3. Results and Discussion

3.1. A tyrosine kinase and its activator control the activity of the CtsR heat shock repressor in *B. subtilis*

Janine Kirstein*, Daniela Zühlke, Ulf Gerth, Kürsad Turgay and Michael Hecker

(2005) The EMBO Journal 24, 3435-3445

* corresponding author

Previous studies demonstrated that all four genes of the *clpC* operon (*ctsR*, *mcsA*, *mcsB* and *clpC*) are involved in the regulation of CtsR. Although it was shown that McsA and McsB exhibit an effect on the DNA binding ability of CtsR (Krüger et al., 2001), the regulation and the underlying mechanisms are poorly understood. Therefore, we conducted a detailed genetic and biochemical analysis to study the precise role of both modulators, McsA and McsB, on the activity of CtsR and hence the regulation of the class III heat shock regulon.

McsB is a protein kinase and CtsR is a bona fide phosphorylation substrate

The protein sequence of McsB exhibits a domain (119 - 253 aa) with high similarity to ATP:guanidino phosphotransferases, originating from eukaryotic phosphagen kinases (Fig. 6). Based on a McsB dependent CtsR modification a potential phosphorylation activity of McsB was already proposed (Krüger et al., 2001). However, a kinase activity of McsB could not be demonstrated yet.

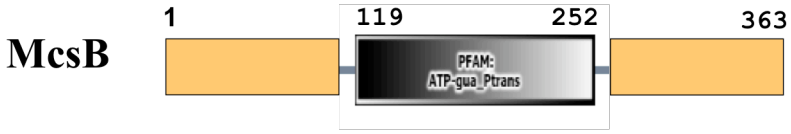

Fig. 6 McsB contains a domain (119 – 252 aa), which is highly conserved among ATP:guanidino phosphotransferases

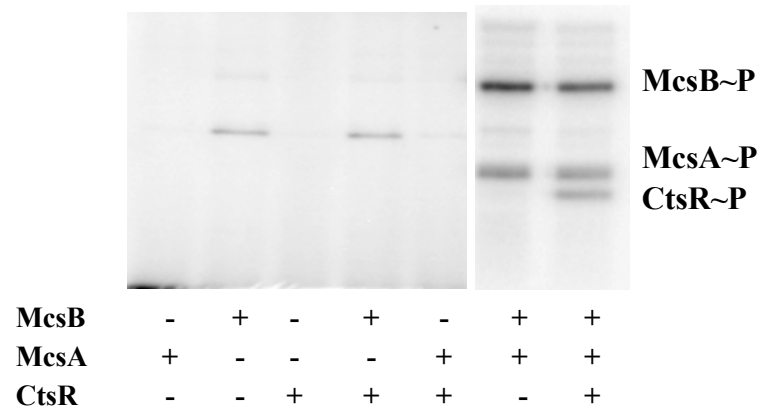

Fig. 7 McsB is a protein kinase, which is stimulated by McsA and phosphorylates CtsR.

We therefore established an *in vitro* phosphorylation assay and could indeed demonstrate kinase activity for McsB. We found that the kinase activity of McsB requires the activation by McsA and results in an autophosphorylation and the phosphorylation of McsA (Fig. 7). In order to verify the previous observation of a post-translational modification of CtsR and to validate its proposed McsB-dependent phosphorylation, CtsR was added into this kinase assay. Once activated by McsA, McsB is enabled to phosphorylate CtsR, which itself, unlike McsA, is not capable to activate the kinase and could be regarded as a bona fide substrate of the McsB kinase (Fig. 2).

McsB utilizes a guanidino phosphotransferase domain for its kinase activity

Although the kinase domain of McsB (119-253 aa) is homologous to guanidino phosphotransferase domains (see below) our biochemical analysis revealed phosphorylation on tyrosine residues. We determined the phosphorylated amino acid of McsA and McsB first by accessing the stability of the phosphorylation by treatment with stringent alkaline, acid and heat conditions. The stability of the phosphorylation under these conditions is consistent with a phosphorylation on a hydroxyamino acid. To dissect between P-Ser, P-Thr and P-Tyr we carried out a two-dimensional thin-layer chromatography analysis of the hydrolyzed amino acids of phosphorylated McsA and McsB. The migration pattern of the phosphoamino acid revealed a

phosphorylation on tyrosine residues for both proteins. In line with the assumption of a phosphorylation on tyrosines is the specific dephosphorylation by the tyrosine phosphatase YwlE. Secondly, we mapped the phosphorylated tyrosine residue by site directed mutagenesis against the structural homologue, phenylalanine. The subsequent *in vitro* analysis revealed that McsB gets phosphorylated on residues Y155 and Y210, both located within the putative kinase domain, and McsA on both of its two tyrosine residues, Y40 and Y103.

Interestingly, McsB shares structural and functional properties of prokaryotic tyrosine kinases such as its autophosphorylation activity and the necessity of an activator protein, as well. But it lacks the conserved P-loop/Walker motif for the ATP binding. Instead, the kinase domain of McsB is highly homologous to arginine/creatine kinases. A structural model of the McsB kinase domain based on the homologous guanidino kinase domain of different arginine and creatine kinases displays all key residues and structural elements (Fig. 8).

Fig. 8 Structural model of the McsB kinase domain (119 – 252 aa) based on homologous guanidine kinase domains of several arginine and creatine kinases (PDB code 1bg0, 1m15, 1p50, 1p52, 1qk1 and 1rl19) and an alignment of the guanidine kinase domain of *L. polyphemus* (KARG) with the McsB kinase domain. The key residues of the catalytic mechanism are highlighted.

These include C271 (in the arginine kinase nomenclature), the NEED segment (223-227 aa), the flexible loop (309-320 aa) and arginines R229, R280 an R309. The activity of arginine kinases is thought to be controlled by a conformational switch (induced fit) upon binding of the substrate arginine and the nucleotide (Yousef et al., 2002; Zhou et al., 1998). The substrate contacts E314 of the flexible loop, E225 of the NEED segment and C271 upon binding of ATP, which is stabilized by the positively charged conserved arginine residues. Y210 of McsB is located within the flexible loop region, whereas Y155 is located on the other side of the ATP-binding domain with C271 and the NEED loop in between. The mutational analysis of these key residues demonstrated that McsB utilizes indeed this domain for its kinase activity. Since the kinase activity of McsB relies on the activation by McsA one could speculate about a similar induced fit model involving a conformational switch from the open into the closed state upon binding of ATP and McsA. Such a conformational change might then also result in a delocalization of Y155 and Y210 to become accessible as substrates. In summary, McsB can be regarded as a new kind of tyrosine kinase utilizing the ATP-binding and γ-phosphate transfer activity of the guanidino kinase domain of arginine and creatine kinases.

CtsR-P does not act as repressor

To address the effect of the phosphorylation of CtsR we analyzed its repressor activity. CtsR binding sites are present in varying copies within the promotor regions of the monocistronic genes *clpP* and *clpE* and the *clpC* operon. We could observe that the DNA binding ability of CtsR decreased under conditions, which allowed a phosphorylation of CtsR. This led to two possible explanations: (i) CtsR-P does not exhibit any DNA binding ability and / or (ii) CtsR-P is sequestered by McsB-P more efficiently than by McsB. Although we cannot exclude the first explanation, our data supports the latter one. We tested a phosphorylation mimicking variant of McsB (see below), McsBY155E, in the DNA gel retardation assay and could show that this mutant protein could diminish the DNA-binding ability of CtsR as efficiently as wt McsB in the presence of McsA and ATP. To confirm the tight interaction with CtsR we used an *in vivo* as well as an *in vitro* approach and could prove the formation of a ternary complex which centers McsB interacting with both proteins, its activator McsA and its substrate CtsR.

Phosphorylation-dependent turnover of CtsR at 50 °C

How does the cell ensure a sufficient de-repression, which allows the expression of the heat shock genes once CtsR is released from the DNA? An accumulation of CtsR in a Δ*clpC* and Δ*clpP* background upon heat shock led to the hypothesis of a proteolytic control (Krüger et al., 2001). Proteolysis is indeed an elegant way to remove the repressor from the equilibrium and thereby to prevent a rebinding and re-repression of the CtsR regulon. We therefore tested the *in vivo* stability of CtsR and could demonstrate that CtsR is stable under normal growth temperatures, which ensures a repression of the class III heat shock genes under these conditions. However, CtsR got rapidly degraded upon heat shock, which was completely abolished in a *mcsA* and *mcsB* mutant background. Taken together, these data strongly support the assumption that the phosphorylation of CtsR is crucial for its degradation.

ClpC mediated regulation of the kinase activity of McsB

Since the fate of CtsR is controlled by McsB, the kinase activity of McsB itself is assumed to be a subject of regulation. The genetic organization of the *clpC* operon including the translational coupling of *mcsA-mcsB-clpC* already suggested an interaction of both modulators with ClpC. Thus, we analyzed the effect of ClpC on McsB and surprisingly ClpC inhibited the kinase activity of McsB. Given that McsB is not the only interaction partner of ClpC we asked if a potential competition with other ClpC interacting proteins might be an integral part in the regulatory circuit of the ClpC mediated control of the McsB kinase activity. ClpC forms together with the adaptor protein MecA a bipartite chaperone complex. Either alone or in conjunction with ClpP they are involved in the disaggregation, refolding and / or degradation of misfolded and aggregated protein species. The accumulation of aggregated proteins upon heat shock could therefore result in a competition between McsA/McsB and MecA for binding to ClpC, substantiating a titration model. To test this hypothesis *in vitro* we included MecA and the model substrate for unfolded proteins, α-casein, into a kinase assay to test for a release of sequestered McsB and subsequently a regain of kinase activity. Indeed, we could observe a MecA and unfolded protein mediated relief of inhibition and subsequent activation of the phosphorylation cascade (Fig. 9 and 10).

The phosphatase YwlE counteracts the kinase activity of McsB

However, the kinase activity of McsB seems to be regulated by at least one additional mechanism. We identified the cognate phosphatase YwlE, a low molecular weight tyrosine phosphatase, which specifically dephosphorylates McsB, McsA and CtsR. Interestingly, YwlE seems not to be co-regulated with its partner kinase. Its presumed constitutive expression suggests a role as a potential shutoff mechanism, leading to a re-repression of the CtsR regulon with cessation of the heat shock conditions (see below).

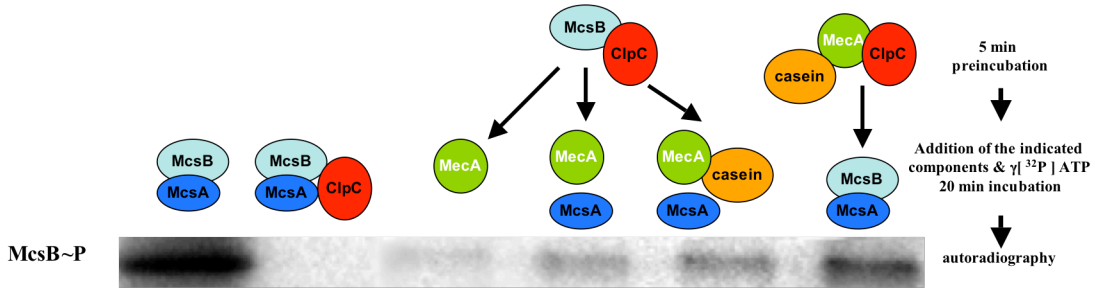

Fig. 9 Regulation of the McsB kinase activity. The negative influence of ClpC could partially be reversed in the presence of competing ClpC substrates.

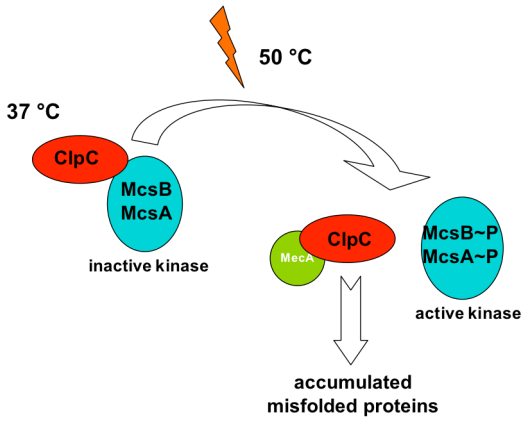

Fig. 10 Titration model of the ClpC governed regulation of the McsB kinase activity.

3.2. Adaptor protein controlled oligomerization activates the AAA+ protein ClpC

Janine Kirstein*, Tilman Schlothauer*, David A. Dougan, Hauke Lilie, Gilbert Tischendorf, Axel Mogk, Bernd Bukau and Kürsad Turgay (2006) The EMBO Journal 25, 1481-1491

* These authors contributed equally

ClpC differs from all other Hsp100/Clp-ATPases in that it requires an adaptor protein for virtually all its activities. We wanted to elucidate the underlying mechanism of the adaptor protein mediated activation of ClpC.

Previous studies revealed that the presence of the adaptor protein MecA was a prerequisite for the induction of the ATPase activity of ClpC. A correlation of ATPase activity and oligomerization could be demonstrated for homologous Hsp100/Clp proteins (Hattendorf and Lindquist, 2002; Mogk et al., 2003; Singh and Maurizi, 1994). The structural features of this AAA+ superfamily substantiate the interrelation of hexamerization and ATPase activity. First, ATP binding occurs at the interface between two subunits within the hexamer of an AAA+ protein. It thereby brings the residues from the ß-sheet of the N-terminal domain and those contributing from the C-terminal domain in close proximity with the adjacent subunit. Secondly, a conserved arginine triggers as arginine finger the conformational changes upon nucleotide binding in the neighbouring subunit of the hexamer.

MecA activates ClpC by facilitating its oligomerization

To address the oligomeric state of ClpC and its dependence on MecA, we first analyzed the assembly of ClpC by size exclusion chromatography using varying conditions (buffer, temperature, column material) in the absence or presence of MecA and / or nucleotides. We could not detect any oligomeric form when analyzing ClpC alone. The addition of MecA led to a nucleotide-independent heterodimer formation. A higher oligomeric form could not be detected. One explanation could be that the interaction is only transient, dissociating upon ATP hydrolysis. To circumvent this

instability, we took two approaches (i) chemical cross-linking of ClpC and MecA and (ii) construction of a double Walker B mutant of ClpC to freeze the ATP-bound state. In either way, we could indeed detect a high molecular weight complex by size exclusion chromatography in the presence of ATP, ADP and ATPγS. The subsequent analysis of the eluted fractions revealed the presence of both, ClpC and MecA, in this assembled complex, suggesting that MecA does not only trigger the oligomerization, but also participates in this high molecular weight complex. An analysis of the molecular weight by multi angle light scattering revealed a size of the whole complex of about 769 kDa, which is consistent with the assumption of a ClpC hexamer interacting with six MecA monomers. In line with this result are the previous data regarding ATPase and chaperone activity of ClpC, exhibiting the highest activity in a 1:1 ratio with MecA (Schlothauer et al., 2003).

To study the complex formation of ClpC and MecA under equilibrium conditions we set out an analytical ultracentrifugation analysis. The equilibrium sedimentation experiments could demonstrate a stably association of ClpC and MecA in the presence of ADP or ATP. The radial distribution could be interpreted as a single homogeneous species with an apparent molecular weight of 747 +/- 19 kDa, which is consistent with the static light scattering data of the ClpC-DWB-MecA complex. In addition to the analytical gel filtration and ultracentrifugation experiments an inspection of negative staining EM images revealed distinct particles resembling in size and shape those of other oligomeric Hsp100/Clp proteins. To summarize, MecA facilitates and participates in the nucleotide-dependent formation of a functional homogeneous ClpC hexamer.

The assembled complex of ClpC and MecA constitutes the active substrate-binding species of this chaperone complex

We further aimed to test for substrate interaction of this assembled chaperone complex. Figure 11 shows the stable association of the substrate ComK with ClpC-DWB and MecA in the presence of ATP by size exclusion chromatography. Similar results were obtained using α-casein as substrate. Notably, (i) no substrate association could be detected in the absence of MecA and (ii) the substrate itself did not facilitate the oligmoerization and thereby activation of ClpC. Taken together, the formation of

the assembled ClpC-MecA chaperone complex is a prerequisite for substrate interaction and constitutes the functional substrate-binding species of this chaperone system.

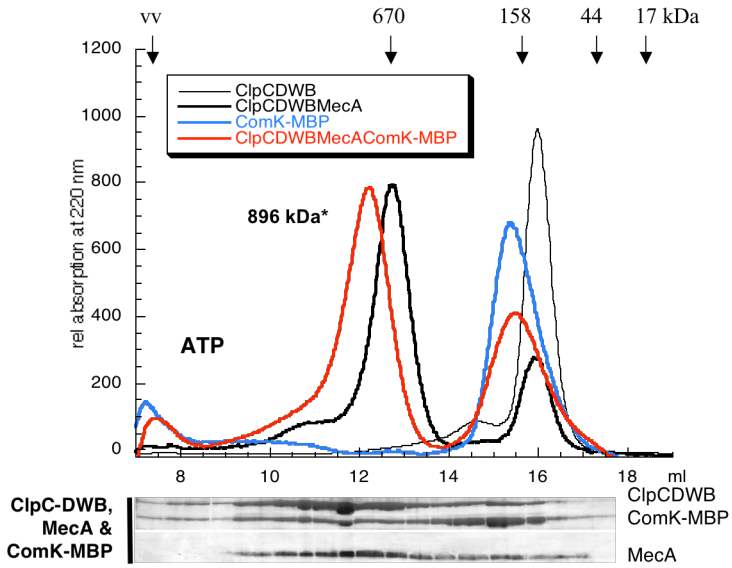

Fig. 11 Substrate interaction of the assembled ClpC-DWB-MecA complex.

The NTD and the Linker domain of ClpC are the interaction sites for MecA

Next, we sought out to determine the interaction sites of MecA on ClpC. The MecA induced ATPase activity of single Walker B ClpC mutants indicated that ATP hydrolysis occured dominantly in the first AAA module. In the presence of ATP only ClpC-WB1 and not ClpC-WB2 could form the stable oligomeric complex with MecA, suggesting that MecA binds to or closely associated to the first AAA domain. Two accessory domains, the NTD and the Linker domain, are directly associated with the first ATPase module and were therefore considered as potential candidates for MecA binding (Fig. 12). Notably, such accessory domains account for the variability regarding their activity in diverse cellular processes, localization and interaction partners. For the latter it could be shown that the NTD of ClpA and ClpX were the binding sites of their adaptor proteins (Dougan et al., 2002a; Dougan et al., 2003; Wah et al., 2003; Zeth et al., 2002). The Linker domain is a unique feature of the ClpC superfamily and therefore became an attractive target for the interaction with adaptor proteins, too.

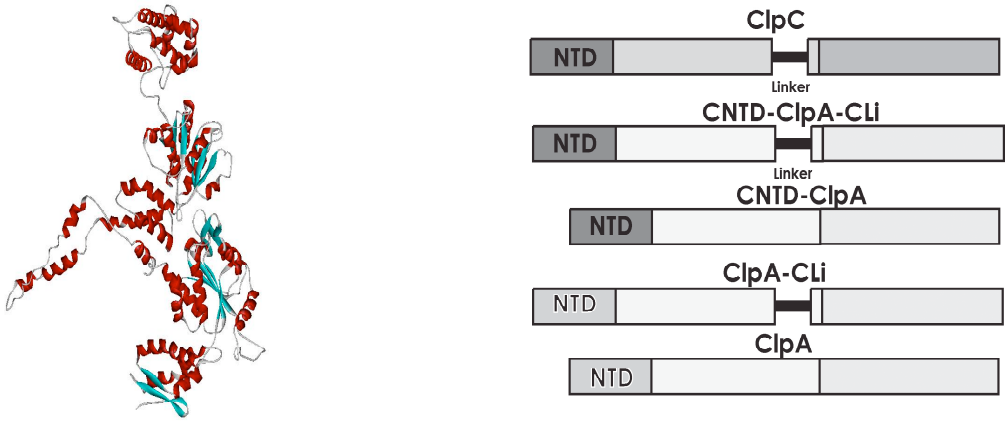

Fig. 12 Structural model of ClpC on the structure of the related HSP100/Clp protein ClpB from *T. thermophilus* (left). Schematic picture of ClpC, ClpA and the ClpA-ClpC hybrid proteins (right).

Indeed, deletion variants of ClpC lacking either the NTD or the Linker domain displayed severe binding defects and subsequently the ATPase activity was no longer inducible by MecA. To verify the role of these two domains as binding sites for MecA, we constructed hybrid proteins, where ClpA, a homologous HSP100 protein from *E. coli*, served as scaffold. (Fig. 12). The addition of MecA stimulated the ATPase activity of the single domain fusion proteins by 30 % and by 70 % for the double hybrid. Direct interaction measurements by surface plasmon resonance confirmed the results of the ATPase activity data, demonstrating an interaction with all hybrid constructs. However, the interaction strength varied from a rather weak interaction with ClpA-CLi to a strong binding with CNTD-ClpA. The interaction with the double construct, CNTD-ClpA-CLi was as strong as with wt ClpC.

Next, we tested the ability of those fusion constructs to support the degradation of MecA and as a next step the MecA-dependent degradation of a substrate protein. As depicted in Fig. 13 the double hybrid protein could degrade α-casein as efficient as wt ClpA, but the presence of both domains enabled now also the recognition and subsequent degradation of MecA by this fusion protein. Although the substrate ComK could be degraded in a MecA dependent manner by the double hybrid, too, it was not as efficient as by wt ClpC. Obviously, the more complex degradation of substrates

requires additional contributions by the AAA modules of ClpC, which are not present in ClpA. Nevertheless, these results clearly demonstrate that the NTD together with the Linker domain of ClpC was sufficient for recognition, targeting and subsequent degradation of MecA and partially also of substrate proteins.

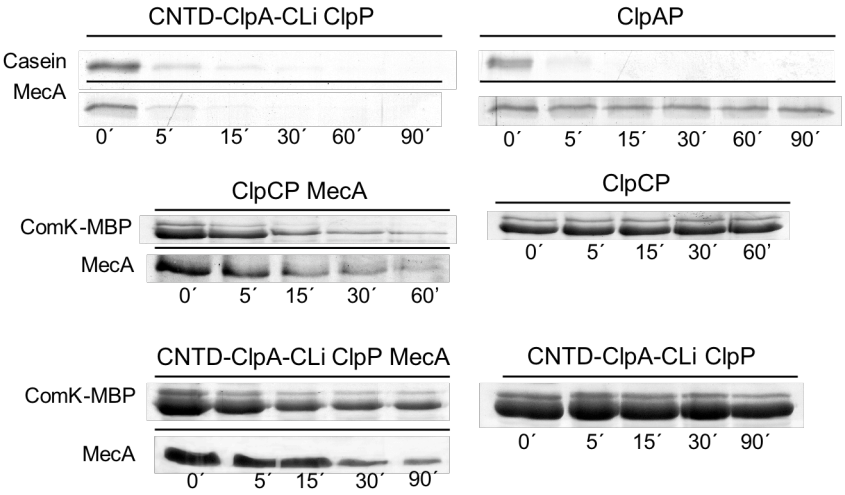

Fig. 13 The NTD and the Linker domain are sufficient for recognition and targeting and subsequent degradation of MecA and the substrate, ComK.

MecA is necessary for the formation of the whole proteolytic ClpCP complex

Previous *in vivo* and *in vitro* data demonstrated the functional interplay between ClpC and ClpP. However, a direct interaction of ClpC and ClpP was not shown, yet. A ClpP-mediated assembly of ClpC, by providing a structural platform could be ruled out (Fig. 14). The more so as we could show that *B. subtilis* ClpP exhibits a unique feature in that it exists solely in its monomeric form. However, once ClpC is hexamerized by MecA it triggers the subsequent assembly of ClpP resulting in the formation of the whole proteolytic complex: ClpCP-MecA (Fig. 14). This hierachical regulation of ClpCP mediated proteolysis by an adaptor protein controlling initially the oligomerization of the cognate ATPase component and the subsequent assembly of ClpP is summarized in Fig. 15. The activation of ClpC by an adaptor protein is an elegant and effective way not only to control its activity, but also to set its substrate specificity. The complete degradation of a certain substrate will subsequently result in the turnover of the adaptor protein, too, leading to the disassembly of ClpC. Another

adaptor protein with e.g. a different substrate repertoire is then enabled to take over to assemble and activate ClpC for its own purpose.

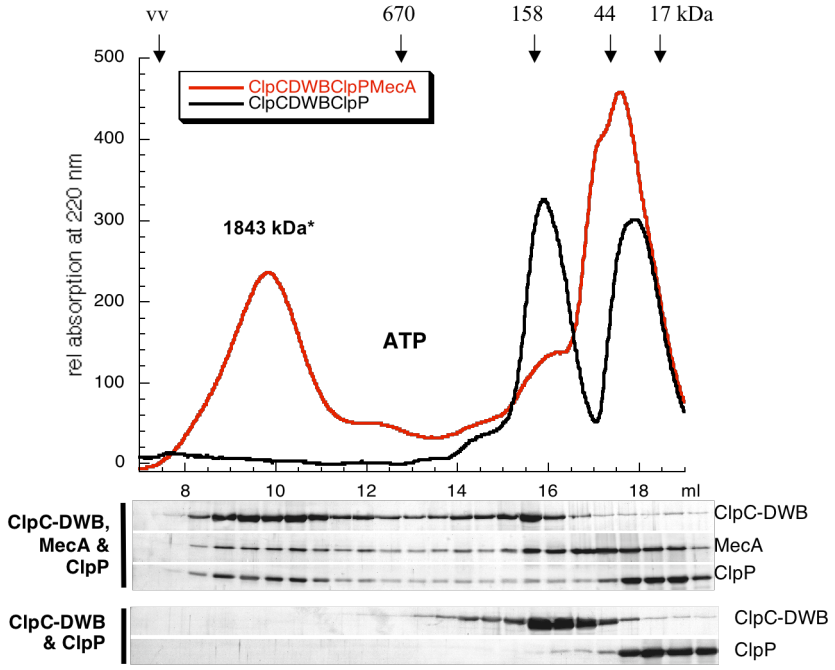

Fig. 14 The assembly of the protease ClpCP requires MecA. The MecA-mediated ClpC hexamerization precedes the formation of the functional proteolytic ClpCP complex.

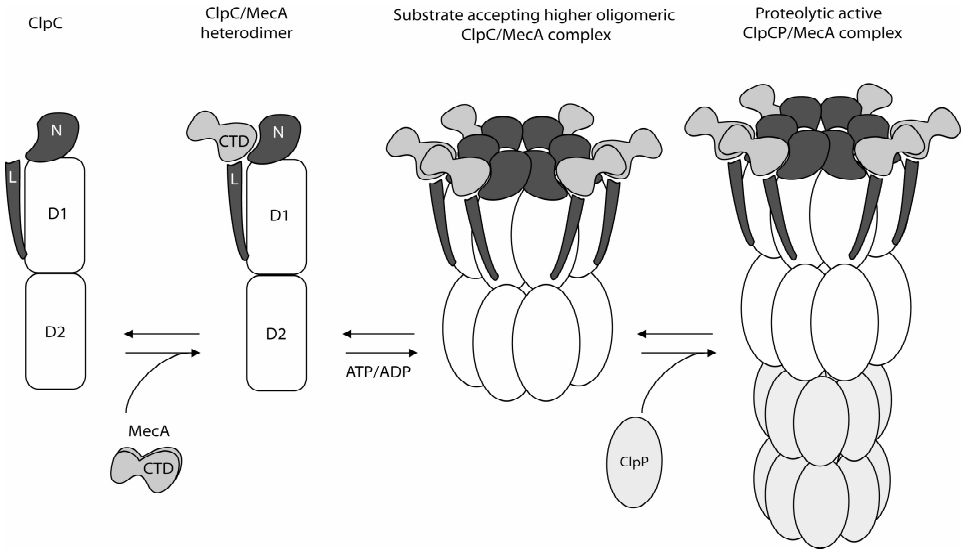

Fig. 15 Model of the adaptor mediated ClpCP activation

3.3. Cyanobacterial ClpC/HSP100 protein displays intrinsic chaperone activity

Fredrik I. Andersson, Robert Blakytny, Janine Kirstein, Kürsad Turgay, Bernd Bukau, Axel Mogk and Adrian K. Clarke (2005) The Journal of Biological Chemistry 281, 5468-5475

The main objective of this study was the characterization of the functional properties of a photobiontic ClpC. Although Clp proteins are highly conserved and found throughout all phyla, those of photosynthetic organisms are higher in number and diversity. ClpC of the cyanobacterium *Synechococcus elongatus* differs from its orthologs e.g. of *B. subtilis* in that it is essential for cell viability even under normal growth conditions.

Cyanobacterial ClpC exhibits autonomous chaperone activity but might employ an adaptor protein as well

This study is the first report demonstrating chaperone activity for a ClpC homolog of a photosynthetic organism. Importantly, *Synechococcus elongatus* ClpC (SyClpC) displays both, holding (prevention of aggregation) and disaggregation activity. Moreover, for its chaperone activity SyClpC does not require the interaction with a co-chaperone as it is the case in *E. coli* for the functional cooperation of ClpB-DnaK nor does it need the activation by an adaptor protein. SyClpC differs most from *B. subtilis* ClpC (BsClpC) with respect to its basal ATPase activity, which is already as high as those of *E. coli* ClpA and ClpB. Nevertheless, it could be demonstrated that the addition of *B. subtilis* MecA (BsMecA), stimulated not only the basal ATPase rate of SyClpC 3 fold, but led also to an enhanced disaggregation and refolding activity. Surprisingly, a structural MecA ortholog does not exist is cyanobacteria or plants. Nonetheless, its specific interaction and the stimulatory effect of BsMecA on the chaperone activity of SyClpC argue for the presence of a functional equivalent. This role could be fulfilled by ClpS, an ortholog to another adaptor protein, ClpS, that exists also in photosynthetic organisms. However, despite a strong interaction, SyClpS was unable to stimulate the ATPase or chaperone activity of SyClpC. SyClpS might nevertheless display adaptor activity e.g. via substrate recognition. The interrelation between SyClpC and SyClpS1 is subject of an ongoing project in the group of Adrian Clarke, Göteborg University (Sweden).

In summary, this study demonstrated that even though ClpC homologs are highly conserved, they differ markedly regarding their interplay with adaptor proteins and emphasizes the need for further studies comparing different ClpC homologs.

3.4. The tyrosine kinase McsB is a regulated adaptor protein for ClpCP

Janine Kirstein, David A. Dougan, Ulf Gerth, Michael Hecker and Kürsad Turgay (2007) The EMBO Journal 26, 2061-2070

In the previous chapter (3.1.) it could be shown that (i) CtsR degradation depends on McsB *in vivo* (ii) McsB interacts with both CtsR and ClpC and (iii) McsB competes with the ClpC adaptor MecA for binding to ClpC. These data raised the question whether McsB could act as adaptor protein for ClpC as well (Kirstein et al., 2005).

The ClpCP mediated degradation of CtsR depends on McsA and McsB *in vivo* and *in vitro*

First, to confirm the interaction of McsB with the ClpCP protease and CtsR *in vivo* we carried out a co-immunoprecipitation using McsB antibodies with the soluble extracts of cells grown at 37 °C and 50 °C. The subsequent western blot analysis verified the interaction of McsB with its activator McsA, the proteolytic components, ClpC and ClpP, and CtsR. Importantly, only the co-precipitated amount of CtsR was enriched under heat shock conditions, reflecting those conditions when CtsR is subject for degradation. Second, we could demonstrate by pulse chase analysis that *in vivo* ClpCP is indeed necessary for the degradation of CtsR. Therefore, we set out an *in vitro* degradation assay to reconstitute the degradation of CtsR with the purified components. As depicted in Fig. 16 CtsR gets degraded, but its proteolysis requires not only the presence of ClpCP and McsB but also the McsB kinase activating protein McsA. Either the turnover of CtsR requires its phosphorylation by McsA and McsB or McsB exhibits only in its phosphorylated state the ability to target CtsR for degradation (see below). Notably, McsB, like the known adaptor protein MecA, is subject for degradation as well, but is stabilized in the presence of its substrate CtsR, too.

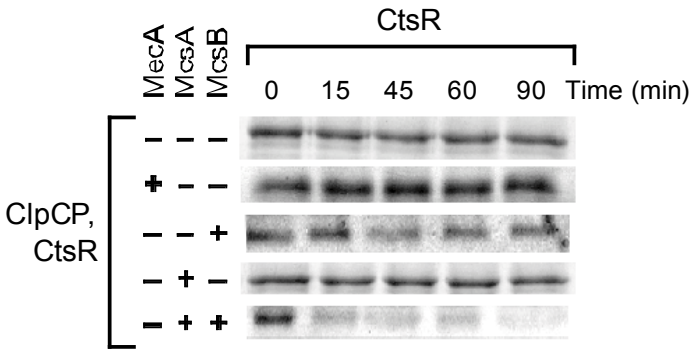

Fig. 16 The ClpCP mediated degradation of CtsR depends on the presence of both modulators, McsA and McsB

McsB is an adaptor protein for ClpC

The result of the *in vitro* degradation assay strongly supported the hypothesis that McsB is an adaptor for ClpC. One hallmark of ClpC adaptor proteins is its ability to induce the ClpC ATPase activity. The addition of McsB alone led only to a moderate increase of the ClpC ATPase activity. However, the additional presence of McsA resulted in a stimulation of the ATPase comparable to the induction by MecA (Fig. 17). In line with that, in the absence of the substrate CtsR, McsB got degraded by ClpCP much faster when McsA was added, too. In sum, McsB-P is indeed a ClpC adaptor protein and the induction of the ATPase implies that McsB-P, analogous to MecA, also facilitates the oligomerization of ClpC. It thereby enables its ATPase and in conjunction with ClpP its proteolytic activity.

McsB exhibits adaptor activity only in its kinase-on state

Both, the *in vivo* and *in vitro* data demonstrated that the adaptor properties of McsB depended on the additional presence of McsA. This raised the question whether the kinase activity of McsB is directly linked to its function as an adaptor for ClpC. To address this issue we first tested the effect of the addition of the cognate phosphatase YwlE on the ClpC ATPase induction by McsA and McsB. As depicted in Fig. 17 the presence of YwlE almost completely abolished the ATPase activity. Consequently, its

presence in an *in vitro* degradation assay resulted in a stabilization of McsB (Fig. 18). We could show that YwlE directly affects McsB: (i) YwlE did not interfere with any ClpC-MecA activity and (ii) we could demonstrate a direct interaction with McsB *in vitro* by surface plasmon resonance.

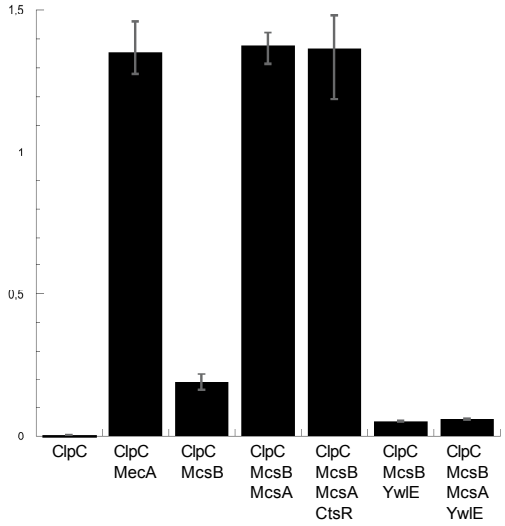

Fig. 17 The kinase activity of McsB is vital for its adaptor function

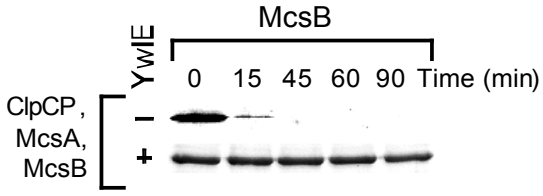

Fig. 18 The dephosphorylation by YwlE stabilizes McsB in an *in vitro* degradation assay

Next, we tested various kinase deficient mutants of McsB (Kirstein et al., 2005) in both assays and as expected, none of them could induce the ClpC ATPase nor did they get degraded, despite the presence of McsA. Taken together, the adaptor function of McsB depends on its kinase activity and thereby its phosphorylation state.

Phosphorylation of CtsR is necessary but not sufficient for its degradation by ClpCP

The McsB dependence of the turnover of CtsR by ClpCP allows two alternative conclusions: (i) McsB is together with McsA only required to phosphorylate and thereby to label CtsR for degradation or (ii) McsB displays beyond the phosphorylation of CtsR also targeting abilities and hands the phosphorylated CtsR over to ClpCP, too. Whereas the latter assumption suggests that McsB fulfills both roles, the labeling and the targeting of CtsR, the former predicts an autonomous recognition of CtsR-P by ClpC. To dissect between these two possibilities, we set out an *in vitro* phosphorylation assay of McsA, McsB and CtsR and subsequently separated CtsR-P from both modulators by size exclusion chromatography. Notably, CtsR-P did not induce the ATPase activity of ClpC, demonstrating that the phosphorylation did not enable CtsR to activate ClpC. We then tested for degradation of CtsR-P by (i) ClpCP alone, (ii) ClpCP + CTD of MecA to test for autonomous substrate recognition of the assembled ClpCP protease, (iii) ClpCP + MecA to test for non-specific substrate recognition and targeting of CtsR-P by MecA and (iv) ClpCP + McsB + McsA to test for specific targeting by McsB-P. As depicted in Fig. 19, CtsR-P is only degraded in the presence of McsB and McsA. Obviously, the phosphorylation of CtsR is necessary, but not sufficient to get degraded solely by ClpCP. Thus, McsB-P is not only required for phosphorylation of CtsR, but also for its subsequent targeting to ClpCP.

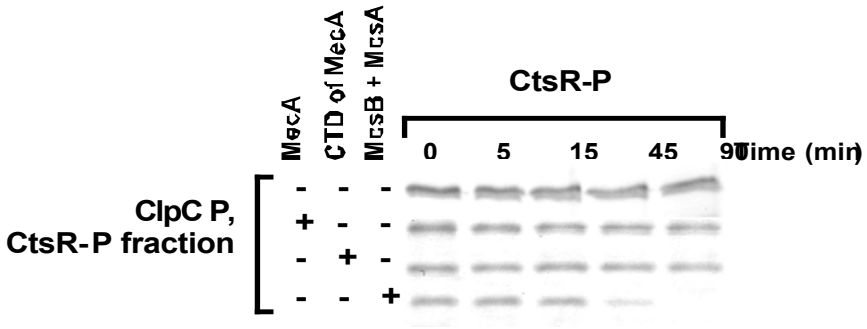

Fig. 19 The degradation of CtsR requires besides its phosphorylation the targeting function of McsB-P

McsB uses the same interaction sites as MecA

Since McsB could compete with MecA for binding to ClpC (Kirstein et al., 2005) and can be regarded as an adaptor protein, we asked whether McsB uses the same binding sites on ClpC. Initially, we analyzed the interaction with the ClpC deletion variants lacking the NTD (ClpC-ΔNTD) and the Linker domain (ClpC-ΔLi). In comparison to full-length ClpC, the absence of the Linker domain resulted in an impaired but much stronger interaction than the deletion of the NTD. In contrast to MecA, the ATPase of ClpC-ΔLi could be still induced by McsB in the presence of McsA (Fig. 20). Moreover, CtsR could be degraded with the same efficiency as full-length ClpC. On the other hand, the interaction of McsB with ClpC-ΔNTD was too weak to support a significant induction of the ATPase activity and resulted only in a very moderate CtsR turnover. Consistent with these findings, the ATPase activity of ClpC-WB1 was more affected than the mutation of the Walker B motif in the second AAA module, because both, NTD and Linker domain, are associated to the first ATPase domain (Fig. 20). Moreover, using the ClpA-ClpC hybrid constructs (Kirstein et al., 2006), we could confirm that McsB interacts with the same domains as MecA, but predominantly with the NTD.

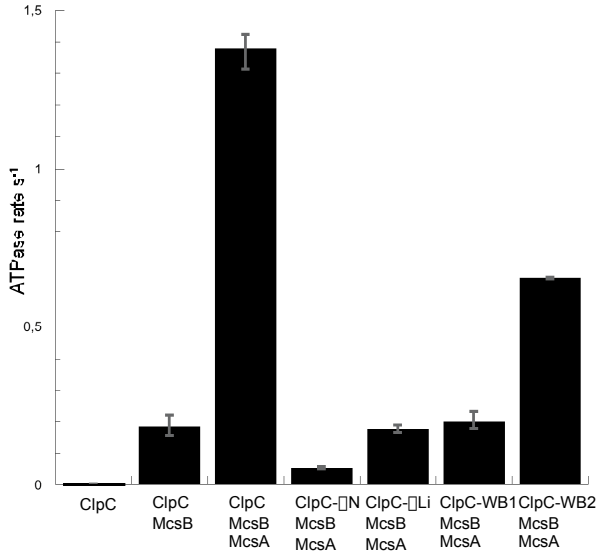

Fig. 20 Induction of the ATPase activity of ClpC and its variants by McsB/McsB-P

Adaptor proteins compete for binding to ClpC

Guided by the result that both adaptor proteins use the same binding sites on ClpC we asked if the formation of a mixed oligomer, consisting of a ClpC hexamer interacting with the two different adaptor proteins might be possible. For that, we compared the degradation kinetics of MecA and McsB separately and in a mixed sample (Fig. 21). Using equimolar concentrations both adaptor proteins were degraded in the mixed sample as fast as alone. However, the additional presence of McsA led to a stabilization of MecA, which could only be reversed by replacing McsB with the kinase deficient McsBY210E. Likewise, MecA could target α-casein for degradation in the presence of equimolar concentrations of McsB, but the further addition of McsA resulted in a stabilization of α-casein, too. This inhibition could be overcome by increasing the amount of MecA about 3 fold, indicating that the interaction of ClpC with its adaptor proteins is reversible (Fig. 22). Such equilibrium of ClpC interacting either with McsB or MecA was already observed and led to a titration model of the class III heat shock regulation (Kirstein et al., 2005).

These results demonstrate that (i) phosphorylated McsB exhibits a higher affinity for ClpC than the non-phosphorylated protein and (ii) McsB-P can certainly out-compete MecA. A concerted action of different adaptor proteins towards one ClpC hexamer is therefore rather unlikely. The competition might be based on the interaction with the NTD of ClpC, which is the major binding site for both adaptor proteins.

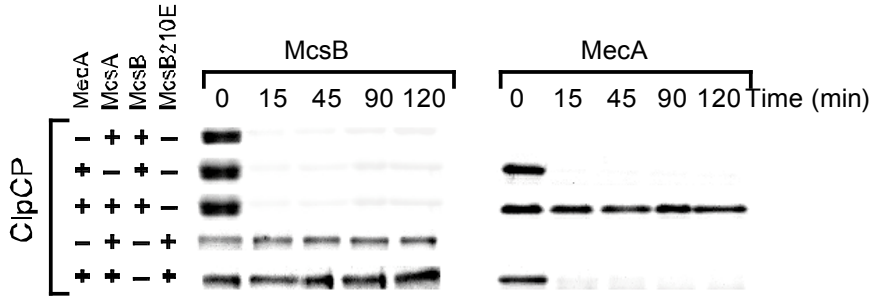

Fig. 21 Competition between the adaptor proteins MecA and McsB - McsB-P can outcompete MecA.

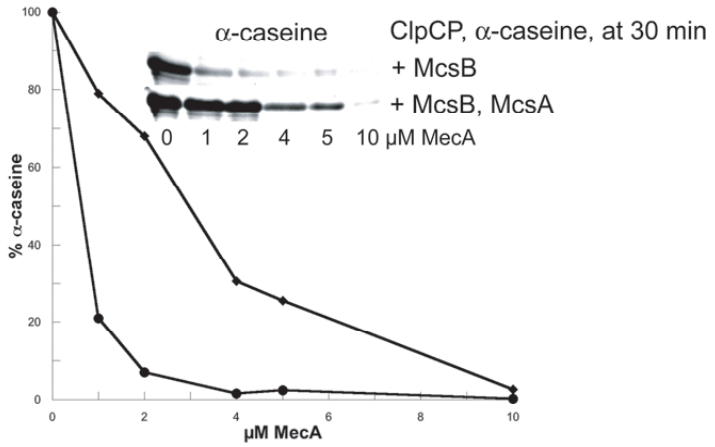

Fig. 22 A 3 fold higher amount of MecA is necessary to allow a degradation of the MecA substrate, a-casein

Extended model of the CtsR-mediated heat shock regulation

The analysis of the adaptor properties of McsB in this manuscript allowed us now to propose a more comprehensive model for the regulation of the class III heat shock regulon (Fig. 23). The presence of different adaptor proteins competing for binding to ClpC builds the center of this regulatory circuit: McsB interacts in two different modes with ClpC. (i) The interaction of ClpC with the non-phosphorylated McsB, which presumably occurs at 37 °C sequesters McsB and thereby keeps the kinase inactive. (ii) The interaction of the phosphorylated McsB with ClpC represents the adaptor active ClpC-McsB species.

The switch from the first into the second interaction mode involves an initial release of ClpC from McsB. Our data suggest that such a release could be due to a competition of McsB with other adaptor proteins for ClpC binding. We could observe a slightly higher affinity of MecA for ClpC when bound to the model substrate for unfolded proteins, α-casein (Kirstein et al., 2005). Indicating that the ClpC-MecA interaction occurs predominantly upon accumulation of unfolded proteins and that MecA gains thereby a sensor function for protein misfolding upon heat shock. This

fits well with the observation that the ClpCP-McsA/B-mediated degradation of CtsR occurs only under heat shock conditions.

The kinase activity of McsB is crucial for the proteolysis of CtsR (i) by phosphorylation and thereby labeling of the substrate and (ii) to exhibit its adaptor function. McsB-P requires a high affinity towards ClpC in order to compete with other adaptor proteins to allow the targeting of CtsR-P. We could demonstrate that McsB-P can indeed out-compete MecA and also the third known adaptor protein, YpbH (unpublished data). The interaction of McsB-P together with its substrate CtsR (+/- McsA) and ClpC must certainly differ from the sequestering interaction in its non-phosphorylated state. However, whether the extent of the interaction with the single NTD and Linker domain of ClpC varies in both modes remains to be elucidated. Likewise, one can assume that different domains of McsB might be involved in the interaction with ClpC depending on its phosphorylation state. The assumed induced fit model of the kinase domain suggests a different conformation of the kinase domain in its open and closed state (Kirstein et al., 2005). This conformational switch might lead to a rearrangement of the overall conformation, thereby contributing to a different interaction with ClpC.

Once McsB-P phosphorylates and releases CtsR from its target DNA, resulting in a de-repression of the CtsR regulon, CtsR is concomitantly targeted for degradation by ClpCP. Despite its degradation *in vitro*, McsB as well as McsA are stable proteins *in vivo*, even under heat shock conditions (unpublished data). Both proteins might therefore immediately phosphorylate and target the next (probably newly synthesized) CtsR protein for degradation. How does the cell ensure a re-repression with cessation of the heat shock condition? We could identify the cognate phosphatase YwlE, which dephosphorylates (i) CtsR, which could thereby rebind its target DNA and (ii) McsA and McsB, which abolishes first any adaptor activity of McsB and second, in their non-phosphorylated state, both modulators could be re-sequestered by ClpC again. YwlE might therefore be an essential component of the shut-off mechanism.

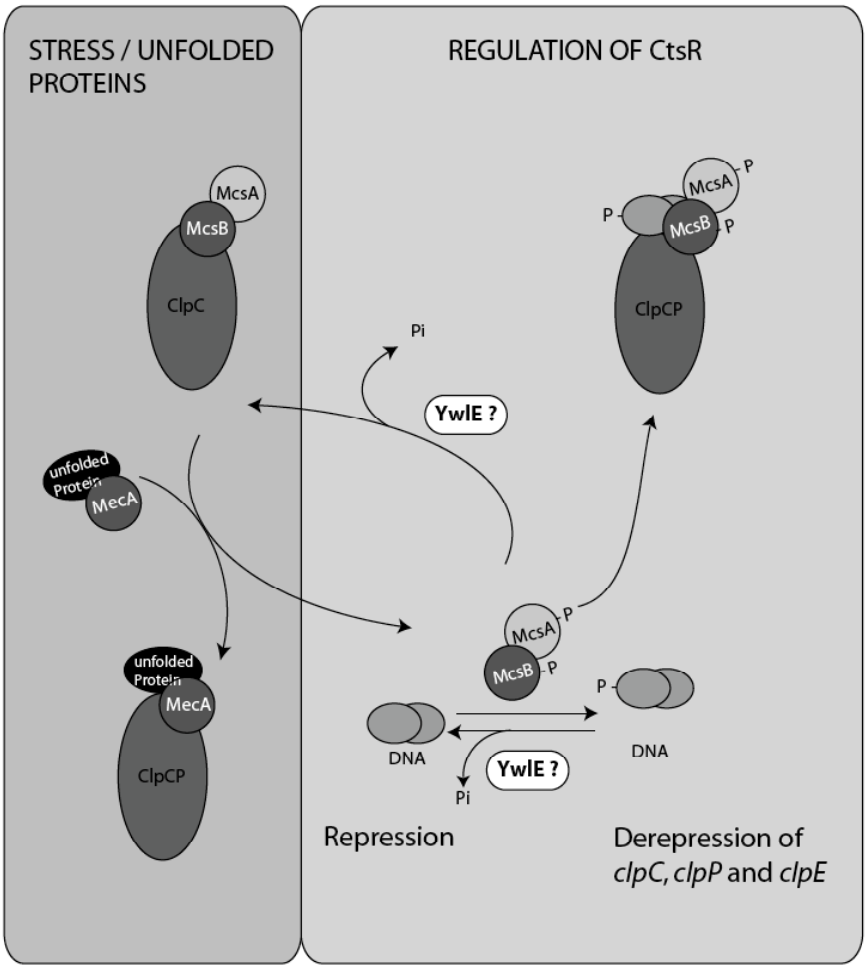

Fig. 23 Model of the regulation of the class III heat shock genes