Molecular Microbiology (1998) **28**(4), 787–802

Stress induction of the *Bacillus subtilis clpP* gene encoding a homologue of the proteolytic component of the Clp protease and the involvement of ClpP and ClpX in stress tolerance

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Summary

The *Bacillus subtilis clpP* gene, encoding the proteolytic component of the Clp or Ti protease, was cloned and sequenced. The amount of *clpP*-specific mRNA increased after heat shock, salt and ethanol stress, as well as after treatment with puromycin. Two transcriptional start sites upstream of the *clpP* structural gene were identified, preceded by sequences resembling the consensus sequences of promoters recognized by σ^A and σ^B transcriptional factors of the *B. subtilis* RNA polymerase respectively. Transcription initiation occurred predominantly at the putative σ^A-dependent promoter in exponentially growing cells and was induced under stress conditions. After exposure to stress, initiation of transcription also increased at the σ^B-dependent promoter, but to a lesser extent, indicating that *clpP* belongs to a double promoter-controlled subgroup of class III general stress genes in *B. subtilis*. In a *sigB* mutant strain, *clpP* remained heat and stress inducible at the σ^A-dependent promoter. BgaB–reporter gene fusions, carrying either the σ^A- or the σ^B-dependent promoter, showed a higher *bgaB* induction at the σ^A-dependent promoter, whereas a significantly lower level of induction was measured at the σ^B-dependent promoter. The σ^A-dependent promoter appeared to be crucial for the heat-inducible transcription of *clpP*. A CIRCE (controlling inverted repeat of chaperone expression) element, the characteristic regulation target of class I heat shock genes such as *dnaK* and *groESL*, was not found between the transcriptional and translational start sites. Mutants lacking either the proteolytic component ClpP or the regulatory ATPase component ClpX were phenotypically distinct from the wild type. Both mutants produced chains of elongated cells and exhibited severely impaired growth under stress conditions and starvation. Comparison of two-dimensional protein gels from wild-type cells with those from *clpP* and *clpX* mutant cells revealed several changes in the protein pattern. Several proteins, such as GroEL, PpiB, PykA, SucD, YhfP, YqkF, YugJ and YvyD, which were found preferentially in higher amounts in both *clpP* and *clpX* mutants, might be potential substrates for the ClpXP protease.

Introduction

Bacteria have evolved highly sophisticated networks to cope with adverse environmental conditions. In response to heat stress, all living organisms including bacteria transiently increase the level of the heat shock proteins. These heat shock proteins comprise at least two important groups: molecular chaperones and ATP-dependent proteases. Chaperones prevent misfolding and aggregation of partially denatured proteins, whereas ATP-dependent proteases are responsible for the degradation of denatured proteins (Craig *et al*., 1994; Visick and Clarke, 1995; Gottesman, 1996).

The expression mechanisms for heat-inducible chaperones and ATP-dependent protease genes in *Escherichia coli* and *Bacillus subtilis* differ significantly. In *E. coli*, the regulation of classical heat shock genes depends mainly on the level of the alternative transcription factor σ^{32} (Yura, 1996). In contrast, *B. subtilis* heat-inducible genes can be divided into at least three classes (Hecker *et al*., 1996). Class I genes, such as the classical chaperone genes *dnaK* and *groESL*, are transcribed from σ^A-dependent promoters and have a highly conserved 9 bp inverted repeat (CIRCE element) between their transcriptional and translational start sites (Zuber and Schumann, 1994). The CIRCE element probably functions as an operator sequence for the HrcA repressor protein, which might be inactivated under heat stress (Yuan and Wong, 1995a,b;

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Schulz *et al*., 1995) and whose activity is modulated by the GroE chaperonine machine (Mogk *et al*., 1997). Class II genes are induced in response to various stresses (e.g. heat, salt or ethanol) or starvation (e.g. glucose and oxygen) and require the alternative sigma factor σ^B for stress induction (Benson and Haldenwang, 1993; Boylan *et al*., 1993; Völker *et al*., 1994; Hecker *et al*., 1996; Yang *et al*., 1996). Class III genes remain inducible in response to several stresses in a *sigB* minus background and encode predominantly ATP-dependent proteases and their regulatory ATPase components. They seem to fall into two subgroups. Genes belonging to subgroup 1 are transcribed from σ^A-like promoters (*lonA*, Riethdorf *et al*., 1994; *ftsH*, Deuerling *et al*., 1995; 1997; *ahpCF*, Antelmann *et al*., 1996), and genes belonging to subgroup 2 are transcribed from a σ^A- as well as a σ^B-dependent promoter (*clpC*, Krüger *et al*., 1996; *clpP*, *trxA*, Scharf *et al*., 1998).

Clp proteins have been highly conserved during evolution. They can function either as proteolysis regulators or as molecular chaperones (Gottesman and Maurizi, 1992; Squires and Squires, 1992; Schirmer *et al*., 1996; Wawrzynow *et al*., 1996). The classical Clp or Ti protease, first described in *E. coli* (Hwang *et al*., 1987; Katayama-Fujimura *et al*., 1987), consists of a large, hetero-oligomeric complex (about 750 kDa), which comprises a proteolytic component (ClpP) and a larger regulatory ATPase component (ClpA).

ClpP forms two rings of seven subunits (Kessel *et al*., 1995; Wang *et al*., 1997). ClpA is composed of six subunits, which are located either on one or on both sides of the ClpP rings (Kessel *et al*., 1995; Shin *et al*., 1996). ClpA, ClpB and ClpC form at least three subfamilies of the HSP100 family, and all have two Walker-type nucleotide-binding domains. These subfamilies can be distinguished based on the length of the spacer region between these two domains (Squires and Squires, 1992). The middle region of ClpA-type proteins is short (about 54 amino acid residues), that of ClpC-type proteins is of intermediate length (about 101–118 amino acid residues) and that of ClpB-type proteins is the longest (about 172–207 amino acid residues; Schirmer *et al*., 1996). The first ATPase site of ClpA in *E. coli* seems to be involved in hexamer formation, whereas the second ATPase site appears to be primarily responsible for the energy-requiring step in the degradation of large proteins (Singh and Maurizi, 1994; Seol *et al*., 1995). ClpB of *E. coli* (Squires *et al*., 1991) and Hsp104 of *S. cerevisiae* (Sanchez and Lindquist, 1990) are necessary for thermotolerance, and ClpC (MecB) is involved in stress tolerance, the development of transformation competence and cell division in *B. subtilis* (Krüger *et al*., 1994; Msadek *et al*., 1994; Turgay *et al*., 1997).

ClpX, another alternative regulatory ATPase subunit of the Clp protease, has only one ATPase site. The proteolytic component ClpP can form a complex with either of the regulatory ATPase components ClpA or ClpX, and the resulting complexes have different substrate specificities (Gottesman *et al*., 1993; Gottesman, 1996). In *E. coli*, *clpP* and *clpX* are transcribed together and belong to the σ^{32} heat shock regulon (Gottesman *et al*., 1993). In *B. subtilis*, both *clpP* and *clpX* are heat-inducible genes (Gerth *et al*., 1996). They are not adjacent on the chromosome.

In this study, we report the cloning, sequencing and transcriptional analysis of *B. subtilis clpP* encoding a homologue of the proteolytic component of the Clp protease. Phenotypical studies and the two-dimensional protein pattern of *clpP* and *clpX* mutants are presented in comparison with wild-type cells.

Results

Nucleotide sequence of the B. subtilis clpP *gene*

First, all bacterial and eukaryotic ClpP protein sequences from the databases were aligned to identify highly conserved regions, and the N-terminal amino acid sequence of the *B. subtilis* ClpP protein, extracted from two-dimensional protein gels, was determined according to Völker *et al*. (1992). This information was used to construct degenerate primers corresponding to the N-terminus (MNLIPTVIE) and to a region located approximately 70 amino acid residues from the N-terminus, which is conserved in all bacterial and eukaryotic ClpP proteins (YLFINSPGG). A 207 bp fragment was amplified, and the translated DNA sequence of the cloned fragment displayed 66.7% identity with the ClpP protein of the Gram-positive *Streptococcus salivarius* (Giffard *et al*., 1993) in a 55 amino acid residue overlap using a BLAST search (Altschul *et al*., 1990). Additional information was obtained from the sequencing of internal ClpP peptides generated by treatment with bromocyan (H. Antelmann and R. Schmid, unpublished). A degenerate primer was synthesized based on the internal amino acid sequence EVMIHQP, and a 370 bp *clpP* polymerase chain reaction (PCR) fragment was amplified and cloned. Vectorette PCR (Arnold and Hodgson, 1991) was used to reclone the region covered by the degenerate primers and to clone the regulatory as well as the C-terminal region of the *clpP* gene. Two PCR products of about 2.7 kb and 1.3 kb were obtained. One contained the regulatory region and the other contained the C-terminal coding region of the *clpP* gene. The DNA of the vectorette PCR fragments was partially sequenced using the primer walking method. Taken together, this gave the entire *B. subtilis clpP* sequence.

Analysis of the nucleotide sequence revealed an open reading frame (ORF) starting with an ATG codon and encoding a putative protein of 197 amino acid residues with a calculated molecular mass of 21 682 Da and an isoelectric point of 5.19. These data are consistent with the localization of ClpP on the two-dimensional protein map

of *B. subtilis* (Völker *et al*., 1994). The start codon was preceded by a potential ribosome binding site, AAGGAGG, which corresponds to the complementary *B. subtilis* 16SrRNA consensus sequence of AGAAAGGAGG (Green *et al*., 1985). The Shine–Dalgarno sequence and the start codon were separated by 6 nucleotides. This is one nucleotide less than the optimal spacing of 7–9 nucleotides determined by Vellanoweth and Rabinowitz (1992). A potential transcriptional terminator structure was identified ($\Delta G = -16.9$ kcal mol^{-1}).

A CIRCE element, a 9 bp inverted repeat most frequently found between the transcriptional and translational start sites of the class I heat shock genes *dnaK* and *groESL*, could not be identified within *B. subtilis clpP*.

The *clpP* gene has recently been located at 301° on the chromosome and sequenced in parallel by F.C. Denizot (Msadek *et al*., 1998) as part of the *B. subtilis* genome sequencing project.

Amino acid sequence of the B. subtilis *ClpP protein*

The alignment of the deduced amino acid sequence with that of known eubacterial and eukaryotic *clpP* genes revealed overall 69% identity with the Gram-negative *E. coli*, 68.3% with *Myxococcus xanthus*, 67.4% and 65.5% with two ClpP proteins of the cyanobacteria *Synechococcus* sp., 58.6% to 66.3% with three ClpP proteins of *Synechocystis* sp., 66.7% with *Helicobacter pylori*, 66.5% with *Yersinia enterocolitica*, 65.6% with *Haemophilus influenzae*, 58.2% with the Gram-positive *Streptococcus salivarius* and 39.3% with *Paracoccus denitrificans*. There was a greater degree of sequence identity between *B. subtilis* ClpP and the human ClpP protein (55.1%) than with a number of plant chloroplast ClpP proteins (approximately 40%). However, *clpP* could not be identified in the genome of the archaeabacterium *Methanococcus jannaschii* (Bult *et al*., 1996).

A high degree of identity occurred throughout the entire length of all aligned sequences (Fig. 1). Several regions within the ClpP protein, including regions surrounding the serine-98 residue, the histidine-123 residue and the aspartate-172 residue, which are thought to constitute the catalytic triad of the serine protease (Maurizi *et al*., 1990a,b; Wang *et al*., 1997), are highly conserved.

Induction of clpP *transcription after stress exposure*

In contrast to *E. coli*, *B. subtilis clpP* and *clpX* are transcribed as monocistronic genes with mRNAs of about 0.8 and 1.4 kb respectively (Gerth *et al*., 1996). Furthermore, unlike some other Gram-negative bacteria, such as *E. coli*, *H. influenzae* and *Y. enterocolitica*, these genes are not adjacent on the *B. subtilis* chromosome. To test the influence of various stress conditions on the expression of *clpP* in *B. subtilis*, slot-blot filters with total RNA isolated before and at different times after stress exposure were hybridized with a digoxigenin-labelled *clpP* antisense RNA probe.

Figure 2 shows a nearly 30-fold increase in *clpP*-specific mRNA after 6 min of heat shock (48°C). Afterwards, the amount of *clpP* mRNA decreased.

Lower induction ratios were measured after 4% (w/v) NaCl and 5% (v/v) ethanol treatment (10-fold), as well as after the application of 20 μg ml^{-1} puromycin to induce the production of aberrant proteins (roughly 20-fold). No significant induction of *clpP* was detected in response to glucose and amino acid starvation (data not shown).

However, the gene remained inducible at least after heat and ethanol stress, even in a *sigB* minus background, but to a lesser extent than in the wild type (data not shown). This result and the absence of a CIRCE element suggested that *clpP* might belong to the class III heat shock genes in *B. subtilis*. These data were in agreement with results obtained by quantitative two-dimensional protein analysis (Bernhardt *et al*., 1997).

Mapping of the transcriptional start sites

Results from two-dimensional PAGE (Bernhardt *et al*., 1997) and data obtained from mRNA slot-blot analysis comparing the wild type with a *sigB* minus strain (data not shown) suggested that *B. subtilis clpP* may also be expressed in a σ^B-dependent manner under stress. To verify this, primer extension experiments were performed.

Two potential transcriptional start sites were mapped for the *clpP* gene using RNA from wild-type *B. subtilis* IS58 cells (Fig. 3A and B). Transcriptional initiation at both sites was heat, salt and ethanol inducible, but to different extents. The sequence upstream of the start site S2 resembled the consensus sequence of a σ^A-dependent promoter with −10 (TATCAT) and −35 (TTGACC) regions (deviations underlined) and with an optimal spacing of 17 nucleotides (see Helmann, 1995). However, the moderately conserved TNTGN motif at the −16 region (Helmann, 1995; Voskuil *et al*., 1995), which is present in other *B. subtilis* class III genes such as *clpX*, *ftsH* or *htpG* (Deuerling *et al*., 1995; Gerth *et al*., 1996; Schulz *et al*., 1997), was not found. The sequence upstream of the start site S1 resembled the consensus sequence of a σ^B-dependent promoter with −10 (GGGAAA) and −35 (GTTTGA) regions separated by 15 nucleotides (deviations underlined). The transcriptional initiation under standard growth conditions as well as after exposure to stress was much stronger at the σ^A-dependent promoter than at the σ^B-dependent promoter. This is in contrast to the *B. subtilis clpC* operon, in which transcriptional initiation is stronger at the σ^B-dependent promoter under stress conditions (Krüger *et al*., 1996).

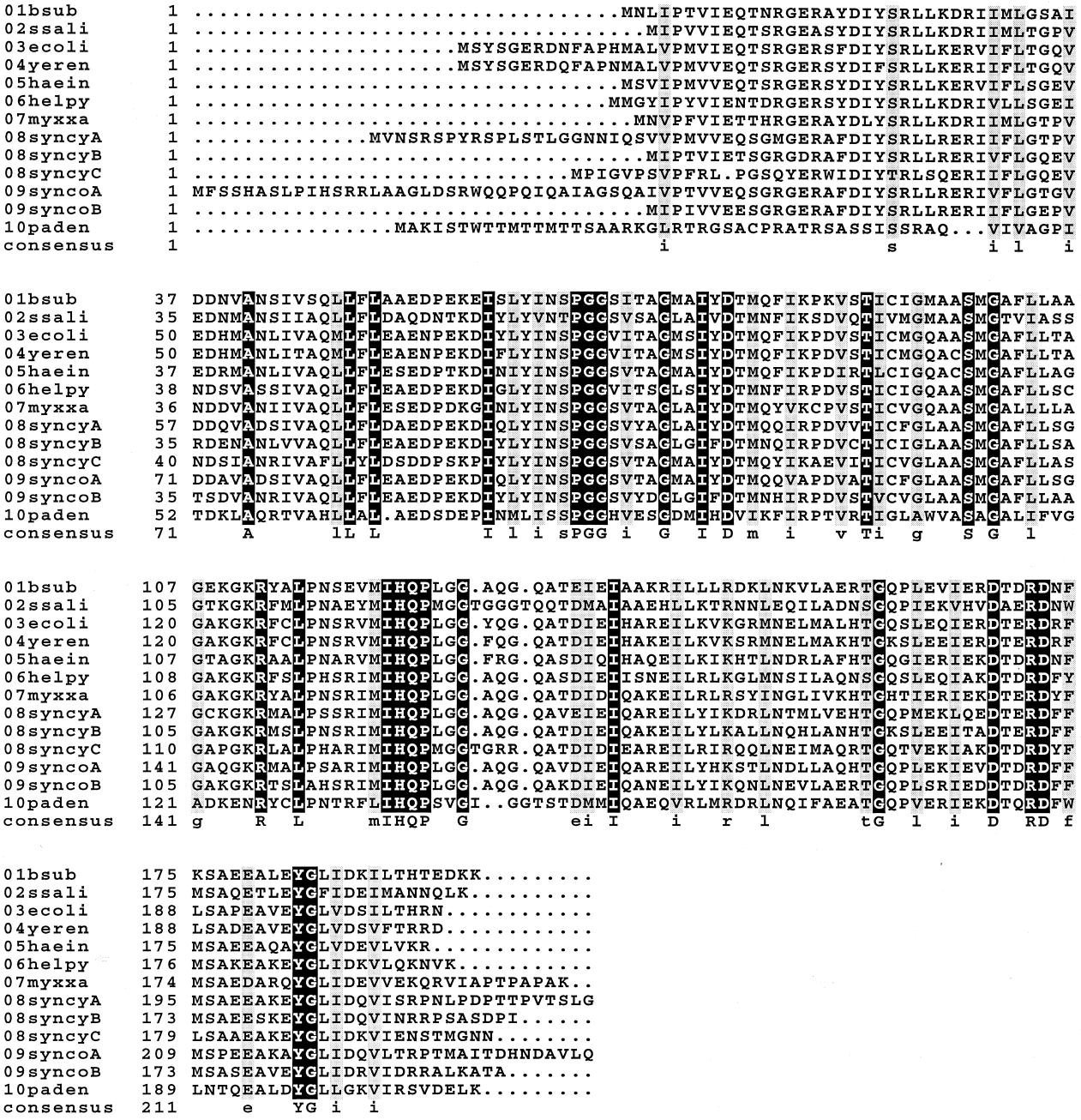


Fig. 1. Alignment of the ClpP proteins of *B. subtilis* (bsub), *Streptococcus salivarius* (ssali), *E. coli* (ecoli), *Yersinia enterocolitica* (yeren), *Haemophilus influenzae* (haein),*Helicobacter pylori* (helpy), *Myxococcus xanthus* (myxxa), *Synechocystis* sp. (syncyABC), *Synechococcus* sp. (syncoAB) and *Paracoccus denitrificans* (paden). Identical residues are labelled with a black background; similar residues are labelled with a grey background.

In a *sigB* mutant strain, transcription was initiated only at the start site S2, proving that the second promoter is indeed σ^B dependent. At least under heat and ethanol stress, a slightly stronger signal at the σ^A-dependent promoter could be recognized in the *sigB* mutant in comparison with wild-type cells, suggesting a weak competition between the RNA polymerases $E\sigma^A$ and $E\sigma^B$ for transcriptional initiation (Fig. 3A).

clpP *promoter deletion analysis using a* bgaB *reporter gene fusion*

To test the contribution of each promoter to the transcriptional induction of *clpP*, different promoter deletion fragments were constructed carrying either the σ^A, σ^B or both promoters (Fig. 4). These constructs were fused to the *bgaB* reporter gene, which encodes a thermostable

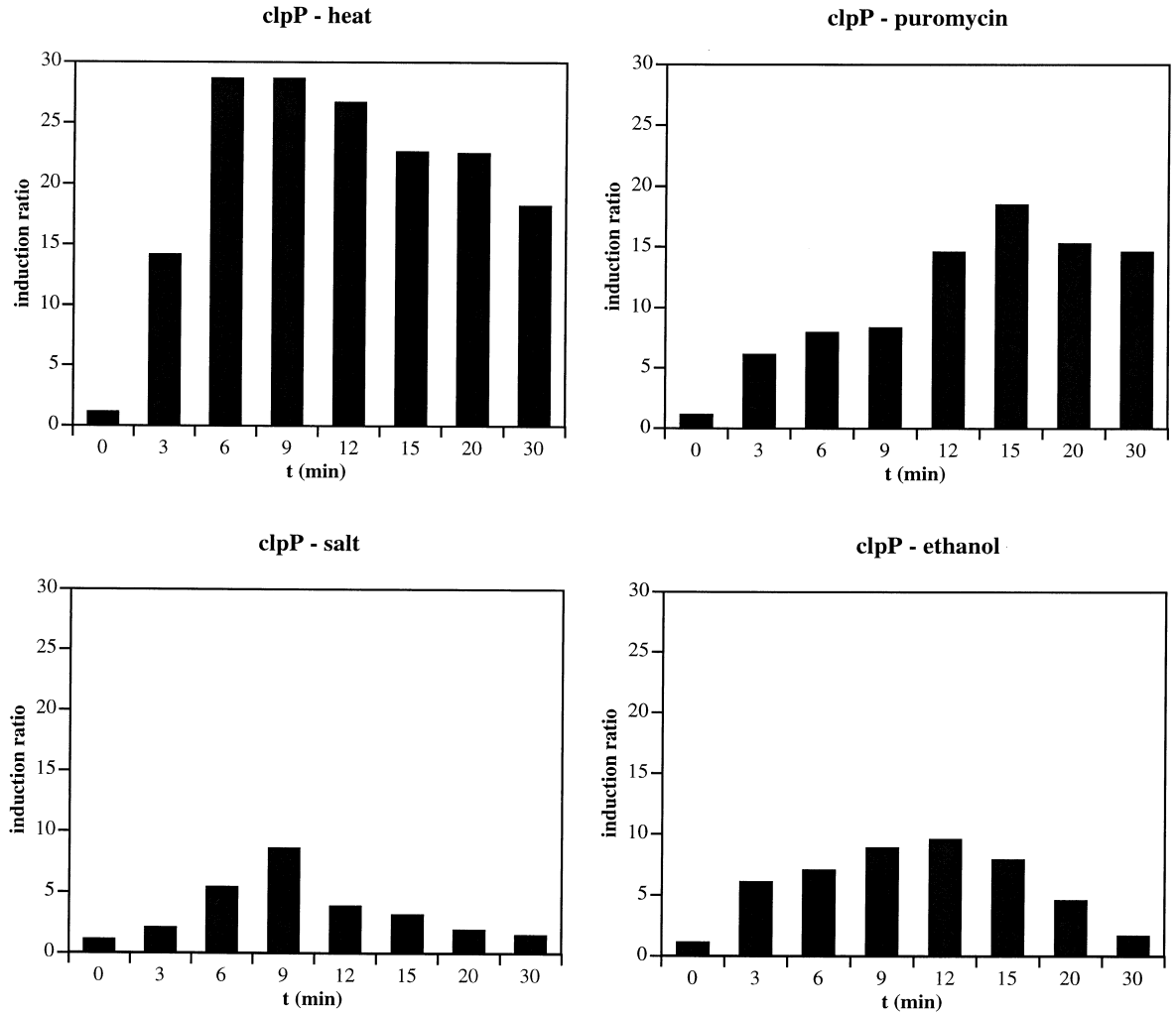


Fig. 2. Schematic representation of the increase in the *clpP* mRNA levels caused by different types of stress. *B. subtilis* IS58 (wild type) was exposed to 48°C heat, 4% (w/v) NaCl, 5% (v/v) ethanol or puromycin (20 μg ml^{-1}). Serial dilutions of total RNA were prepared before (0 min) and after different exposure times.

β-galactosidase using the pDL plasmid (Yuan and Wong, 1995a). The nucleotide sequence of the constructs was then confirmed by DNA sequencing. The different deletion fragments were introduced into the dispensable *B. subtilis amyE* locus by a double cross-over event.

Transcriptional induction was measured by BgaB activity after heat stress (Fig. 4). As expected, the highest BgaB activity was detected after heat shock (48°C) using fragment 1 comprising both promoters. Only a slightly lower BgaB activity was measured with the σ^A-dependent promoter fragment, indicating that the σ^A-dependent promoter seems to be responsible for most of the heat induction of *clpP*. A drastic decrease in BgaB activity was detected in strains containing only the σ^B-dependent promoter fragment, confirming the primer extension results (see Fig. 3A). The reasons for the rather low activity of the σ^B-dependent promoter are not known. Perhaps this is a result of the spacing between the −35 and −10 regions of the *clpP* σ^B promoter not being of an optimal length compared with that of other σ^B-dependent promoters (15 instead of 12–14 bases). Therefore, BgaB activity was tested using a σ^B-dependent *clpP* promoter fragment with a spacing of 14 bases changing the fourth of the four Gs to an A in the −10 region. However, the BgaB expression level did not increase in this strain (data not shown). These results also indicate that the relatively weak activity of the σ^B-dependent promoter was not influenced by the binding and competition of Eσ^A at the stronger σ^A-dependent promoter, as suggested earlier. Furthermore, the activity of the σ^A-dependent promoter did not increase when the measurements were performed in a *sigB* minus background (data not shown).

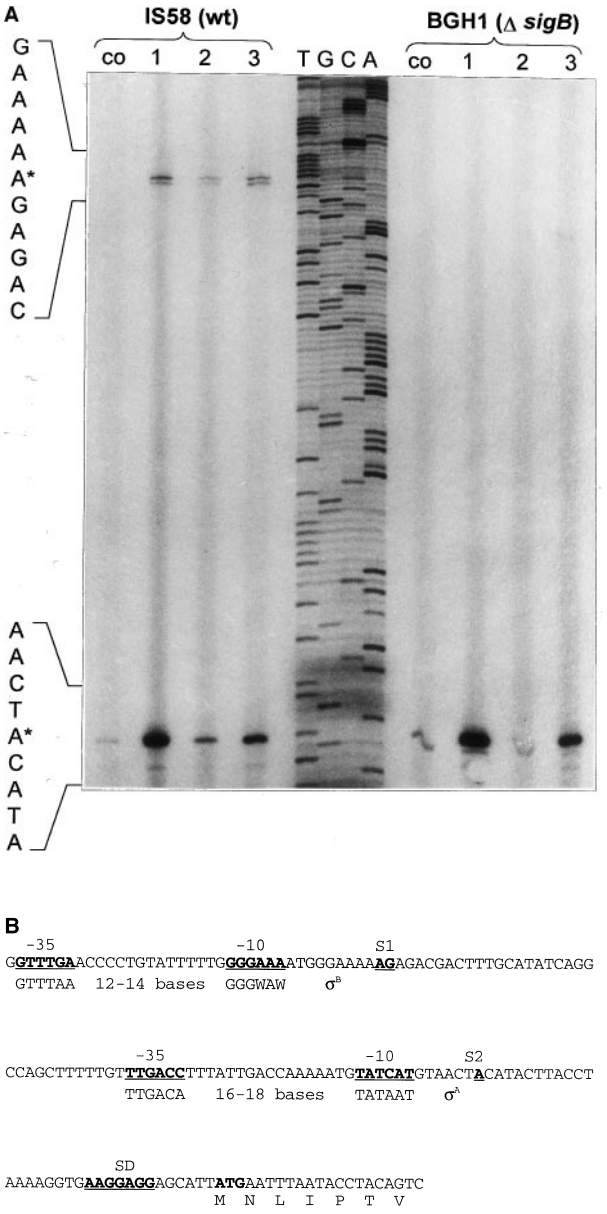


Fig. 3. A. Mapping of the 5′ end of the *clpP* mRNA by primer extension analysis. RNA was isolated from *B. subtilis* IS58 (wild type) and BGH1 (*sigB* mutant) before (control, 37°C) and after 12 min of stress exposure [1, heat (48°C); 2, salt (4% NaCl); 3, ethanol (5%)]. Equal amounts of total RNA (10 μg) were used for the primer extension analysis. The potential transcription start sites are marked with asterisks. Lanes T, G, C and A show the dideoxy sequencing ladder obtained with ΔTaq polymerase (US Biochemical) using the same primer as was used for the primer extension reaction. The sequence displayed is complementary to the coding strand.

B. Sequence of the *clpP* promoter region. Potential −35 and −10 regions and the Shine–Dalgarno (SD) sequence are underlined. The transcriptional start sites and translational start codons are indicated in bold letters. The consensus sequence for σ^A- and σ^B-dependent promoters with their appropriate spacers are shown (W = A or T).

Effect of the inactivation of clpP *and* clpX *on growth under stress conditions*

Although neither Clp nor Lon, another well-characterized ATP-dependent protease of *E. coli*, are essential for *E. coli* growth, cells lacking ClpA or Lon have significantly reduced turnover levels for abnormal proteins (Gottesman *et al*., 1990). The disruption of heat-inducible *clp* genes in *E. coli*, *S. cerevisiae* and *B. subtilis* revealed that the encoded proteins, such as ClpB (Hsp104) and ClpC, were required for stress tolerance (Squires and Squires, 1992; Schirmer *et al*., 1996).

The induction of *clpP* (Figs 2 and 3A) and *clpX* (Gerth *et al*., 1996) mRNA at elevated temperatures suggested that the ClpP and ClpX proteins are necessary in higher amounts during heat shock than during vegetative growth. To address the question of whether mutants lacking either ClpP or ClpX have an altered phenotype with respect to stress tolerance, mutants in *clpP* (Msadek *et al*., 1998) and *clpX* were constructed.

Because of the observation that both mutants lysed on DSM sporulation plates and suppressors emerged after a few days of incubation, fresh transformants or fresh glycerol stocks were used in order to minimize the risk of suppressor selection. The growth of wild-type bacteria and the mutants at normal temperatures and after the imposition of stress were compared. Frozen glycerol stocks were diluted into LB medium, grown to an optical density of 0.1 and then either shifted from 37°C to 50°C or exposed to 6% (w/v) NaCl or 5% (v/v) ethanol.

Mutants generally displayed an extended lag phase but, at 37°C in LB, they grew at similar rates to the wild type (Fig. 5A). However, mutants grew very poorly or not at all in a synthetic medium (data not shown). A diminished growth rate of both mutants could be observed after salt and ethanol stress in comparison with the growth of the wild type (Fig. 5B and C). As shown for the *clpP* mutant (Msadek *et al*., 1998), the *clpX* mutant was not able to grow after the transfer from 37°C to 50°C (Fig. 5D). Wild-type cells were still able to grow, albeit with a slightly reduced rate, after the imposition of 6% (w/v) NaCl, 5% (v/v) ethanol or after a temperature shift to 50°C (Fig. 5A–D). These results argue strongly for a role for both proteins in stress tolerance.

Morphological features of clpP *and* clpX *mutants*

The differences observed in stress tolerance between both mutants and the wild type prompted the investigation of the morphology of the three strains during exponential growth. Using light microscopy, the *B. subtilis clpP* mutant was observed to be filamentous. Electron microscopic studies revealed that both mutants formed elongated cells with hook-like structures (Fig. 6B and C), whereas the

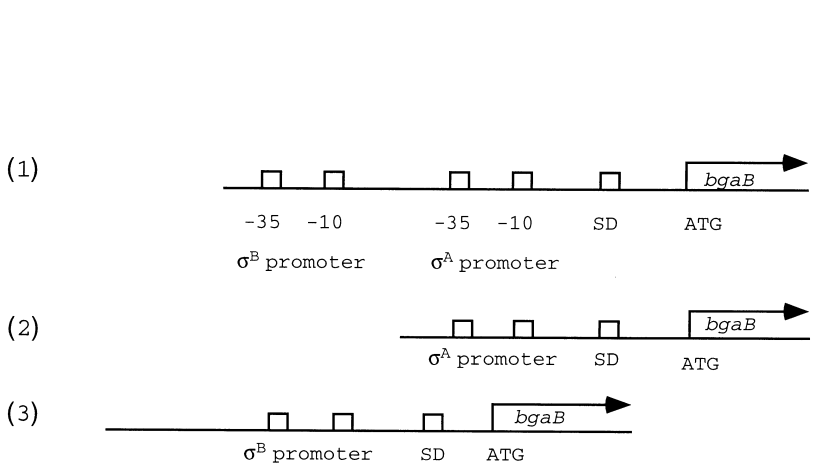


Fig. 4. Schematic representation of the different *clpP* promoter fragments fused to the *bgaB* reporter gene. Transcription of *clpP* was measured as BgaB activity. The −35 and −10 boxes of the promoters, the Shine–Dalgarno sequence (SD) of the *spoVG* gene and the translational start codon (ATG) of *bgaB* are indicated. (1) Large DNA fragment carrying both σ^A- and σ^B-dependent promoters (wild-type situation; BUG3); (2) DNA fragment with the σ^A-dependent promoter (BUG4); (3) DNA fragment with the σ^B-dependent promoter (BUG5).

wild-type strain produced normal rods during growth (Fig. 6A). These phenotypes seem to be pronounced under stress and stationary phase conditions (data not shown).

In addition to the sporulation-deficient phenotype of the *clpP* mutant described by Msadek *et al*. (1998), it was noted that the *clpX* mutant lysed, especially on DSM sporulation plates after a few days of incubation. Therefore, the ability of the mutant to sporulate was tested. A sporulation assay, as described by Nicholson and Setlow (1990), revealed that the *clpX* mutant is also sporulation deficient (Table 1). Furthermore, for both mutants, there was a decreased number of colonies on LB plates in comparison with the wild-type cells after liquid incubation in LB (Table 1). Both ClpP and ClpX seem to be involved in the regulation of genes necessary for starvation survival in the stationary phase, which may also explain the sporulation-deficient phenotype.

Two-dimensional PAGE of clpP *and* clpX *mutants*

A two-dimensional PAGE approach was used for the identification of potential substrates for the ClpXP protease in *B. subtilis*. The protein pattern of wild-type cells was compared with *clpP* and *clpX* mutants with the aim of finding proteins that were present in higher amounts in both mutants. The cells were grown at 37°C in LB to an optical density of 1.5, harvested, and two-dimensional PAGE was performed. ClpP (MW 21.7 kDa, pI 5.2) did not appear on the corresponding *clpP* mutant gels as expected. However, ClpX (MW 46.4 kDa, pI 4.8) remains to be localized on the two-dimensional map (Fig. 7A–C; see Antelmann *et al*., 1997). Several differences in the protein pattern were observed when comparing gels from wild-type and mutant cells. The proteins found in larger amounts in both mutants relative to the wild-type cells might represent potential substrates for the ClpXP protease.

Several proteins, such as GroEL (MKEI), PpiB (MKTG), PykA (MRKTKIV), SucD (MSVFINKD), YhfP (MSTLFQAL), YqkF (MRKRKLG), YugJ (MQNFTY) and YvyD (MNYNIRG), were identified by their N-termini as proteins present in larger quantities in the *clpP* and *clpX* mutants than in the wild-type cells. Whether these proteins are indeed substrates for the ClpXP protease or accumulate for other reasons needs to be clarified. Furthermore, there are several proteins that were no longer detectable in *clpP* and *clpX* mutant strains (see Fig. 7A–C).

Discussion

We have described the cloning, sequencing and transcriptional analysis of the *B. subtilis clpP* heat shock gene, which encodes the proteolytic component of the Clp protease.

Table 1. Survival in the stationary phase and sporulation-deficiency assay.

B. subtilis IS58	Stationary phase survival (cfu ml^{-1} after 2 days incubation in LB)	Sporulation assay (cfu ml^{-1} after 2 days incubation in DSM, plating before and after heat treatment)	Sporulation (%)
Wild type	3.0×10^{10}	7.4×10^{8} 4.8×10^{8}	65
clpP mutant	4.0×10^{6}	ND (see Msadek *et al*., 1998)	
clpX mutant	1.4×10^{7}	2.3×10^{6} 8.0×10^{4}	3.5

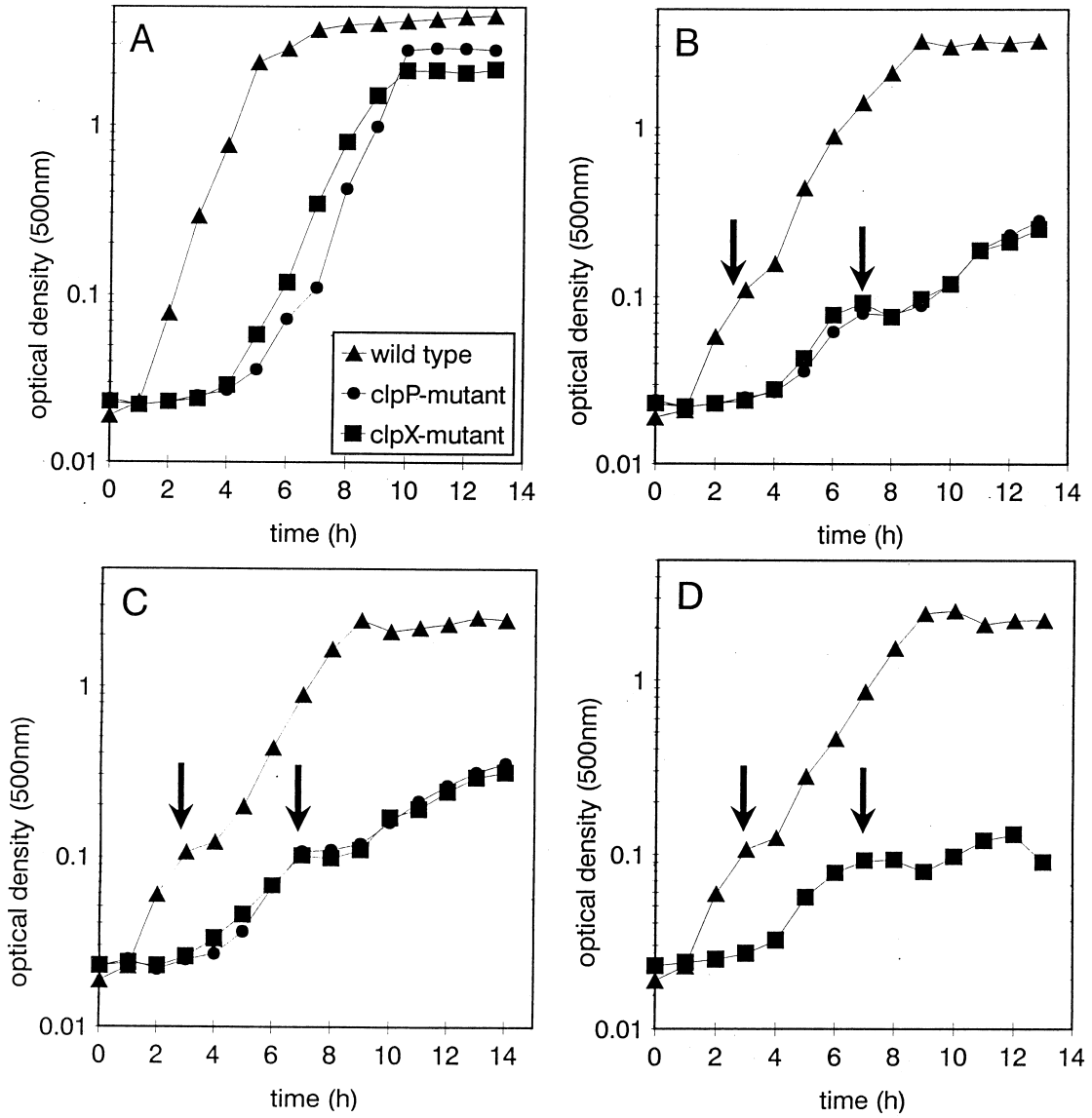
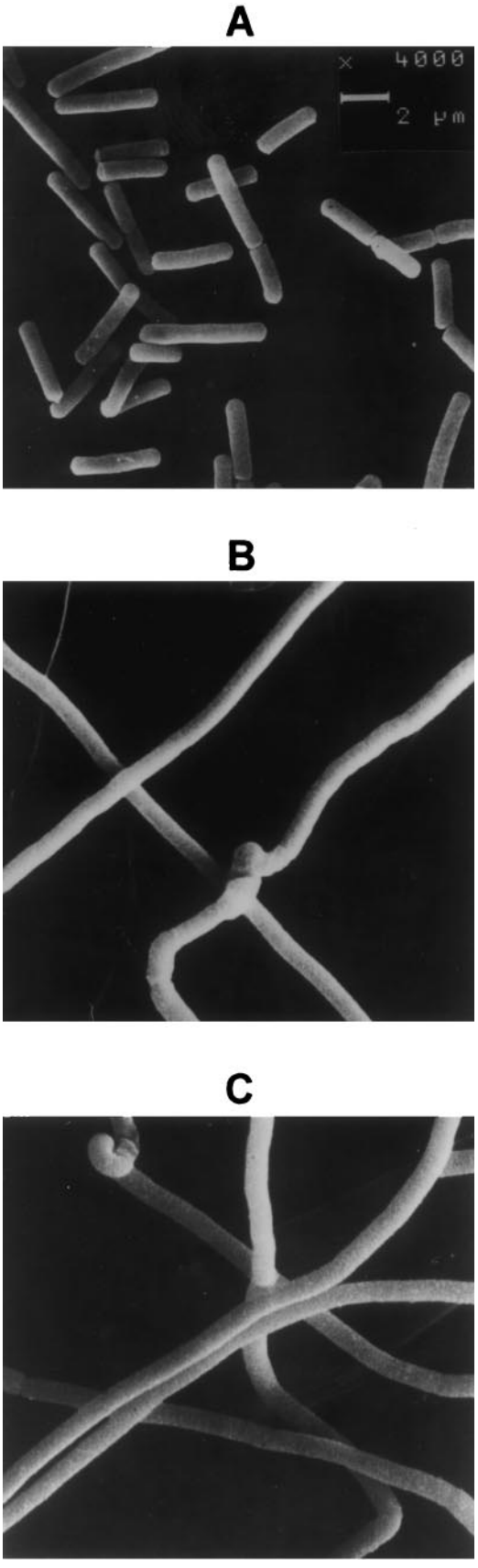


Fig. 5. Growth curves of *B. subtilis* IS58 (wild type, ▲), the *clpP* (●) and *clpX* (■) mutant in LB at 37°C (A), after the addition of 6% (w/v) NaCl (B), 5% (v/v) ethanol (C) or during 50°C heat stress (D). Stress exposure started at an optical density of 0.1 as indicated by an arrow. Typical results of five independent experiments are shown.

In *E. coli*, *clpP* and *clpX* belong to the σ^{32} heat shock regulon. The genes are co-transcribed as a single heat-inducible 2.2 kb mRNA, with the *clpP* as the promoter proximal gene (Kroh and Simon, 1990; Gottesman *et al*., 1993). Two promoters were described for this bicistronic operon, a σ^{70}- and a σ^{32}-dependent promoter, suggesting an important role for the protease under normal growth conditions as well as under heat shock conditions (Squires and Squires, 1992). However, Yoo *et al*. (1994) have reported that, under some conditions, *clpP* and *clpX* can be expressed independently in *E. coli*. Additionally, a possible rho factor-dependent terminator between *clpP* and *clpX* was described by Maurizi *et al*. (1990a).

In this report, it has been demonstrated that the *B. subtilis clpP* gene is induced after stress exposure (puromycin, salt or ethanol), but most prominently after heat shock, suggesting that ClpP is involved in the degradation of aberrant proteins occurring after heat shock and puromycin treatment.

As shown for the *clpC* operon and the *trxA* gene in *B. subtilis*, two stress-inducible promoters were found upstream of the *clpP* coding region resembling promoters recognized by the $E\sigma^A$ and $E\sigma^B$ polymerases. A CIRCE element, the characteristic regulation target between the transcriptional and translational start sites of class I heat shock genes such as *dnaK* and *groESL*, could not be



identified within *B. subtilis clpP*. Therefore, *clpP* was classified as a member of the double promoter-controlled subgroup of class III heat-inducible *B. subtilis* genes. A stronger stress induction was observed at the σ^A-dependent promoter. The reasons for the rather weak σ^B-dependent *clpP* induction are not known, especially in view of the relatively high similarity of the σ^B promoter region to the consensus sequence.

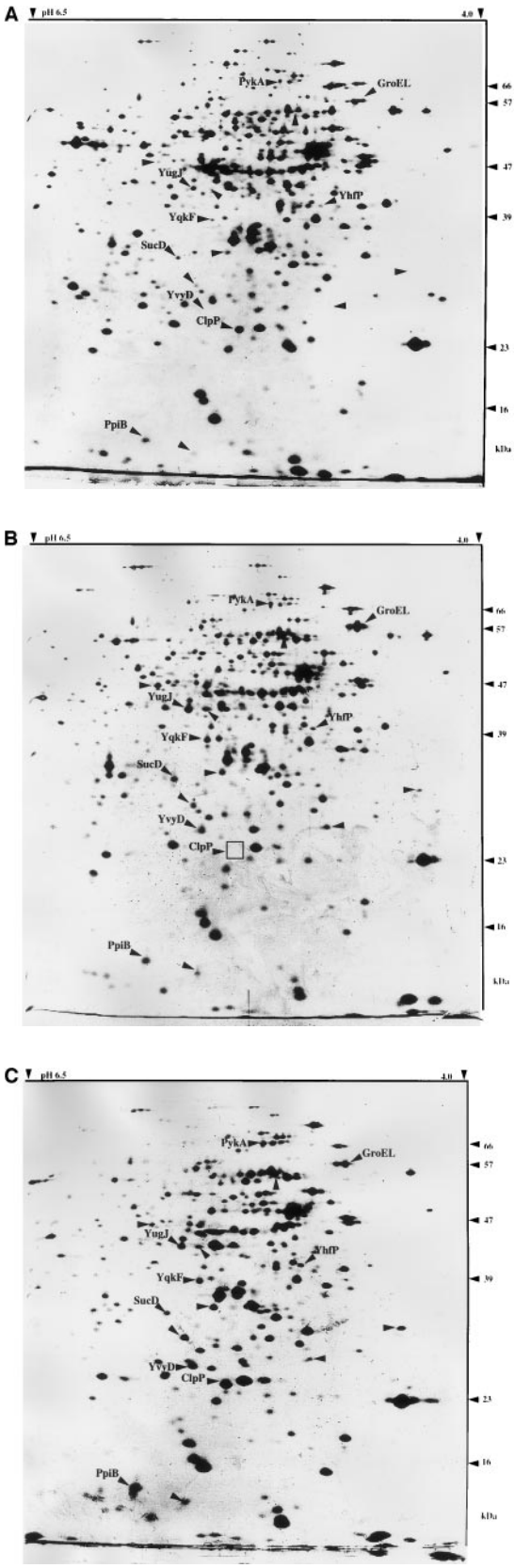
This is in contrast to the *B. subtilis clpC* operon, in which the σ^B-dependent promoter is stronger, but deviates from the consensus sequence in an apparently essential position in the −10 region (TGGAAA, underlined). We have suggested that the competition of $E\sigma^A$ and $E\sigma^B$ for promoter binding could be a reason for the relatively weak σ^B dependence of *clpP* because of the relatively short distance between both promoters, indicating that $E\sigma^A$ might be the stronger competitor. However, when the activity of $E\sigma^A$ and $E\sigma^B$ on both promoters was determined separately after heat shock, only a slight BgaB activity (about 24% of the total activity) was measured with a fragment carrying the σ^B promoter alone. The activity with the σ^A promoter-containing fragment was several-fold higher (about 80% of the total activity). Therefore, the suggestion that a strong competition between $E\sigma^A$ and $E\sigma^B$ RNA polymerases resulted in a weak σ^B promoter activity should be disregarded. Consequently, the σ^A-dependent promoter is most responsible for the accumulation of ClpP under heat shock conditions.

The interaction of ClpP with ATPase components, other than ClpX or ClpA of the Clp protein family, such as ClpB in *E. coli* or ClpC in *B. subtilis* is suggested, because all regulatory ATPase subunits share the same conserved C-terminal amino acid motif that is believed to interact with ClpP (Gottesman *et al*., 1993). ClpA or ClpB homologous proteins were not identified in *B. subtilis*. Whether ClpP can interact with ClpC, ClpX or the recently identified ClpE ATPase (T. Msadek, personal communication) is currently under investigation.

The ClpAP protease of *E. coli* has a structural organization similar to the eukaryotic 26S proteasome (Kessel *et al*., 1995). Interestingly, homologous proteins of the 20S proteasome (Seemüller *et al*., 1995; 1996) constituting the catalytic core of a threonine protease (ClpQ; HslV) flanked by regulatory ATP-binding proteins (ClpY; HslU) can also be found in *B. subtilis* (Slack *et al*., 1995), *E. coli* (Chuang *et al*., 1993; Missiakas *et al*., 1996; Rohrwild *et al*., 1996) and other eubacteria. However, little is known about the differences in substrate recognition of these proteolytic systems in *B. subtilis*.

Mutants lacking either the ClpP or the ClpX protein are impaired in their growth under stress conditions, indicating

Fig. 6. Scanning electron micrographs of *B. subtilis* IS58 (A), the *clpP* (B) and *clpX* (C) mutant grown exponentially.



that they play an important role as proteases or chaperones in stress tolerance. Both mutants produce elongated cells, suggesting that proteins involved in cell morphology or cell division are substrates for the ClpXP protease in *B. subtilis*. It is tempting to speculate that ClpP and ClpX might constitute a main proteolytic complex in *B. subtilis*. Remarkably, *clpP* (Msadek *et al*., 1998) and *clpX* mutants were impaired in stationary phase survival and had a sporulation-deficient phenotype.

Several substrates were described for *E. coli* ClpXP (Wojtkowiak *et al*., 1993; Mhammedi-Alaoui *et al*., 1994; Lehnherr and Yarmolinsky, 1995; Frank *et al*., 1996; Gottesman, 1996; Schweder *et al*., 1996; Levchenko *et al*., 1997), but nothing is known about substrate recognition and degradation by *B. subtilis* ClpXP.

Therefore, two-dimensional protein patterns of *B. subtilis* wild-type cells were compared with both *clpP* and *clpX* mutant cells, and several proteins were found in larger amounts in both mutants. These proteins that were thought to be potential candidates for ClpXP substrates were identified as follows: GroEL, the chaperonine; PpiB, a peptidyl-prolyl *cis–trans* isomerase; PykA, the glycolytic pyruvate kinase; SucD, the succinyl-CoA synthetase alpha chain; YhfP, a protein similar to *E. coli* YhdH and to many alcohol dehydrogenases; YqkF, another hypothetical protein showing no significant similarities to other proteins; YugJ, a potential NADH-dependent butanol dehydrogenase; and YvyD (Antelmann *et al*., 1997), a protein of unknown function with similarity to σ^{54} modulation factors. Some proteins, such as YugJ, YhfP and YqkF, show a relatively high content of lysine, leucine and aspartic acid residues at their C-termini, which could be sufficient for targeting, at least in eukaryotic cells (Gottesman, 1996).

Experimental evidence that the proteins marked in Fig. 7 are really degraded by ClpXP is still lacking, and indirect effects cannot be excluded. An increased level of GroEL and PpiB might be the result of an accumulation of misfolded proteins that can no longer be degraded by the ClpXP protease. Additionally, it could also be suggested that positive regulators or alternative sigma factors are stabilized in the mutants, leading to an increased transcription of the corresponding genes (indirect effects). The level of YvyD, whose gene is partially dependent on σ^{H} (Antelmann *et al*., 1997), is enhanced in both mutants. It would be attractive to test the hypothesis as to whether ClpXP is involved in the degradation of σ^{H}.

However, the only way to demonstrate conclusively that

Fig. 7. Two-dimensional protein gels of *B. subtilis* IS58 (A), the *clpP* mutant (B) and the *clpX* mutant (C). The cells were grown in Luria–Bertani medium to an optical density (OD_{540}) of 1.5. Silver-stained proteins that are present in higher amounts preferentially in both *clpP* and *clpX* mutants in comparison with the wild-type cells are marked by arrows.

Table 2. Bacterial strains and plasmids.

Strain or plasmid	Genotype or description	Reference(s) or source
Strains		
E. coli		
DH5α	F⁻ F80d*lacZ*DM15 D(*lacZYA-argF*) *U169 deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA69*	Sambrook *et al*. (1989)
RR1	F⁻ *mcrB mrr hsdS20* (r_B^- m_B^-) *ara14 proA2 lacY1 leu galK2 rpsL20* (Sm^r) *xyl5 mtl1 supE44*	Sambrook *et al*. (1989)
B. subtilis		
IS58	*trpC2 lys-3*	Smith *et al*. (1980)
BGH1	*trpC2 lys-3 sigB*::*Δ(Hin*dIII–*Eco*RV)::*cat*	Maul *et al*. (1995)
QB4916	*trpC2 ΔclpP*::*spec*	Msadek *et al*. (1998)
BUG1	*trpC2 lys-3 ΔclpP*::*spec*	QB4916 → IS58
BUG2	*trpC2 lys-3 clpX*::*pMutin4*	pMutin4clpX → IS58
BUG3	*trpC2 lys-3 amyE*::*(clpP′Δ1-bgaB cat)*	QB4970 → IS58
BUG4	*trpC2 lys-3 amyE*::*(clpP′Δ2-bgaB cat)*	QB4972 → IS58
BUG5	*trpC2 lys-3 amyE*::*(clpP′Δ3-bgaB cat)*	pDLclpP330 → IS58
Plasmids		
pBluescript-II KS+	Cloning vector, Ap^r	Stratagene
pMutin4	Insertion vector, Erm^r	Vagner *et al*. (1995)
pDL	Insertion vector, Cm^r	Yuan and Wong (1995a)
pDLclpP330	pDL containing the 330 bp *clpP* σ^B promoter PCR fragment in the *Eco*RI and *Bam*HI sites	This study
pKSclpP1	pBluescript-II KS+ containing the 207 bp *clpP* PCR fragment	This study
pKSclpP2	pBluescript-II KS+ containing a 370 bp *clpP* PCR fragment	This study
pKSclpP3	pBluescript-II KS+ containing the 1.3 kb C-terminal *clpP* PCR fragment	This study
pKSclpP4	pBluescript-II KS+ containing the N-terminal 450 bp *clpP* PCR fragment	This study
pMutin4clpX	pMutin4 containing a 490 bp *clpX* PCR fragment in the *Hin*dIII–*Bam*HI sites	This study

some of the proteins mentioned above are indeed substrates for ClpXP would be an *in vitro* degradation assay with purified proteins. Additionally, several proteins were no longer detectable in *clpP* and *clpX* mutant strains, leading to the suggestion that ClpXP could also be involved in the degradation of repressor proteins.

Experimental procedures

Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids are listed in Table 2. *E. coli* strains DH5α and RR1 were grown routinely in Luria–Bertani (LB) medium and used as hosts for DNA manipulation. *B. subtilis* was cultivated under vigorous agitation at 37°C in a synthetic medium described previously (Stülke *et al*., 1993). The different stress conditions were established as described earlier (Völker *et al*., 1994). The culture was divided quickly 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 48°C, 50°C or 20 μg ml^{-1} puromycin to induce the production of aberrant proteins or to 4% (w/v) sodium chloride or 5% (v/v) ethanol. Samples were taken during the exponential growth phase immediately before stress exposure and at the time indicated in Fig. 2. The time of the shift was set to zero. For the phenotypical studies, a stock of glycerol cultures was inoculated into fresh LB medium and transferred to 50°C. NaCl or ethanol were added to final concentrations of 6% (w/v) and 5% (v/v) respectively.

PCR, cloning and sequencing

A 207 bp *B. subtilis clpP* fragment was amplified with primers derived from the N-terminal protein part (5′-ATGAAYYT-NATHCCNACNGTNATHGA-3′) of the *B. subtilis* ClpP protein and from a conserved region of all existing ClpP proteins in the databases (5′-CCNCCNGGNSWRTTDATRAANARRTA-3′). The 207 bp PCR fragment was cloned into the *Eco*RV site of pBluescript-II KS+ (pKSclpP1). Another degenerate primer (5′-GGYTGRTGDATCATNACRTC-3′) was generated from an internal amino acid sequence of the *B. subtilis* ClpP protein (H. Antelmann and R. Schmid, unpublished), and a 370 bp *clpP* PCR fragment was obtained. This fragment was also cloned into the *Eco*RV site of pBluescript-II KS+ (pKSclpP2). DNA sequence information, especially from the regulatory and C-terminal region of the *clpP* gene, was obtained using the vectorette PCR (Genosys Biotechnology). *B. subtilis* IS58 chromosomal DNA (100 ng) was digested with *Hin*dIII and ligated to *Hin*dIII vectorette units. PCR using the vectorette primer

and the internal *clpP* primer invclpPI (5′-GTTCGCAACGTTGTCATCAATCGCAGATCC-3′) generated a 2.7 kb product containing the regulatory region of the *clpP* gene. A 1.3 kb PCR product containing the C-terminal coding region of the *clpP* gene, using the vectorette primer and the internal *clpP* primer invclpPII (5′-CCATCGTGTCACAGCTTTTATTCTTAGCAGC-3′), was amplified and cloned into pBluescript-II KS+ (pKSclpP3). A 450 bp PCR fragment containing the entire promoter region of the *clpP* gene could be obtained using the *clpP* promoter primer (5′-GCCGAAGCTTGGCAATCTGACATACGATGG-3′) and an internal primer (5′-CGGGATCCCGGCAGCCATACCGATACAAATTG-3′). The fragment was cloned into the *Hin*dIII and *Bam*HI sites of pBluescript-II KS+ (pKSclpP4) and was used as template for the primer extension analysis. PCR was carried out in a reaction volume of 50 μl with 200 μM dNTPs, 1.5 mM $MgCl_2$, 50 pmol of each primer, 5 μg of chromosomal DNA of *B. subtilis* IS58 and 2.5 units of *Taq* DNA polymerase (Promega) in 1× reaction buffer. The annealing temperature was 50°C using degenerated primers and 5°C below the melting temperature using all other primers. Thirty cycles were performed. PCR products were purified using the gene clean procedure (BIO101) and cloned into the *Eco*RV site of the vector pBluescript-II KS+. DNA sequencing of the recombinant plasmids was performed according to the *Taq* cycle or Sequenase sequencing method (US Biochemical).

Analysis of mRNA transcription by slot-blotting

Total RNA of *B. subtilis* strains was isolated from exponentially growing or stressed cells by the acid phenol method of Majumdar *et al*. (1991), with modifications according to Völker *et al*. (1994). Serial dilutions of total RNA were prepared, transferred onto a positively charged nylon membrane (Hybond N^+; Amersham-Buchler) by slot-blotting and hybridized with a digoxigenin-labelled antisense RNA probe as recommended by the manufacturer (Boehringer Mannheim). The chemiluminograms were quantitated using a personal densitometer (Molecular Dynamics). The induction ratios were calculated by setting the value of the control to 1. Hybridization specific for *clpP* mRNA was conducted with a digoxigenin-labelled RNA probe synthesized *in vitro* with T3 RNA polymerase producing anti-sense RNA from linearized plasmid pKSclpP1. The RNAs synthesized *in vitro* from the coding strand were used as negative controls and did not yield any specific hybridization signal (data not shown).

Mapping of the transcriptional start sites

A primer complementary to the 5′ coding region of *clpP* (5′-GTTCGCAACGTTGTCATCAATCGCAGATCC-3′; 100 ng μl^{-1}) was labelled with 4 units of polynucleotide kinase (Boehringer Mannheim) and 1.85 kBq of [γ-^{32}P]-ATP (111 TBq $mmol^{-1}$; NEN DuPont). Total RNA was isolated from *B. subtilis* IS58 and BGH 1 (Maul *et al*., 1995) before (co) and 12 min after the imposition of stress [heat shock, 48°C; salt stress, 5% (w/v), ethanol treatment, 5% (v/v)]. The labelled primer was annealed to 10 μg of cellular RNA in 10 μl of hybridization buffer (10 mM Tris-HCl, pH 7.9, 1 mM EDTA and 250 mM KCl) at 55°C for 1 h, and the extension reaction was performed in reverse transcriptase buffer [10 mM Tris-HCl, pH 8.7, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.4 mM dNTPs, 20 U ml^{-1} RNase inhibitor (Promega)] using 20 units of AMV reverse transcriptase (Stratagene) at 37°C for 30 min. The samples were precipitated with ethanol, vacuum dried and resuspended in 10 μl of stop mixture (US Biochemical). After heat denaturation, 2 μl samples were immediately loaded onto a 6% polyacrylamide–urea sequencing gel for electrophoresis.

Construction of bgaB *fusions and the determination of the BgaB activity*

BgaB reporter gene fusions to *clpP* were constructed by cloning *Eco*RI–*Bam*HI-digested PCR fragments amplified using *Taq* polymerase and the following primers: (i) TM-196 (5′-GAAGAATTCATACGATGGTTTGAACCCCTG-3′) and TM-187 (5′-GGAGGATCCTTTTAGGTAAGTATGTAG-3′) to generate fragment (1) carrying both σ^B- and σ^A-dependent promoters; (ii) TM-197 (5′-GAAGAATTCAGGCCAGCTTTTTGTTTGACC-3′) and TM-187 (5′-GGAGGATCCTTTTAGGTAAGTATGTAG-3′) to generate fragment (2) carrying only the σ^A-dependent promoter; (iii) TM-186 (5′-GAAGAATTCGACAAGAGCGGCAAATCGCC-3′) and PclpPR primer (5′-GGAGGATCCGTCAAACAAAAAGCTGGCCTG-3′) to generate fragment (3) carrying only the σ^B-dependent promoter into the vector pDL (Yuan and Wong, 1995a).

Recombinant plasmids were linearized by *Pst*I digestion and transformed into *B. subtilis* for integration at the *amyE* site of the chromosome by a double cross-over. Chromosomal *bgaB* fusions in *amyE* were screened for the expression of the thermostable β-galactosidase at 50°C and a deficiency of α-amylase activity. Transformants were plated on LB agar containing 1% (w/v) starch. Starch degradation was detected by pouring iodine onto the plates. BgaB activity was visualized on plates by 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal; 100 μg ml^{-1}) hydrolysis.

Bacteria (strain IS58) carrying the different deletion fragments were grown under selective conditions (5 μg ml^{-1} chloramphenicol) in LB to an optical density of $OD_{540} = 0.4$, then 10 ml of the cells were exposed to heat (30 min at 48°C), and 1 ml samples were harvested from these as well as from the control (37°C) cells. Bacteria were resuspended in 800 μl of assay buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mM $MgCl_2$, 0.27% mercaptoethanol) treated with chloroform (60 μl) and SDS (30 μl, 0.1%). The enzyme reaction was started after 10 min preincubation at 65°C using 200 μl of ONPG (4 mg ml^{-1}). The reaction was stopped using 500 μl of Na_2CO_3, and the activity of BgaB was measured at 420 nm.

Construction of the clpX *insertional mutant*

To construct an insertional mutant with a disruption in the *B. subtilis clpX* gene, a 490 bp PCR fragment (Xfor primer: 5′-CCCAAGCTTGGGGTGACGAATGTATCGAACTCTGC-3′; Xrev primer: 5′-CGGGATCCCGCATCACGTGTGATAGACGGG-3′) was amplified and cloned after digestion into the *Hin*dIII and *Bam*HI sites of the *B. subtilis* integrational vector pMutin4 (Vagner *et al*., 1995). Naturally competent cells of *B. subtilis* IS58 were transformed with about 1 μg of the resulting plasmid pMutin4clpX. Transformants were selected on agar

plates containing 1 μg ml^{-1} erythromycin and 25 μg ml^{-1} lincomycin. Integration of the recombinant plasmid was confirmed by PCR and Southern hybridization.

Preparation of samples for scanning electron microscopy

B. subtilis IS58 wild-type cells together with *clpP* and *clpX* mutants were grown in LB and harvested by centrifugation during exponential growth. Bacteria were washed, resuspended in synthetic medium and applied to small glass slides covered with Alcian blue. Bacteria were fixed for 2 h at room temperature with glutaraldehyde [3% (v/v) in 0.1 M cacodylate buffer, 10 mM calcium chloride, pH 7.4], washed with the same buffer and post-fixed with 2% (v/v) osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4). The samples were dehydrated using a series of ethanol solutions followed by treatment with isoamylacetate and critical point drying. The sputtered samples were observed at 15 kV using a DSM 940A microscope (Zeiss).

Two-dimensional protein gel electrophoresis

B. subtilis IS58 and the isogenic *clpP* and *clpX* mutants were grown at 37°C in LB medium and harvested at an OD_{540} = 1.5. The cells were washed and resuspended in TE buffer containing 65 mM DTT lysed using a French press (20 000 psi cell^{-1}; SLM Aminco). The protein content was determined according to the Bradford assay (Bio-Rad) using BSA as standard. Immobiline dry strips (Pharmacia), IPG 4–7 linear, 180 mm, were used for the separation in the first dimension. A sample of 100 μg of protein was dissolved in 500 μl of rehydration solution [8 M (w/v) urea, 1% (w/v) CHAPS, 0.2% (w/v) DTT and 0.5% (v/v) Pharmalyte 3–10] and incubated together with one IPG strip at room temperature for at least 6 h. Isoelectric focusing was performed at 20°C using a Multiphor II electrophoresis system according to the manufacturer's instructions. SDS–PAGE (12% polyacrylamide) was performed in the second dimension using a Protean II xi Multicell (Bio-Rad). Immobiline dry strips were first equilibrated for 15 min in buffer A [50 mM Tris-HCl, pH 6.8, 6 M (w/v) urea, 30% (v/v) glycerol, 4% (w/v) SDS, 0.35% (w/v) DTT] and then for 15 min in buffer B (same composition as buffer A, but contains bromophenol blue and 4.5% (w/v) iodoacetamide instead of DTT).

Immediately after equilibration, the IPG strips were cut at the pH 7 end to a size of 15.5 cm and fixed on the top of the PAGE gels using 0.8% agarose. The PAGE gels were run overnight at room temperature at 10 mA per gel. After electrophoresis, the gels were stained with a commercially available protein silver-staining kit (Pharmacia) and an automated gel stainer (Hoefer).

For preparative two-dimensional protein electrophoresis, 500–1000 μg of protein extract was separated using the same electrophoresis equipment. N-terminal microsequencing of proteins was carried out after cutting out Coomassie-stained protein spots from 30–40 two-dimensional gels. The collected pieces were blotted after one-dimensional protein electrophoresis onto a polyvinylidene difluoride membrane by electroblotting with CAPS buffer (10 mM CAPS, 20% methanol, pH 10.5), stained with Coomassie and sequenced using an Applied Biosystems 610A protein sequencer.

General methods

Plasmid isolation, restriction enzyme analysis, transformation of *E. coli* competent cells, filling in of recessed 3′ termini, and 5′–3′ exonuclease digestion by Klenow fragment of DNA polymerase I were performed as described by Sambrook *et al*. (1989). Chromosomal DNA from *B. subtilis* was isolated according to Meade *et al*. (1982). Transformation of naturally competent *B. subtilis* cells was carried out by the two-step protocol of Hoch (1991).

Computer analysis of sequence data

The GCG sequence analysis software package (version 7.0), the BOXSHADE program (version 3.21) and the GENE INSPECTOR (Textco) were used for all DNA and protein sequence comparisons.

Nucleotide sequence accession number

These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number U59754.

Acknowledgements

We thank Roland Schmid (University of Osnabrück) and Holger Hippe (TTB Greifswald) for protein sequencing, Jörg Mostertz for the construction of the *B. subtilis clpX* integrational mutant, Holger Ludwig for his help in performing two-dimensional protein gels, Veronika Grapentin, and especially Annette Tschirner for her excellent technical assistance. Furthermore, we are grateful to Renate Hanschke and Hartmut Fischer for support in electron microscopic studies. We thank David Dubnau (Public Health Research Institute, NYC, USA) for helpful advice, Uwe Völker (University of Marburg) and Moira Messenger (University of Western Ontario, Canada) for critical reading of the manuscript, and Georges Rapoport, in whose laboratory part of this work was carried out. This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie to M.H.

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