The ClpXP protease is responsible for the degradation of the Epsilon antidote to the Zeta toxin of the streptococcal pSM19035 plasmid*

Iwona Brzozowska, Urszula Zielenkiewicz

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Pawińskíego 5A, 02-106 Warsaw, Poland

Running title: Epsilon antitoxin degradation by ClpXP protease

To whom correspondence should be addressed: Urszula Zielenkiewicz, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Pawińskíego 5A, 02-106 Warsaw, Poland, Tel.: (48) 225921307; Fax: (48) 226584636; E-mail: ulazet@ibb.waw.pl

Background: Epsilon is an antitoxin of the ε/ζ toxin-antitoxin (TA) system family, which is widespread in pathogenic bacteria.
Results: *In vitro* and *in vivo* degradation of the Epsilon protein is shown.
Conclusion: ClpXP protease is responsible for the degradation of the Epsilon antitoxin.
Significance: The first demonstration of *in vitro* degradation of the antitoxin from Gram-positive bacteria.

ABSTRACT

Most bacterial genomes contain different types of toxin–antitoxin (TA) systems. The ω-ε-ζ proteinaceous type II TA cassette from the streptococcal pSM19035 plasmid is a member of the ε/ζ 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 *in vivo* and *in vitro* analyses, that the ClpXP protease is responsible for Epsilon antitoxin degradation. Using *in vivo* studies, we examined the stability of the plasmids with active or inactive ω-ε-ζ TA cassettes in *B. subtilis* mutants that were defective for different proteases. Using *in vitro* assays, the degradation of purified His$_6$-Epsilon by the His$_6$-Lon$_{Bs}$, ClpP$_{Bs}$ and ClpX$_{Bs}$ proteases from *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. Due to 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 antidotes. The genetic organization of TA operons ensures a higher transcription level of the antidote 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 *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 antidote can be carried out by more than one protease (12–15). In contrast to the *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 MazE*sa*, Axe1, and Axe2 antitoxins from the three known TA systems in *Staphylococcus aureus* are rapidly degraded *in vivo* by the ClpCP protease.

The ε/ζ 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 ε/ζ system from the pSM19035 plasmid, which was originally isolated from the clinical strain of *Streptococcus pyogenes* (24) and was expanded by studies on the homologous PezAT system in *Streptococcus pneumoniae* (25, 26). The ω-ε-ζ cassette plays a major role in the stable inheritance of pSM19035 in *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 ω gene constitutes a transcriptional unit with the downstream ε and ζ genes, which are tightly regulated by the ω promoter. Interestingly, the ω gene also forms an atypical two-cistronic partition system together with the δ gene (30).

The inactive $\varepsilon_2\zeta_2$ complex forms a unique heterotetramer, with the two Epsilon proteins sandwiched between the Zeta 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 the formation of the $\varepsilon_2\zeta_2$ 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$_6$-Epsilon antitoxin, the Zeta toxin and the His$_6$-LonA$_{Bs}$, ClpP$_{Bs}$ and ClpX$_{Bs}$ proteases were purified and used in *in vitro* degradation assays. Our *in vitro* proteolysis tests confirmed that ClpXP is the protease responsible for the degradation of the Epsilon antitoxin.

EXPERIMENTAL PROCEDURES

Bacterial strains, media and growth conditions–All bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α strain was used for plasmid construction. Bacteria were grown in Luria-Bertani (LB or LBA) or 2YT media (41) and in minimal SMM media (42) supplemented with the appropriate antibiotics at the following concentrations (μg ml^{-1}): ampicillin (Ap), 100; spectinomycin (Spc), 60 or 100; erythromycin (Erm), 5; chloramphenicol (Cm), 30 for *E. coli* and 5 for *B. subtilis*; neomycin (Neo), 5; kanamycin (Km), 50; tetracycline (Tet), 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 pTZ57R/T vector from the InsT/Aclone^{TM} PCR Product Cloning Kit. The oligonucleotides 5'-TGGTTCATACTAAAGTCACGG-3' and 5'-GGTACTGTTCCGGTTTTACTGC-3' 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 pHP13 vector that encompasses the *cat* gene. To construct the chromosomal deletion of the *clpX* gene, the corresponding DNA fragment was generated by PCR using the oligonucleotides 5'-GAATGTGCAAGTCAGAAAC-3' and 5'-AGGTTTGTGCTTATC-3' and YB886 chromosomal DNA. This blunted fragment, which was obtained with the Pfu DNA polymerase, was cloned into the pUC18 vector lacking of the MCS sequence between the PvuII sites. The SauI/MunI (both ends blunted) internal fragment of the *clpX* sequence was replaced with the SspI/PvuII DNA fragment of the pHP13 vector that encompassed the *cat* gene. The

pTZ57R/T*lonA::cat* and pUC18*clpX::cat* plasmids were linearized at the unique ScaI 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 in 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 (BioRad) 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 F_{epsi} (5'-GCCGGATCCATGGCAGTTACGTATG-3') and R_{epsi} (5'-CTCGGATCCTTAAGCCACTTTCTCTTTATTCAA-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_6-Epsilon overproduction. The proper orientation of the inserted fragment was verified by PCR with the F_{T7} universal primer and the R_{epsi} primer. The F_{zeta} (5'-GGTGGTCATATGGCAAATATAGTCAATTTTAC-3') and R_{zeta} (5'-GGTGGTTGCTCTTCCGCAAATACCTGGAAGTTTAGG-3') primers were used to amplify the *zeta* gene using the pBT286 plasmid as a template. The PCR product was cloned into the NdeI and SapI sites of the pTXB1 vector (New England Biolabs), creating the pTXB1*zeta* plasmid for the overproduction of Zeta toxin. The *spx* gene was amplified using the F_{spx} (5'-GCGAATTCATGGTTACACTATACACATCA-3') and R_{spx} (5'-GCGTCGACTTAGTTTGCCAAACGCTGT-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_6-Spx protein was obtained. The full-length *lonA* gene from *B. subtilis* YB886 was amplified using the F_{lon} (5'-GCGAATTCATGGCAGAAGAATTAAAA-3') and R_{lon} (5'-GCGTCGACTCATTTCTTCTCTCCTAC-3') primers. The PCR product was cloned into the pET28a(+) plasmid at the EcoRI and SalI sites to create a plasmid for the overproduction of His_6-LonA$_{Bs}$. The *clpP* and *clpX* genes from *B. subtilis* YB886 were amplified using the F_{clpP} (5'-GGTGGTCATATGAATTTAATACCTACAGTC-3') and R_{clpP} (5'-GGTGGTTGCTCTTCCGCACTTTTTGTCTTCTGTGTG-3') or the F_{clpX} (5'-GGTGGTCATATGTTTAAATTTAACGAGGAA-3') and R_{clpX} (5'-GGTGGTTGCTCTTCCGCATGCAGATGTTTTATCTTG-3') primers, respectively. The PCR products were digested with NdeI and SapI and were ligated into the NdeI and SapI sites of pTXB1, creating the pTXB1*clpP$_{Bs}$* and pTXB1*clpX$_{Bs}$* plasmids for the overproduction of ClpP$_{Bs}$ or ClpX$_{Bs}$, respectively. Sequences of all cloned genes were verified by sequencing.

Proteins purification–All proteins were overproduced in *E. coli* BL21 (DE3) cells. The His_6-Epsilon, His_6-Spx and His_6- LonA$_{Bs}$, N-terminally hexa-histidine 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 Km and were incubated at 37°C with shaking. Protein expression was induced by the addition of 0.6 mM IPTG to the cultures at an OD_{660} 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 Na_2HPO_4, 1.8 mM KH_2PO_4, 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 Na_2HPO_4,

16.18 mM NaH_2PO_4, 0.1 mM EDTA, 10% glycerol). After dialysis, the His_6-Epsilon and His_6-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-cleavable system, was used to purify the $ClpX_{Bs}$ and $ClpP_{Bs}$ proteins (46, 47), as well as the Zeta toxin (this work). $ClpX_{Bs}$ was further purified from a HiTrap Q HP column (GE Healthcare) by elution with a 100-600 mM KCl gradient. The fraction containing pure $ClpX_{Bs}$ was concentrated using the Amicon filter device (Millipore). Purified $ClpX_{Bs}$ and $ClpP_{Bs}$, which contained no extra residues, were dialyzed against the storage buffer (25 mM Tris pH 8, 100 mM KCl, 5 mM DTT, 1 mM $MgCl_2$, 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 pTXB1*zeta* plasmid. Overnight cultures were diluted 1:100 in 800 ml of LB supplemented with Ap and Tet and were incubated at 37˚C with shaking until an OD_{660} of 0.4 was reached. After induction of *zeta* gene expression using 0.6 mM IPTG, the cells were incubated for an additional 3 h at 28˚C; then, the cells were pelleted by centrifugation and stored at -20˚C until use. All subsequent purification steps were carried out at 4˚C. The frozen cells were resuspended in 40 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 and 75 ml of buffer B (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 Δ*clpP* 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 (Δ*clpP*, Δ*clpX*, Δ*clpC*, Δ*clpE* and Δ*lonA*) were prepared from 5 ml cultures of OD_{660} 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.

His_6-Epsilon stability in cell extracts of mutant strains–Five micrograms of His_6-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 4x Laemmli buffer and were analyzed by SDS-PAGE, followed by Coomassie blue staining.

In vitro proteolysis reactions–Catalysis by $LonA_{Bs}$: Standard proteolysis reactions of 25 μl contained 0.5 μg of casein or various amounts of His_6-Epsilon protein (0.115 μg, 0.27 μg, or 0.4 μg) as a substrate and 0.5 μg of His_6-$LonA_{Bs}$ protease in the reaction buffer (48) supplemented with an ATP-regenerating system (5 mM creatine phosphate, 0.05 μg μl^{-1} creatine kinase; Roche). The reactions were carried out for 2 h at 32˚C and were terminated by the addition of 4x Laemmli buffer and incubation at 99˚C for 5 min. The proteins were separated by SDS-PAGE using 14% Tris-tricine gels and were stained with Coomassie blue.

Catalysis by $ClpXP_{Bs}$: *In vitro* degradation of the His_6-Spx protein by purified $ClpXP_{Bs}$ was performed in a reaction volume of 60 μl containing 2.56 μg of $ClpX_{Bs}$ and 1.92 μg of $ClpP_{Bs}$ pre-incubated in degradation buffer (49) for 7 min at 30˚C. Following the addition of 2.4 μg of His_6-Spx, the reaction was carried out at 30˚C. At the indicated time intervals, 15 μl of the

reaction mixture was collected, treated with 4x Laemmli buffer and heated for 5 min at 99˚C.

The *in vitro* degradation assay of purified His_6-Epsilon was carried out in a reaction volume of 105 µl containing degradation buffer (49); 4.5 µg of the $ClpX_{Bs}$ and 3.36 µg of the $ClpP_{Bs}$ proteases were pre-incubated in the buffer for 7 min at 30˚C. Then, 5 µg of His_6-Epsilon was added, and the reaction mixture was incubated at 30˚C. When indicated, His_6-Epsilon was pre-incubated with 5 µg of Zeta protein. At the indicated time intervals, 15 µl of the reaction mixture was collected, treated with 4x Laemmli buffer and heated for 5 min at 99˚C. The collected samples were analyzed using 14% Tris-tricine SDS-PAGE gels, followed by Coomassie blue staining. The levels of Epsilon protein were determined after proteolysis from three independent experiments using Multi Gauge V3.0 software (FujiFilm). The intensity of the Epsilon protein in the 0-min reaction was set to 100%.

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 reduced with 100 mM DTT (for 30 minutes at 56°C), alkylated with 0.5 M iodoacetamide (45 minutes in darkroom at room temperature). Peptide mixture was applied to RP-18 precolumn (nanoACQUITY Symmetry® C18 – Waters 186003514) using water containing 0,1% TFA as mobile phase and then transferred to nano-HPLC RP-18 column (nanoACQUITY BEH C18 - Waters 186003545) using an acetonitrile gradient (0% - 35% AcN in 160 minutes) in the presence of 0,1% formic acid with the flow rate of 300 nl/min. Column outlet was directly coupled to the ion source of the spectrometer working in the regime of data dependent MS to MS/MS switch. A blank run ensuring lack of cross contamination from previous samples preceded each analysis. Acquired raw data were processed by Mascot Distiller followed by Mascot Search (Matrix Science, London, UK, on-site license) against SwissPROT database (20110124). Search parameters for precursor and product ions mass tolerance were 20 ppm and 50 mmu, respectively, enzyme specificity: none; missed cleavage sites allowed: 0; variable modification of methionine oxidation and cysteine carbamidomethylation. Peptides with Mascot Score exceeding the threshold value corresponding to < 5% False Positive Rate, calculated by Mascot procedure, and with the Mascot score above 30 were considered to be positively identified. Mascot results were analyzed by MScan – in house software (50).

RESULTS

Involvement of the ClpP protease in ω-ε-ζ addiction activity–The presence of the ω-ε-ζ 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 antidote of the pSM19035 plasmid TA system, the stabilities of the pBT233-1 (containing an intact ω-ε-ζ operon) and pBT233-1S (containing a non-functional 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 ω-ε-ζ stability gene operon, together with the erythromycin resistance determinant (39). It behaves in 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 SpeI restriction site (40). The resulting plasmid is unstable due to the non-functionality 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* Δ*clpP* mutant QB4916 is deficient in DNA uptake (34), to obtain the proper strains, the plasmids containing $YB886_{pBT233-1}$ and $YB886_{pBT233-1S}$ were transformed with chromosomal DNA from QB4916 (Table 1). All resultant strains were tested for plasmid stability as described in the Methods section. 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. Comparison of the generational plasmid retention of the different strains is presented in Fig. 1A and shows that the *B. subtilis lon* 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 $\Delta clpP$ 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 antidote degradation–ClpP is an ATP-dependent protease. In contrast to the Lon protease, the proteases of the Clp family form complexes with different ATPases to promote protein degradation 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 antidote, 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 non-selective 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 30ºC. 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 His$_6$-Epsilon was assayed after incubation for 1 h with lysates obtained from the *B. subtilis* protease-deficient strains, including the $\Delta clpP$, $\Delta lonA$ and the ATPase deficient strains $\Delta clpX$, $\Delta clpC$ and $\Delta clpE$. Lysate from the wild-type *B. subtilis* YB886 cells was used as a control for this experiment. Figure 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 $\Delta lonA$ 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$_{Bs}$–Our *in vivo* experiments strongly indicated that the ClpXP$_{Bs}$ 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 Lioy 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, as its *in vitro* proteolysis by ClpXP$_{Bs}$ has previously been demonstrated (49). Purified ClpXP$_{Bs}$ protease exhibited enzymatic activity against purified His$_6$-Spx protein in the *in vitro* degradation assays. In contrast, incubation of His$_6$-Spx in reaction buffer without protease did not affect Spx stability (Fig. 3E). Stability of the purified His$_6$-Epsilon protein was verified following its incubation for 1 h at 4, 20, 30 and 37°C. As demonstrated in Fig. 3D, Epsilon degradation did not occur in the absence of additional proteins. The *in vitro* proteolysis assay of the His$_6$-Epsilon protein in the presence of the ClpXP$_{Bs}$ protease at 30°C clearly showed His$_6$-Epsilon degradation. His$_6$-Epsilon decay was very fast since; after 15 min, only 6.7% of the His$_6$-Epsilon protein remained intact (Fig.

3A). The same results were obtained with the Epsilon protein lacking the His$_6$ tag (data not shown). Epsilon degradation did not occur in the presence of either ClpP or ClpX alone (Fig. 3C). Therefore, the active ClpXP complex, which is composed of the protease and the chaperon 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$_{Bs}$ degrades the Zeta-bound Epsilon. As shown in Fig. 3B, the presence of equal amounts of purified Zeta protein significantly reduced the rate of Epsilon degradation. During the first 10 min, the gradual degradation of Epsilon was observed (up to ~20%), while further degradation of Epsilon proceeded very slowly. Even after prolonged incubation (up to 60 minutes), approximately 40% of the Epsilon protein remained (data not shown). We verified that Zeta did not affect Spx degradation by ClpXP$_{Bs}$ (data not shown). Therefore, the lack of rapid degradation of Epsilon in the presence of Zeta was a consequence of the TA complex formation. We did not observe any degradation of the Zeta protein by ClpXP$_{Bs}$ (data not shown).

To check, whether the ClpXP protease has preferable digestion sites, reaction sample after 5 minutes 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 His$_6$-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 His$_6$-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-positive bacteria (28, 59), the specific protease responsible for Epsilon antitoxin degradation has thus far been unknown. As 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 unspecific proteolytic degradation of the Epsilon antidote when compared to 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* $\Delta clpP$ 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 approximately 60 generations. This indicates that the $\Delta clpP$ 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 MazE*sa*, 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 His$_6$-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 due to non-specific 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 due to its direct interaction with the antidote. The stability of the antitoxin protein increases due to 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 partial shielding the C-terminal fragment of the antitoxin from the solvent (61). Although the Epsilon antidote 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 ε$_2$ζ$_2$ complex, as it was previously shown (27) that the ε$_2$ζ$_2$ complex is stable between pH 5 and 9 and in high salt (2 M).

However, it is possible that some ε and ζ 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 ε$_2$ζ$_2$, 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* Δ*clpC*, Δ*clpX*, Δ*clpE,* Δ*clpP* or Δ*lonA* 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 Δ*lonA* cells. The plating efficiency of the Δ*clpX* showed an intermediate phenotype, which in our opinion is inexplicable due to the lack of any effect in the Δ*clpP* strain. Furthermore, the known pleiotropic effects of Δ*clpP* 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 Δ*lonA* 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 purified 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.

REFERENCES

1. Yamaguchi, Y., Park, J.H., Inouye, M. (2011) Toxin–antitoxin systems in bacteria and archaea. *Annu. Rev. Genet.* **45,** 61–79.
2. Roberts, R.C., Strom, A.R., Helinski, D.R. (1994) The *parDE* operon of the broad-host-range plasmid RK2 specifies growth inhibition associated with plasmid loss. *J. Mol. Biol.* **237,** 35–51.
3. Van Melderen, L., Thi, M.H., Lecchi, P., Gottesman, S., Couturier, M., Maurizi, M.R. (1996) ATP-dependent degradation of CcdA by Lon protease. Effects of secondary structure and heterologous subunit interactions. *J. Biol. Chem.* **271,** 27730–27738.
4. Smith, A.S.G., Rawlings, D.E. (1998) Efficiency of the pTF-FC2 pas poison-antidote stability system in *Escherichia coli* is affected by the host strain, and antidote degradation requires the Lon protease. *J. Bacteriol.* **180,** 5458–5462.
5. Christensen, S.K., Mikkelsen, M., Pedersen, K., Gerdes, K. (2001) RelE, a global inhibitor of translation, is activated during nutritional stress. *Proc. Natl. Acad. Sci. USA* **98,** 14328–14333.
6. Christensen, S.K., Maenhaut-Michel, G., Mine, N., Gottesman, S., Gerdes, K., Van Melderen, L. (2004) Overproduction of the Lon protease triggers inhibition of translation in *Escherichia coli*: involvement of the *yefM-yoeB* toxin-antitoxin system. *Mol. Microbiol.* **51,** 1705–1717.
7. Jørgensen, M.G., Pandey, D.P., Jaskolska, M., Gerdes, K. (2009) HicA of *Escherichia coli* defines a novel family of translation-independent mRNA interferases in Bacteria and Archaea. *J. Bacteriol.* **191,** 1191–1199.
8. Wang, X., Kim, Y., Hoon Hong, S., Ma, Q., Brown, B.L., Pu, M., Tarone, A.M., Benedik, M.J., Peti, W., Page, R., Wood, T.K. (2011) Antitoxin MqsA helps mediate the bacterial general stress response. *Nat. Chem. Biol.* **7,** 359–366.
9. Hansen, S., Vulic, M., Min, J., Yen, T.J., Schumacher, M.A., Brennan, R.G., Lewis, K. (2012) Regulation of the *Escherichia coli* HipBA toxin-antitoxin system by proteolysis. *PLoS One* **7,** e39185.
10. Winther, K.S., Gerdes, K. (2012) Regulation of enteric *vapBC* transcription: induction by VapC toxin dimer-breaking. *Nucleic Acids Res.* **40,** 4347–4357.
11. Lehnherr, H., Yarmolinsky, M.B. (1995) Addiction protein Phd of plasmid prophage P1 is a substrate of the ClpXP serine protease of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **92,** 3274–3277.
12. Aizenman, E., Engelberg-Kulka, H., Glaser, G. (1996) An *Escherichia coli* chromosomal ''addiction module'' regulated by guanosine 3′, 5′- bispyrophosphate: a model for programmed bacterial cell death. *Proc. Natl. Acad. Sci. USA* **93,** 6059–6063.
13. Christensen, S.K., Pedersen, K., Hansen, F.G., Gerdes, K. (2003) Toxin-antitoxin loci as stress-response-elements: ChpAK/MazF and ChpBK cleave translated RNAs and are counteracted by tmRNA. *J. Mol. Biol.* **332,** 809–819.
14. Prysak, M.H., Mozdzierz, C. J., Cook, A.M., Zhu, L., Zhang, Y., Inouye, M., Woychik, N.A. (2009) Bacterial toxin YafQ is an endoribonuclease that associates with the ribosome and blocks translation elongation through sequence-specific and frame-dependent mRNA cleavage. *Mol. Microbiol.* **71,** 1071–1087.
15. Koga, M., Otsuka, Y., Lemire, S., Yonesaki T. (2011) *Escherichia coli* rnlA and rnlB compose a novel toxin–antitoxin system. *Genetics* **187,** 123–130.
16. Donegan, N.P., Thompson, E.T., Fu, Z., Cheung, A.L. (2010) Proteolytic regulation of toxin-antitoxin systems by ClpPC in *Staphylococcus aureus*. *J. Bacteriol.* **192,** 1416–1422.

17. Brantl, S., Behnke, D., Alonso, J.C. (1990) Molecular analysis of the replication region of the conjugative *Streptococcus agalactiae* plasmid pIP501 in *Bacillus subtilis*. Comparison with plasmids pAMb1 and pSM19035. *Nucleic Acids Res.* **18,** 4783–4790.

18. Schwarz, F.V., Perreten, V., Tauber, M. (2001) Sequence of the 50-kb conjugative multiresistance plasmid pRE25 from *Enterococcus faecalis* RE25. *Plasmid* **46,** 170–187.

19. Sletvold, H., Johnsen, P.J., Hamre, I., Simonsen, G.S., Sundsfjord, A., Nielsen, K.M. (2008) Complete sequence of *Enterococcus faecium* pVEF3 and the detection of an omega-epsilon-zeta toxin–antitoxin module and an ABC transporter. *Plasmid* **60,** 75–85.

20. Holden, M.T., Hauser, H., Sanders, M., Ngo, T.H., Cherevach, I., Cronin, A., *et al*. (2009) Rapid evolution of virulence and drug resistance in the emerging zoonotic pathogen *Streptococcus suis*. *PLoS One* e6072. doi:10.1371/ journal.pone.0006072.

21. Mutschler, H., Meinhart, A. (2011) ε/ζ systems: their role in resistance, virulence, and their potential for antibiotic development. *J. Mol. Med.* **89,** 1183–1194.

22. Pachulec, E., van der Does, C. (2010) Conjugative plasmids of *Neisseria gonorrhoeae*. *PLoS One* **5,** e9962. doi:10.1371/ journal.pone.0009962.

23. Van Melderen, L., Saavedra De Bast, M. (2009) Bacterial toxin–antitoxin systems: more than selfish entities? *PLoS Gen*. **5,** e1000437. doi:10.1371/journal.pgen.1000437.

24. Behnke, D., Malke, H., Hartmann, M., Walter, F. (1979) Post-transformational rearrangement of an in vitro reconstructed group-A streptococcal erythromycin resistance plasmid. *Plasmid* **2,** 605-616.

25. Khoo, S.K., Loll, B., Chan, W.T., Shoeman, R.L., Ngoo, L., Yeo, C.C., Meinhart, A. (2007) Molecular and structural characterization of the PezAT chromosomal toxin–antitoxin system of the human pathogen *Streptococcus pneumoniae*. *J. Biol. Chem.* **282,** 19606–19618.

26. Mutschler, H., Gebhardt, M., Shoeman, R.L., Meinhart, A. (2011) A novel mechanism of programmed cell death in bacteria by toxin–antitoxin systems corrupts peptidoglycan synthesis. *PLoS Biol.* **9,** e1001033. doi:10.1371/journal.pbio.1001033.

27. Camacho, A.G., Misselwitz R., Behlke, J., Ayora, S., Welfle, K., Meinhart, A., Lara, B., Saenger, W., Welfle, H., Alonso, J.C. (2002) *In vitro* and *in vivo* stability of the ε2ζ2 protein complex of the broad host-range *Streptococcus pyogenes* pSM19035 addiction system. *Biol. Chem.* **383,** 1701–1713.

28. Zielenkiewicz, U., Cegłowski, P. (2005) The toxin–antitoxin system of the streptococcal plasmid pSM19035. *J. Bacteriol.* **187,** 6094–6105.

29. de la Hoz, A.B., Ayora, S., Sitkiewicz, I., Fernandez, S., Pankiewicz, R., Alonso, J.C., Cegłowski P. (2000) Plasmid copy-number control and better than-random segregation genes of pSM19035 share a common regulator. *Proc. Natl. Acad. Sci. USA* **97,** 728–733.

30. Dmowski, M., Sitkiewicz, I., Cegłowski, P. (2006) Characterization of a novel partition system encoded by the δ and ω genes from the Streptococcal plasmid pSM19035. *J. Bacteriol.* **188,** 4362–4372.

31. Meinhart, A., Alonso, J.C., Strater, N., Saenger, W. (2003) Crystal structure of the plasmid maintenance system epsilon/zeta: functional mechanism of toxin zeta and inactivation by ε2ζ2 complex formation. *Proc. Natl. Acad. Sci. USA* **100,** 1661–1666.

32. Zielenkiewicz, U., Kowalewska, M., Kaczor, C., Cegłowski, P. (2009) *In vivo* interactions between toxin–antitoxin proteins epsilon and zeta of streptococcal plasmid pSM19035 in *Saccharomyces cerevisiae*. *J. Bacteriol.* **191,** 3677–3684.

33. Friedman, B.M., Yasbin, R.E. (1983) The genetics and specificity of the constitutive excision repair system of *Bacillus subtilis*. *Mol. Gen. Genet.* **190**, 481–486.

34. Msadek, T., Dartois, V., Kunst, F., Herbaud, M.L., Denizot, F., Rapoport, G. (1998) ClpP of *Bacillus subtilis* is required for competence development, motility, degradative enzyme synthesis, growth at high temperature and sporulation. *Mol. Microbiol.* **27,** 899-914.

35. Slack, F.J., Serror, P., Joyce, E., Sonenshein, A.L. (1995) A gene required for nutritional repression of the *Bacillus subtilis* dipeptide permease operon. *Mol. Microbiol.* **15,** 689–702.

36. Msadek, T., Kunst, F., Rapoport, G. (1994) MecB of *Bacillus subtilis*, a member of the ClpC ATPase family, is a pleiotropic regulator controlling competence gene expression and growth at high temperature. *Proc. Natl. Acad. Sci. USA* **21,** 5788-92.

37. Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J. Mol.Biol.* **166,** 557-580.

38. Haima, P., Bron, S., Venema, G. (1987) The effect of restriction on shotgun cloning and plasmid stability in *Bacillus subtilis* Marburg. *Mol. Gen. Genet.* **209**, 335–342.

39. Ceglowski, P., Boitsov, A., Karamyan, N., Chai, S., Alonso, J.C., 1993. Characterization of the effectors required for stable inheritance of *Streptococcus pyogenes* pSM19035-derived plasmids in *Bacillus subtilis*. *Mol. Gen. Genet.* **241,** 579-585.

40. Brzozowska, I., Brzozowska. K., Zielenkiewicz, U. (2012) Functioning of the TA cassette of streptococcal plasmid pSM19035 in various Gram-positive bacteria. *Plasmid* **68,** 51–60.

41. Sambrook, J., Fritsch, E.F., Maniatis, T. (1989) Molecular cloning: a laboratory manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

42. Spizizen, J. (1958) Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. *Proc. Natl. Acad. Sci. USA* **44,** 1072-1078.

43. Burdett, V., Inamine, J., Rajagopalan, S. (1982) Heterogeneity of tetracycline resistance determinants in *Streptococcus*. *J. Bacteriol.* **149,** 995-1004.

44. Bron, S. (1990) Plasmids. In *Molecular Biological Methods for Bacillus*, pp. 75-174. Edited by C. R. Harwood and S. M. Cutting, Willey & Sons Ltd, England.

45. Rottlander, E., Trautner, T.A. (1970) Genetic and transfection studies with *B. subtilis* phage SP 50. I. Phage mutants with restricted growth on *B. subtilis* strain 168. Mol *Gen. Genet.* **108,** 47-60.

46. Liu, J., Cosby, W.M., Zuber, P. (1999) Role of Lon and ClpX in the posttranslational regulation of a sigma subunit of RNA polymerase required for cellular differentiation in *Bacillus subtilis*. *Mol. Microbiol.* **33,** 415–428.

47. Nakano, S., Zheng, G., Nakano, M. M., Zuber, P. (2002) Multiple pathways of Spx (YjbD) proteolysis in *Bacillus subtilis. J. Bacteriol.* **184,** 3664–3670.

48. Kubik, S., Wegrzyn, K., Pierechod, M., Konieczny, I. (2012) Opposing effects of DNA on proteolysis of a replication initiator. *Nucleic Acids Res*. **40,** 1148–1159.

49. Nakano, S., Nakano, M.M, Zhang, Y., Leelakriangsak, M., Zuber, P. (2003) A regulatory protein that interferes with activator-stimulated transcription in bacteria. *Proc. Natl. Acad. Sci. USA* **100,** 4233–4238.

50. Bakun, M., Karczmarski, J., Poznanski J., Rubel, T., Rozga, M., Malinowska, A., Sands, D., Hennig, E., Oledzki, J., Ostrowski, J., Dadlez, M. (2009) An integrated LC-ESI-MS platform for quantitation of serum peptide ladders. Application for colon carcinoma study. *Proteomics Clin Appl* **3,** 932-946.

51. Gottesman, S. (1996) Proteases and their targets in *Escherichia coli*. *Annu. Rev. Genet.* **30,** 465-506.

52. Gerth, U., Kirstein, J., Mostertz, J., Waldminghaus, T., Miethke, M., Kock, H., Hecker, M. (2004) Fine-tuning in regulation of Clp protein content in *Bacillus subtilis*. *J. Bacteriol.* **186,** 179-191.

53. Gerth, U., Wipat, A., Harwood, C.R., Carter, N., Emmerson, P.T., Hecker, M. (1996) Sequence and transcriptional analysis of clpX, a class-III heat-shock gene of *Bacillus subtilis*. *Gene* **181,** 77-83.

54. Lioy, V.S., Martin, T.M., Camacho, A.G., Lurz, R., Antelmann, H., Hecker, M., Hitchin, E., Ridge, Y., Wells, J.M., Alonso, J.C. (2006) pSM19035-encoded ζ- toxin induces stasis followed by death in a subpopulation of cells. *Microbiol.* **152,** 2365–2379.

55. Brzozowska, I., Zielenkiewicz, U. (2013) Regulation of toxin–antitoxin systems by proteolysis. *Plasmid* **70,** 33–41.

56. Jensen, R.B., Gerdes, K. (1995) Programmed cell death in bacteria: proteic plasmid stabilization systems. *Mol. Microbiol.* **17:** 205–210.

57. Overgaard, M., Borch, J., Gerdes, K. (2009) RelB and RelE of *Escherichia coli* form a tight complex that represses transcription via the ribbon–helix–helix motif in RelB. *J. Mol. Biol.* **394,** 183–196.

58. Diago-Navarro, E., Hernández-Arriaga, A.M., Kubik, S., Konieczny, I., Díaz-Orejas, R. (2013) Cleavage of the antitoxin of the parD toxin–antitoxin system is determined by the

ClpAP protease and is modulated by the relative ratio of the toxin and the antitoxin. *Plasmid* **70,** 78–85.

59. Lioy, V.S., Pratto, F., de la Hoz, A.B., Ayora, S., Alonso, J.C. (2010) Plasmid pSM19035, a model to study stable maintenance in Firmicutes. *Plasmid* **64,** 1–17.

60. Takagi, H., Kakuta, Y., Okada, T., Yao, M., Tanaka, I., Kimura, M. (2005) Crystal structure of archaeal toxin–antitoxin RelE–RelB complex with implications for toxin activity and antitoxin effects. *Nat. Struct. Mol. Biol.* **12,** 327–331.

61. Schumacher, M.A., Piro, K.M., Xu, W., Hansen, S., Lewis, K., Brennan, R.G. (2009) Molecular mechanisms of HipA-mediated multidrug tolerance and its neutralization by HipB. *Science* **323,** 396–401.

FOOTNOTES

[1] To whom correspondence should be addressed: Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Pawińskiego 5A, 02-106 Warsaw, Poland, Tel.: (48) 225921307; Fax: (48) 226584636; E-mail: ulazet@ibb.waw.pl
[2] The abbreviations used are: TA, toxin-antitoxin; CK, creatine kinase.

FIGURE LEGENDS

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 pBT233-1S plasmid; YB886 (wt; ▲,△), QB4916 (*clpP*; ●,○), 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 pBT233-1S (○) 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 pBT233-1S plasmid in QB4756 (△), QB8023 (□) and PS28 (◊).

FIGURE 2. Epsilon stability in cell extracts of the mutant strains. SDS–PAGE gel stained with Coomassie blue. Five micrograms of purified His$_6$-Epsilon was incubated for 1 h at 37°C with lysates from the *B. subtilis* protease-deficient strains, including the Δ*clpP* and Δ*lonA* strains and the ATPase deficient strains Δ*clpX*, Δ*clpC*, and Δ*clpE*. Control: lysate from the wild-type *B. subtilis*. Protein mass ladder: Multicolor Low Range (Fermentas).

FIGURE 3. *In vitro* degradation of the Epsilon antidote by ClpXP$_{Bs}$. (A) Time-course degradation of His$_6$-Epsilon. ClpX$_{Bs}$ (4.5 μg) and 3.36 μg of ClpP$_{Bs}$ were pre-incubated in buffer for 7 min at 30°C. Then, 5 μg of His$_6$-Epsilon was added to the reaction mixture and incubated at 30°C. At the indicated time intervals, 15 μl of the samples was collected and subjected to SDS-PAGE and Coomassie blue staining. The data shown on the corresponding graph are the averages of three independent experiments, and the standard deviation is also shown. (B) Time-course degradation of His$_6$-Epsilon in the presence of purified Zeta toxin. Five micrograms of His$_6$-Epsilon was pre-incubated with 5 μg of purified Zeta toxin for 7-10 min at 30°C and was then added to the reaction mixture containing the ClpXP$_{Bs}$ protease. At the indicated time intervals, 15 μl of the samples was collected and subjected to SDS-PAGE and Coomassie blue staining. The data shown on the corresponding graph are the averages of three independent experiments, and the standard deviation is also shown. (C) Epsilon degradation in presence/absence of the ClpP$_{Bs}$ or ClpX$_{Bs}$ protease subunits. The reaction conditions were the same as in A. (D) Stability of purified His$_6$-Epsilon (1.5 μg) incubated for 1 h at different temperatures. Protein mass ladder (in C and D): Multicolor Low Range (Fermentas). (E) Time-course degradation of His$_6$-Spx by ClpXP$_{Bs}$. ClpX$_{Bs}$ (2.56 μg) and 1.92 μg of ClpP$_{Bs}$ were pre-incubated in buffer for 7 min at 30°C, and 2.4 μg of His$_6$-Spx was then added to the reaction mixture and incubated at 30°C. At the indicated time intervals, 15 μl of the samples was collected and subjected to SDS-

PAGE and Coomassie blue staining. Control: His$_6$-Spx protein incubated in the presence of reaction buffer for 60 min at 30˚C. CK is the creatine kinase from the ATP-regenerating system.

FIGURE 4. LC/MS analysis of protein Epsilon after ClpXP protease degradation. Table represents list of identified peptides and respective Mascot Score. For clarity, duplicate peptides were removed. The same data are presented as percentages of peptides with certain C-terminus amino acid. Protease preferably hydrolyzes peptide bond following leucine and asparagine residues. This tendency however is not very specific, as other sites are also cleaved.

FIGURE 5. *In vitro* degradation assay catalyzed by LonA$_{Bs.}$ Casein (0.5 μg) or various amounts of Epsilon (0.115, 0.27 or 0.4 μg) were incubated with 0.5 μg of His$_6$-LonA$_{Bs}$ protease at 32˚C for 2 h.; 60% of the reaction volume was loaded onto the gel. Protein mass ladder: Multicolor Low Range (Fermentas). CK is the creatine kinase from the ATP-regenerating system.

TABLE 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
B. subtilis		
YB886	*trp*C2 *met*B5 *xin*-1 *att*SPβ *amy*E	33
QB4916	*trp*C2 Δ*clp*P::*spc*	34
PS28	*trp*C2 *unk*U::*spc* Δ*cod*X	35
QB4756	Δ*mec*B::*spc*	36
QB8023	Δ*clp*E::*spc*	T. Msadek
YBX01	YB886 Δ*clp*X::*cat*	this work
YBL01	YB886 Δ*lon*A::*cat*	this work
E. coli		
DH5α	*rec*A1*end*A1 *gyr*A96 *thi*1 *hsd*R17 (r^-_k m^-_k) *sup*E44 *rel*A1 *deo*R F$^-$(Φ80 *lac*ZΔM15) Δ*lac*(ZYA-*arg*F)U169	37
BL21(DE3)	F-, ompT hsdSB (rB-mB-) gal dcm (λDE3)	Novagen
Plasmids		
pUC18	cloning vector, Ap^R	Pharmacia
pHP13	shuttle vector, Er^R Cm^R	38
pTZ57R/T	PCR cloning vector	Fermentas
pBT286	source of *ω*, *ε* and *ζ* genes sequences for PCR amplification	28
pBT233-1	derivative of pSM19035 containing *ω*-*ε*-*ζ* genes, Er^R	39
pBT233-1S	derivative of pBT233-1; filling in of SpeI restriction site (pos. 7116 according to EMBL X64695) resulting in truncation of *ζ* ORF, Er^R	40

pACE 1	the EcoRI-XmnI fragment of pBT286 (pos. 5666-6950 encompassing the *ω-ε* genes); ligated to EcoRI-ScaI sites of pACYC184 vector	28
pET28a(+)	*E. coli* expression vector, Km^R	Novagen
pTXB1	*E. coli* expression vector, Ap^R	New Biolabs England
pET28*epsilon*	plasmid for overexpression of Epsilon antitoxin, Km^R	this work
pET28*spx*	plasmid for overexpression of Spx protein, Km^R	this work
pET28*lonA*$_{Bs}$	plasmid for overexpression of LonA $_{Bs}$ protease, Km^R	this work
pTXB1*zeta*	plasmid for overexpression of Zeta toxin, Ap^R	this work
pTXB1*clpP*$_{Bs}$	plasmid for overexpression of ClpP$_{Bs}$ protease, Ap^R	this work
pTXB1*clpX*$_{Bs}$	plasmid for overexpression of ClpX$_{Bs}$ chaperone, Ap^R	this work

Figure 1

Figure 2

lysate	ΔclpP			ΔclpX	ΔclpC	ΔclpE	−	ΔlonA		wt	
Epsilon	+	−		+	−	+	−	+	−	+	−		+	−

Figure 3

A

B

C

Epsilon	+	+	+	+
ClpP_{Bs}	+	−	+	−
ClpX_{Bs}	+	−	−	+

D

E
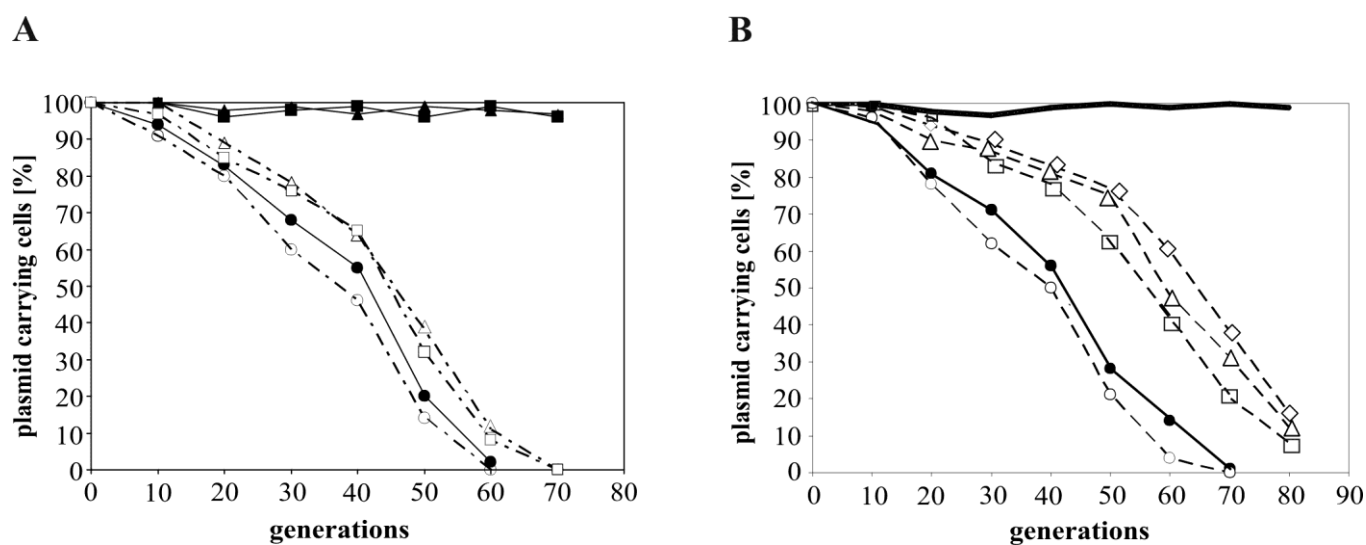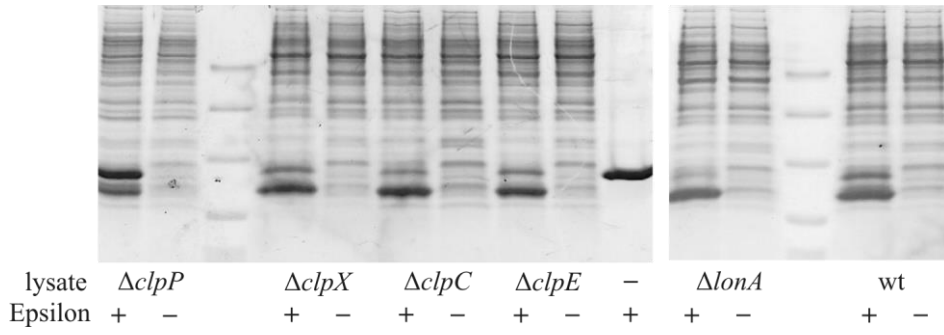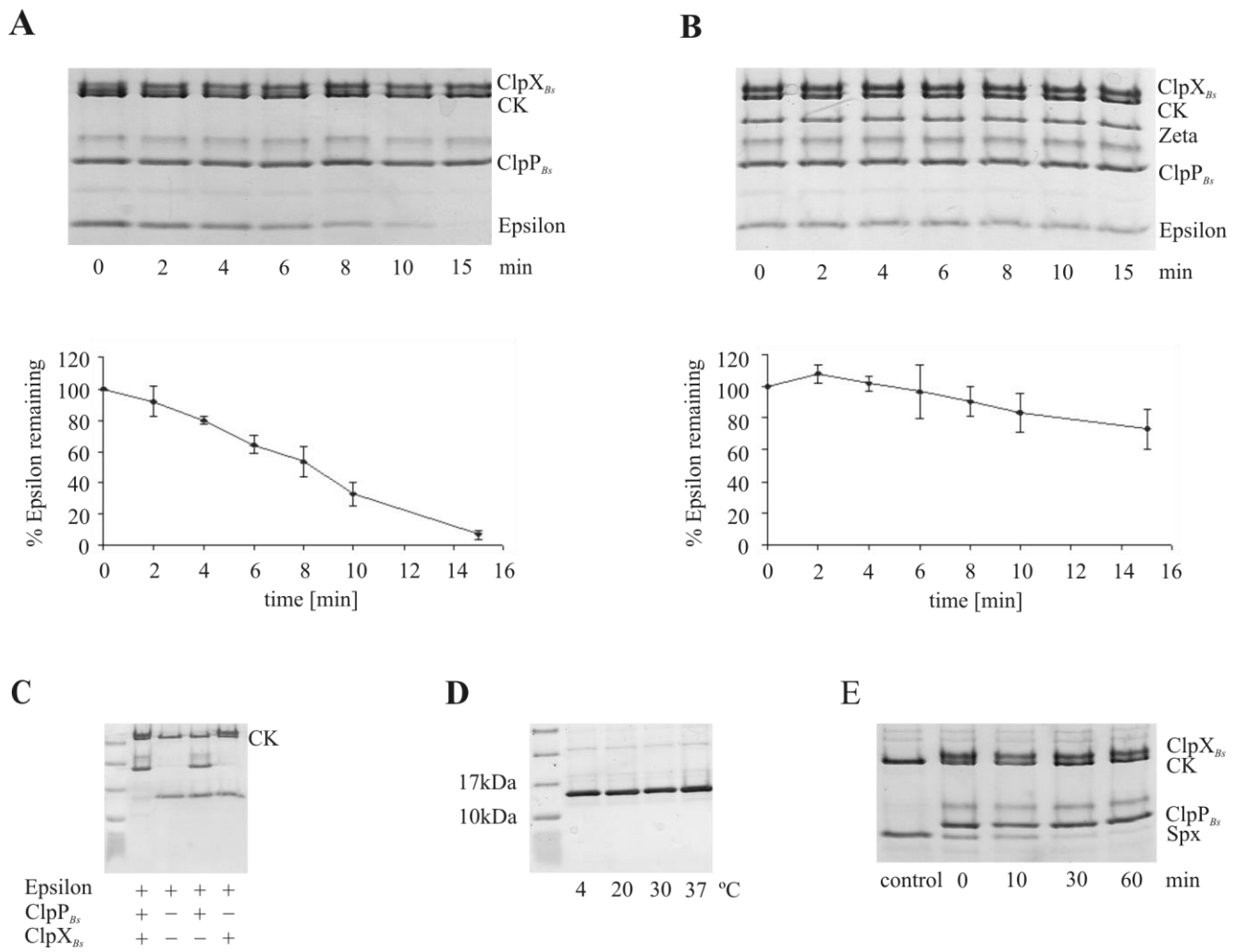
Figure 4

No	Sequence	Mass	Mascot Score	Start - End		
1	EKTFEIEIIN	1234,6445	57	6	-	15
2	ELSASVYNRVLN	1363,7095	75	16	-	27
3	ELSASVYNRVLNY	1526,7729	68	16	-	28
4	LSASVYNRVLN	1234,667	43	17	-	27
5	SASVYNRVLN	1121,5829	58	18	-	27
6	SASVYNRVLNY	1284,6462	51	18	-	28
7	SVYNRVLN	963,5138	42	20	-	27
8	SVYNRVLNY	1126,5771	52	20	-	28
9	VYNRVLN	876,4817	40	21	-	27
10	RVLNYVL	875,5229	44	24	-	30
11	RVLNYVLN	989,5658	44	24	-	31
12	RVLNYVLNH	1126,6247	41	24	-	32
13	YVLNHELNKNDS	1444,6946	36	28	-	39
14	YVLNHELNKNDSQLLEVN	2141,0753	103	28	-	45
15	YVLNHELNKNDSQLLEVNL	2254,1593	141	28	-	46
16	VLNHELN	837,4344	33	29	-	35
17	VLNHELNKN	1079,5723	56	29	-	37
18	VLNHELNKNDSQLLEVN	1978,0119	116	29	-	45
19	VLNHELNKNDSQLLEVNL	2091,096	126	29	-	46
20	VLNHELNKNDSQLLEVNLN	2318,223	36	29	-	48
21	NHELNKN	867,4198	47	31	-	37
22	NHELNKNDSQL	1310,6215	37	31	-	41
23	NHELNKNDSQLLEVN	1765,8595	123	31	-	45
24	NHELNKNDSQLLEVNL	1878,9435	137	31	-	46
25	HELNKNDSQLLEVN	1651,8165	59	32	-	45
26	HELNKNDSQLLEVNL	1764,9006	100	32	-	46
27	ELNKNDSQLLEVNL	1627,8417	39	33	-	46
28	NKNDSQLLEVN	1272,631	53	35	-	45
29	NKNDSQLLEVNL	1385,715	49	35	-	46
30	KNDSQLL	816,4341	48	36	-	42
31	KNDSQLLEV	1044,5451	35	36	-	44
32	KNDSQLLEVN	1158,588	64	36	-	45
33	KNDSQLLEVNL	1271,6721	79	36	-	46
34	KNDSQLLEVNLLN	1498,7991	44	36	-	48
35	DSQLLEVN	916,4502	32	38	-	45
36	DSQLLEVNL	1029,5342	55	38	-	46
37	QLLEVNLLN	1054,6022	31	40	-	48
38	EVNLLNQLKL	1182,6972	46	43	-	52
39	NLLNQLKL	954,5862	36	45	-	52
40	LLNQLKL	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	AKRVNLFDYSLEELQA	1894,9788	78	53	-	68
47	LFDYSLEELQA	1326,6343	35	58	-	68
48	LFDYSLEELQAVHE	1691,8042	108	58	-	71
49	FDYSLEELQA	1213,5503	34	59	-	68
50	FDYSLEELQAVHE	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	EELQAVHE	953,4454	48	64	-	71
57	ELQAVHE	824,4028	35	65	-	71
58	VHEYWRSMN	1220,5397	42	69	-	77
59	RYSKQVLN	1006,556	44	78	-	85
60	VLNKEKVA	899,544	45	83	-	90

Figure 5

LonA$_{Bs}$	+	−		+	−	+	−	+	−
Casein	+	+		−	−	−	−	−	−
Epsilon	−	−		+	+	+	+	+	+