoding genes by bioinformatic analysis. The availability of the B. anthracis genome sequence allowed us to search for genes encoding proteins with homology to the Rap proteins of B. subtilis (37). The amino acid sequence of the B. subtilis RapA protein involved in the control of the sporulation phosphorylase was used as a query in a BLAST search against the genome sequence of B. anthracis strain A2012. Five genes were identified encoding proteins with approximately the same molecular weight as that of RapA and sharing with it an average of 24% identical residues. The genes were annotated as BA1582, BA3790, BA3016, BA4060, and BXA0205 (NCBI accession number NC_003909) (Fig. 1A and Table 1). An additional gene, annotated as BA3760, was found when the same search was carried out on the B. anthracis Ames genome sequence (NCBI accession number NC_003997). Using the sequence of BA3760 to search the B. anthracis A2012 genome, we noted that an identical gene was present in this strain but that its product seemed to be frame-shifted, giving rise to two open reading frames (annotated as BA4239 and BA4240), none of which would have sufficient significant similarity to the B. subtilis RapA protein to be identified by our first BLAST search. The nomenclature BA3760 will be used hereafter to indicate this gene, while for the others the strain A2012 nomenclature will be used. The identified genes and their nomenclature are summarized in Table 1.

Among themselves, the six B. anthracis Rap proteins share between 37 and 54% identical residues. The gene encoding BXA0205 is located on the pX01 virulence plasmid, while the remaining five genes are all located on the chromosome.

Curiously, the sequence of BA4060 was found to be interrupted by a stop codon at position 225. This stop codon is present in the orthologues of BA4060 identified in the available genome sequences of all B. anthracis strains but one, A1055, in which the stop codon is replaced by a glutamine residue. The B. cereus orthologue of BA4060, BCE3523 (NCBI accession number NC_003909), is also uninterrupted because of a glutamine residue in place of the stop codon. BCE3523 was used instead of BA4060, in order to test the role of this Rap protein on sporulation.

Analysis of the nucleotide sequence at the 3' end of each gene revealed the presence of open reading frames encoding putative Phr-like proteins. Some of them were annotated in the database as BA3791, BA4061, BA3015, and BA3759. We have named the remaining ORFs BA1582phr, BXA0205phr, and BCE3523phr (Fig. 1B).

Analysis of each Phr protein with the SignalP program (30) indicated structural organizations typical of secreted proteins as previously described for the Phr proteins of B. subtilis (39). The amino-terminal N domain contains positively charged amino acids, the H domain is formed by hydrophobic residues, and the C domain contains the residues presumably recognized by the signal peptidase for the cleavage reaction (57).

Overexpression of B. anthracis Rap proteins in B. subtilis. The B. subtilis Rap proteins affecting the phosphorylase for sporulation initiation were identified as negative regulators by their ability to inhibit the sporulation process when overexpressed from a multicopy vector. We applied this strategy in order to determine whether any of the Rap proteins of B. anthracis had a role in modulating sporulation in this organism. Since the Spo0F, Spo0B, and Spo0A proteins of the sporulation phosphorylase in B. anthracis are highly homologous to their counterparts in B. subtilis (49), we assumed that if a Rap protein were active on Spo0F of the former it would also be active on the orthologue of the latter organism. Thus, we carried out the initial phenotypic analysis of Rap proteins in B. subtilis.

Each Rap-encoding gene was amplified by PCR as a fragment including approximately 200 to 400 bp at the 5' end, in order to amplify the promoter region as well. At the 3' end, each fragment terminated immediately downstream from the stop codon of the rap genes in order not to include the phr gene. The fragments were cloned in the multicopy vector pHT315, which is presumably present at approximately 15 copies per cell (2). The plasmids obtained were transformed in the B. subtilis wild-type strain JH642, and the phenotype of the transformants was analyzed on Schaeffer's sporulation agar plates. Visual analysis on agar plates of the transformants obtained indicated that the expression of BXA0205 and BA3790 inhibited sporulation of B. subtilis JH642. The remaining B. anthracis Rap proteins did not seem to affect the sporulation phenotype. In order to quantitate the effect of the overexpression of the B. anthracis Rap proteins on sporulation, we carried out a liquid sporulation assay. The results shown in Table 2 indicated that BXA0205 severely inhibited sporulation, while BA3790 caused a 2.5-fold reduction in efficiency. The other Rap proteins did not significantly affect the effi-
TABLE 1. Summary of Rap-Phr ORF annotations in two
B. anthracis sequenced genomes

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>ORF</th>
<th>Accession no.</th>
<th>ORF</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC_003995</td>
<td>BA3790-3791</td>
<td>NC_003997</td>
<td>BA3284-3285</td>
</tr>
<tr>
<td>NC_003995</td>
<td>BA4060p/4061-4062</td>
<td>NC_003997</td>
<td>BA3569p-3570</td>
</tr>
<tr>
<td>NC_003995</td>
<td>BA1582d</td>
<td>NC_003997</td>
<td>BA1017-1018</td>
</tr>
<tr>
<td>NC_003995</td>
<td>BA2016-2015</td>
<td>NC_003997</td>
<td>BA2522d</td>
</tr>
<tr>
<td>NC_003995</td>
<td>BA4239-4240</td>
<td>NC_003997</td>
<td>BA3760-3759</td>
</tr>
<tr>
<td>NC_003980</td>
<td>BXA0205d</td>
<td>NC_007322</td>
<td>GBAA_pXO1-0205d</td>
</tr>
</tbody>
</table>

*This ORF is interrupted by a stop codon in both organisms. (This is not a sequencing error.)

*This ORF is frameshifted in the A2012 strain only. (This could be a sequencing error.)

*This ORF is missing 196 amino acids at the amino-terminal end due to a frameshift. (This could be a sequencing error, since the clone we recovered from strain 34F2 does not have this error.)

*d In the database, these ORFs do not have an annotated phr gene associated with them.

e This gene is called ORF136 by Okinaka et al. (32).

Microscopic analysis of the strains grown on solid medium revealed that none of the remaining Rap proteins affected sporulation significantly, despite the variability observed by the liquid sporulation assay shown in Table 3.

These results are consistent with the conclusion that only two of the six Rap proteins of B. anthracis, BXA0205 and BA3790, affected sporulation in vivo in B. subtilis and B. anthracis.

In vitro biochemical characterization of B. anthracis Rap proteins. In order to confirm the results of the in vivo analysis on the role of B. anthracis Rap proteins on sporulation, we overexpressed in E. coli and purified four full-length B. anthracis proteins (BXA0205, BA3016, BA3790, and BA1582) as N-terminal histidine-tagged derivatives. The ability of each protein to modulate the sporulation phosphorelay was tested against the KInA-dependent phosphorylation reaction of Spo0F or specifically against the purified Spo0F phosphoform of RapA, RapB, and RapE of B. subtilis (18, 38).

The results shown in Fig. 2 and 3 indicate that the BXA0205 and BA3790 proteins are capable of dephosphorylating the Spo0F~P protein of the phosphorelay, while BA1582 and BA3016 are not, thus confirming the results obtained by the in vivo analysis. The seemingly more efficient dephosphorylation of Spo0F~P by BXA0205 and BA3790 observed in the reactions in Fig. 2A and B (compared to the ones in Fig. 3A and B) are most likely the result of the relative concentration of Spo0F, Spo0F~P, and Rap proteins in the reaction. Alternatively, there may be a stronger affinity of the Rap proteins for the unphosphorylated Spo0F than the affinity previously observed for Spo0F by the B. subtilis RapA (17).

![FIG. 1. Amino acid sequence alignment of the Rap and Phr proteins of B. anthracis. (A) The Rap proteins of B. anthracis were aligned against the RapA protein of B. subtilis. The six TPR domains in RapA are underlined. The gray box in the BA3760 sequence indicates a duplication in the nucleotide sequence of the 34F2 strain used in this study that is not present in the database at accession number NC_003997. The dash replacing residue 226 in the sequence of BA4060 indicates the position of the frame shift that inactivates this gene product. The alignment was obtained by the ClustalW program. (B) Amino acid sequences of the Phr proteins of B. anthracis and the PhrA protein of B. subtilis. Gray boxes in the BsPhrA and BXA0205phr sequences indicate the sequence of the active pentapeptide inhibitor. The gray box in the BA3791 sequence indicates the region presumably containing the pentapeptide inhibitor. The amino-terminal positively charged domain (N), the hydrophobic domain (H), and the putative signal peptidase cleavage domain (C) of PhrA are indicated. The dashes indicate the putative signal peptidase cleavage site identified by the SignalP program (30). ¥, identical residue; ·, conserved residue.](http://jb.asm.org/)
Identification of the active Phr pentapeptide inhibitors. The activity of the *B. subtilis* Rap proteins is inhibited by a specific pentapeptide that is generated from the precursor Phr protein through an export-import pathway and multiple processing events (35, 39). In most cases analyzed thus far, the Phr pentapeptide inhibitor is in the C-terminal five amino acids of the protein encoded by the *phr* gene (5, 22, 31). One exception is the PhrE pentapeptide that is generated from within the carboxy-terminal domain of its precursor protein, up nine amino acids from the terminal residue (18).

The *phr* genes associated with the *B. anthracis* BXA0205 and BXA3790 *rap* genes encoded proteins capable of inhibiting the phosphatase activity of their corresponding Rap proteins in vivo. This phenotype allowed us to search in vivo for the specific pentapeptide sequence with inhibitory activity toward BXA0205 and BA3790.

A new derivative of pHt315 was constructed, pHt315-BXA0205phr2, carrying the BXA0205 gene and its *phr*-associated gene with a premature stop codon that deleted the last five codons (GHTGG). When this plasmid was transformed into JH642, the resulting colonies showed a strong sporulation-deficient phenotype comparable to the phenotype generated by plasmid pHt315-BXA0205 (data not shown). This suggested that the five amino acids at the carboxy-terminal end of BXA0205phr could be the inhibiting pentapeptide. A synthetic GHTGG pentapeptide was then tested in vitro for its ability to inhibit the BXA0205 phosphatase activity against Spo0F/P.

As shown in Fig. 4, the presence of the synthetic pentapeptide inhibited BXA0205, indicating that GHTGG is the inhibitor sequence.

A similar strategy was applied to the search for the pentapeptide inhibitor of BA3790. Premature stop codons were generated that resulted in the deletion of the last 5, 10, 15, and 20 amino acids of the BA3791 gene product (plasmids pHt315-3790phr 3, -4, -5, and -6, respectively). The pHt315 plasmid derivatives carrying the BA3790 gene with each of these 3’ end deletions in BA3791 were transformed into JH642 for phenotypic analysis. A quantitative analysis of the sporulation efficiency generated by each of these constructs, shown in Table 4, indicated that the active pentapeptide was within the sequence that is located 10 to 20 amino acids from the carboxy-terminal end (GHYPVPTYSV). In fact, deletion of the last 10 amino acids still resulted in inhibition of BA3790 activity, while deletion of the last 20 amino acids completely eliminated the inhibition of phosphatase activity. Surprisingly, a partial loss of inhibition was observed with the construct with the last 15 amino acids deleted, thus not allowing us to clearly outline the sequence of the inhibitor and carry out in vitro studies.

**TABLE 3. Efficiency of sporulation of *B. anthracis* 34F2 derivative strains expressing the Rap proteins in the pHt315 multicopy plasmid.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Insert</th>
<th>Viable cells</th>
<th>Spore count</th>
<th>% Sporulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHt315</td>
<td>None</td>
<td>3.2 × 10^8</td>
<td>3.4 × 10^7</td>
<td>10.6</td>
</tr>
<tr>
<td>pHt315-BXA0205</td>
<td>BXA0205</td>
<td>2.4 × 10^8</td>
<td>2.8 × 10^7</td>
<td>1.2 × 10^-7</td>
</tr>
<tr>
<td>pHt315-BXA0205 phr</td>
<td>BXA0205 phr</td>
<td>2.4 × 10^8</td>
<td>1.5 × 10^7</td>
<td>5.8</td>
</tr>
<tr>
<td>pHt315-1582</td>
<td>BA1582</td>
<td>1.3 × 10^8</td>
<td>4.9 × 10^6</td>
<td>3.7</td>
</tr>
<tr>
<td>pHt315-3790</td>
<td>BA3790 phr</td>
<td>2.2 × 10^8</td>
<td>1.2 × 10^7</td>
<td>5.4</td>
</tr>
<tr>
<td>pHt315-3016</td>
<td>BA3016</td>
<td>2.5 × 10^8</td>
<td>1.5 × 10^7</td>
<td>6</td>
</tr>
<tr>
<td>pHt315-4060</td>
<td>BA4060</td>
<td>3.1 × 10^8</td>
<td>1.3 × 10^7</td>
<td>4.2</td>
</tr>
<tr>
<td>pHt315-3523</td>
<td>BCE3523</td>
<td>1.8 × 10^8</td>
<td>1.5 × 10^7</td>
<td>8</td>
</tr>
<tr>
<td>pHt315-3523 phr</td>
<td>BCE3523 phr</td>
<td>2.2 × 10^8</td>
<td>1.9 × 10^7</td>
<td>9</td>
</tr>
</tbody>
</table>

* Growth was carried out in Schaeffer’s sporulation medium for 30 hours at 37°C. Data are representative of three independent experiments.

**FIG. 2.** In vitro activity assay of *B. anthracis* Rap proteins against the *B. subtilis* phosphorelay components. Purified *B. anthracis* Rap proteins were assayed in a reaction containing KinA (0.1 μM), Spo0F (2.5 μM), and [γ-32P]ATP. Each Rap protein was added at 5 μM final concentration. Time course experiments were carried out and aliquots withdrawn at the indicated time points. The positions of the KinA and Spo0F proteins in each gel are indicated by the bars. The labeled band denoted by the asterisk is a dimer form of Spo0F.
Deletion analysis of BXA0205 and BXA0205phr in B. anthracis. In order to confirm in vivo the physiological roles of the BXA0205 and BXA0205phr proteins inferred from the in vitro studies, we carried out a deletion analysis of their coding genes on the genome of B. anthracis. The genes encoding the BXA0205 and BXA0205phr proteins were inactivated with a spectinomycin resistance cassette inserted via a double-cross-over recombination event (see Materials and Methods). The strains obtained, Ba34F2Δ0205 and Ba34F2Δ0205phr, were tested for sporulation efficiency in Schaeffer’s sporulation medium. The results shown in Table 5 confirmed the roles of these proteins in regulating sporulation. In fact, while the deletion of the BXA0205 Rap protein increased the efficiency of sporulation, the deletion of the gene encoding the inhibitor Phr peptide severely inhibited spore formation. Therefore, the BXA0205 Rap protein dephosphorylates the Spo0F response regulator in vivo in B. anthracis, as well as in vitro.

Transcription analysis of the B. anthracis rap promoters. In order to determine whether the B. anthracis rap genes were expressed in B. subtilis in the assay conditions used, a promoter fusion to the E. coli lacZ gene was constructed for each of them (see Materials and Methods). Each construct was introduced in the B. subtilis chromosome of strain JH642 at the amyE locus, and promoter activity was measured by means of β-galactosidase activity assays. The results shown in Fig. 5 indicated that the promoter of the pXO1-located rap gene, BXA0205, was the most active of all, reaching the highest level of activity in mid-late exponential phase and then decreasing sharply during stationary phase. The remaining five promoters were significantly less active than BXA0205, with BA3016 seeming to be most active early in exponential phase while BA4060 and BA3790 were transcribed more efficiently in late-exponential phase and during the transition to stationary phase. The transcription of BA3760, on the contrary, seemed to be increasing during growth, although it remained at very low levels throughout. The promoter of BA1582 did not seem to be transcribed at detectable levels, since the β-galactosidase activity never exceeded the background level. Also, transcription activity was not detected in B. subtilis from a 725-bp fragment carrying the 3′ end of the BXA0205 gene and the first 33 bp of the BXA0205phr gene (Fig. 5B). However, a very low level of transcription was detected when the same fragment cloned in pTCVlac was analyzed in B. anthracis. Similar results were obtained for the BA3791phr gene when analyzed in B. subtilis, although a low level of transcription was detected when the analysis was carried out in B. anthracis (data not shown). Differential transcription regulatory mechanisms between the two organisms must exist, and they will be investigated in future studies.

Thus, the patterns of transcription of the B. anthracis rap genes in general, and of the two involved in sporulation in particular, differ significantly, indicating that different regulatory mechanisms may be governing Rap protein production. Distinctive pathways of gene expression have been previously identified for the rap genes of B. subtilis, each pathway indicative of regulatory mechanisms governing Rap protein production (29, 38).

Effect of BAXA0205 Rap and Phr on transcription of abrB, atxA, and pagA genes. Because of the location of the BAXA0205 rap and phr genes on the pXO1 virulence plasmid that also carries the genetic determinants for B. anthracis toxin synthesis, we wondered whether their products would affect toxin production or the transcription of the genes encoding the toxin regulators AtxA and AbrB. In B. subtilis, deregulation of RapA due to a deletion of the phR gene results in reactivation of abrB transcription after the transition to stationary phase (38). In B. anthracis, AbrB was shown to repress atxA transcription during exponential phase when cells were grown in LB medium in the presence of CO₂ (43). AtxA is known to be the essential transcriptional activator of the pagA, cya, and lef genes encoding the toxin components (9, 43, 56). Thus, repression of AbrB production by the phosphorelay is thought to be required for activation of AtxA and toxin synthesis.

We constructed abrB- and atxA-lacZ transcriptional fusions in the pTCVlac replicative vector and assayed their transcription-
tional activities in the parental strain 34F2 and in the BXA0205 rap or phr deletion strains. The results shown in Fig. 6 indicated that the sporulation-deficient phenotype induced by the deletion of the BXA0205phr gene also resulted in extended transcription of the abrB gene during the stationary phase of growth, while in the parental strain the abrB promoter is turned off at around the transition time (time zero). No significant effect was observed on the transcription of pagA (data not shown) by the deletion of either the BXA0205 rap or phr genes. Both genes were maximally transcribed only until mid-exponential phase in our growth conditions (Schaeffer’s sporulation medium), supporting the notion that the initiation of sporulation inhibits toxin production.

**DISCUSSION**

The ability of B. anthracis to cause disease is associated with its plasmid content. Plasmid pXO1 carries the genes encoding the toxin components, pagA (protective antigen), cya (edema factor), and lef (lethal factor). Plasmid pXO2 contains the cap genes necessary for capsule production. While the toxin is a required virulence factor that contributes to the pathogenesis of the organism, the capsule provides bacterial resistance to host phagocytosis. Growth and persistence of the microorganism in the host are additional contributing factors in the virulence of B. anthracis. After germination of the infecting spores, rapid growth of capsulated vegetative cells in the bloodstream gives rise to the synergistic effects of bacillemia and toxemia that are characteristic of fatal anthrax infection (for a review, see reference 27).

In this communication we report the identification on the pXO1 plasmid of genes that regulate sporulation initiation in B. anthracis and therefore may contribute to the infectivity of this organism. The BXA0205 gene was found to encode a Rap protein with an aspartyl phosphate phosphatase activity against the Spo0F−P intermediate response regulator of the phosphorelay for sporulation initiation. An associated gene called BXA0205phr was shown to encode a Phr peptide whose carboxy-terminal five amino acids (GHTGG) acted as an inhibitor of the BXA0205 Rap protein. Deregulation of the Rap activity, either by its overexpression or by deletion of the Phr-encoding gene, was shown to inhibit sporulation of B. anthracis.

The reduced ability of B. anthracis to initiate the sporulation process in the absence of the BXA0205 peptide may have relevance in the pathogenesis of the organism. To establish the disease, germinated spores must be able to rapidly grow and maintain themselves in the vegetative form which is likely to be the most favorable for survival. Host defenses can eliminate the spores, but the encapsulated vegetative cells can avoid ingestion by host phagocytes (15). Physiological conditions that inhibit spore formation but allow toxin production, a prerequisite for effective infection, may then be very advantageous to the bacterial cells. In the absence of the BXA0205phr peptide, these conditions are met, indeed: since the activation of the inhibiting Phr pentapeptide involves an export-import processing pathway (35), any event that reduces the efficiency of pentapeptide reimportation will result in inhibition of sporulation. B. anthracis growth in the bloodstream may result in dilution and reduced concentrations of peptide available for reimportation, thus contributing to maintaining cells in vegetative growth.

The hypothesis that the selective ability to avoid sporulation in the body while retaining the capability to sporulate in other environments (such as laboratory conditions or soil) is advantageous to the cells has been recently proposed by Brunsing et al. (6). The hypothesis was based on the observation that of the nine potential histidine sensor kinase-coding genes for sporulation initiation identified on the B. anthracis genome, some

**TABLE 4. Sporulation efficiency of B. subtilis strains carrying carboxy-terminal deletions of the BA3791 Phr protein**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Viable count</th>
<th>Spore count</th>
<th>% Sporulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHT315</td>
<td>1.9 x 10^8</td>
<td>1.3 x 10^8</td>
<td>68.4</td>
</tr>
<tr>
<td>pHT315-3790</td>
<td>3.9 x 10^8</td>
<td>9.2 x 10^7</td>
<td>23.6</td>
</tr>
<tr>
<td>pHT315-3790phr</td>
<td>3.3 x 10^8</td>
<td>1.0 x 10^8</td>
<td>31</td>
</tr>
<tr>
<td>pHT315-3790phr3</td>
<td>2.9 x 10^8</td>
<td>1.1 x 10^8</td>
<td>37.9</td>
</tr>
<tr>
<td>pHT315-3790phr4</td>
<td>2.6 x 10^8</td>
<td>1.0 x 10^8</td>
<td>39.2</td>
</tr>
<tr>
<td>pHT315-3790phr5</td>
<td>3.5 x 10^8</td>
<td>9.8 x 10^7</td>
<td>28</td>
</tr>
<tr>
<td>pHT315-3790phr6</td>
<td>5.1 x 10^8</td>
<td>8.2 x 10^7</td>
<td>16</td>
</tr>
</tbody>
</table>

*JH642 derivative strains were grown for 36 hours in Schaeffer’s sporulation medium containing erythromycin at 5 µg/ml. The data are representative of two independent experiments.

**TABLE 5. Sporulation efficiency of B. anthracis strains carrying deletions of BXA0205 or BXA0205phr genes encoding the Rap and Phr proteins, respectively, of plasmid pXO1**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Viable cells</th>
<th>Spore count</th>
<th>% Sporulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>34F2</td>
<td>1.95 x 10^8</td>
<td>3.0 x 10^7</td>
<td>15.4</td>
</tr>
<tr>
<td>34F2ΔBXA0205</td>
<td>1.35 x 10^8</td>
<td>3.8 x 10^7</td>
<td>28.1</td>
</tr>
<tr>
<td>34F2ΔBXA0205phr</td>
<td>1.9 x 10^6</td>
<td>4.25 x 10^3</td>
<td>0.22</td>
</tr>
</tbody>
</table>

*Cells were grown in Schaeffer’s sporulation medium for 24 hours at 37°C. Data are the averages of two independent experiments.

**FIG. 5. Transcription analysis of the B. anthracis rap promoters.**

β-Galactosidase assays were carried out on B. subtilis strains carrying rap-promoter fusions to the E. coli lacZ gene. Each fusion was integrated at the amyE locus via a double-crossover event. (A) β-Galactosidase activity of the BXA0205 promoter (●), (B) β-galactosidase activity of the BA4060 (▲), BA3016 (■), BA3790 (●), and BA3760 (●), and BXA0205phr (□) promoters. Cultures were carried out in Schaeffer’s sporulation medium. The time zero on the x axis represents the time of transition from exponential to stationary phase. The data are representative of multiple independent experiments.
The BXA0205 pentapeptide did not seem to affect the progression to its often fatal outcome. Conditions optimal to establish the infection and allow its progression, and therefore they would be advantageous for the conditions for which the bacterial cells were selected (21, 24). Although the BXA0205 genes of the pXO1 plasmid of B. anthracis are not located within the pathogenicity island (carrying, among others, the toxin genes, atxA, and the gerX operon for germination), the extensive sequence conservation observed within pXO1 ORFs of the Bacillus anthracis/Bacillus cereus/Bacillus thuringiensis group indicates that they are in fact important for the biology of these species (33).

Although the sporulation defect brought about by the lack of the BXA0205 pentapeptide did not seem to affect the production of toxin or toxin regulator, as seen by β-galactosidase transcription analyses of the pagA and atxA promoters, a deregulation of abrB transcription was observed (Fig. 6). AbrB is a transition state transcription regulator known in B. subtilis to generally repress transcription, during the exponential phase of growth, of a number of genes whose products are required during the transition from active growth to stationary phase and/or for sporulation (51). One of the genes repressed by AbrB in B. anthracis is atxA, and a deletion of the abrB gene was shown to result in earlier and higher expression of atxA and therefore pagA, cya, and lef when cells were grown in LB medium in the presence of 5% CO₂ (43, 52). Because of this observation, we expected to see a stronger repression of atxA transcription in the BXA0205phr-deleted strain. However, our transcriptional analysis, carried out in Schaeffer’s sporulation medium, showed that in the parental strain, atxA and pagA were transcribed only until mid-exponential phase and then their transcription was essentially turned off. In the sporulation-deficient Ba34F2Δ0205phr strain, this transcription pattern was not affected. These results could be interpreted to mean that the Schaeffer’s sporulation medium, which causes earlier and faster development of spores than the LB medium, represses atxA and toxin production before the transition phase, while in LB medium with CO₂, transcription is still maximal at the beginning of stationary phase. This repression is not increased by the higher level of AbrB observed after the transition phase (T0) in the strain lacking the BXA0205phr peptide gene, suggesting that the regulation of atxA transcription by AbrB may be limited to the exponential phase of growth, while the turnoff at around late-exponential phase occurs independently of the level of AbrB. Clearly, different growth conditions differentially affect transcription regulation of the genes so far known to regulate B. anthracis toxin production, and nothing is known about the regulation of gene transcription when bacteria grow in the bloodstream, in which

![Graph](http://jb.asm.org/): Transcription analysis of the abrB and atxA promoters in B. anthracis BXA0205 and BXA0205phr mutant strains. (A) β-Galactosidase analysis of an abrB promoter-lacZ fusion construct in the parental strain 34F2 (●), in the BXA0205 mutant (○), and in the BXA0205phr mutant (▲). (B) β-Galactosidase analysis of the atxA promoter-lacZ fusion construct. Strains and symbols are as for panel A. Strains were grown in Schaeffer’s sporulation medium. The time zero on the x axis indicates the time of transition from exponential to stationary phase. The growth curve of one representative strain for each panel is shown by the open triangles. OD₅₂₅, optical density at 525 nm.
presumably toxins are favorably expressed and sporulation does not occur. Since environmental growth conditions play a critical role in regulating atxA and toxin gene transcription, a more in-depth analysis is necessary in order to clearly understand the nuances of the regulatory pathway involving the phosphorelay, AbrB, AtxA, and toxin production.

The activity of the BXA0205 Rap protein is inhibited by the carboxy-terminal pentapeptide of the BXA0205Phr protein (GHTGG) both in vivo and in vitro, although with limited efficiency compared to the 1:1 stoichiometry observed with the RapA-PhrA system of B. subtilis (Tables 2 and 3 and Fig. 4) (17). The observation that the phr gene is cotranscribed with the rap gene and thus does not get transcriptionally amplified by an additional specific promoter, as seen in several rap-phr systems of B. subtilis (8, 23), also indicates that an advantage must exist in keeping the Rap protein active by producing little and/or a poorly active pentapeptide.

The role of BXA0205 Rap and Phr proteins in B. anthracis sporulation is likely to provide a rationale for the observation made by Aronson et al. on regulation of extracellular protease production by these proteins, by them identified as Cot43 and Cot43p (3). If transcription regulation of the proteases analyzed by their work is somehow dependent on the regulation of the transition phase (involving the phosphorelay and AbrB), then changes are expected if the BXA0205rap gene is either deleted or cloned in a multicopy plasmid. Effects on the production of the subtilisin protease of B. subtilis are observed when the rapA gene is either deleted or overexpressed, and an effect on extracellular proteases has been recently reported upon expression of a plasmid-borne Rap-Phr system (M. Perego, unpublished data) (21).

A second Rap-Phr system affecting sporulation initiation, BA3790-3791, is chromosomally located and highly conserved (100% identity) among B. cereus strains but only 45 to 53% conserved with its orthologues (based on chromosomal mapping) of B. cereus strains. In this system, as previously discussed for the BXA0205 system, the phr gene is cotranscribed with the rap gene but also has a specific promoter. Coexpression of BA3791 with BA3790 resulted in efficient inhibition of the Rap activity in vivo. Unfortunately, we could not clearly define the active pentapeptide sequence in order to test it in vitro. Curiously, this active pentapeptide is not in the sequence GDHGG that strongly resembles the GHTGG of the BXA0205Phr inhibitor (Fig. 1B). This observation suggests caution in attempting to identify Phr pentapeptide inhibitors based solely on sequence homology. As inferred by the sequence analysis of the B. anthracis Rap-Phr systems (Fig. 1A and B), evolution within this species from a common ancestor has resulted in highly homologous proteins with, however, differential specificity in protein-protein and protein-ligand interactions. Avoiding Rap-Phr cross-reactivity among the different systems must be essential in order to avoid physiologically detrimental interferences.

Four additional Rap-Phr systems were identified on the chromosome of B. anthracis. Three of them did not show any involvement in the sporulation process by means of in vivo or and/or in vitro analysis. The fourth one (BA4060) carries a rap gene with a frameshift mutation that generates a truncated protein. However, the orthologue from B. cereus is uninterrupted and, when tested, did not affect sporulation in vivo (Table 3). A physiological function for these systems is unknown at this time. A recent report assigned the function of regulator of an integrative and conjugative element (the mobile genetic element ICEBs1) to RapI and PhrI, the B. subtilis orthologues of BA3760 and BA3759, suggesting that these proteins may have identical functions in B. anthracis (4).

The Rap-Phr systems have so far been identified only in members of the genus Bacillus. Rap proteins seem to have evolved to carry out different regulatory functions based on specific protein-protein interactions and modulation by an inhibitory pentapeptide ligand. The structural basis for these interactions is provided by the Rap organization in TPR (37). TPR are structural modules of 34 amino acids that assume an α-helix-turn-α-helix conformation. The α helices are antiparallel and pack with an angle of approximately 24° between the helix axes. These motifs occur in tandem arrays, the number ranging from 3 to 16, in a variety of different proteins, both prokaryotic and eukaryotic (10, 11, 14). These arrays function as molecular scaffolds known to mediate protein-protein or protein-ligand interactions. Rap proteins contain six TPR motifs whose packing in a typical parallel fashion generates a right-handed superhelical structure with two major surfaces: the outer convex and the inner concave (37). The latter surface has been shown to provide a docking site for linear peptides, and this is most likely the site of interaction of the Phr pentapeptides with the Rap proteins (46).

A related family of TPR-containing proteins includes the members of the PlcR group of transcription regulators characteristic of the B. cereus group of bacteria (48). These proteins carry an additional DNA-binding domain at their amino-terminal ends and, unusually, only two TPR domains (according to PFAM analysis [http://www.sanger.ac.uk]). The work on the B. subtilis Rap-Phr protein has been instrumental in finding that a pentapeptide generated from an export-import processing pathway of an associated gene product is required to activate the transcriptional function of PlcR proteins (47). It has been observed that in B. anthracis, PlcR is inactive because of a nonsense mutation in the plcR gene, and this mutation is ubiquitous among an extensive collection of genetically distinct B. anthracis strains (12, 25). Complementation of the B. anthracis plcR gene with the orthologous B. thuringiensis gene revealed that, although PlcR and its regulon have no influence on virulence, the concurrent expression of the AtxA virulence regulator and/or the presence of the pXO1 plasmid resulted in a sporulation-deficient phenotype (25). Since the inability to sporulate is counterproductive for the survival of the bacteria, a nonfunctional PlcR must have been counterselected in this species. It remains to be established whether the BXA0205 Rap and Phr proteins may have any role in this so-called incompatibility of PlcR and AtxA in B. anthracis by means of their role in regulating the sporulation process.

**ACKNOWLEDGMENTS**

This work was supported in part by grant GM55594 from the National Institute of General Medical Sciences and grant AI55860 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Oligonucleotide syntheses and DNA sequencing reactions were supported in part by the Stein Beneficial Trust. This report is manuscript number 17743-MEM from The Scripps Research Institute.