

## Distinct *clpP* Genes Control Specific Adaptive Responses in *Bacillus thuringiensis*

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ClpP and ClpC are subunits of the Clp ATP-dependent protease, which is ubiquitous among prokaryotic and eukaryotic organisms. The role of these proteins in stress tolerance, stationary-phase adaptive responses, and virulence in many bacterial species has been demonstrated. Based on the amino acid sequences of the *Bacillus subtilis* *clpC* and *clpP* genes, we identified one *clpC* gene and two *clpP* genes (designated *clpP1* and *clpP2*) in *Bacillus thuringiensis*. Predicted proteins ClpP1 and ClpP2 have approximately 88 and 67% amino acid sequence identity with ClpP of *B. subtilis*, respectively. Inactivation of *clpC* in *B. thuringiensis* impaired sporulation efficiency. The *clpP1* and *clpP2* mutants were both slightly susceptible to salt stress, whereas disruption of *clpP2* negatively affected sporulation and abolished motility. Virulence of the *clp* mutants was assessed by injecting bacteria into the hemocoel of *Bombyx mori* larvae. The *clpP1* mutant displayed attenuated virulence, which appeared to be related to its inability to grow at low temperature (25°C), suggesting an essential role for ClpP1 in tolerance of low temperature. Microscopic examination of *clpP1* mutant cells grown at 25°C showed altered bacterial division, with cells remaining attached after septum formation. Analysis of *lacZ* transcriptional fusions showed that *clpP1* was expressed at 25 and 37°C during the entire growth cycle. In contrast, *clpP2* was expressed at 37°C but not at 25°C, suggesting that ClpP2 cannot compensate for the absence of ClpP1 in the *clpP1* mutant cells at low temperature. Our study demonstrates that ClpP1 and ClpP2 control distinct cellular regulatory pathways in *B. thuringiensis*.

Living organisms have evolved complex regulatory networks to maintain cell viability under adverse environmental conditions (nutrient starvation; changes in temperature, humidity, or osmolarity; oxidative stress, host defense systems; etc.). An essential element of these networks is the induction of targeted intracellular proteolysis performed by energy-dependent proteases, such as the Clp ATP-dependent proteases (18, 49).

Proteins of the Clp ATPase family are highly conserved and ubiquitous in prokaryotes and higher organisms and are classified into two groups; proteins of the first group (ClpA, ClpB, ClpC, and ClpD), also known as the Hsp100 family, contain two distinct ATP-binding domains, while those of the second group (ClpM, ClpN, ClpX, and ClpY) contain smaller-size proteins with only one such domain (58). In *Escherichia coli*, ClpA or ClpX ATPase subunits associate with the ClpP proteolytic subunit to form the Clp ATP-dependent protease (21). This complex is involved in the degradation of several substrates such as endogenous regulator of programmed cell death MazE (3), stationary-phase transcriptional factor RpoS (61), and SsrA-tagged proteins (20). Clp ATPases can also function as molecular chaperones, preventing polypeptides from misfolding and aggregating (19, 21, 67, 69).

In *E. coli*, the majority of the *clp* genes are heat inducible, and their regulation depends mainly on the  $\sigma^{32}$  sigma factor (70). In *Bacillus subtilis*, *clpP* and *clpC* genes belong to the class III group of heat shock genes, whose expression is negatively

regulated by the CtsR repressor, encoded by the first gene of the *clpC* operon (10, 29).

ClpP and ClpC of *B. subtilis* have been shown to play essential roles in growth at high temperature and stress tolerance (salt, ethanol, puromycin), presumably due to their role in the degradation of misfolded proteins generated by these types of stress (16, 30, 43, 45). Similar observations were reported for *Lactococcus lactis* and *Listeria monocytogenes* (14, 15, 55, 56).

Besides stress tolerance, ClpP and ClpC have been linked to many physiological, morphological, and developmental processes in bacteria. For instance, in *B. subtilis* they are required for motility, division, degradative enzyme synthesis, sporulation, and competence development (42). ClpP is required for the mycelium formation of filamentous soil bacterium *Streptomyces lividans* (9) as well as for the initiation of biofilm formation of surface-attached microorganism *Pseudomonas fluorescens* (48).

Clp proteins, including ClpX of *Staphylococcus aureus* (41), ClpE and ClpC ATPases of *Streptococcus pneumoniae* (31, 51), and ClpP, ClpC, and ClpE of *Listeria monocytogenes* (15, 47, 55), have been shown to play a major role in the virulence of several pathogens.

*Bacillus thuringiensis*, a gram-positive spore-forming bacterium well known for its entomopathogenic properties, is a member of the *Bacillus cereus* group, which also includes the closely related species *B. cereus sensu stricto*, *Bacillus anthracis*, and *Bacillus mycoides* (8, 25). The insecticidal activity of *B. thuringiensis* resides mainly in the proteinaceous crystal of  $\delta$ -endotoxins produced during the stationary phase, which is lethal to susceptible insects upon ingestion (59). Many studies have shown that *B. thuringiensis* and *B. cereus* spores have a

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TABLE 1. Primer sequences used in this study

Name	Nucleotide sequence <sup>a</sup> (5'–3')	Restriction site
SH1	GAAGAATTCGTTTGGCTCGTAATTTAAAAAATATCAATTC	<i>Eco</i> RI
SH2	GGAGGATCCGAAATCGCCTCCTACTGCGCTTAGTTTTTTC	<i>Bam</i> HI
SH3	GAAGAATTCGTATAGACAAACTAAGAGGGCTACGAGATAGCC	<i>Eco</i> RI
SH4	AAGAAGCTTTCTTGTTTCCTTCCATTGAAGCAACCACTGTTG	<i>Hind</i> III
Sp1	CGCGGATCCGGATCTTCACCTAGATCC	<i>Bam</i> HI
Sp2	CCGGAATTCGTTACAAATTGTTTCACTAAATTAAG	<i>Eco</i> RI
Pi2	CCCAAGCTTGCTGTCATGAAGTTGTGCGGG	<i>Hind</i> III
Pi3	CGCGGATCCGGTATGGCTGCATCTATGGGTGC	<i>Bam</i> HI
Pi4	CCCAAGCTTGTTGACCTGTGCGGTTCAGC	<i>Hind</i> III
P2.i3	CGCGGATCCAATAGCCCCGGCGGTTCAACG	<i>Bam</i> HI
P2.i4	CCCAAGCTTGCCCTTGTGCACCACCAAGC	<i>Hind</i> III
PclpP1	AAACTGCAGCCCTAGAAACAAAGTCGAAATC	<i>Pst</i> I
PclpP2	CGCGGATCCCGTAAGCGCGTTCTCCACG	<i>Bam</i> HI
pP2.1	AAACTGCAGCACATCAAAAGTTCCCGACC	<i>Pst</i> I
pP2.5	CGCGGATCCGAGATCTTCCCCATCCCATTGT	<i>Bam</i> HI
P8	GCTGTCTTAGAACCGAGCGGAG	
P9	CGTTTAGTGAATTGCGAAACAGC	
clpP2.1	CGTCGTATATAAAATAGCTAG	
clpP2.2	CGGTTGCCCTGTTTTTCTGC	
clpP2.3	CCAGACGTGCAAACGCTATGC	
clpP2.6	CTTGTCCAATCTTCTGAAGCTGC	

<sup>a</sup> Restriction sites are underlined.

synergistic effect when fed in association with  $\delta$ -endotoxins to susceptible larvae (26, 37, 57). In addition, it was shown that *B. thuringiensis* and *B. cereus* cells are highly pathogenic when injected into the hemocoel of the insect host (23, 24, 57, 62, 71). Several putative virulence factors, including phospholipases C, enterotoxins, hemolysins, immune inhibitors, and flagella, have been identified in *B. thuringiensis* and *B. cereus*, and there has been speculation that they facilitate the development of these bacteria within the host (22, 60, 71). However, none of these factors on its own has been confirmed to be essential for the pathogenic mechanisms leading to systemic septicemia.

To identify genes responsible for the virulence of *B. thuringiensis* in insects, we focused our interest on the *clpP* and *clpC* genes of this bacterium, since as mentioned above their role in the virulence of many pathogens has been established. Here we report that, in *B. thuringiensis*, there are two copies of the *clpP* gene, named *clpP1* and *clpP2*. Mutants with *clpC* and both *clpP* genes inactivated were constructed and assayed for their phenotypical characteristics with respect to stress tolerance and virulence against *Bombyx mori* larvae. Results indicate that ClpP1 and ClpP2 control distinct cellular regulatory pathways.

MATERIALS AND METHODS

**Bacterial strains, transformation, and culture conditions.** Sporogenous acrystalliferous *B. thuringiensis* strain 407 Cry<sup>−</sup>, belonging to serotype 1 (36), was used throughout this study. *E. coli* K-12 strain TG1 [ $\Delta$ (*lac-proAB*) *supE* *thi* *hsd* $\Delta$ 5 (*F'* *traD36* *proA*<sup>+</sup> *proB*<sup>+</sup> *lacF*<sup>+</sup> *lacZ* $\Delta$ M15)] (17) and *E. coli* K-12 strain TG1 RepA<sup>+</sup> (33) were used as hosts for plasmid construction. *E. coli* strains SCS 110 [*rpsL* (*Str*<sup>r</sup>) *thr* *leu* *endA* *thi-1* *lacY* *galK* *galT* *ara* *tonA* *tsx* *dam* *dcm* *supE*44  $\Delta$ (*lac-proAB*) (*F'* *traD36* *proA*<sup>+</sup> *proB*<sup>+</sup> *lacF*<sup>+</sup> *lacZ* $\Delta$ M15)] (Stratagene, La Jolla, Calif.) and ET12567 (*F*<sup>−</sup> *dam-13*::Tn9 *dcm-6* *hsdM* *hsdR* *recF*143 *zjj-202*::Tn10 *galK2* *galT22* *ara14* *pacY1* *xyl-5* *leuB6* *thi-1*) were used to generate unmethylated plasmid DNA prior to transformation of *B. thuringiensis*. Conventional CaCl<sub>2</sub> (7) or electroporation procedures (11) were used for *E. coli* transformation. *B. thuringiensis* strain 407 Cry<sup>−</sup> was transformed by electroporation as previously described (36).

*E. coli* and *B. thuringiensis* cells were routinely grown in Luria broth (LB) medium under vigorous agitation at 37 and 30°C, respectively. Antibiotic con-

centrations used for bacterial selection were as follows: ampicillin, 100  $\mu$ g ml<sup>−1</sup> for *E. coli*; spectinomycin, 100  $\mu$ g ml<sup>−1</sup> for *E. coli* and 300  $\mu$ g ml<sup>−1</sup> for *B. thuringiensis*; erythromycin, 10  $\mu$ g ml<sup>−1</sup> for *B. thuringiensis*.

For the phenotypical studies, different stress conditions were established as follows. Frozen glycerol stocks (exponentially growing cells; optical density at 600 nm [OD<sub>600</sub>] = 1) of the different strains were diluted 100-fold into LB medium and grown under vigorous shaking at 37°C. At an OD<sub>600</sub> of 0.1, the culture was divided and one half was grown at 37°C whereas the other half was either shifted from 37 to 43°C or exposed to a final concentration of 6% (wt/vol) sodium chloride. Motility assays were performed on LB soft agar swarm plates (0.3% agar final concentration) followed by 16 h of incubation at 37°C. Columbia medium agar plates (Biomérieux) containing 5% sheep blood were used to evaluate the hemolytic activity of *B. thuringiensis* strains. Sporulation assays were performed as follows. Frozen glycerol stocks of exponentially growing cells were diluted 100-fold into sporulation-specific (HCT) medium (32) and grown for 3 days at 39°C. Serial dilutions of sporulating cells were plated at 24-h intervals before and after heat treatment (80°C; 12 min). Sporulation frequencies were established on the basis of viable-cell and spore counts.

**DNA manipulations.** Plasmid DNA was extracted from *E. coli* by standard alkaline lysis with QIAprep spin columns (Qiagen). Chromosomal DNA was extracted from *B. thuringiensis* cells harvested in mid-log phase as described previously (44). Restriction enzymes and T4 DNA ligase were used as recommended by the manufacturer (New England Biolabs). Oligonucleotide primers (Table 1) were synthesized by Genset (Paris, France). PCRs were performed in a GeneAmp PCR system 2400 thermal cycler (Perkin-Elmer). Amplified DNA fragments were purified with the QIAquick PCR purification kit (Qiagen) and separated on 0.7% agarose gels after digestion. Digested DNA fragments were eluted from agarose electrophoresis gels with a centrifugal filter device (Ultra-free-DA; Amicon Laboratories).

**Plasmid and mutant strain constructions.** Plasmids pGhost9 (38) and pRN5101, a pE194 derivative (65), conferring resistance to erythromycin in gram-positive hosts and to ampicillin in *E. coli*, were used for gene deletion and gene disruptions, respectively. These vectors contain a thermosensitive origin of replication that prevents them from replicating in gram-positive hosts at non-permissive temperatures ( $\geq 37^\circ\text{C}$ ).

Deletion of the *clpC* gene was carried out as follows. A 996-bp *Eco*RI/*Bam*HI DNA fragment and a 1,010-bp *Eco*RI/*Hind*III DNA fragment, corresponding to the DNA chromosomal regions located immediately upstream and downstream from the *clpC* gene, respectively, were generated by PCR using *B. thuringiensis* strain 407 Cry<sup>−</sup> chromosomal DNA as a template and oligonucleotide pairs SH1-SH2 and SH3-SH4, respectively (Table 1). The primers were designed from the sequence of the *clpC* operon identified in the *B. anthracis* incomplete genome sequence. A 1,134-bp *Bam*HI/*Eco*RI DNA fragment carrying the *S. aureus* spectinomycin resistance gene *spc* (46) was amplified with primer pair Sp1-Sp2 (Table

1) and *B. subtilis* strain QB4756 chromosomal DNA (45) as a template. The amplified DNA fragments were digested by the appropriate restriction enzymes and cloned between the internal *HindIII* and *EcoRI* sites of vector pGhost9 to give plasmid pGHost9 $\Delta$ clpC::spc, which requires *E. coli* strain TG1 RepA<sup>+</sup> to replicate (33). The resulting plasmid was introduced into *B. thuringiensis* strain 407 Cry<sup>-</sup> by electroporation, and the deletion of the *clpC* gene was obtained by a double-crossover event as described previously (34). The complete deletion of the gene was verified by PCR using additional oligonucleotides located further upstream and downstream from the original fragments. The corresponding mutant strain was designated 407 Cry<sup>-</sup>  $\Delta$ clpC.

The chromosomal *B. thuringiensis* *clpP1* and *clpP2* genes were disrupted by insertion, through homologous recombination, of derivatives of plasmid pRN5101. Constructions were performed as follows. DNA fragments corresponding to internal regions of the *clpP1* and *clpP2* genes (203 and 205 bp, respectively) were generated by PCR using primer pairs Pi3-Pi4 and P2.i3-P2.i4 (Table 1) and cloned between the *BamHI*/*HindIII* sites of pRN5101. The resulting plasmids were introduced into *B. thuringiensis* strain 407 Cry<sup>-</sup> by electroporation. Transformants were grown at 30°C and then transferred to a nonpermissive temperature (39°C) and finally plated onto LB agar plates supplemented with erythromycin (10  $\mu$ g ml<sup>-1</sup>) at 39°C. Integration of the recombinant plasmid was confirmed by PCR and Southern hybridization. The corresponding insertional mutant strains were designated 407 Cry<sup>-</sup>  $\Delta$ clpP1 and 407 Cry<sup>-</sup>  $\Delta$ clpP2 and were found to be highly stable during growth at the nonpermissive temperature.

**Construction of *clpP1'-lacZ* and *clpP2'-lacZ* transcriptional fusions.** A *clpP1'-lacZ* transcriptional fusion was constructed by cloning a 395-bp *BamHI*/*PstI* DNA fragment, corresponding to the chromosomal DNA region upstream from the *clpP1* gene, between the *BamHI* and *PstI* sites of plasmid pHT304-18'Z (2) to give plasmid pHT304 $\Omega$ clpP1'Z. This DNA fragment was generated by PCR using synthetic oligonucleotides PclpP1 and PclpP2 (Table 1).

A *clpP2'-lacZ* transcriptional fusion was constructed by amplifying a 326-bp *BamHI*/*PstI* DNA fragment by PCR using synthetic primers pP2.1 and pP2.5 (Table 1), designed on the available *B. cereus* nucleotide sequence. This fragment contains 227 bp upstream from the extracytoplasmic function (ECF) RNA polymerase  $\sigma$  factor gene as well as the first 100 bp of the coding sequence. The fragment was cloned between the *BamHI* and *PstI* restriction sites of the pHT304-18'Z vector to give plasmid pHT304 $\Omega$ clpP2'Z.

The recombinant plasmids were introduced into strain 407 Cry<sup>-</sup> by electroporation to give strains 407 Cry<sup>-</sup> [pHT304 $\Omega$ clpP1'Z] and 407 Cry<sup>-</sup> [pHT304 $\Omega$ clpP2'Z].

**$\beta$ -Galactosidase activity.** Colonies expressing *lacZ* fusions were detected on media containing 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal; 40  $\mu$ g ml<sup>-1</sup>) and erythromycin (10  $\mu$ g ml<sup>-1</sup>). Cells were grown in LB medium devoid of antibiotics at 25 and 37°C with vigorous shaking and assayed for  $\beta$ -galactosidase-specific activities as described previously (44).

**PCR amplification and sequencing of *clpP1* and *clpP2* genes.** Four pairs of synthetic oligonucleotides (p1 [P8-Pi2], p2 [P9-Pi3], p3 [clpP2.1-clpP2.2], and p4 [clpP2.3-clpP2.6]) (Table 1) were designed from the sequence of *B. anthracis* genomic regions encompassing the *clpP1* and *clpP2* genes. The p1, p2, p3, and p4 oligonucleotide pairs were used to amplify four overlapping fragments, corresponding to the *clpP1* and *clpP2* regions, from positions -640 to +900 and -470 to +392 with respect to the ATG start codon and the TAA terminal codons of *clpP1* and *clpP2*, respectively. PCRs were carried out in a reaction volume of 100  $\mu$ l containing 200  $\mu$ M deoxynucleoside triphosphates, 2.5 mM MgSO<sub>4</sub>, 50 pmol of each primer, 0.5  $\mu$ g of *B. thuringiensis* strain 407 Cry<sup>-</sup> chromosomal DNA, and 0.5 U of *Pwo* DNA polymerase (Roche Boehringer). Purified PCR products were sequenced on both strands by Genome Express (Paris, France).

**Database comparisons and sequence analysis.** Alignment and sequence comparisons with the GenBank database were performed by using the National Center for Biotechnology Information BLAST2 (4) network server with the default parameter values provided. *B. subtilis* sequences were from Subtilist (<http://genolist.pasteur.fr/Subtilist/>), and *B. cereus* sequences were from [http://ergo.integratedgenomics.com/B\\_cereus.html](http://ergo.integratedgenomics.com/B_cereus.html). Unfinished *B. anthracis* genome sequences were kindly provided by The Institute for Genomic Research website (<http://www.tigr.org>).

**Insects and in vivo experimental infections.** Eggs of *B. mori* strain nistari provided by the Institut National de la Recherche Agronomique (Unité Nationale Séricicole, Lyon, France) were incubated at 25°C. The resulting larvae were reared on a commercially available artificial diet (Fukui and Co., Ltd., Yokohama, Japan).

Pathogenicity assays of *B. thuringiensis* vegetative cells were carried out as follows. Cells of wild-type and mutant strains of *B. thuringiensis* were grown in LB medium devoid of antibiotics at 30°C and with shaking. Bacterial concentrations were monitored by optical density measurement at 600 nm and verified by plating dilutions onto LB agar plates. Different dilutions of exponentially growing *B.*

*thuringiensis* cells were inoculated into groups of 30 *B. mori* larvae (10  $\mu$ l larva<sup>-1</sup>). The control group was injected with sterile water. *B. mori* larvae were on the first day of the fourth instar and weighed about 150 to 200 mg. *B. thuringiensis* cell suspensions were injected through the intersegmental membrane between the fourth and the fifth abdominal legs of the larva with a 1-ml Terumo syringe and a microapplicator (Buckard type LV 65). Inoculated larvae were incubated individually in plastic containers at 25°C. Mortality was recorded daily over a 3-day period.

**In vitro experimental infections.** In vitro experimental infections were performed in insect hemolymph pooled from fifth-instar *B. mori* larvae. The surfaces of the larvae were cleaned with 70% ethanol and dried. Cell-free hemolymph was collected by capillary action in a sterile, ice-cooled Eppendorf tube by cutting off the abdominal legs of the larvae. Cell-free hemolymph was prepared by centrifugation at 16,000  $\times g$  for 2 min. Twenty microliters of a dilution of a *B. thuringiensis* culture were added to 200  $\mu$ l of hemocoel, and the mixture was incubated at 28 or 37°C for 3 h. During this period, 20- $\mu$ l aliquots were withdrawn at 30-min intervals and spread on LB plates for viable-cell counts. Experiments were repeated three times for each test.

**Statistical analysis.** Mortality data were analyzed by calculating 50% lethal doses (LD<sub>50</sub>s) with the Log-Probbit program (12; M. Raymond, G. Prato, and D. Ratsira, PROBIT analysis of mortality assays displaying quantal response, license no. L93019-Avenix [Praxeme, Montpellier, France], 34680 St. George d'Orques, France, 1993).

**Nucleotide sequence accession numbers.** The nucleotide sequences of *clpP1* and *clpP2* from *B. thuringiensis* reported in this paper have been submitted to the GenBank database under accession no. AF454757 and AF454758, respectively.

## RESULTS

**Nucleotide and amino acid sequence analysis of *B. thuringiensis* *clpP* genes.** The *B. subtilis* ClpP protein sequence (43) was used to screen the incomplete *B. anthracis* genome sequence. This revealed the presence of two highly similar gene products, having approximately 88 and 66% identity with ClpP of *B. subtilis*. Primer pairs were designed from the *B. anthracis* sequence and used to amplify the corresponding DNA regions from *B. thuringiensis* strain 407 Cry<sup>-</sup> (see Materials and Methods). The nucleotide sequences of the PCR products were determined on both strands, and the *B. thuringiensis* DNA regions encompassing both open reading frames (ORFs) were entirely reconstituted. Analysis of the nucleotide sequence of the first DNA fragment (2,121 bp) revealed an ORF starting with an ATG codon and predicted to encode a protein of 194 amino acid residues having 88% identity with ClpP of *B. subtilis* (Fig. 1). The ATG codon is preceded by a typical ribosome binding site sequence (GGAGG) at an appropriate distance, and a potential promoter sequence with significant similarity to  $\sigma^A$ -type -35 and -10 promoter recognition sequences (TTG ACCN<sub>17</sub>TATTAT) was found 35 bp upstream from the start codon. A CtsR consensus sequence (A/GGTCAAANANA/GGTCAAA), reported in the promoter regions of several *clp* genes of various bacterial species (10), in the reverse orientation (5'-GGTCAATAAAGGTCAAA-3') was identified 66 bp upstream from the translational start site, overlapping the potential -35 sequence. A likely rho-independent transcription terminator stem-loop sequence (GAAGCGCCCTATAATTG GGCGCTTC,  $\Delta G = -103.7$  kJ mol<sup>-1</sup>) followed by a poly(T) stretch is located 135 bp after the TAA stop codon. This ORF appeared to be organized as a single transcriptional unit and was designated *clpP1*.

The second DNA fragment contained two ORFs that appear to be organized as an operon. The second ORF, beginning with an ATG codon, encodes a 193-amino-acid protein displaying 67% identity with ClpP of *B. subtilis* (Fig. 1). This ORF is



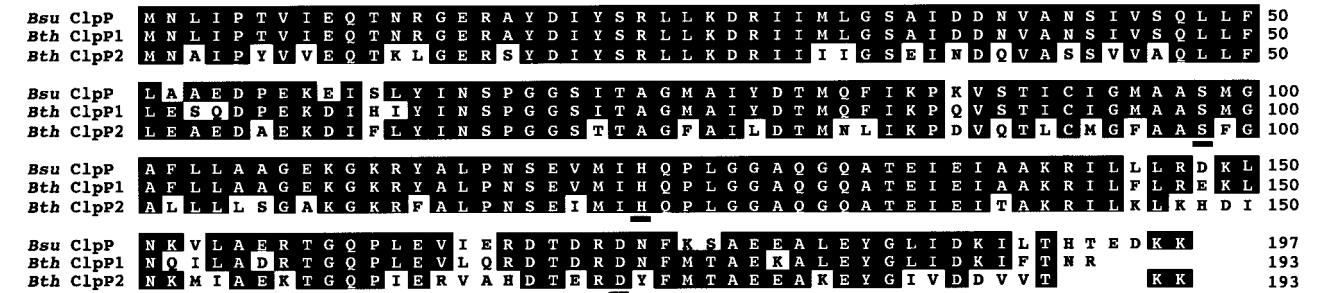


FIG. 1. Alignment of the *B. thuringiensis* (*Bth*) ClpP1 and ClpP2 amino acid sequences with that of *B. subtilis* ClpP. The *B. subtilis* (*Bsu*) ClpP sequence is as described by Msadek and colleagues (43). Numbers indicate positions in the amino acid sequence. Identical residues are shaded. The conserved catalytic Ser-98, His-123, and Asp-172 residues are underlined.

therefore referred to as *clpP2*. The fact that the stop codon of the upstream ORF and the ATG codon of the *clpP2* ORF are separated by only 21 bases and the absence of a potential stem-and-loop structure in the intergenic region suggest that the two ORFs are organized as an operon. The *clpP1* and *clpP2* genes are organized similarly in the *B. cereus* strain ATCC 14579 and *B. anthracis* genomes. The deduced amino acid sequence encoded by the first ORF of the *B. cereus clpP2* operon displayed similarities with an RNA polymerase ECF-type sigma factor from *Bacillus halodurans* (63). No CtsR box could be identified in the upstream region of the *clpP2* operon in *B. cereus*. The high degree of identity over the entire length of the aligned sequences (*B. thuringiensis clpP1*, *B. thuringiensis clpP2*, and *B. subtilis clpP*; Fig. 1) indicates that the *B. thuringiensis* ClpP proteins are members of the ClpP family of proteolytic subunits (40). The serine-98 residue, the histidine-123 residue, and the aspartate-172 residue, which constitute the catalytic triad of the serine protease (39, 40, 66), are conserved (Fig. 1).

Since a CtsR consensus sequence was found upstream of *clpP1* ORF, we searched for a *ctsR*-like gene in *B. cereus* and *B. anthracis* genomes. This led to the identification, in the genomes of both species, of an ortholog of the *ctsR* gene organized as in *B. subtilis*, with *ctsR* and *clpC* as the first and last genes of a four-gene operon.

**Phenotypical features of the mutant strains.** To address the question of whether the ClpP1, ClpP2, and ClpC proteins are required for stress tolerance, mutations inactivating the corresponding genes were generated and the mutants were assessed for altered phenotypes with respect to high temperature (43°C) and salt concentration (6% NaCl) (see Materials and Methods).

Regarding stress tolerance, all mutants displayed growth rates when grown at 37°C that were the same as those when they were shifted from 37 to 43°C, the upper limit for growth of *B. thuringiensis* 407 Cry<sup>-</sup> (data not shown). However, diminished growth rates of the  $\Delta clpP1$  and  $\Delta clpP2$  strains were observed in the presence of 6% NaCl, with a larger lag phase for the  $\Delta clpP1$  mutant strain (Fig. 2). These results indicate a possible role for the *B. thuringiensis clpP2* gene in salt tolerance.

As shown in Fig. 3, the  $\Delta clpP2$  cells were severely impaired in motility whereas all the other mutants were as motile as the parental strain. Further confirmation of the nonmotile pheno-

type of  $\Delta clpP2$  cells was obtained by microscopic examination, indicating that ClpP2 is required for motility. Sporulation assays were assessed by establishing sporulation frequencies after 24 h of incubation in HCT medium. As shown in Table 2, the sporulation frequencies of the  $\Delta clpC$  and the  $\Delta clpP2$  mutants were diminished approximately 10<sup>-3</sup>- and 10<sup>-5</sup>-fold, respectively, whereas that of the  $\Delta clpP1$  mutant was lowered only 1.4-fold. These results suggest that ClpP2 and ClpC play an important role in the sporulation pathway whereas ClpP1 does not.

*B. thuringiensis* genes encoding extracellular virulence factors (including phospholipases C PlcA and PlcB, enterotoxins Hbl and Nhe, hemolysins and proteases) are positively regulated by the PlcR pleiotropic regulator (1, 35). Inactivation of the *plcR* gene prevents the hemolytic activity of *B. thuringiensis* and *B. cereus* cells (57). All the mutant strains ( $\Delta clpP1$ ,  $\Delta clpP2$ , and  $\Delta clpC$  strains) were fully hemolytic on sheep erythrocytes (data not shown), suggesting that the PlcR regulon is not affected in the *clp* mutant backgrounds.

**Virulence of *B. thuringiensis clp* mutants in insects.** The virulence of the different *B. thuringiensis clp* mutants was assessed by injecting exponentially growing cells into the hemo-

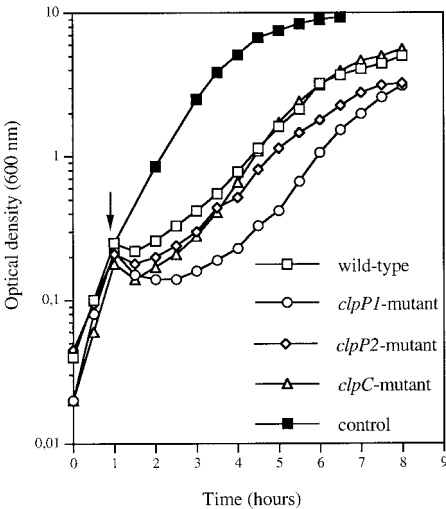


FIG. 2. Growth curves of *B. thuringiensis* strains in LB at 37°C after the addition of 6% (wt/vol) NaCl. Arrow, OD at which the cultures were exposed to the salt stress. The control indicates the growth curve of the wild type at 37°C in LB devoid of NaCl.

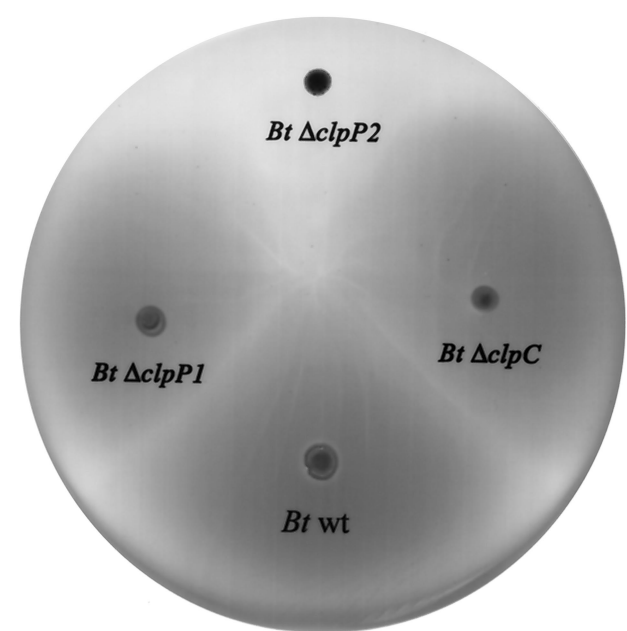


FIG. 3. The *clpP2* gene is required for motility. The *B. thuringiensis* (*Bt*) strains were incubated on LB soft agar as described in Materials and Methods.

coel of fourth-instar *B. mori* larvae. This lepidopteran species is a useful model because the larvae are highly susceptible to the parental-strain cells ( $LD_{50} < 5$  vegetative cells/larva; Table 3). As shown in Table 3,  $LD_{50}$ s of the  $\Delta clpC$  and  $\Delta clpP2$  mutants were not significantly different from that of the 407 Cry<sup>-</sup> strain, suggesting that the ClpP2 and ClpC proteins are not required for expression of virulence. In contrast, the virulence of the *clpP1*-deficient mutant was severely attenuated since no dead larvae were obtained from the insects infected with 100 cells of the mutant strain.

To determine whether the avirulent phenotype of the  $\Delta clpP1$  strain was due to the loss of specific pathogenic properties or to the inability of the mutant to survive within the host, we tested bacterial survival in vitro over a 3-h period of incubation in *B. mori* hemolymph (see Materials and Methods). The test was performed at 28°C without shaking to mimic the in vivo temperature and partially anaerobic conditions. The  $\Delta clpP1$  mutant was unable to grow in the hemolymph in contrast to the wild type (Fig. 4A). These results suggest that the reduced virulence of the *clpP1*-deficient mutant was due to the restricted in vivo development of the strain.

**ClpP1 is required for growth at low temperature.** To investigate the altered growth of the  $\Delta clpP1$  strain, we first assumed that the partially anaerobic conditions encountered in the insect hemolymph could prevent the growth of the mutant strain. However, both wild-type and mutant strains grew similarly at 37°C in anaerobiosis either in LB (data not shown) or in hemolymph in vitro (Fig. 4B), suggesting that anaerobiosis is not the reason that the  $\Delta clpP1$  mutant is unable to grow in vivo.

At 30°C, the  $\Delta clpP1$  mutant gave small-size colonies, suggesting that this strain presented a sensitivity to low growth temperatures. To test this hypothesis, we compared the growth rates of the  $\Delta clpP1$  mutant and the wild-type strains in LB

TABLE 2. Effects of *clpP* and *clpC* mutations on sporulation

<i>B. thuringiensis</i> strain	Sporulation frequency (CFU ml <sup>-1</sup> ) after 24 h incubation in HCT, plating:		% Sporulation
	Before heat treatment	After heat treatment	
407 Cry <sup>-</sup>	$2.88 \times 10^8$	$2.5 \times 10^8$	86
407 Cry <sup>-</sup> $\Delta clpC$	$1.72 \times 10^8$	$1.4 \times 10^5$	0.072
407 Cry <sup>-</sup> $\Delta clpP1$	$2.84 \times 10^8$	$1.71 \times 10^8$	60.2
407 Cry <sup>-</sup> $\Delta clpP2$	$4 \times 10^7$	300	0.00075

medium at 30, 28, and 25°C, the last temperature being that routinely used for in vivo infection tests. As shown in Fig. 5, the ability of  $\Delta clpP1$  mutant cells to grow decreased at lower growth temperatures. At 25°C, the cell concentration did not exceed that corresponding to an OD<sub>600</sub> of 1.5 while the OD<sub>600</sub> for the wild type reached a value of 10 (Fig. 5C). Furthermore, microscopic examination revealed a filamentous phenotype for the mutant cells during growth at 25°C, which was not observed for the wild-type cells. This suggests that ClpP1 is essentially required for cell separation at low temperature.

To determine whether low temperature is the only factor that might affect in vivo septicemia due to the  $\Delta clpP1$  strain, we assessed the virulence of the mutant strain at 37°C. A dose of 20 cells of the strain was inoculated into groups of 30 *B. mori* larvae. Nonentomopathogenic bacterium *B. subtilis* 168 was used as a negative control. By 24 h of incubation of infected insects at 37°C, we recorded the same mortality values for both wild-type and  $\Delta clpP1$  mutant strains (90 to 95% mortality). The *B. subtilis* control used did not induce any lethality even at a dose of 100 cells, indicating that the mortality was not due to the relatively high temperature of incubation. This suggests that the loss of virulence of the  $\Delta clpP1$  strain resulted from its reduced growth at low temperatures such as those present in the insect host.

**Analysis of *clpP1* and *clpP2* expression in *B. thuringiensis* during growth at low temperatures.** The expression of the *clpP* genes at different temperatures was analyzed by using transcriptional fusions with the *lacZ* gene. Plasmid pHT304 $\Omega$ *clpP1'*Z was introduced into *B. thuringiensis* strain 407 Cry<sup>-</sup>, and the  $\beta$ -galactosidase activity of the recombinant strain was monitored during growth in LB medium at 37 and 25°C (Fig. 6A). *clpP1'-lacZ* was expressed during the exponential growth phase: about 200 to 300 U of  $\beta$ -galactosidase mg<sup>-1</sup> was produced before the onset of stationary phase (time zero) at each temperature. As reported for the *B. subtilis clpP* gene (43), induction of *clpP1*

TABLE 3. Effects of *clpP* and *clpC* mutations on the virulence of *B. thuringiensis* in *B. mori* larvae

Strain	LD <sub>50</sub> <sup>a</sup> (CFU/injected larva)
407 Cry <sup>-</sup> .....	4.79 (3.28–6.12)
407 Cry <sup>-</sup> $\Delta clpC$ .....	5.27 (3.57–6.95)
407 Cry <sup>-</sup> $\Delta clpP1$ .....	>100 <sup>b</sup>
407 Cry <sup>-</sup> $\Delta clpP2$ .....	7.094 (3.56–9.91)

<sup>a</sup> LD<sub>50</sub>s were calculated 1 day postinfection by Probit analysis (Raymond et al., license no. L93019-Avenix). Values in parentheses are 95% confidence intervals (12).

<sup>b</sup> Injection of 100 cells of a *clpP*-deficient mutant causes 0% mortality.

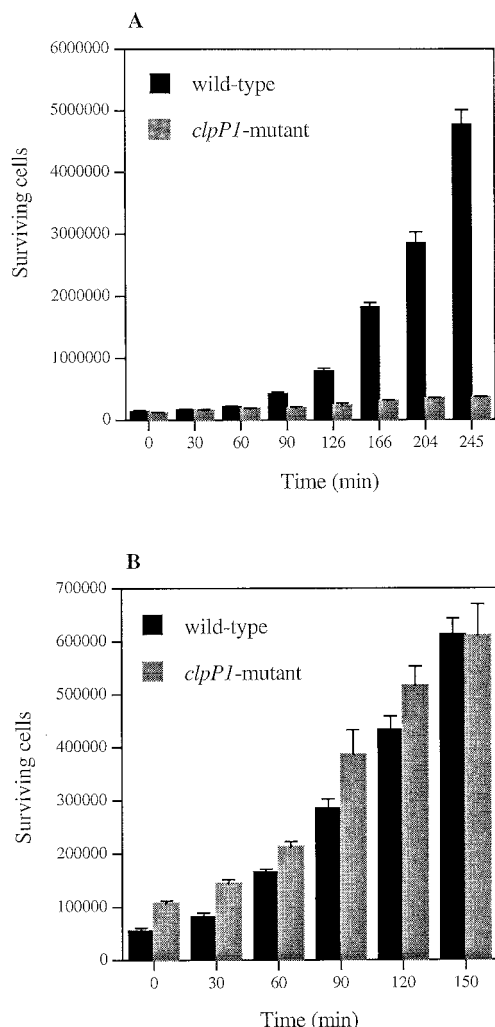


FIG. 4. Surviving *B. thuringiensis* wild-type and  $\Delta clpP1$  cells after incubation with hemolymph from *B. mori* larvae. Two hundred microliters of hemolymph and 20  $\mu$ l of a dilution containing about  $10^5$  *B. thuringiensis* viable cells were mixed. The reaction mixture was incubated without shaking at 28 (A) and 37°C (B). Aliquots were withdrawn at the time points indicated, and the surviving bacteria were counted. Experiments were repeated three times. Error bars, standard errors of the means.

expression is observed upon entry into stationary phase. In cells grown at 25 and 37°C, the transcription patterns were similar. The significant level of *clpP* expression during the exponential growth phase at low temperature is in agreement with its requirement for low-temperature growth of *B. thuringiensis*.

A transcriptional fusion between a DNA fragment carrying the 370 bp directly upstream from *clpP2* and *lacZ* was not significantly expressed in *B. thuringiensis* (data not shown), in agreement with the proposed operon structure of the gene. We therefore analyzed *clpP2* expression by fusing the region upstream from the first gene of the operon to the *lacZ* reporter gene (plasmid pHT304 $\Omega$ *clpP2'**Z*) (Fig. 6B). A relatively weak *clpP2* operon-directed  $\beta$ -galactosidase activity was noted at 37°C, and no  $\beta$ -galactosidase production ( $<10$  U  $\text{mg}^{-1}$ ) was detected during growth at 25°C.

## DISCUSSION

This is the first study reporting an analysis of *clp* genes in the entomopathogenic bacterium *B. thuringiensis* and their involvement in stress tolerance, stationary-phase responses, and virulence.

We have shown that, in contrast to *B. subtilis*, *B. thuringiensis* contains two copies of the *clpP* gene (*clpP1* and *clpP2*). The *clpP1* gene appears to belong to a monocistronic transcription unit, whereas *clpP2* is organized as an operon with a gene encoding an ECF RNA polymerase sigma factor homologue. The *B. thuringiensis* ClpP1 and ClpP2 proteins were typical of the highly conserved family of ClpP proteolytic subunits (40). Most bacteria (e.g., *E. coli*, *B. subtilis*, and *Yersinia enterocolitica*) possess a single *clpP* gene; however, some have recently been shown to contain more than one copy. For instance, *Mycobacterium tuberculosis* has two ClpP proteins (28) and cyanobacteria *Synechocystis* sp. strain PCC6803 (27) and *Synechococcus* sp. strain PCC7942 (6) possess three distinct ClpP isoenzymes. *S. lividans* has been recently reported to possess a *clpP* multigenic family with four *clpP* representatives (9, 64). Since *B. thuringiensis* and *B. subtilis* are closely related species, it was unexpected to find two *clpP* copies in *B. thuringiensis*.

In *B. subtilis*, transcriptional repressor CtsR negatively regulates the expression of class III heat shock genes (*clpP*, *clpE*, and *clpC* operon) by binding to a directly repeated heptanucleotide operator sequence (A/GGTCAAA NAN A/GGTCAAA) which has been identified upstream of many bacterial *clp* genes (10). A gene encoding a CtsR homologue exists in the *clpC* operon in the genomes of *B. cereus* ([http://ergo.integratedgenomics.com/B\\_cereus.html](http://ergo.integratedgenomics.com/B_cereus.html)) and *B. anthracis* (<http://www.tigr.org>), and a CtsR consensus sequence was found in the promoter region of *clpP1* in *B. thuringiensis* but

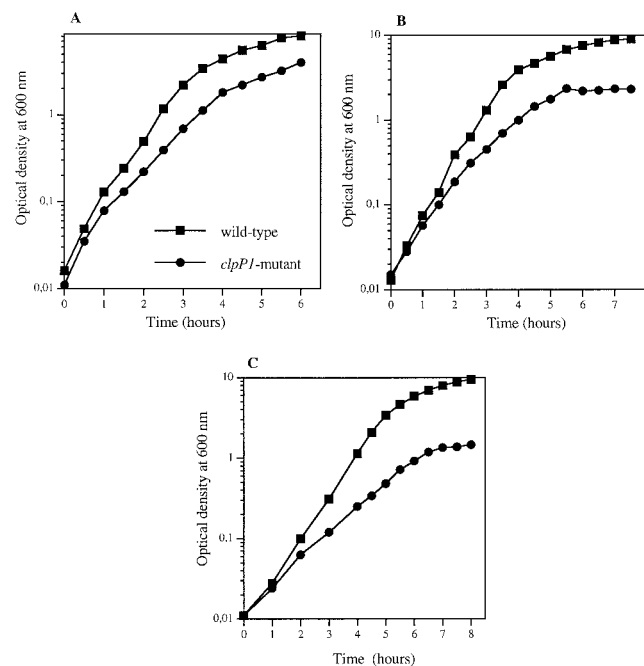


FIG. 5. Growth curves of *B. thuringiensis* wild-type and *clpP1* mutant strains in LB medium at 30 (A), 28 (B), and 25°C (C).

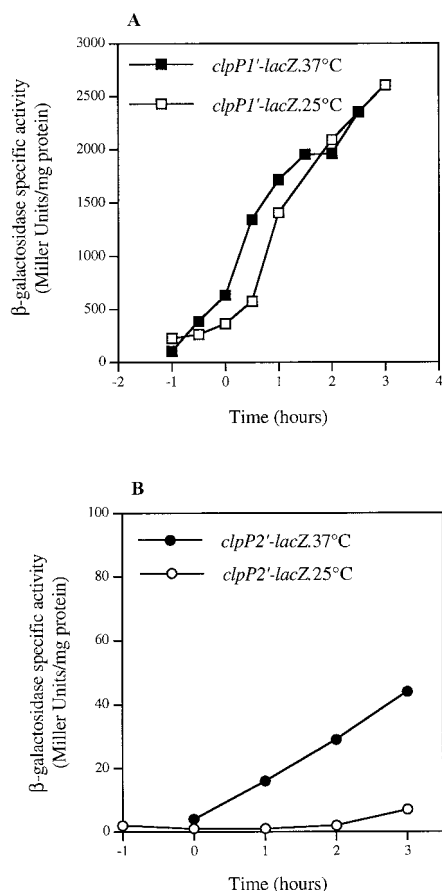


FIG. 6. Expression of *B. thuringiensis* *clpP* genes in strain 407 Cry<sup>-</sup>. (A) Expression of *clpP1'*-lacZ at 37 and 25°C. (B) Expression of *clpP2'*-lacZ at 37 and 25°C. Cells were grown in LB medium. Time zero indicates the onset of the stationary phase.

not in that of the *clpP2* operon. This suggests that *B. thuringiensis* *clpP1* is negatively controlled by CtsR.

The role of ClpP and ClpC in the stress survival of several gram-positive bacteria, including *B. subtilis*, *L. monocytogenes*, *L. lactis*, *Synechococcus*, and *S. pneumoniae*, has previously been reported (5, 54). In *B. subtilis* and *L. monocytogenes*, ClpP and ClpC were shown to be required for growth at high temperature and stress tolerance (exposure to salt, ethanol, or puromycin) (15, 16, 30, 56). Furthermore, *B. subtilis* Clp proteins were found to play central roles in many cellular developmental processes such as cell division, motility, degradative enzyme synthesis, sporulation, and genetic competence (43, 45). To study the involvement of ClpP1, ClpP2, and ClpC proteins of *B. thuringiensis* in stress tolerance and stationary-phase adaptive responses, we constructed mutants by inactivating the corresponding genes. Our results showed that, in contrast to what was found for *B. subtilis*, inactivation of *clpC* did not lead to pleiotropic effects. The *clpC*-deficient mutant displayed only a sporulation defect. Moreover, none of the mutant strains was sensitive to the different forms of stress imposed (high temperature and elevated salt concentrations) except for a slight sensitivity to high osmolarity for the *clpP1* mutant. The  $\Delta$ *clpP2* mutant was the most affected since it was

both nonmotile and deficient in sporulation. These results pointed out the involvement of *B. thuringiensis* ClpP1 in salt tolerance, of ClpP2 in both sporulation and motility, and of ClpC in sporulation. Our results also emphasize the functional diversity of the ClpP and ClpC proteins among prokaryotic organisms since the phenotypical features of the mutants vary strongly among bacteria.

Clp proteases have been shown to play a major role in the virulence of many bacterial pathogens including *L. monocytogenes*, *Y. enterocolitica*, and *Salmonella enterica* serovar Typhimurium (15, 50, 55, 68). We therefore assessed the pathogenicity of  $\Delta$ *clpC*,  $\Delta$ *clpP1*, and  $\Delta$ *clpP2* mutants against *B. mori* larvae and found that the virulence was markedly decreased for *clpP1*-deficient bacteria. The in vitro growth measurement data and the in vivo infection experiments performed at 37°C demonstrated that the avirulent phenotype of the *clpP1* mutant strain was due to a lower growth rate at temperatures less than 30°C. The importance of ClpP1 in tolerance of low temperature might reflect a possible mechanism allowing the bacterium to adapt to the physical conditions encountered in the insect host since invertebrates are not able to regulate their internal temperature. Furthermore, the loss of ClpP1 impaired cell separation after septum formation during the exponential growth phase at 25°C, indicating an involvement of ClpP1 in normal cell division at low temperatures. The characteristics of the *B. thuringiensis*  $\Delta$ *clpP1* mutant with regard to the effect of exposure to low temperature are reminiscent of the ClpP1<sup>-</sup> and ClpB<sup>-</sup> phenotypes in *Synechococcus* sp. strain PCC7942. In this organism, ClpP1 and ClpB were shown to play critical roles during acclimation to low temperature (52, 53). Indeed, cyanobacterial *clpP1* and *clpB* mutant cells were arrested during cell division at 25°C and lost viability after prolonged cold treatment. In addition, as for the *B. thuringiensis*  $\Delta$ *clpP1* mutant, the loss of ClpB in *Synechococcus* sp. strain PCC7942 led to altered bacterial division, with the cells remaining attached after septum formation. On the other hand, the inactivation of cyanobacterial *clpP1* caused the formation of severely elongated cells, a morphological phenotype previously reported for the *B. subtilis* *clpP* mutant (6, 43).

The phenotypical changes resulting from the disruption of the *B. thuringiensis* *clpP1* gene prompted the investigation of *clpP1* expression at different growth temperatures. Analysis of *clpP1'*-lacZ expression showed that *clpP1* is expressed at both temperatures tested (25 and 37°C). This result suggests that the ClpP1 protein is present in the *B. thuringiensis* cells during vegetative growth at low temperature, in agreement with its requirement for growth. An increase in ClpP1 content of *Synechococcus* wild-type cultures shifted from 37 to 25°C has been reported (53). It has been suggested that one likely function for the cyanobacterial ClpP1 protein would be to maintain the correct extracellular protein environment through its proteolytic activity in the cold. Indeed, as for high temperature, the abrupt shift to low temperature is considered to be a form of thermal stress that causes extensive protein denaturation and subsequent aggregation, whose magnitudes depend on the range of the temperature shift (13). *B. thuringiensis* ClpP1 may play the same role as that suggested for *Synechococcus* sp. ClpP1.

The finding that *clpP2* was not expressed at 25°C suggests that the loss of ClpP1 in the  $\Delta$ *clpP1* mutant is not compensated



by ClpP2 at this temperature. This is consistent with the presence of ClpP1 being crucial for the cell's tolerance of low temperature.

A major finding of this work is that the two related ClpP proteins of *B. thuringiensis* (ClpP1 and ClpP2) control distinct cellular regulatory pathways. While ClpP1 seems to be essential for normal cell division at low temperature, ClpP2 is required for motility and sporulation but is unnecessary for low-temperature growth. The apparent functional difference between ClpP1 and ClpP2 might explain the significance of the duplication of *clpP* genes in *B. thuringiensis*.

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