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The Clp Proteases of *Bacillus subtilis* Are Directly Involved in Degradation of Misfolded Proteins

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The presence of the heat stress response-related ATPases ClpC and ClpX or the peptidase ClpP in the cell is crucial for tolerance of many forms of stress in *Bacillus subtilis*. Assays for detection of defects in protein degradation suggest that ClpC, ClpP, and ClpX participate directly in overall proteolysis of misfolded proteins. Turnover rates for abnormal puromycyl peptides are significantly decreased in *clpC*, *clpP*, and *clpX* mutant cells. Electron-dense aggregates, most likely due to the accumulation of misfolded proteins, were noticed in studies of ultrathin cryosections in *clpC* and *clpP* mutant cells even under nonstress conditions. In contrast, in the wild type or *clpX* mutants such aggregates could only be observed after heat shock. This phenomenon supports the assumption that *clpC* and *clpP* mutants are deficient in the ability to solubilize or degrade damaged and aggregated proteins, the accumulation of which is toxic for the cell. By using immunogold labeling with antibodies raised against ClpC, ClpP, and ClpX, the Clp proteins were localized in these aggregates, showing that the Clp proteins act at this level in vivo.

In bacteria, as in eukaryotic cells, heat shock proteins are part of the cellular machinery for protein folding, repair, and degradation. Important energy-dependent and heat response-related proteases in *Escherichia coli* are the Clp proteases, ClpAP and ClpXP, consisting of separately encoded ATPase and peptidase subunits. Different substrate specificity is determined by association of the proteolytic component ClpP with either ClpA or ClpX as a regulatory ATPase (for a review, see references 9 and 11). The resulting complexes exhibit a native molecular architecture of two rings of a ClpP heptamer, stacking back to back. A hexamer of the Clp ATPase is located either on one or on both sides of the ClpP rings. For this complex, a structural similarity to the eukaryotic proteasome has been discussed (12, 16, 41, 52).

It has been accepted that the conserved and ubiquitous Clp ATPases can function as either proteolysis regulators or molecular chaperones (for recent reviews, see references 10, 11, 39, and 43). Chaperone or disaggregase function has been shown or suggested for the ClpA and ClpB, as well as for the ClpX members of the HSP100 family of Clp ATPases (33, 44, 53, 54). Participation in overall proteolysis of misfolded proteins has also been demonstrated for the ClpYQ (HslUV) protease. ClpQ, the proteolytic subunit, shares a very high degree of similarity with members of the β-type subunit constituting the catalytic core of the eukaryotic 20S proteasome, whereas ClpY also belongs to the Hsp100 ATPase family (1, 27, 35, 36). Besides Clp in *E. coli*, the ATP-dependent protease Lon plays an important role in cellular processes by modulating the availability of certain regulatory proteins or degrading abnormally folded proteins (for a review, see reference 9). Regulatory proteins, such as the cell division inhibitor SulA or RcsA, involved in capsule synthesis, were shown to be degraded by the Lon protease (4, 48).

In the gram-positive soil bacterium *Bacillus subtilis*, deletion or disruption of either *clpC*, *clpP*, or *clpX* causes a very pleiotropic phenotype. The presence of ClpC, ClpP, or ClpX in the cell is essential for stress tolerance, because *clp* mutants cannot grow under several stress conditions (7, 21, 29). Furthermore, *B. subtilis* Clp proteins were found to be required for cell division and several stationary-phase phenomena, such as motility and degradative enzyme synthesis, as well as the development of sporulation and genetic competence (7, 17, 21, 29, 30, 31, 49, 50).

Our experiments on the role of Clp proteins in protein degradation revealed a direct participation of ClpC, ClpX, and ClpP in overall proteolysis of heat-damaged proteins in *B. subtilis*. The decreased breakdown of damaged proteins, evidenced by accumulation of protein aggregates in *clpC* and *clpP* mutants, occurred even under nonstress conditions. By immunocytochemical methods, we could localize Clp proteins at these protein aggregates, suggesting that they most likely act there in vivo in resolubilizing and/or degrading damaged proteins.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. *E. coli* and *B. subtilis* cells were routinely cultivated under vigorous agitation at 37°C in Luria-Bertani medium. The different stress conditions were induced as described earlier (51). The culture was divided during exponential growth, and one half of the culture was grown at 37°C (control), whereas the other half of the culture was exposed to heat shock at 50°C or treated with puromycin. Since *clp* mutant cells showed impaired growth in minimal medium (7, 21), the culture was supplemented with 0.05% (wt/vol) yeast extract. The media were supplemented with the following antibiotics if necessary: ampicillin (100 μg/ml), chloramphenicol (5 μg/ml for *B. subtilis* or 25 μg/ml for *E. coli*), kanamycin (10 μg/ml), spectinomycin (100 μg/ml), erythromycin (1 μg/ml), and lincomycin (25 μg/ml).

General methods. DNA manipulations and transformation of *E. coli* were done according to standard protocols (37, 38). Some oligonucleotides used for PCR included mismatches, allowing creation of restriction sites. Chromosomal DNA from *B. subtilis* was isolated using the Wizard genomic DNA purification kit (Promega, Inc.). Transformation of *B. subtilis* with plasmid or chromosomal

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TABLE 1. Bacterial strains used in this study

Strain	Genotype	Reference
E. coli		
RR1	F^- *mcrB mrr hsdS20*(r_B^- m_B^-) *ara-14 proA2 lacY1 leuB2 galK2 rpsL20* (Sm^r) *xy1-5 mtl-l supE44*	2
DH5α	F^- ϕ80d*lacZ*Δ M15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 gyrA96*	12
BL21(DE3)/pLysS	F^- *lon hsdS*$_B$ (r_B^- m_B^-) with DE3, a λ prophage carrying the T7 RNA polymerase gene, and pLysS plasmid containing the T7 phage lysozyme gene	45
B. subtilis		
IS58	*trpC2 lys-3*	42
BGWLON1	*trpC2 lys-3 lonA*::*pJH101*	34
BUG1	*trpC2 lys-3* Δ*clpP*::*spec*	6
BUG2	*trpC2 lys-3 clpX*::*pMUTIN4*	6
BEK4	*trpC2 lys-3* Δ*clpC*::*spec*	19

DNA was carried out by using a two-step protocol (14). Analysis of transcription by mRNA slot blotting has been described previously (21). Protein extracts were electrophoresed with standard sodium dodecyl sulfate-polyacrylamide gels (24). The protein concentrations of crude extracts were determined by the Bio-Rad protein assay (3). Western blotting was performed by transferring the proteins to polyvinylidene difluoride membranes (Bio-Rad Laboratories). For immunodetection, the membranes were blocked for 1 h in BLOTTO buffer (50 mM Tris [pH 7.6], 150 mM NaCl, 2 mM NaN_3, 2.5% [wt/vol] skim milk powder, 0.5% [vol/vol] Tween 20); incubated overnight with the polyclonal antisera for ClpC (1:8,000) (17), ClpX (1:20,000), and ClpP (1:10,000) diluted in BLOTTO; washed twice for 20 min in BLOTTO; and processed with a goat anti-rabbit or a goat anti-guinea pig alkaline phosphatase conjugate (Sigma). Cross-reacting material was visualized by chemiluminescense with CDP-Star as a substrate of the alkaline phosphatase. The Lumi-Imager system (Roche Diagnostics) was used for documentation and quantitation.

Purification of proteins and antibody production. For overproduction and purification of ClpP and ClpX in *E. coli*, the entire genes were amplified by PCR using primers PRCLPPF (GGAGGATCCATGAATTTAATACCTACAGTC) and PRCLPPR (CGGAATTCTTACTTTTTGTCTTCTGTGTG), as well as PRXFOR (GGAGGATCCATGTTTAAATTTAACGAGGA) and PRXREV (CGGGGTACCTTATGCAGATGTTTTATCTT), and cloned as a *Bam*HI/*Eco*RI or *Bam*HI/*Kpn*I fragment into pRSETA (Invitrogen, Inc.). This plasmid allowed an in-frame fusion of the *clpP* and the *clpX* gene to six histidine codons at the N terminus and transcription from a T7 promoter. Overproduction of His_6-ClpP and His_6-ClpX proteins with T7 RNA polymerase in the *E. coli* strain BL21(DE3) (45) and purification under native conditions by Ni-nitriloacetic acid affinity chromatography (Qiagen, Inc.) was done as previously described (19). The His_6-ClpP and His_6-ClpX proteins were used for custom antibody production in rabbits (Eurogentec, Liege, Belgium).

Measurement of degradation of puromycyl peptides. *B. subtilis* wild type and the isogenic *clpC*, *clpP*, *clpX*, and *lonA* mutant strains were grown at 37°C in synthetic medium (46) until the optical density reached 0.4. Puromycin was added to a final concentration of 40 μg/ml, while the control culture contained no puromycin. After 15 min of incubation at 37°C, [^{3}H]leucine was added to a final concentration of 20 μCi/ml. After 5 min, the cells were collected by centrifugation and washed twice. They were then grown at 37°C for 1 h in synthetic medium supplemented with leucine. Samples were taken at intervals, applied to filter disks, and precipitated with 10% trichloroacetic acid. The radioactivity of the acid-insoluble fraction was measured by liquid scintillation counting (8).

Preparation of *B. subtilis* cells for cryosectioning and immunocytochemistry. Cells were harvested at an optical density of 0.3 before (control) and after heat shock at 50°C. After a fixation step (30 min in 0.2% glutaraldehyde, 2% [wt/vol] paraformaldehyde, 100 mM cacodylate buffer [pH 7.4], 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 25 mM NaN_3), the cells were washed for 5 min in the same buffer without aldehydes, quenched for 15 min in glycine–Tris-buffered saline (TBS) (50 mM glycine, 20 mM Tris-HCl [pH 8.0], 2.5 mM KCl, 135 mM NaCl, 20 mM NaN_3), washed for 5 min in TBS, and soaked in a mixture of 25% (wt/vol) polyvinylpyrrolidone (M_r, 10,000; Sigma-Aldrich) and 1.6 M sucrose according to the method of Tokuyasu (47). Samples were mounted on specimen holders, frozen in liquid nitrogen, and sectioned with a diamond knife at −100°C with an ultracut S/FCS cryoultramicrotome (Leica). Ultrathin thawed cryosections were placed on Formavar-carbon-coated copper grids (400 mesh), floated sections down six times for 10 min each time on drops with glycine-TBS, for 15 min on 5% (vol/vol) goat serum in incubation buffer (1% skim milk powder [wt/vol], 0.01% Tween 20 in TBS), for 16 h on polyclonal antiserum against ClpC, ClpX, and ClpP (1:125, 1:1,000, and 1:1,000, respectively) diluted in incubation buffer, six times for 2 min each time on incubation buffer, and for 60 min on goat anti-rabbit or goat anti-guinea pig 10-nm-diameter gold conjugates (British BioCell International) diluted 1:25 in incubation buffer. After extensive washes with TBS and double-distilled water, the sections were stabilized with 2% methyl cellulose (25 cps) containing 0.3% uranyl acetate and analyzed with a Zeiss EM 906 electron microscope at 60 kV. Incubations with primary antibodies took place at 4°C; all other incubation steps were carried out at room temperature. The specificities of immune reactions were demonstrated by omitting the primary antibodies. No gold particles were detected in the negative controls.

RESULTS

Intracellular levels of ClpC, ClpP, and ClpX. The transcription patterns of the *clpC* operon, as well as those of the *clpP* and *clpX* genes, showed an mRNA induction of all three genes after stress conditions producing nonnative proteins in the cell (6, 7, 20, 28). To find out more about the intracellular concentrations of the ClpC, ClpP, and ClpX proteins under stress conditions, Western blot experiments were performed with protein extracts of control and heat-shocked cells. As expected, significantly higher intracellular amounts of ClpC and ClpP were observed after heat shock in comparison to the control level before heat shock, also indicating increased synthesis of these proteins under stress conditions (Fig. 1). Interestingly, Western blots with the ClpP antibody revealed two specific signals with a mass difference of approximately 5 kDa, indicating that the ClpP protein might be present in two different forms in the cell. This has also been observed in *E. coli*, where the first 14 amino acids are autocatalytically processed (26). Inspection of the *clpP* sequence revealed a second, theoretical translation start at position 91 of the coding sequence preceded by a putative Shine-Dalgarno sequence (coding sequence position 76) (data not shown), indicating reinitiation of translation rather than processing. Surprisingly, there was no obvious difference in the level of ClpX in nonstressed and heat-shocked cells (6) (Fig. 1). This result did not agree with our transcriptional data, which showed a moderate heat shock induction of the *clpX* gene.

ClpC, ClpP, and ClpX participate in overall proteolysis of misfolded proteins. Much data is available concerning the role of ClpAP and ClpXP proteases in ATP-dependent proteolysis in *E. coli* (for a review, see references 9 and 11). In contrast to *E. coli*, little is known about the importance of Clp-mediated protein degradation in gram-positive bacteria. Genes encoding ClpA- or ClpB-type ATPases were not found in the *B. subtilis* genome (22). However, ClpX and ClpC appeared to be good candidates for direction of energy-dependent proteolysis in association with the ClpP peptidase subunit during stress. To look more closely at the function of putative ATP-dependent proteases in *B. subtilis*, we examined the abilities of the *clpC*, *clpP*, and *clpX* mutants as well as the *lonA* mutant to degrade prematurely released puromycyl polypeptides. In general, a low protein turnover was observed in the absence of puromycin (data not shown). As expected, the turnover rates rose significantly after the addition of puromycin, which induces the

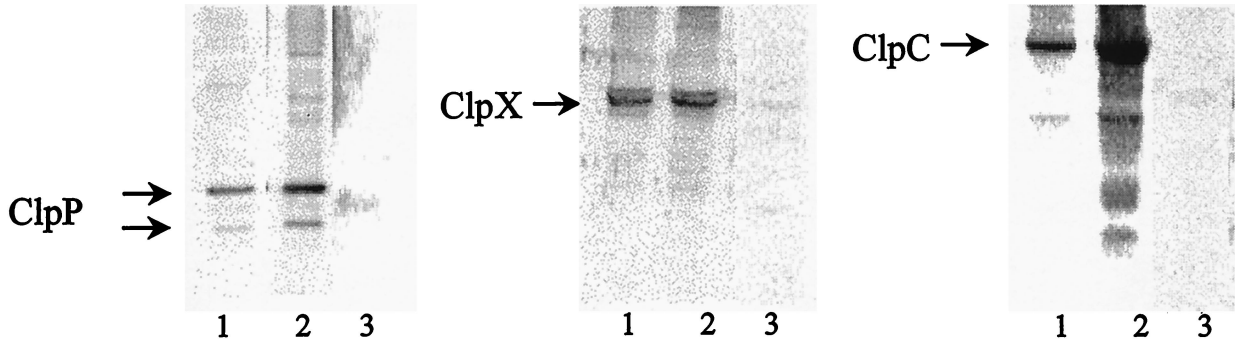

FIG. 1. Amount of ClpC, ClpP, and ClpX in exponentially growing and heat-shocked cells. Samples were taken before (lanes 1) and 15 min after (lanes 2) 50°C heat shock and were analyzed by Western blotting using an antibody against ClpC, ClpP, or ClpX. Crude extracts of the mutants for the appropriate target proteins ClpP (BUG1), ClpX (BUG2), and ClpC (BEK4) were examined as specificity controls for the respective antibodies (lanes 3).

synthesis of abnormal proteins. The results, presented in Fig. 2, show that the *clpC*, *clpP*, and *clpX* mutants degraded puromycyl polypeptides both at a reduced rate and to a much lower overall extent than the wild-type strain, reflecting the important role of Clp proteases in protein degradation. Deletion of *clpC* and *clpP* affected protein breakdown more than a *clpX* mutation did (Fig. 2). Unexpectedly, there was no difference between the turnover rates of the wild type and the *lonA* mutant (Fig. 2). In contrast to *E. coli lon* mutants, which exhibit strongly diminished decay rates for abnormal proteins (25), the *B. subtilis* LonA did not participate in overall proteolysis of puromycyl polypeptides.

Subcellular localization of Clp proteins. Ultrastructural analysis of heat-shocked *Saccharomyces cerevisiae* cells revealed electron-dense particles, which were considered to be aggregates of heat-denatured proteins (33). In order to get more information about general heat shock damage and the subcellular localization of the ClpC, ClpP, and ClpX proteins in *B. subtilis* cells, we performed electron microscopic experiments in combination with the immunogold labeling technique. Cryosections of exponentially growing or heat-shocked cells were processed with antibodies against one of the three Clp proteins and with a secondary antibody-gold conjugate to visualize the proteins by electron microscopy.

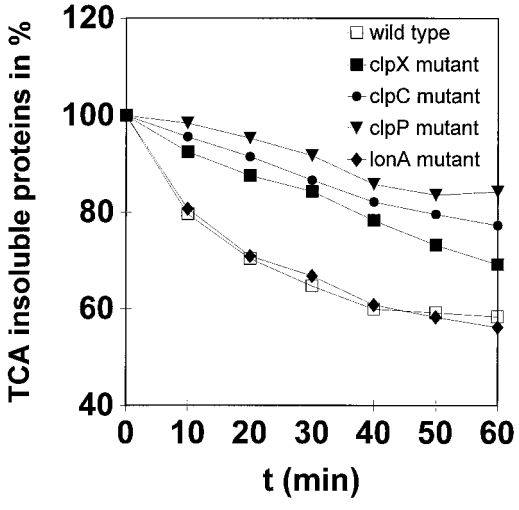

FIG. 2. Overall cellular proteolysis of *clpC*, *clpP*, *clpX*, and *lonA* mutants. Relative puromycylpolypeptide degradation in the wild type and the Δ*clpC*, Δ*clpP*, Δ*clpX*, and *lonA* mutants is shown. Cellular protein was labeled with L-[^{3}H]leucine (20 μCi/ml) in the presence of puromycin (40 μg/ml) and then chased with nonradioactive L-leucine in the absence of puromycin. Radioactivity of the trichloroacetic acid (TCA)-insoluble fraction was measured by liquid scintillation counting as described in Materials and Methods.

Consistent with the yeast data, equivalent aggregates could be observed in electron microscopic studies of wild-type cells heat shocked for 10 min at 50°C (Fig. 3). Generally, treatment with 20 μg of puromycin/ml gave similar results (not shown). Similar to *S. cerevisiae*, general heat damage of *B. subtilis* cells was accompanied by aggregation of heat-damaged proteins, which can be visualized by electron microscopy. These aggregates did not show a preferred localization but were randomly distributed in the cell. Immunocytochemical experiments with exponentially growing wild-type cells showed that ClpP was spread inside the cells, as were some of the ClpC and ClpX proteins (Fig. 3A to C). However, gold particles corresponding to the ClpC and ClpX ATPases were also found at the cell envelope, indicating that they might also have functions there (Fig. 3A and C). In heat-shocked cells, all three proteins could be detected at the electron-dense aggregates (Fig. 3D to F). Thus, it was possible to show that ClpC, ClpP, and ClpX indeed adhere to these aggregates in vivo, most likely for resolubilization and degradation of heat-damaged and aggregated proteins. Table 2 shows a quantitation of the immunogold particles in cryosections of wild-type cells before and after heat shock.

***clpC* and *clpP* mutants accumulate aggregates of denatured proteins under nonstress conditions.** Yeast with mutations in the *hsp104* gene, encoding a B-type Hsp100 ATPase, failed to recover from aggregation damage (33). Since *clpC*, *clpP*, and *clpX* mutants exhibit heat-sensitive phenotypes and defects in overall protein breakdown (see above), electron microscopic studies with these mutants were performed in the next step. Ultrathin sections of heat-shocked wild-type cells revealed that aggregation damage was significantly decreased or completely disappeared after 30 min of incubation at 50°C, whereas after 30 min *clpC* and *clpP* mutants were as damaged as immediately after stress. This phenomenon was less pronounced in *clpX* mutant cells (not shown). No aggregation damage was observed in nonstressed exponentially growing wild-type and *clpX* mutant cells (Fig. 3A to C and 4C). In contrast, in *clpC* and *clpP* mutant cells, accumulation of electron-dense material could also be detected under nonstress conditions (Fig. 4A and B). These observations support the conclusion that *B. subtilis* ClpC and ClpP play a crucial role in protein turnover under nonstress as well as under stress conditions.

Changes in the localization of ClpP were observed in *clpC* mutant cells. Gold particles corresponding to ClpP found at electron-dense aggregates were considerably diminished in ex-

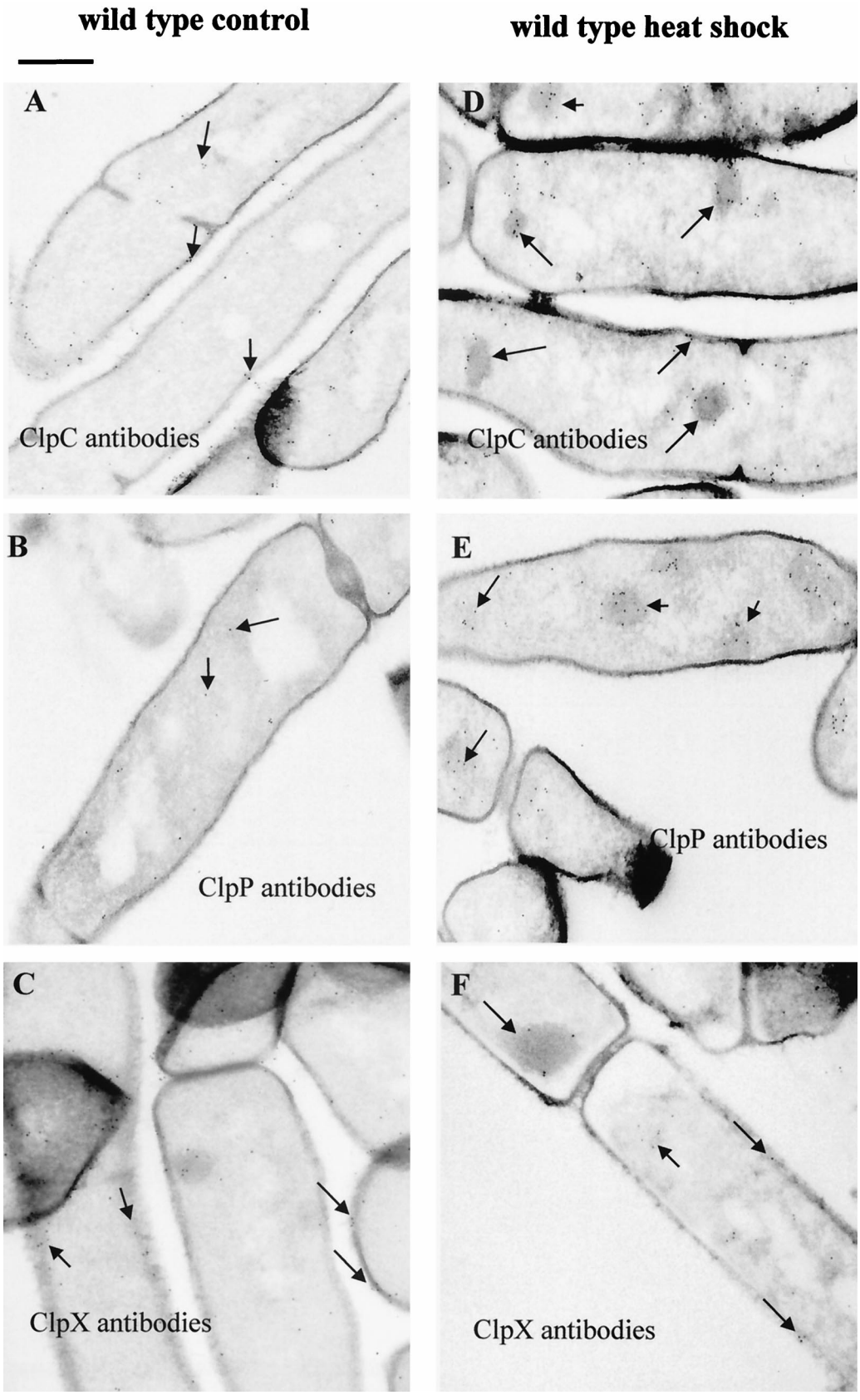

FIG. 3. Subcellular localization of Clp proteins using the immunogold labeling technique. Cryosections of exponentially growing (A, B, and C) or heat-shocked (D, E, and F) wild-type cells were treated with antibodies against ClpC (A and D), ClpP (B and E), and ClpX (C and F) with a secondary antibody-gold conjugate to visualize the localization of the proteins. The localization of gold particles corresponding to Clp proteins is indicated by arrows. Bar, 0.5 μm.

TABLE 2. Quantitation of immunogold particles in ultrathin cryosections of *B. subtilis* wild-type cells before and after heat shock

Parameter	Value[a]					
	ClpP antibody		ClpC antibody		ClpX antibody	
	Control[b]	Heat shock[c]	Control[b]	Heat shock[c]	Control[b]	Heat shock[c]
% Cells with inclusions bodies	0	99.2	0	100	0	98.3
No. (± SD) of gold particles in cytoplasm per cell	12.6 ± 6.6	17.0 ± 6.9	26.4 ± 9.2	39.4 ± 11.5	15.9 ± 5.1	11.9 ± 4.9
No. (± SD) of gold particles at protein aggregates per cell	0	14.0 ± 6.7	0	30.4 ± 10.2	0	7.2 ± 4.1

[a] At least 100 cells were evaluated.
[b] Cells were harvested during exponential growth.
[c] Cells were harvested 10 min after heat shock at 50°C.

ponentially growing *clpC* mutant cells, whereas deletion of *clpP* had no effect on localization of ClpC at electron-dense material (data not shown). The distribution of ClpX in *clpP* mutant cells and that of ClpP in *clpX* mutants resembled the wild-type situation (not shown). In summary, these results suggest that ClpC could direct ClpP in the degradation of denatured proteins.

DISCUSSION

Energy-dependent degradation of misfolded proteins in *E. coli* is assigned to the ClpAP, the ClpYQ (HslUV), and the Lon proteases (for a review, see reference 9). Despite the conservation and ubiquity of Clp proteins in bacteria and higher organisms, little is known about the importance of Clp-mediated proteolysis in organisms other than *E. coli*. *Lactococcus lactis* cells lacking ClpP had a reduced ability to degrade puromycyl-containing peptides (5). No A-type Clp ATPases were apparent from the complete sequence of the *B. subtilis* genome (22). Instead, a direct participation in the overall proteolysis of misfolded proteins was shown for a member of the ClpC subfamily in *B. subtilis*, most prominently found in gram-positive bacteria and plants. Besides its global function in removal of damaged proteins, CtsR, the global repressor of *clp* gene expression in gram-positive bacteria, has been proved to be a specific substrate of the ClpCP protease (our unpublished observation).

The ClpX-type ATPase of *B. subtilis* is also involved in degradation of damaged proteins, but to a lesser extent (Fig. 2). Recently, potential specific substrates have been determined by a two-dimensional-gel approach (7), suggesting that ClpX has a regulatory function. Interestingly, homologous proteins of components of the ClpYQ protease, found in *E. coli* (27, 35) and other eubacteria, also exist in *B. subtilis* (22). However, nothing is known about the contribution to energy-dependent proteolysis of this proteolytic system in *B. subtilis*.

Lon was reported to be the primary protease in *E. coli* for degrading abnormally folded proteins (9). Although Lon is very well conserved, *B. subtilis* LonA was obviously not involved in degradation of misfolded proteins. A second *lon* gene, *lonB*, has been identified upstream of *B. subtilis lonA* and shown to increase total cellular ATP-dependent protease activity, but only after overproduction (R. Ye and S.-L. Wong, Abstr. Proc. 8th Conf. Bacilli, abstr. T31, p. 78, 1995). Possibly, the Lon proteins can compensate for one another. However, our data strongly suggest that the dominant portion of energy-dependent proteolysis in vivo is executed by the ClpCP and ClpXP proteases in *B. subtilis* (Fig. 2). It should be mentioned, however, that the interaction of ClpP with ClpX has not yet been proven. Whereas mutations in the *lon* gene of *E. coli* exhibit various phenotypes, such as filamentation or mucoidy (4, 48), no growth defect has been described so far for *clpA*, *clpX*, or *clpP* mutants (15, 26). Conversely, a single mutation in any of the *clp* genes in *B. subtilis* has profound effects on cell morphology and growth even under standard conditions (7, 18, 21, 23, 29). This phenomenon can be due to the deficiency in the ability of the *clpC* and *clpP* mutants to remove damaged and aggregated proteins and may cause the extreme sensitivity of those mutants, not only during stress (7, 21, 29, 30). Consequently, they are unable to recover from stress (7, 21, 29) (Fig. 2 and 4). From a functional point of view, ClpC seems to combine properties of ClpA as well as ClpB ATPase in the direction of proteolysis while also protecting the cell from stress by resolubilization of protein aggregates (for a review, see references 9, 10, 11, and 43). In this context, it is interesting

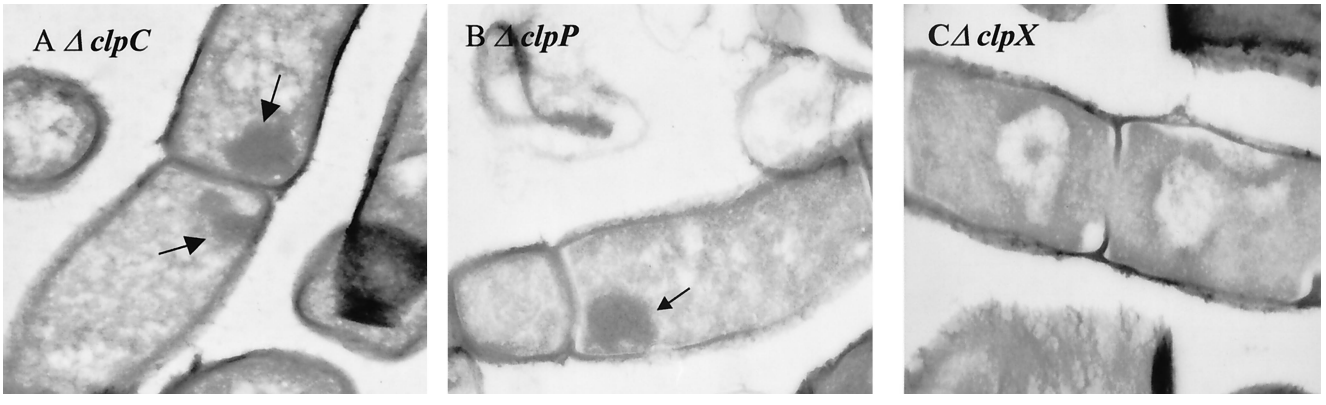

FIG. 4. Ultrastructural analysis of *clp* mutants. Ultrathin sections of exponentially growing cells of Δ*clpC* (A), Δ*clpP* (B), and *clpX* (C) mutants are shown. Accumulations of electron-dense material in the *clpC* and *clpP* mutants are indicated by arrows. Bar, 0.5 μm.

to note that double *clp* gene mutants in any combination do not appear viable (our unpublished observation).

Our experiments indicate that ClpC, ClpP, and ClpX adhere to aggregates of damaged proteins generated by heat shock, presumably acting as disaggregases and/or proteases in vivo (Fig. 3 and 4). So far, however, we cannot exclude the possibility that the Clp proteins themselves aggregate under stress conditions, thus explaining their presence in denatured protein aggregates, but this is probably not the main reason for their localization at these aggregates. Similarly, Clp proteins were detected at inclusion bodies caused by an overproduction of a foreign protein in *B. subtilis* (B. Jürgen, M. Hecker, and T. Schweder, unpublished observation). Immunocytochemical experiments with *clpP* mutants showed that both ATPases, ClpC and ClpX, are located alone at these aggregates. This may support the assumption of disaggregase capacity that has also been suggested for the yeast Hsp104 ATPase (33). Localization of the ATPases at the cell envelope implies further specific and possibly chaperone functions, e.g., protein transport or translocation, as already shown in eukaryotic systems for a chloroplastic ClpC and for yeast Hsp78 in mitochondria (32, 40).

In summary, these data provide evidence of synergistic roles of the ClpCP and ClpXP proteases of *B. subtilis* in energy-dependent protein degradation. In contrast to *E. coli*, the primary proteolytic activity in degrading heat-damaged and abnormally folded proteins of *B. subtilis* can be assigned not to the Lon protease but to the ClpCP protease. Our future projects will focus on investigation of specific substrates of the Clp proteases in *B. subtilis* under different physiological conditions to determine the participation of proteolysis in regulatory pathways.

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