The ClpXP Protease Is Responsible for the Degradation of the Epsilon Antidote to the Zeta Toxin of the Streptococcal pSM19035 Plasmid

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Most bacterial genomes contain different types of toxin-antitoxin (TA) systems. The \( \omega-e-\zeta \) proteinaceous type II TA cassette from the streptococcal pSM19035 plasmid is a member of the \( e/\zeta \) family, which is commonly found in multiresistance plasmids and chromosomes of various human pathogens. Regulation of type II TA systems relies on the proteolysis of antitoxin proteins. Under normal conditions, the Epsilon antidote neutralizes the Zeta toxin through the formation of a tight complex. In this study, we show, using both \textit{in vivo} and \textit{in vitro} analyses, that the ClpXP protease is responsible for Epsilon antitoxin degradation. Using \textit{in vitro} studies, we examined the stability of the plasmids with active or inactive \( \omega-e-\zeta \) TA cassettes in \textit{B. subtilis} mutants that were defective for different proteases. Using \textit{in vitro} assays, the degradation of purified His\(_{6}\)-Epsilon by the His\(_{6}\)-Lon\(_{Bt}\), ClpP\(_{Bt}\), and ClpX\(_{Bt}\) proteases from \textit{B. subtilis} was analyzed. Additionally, we showed that purified Zeta toxin protects the Epsilon protein from rapid ClpXP-catalyzed degradation.

Toxin-antitoxin (TA) systems are widely found in plasmids and chromosomes of many bacteria, despite the fact that they are not essential for normal cell growth. Because of their role in the maintenance of mobile genetic elements and the response to specific stresses, the presence of TA systems seems advantageous for cell survival in the natural environment (1). In type II TA systems, where both components are proteins, the toxin is neutralized by direct interaction with the cognate antitoxin. These systems rely on the different decay rates of the two proteins that are involved, and the toxins are often much more stable than the antitoxins. The genetic organization of TA operons ensures a higher transcription level of the antitoxin than the toxin genes, which results in the inactivation of the toxins. Regulation of type II TA systems, which relies on the proteolysis of antitoxin proteins, has been studied mainly in \textit{Escherichia coli}. It has been shown that, in many cases, the unfolded nature of the antitoxin protein or its unstructured C-terminal domain is the cause of its vulnerability to degradation by intracellular proteases. In most cases, the Lon protease, which represents a major class of ATP-dependent proteases, is involved in antitoxin degradation (2–10). There are also known TA systems that use the two-component protease ClpP, which cooperates with the ATPase-active chaperones ClpA or ClpX (11, 12). Moreover, degradation of the antitoxin can be carried out by more than one protease (12–15). In contrast to the \textit{E. coli} antitoxins, knowledge of the proteolysis of antitoxins from other bacterial species is scarce. Until now, only Donegan et al. (16) had described this process in Gram-positive bacteria. These authors demonstrated that the MazE3a, Axe1, and Axe2 antitoxins from the three known TA systems in \textit{Staphylococcus aureus} are rapidly degraded \textit{in vivo} by the ClpCP protease.

The \( e/\zeta \) TA family is commonly found on plasmids (17–20) and chromosomes of many human pathogens (21), including both Gram-positive and Gram-negative bacteria (22, 23). Information concerning this TA family has been gathered from many years of studies on the \( e/\zeta \) system from the pSM19035 plasmid, which was originally isolated from the clinical strain of \textit{Streptococcus pyogenes} (24) and was expanded by studies on the homologous PezAT system in \textit{Streptococcus pneumoniae} (25, 26). The \( \omega-e-\zeta \) cassette plays a major role in the stable inheritance of pSM19035 in \textit{Bacillus subtilis} cells and acts as a post-segregational killing system (27, 28). An unusual feature of this system, which is located on a plasmid, is the lack of transcriptional regulation by the free antitoxin or the antitoxin in complex with the toxin. Autorepression is ensured by a third regulatory component, the Omega protein, which is a global regulator of other functions connected with plasmid replication and copy number control (29). The \( \omega \) gene constitutes a transcriptional unit with the downstream \( e \) and \( \zeta \) genes, which are tightly regulated by the \( \omega \) promoter. Interestingly, the \( \omega \) gene also forms an atypical two-cistronic partition system together with the \( \delta \) gene (30).

The inactive \( e_2\zeta_2 \) complex forms a unique heterotetramer, with the two Epsilon proteins sandwiched between the Zeta proteins. The ClpXP protease is responsible for the degradation of the Epsilon antitoxin. Additionally, we showed that purified Zeta toxin protects the Epsilon protein from rapid ClpXP-catalyzed degradation.
monomers (31). Analysis of the two-hybrid interaction between the N-terminal part of Zeta and the N-terminal region of Epsilon showed that these regions are involved in the formation of the εζ complex (32). The estimated in vivo half-life of the Epsilon protein is ~18 min, whereas the half-life of Zeta is over 1 h (27).

To identify the protease(s) responsible for the degradation of the antidote Epsilon protein, lon and clpX deficient mutants of B. subtilis were constructed and were used together with the clpP, clpC, clpE, and codX mutants to test the maintenance of the shortened derivatives of the pSM19035 plasmid. The data indicate that the ClpXP protease is the enzyme involved in the degradation of the antidote Epsilon in growing B. subtilis cells. The His_{56}-Epsilon antidote toxin, the Zeta toxin, and the His_{56}-LonA_{BS}, ClpP_{BS}, and ClpX_{BS} proteases were purified and used in vitro degradation assays. Our in vitro proteolysis tests confirmed that ClpXP is the protease responsible for the degradation of the Epsilon antidote.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Media, and Growth Conditions**—All bacterial strains and plasmids used in this study are listed in Table 1. E. coli DH5α strain was used for plasmid construction. Bacteria were grown in Luria-Bertani (LB or LBA) or 2YT (2× yeast extract and Tryptone) (41) and in SMM (Spizizen minimal) medium (42) supplemented with the appropriate antibiotics at the following concentrations (μg ml⁻¹): ampicillin, 100; spectinomycin, 60 or 100; erythromycin, 5; chloramphenicol, 30 for E. coli and 5 for B. subtilis; neomycin, 5; kanamycin, 50; and tetracycline, 10.

**Construction of B. subtilis lon and clpX Mutants**—To delete the chromosomal lon gene, the corresponding DNA fragment was generated by PCR and was cloned into the pT257R/T vector from the InstI/Aclone™ PCR product cloning kit. The oligonucleotides 5'-TGGTTCTACTAAAGTCACGG-3' and 5'-GGTTACCTGTAATTTCTCCTTCC-3' digested pET28a(+) and the YB886 chromosomal DNA were used to amplify the lonA sequence. The EcoO109I/MunI internal fragment of the lonA sequence was then replaced with the SspI/PvuII DNA fragment of the pHPI3 vector that encodes the cat gene. To construct the chromosomal deletion of the clpX gene, the corresponding DNA fragment was generated by PCR using the oligonucleotides 5’-GGAATTCATGGTTACACATCA-3’ and 5’-AGGTTTTGTGCCTTATC-3’ and YB886 chromosomal DNA were used to amplify the lonA sequence. The EcoO109I/MunI internal fragment of the lonA sequence was then replaced with the SspI/PvuII DNA fragment of the pHPI3 vector that encodes the cat gene. The pT257R/TlonA::cat and pUC18clpX::cat plasmids were linearized at the unique Scal restriction sites and used to transform YB886. Chloramphenicol-resistant integrants (in lonA or clpX sequences) were verified by restriction analysis of the PCR products that were generated from their chromosomal DNA using oligonucleotides corresponding to the lonA or clpX sequences, respectively.

**DNA Manipulations**—Routine DNA recombinant techniques were performed as described by Sambrook et al. (41). Restriction enzymes and other enzymes were used according to the supplier’s instructions. B. subtilis chromosomal DNA was isolated as described by Burdett et al. (43). E. coli transformation was performed using the standard calcium chloride method or by electrotransformation with a Gene-Pulser (Bio-Rad) according to the protocol described by Sambrook et al. (41). Electrotransformation of B. subtilis and protoplasts with plasmid DNA was performed as described by Bron (44), whereas the transformation of competent B. subtilis cells with chromosomal or ligated DNA was performed according to the protocol described by Rottländer and Trautner (45).

**Construction of Plasmids for Overproduction of Proteins**—The Feps (5'-GGCTGATCCATGGGCAAGATGTTAC-3') and Reps (5'-CCGGATCTCCTTCTCCTTTATTG-3') primers were used to amplify the epsilon gene from the pBT286 plasmid. The PCR product was digested with BamHI and was ligated into the BamHI site of the pET28a(+) plasmid (Novagen) for His_{56}-Epsilon overproduction. The proper orientation of the inserted fragment was verified by PCR with the F Capitol universal primer and the Reps primer. The Feps (5'-GGTTGTTGCTCTTCCGCAAATACCTGGAAGTTTACACATC-3') and Reps (5'-GGTTGTTGCTCTTCCGCAAATACCTGGAAGTTTACACATC-3') primers were used to amplify the zeta gene using the pBT286 plasmid as a template. The PCR product was cloned into the Ndel and SapI sites of the pTXB1 vector (New England Biolabs), creating the pTXB1zeta plasmid for the overproduction of Zeta toxin. The spx gene was amplified using the Fspx (5'-GGCTGATCCATGGGCAAGATGTTAC-3') and Rspx (5'-GGCTGATCCATGGGCAAGATGTTAC-3') primers and YB886 chromosomal DNA. The PCR product was digested with EcoRI and SalI and was cloned into a similarly digested pET28a(+) plasmid. As a result, a plasmid overproducing the His_{56}-Spx protein was obtained. The full-length lonA gene from B. subtilis YB886 was amplified using the Fon (5’-GGCAATTCTAGGCTATAGCTACTA-3’) and Ron (5’-GGCTGATCCATGGGCAAGATGTTAC-3’) primers and YB886 chromosomal DNA. The PCR product was digested with EcoRI and SalI and was cloned into a similarly digested pET28a(+) plasmid. Expression was induced by the addition of 0.6 mM isopropyl-β-D-thiogalactopyranoside to the cultures at an optical density of 0.3.

**Protein Purification**—All proteins were overproduced in E. coli BL21 (DE3) cells. The His_{56}-Epsilon, His_{56}-Spx, and His_{56}-LonA_{BS} N-terminally hexahistidine-tagged proteins were purified using Ni-TED columns (Macherey-Nagel), and the same purification procedure was used for these proteins. Overnight cultures were diluted 1:100 in 150 ml of LB supplemented with kanamycin and were incubated at 37 °C with shaking. Protein expression was induced by the addition of 0.6 mM isopropyl-β-D-thiogalactopyranoside to the cultures at an A_{600} of ~0.3,
and the cultures were incubated for 3 h at 28 °C. The bacteria were then harvested and stored at −20 °C until use. Frozen cells were resuspended in 3 ml of phosphate-buffered saline (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 140 mM NaCl, 2.7 mM KCl, pH 7.4) with 1 mg ml⁻¹ lysozyme and were incubated on ice for 30 min, followed by lysis by sonication. All subsequent purification steps were performed under native conditions following the manufacturer’s instructions. Purified proteins were dialyzed against the storage buffer (16.18 mM Na₂HPO₄, 16.18 mM KH₂PO₄, 0.1 mM EDTA, 10% glycerol). After dialysis, the His₆-Epsilon and His₆-Spx protein samples were centrifuged at 4 °C at 7,000 × g for 3 min to remove the insoluble fraction. Purified proteins were stored in small aliquots at −80 °C.

The IMPACT affinity tag system (New England Biolabs), which is a self-cleaveable system, was used to purify the ClpX₆₅ and ClpP₆₅ proteins (46, 47), as well as the Zeta toxin (this work). ClpX₆₅ was further purified from a Hitrap Q HP column (GE Healthcare) by elution with a 100–600 mM KCl gradient. The fraction containing pure ClpX₆₅ was concentrated using the Amicon filter device (Millipore). Purified ClpX₆₅ and ClpP₆₅ proteins, which contained no extra residues, were dialyzed against the storage buffer (25 mM Tris, pH 8, 100 mM KCl, 5 mM DTT, 1 mM MgCl₂, 50% glycerol) and were stored in aliquots at −20 °C. To purify the Zeta protein, BL21 (DE3) cells harboring the pACE1 plasmid, which carries the epsilon gene, were transformed with the pTXB1zeta plasmid. Overnight cultures were diluted 1:100 in 800 ml of LB supplemented with ampicillin and tetracycline and were incubated at 37 °C with shaking until an O.D.₆₆₀ of 0.5–0.6. After centrifugation at 14,000 × g for 30 min, the supernatants were centrifuged at 15,000 × g for 30 min, the clear supernatant was incubated with 5 ml of chitin resin for 1 h with gentle shaking. Then the chitin column was washed with 150 ml of buffer A (50 mM Tris-HCl, pH 7.5, 10% glycerol) and were lysed by sonication. After centrifugation at 15,000 × g for 30 min, the clear supernatant was incubated with 5 ml of chitin resin for 1 h with gentle shaking. Then the chitin column was washed with 150 ml of buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol). Zeta protein was released from the chitin beads by overnight incubation with 10 ml of cleavage buffer containing thiol (buffer B with 50 mM DTT). The eluted Zeta protein, with no extra residues, was concentrated using an Amicon filter device (Millipore), dialyzed against the storage buffer (50 mM Tris-HCl, pH 7.5, 50% glycerol), and stored at −20 °C.

The purities of all the proteins were verified by SDS-PAGE with Coomassie Blue staining. Protein concentrations were determined by comparing the intensity of the protein bands with that of the bovine serum albumin standard using Multi Gauge V3.0 software (FujiFilm).

**Plasmid Stability Assay**—The apparent plasmid stability was determined as previously described (28). *B. subtilis* cells containing the tested plasmid were grown overnight at 30 °C in liquid SMM medium supplemented with the appropriate antibiotics, including erythromycin and the proper chromosomal markers. The plasmid stability tests for the ΔclpP mutant were performed essentially in the same way, except that 2YT medium was used, and the growth cycle was 12 h to avoid the stationary phase of growth.

**Preparation of Cell Extracts**—Cell extracts of B. subtilis YB886 and the B. subtilis protease mutants (ΔclpP, ΔclpX, ΔclpC, ΔclpE, and ΔlonA) were prepared from 5-ml cultures of A₆₆₀ of 0.5–0.6. After centrifugation at 14,000 × g for 1 min, the cells were resuspended in 43 μl of PBS and were lysed by sonication. The insoluble material was removed by centrifugation at 5,000 × g for 5 min at 4 °C. The supernatants were collected into new Eppendorf tubes and were kept on ice.

**His₆-Epsilon Stability in Cell Extracts of Mutant Strains**—Five micrograms of His₆-Epsilon protein was mixed with 3.5 μl of cell extracts in a final reaction volume of 15 μl and was incubated for 1 h at 37 °C. The samples were boiled at 99 °C for 5 min in 4× Laemmli buffer and were analyzed by SDS-PAGE, followed by Coomassie Blue staining.

**In Vitro Proteolysis Reactions**—For catalysis by LonA₆₅, standard proteolysis reactions of 25 μl contained 0.5 μg of casein or various amounts of His₆-Epsilon protein (0.115, 0.27, or 0.4 μg) as a substrate and 0.5 μg of His₆-LonA₆₅ protease in the reaction buffer (48) supplemented with an ATP-regenerating system (5 mM creatine phosphate, 0.05 mM MgCl₂, 50% glycerol) and were stored in aliquots at −80 °C. After induction of zeta protein expression using Multi Gauge V3.0 software (FujiFilm). The intensity of the Epsilon protein in the 0-min reaction was set to 100%.

**In Vitro Degradation of His₆-Epsilon**—For catalysis by ClpXP₆₅, in vitro degradation of the His₆-Epsilon protein by purified ClpXP₆₅ was performed in a reaction volume of 60 μl containing 2.56 μg of ClpX₆₅ and 1.92 μg of ClpP₆₅ preincubated in degradation buffer (49) for 7 min at 30 °C. Following the addition of 2.4 μg of His₆-Spx, the reaction was carried out at 30 °C. At the indicated time intervals, 15 μl of the reaction mixture was collected, treated with 4× Laemmli buffer, and heated for 5 min at 99 °C.

**Mass Spectrometry**—Peptides resulting from Epsilon protein digestion with protease ClpXP were analyzed by LC-MS/MS/MS (liquid chromatography coupled to tandem mass spectrometry) using Nano-Acquity (Waters) LC system and Orbitrap Velos Pro mass spectrometer (Thermo Electron Corp., San Jose, CA). Prior to the analysis, peptides were diluted with 100 mM DTT (for 30 min at 36 °C) and alkylated with 0.5 mM iodoacetamide (45 min in a dark room at room temperature). Peptide mixture was applied to RP-18 precolumn...
Involvement of the ClpP Protease in ClpP Addiction Activity—

The presence of the \( \omega-\xi \) addiction gene cassette efficiently stabilizes plasmids in \( B. subtilis \) via postsegregational killing of the cells that have not received a copy of the plasmid (28). To determine the protease(s) involved in the degradation of the Epsilon anti- 
dote of the pSM19035 plasmid TA system, the stabilities of the pBT233-1 (containing an intact \( \omega-\xi \) operon) and pBT233-1S (containing a nonfunctional Zeta toxin) plasmids were tested in \( B. subtilis \) mutants defective in different proteases. The pBT233-1 plasmid is a deletion derivative of the pSM19035 plasmid and contains a replication operon and the \( \omega-\xi \) operon, together with the erythromycin resistance determinant (39). It behaves in a manner that is similar to the parental plasmid, except for an increased number of copies, which can reach up to 15 copies per cell (40). The pBT233-1S version differs from pBT233-1 only in the zeta gene sequence; a stop codon was created in the by pBT233-1S plasmid by filling in the Spel restriction site (40). The resulting plasmid is unstable because of the nonfunctionality of the Zeta protein, which is truncated after 79 amino acids. The \( B. subtilis \) mutant strains used in this study were separately transformed with the pBT233-1 and pBT233-1S plasmids that contain the erythromycin resistance that is conferred by derivatives of the pSM19035 plasmid. Because the \( B. subtilis \Delta clpP \) mutant strain Q4916 is deficient in DNA uptake (34), to obtain the proper strains, the plasmids containing YB886\textsubscript{pBT233-1} and YB886\textsubscript{pBT233-1S} were transformed with chromosomal DNA from Q4916 (Table 1). All resultant strains were tested for plasmid stability as described under “Experimental Procedures.” The wild-type YB886 strain carrying the pBT233-1 or pBT233-1S plasmid was used as a positive and negative control, respectively. To maintain the logarithmic phase of bacterial growth, the strains were grown in minimal SMM medium at a low temperature. Because the \( \Delta clpP \) mutation confers pleiotropic effects on the physiology of \( B. subtilis \) cells and prevents growth in minimal media by surviving in stationary phase (34), the stability tests for this mutant were performed using the rich 2YT medium with short periods between the subsequent culture dilutions. A comparison of the generational plasmid retention of the different strains is presented in Fig. 1A and shows that the \( B. subtilis \) lon
Epsilon Antitoxin Degradation by ClpXP Protease

FIGURE 1. Plasmid maintenance in the B. subtilis mutant strains. A, plasmid maintenance in the protease-deficient strains. The curves represent plasmid retention during growth without selection pressure. Continuous lines with filled symbols show the pBT233-1 plasmid, and the dashed lines with open symbols represent the pBT233-15 plasmid. Triangles, YB886 (wt); circles, QB4916 (clpP); squares, YBL01 (lonA). B, plasmid maintenance in the ATPase-deficient strains. The curves represent plasmid retention during growth without selection pressure. Lines with circles show the data for the clpX mutant YBX01 carrying the pBT233-1 (●) or the pBT233-15 (○) plasmid. The thick continuous lines represent the QB4756 (clpC), QB8023 (clpE), and PS28 (codX) deletion strains carrying the pBT233-1 plasmid, and the dashed lines with open symbols represent the maintenance of the pBT233-15 plasmid in QB4756 (△), QB8023 (□), and PS28 (▲).

mutants with both plasmids behave similar to the wild-type isogenic strain; the pBT233-1 plasmid was stably maintained for up to 100 generations, whereas the pBT233-1S plasmid was lost very quickly from the dividing bacterial cells. In contrast, the loss rate of both plasmids was essentially the same in the ΔclpP mutant, proving that the absence of the ClpP protease prevented Zeta toxin activity. These results indicate that ClpP, and not Lon, is involved in the functioning of the ω-ε-ζ addiction system.

The ClpX ATPase Is Required for Epsilon Antitoxin Degradation—ClpP is an ATP-dependent protease. In contrast to the Lon protease, the proteases of the Clp family form complex processes (51). Moreover, there is no competition between the different ATPases for the proteolytic ClpP subunits in B. subtilis, either under standard or stressed growth conditions (52). To define the ATPase subunit involved in the degradation of the Epsilon antitoxin, different B. subtilis mutants defective in one of these proteins were examined for the stability of the plasmid bearing the ω-ε-ζ operon. The strains deficient in ATPases, clpY (PS28), clpC (QB4756), clpE (QB8023), and clpX (YBX01), were transformed with the pBT233-1 or pBT233-1S plasmids, and their stable maintenance under nonselective growth conditions was tested. Taking into account the requirement of clpC and clpX for growth at high temperatures (36, 53), the stability tests for all of the ATPase mutants were performed at 30 °C. The collective data for all of the ATPase mutants are presented in Fig. 1B. The stability of the pBT233-1 plasmid was affected only in the clpX defective strain, showing a loss rate similar to that of pBT233-1S, which does not produce an active Zeta toxin. These results indicate that ClpX is the ATPase subunit that cooperates with the catalytic ClpP protease to degrade Epsilon.

Epsilon Stability in Cell Extracts of the Mutant Strains—The stability of the purified His6-Epsilon was assayed after incubation for 1 h with lysates obtained from the B. subtilis protease-deficient strains, including ΔclpP, ΔlonA, and the ATPase-deficient strains ΔclpX, ΔclpC, and ΔclpE. Lysate from the wild-type B. subtilis YB886 cells was used as a control for this experiment. Fig. 2 shows that the Epsilon protein was more stable only after incubation with lysate from the ClpP protease-deficient strain. This suggests the involvement of ClpP in antitoxin proteolysis. Incubation of Epsilon with lysates from the ClpX, ClpC, and ClpE mutant strains resulted in the proteolysis of the antitoxin (Fig. 2). The degradation patterns of these lysates were similar to that observed with the wild-type strain lysate. The results presented in Fig. 2 show that the LonA protease is most likely not responsible for antitoxin proteolysis because the ΔlonA lysate resulted in the degradation of Epsilon at a rate similar to that of the wild-type strain.

Rapid in Vitro Degradation of Epsilon by ClpXP B. subtilis—Our in vivo experiments strongly indicated that the ClpXP B. subtilis protease is involved in Epsilon degradation. To perform in vitro degradation analysis, we purified the B. subtilis protease: ClpP Bs, ClpX Bs, and LonA Bs. We included the LonA Bs protease in the in vitro analysis because the genetic results obtained by Liyo et al. (54) suggested that the Epsilon antitoxin was not specifically degraded in the B. subtilis null lonA mutant strain and, to a minor extent, in null clpX cells. The proteolytic activity of the purified proteases was verified in reactions with specific substrates. Spx protein, which is a global transcription regulator in B. subtilis, was used in the control reaction with the ClpXP Bs protease, because its in vitro proteolysis by ClpXP Bs has previously been demonstrated (49). Purified ClpXP Bs protease exhibited enzymatic activity against purified His6-Spx protein in the in vitro degradation assays. In contrast, incubation of His6-Spx in reaction buffer without protease did not affect Spx stability (Fig. 3E).

Stability of the purified His6-Epsilon protein was verified following its incubation for 1 h at 4, 20, 30, and 37 °C. As demon-
ClpXP shown). We verified that Zeta did not affect Spx degradation by absence of additional proteins. The His6-Epsilon protein in the presence of the ClpXP results were obtained with the Epsilon protein lacking the His6 tag (data not shown). Epsilon degradation did not occur in the presence of either ClpP or ClpX alone (Fig. 3A). Therefore, the active ClpXP complex, which is composed of the protease and the chaperone subunits, is required for degradation to occur. Under normal growth conditions, Epsilon forms a tight tetrameric complex with Zeta, thus inhibiting its toxicity. We tested whether ClpXP protease has preferable digestion sites, reaction sample after 5 min of incubation was directly measured by LC/MS. Database search identified protein Epsilon with high identity reaching 94% protein coverage. Mass spectrometry analysis revealed that protease intensively digests the protein, either without amino acid or sequence position preference, generating overlapping peptides and peptides often longer by one amino acid (Fig. 4).

Despite changing the reaction conditions, including different buffer compositions or protein concentrations, no degradation of His6-Epsilon occurred in the presence of the LonA_Bs protease. At the same time, casein, which is a known substrate of the Lon protease, was degraded by 50% by the His6-LonA_Bs protease after 2 h at 32 °C, proving that an active protease was used in the in vitro assays (Fig. 5).

**DISCUSSION**

The function of type II TA systems is regulated by the proteolysis of antitoxin proteins (55). Antitoxin vulnerability to degradation by cellular proteases constitutes the molecular basis of TA systems (56). Antitoxin proteolysis has been examined in many E. coli TA systems, generally only using in vivo analysis. In the cases of the RelB (57), CcdA (3), HipB (9), and Kis (58) antitoxins, this process has been further elucidated using in vitro degradation assays. Still, little is known in this field for bacteria other than E. coli. Although the ω-ε-ζ cassette from pSM19035 is one of the best described TA systems in Gram-negative bacteria (28, 59), the specific protease responsible for Epsilon antitoxin degradation has thus far been unknown. As
FIGURE 4. LC/MS analysis of protein Epsilon after ClpXP protease degradation. The table represents a list of identified peptides and respective Mascot scores. For clarity, duplicate peptides were removed. The same data are presented as percentages of peptides with certain C-terminal amino acid. Protease preferably hydrolyzes peptide bond following leucine and asparagine residues. This tendency, however, is not very specific, because other sites are also cleaved.

| No | Sequence     | Mass     | Mascot Score | Start - End |
|----|--------------|----------|--------------|-------------|
| 1  | EKTFEIEIN    | 1234,6445 | 57           | 6 - 15      |
| 2  | ELASASVYNRLN | 1363,7095 | 75           | 16 - 27     |
| 3  | ELASASVYNRLNY | 1526,7729 | 68           | 16 - 28     |
| 4  | LSASVYNRLN  | 1234,667  | 43           | 17 - 27     |
| 5  | SASVYNRLN   | 1121,5829 | 58           | 18 - 27     |
| 6  | SASVYNRLNY  | 1284,6462 | 51           | 18 - 28     |
| 7  | SVYNRLN     | 963,5138  | 42           | 20 - 27     |
| 8  | SVYNRLNY    | 1126,5771 | 52           | 20 - 28     |
| 9  | VYNRLN      | 876,4817  | 40           | 21 - 27     |
| 10 | RVLYVL      | 875,5229  | 44           | 24 - 30     |
| 11 | RVLYVNL     | 989,5658  | 44           | 24 - 31     |
| 12 | RVLYVNLH    | 1126,6247 | 41           | 24 - 32     |
| 13 | YVNLHKNKNS  | 1444,6946 | 36           | 28 - 39     |
| 14 | YVNLHKNKNSQLELEVN | 2141,0753 | 103        | 28 - 45     |
| 15 | YVNLHKNKNSQLELEVN | 2254,1593 | 141        | 28 - 46     |
| 16 | VLNHELN     | 837,4344  | 33           | 29 - 35     |
| 17 | VLNHELKN    | 1079,5723 | 56           | 29 - 37     |
| 18 | VLNHELKNKNSQLELEVN | 1978,0119 | 116       | 29 - 45     |
| 19 | VLNHELKNKNSQLELEVN | 2091,096  | 126        | 29 - 46     |
| 20 | VLNHELKNKNSQLELEVN | 2318,223  | 36           | 29 - 48     |
| 21 | NHENLNK     | 867,4198  | 47           | 31 - 37     |
| 22 | NHENLNKNSQLELN | 1310,6215 | 37         | 31 - 41     |
| 23 | NHENLNKNSQLELN | 1765,8595 | 123       | 31 - 45     |
| 24 | NHENLNKNSQLELN | 1878,9435 | 137       | 31 - 46     |
| 25 | HELNKNSQLELN | 1651,8165 | 59          | 32 - 45     |
| 26 | HELNKNSQLELN | 1764,9006 | 100        | 32 - 46     |
| 27 | ENKNSQLELN  | 1627,8417 | 39          | 33 - 46     |
| 28 | NKDSQLELN   | 1272,631  | 53          | 35 - 45     |
| 29 | NKDSQLELN   | 1385,715  | 49          | 35 - 46     |
| 30 | KNSQQL      | 816,4341  | 48          | 36 - 42     |
| 31 | KNSQLELV    | 1044,4545 | 35          | 36 - 44     |
| 32 | KNSQLELVN   | 1158,588  | 64          | 36 - 45     |
| 33 | KNSQLELVN   | 1271,6721 | 79          | 36 - 46     |
| 34 | KNSQLELVNLLN | 1498,7991 | 44          | 36 - 48     |
| 35 | DSQOLEVN    | 916,4502  | 32          | 38 - 45     |
| 36 | DSQOLEVN    | 1029,5342 | 55          | 38 - 46     |
| 37 | QLEVLNLN    | 1054,6022 | 31          | 40 - 48     |
| 38 | EVNLLNQLKL  | 1182,6972 | 46          | 43 - 52     |

No | Sequence     | Mass     | Mascot Score | Start - End |
|----|--------------|----------|--------------|-------------|
| 39 | NLLNQLKL     | 954,5862 | 36           | 45 - 52     |
| 40 | LLLNQLKL     | 840,5433 | 53           | 46 - 52     |
| 41 | LNQLKL       | 727,4592 | 32           | 47 - 52     |
| 42 | AKRVNLF      | 846,5076 | 35           | 53 - 59     |
| 43 | AKRVNLFD     | 961,5345 | 42           | 53 - 60     |
| 44 | AKRVNLFDY    | 1124,5978 | 57        | 53 - 61     |
| 45 | AKRVNLFDYSL  | 1324,7139 | 41        | 53 - 63     |
| 46 | AKRVNLFDYSL  | 1894,9788 | 78        | 53 - 68     |
| 47 | LFDYSLEELQA  | 1326,6343 | 35        | 58 - 68     |
| 48 | LFDYSLEELQA  | 1691,8042 | 108       | 58 - 71     |
| 49 | FDYSLEELQA   | 1213,5503 | 34        | 59 - 68     |
| 50 | FDYSLEELQA   | 1578,7202 | 118       | 59 - 71     |
| 51 | DYSLEELQAVHE | 1431,6518 | 58        | 60 - 71     |
| 52 | YSLEELQAVHE  | 1316,6248 | 83        | 61 - 71     |
| 53 | SLEELQA      | 788,3916  | 43          | 62 - 68     |
| 54 | SLEELQAVHE   | 1153,5615 | 61          | 62 - 71     |
| 55 | SLEELQAVHEYWRSMN | 1990,9207 | 91          | 62 - 77     |
| 56 | EEQLAQAVHE   | 953,4454  | 48          | 64 - 71     |
| 57 | ELQAVHE      | 824,4028  | 35          | 65 - 71     |
| 58 | VEYWRSMN     | 1220,5397 | 42          | 69 - 77     |
| 59 | RYSKQVLN     | 1006,556  | 44          | 78 - 85     |
| 60 | VLNKEKVA     | 899,544   | 45          | 83 - 90     |
expected for the antitoxin proteins, Epsilon has a shorter in vivo half-life (18 min) than the Zeta toxin (60 min) (27). In vitro analysis showed the higher stability against urea-induced unfolding and the higher resistance against unspliced proteolytic degradation of the Epsilon antitoxin when compared with the Zeta toxin. Moreover, the Epsilon protein, unlike many other antitoxin proteins, does not have an unfolded structure that results in physiological instability but is instead folded into a three-helix bundle (31).

The data presented in this paper demonstrate that Epsilon is a substrate of the ClpXP protease, which was confirmed both in vivo and in vitro. In the B. subtilis ΔclpP mutant, an active ω-ε-ζ cassette did not stabilize the pBT233-1 plasmid, which was lost at a similar rate as the plasmid containing an inactive cassette. Both of these plasmids were no longer present in the tested cells after ~60 generations. This indicates that the ΔclpP mutant did not contain the protease necessary for the proper addiction function of the examined TA cassette. Lack of ClpP most likely resulted in a higher stability of the Epsilon protein; therefore, the toxin was effectively neutralized, even when the plasmid was lost. ClpP is a two-component protease, which requires an ATPase-active chaperone responsible for substrate recognition for proper activity, unfolding, and translocation into the proteolytic chamber. In Gram-positive bacteria, the chaperones ClpX, ClpC, ClpE, and ClpY interact with the ClpP protease core. In a previous paper, the ClpC chaperone was shown to be essential for the ClpP-mediated degradation of the MazEα, Axe1, and Axe2 antitoxins in S. aureus, a Gram-positive human pathogen (16). Increased stability of these antitoxins was observed only in S. aureus strains that were deficient for clpC or clpP. Until now, this was the first and only publication concerning the degradation of antitoxin in Gram-positive bacteria.

We found that the ClpX chaperone, together with ClpP, contributes to the degradation of the streptococcal antitoxin Epsilon. In contrast with the clpX mutant, the pBT233-1 plasmid was stably maintained in the ClpE, ClpC, and ClpY deficient strains, suggesting that these chaperones are dispensable for Epsilon proteolysis. The lack of good quality anti-Epsilon antibodies precluded the verification of Epsilon stability in the protease mutant strains by Western blot analysis. Therefore, we attempted to indirectly verify the antitoxin stability by incubating purified His6-Epsilon with a total pool of proteins obtained from protease-deficient strains. Although a higher stability of the Epsilon protein was observed following incubation with a lysate lacking the ClpP protease, Epsilon proteolysis was observed in all of the tested extracts probably because of nonspecific degradation. Therefore, this analysis did not result in the identification of the chaperone that cooperates with ClpP during Epsilon degradation.

In vitro proteolysis assays confirmed our in vivo findings that the ClpXP protease was involved in Epsilon degradation. A drastic, rapid reduction in the antitoxin level was observed; the major degradation of Epsilon occurred in less than 15 min. We estimated that Epsilon is degraded by ClpXP with a $T_{1/2}$ of ~8 min in vitro, which is similar to the degradation of Kis antitoxin by the ClpAP protease in in vitro proteolysis assays (58).

In a TA complex, the toxin remains inactive because of its direct interaction with the antidote. The stability of the antitoxin protein increases because of structural changes that bring the disordered structure into order upon toxin binding (11, 60). When the unfolded domain does not adopt an ordered structure, the toxin blocks the access of the protease to the antitoxin by partially shielding the C-terminal fragment of the antitoxin from the solvent (61). Although the Epsilon antitoxin does not have a disordered structure or unfolded C-terminal domain, its rapid degradation was observed in the presence of the ClpXP protease. As expected, the presence of the Zeta toxin in the in vitro degradation assay stabilized Epsilon, most likely as a result of complex formation between these two proteins. The conditions used in our in vitro experiments enabled the formation of stable $\epsilon_6\zeta_2$ complex, because it was previously shown (27) that the $\epsilon_6\zeta_2$ complex is stable between pH 5 and 9 in high salt (2 M). However, it is possible that some $\epsilon$ and $\zeta$ molecules remained unbound in our in vitro assay. Therefore, the decrease in Epsilon quantity observed during the first 10 min most likely reflects the presence of free antitoxin molecules. In heterotetramer $\epsilon_6\zeta_2$, in which two molecules of the Epsilon protein are surrounded by two monomers of the Zeta protein, Zeta protects Epsilon from the ClpXP protease.

Lioy et al. (54) reported that LonA is the putative protease responsible for the degradation of the Epsilon antidote, but the presented data were inconsistent and unconvincing. This was inferred from the plating efficiency of the B. subtilis ΔclpC, ΔclpX, ΔclpE, ΔclpP, or ΔlonA cells that contained plasmids expressing the ω-ε-ζ genes upon exposure to 50 μg ml$^{-1}$ rifampicin because no reduction in the plating efficiency was observed for the ΔlonA cells. The plating efficiency of the ΔclpX showed an intermediate phenotype, which in our opinion is inexplicable because of the lack of any effect in the ΔclpP strain. Furthermore, the known pleiotropic effects of ΔclpP deletion on the B. subtilis phenotype (34) preclude the rational evaluation of the consequences of specific factors, such as Zeta toxin activity in this case. Although the authors mentioned that the level of Epsilon protein in the ΔlonA strain remained constant during the first 120 min, concrete data unfortunately were not shown. In our study, the involvement of the Lon protease in Epsilon degradation was excluded by three different methods. The results of the plasmid stability tests, incubation of the puri-

**Epsilon Antitoxin Degradation by ClpXP Protease**
Epsilon Antitoxin Degradation by ClpXP Protease

vided Epsilon protein with lysate depleted of Lon protease, and the in vitro degradation assay clearly showed that the LonA protease does not play any role in the regulation of the ω-ε-ζ TA system in B. subtilis.

It was previously shown that the efficiency of the ω-ε-ζ cassette differs in Gram-positive bacteria with a low DNA G/C content (40). The observed results did not depend on the plasmid copy number but were most likely connected to the host-dependent cellular factors. One reason for this could be a different proteolytic regulation of this TA system. It was demonstrated for the mazEF system that homologous antidotes were degraded by various proteases in the host strains (12, 16), which were also dependent on environmental conditions (13).

The hypothesis concerning the differences in the functioning of the TA systems in various bacteria and their proteolytic regulation could be verified by similar studies with protease mutant strains or in vitro degradation analyses. The gained knowledge might be applied to the development of new strategies for coping with pathogens containing TA systems.

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Epsilon Antitoxin Degradation by ClpXP Protease

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