# Dissertation

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Oral examination:

# Role of the MecA adaptor protein in regulation of the $AAA^+$ chaperone ClpC of *Bacillus subtilis*

Referees: Prof. Dr. Bernd Bukau

Prof. Dr. Brunner

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# 1A SUMMARY

ClpC, a member of the AAA+ protein superfamily from *Bacillus subtilis*, is forming with ClpP a proteolytic system, that is part of the protein quality control system and involved in general proteolysis of misfolded and aggregated proteins. In addition ClpCP together with the adaptor MecA is necessary for the regulated proteolysis of the transcription factor ComK in competence development of *B. subtilis*. The ClpCP mediated regulatory proteolysis controls also stress response and sporulation in *B. subtilis*.

In this work the *in vitro* chaperone activity of ClpC was investigated. It was discovered that the presence of the adaptor protein MecA is essential for the chaperone activity of ClpC, because it targets substrate to ClpC and activates ClpC by assisting the oligomerisation of ClpC.

In particular MecA enabled ClpC to disaggregate and refold previously heat aggregated Luciferase and Malate Dehydrogenase. In the presence of ClpP, MecA enabled the subsequent degradation of unfolded or previously heat-aggregated proteins by ClpCP while native proteins were not degraded. In addition it was demonstrated that the MecA paralogue YpbH, which is not involved in the regulatory proteolysis in *B. subtilis*, displayed comparable chaperone activities. Therefore MecA and YpbH may have a general and complementary function in protein quality control. These and other experiments suggested that MecA can coordinate substrate targeting with ClpC activation and that the ATPase induction of ClpC by MecA was necessary but not sufficient for this activation.

The question why MecA is necessary for the general activation of ClpC was addressed in more detail. It could be demonstrated that in the presence of ATP MecA assists the assembly of an active higher oligomer of ClpC via formation of a ClpC-MecA heterodimer. This higher oligomeric complex is a prerequisite for all the activities of AAA+ proteins and consists presumably of a hexamer of ClpC interacting with up to six MecA molecules. The N-terminal and the Linker domain of the first AAA+ domain of ClpC were identified as MecA interaction sites and structural determinants necessary for this process.

Controlling the ability of an AAA+ protein to form an active ring is an important functional aspect by which the activity of this protein family can be specifically regulated by an adaptor protein.

# 1B ZUSAMMENFASSUNG

ClpC, ein Protein der AAA+-Superfamilie aus *Bacillus subtilis,* ist zusammen mit ClpP ein Teil der zellulären Protein-Qualitätskontrolle und für die generelle Proteolyse von missgefalteten und aggregierten Proteinen zuständig. ClpCP ist außerdem wichtig für die gerichtete Degradation von regulatorischen Proteinen, welche die Stressantwort, die Sporulation und die Kompetenzentwicklung in *B. subtilis* kontrollieren. Der genaue Mechanismus dieser Regulation konnte für die Kompetenzentwicklung genauer untersucht werden. Es konnte gezeigt werden, dass das Adaptorprotein MecA notwendig ist, um zusammen mit ClpCP den Transkriptionsfaktor ComK der Kompetenzentwicklung in *B. subtilis* gezielt zu degradieren, und dass MecA gleichzeitig die ATP-Hydrolyse von ClpC stimuliert.

In dieser Arbeit wurden die *in vitro* Chaperon-Eigenschaften von ClpC untersucht. Es konnte gezeigt werden, dass die Anwesenheit des Adaptorproteins MecA essentiell für die ATPase und die Chaperon-Aktivität von ClpC ist.

Diese Arbeit legt dar, dass MecA die Disaggregation und Rückfaltung der hitzeaggregierten Modellsubstrate Luciferase und Malatdehydrogenase durch ClpC ermöglicht. Weiterhin ermöglicht MecA auch die Degradation von ungefalteten und aggregierten Proteinen durch ClpCP. Zusätzlich konnte gezeigt werden, dass das MecA-Paralog YpbH, welches nicht an der regulierten Proteolyse in *B. subtilis* beteiligt ist, mit MecA vergleichbare Chaperon-Aktivitäten besitzt. Daher können MecA und YpbH generelle und komplementäre Funktionen in der Protein-Qualitätskontrolle haben. Diese Experimente schlagen eine durch MecA koordinierte Substrat-Erkennung und ClpC-Aktivierung vor, für welche die ATPase-Aktivität von ClpC zwar notwendig aber nicht allein ausreichend ist.

Der Mechanismus der generellen Aktivierung von ClpC durch MecA wurde genauer untersucht. Es konnte nachgewiesen werden, dass ClpC ohne Adaptor ein inaktives Monomer ist, welches durch Zugabe von MecA über ein Heterodimer in Anwesenheit von ATP zu einem aktiven hexameren Komplex oligomerisiert wird. Weitere Experimente zeigten, dass für die Interaktion von ClpC mit MecA die N- und die Linker-Domäne notwendig sind. Die Kontrolle der Ringbildung und somit der ATPase- und Chaperon-Aktivität von ClpC durch ein Adaptorprotein stellt einen neuen Kontrollmechanismus von AAA+-Proteinen dar.

# 2 INTRODUCTION

## 2.1 Protein folding *in vitro* and *in vivo*

Newly synthesized protein chains must fold into defined three-dimensional structures, to become functionally active. All information about the structure of a protein is encoded in its aminoacid sequence. Proteins fold within the range of seconds or even milliseconds, although the number of theoretical possible but wrong conformations is extraordinary high. The mechanism of the protein folding process is currently described by folding funnels of energy landscapes (Schultz, 2000). All non-native conformations of a protein possess a higher energy and protein folding can be imagined as a ball rolling down the slope of the energy landscape into a funnel. The native structure represents the thermodynamically most favored status at the bottom of the folding funnel. The folding funnel of a protein represents many different folding pathways that can be used by the unfolded protein to reach the energy minimum (native structure). This process can be fast or slow, depending on the shape and the gradient of the funnel.

Folding processes inside the cell are much more complex than the refolding of denatured model proteins *in vitro*. Proteins are synthesized *in vivo* at the ribosomes in a vectorial manner from the N to the C terminus (Figure 1). In contrast to *in vitro* experiments, which are performed with diluted (< 1 g/l protein) and complete polypeptide chains, nascent polypeptides emerging from the ribosome do not contain the complete information necessary for folding. In addition, the concentration of cytosolic macromolecules including ribosomes, nucleic acids and proteins, is very high (340 g/l) (Goodsell, 1991).

In this macromolecular crowded environment exposed hydrophobic amino acids of nascent polypeptides and folding intermediates may interact inappropriately leading to protein misfolding and aggregation. A number of proteins are also structurally labile and their folding status is even more susceptible to changes of the cellular environment. Stress conditions, like a sudden increase in temperatures, can therefore lead to unfolding and aggregation of many proteins (Figure 1).

## 2.2 The chaperone network

### *2.2.1 De novo folding*

To optimize cellular protein folding, protective systems have developed in the course of evolution. These systems consist of families of highly conserved proteins so called molecular chaperones. Chaperones are found in high concentrations in all cells, from bacteria to humans. They guide a large variety of folding processes throughout the life cycle of proteins from synthesis to their degradation (Figure 1). The various chaperones protect non-native protein chains from misfolding and aggregation, but apparently do not contribute conformational information to the folding process. Chaperones were first identified as stress- or heat-shock proteins (HSPs). They assist folding processes at different stages throughout the lifetime of proteins, from synthesis to degradation (Figure 1).

Depending on their function, chaperones can be classified into "holder" or "folder" chaperones. Holder chaperones (e.g. small HSPs) can prevent the aggregation of misfolded protein species independent of ATP without mediating protein reactivation. Folder chaperones (e.g. Hsp60/GroEL, Hsp70/DnaK) can support in addition substrate refolding, which is ATP dependent.

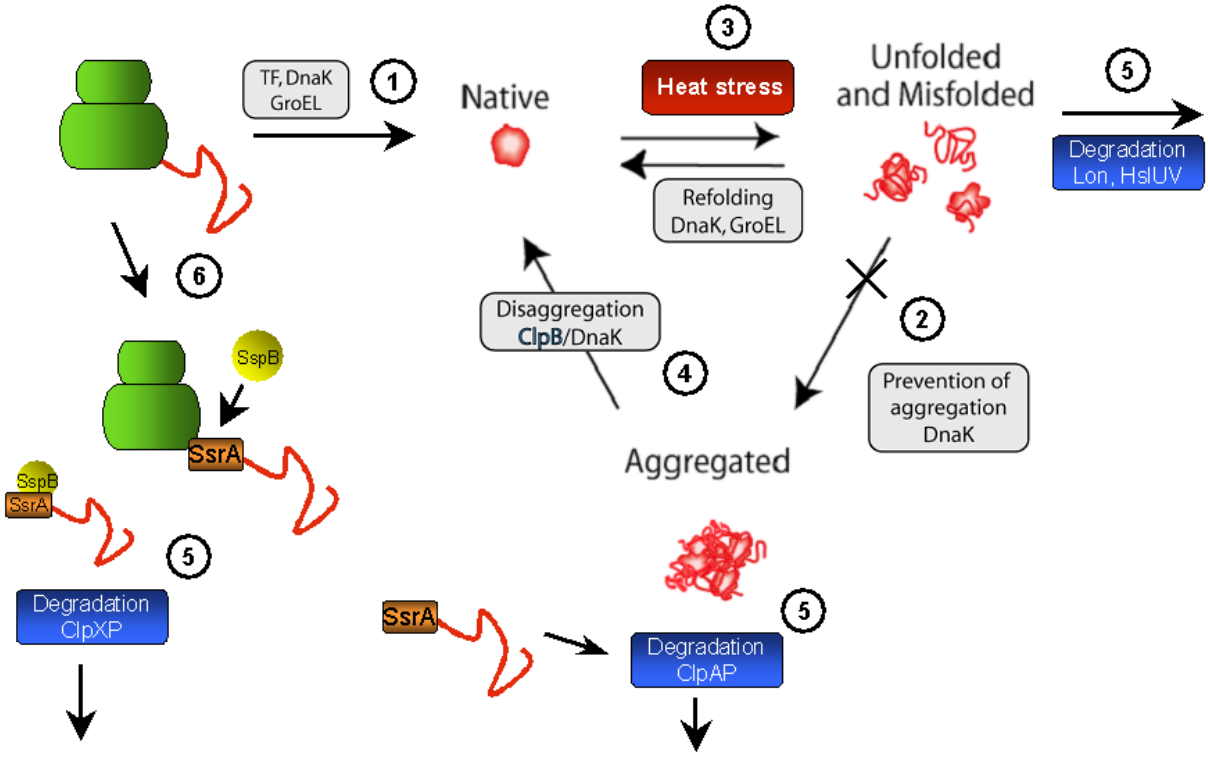



**Figure 1.** Chaperone network and protein quality control system in *E.coli*. The figure illustrates the function of different chaperone and protease systems. Chaperones assist the de novo protein folding (1), the refolding of misfolded proteins (3) and/or prevent protein misfolding and aggregation (2). Once proteins are aggregated they

can be disaggregated and refolded (4). Alternatively misfolded and aggregated proteins can be degraded by proteolytic systems (5). For details see text.

Newly synthesized proteins of bacteria associate with the chaperone trigger factor, as soon as they leave the exit tunnel of the ribosome (see Figure 1 step 1)(Deuerling et al., 1999; Teter et al., 1999). Exposed hydrophobic side chains of the newly synthesized polypeptides are most likely protected from wrong interactions by association with trigger factor. In addition, trigger factor may prevent premature folding until a complete domain has emerged from the ribosomal exit site. (Bukau et al., 2000)

It is assumed that after release of trigger factor the newly synthesized polypeptide chain can start or continue its folding to the native state. The kinetics of the folding process and the yield of correctly folded proteins differ from protein to protein. Small proteins (< 30 kDa) can, in general, fold rapidly and without guidance of chaperones into their native structure (~65 to 80% of cytosolic proteins). Larger proteins (10 to 20% of cytosolic proteins) fold more slowly and can interact during their folding process either with the GroEL or the DnaK chaperone system. DnaK associates with both, nascent polypeptides and polypeptides released from the ribosome (see Figure 1 step 1), in contrast to the GroEL machine, which is supposed to associate with newly synthesized proteins exclusively after their release from the ribosome.(Deuerling et al., 1999; Ewalt et al., 1997; Patzelt et al., 2001; Teter et al., 1999)

### *2.2.2 Repair of misfolded proteins*

Stress conditions like a sudden increase in temperature, can lead to protein unfolding and aggregation (Figure 1, step 2 + 3). Different chaperone systems function as a protective system that prevents protein aggregation by binding to the misfolded proteins, e.g. the DnaK (HSP70) and GroEL (HSP60) systems. The DnaK system consisting of DnaK, DnaJ and GrpE cooperates in addition with another family of chaperones the small heat shock proteins (sHSP`s). These substrates can subsequentlybe refolded by DnaK and/or GroEL(Figure 1 step 3). DnaK consists of an N-terminal ATPase domain and a C-terminal substrate-binding domain. Substrate binding and release is controlled by the nucleotide state and co-chaperones DnaJ and GrpE.

The barrel shaped GroEL chaperon consists of two stacked rings of seven subunits each. After ATP binding substrates are enclosed in a “folding chamber” by the association of the GroES co-chaperone.

The importance of molecular chaperones can be demonstrated by analysing the phenotypes of *E. coli* cells missing a specific chaperone system. For example *E. coli* cells lacking the HSP70 chaperone DnaK exhibit a temperature-sensitive growth phenotype (Paek and Walker, 1987) and accumulate large amounts of aggregated proteins at high temperatures (Hesterkamp and Bukau, 1998).

### *2.2.3 Refolding of aggregated proteins*

During severe stress high substrate load can overburden the cellular chaperone capacity, leading to accumulation of protein aggregates. Studies using the model systems bakers yeast *Saccharomyces cerevisiae*, the bacterium *E. coli* and the plant *Arabidopsis thaliana*, demonstrated that pre-existing protein aggregates can be successfully disaggregated and even refolded to the active conformation (Glover and Lindquist, 1998; Hong and Vierling, 2000; Lee et al., 1997; Mogk et al., 1999). Protein disaggregation is achieved by a bi-chaperone system, consisting of ClpB (Hsp104) and the DnaK (Hsp70) machine (see figure 2 arrow 4). Importantly only the combination of both chaperone systems is active in resolubilization and refolding of aggregated proteins. This activity of the bi-chaperone system is directly linked to the survival of the mentioned organisms at very high temperatures (Hong and Vierling, 2000; Sanchez and Lindquist, 1990). Disaggregation of protein aggregates was demonstrated *in vivo* and *in vitro* using thermolabile malate dehydrogenase (MDH) as a reporter enzyme.(Goloubinoff et al., 1999) Large aggregates of MDH can be solubilized *in vitro* and MDH is subsequently refolded into its native structure.

## 2.3 The AAA+ superfamily

The AAA+ superfamily (ATPase associated with a variety of cellular activities) of ATPases, which contain a homologous ATPase module, are found in all kingdoms of living organisms where they participate in diverse cellular processes.
Hsp104/ClpB is a member of the conserved Hsp100/Clp protein family, a group of ring-forming ATPases (Schirmer et al., 1996).
Neuwald and colleagues found that Hsp100/Clp proteins and AAA proteins share conserved motifs involved in ATP binding and hydrolysis and they combined both protein classes to generate a new protein superfamily: the AAA+ proteins (Lupas and Martin, 2002; Neuwald et al., 1999). Generally, AAA+ proteins remodel target substrates in an ATP-dependent manner,

an activity that is of central importance for a large number of cellular processes (Karata et al., 2001; Vale, 2000).

The structural basis of this superfamily was confirmed by determining the structure of various AAA+ proteins (Bochtler et al., 2000; Guo et al., 2002; Lenzen et al., 1998; Li and Sha, 2002; Yu et al., 1998; Zhang et al., 2000).

AAA+ proteins share a common ATPase domain with a conserved sequence of 230-250 amino acid residues referred to as the AAA+ domain. Each AAA+ domain consists of a core ATPase domain, containing the classic Walker A and B motifs, and a C-terminal α-helical domain (Figure 2). The superfamily can be further divided into two distinct classes, based on the number of AAA+ domains present in the protein (Schirmer et al., 1996). Class I proteins (e.g. ClpA, ClpB, and ClpC) contain two AAA domains, referred to as AAA-1 and AAA-2, separated by a linker sequence of variable length (Fig. 3). In contrast, class II proteins (e.g. ClpX and HslU(ClpY)) contain only one AAA domain (homologous to AAA-2).

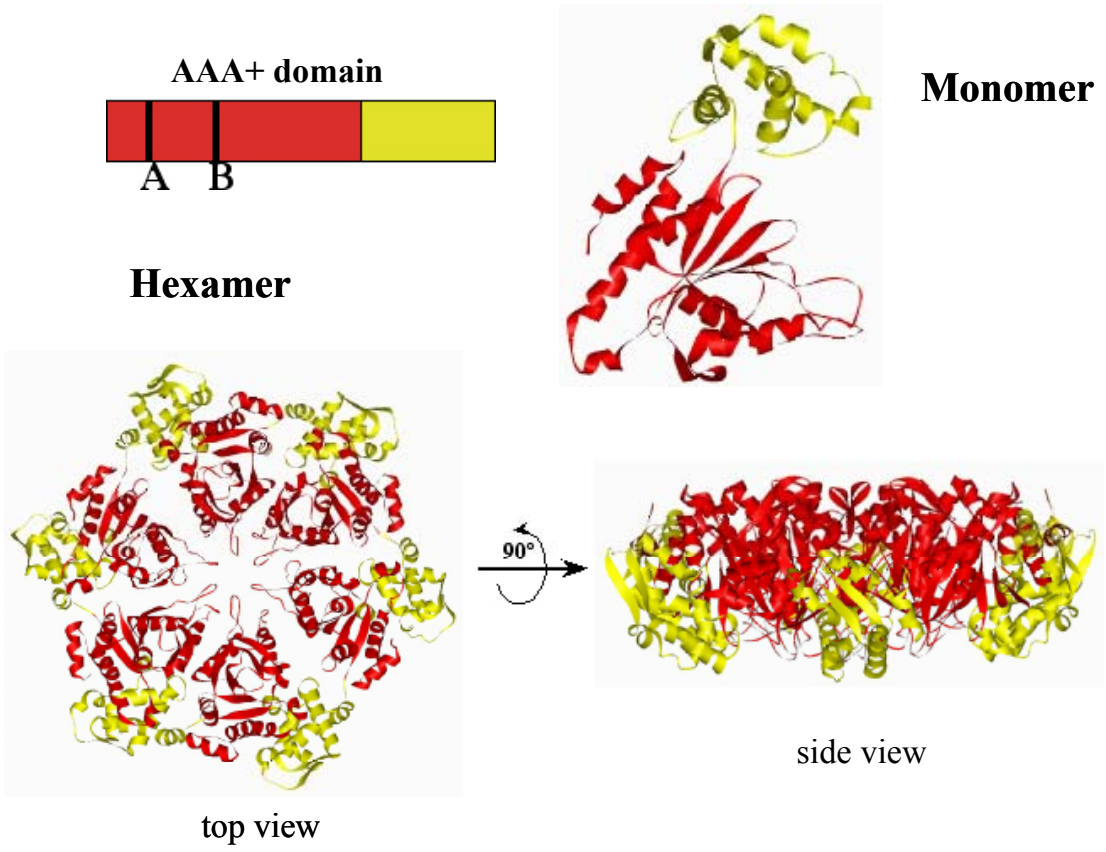



**Figure 2** Common structural features of AAA+ proteins. The AAA domain consists of a core domain with a RecA-like fold (red) and a α-helical subdomain (yellow) The core domain contains the Walker A ($GX_4GKT$) and Walker B (HyDE) nucleotide binding motifs, where X= any amino acid and Hy = hydrobphobic amino acids (Top left). The structures of an HslU monomer and HslU hexamer are shown.

### 2.3.1 AAA+/HSP100 proteins involved in protein degradation

The removal of non-native proteins by proteolytic systems is another pathway of the protein quality control system. A number of AAA+ proteins (e.g. ClpA, ClpX, HslU) associate with proteolytic components (ClpP, HslV) to form ATP-dependent proteases. Some AAA+ proteins (e.g. Lon) also contain a peptidase domain within a single polypeptide (Fig. 3). Proteins of the Hsp100/Clp family are the best-studied proteolytic systems. ClpAP, ClpXP and HslUV form barrel-like structures with two heptameric/hexameric rings of ClpP/HslV flanked on either side by hexameric rings of ClpX, ClpA or HslU (Beuron et al., 1998; Sousa et al., 2000). The active sites of ClpP and HslV are located in a chamber inside the barrel and are therefore isolated from the surrounding medium and inaccessible to folded proteins (Bochtler et al., 2000; Sousa et al., 2000; Wang et al., 1997). The access of substrates to the proteolytic chambers occurs through narrow pores, which are not wide enough to allow passage of globular proteins (Wang et al., 1997). Therefore, substrate proteins must be unfolded by the AAA+ component and threaded into the protease for degradation. Consequently, substrate recognition by the AAA+ components represents the first step in protein degradation and must be highly controlled in order to protect cells from uncontrolled proteolysis.

In order to fulfill their function in protein quality control, AAA+ proteins must be able to distinguish misfolded from properly folded proteins. In this process, AAA+ proteins compete and/or cooperate with other chaperone systems in binding unfolded proteins (Gottesman et al., 1997).

AAA+ proteins also play a key role in the removal of truncated polypeptides, which cannot reach the native state (Keiler et al., 1996). When protein synthesis is stalled at eubacterial ribosomes caused by e.g. truncated mRNAs that are lacking a stop codon a short peptide tag is coupled to the protein (Fig.1 step 6). The peptide tag is encoded by a RNA, which has properties of both transfer and messenger RNA. This 11-residue peptide (AANDENYALAA), the SsrA tag, is added to the C terminus of the incomplete polypeptide and directs the protein to specific multisubunit protease complexes, ClpXP or ClpAP. The SsrA-tagging system not only permits the dissociation of the truncated polypeptide from the ribosome but also targets the unwanted protein for degradation by several proteolytic machines including ClpXP and ClpAP (Gottesman et al., 1998).

Besides degrading misfolded proteins, AAA+ proteins are also involved in regulatory proteolysis. Selective removal of regulatory proteins, like transcription factors or signal

transduction proteins, is an efficient and fast strategy to control checkpoints for e.g. development or cell differentiation (Jenal and Hengge-Aronis, 2003). Unlike misfolded substrates, regulatory proteins are properly folded and can possess different destruction tags for their recognition by AAA+ proteins. Although the recognition motifs of several regulatory substrates (e.g. RepA, MuA and λ O) have been identified, the substrate specificity of ClpX has been most extensively studied. Using a proteolytic inactive variant of ClpP Baker and co-workers were able to trap a large number of ClpX substrates *in vivo*. From this analysis they were able to identify five different degradation signals, located either at the N- or C-termini. C-terminal recognition signals were either similar to the SsrA-tag (Leu-Leu-Ala-COOH) or to the MuA recognition signal (enrichement of basic residues), while N-terminal degradation motifs were more variable (Flynn et al., 2003).

### 2.3.2 Extra domains of AAA+/HSP100 proteins

In order to carry out their chaperone and regulatory functions AAA+ proteins must exhibit diverse substrate specificities. Thereby AAA+ proteins may posses multiple recognition sites within a single molecule, some of which may be common to all AAA+ proteins while others are unique to individual AAA+ proteins. One possibility to ensure substrate specificity is to recognize substrates via extra domains, which are missing in other members of the protein superfamily (Figure 3).

Extra domains are usually encoded N-terminal to the AAA domain, but can also be inserted within an AAA domain. They are located at the distal end of the AAA+ oligomer and thereby placed in an ideal position to make initial contacts with substrates (Bochtler et al., 2000; Guo et al., 2002; Sousa et al., 2000).

The N-domains of ClpA and ClpB are independently folded domains, which are attached to the AAA domain via a flexible linker (Lo et al., 2001; Singh et al., 2001; Tek and Zolkiewski, 2002)

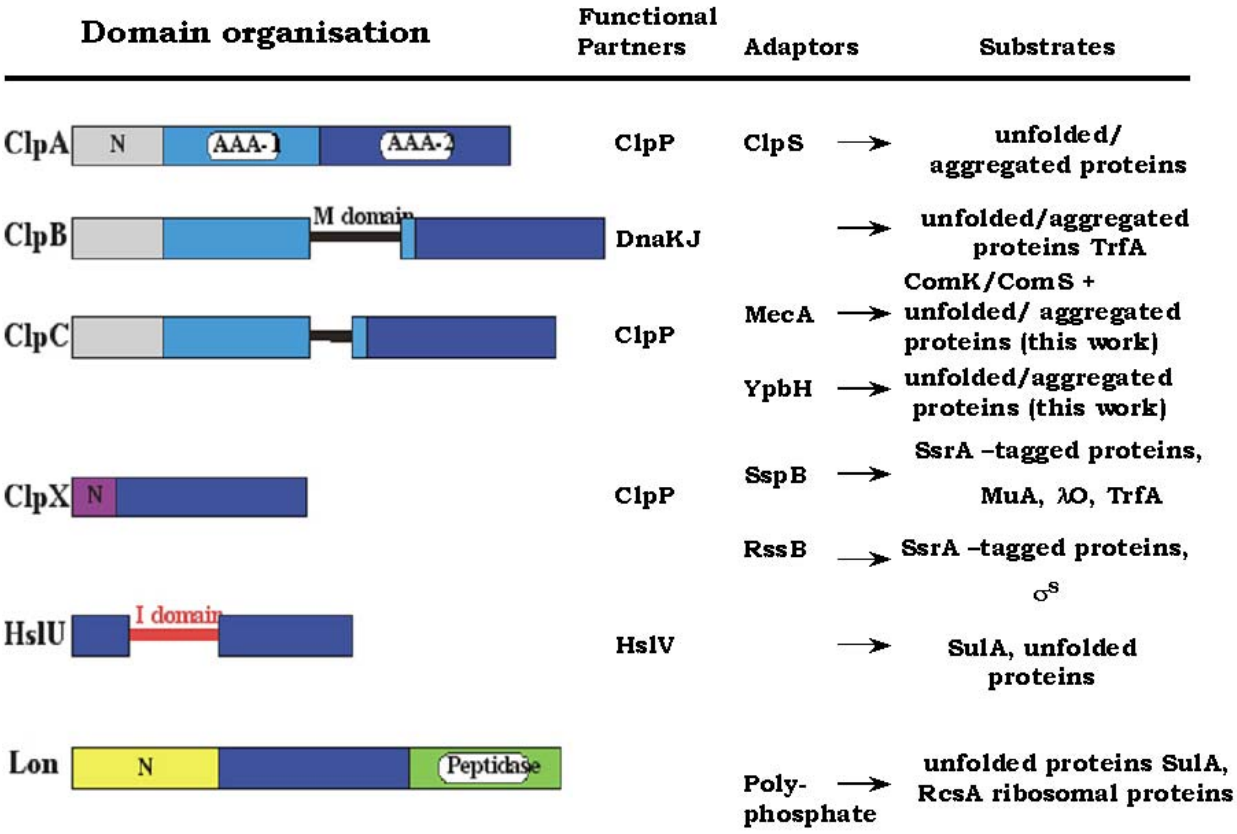



**Figure 3** Structural features of prokaryotic AAA+ proteins. The AAA domains, although highly conserved, contain significant differences and can be classified accordingly (AAA-1, AAA-2). N-terminal domains of AAA+ proteins are diverse and are in most cases involved in substrate recognition, either directly or indirectly by serving as a binding platform for adaptor proteins. The AAA domain of HslU is interrupted by the specialized I-domain, which is proposed to mediate SulA binding. Similarly a middle domain (M domain) of variable length is inserted into the AAA-1 domains of ClpB and ClpC. The middle domain of ClpB has an essential, however yet unknown function. Lon also contains an additional proteolytic domain. Adaptors/functional partners and identified substrates of the corresponding AAA+ proteins are given. The targeted substrates are indicated by an arrow.

The N-domain of ClpX is different to the N-domains of ClpA and ClpB. It forms a stable dimer and is complexed with Zn (II) cations via a zinc finger motif (Banecki et al., 2001; Wojtyra et al., 2003). ΔN-ClpX is still able to bind SsrA-tagged proteins and to support GFP-SsrA degradation *in vitro* and *in vivo* (Dougan et al., 2003; Singh et al., 2001; Wojtyra et al., 2003). However, ΔN-ClpX is affected in MuA degradation in vitro and, consistently, ΔN-ClpX cannot function in disassembling MuA-DNA complexes in vivo (Wojtyra et al., 2003). Consistently MuA, but not GFP-SsrA, were recently demonstrated to bind to the isolated N-domain of ClpX. Thus it appears that ClpX contains at least two different binding sites: one is present in the N-domain and is required for binding MuA, while the second resides in the

AAA domain and mediates interaction with GFP-SsrA. This demonstrated that an N-domain could be involved in specific substrate interaction (Wojtyra et al., 2003).
The Linker (or middle domain) is proposed to form a coiled coil, which is repeated, two times in ClpB and once in ClpC. Its function is poorly studied although as a coiled coil, it is likely to play a role in protein-protein interactions, either in protein oligomerisation or perhaps in substrate binding. (Dougan et al., 2002a).
HslU contains a discrete domain (the I-domain), which is inserted between the Walker A and B motifs of the AAA domain (Fig. 3). The I-domain appears as a flexible region with coiled helices and is located at the distal end of the HslU oligomer (Bochtler et al., 2000; Sousa et al., 2000). Deletion of the I-domain does not affect HslU hexamerization or the association with the HslV peptidase (Kwon et al., 2003; Song et al., 2000). However, ΔI-HslU did no longer prepare SulA, a cell division inhibitor protein, for degradation by HslV. Since degradation of casein was not affected in this deletion variant, the I-domain may play a specific role in SulA binding (Song et al., 2000). In addition extra loops, which confer the interaction of ClpA, ClpX and ClpC with ClpP had been identified (Dougan et al., 2002b).

## 2.4 AAA+ adaptor proteins

An alternative way to ensure substrate specificity of AAA+ chaperones is the presence of adaptor proteins, which assert their effects through specific binding to both, substrates and AAA+ partner proteins and deliver specific substrates in a trans-targeting mechanism to the AAA+ proteins (Fig. 2) (Dougan et al., 2002a). Currently there is an increasing number of adaptor proteins reported not only in bacteria but also in higher organisms. Many adaptor proteins modulate the activity of their parent AAA+ chaperone by binding to the N-terminal domain (Dougan et al., 2002b; Dougan et al., 2003; Yuan et al., 2001). This mode of binding positions the adaptor proteins at the side or on the top of the AAA+ ring, placing them in an ideal position to hand over the bound substrates to the AAA+ partner protein.

### *2.4.1 SspB and RssB*

A simple strategy to enlarge the substrate pool of AAA+ proteins is the usage of an individual adaptor protein, which is delivering the bound cargo upon binding to its AAA+ partner protein, recognizes specific substrates. The first such adaptor protein identified in *E. coli* for ClpX include SspB (stringent starvation protein B) and RssB (Regulator of Sigma S). SspB was identified as a specificity factor of ClpX, which enhances the degradation of SsrA-tagged

proteins by ClpXP (Levchenko et al., 2000). Tagged polypeptides are directly transferred by SspB to ClpXP. SspB is not essential for the degradation of SsrA-tagged substrates by ClpXP, but becomes especially important at low substrate concentrations, which are probably physiologically relevant (Wah et al., 2002).

RssB is the second adaptor protein of ClpX and is essential for the degradation of the starvation sigma factor $\sigma^S$ by ClpXP. Several stresses increase cellular $\sigma^S$ levels by inhibiting proteolysis of $\sigma^S$, which under non-stress conditions is a highly unstable protein. Proteolysis of $\sigma^S$ requires the response regulator RssB. RssB can directly interact with $\sigma^S$ and can target its substrates to ClpXP. The interaction of RssB with $\sigma^S$ is dependent on RssB phosphorylation and thereby the phosphorylation status of RssB controls $\sigma^S$ stability. The in vivo signal transduction cascade that leads to RssB phoshorylation is still unknown (Becker et al., 1999; Muffler et al., 1996; Pratt and Silhavy, 1996; Zhou et al., 2001).

### *2.4.2 ClpS*

Besides targeting specific substrates, adaptor proteins can also play a different role in controlling the activity of AAA+ partner proteins. One adaptor proteins, which did not only target specific substrate, is ClpS, a ClpA-specific adaptor protein (Dougan et al., 2002c; Zeth et al., 2002b). ClpS modulates ClpA substrate specificity by binding to the N-domain of its AAA+ partner protein. (Guo et al., 2002; Zeth et al., 2002a). ClpS was shown to regulate the degradation of two different Clap-mediated substrates. Recognition and consequently degradation of aggregated proteins was enhanced by the presence of ClpS. In contrast, ClpS was shown to inhibit both binding and degradation of SsrA-tagged substrates. The mechanism of the ClpS-mediated switch of ClpA activity and the modulation of ClpA substrate specifity is largely unknown. Since ClpS and SsrA-tagged substrates bind to different regions of ClpA, the inhibitory activities of ClpS towards this substrate class cannot be explained by competition for the binding to the same site within ClpA. However, ClpS binding can trigger the release of pre-bound SsrA-tagged substrates (Dougan et al., 2002c). It was therefore proposed that binding of ClpS to the N-domains of ClpA causes conformational changes in ClpA, resulting in the shielding and exposure of substrates binding site for SsrA-tagged substrates and protein aggregates, respectively (Zeth et al., 2002b). In addition ClpS inhibits the autodegradation of ClpA *in vitro* and *in vivo* (Dougan et al., 2002c).

## 2.5 AAA+ chaperones of *Bacillus subtilis*

The gram-positive soil bacterium *Bacillus subtilis* is next to *E. coli* the best-studied prokaryotic organism. *B. subtilis* possesses four different Clp ATPases, ClpC, ClpE, ClpX and ClpY (Fig. 4). ClpX and ClpY are orthologues of the E. coli ClpX and ClpY proteins. The E. coli HSP100 proteins ClpA and ClpB are missing in *B. subtilis*. ClpC possesses an N-terminal domain homologous to ClpA and ClpB and a middle domain half the size of the ClpB middle domain. ClpC and ClpE are exclusive in *B. subtilis* and most of the other fully sequenced gram-positive organisms.

The ClpE protein is a prototype of a novel subfamily among the Clp ATPases. It consists of two AAA domains, a domain that is inserted into AAA-1 and an N-terminal domain that is homologous to the N-domain of ClpX from *E. coli* and *B. subtilis*.

The *B. subtilis* HSP100 proteins form complexes with subunits (ClpP or HslV), like their *E. coli* counterparts.

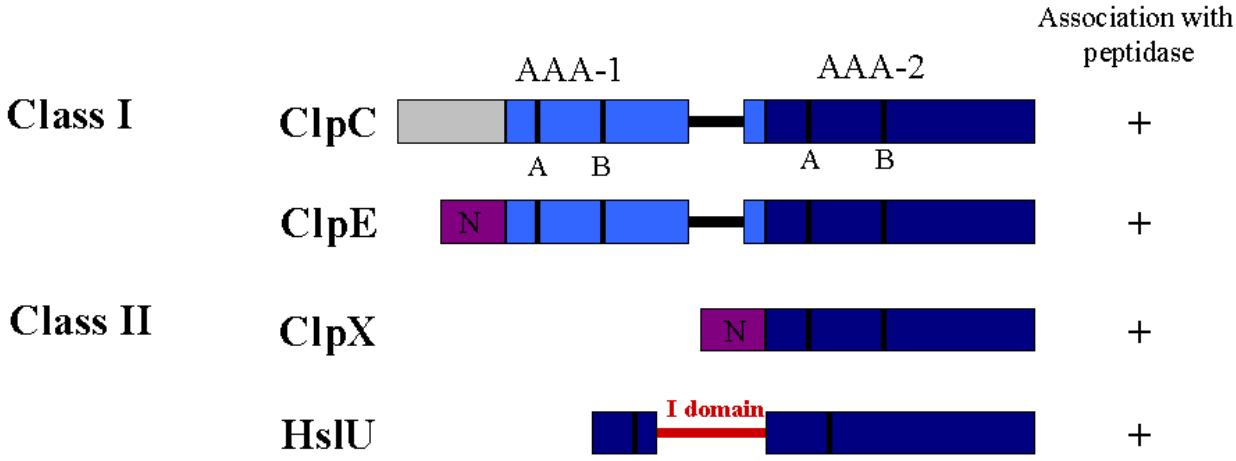



**Figure 4** A comparison of the *B. subtilis* HSP100/Clp proteins. Class I proteins contain a variable N-terminal (in gray) and two AAA domains (AAA-1 and AAA-2) separated by a linker domain of variable length. Class II proteins contain only one AAA domain. N-terminal domains with a Zinc binding motif are marked in magenta (ClpE and ClpX).

## 2.6 Role of ClpC and the adaptor MecA in regulated and general proteolysis in *B. subtilis*

### *2.6.1 Regulated proteolysis in B. subtilis*

Adaptor proteins are not only found in *E. coli*. In fact, the first characterized adaptor protein, MecA, was discovered in *B. subtilis.* The adaptor protein MecA of *B. subtilis* was identified together with ClpC in a genetic screen for repressors of competence development (Roggiani et al., 1990). During the last years it has been shown that ClpCP is also directly involved in the regulation of several cellular processes and as a part of the protein quality control system. ClpCP is responsible for the degradation of key regulators, as shown in the following chapters for ComK, SpoIIAB, Spx, MurAA and CtsR

#### *2.6.1.1 Competence development*

MecA is a negative regulator of competence and acts together with ClpC to target the master regulator for competence development (ComK) for proteolysis by ClpCP (Persuh et al., 1999; Turgay et al., 1998; Turgay et al., 1997). Signalling through a quorum sensing system results in the expression of a small protein ComS, which like ComK, interacts with the N-terminal domain of MecA leading to the release and stabilization of ComK. The released ComK activates not only its own transcription but also the transcription of all the genes necessary for the competent state. Once the signal (ComS) is removed, MecA is able to rebind the highly abundant ComK and targets it for degradation by ClpCP until the previously competent cells return to a normal physiological state (see Figure 4) (Dubnau, 1999; Dubnau and Turgay, 2000; Turgay et al., 1998). Interestingly, a homologue of MecA known as YpbH also interacts with ClpC. YpbH, however, is not involved in the control of ComK activity (Persuh et al., 2002). These data suggest that MecA is a specific adaptor protein for competence regulation. However homologues of MecA and ClpC are also widespread in other bacterial species, which lack ComK homologues. This suggests that the roles of MecA and ClpC are not only limited to controlling competence development.

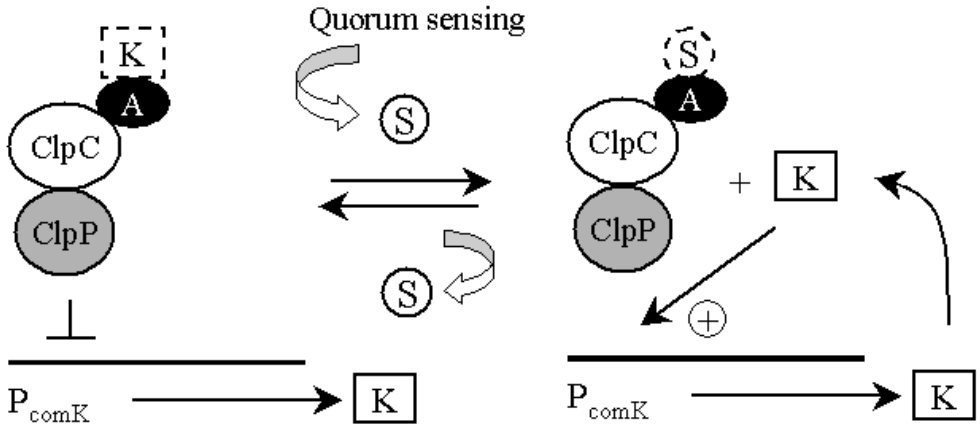



**Figure 5.** The competence switch. The dotted outlines indicate degradation. The circled + symbol indicates that ComK operates positively on its own promotor. A, K and S represent MecA, ComK and ComS, respectively (Dubnau and Turgay, 2000).

*2.6.1.2 The anti-sigma factor SpoIIAB is degraded by ClpCP*

Transcriptional regulation in bacteria can be controlled by factors acting to alter promotor utilization. The process of transcription begins when RNA polymerase (RNAP) holoenzyme (core + σ) binds to a specific promoter region (deHaseth et al., 1998). In *B. subtilis*, one notable class of regulators are the alternative σ-factors that bind to the core enzyme (in place of the primary σ-factor) and thereby direct the RNA polymerase to recognize new promoter sites (Haldenwang, 1995). Some sigma factors (i.e. sigma B, sigma F, and sigma G) are inhibited by "anti-sigma factor" which block their ability to form RNA polymerase holoenzymes. The transcription factor σF, which is activated in a cell-specific manner during sporulation in *B. subtilis*, is initially held in an inactive complex by the anti-sigma factor SpoIIAB. The anti-anti-sigma factor SpoIIAA interacts with SpoIIAB−σF and induces the release of free σF and free SpoIIAB. Free SpoIIAB is subject to proteolysis while it is protected from degradation by σF in the SpoIIAB-σF complex and by SpoIIAA in an alternative complex. Proteolysis of SpoIIAB requires residues located near the extreme C terminus of SpoIIAB and is dependent upon the ClpCP protease (Pan and Losick, 2003). In a *clpC* null mutant SpoIIAB is stabilized and as a consequence these mutants are deficient in sporulation. The interaction of SpoIIAA with SpoIIAB-σF and the resulting degradation of newly released SpoIIAB could set up a self-reinforcing cycle that locks on the activation of σF (Pan et al., 2001).

*2.6.1.3 ClpC and MecA degrade the transcriptional regulator Spx (YjbD)*

The stability of a newly discovered stress dependent transcriptional regulator Spx (YjbD) is controlled by ClpXP but can also get degraded by ClpCP in presence of MecA or YpbH. Spx is an "anti-α" factor (α-factors interact with the C-terminal domain of the α-subunit of RNAP), which blocks transcriptional activation by binding to the α-subunit, thereby interfering with the capacity of RNAP to respond to certain activator proteins. It has been shown that Spx suppress defects in competence development conferred by *clpP* and *clpX* mutants. The levels of Spx protein in wild-type cells grown in competence medium are low, while those in *clpP* mutants are high.
An *in vitro* proteolysis experiment using purified proteins demonstrated that Spx was degraded by ClpCP, but only in the presence of one of the ClpC adapter proteins, MecA or YpbH. (Nakano et al., 2003b; Nakano et al., 2002b). The in vivo relevance of this degradation is not clear, however Spx was shown to be involved in stress related induction of MecA transcription (Nakano et al., 2003a).

*2.6.1.4 Degradation of MurAA by ClpCP*

MurAA which catalyses the first committed step in the peptidoglycan biosynthesis pathway has been recently identified as another possible substrate of ClpCP. Peptidoglycan constitutes an essential component of the bacterial cell wall, providing mechanical resistance to the internal osmotic pressure and determining cell shape. Stabilisation of MurAA was observed in Δ*clpC*-mutant strains and the in vivo stability of MurAA depended on the presence of ClpC and ClpP. This Clp dependent degradation is especially enhanced upon entry into stationary phase. To control growth of *B. subtilis* the MurAA protein could be a target for regulatory proteolysis by ClpCP (Kock et al., 2003).

*2.6.1.5 Degradation of CtsR*

Only parts of the stress response regulation of *B. subtilis* are understood yet. Based on distinct regulatory features five different classes of stress-inducible genes have been defined. The transcription of Class III heat shock genes, which encode HSP100/Clp proteins, is controlled by the CtsR repressor (class three stress gene repressor). CtsR negatively regulates the

expression of *clpP*, *clpE* and the *clpC* operon, by binding to operator sequences in front of these operons (Derre et al., 1999a). CtsR is stable during non-stressed conditions, but is degraded upon temperature upshift by ClpCP. Thereby, ClpC acts as a positive regulator of the heat shock response in *B. subtilis* The activity of CtsR is controlled by the two modulatory proteins, McsA and McsB, whose genes are encoded by the *clpC* operon (*ctsR*, *mcsA*, *mcsB* and *clpC*). McsA contains zinc finger motifs and interacts with McsB under non-stressed conditions. McsB, a putative tyrosine kinase, remove the repressor from the DNA and target CtsR for degradation by the ClpCP protease during stress (Krüger et al., 2001).

### *2.6.2 General proteolysis in B. subtilis*

ClpC functions as a general stress protein, its expression is heat shock regulated and a *clpC* deletion strain is thermosensitive and less thermotolerant (Krüger et al., 1994; Msadek et al., 1994). Krüger et al. showed, that the presence of ClpC is also necessary for the general protein quality control system in *B. subtilis*. Turnover rates for abnormal puromycyl peptides are significantly decreased in *clpC, clpX* and *clpP* mutant cells. This demonstrated that ClpC, ClpX and ClpP participate directly in general proteolysis of misfolded proteins. Electron-dense aggregates, most likely caused by the accumulation of misfolded proteins, were noticed in studies of ultrathin cryosections in heat shocked cells and even under non-stressed conditions in *clpC, clpX* and *clpP* mutant cells. Furthermore *clpC, clpX* and *clpP* mutant cells are deficient in the ability to solubilize or degrade damaged and aggregated proteins. ClpC like ClpX and ClpP also localizes to protein aggregates in heat-shocked cells and to inclusion bodies formed by overproduced insoluble heterologous proteins (Jürgen et al., 2001; Krüger et al., 2000). Based on these findings it is assumed that ClpCP plays an important role in the removal of unfolded and aggregated proteins in *B. subtilis*.

## 2.7 Aim of this work

To elucidate the chaperone functions and the mechanism of the AAA+ protein ClpC, the role of its adaptor protein MecA and other adaptor proteins should be examined.

- MecA, necessary for ClpC chaperone activity

The chaperone activities of ClpC and MecA in prevention of protein aggregation, protein refolding and protein disaggregation should be examined *in vitro* with the model substrates Luciferase and Malate Dehydrogenase (MDH). It will be tested whether MecA is required for the ClpCP mediated degradation of model substrates.

- MecA controlled activation of ClpC

MecA can target proteins to ClpC but is also necessary to activate ClpC. To elucidate the molecular mechanism of this additional function of MecA the influence of MecA on the oligomerisation of ClpC should be examined. Therefore ClpC and different ClpC variants will be analysed by gelfiltration and chemical crosslinking experiments. Additionally the MecA induced ATPase of ClpC and different ClpC variants need to be determined.

- MecA-ClpC interaction

The identification of the ClpC domains necessary for the ClpC-MecA interaction would provide further insight into the mechanism of MecA mediated ClpC activation.
Therefore ClpC, mutated in the Walker motifs of the first and the second AAA+ domain, which are able to bind but not hydrolyse ATP will be analysed for MecA interactions and MecA mediated activity. In addition deletions of possible MecA binding sites in ClpC, such as the N-terminal domain (NTD) and the Linker, should be investigated in this work.

- Other ClpC adaptors

Recently YpbH has been identified to be a paralog of MecA (Persuh et al., 2002). In collaboration with the group of Prof. M.Hecker, J.Kirstein showed that McsB, a modulator of CtsR repression, is needed for ClpCP mediated degradation of CtsR. In this work the newly identified adaptors will be compared to MecA in ATPase stimulation, disaggregation and refolding abilities and oligomerisation of ClpC.

# 3 MATERIAL AND METHODS

## 3.1 Materials

### *3.1.1 Equipment*

| | |
|---|---|
| Analytical balance | Mettler AE 100 |
| Centrifuges | Heraeus, Kendrow, Sorvall, Beckmann |
| Circulardichroismus-spectrometer | J715 Jasco |
| Fluoro/PhosphoImager | FLA3000 FujiFilm |
| Fluorescence-/absorptions spectrometer | LS50B, PerkinElmer/Applied Biosystems |
| FPLC + columns | Amersham Biosciences |
| Fraction collector | Amersham Biosciences |
| French Press | SLM-AMin.co |
| Shaker (Gels) | GFL |
| Gradient mixer for Polyacrylamidegels | self-construction of the institute's workshop |
| HPLC + columns | Applied Biosystems (BioCad) |
| Incubators | Heraeus |
| Balance | Mettler PM 2000 |
| Lumat | LB9501 Berthold |
| Minigelapperatus | BioRAD® |
| Phosphoimager plates | Raytest |
| Power supply | Amersham Bioscience |
| Stirrer | Heidolph |
| Sonifier | 450 Branson |
| Spectrometer | UV-1601 Shimadzu |
| Static Lightscattering | Wyatt Technology |
| Scintillation counter | 2500TR Canberra-Packardt |

| | |
|---|---|
| Thermoblock | Eppendorf, Techne |
| Vortex | Ikamag |
| Waterbath | Bioblock Scientific |

*3.1.2 Chemicals*

| | |
|---|---|
| Acrylamide | Roth (Karlsruhe, GER) |
| Adenosine triphosphate, disodium salt | Sigma-Aldrich (Steinheim, GER) |
| Adenosine diphosphate, disodium salt | Sigma-Aldrich (Steinheim, GER) |
| Agarose | Roth (Karlsruhe, GER) |
| Ammonium peroxodisulfate (APS) | Merck (Darmstadt, GER) |
| Ampicillin | Roth (Karlsruhe, GER) |
| Bacto agar | Difco Laboratories (Detroit, USA) |
| Bacto trypton | Difco Laboratories (Detroit, USA) |
| Bacto yeast extract | Difco Laboratories (Detroit, USA) |
| Chloramphenicol | Sigma-Aldrich (Steinheim, GER) |
| Coomassie brilliant blue G-250 | Roth (Karlsruhe, GER) |
| Ethidium bromide | AppliChem (Darmstadt, GER) |
| Glutaraldehyde, 25 % in water | Roth (Karlsruhe, GER) |
| Guanidinium hydrochloride | Roth (Karlsruhe, GER) |
| Isopropyl-β-D-thiogalactopyranoside (IPTG) | AppliChem (Darmstadt, GER) |
| β-mercaptoethanol | Roth (Karlsruhe, GER) |
| o-nitrophenyl-β-D-galactopyranoside | Sigma-Aldrich (Steinheim, GER) |
| Phenyl-methyl-sulfonyl-fluoride (PMSF) | AppliChem(Darmstadt,GER) |
| Polyoxyethylene sorbitan-monolaurate (Tween 20) | Roth (Karlsruhe, GER) |
| Phosphoenolpyuruvate | Sigma-Aldrich (Steinheim, GER) |
| Sodium dodecylsulfate (SDS) | Roth (Karlsruhe, GER) |
| Tetracycline (tet) | Merck (Darmstadt, GER) |
| N, N, N', N'-Tetramethylethylendiamine (TEMED) | Sigma-Aldrich (Steinheim, GER) |
| *N*-succinimidyl-[2,3-3H]propionate | Amersham |

All other chemicals were analytical grade and obtained from Roth, Sigma or Merck. Only High purity solvents were used for Äkta purifier gelfiltration runs.

### *3.1.2 Standards and Kits*

#### *3.1.2.1 Protein standard*

*Premixed Protein Molecular Weight Marker (low range)*

Roche Diagnostics (1.4, 21.5, 26.6, 39.2, 66.2, 97.4 kDa)

*Prestained SDS Molecular Weight Markers*
Sigma (33.5, 38.5, 56, 69, 87,112, 205 kDa)

*Molecular weight standard*

Biorad (670, 158, 44, 17, 1.35 kDa)

#### *3.1.2.2 Kits*

QIAprep Spin Miniprep Kit Qiagen
QIAquick-Plasmid-Präparation-Kit Qiagen
QIAquick-Gel-Extraction-Kit Qiagen
Ni-NTA-Spincolumn Kit Qiagen

### *3.1.3 Expendable items*

| | |
|---|---|
| Centricon | Amicon |
| Chromatography paper, | Whatmann 3MM Vetter, St. Leon-Rot |
| Dialysis membrane | Medicell International Ltd. |
| Immobilon P$^{TM}$ PVDF- | Membran Millipore |
| Membrane filter | Schleicher & Schleicher |
| Nitrocellulose Membrane | Roth (Karlsruhe, GER) |
| Protein Assay | BioRAD® |
| Scintillation vials | Roth (Karlsruhe, GER) |

*3.1.4 Proteins and Enzymes*

| | |
|---|---|
| α-casein (bovine milk) | Sigma-Aldrich (Steinheim, GER) |
| bovine serum albumin (BSA) | Sigma-Aldrich (Steinheim, GER) |
| Malate dehydrogenase | Roth (Karlsruhe, GER) |
| Luciferase | Sigma-Aldrich (Steinheim, GER) |
| Pyruvatkinase | Roth (Karlsruhe, GER) |
| Lysozyme | Roth (Karlsruhe, GER) |

*3.1.5 Media and antibiotics*

LB broth:
10 g/l Bacto trypton
5 g/l Bacto yeast extract
5 g/l NaCl
15 g/l Bacto agar (only for culture plates)

2xYT:
16 g/l trypton
10 g/l yeast extract
5 g/l NaCl

Special media like SPIZ II are described in the according protocol they are used for.

Antibiotic stock solutions (for *E.coli*):

| | |
|---|---|
| Ampicilline | 100 mg/ml in $H_2O$ (store at –20°C) |
| Chloramphenicol | 30 mg/ml in 70% (v/v) ethanol (store at –20°C) |
| Kanamycine | 30 mg/ml in $H_2O$ (store at –20°C) |
| Tetracycline | 20 mg/ml in 70% (v/v) ethanol (store in the dark at –20°C) |

Antibiotic stock solutions (for *B.subilis*):

| | |
|---|---|
| Ampicilline | 30 mg/ml in $H_2O$ (store at –20°C) |
| Chloramphenicol | 10 mg/ml in 70% (v/v) ethanol (store at –20°C) |

| | |
|---|---|
| Kanamycine | 10 mg/ml in H2O (store at –20°C) |
| Tetracycline | 10 mg/ml in 70% (v/v) ethanol |

The antibiotics are added to the media in a dilution of 1:1000 .

*3.1.6 Materials for chromatography*

- S200 HR10-30 (pharmacia)
- S300, 120 ml, (pharmacia) /HiPrep 16/60 Sephacryl S-300 HR

## 3.2 Molecular cloning techniques

*3.2.1 Bacterial strains and plasmids*

**Table I**

| Strain | Relevant genotype or properties | Source and/or reference |
|---|---|---|
| *Escherischia coli* | | |
| SG22189 | *MC4100 ΔclpA clpP::cat malP::lacI$^{Q}$* | S.Gottesman |
| ER2566 | *F$^{-}$λ$^{-}$ fhuA2 [lon] ompT lacZ::T7 gene1 gal sulA11 Δ(mcrC-mrr)114::IS10 R(mcr-73::miniTn10-TetS)2 R(zgb-210::Tn10)(TetS) endA1 [dcm]* | New England BioLabs |
| M15 | *nal$^{S}$,str$^{S}$, rif$^{S}$, lac, ara, gal, mtl, F$^{-}$;* | (Zamenhof and Villarejo, 1972) |
| *Bacillus subtilis* | | |
| IS75 | *his leu metB5* | I. Smith |
| BD1991 | *IS75 amyE::comK-lacZ cat* | (Hahn et al., 1995) |
| *clpC-WB1* | *BD1991 (clpC-E280A)* | K. Turgay/ C.Escher |
| *clpC-WB2* | *BD1991 (clpC-E618A)* | K. Turgay/ C.Escher |
| *clpC-DWB* | *BD1991 (clpC-E280A/E618A)* | K. Turgay/ C.Escher |
| *clpC-ΔLinker* | *BD1991(clpC-ΔP412-E471 replaced by two amino acids)* | K. Turgay/ C.Escher |

| plasmid | Relevant genotype or properties | Source and/or reference |
|---|---|---|
| pTYB2 | Cloning vector for IMPACT* CN system | New England BioLabs |
| pClpC | pTYB2 with *clpC* | (Nakano et al., 2002a) |
| pClpC-WB1 | pTYB2 with *clpC-WB1* | K. Turgay/ C.Escher |
| pClpC-WB2 | pTYB2 with *clpC-WB2* | K. Turgay/ C.Escher |
| pClpC-DWB | pTYB2 with *clpC-DWB* | K. Turgay/ C.Escher |
| pClpC-ΔN | pTYB2 with *clpC-ΔN* | K. Turgay/ C.Escher |
| pClpC-ΔLi | pTYB2 with *clpC-ΔLi* | K. Turgay/ C.Escher |
| pQMA | pQE60 with *mecA-His$_6$* | (Turgay et al., 1997) |
| pQYH | pQE60 with *ypbH-His$_6$* | K. Turgay |
| pNTD | pQE60 with *mecA(1-92)-His$_6$* | (Persuh et al., 1999) |
| pCTD | pQE60 with *mecA(87-218)-His$_6$* | (Persuh et al., 1999) |
| pClpP11 | pQE70 with *clpP-His$_6$* | (Turgay et al., 1998) |

(*Intein Mediated Purification with an Affinity Chitin-binding Tag)

The mutations ClpC E280A referred to as ClpC-WB1, ClpC E618A referred to as ClpC-WB2 and ClpC E280A/E618A referred to as ClpC-DWB, ClpC lacking the N-domain (referred to as ClpC-ΔN; in which amino acids M2-L141 were removed) or the linker domain (referred to as ClpC-ΔLi; in which amino acids P412-E471 were replaced by a two amino acid linker) were introduced into *clpC* by standard molecular biological methods including site directed mutagenesis, PCR, and fusion PCR. The cloning vector for the IMPACT CN system was used for these manipulations and the introduced changes were confirmed by sequencing. ClpC-WT protein and all other ClpC constructs were over expressed using the *E. coli* strain ER2566. MecA, YpbH, the C-terminal domain (CTD) of MecA and the N-terminal domain (NTD) of MecA were over expressed using the *E. coli* strain M15. ClpP expression was performed using the strain SG22189 (see Table I)

The respective *B. subtilis* mutant strains *clpC-DWB*, *clpC-WB, clpC-WB2* and *clpC-ΔLinker* were constructed like the respective WT strain BD 2827 on the basis of *B. subtilis* BD 1991 and confirmed as described previously (Turgay et al., 2001).

*3.2.2 Competent cells of E. coli*

1 ml overnight culture was added to 100 ml pre-warmed LB medium containing the relevant antibiotic(s) in a 500 ml flask and shaken at 37°C until an $OD_{600}$ of 0.5 is reached (approximately 90–120 min). The culture was cooled on ice for 5 min and transferred to a sterile, round-bottom centrifuge tube. The cells were harvested by centrifugation at low speed (5 min, 4000 x *g*, 4°C). The supernatant was discarded carefully. The cells were always kept on ice. The cells were resuspended gently in cold (4°C) TFB1 buffer (30 ml for a 100 ml culture) and the suspension was kept on ice for an additional 90 min. After that the cells were centrifuged again (5 min, 4000 x *g*, 4°C) and the supernatant was discarded carefully. The cells were resuspended carefully in 4 ml ice-cold TFB2 buffer and aliquoted into 100–200 µl in sterile micro centrifuge tubes and frozen in liquid nitrogen or a dry-ice ethanol mix. The competent were stored cells at –70°C.

Buffers:

TFB1 buffer:

(sterile filtered)

| | |
|---|---|
| 100 mM | Rubidiumchloride |
| 50 mM | Manganesechloride |
| 30 mM | Potassiumacetate |
| 10 mM | Calciumchloride |
| 15 %(v/v) | Glycerine |
| | HCl pH 5.8 |

TFB2 buffer:

(Autoclaved)

| | |
|---|---|
| 10 mM | Mops, pH 8.0 |
| 10 mM | Rubidium chloride |
| 75 mM | Calciumchloride |
| 15 %(v/v) | Glycerine |

### *3.2.3 Isolation of plasmid DNA from E. coli*

#### *3.2.3.1 Purification Protocol*

Plasmid DNA purification was performed by using Qiagen "*QIAprep Spin Miniprep Kit*" centrifugation columns that uses the highly specific binding of DNA to a silica gel membrane matrix. DNA extraction was performed as described, according to the procedure suggested in the supplemented Qiagen handbook (*QIAprep Miniprep Handbook*, Qiagen 1999).
This protocol is for Mini (up to 20 µg) preparations of high-copy plasmid DNA from cultures of *E. coli*. Bacterial cells from a 6 ml overnight culture were harvested by quick centrifugation (13,000 rpm 1 min, at RT), resuspended in 250 µl resuspension buffer (buffer P1), and opened by alkaline lysis adding 250 µl buffer P2. The addition of 300 µl neutralization buffer (buffer N3) caused precipitation of cell debris, cellular proteins and chromosomal DNA, which were subsequently removed by centrifugation (13,000 rpm 10 min, at RT). The supernatant containing the dissolved plasmid DNA was applied directly onto the Qiagen silica gel spin columns provided and centrifuged (13,000 rpm 1 min, at RT) to rid it from other soluble cellular components. The bound DNA was washed twice using 750 µl PE buffer (13,000 rpm 1 min, at RT), spun dry (13,000 rpm 1 min, at RT) and eluted in 30 µl EB buffer (13,000 rpm 1 min, at RT).

Buffers:

P1 (suspension)

50 mM Tris/HCl pH 8.0
10 mM EDTA
100 µg/ml Rnase A

P2 (cell lysis)

200 mM sodium hydroxide
1 %(v/v) SDS

N3 (neutralisation)

2.55 M potassium acetate

PE (wash)

1 M sodium chloride
50 mM MOPS pH 7.0

15 % (v/v) ethanol

EB (buffer)

10 mM Tris/HCl, pH 8.5

*3.2.4 Cleavage of DNA with restriction endonucleases*

For restriction analysis, typically 1µg of plasmid DNA was mixed with 5-10 units of the restriction enzyme. The volume of the restriction enzyme stock solution (50 % w/v glycerol) should not exceed 1/10 of the reaction mixture. The restriction assay was incubated for 1-3 hours at the temperature optimum of the enzyme. The cleavage products are analyzed by agarose gel electrophoresis.

*3.2.5 Gel electrophoresis of DNA*

Agarose gel electrophoresis (1 % w/v) is applied to separate double-stranded DNA molecules according to their molecular weight after enzymatic restriction. Agarose is dissolved in TB buffer by heating in a microwave oven. After the solution is cooled to < 50°C, it is mixed with ethidium bromide (final concentration 0.1 µg/ml). The samples (10-20µl) are mixed with 1/10 of their volume with agarose gel sample buffer and subjected to electrophoresis for 45 min at 110 V. The running buffer is TB buffer. After electrophoresis, the intercalated ethidium bromide is detected at 302 nm.

Buffers:

TB:

90 mM Tris-base

90 mM Boric acid

Agarose gel sample buffer:

0.25 % (w/v) bromphenol blue

0.25 % (w/v) xylene-cyanol

15 % (w/v) Ficol-400

## 3.3 Biochemical techniques

### *3.3.1 Preparation of ClpC-intein fusion proteins*

A ClpC-WT–intein (or ClpC-DWB-, ClpC-WB1-, ClpC-WB2-, ClpC-ΔN- ClpC-ΔLi-intein) construct in pTYB2 (New England Biolabs) was used for expression, and the ClpC was purified as described below. This IMPACT-system (NEB) is a novel protein purification system, which utilizes the inducible self-cleavage activity of a protein splicing element (named intein) to separate the target protein from the affinity tag. It distinguishes itself from all other purification systems by its ability to purify, in a single chromatographic step, a native recombinant protein without the use of a protease. A target protein is fused to a tag consisting of the intein and the chitin-binding domain, which allows affinity purification of the fusion precursor on a chitin column. In the presence of thiols such as DTT, β- mercaptoethanol or cysteine, the intein undergoes specific self-cleavage, which releases the target protein from the chitin-bound intein tag resulting in a single-column purification of the target protein.

#### *3.3.1.1 Growth of bacteria*

A culture (1500 mL 2xYT with Amp, 37°C, in 5l flasks) was inoculated from cells grown overnight of *E. coli* (ER2566 from Biolabs bearing the plasmid pClpC). The culture was grown to an $OD_{600}$ of ~ 0.6. At this optical density 0.5 mM (final concentration) isopropyl-ß,-D-thiogalactoside (IPTG) was added. The flasks were then placed into a precooled incubator-shaker at 15°C and shaken over night at this temperature. The cells were harvested by centrifugation (4500 rpm, 4°C, 15 min, Sorvall F7S rotor), resuspended in cell-lysisbuffer with the smallest possible volume. Finally, the pellets were frozen and stored at –20°C.

##### *3.3.1.1.1 Purification of ClpC*

The solubilised pellets were thawn and lysed in a French Pressure cell (1200 PSI). The extract was centrifuged (18000 rpm, 4°C, 45 min in an SS34 rotor). The soluble fraction was mixed with 1 ml of Chitin beads (previously washed with water and equilibrated with the column buffer) per 1500 ml culture. When the cell extract was viscous, it was necessary to add a small amount of DNAse I (Roche). This mixture was than incubated over night in an

overhead shaker. The next day the beads were poured into a column (biorad 20 ml) and the flowthrough was collected for SDS-gel analysis. The column was washed with 10-20 CV (Column volumes) with column buffer. In addition, the first and the last ml of this washing procedure were collected for SDS-gel analysis. Subsequently two CV of cleavage buffer were added separately to the column and collected for SDS-gel analysis. All this, starting with the celllysis was done in the cold-room and/or on ice. The column with the cleavage buffer was kept over night in the cold room for the self-splicing reaction. The next day (about 16 h later) the protein was eluted with the elution buffer and collected. All samples were prepared with the 3x SDS-PAGE Sample buffer and analysed by SDS-gel, withe subsequent coomassie stain. The fractions containing the protein were pooled and dialysed.

Buffers:

Column & Elution Buffer:

500 mM NaCl
20 mM Tris-HCl pH 8.0
1mM EDTA

Cell Lysis Buffer:

500 mM NaCl
20 mM Tris-HCl pH 8.0
1 mM EDTA
0,1% Triton X 100

+ complete (Proteinase inhibitors from Boehringer, 1 Tablet/50 ml)

Cleavage Buffer:

500 mM NaCl
50 mM DTT
20 mM Tris-HCl pH 8.0
1 mM EDTA

Dialysisbuffer:

200 mM KCl
33 mM Bicin pH 8.9
5 mM $MgCl_2$
1 mM DTT

3x SDS-PAGE Sample buffer:

187.5 mM Tris-HCl pH 6.8
6% SDS
30% Glycerol
0.03% Bromphenol Blue

*3.3.1.1.2 Second purification step of ClpC*

The next step of the ClpC purification always was an anion exchange with a ResourceQ 1ml (Amersham Pharmacia). For unknown reasons only active ClpC- molecules bind to this column. The pooled and dialysed fraction from the Chitin column was then diluted with an equal volume of buffer A and loaded on a resource Q. The ClpC was eluted with a linear gradient (0-1000 mM KCL) of buffer A and buffer B. ClpC and the ClpC mutant proteins eluted at 180 mM KCl.
A final protein concentration of 1- 3 mg/ml was obtained using this standard purification. The protein was aliquoted and frozen in liquid nitrogen and stored at –80°C.

Buffers:
Buffer A:

50 mM Bicin pH 8.9
5mM $MgCl_2$
1mM DTT
125 µM EDTA

Buffer B:

1M KCl
50 mM Bicin pH 8.9
5mM $MgCl_2$
1mM DTT
125 µM EDTA

### *3.3.2 Preparation of* His-tag fusion proteins

#### *3.3.2.1 Growth of bacteria*

Cells of the *E.coli* strain M15 (for ClpP SG22189) containing the expression plasmid pQMA were grown over night at 37°C in a sterile tube containing 2xYT and ampicillin (100µg/ml). Then a 2l flask containing 1l of 2xYT and ampicillin (100µg/ml) was inoculated with the cell culture. The flask was shaken moderately. At an $OD_{600}$ of 0.6 IPTG was added to a final concentration of 0.5 mM and the cells were grown an additional 4 hours. Then the cells were harvested by centrifugation (4500 rpm, 4°C, 15 min), dissolved in buffer A containing protease inhibitors (from Boehringer, 1 Tablet/50 ml) and lysed in a French Press cell (as described above). The extract was centrifuged (18000 rpm, 4°C, 45 min) the soluble fraction was diluted into 50 ml and applied onto a nickel-nitrilotriacetic acid (NTA) agarose column (2 ml equilibrated with bufferA on a FPLC System (Pharmacia)). The column was washed with buffer A and then with buffer A containing 40 mM imidazol and the protein was then eluted with a gradient of 40-250 mM imidazol in buffer A. The fractions with the highest concentrations of proteins (judged by SDS-PAGE) were pooled and dialysed. {Irgendwo sagen welche Proteine so gereinigt werden, vielleicht hier}For concentration and as a final purification step of MecA and YpbH an anion exchange column run (resource Q) was performed as described for the ClpC purification. In this case, buffer A and B were adjusted to a pH of 8.5. The protein was aliquoted and stored at –80°C.

### *3.3.3 Other Proteins*

Purifications of DnaK, DnaJ, GrpE and ClpB, were performed according to published procedures (Goloubinoff et al., 1999). Protein concentrations were determined using the Bio-Rad Bradford assay with BSA as a standard.

## 3.4 Gel electrophoresis and protein detection

### *3.4.1 Gel electrophoresis of proteins*

The discontinuous sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (Laemmli, 1970).
The following solutions were used for the preparation of 15% polyacrylamide mini gels (11 cm x 7 cm x 0,1 cm)

Separating gel (20 ml):

10 ml 30% acrylamide-bisacrylamide (37:1)
5 ml 1.5 M Tris/HCl pH 8.8
5 ml dd$H_2O$
200 µl 10% (w/v) SDS
40 µl TEMED
(N, N, N', N'-tetramethylethylendiamine)
100 µl 10% (w/v) APS (ammonium peroxodisulfate)

Stacking gel (10ml):

1,5 ml 30% acrylamide-bisacrylamide (37:1)
2,5 ml 0.5 M Tris/HCl pH 6.8
5,5 ml dd$H_2O$
100 µl 10% (w/v) SDS
30 µl TEMED
30 µl 10% (w/v) APS

Running buffer:

50 mM Tris, 192 mM glycine, 0.1 % (w/v) SDS

Sample buffer (4x)

40 % glycerol
500 mM Tris/HCl pH 6.8
8 % (w/v) SDS
0.6% (w/v) bromphenol blue
20 % (v/v) β-mercaptoethanol

Protein samples were mixed with 1/3 of their volume with sample buffer and heated for 5 min at 95°C.
The mini gel runs were performed at a electric current of 20 mA per gel for the stacking gel and 40 mA for the separating gel.
Large protein gels (12 cm x 28 cm x 0,2 cm) were run at a constant voltage of 150 V for the stacking gel and at max. 250V for the separating gel.
If the runs of large protein gels were performed during the night, the used voltage should not exceed 40V. Standard proteins for SDS-PAGE were from Roche Diagnostics.
The gels were stained with Coomassie solution or with silver staining.

### *3.4.2 Coomassie staining*

The gel was carefully removed from the glass plates after the electrophoresis was completed and transferred to a petri dish for staining. The gel was completely covered with staining solution, warmed shortly in the microwave for one minute at a low voltage and incubated for twenty more minutes while shaking on a shaking plate. Once the gel was evenly stained, it was washed briefly with water before transferring it to the destaining solution.

Staining solution:

25% (v/v) Ethanol
10% (v/v) acetic acid
0.25% (w/v) Coomassie brilliant blue R250
60% (v/v) dd$H_2O$

Destaining solution:

20% (v/v) Ethanol
10% (v/v) acetic acid
70% (v/v) dd$H_2O$

*3.4.3 Silver stain*

Silver staining is a much more sensitive method to detect proteins. Typically less than 10 ng of protein can be detected with silver staining, whereas an about 50 times higher amount of protein is needed to be detectable by Coomassie staining.
The SDS-gel must be incubated in the fixing solution over night or a minimum of 60 min. Then the gel has to be washed two times with water and treated with $Na_2S_2O_3$ solution for one minute. The staining occurs for 20 minutes in the $AgNO_3$-solution. Then the gel has to be washed again 4-6 times with double distilled water. As a next step the gel is developed in the $Na_2CO_3$ solution. When the bands become visible the buffer has to be exchanged with stop solution.

Buffers:
Fix solution:

500 ml Ethanol (prep. Grade)
400 ml $ddH_2O$
100 ml acetic acid
0.5 ml formaldehyde (37%)

Wash solution:

500 ml $ddH_2O$
500 ml Ethanol (prep. Grade)

Pretreatment solution:

0.1 g $Na_2S_2O_3x5H_2O$
500 ml $ddH_2O$

Stainer: solution

0.4 g $AgNO_3$ (Fluka)
0.15 ml Formaldehyd (37%)
200 ml $ddH_2O$

Developer solution:

30 g $Na_2CO_3$ (Fluka)
0.5 ml Formaldehyd (37%)
2 mg $Na_2S_2O_3x5H_2O$
500 ml $ddH_2O$

Stop solution:

220 ml $ddH_2O$
220 ml Ethanol (prep. Grade)
60 ml acetic acid

*3.4.4 Immunological methods*

*3.4.4.1 Production of antisera:*

A rabbit was immunized with 0.5 mg of pure MecA, YpbH or ClpC diluted 1:1 in complete Freund's adjuvans. A subsequent booster injection was given after six weeks with 0.5 mg pure MecA (YpbH) diluted 1:1 into incomplete Freund's adjuvans. 30 ml of blood were harvested two weeks after the boost and then incubated at 37°C for one hour. The resulting clot of blood is incubated for five hours at 4°C. The solution was spun at 4500 rpm for 15 min at 4°C (Sorvall, SS34). The Serum was stored at –20°C.

*3.4.4.2 Immunoblotting:*

Western blot analyses were performed in order to histochemically identify a protein of interest from a complex protein mixture that has previously been separated by its molecular weight by SDS-PAGE and transferred to a membrane {!!!}. Two different antibodies are required, the first (primary) antibody interacting directly with the proteins, which was raised against (MecA, YpbH, ClpC), followed by the application of a secondary antibody detecting the first antibodies at their constant regions of the immunoglobulins' heavy chains ($F_C$).
The protein-samples were subjected to electrophoresis in 15% SDS-polyacrylamide gels. Dilutions for the antisera were as follows: MecA, YpbH 1:20000, ClpC 1:10000, McsB 1:10000.
Before usage the PVDF membrane has to be washed with Methanol p.a. for 5 min. Then the membrane and six sheets of Whatman paper were incubated in blotting buffer for 5 min.
After that all components were assembled as follows:

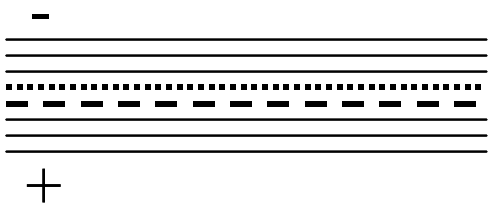
Cathode

3 layers of Whatman paper

SDS gel

PVDF membrane

3 layers of Whatman paper



Anode

The gel was blotted onto the PVDF membrane with 1.5 mA/cm (45 min). After that the PVDF membrane was incubated in blocking solution for 1h at room temperature with gentle shaking or over night at 4°C. Next, the PVDF membrane was washed with fresh TBS-T buffer (3 x 10 min). The primary antibody was diluted in TBS-T (add a small amount of acetone powder to inhibit unspecific reactions) and after that the blot was incubated with the diluted primary antibody for 1h at room temperature with gentle shaking. Next, the blot was washed in 100-150 ml of TBS-T: (3x 10´). Meanwhile the fluorochrome-conjugated secondary antibody was diluted into TBS-T (1:10000). The blot was incubated with the diluted secondary antibody for 1 h at room temperature with gentle shaking. Following the blot was washed again in 100-150 ml of TBS-T: (3x 10´) and the procedure continued with chemifluorescence substrate incubation.

Buffers:

Blotting Buffer (pH 9.2):

48 mM Tris/HCl pH 9.2
39 mM Glycin
1.3 mM SDS
20% (v/v) Methanol

TBS-T Buffer (pH 7.6):

20 mM Tris/HCl pH 7.6
137 mM sodium chloride
0.1 % Tween 20

TBS Buffer (pH 7.6):

20 mM Tris/HCl pH 7.6
137 mM sodium chloride

Blocking solution:

TBS-T buffer containing 3% (w/v) nonfat-dry milk

ECF-solution:

ECF Western Bloting Kit (Amersham Pharmacia)

*3.4.4.3 Imaging:*

The secondary antibodies are covalently linked to an alkaline phosphatase that is used for detection purposes. It reacts with ECF solution releasing a fluorophore that can be detected by its emission of light at 540-560 nm upon the stimulation with light at a wavelength of 473 nm. The washed blot was placed (protein-side up) onto a cleaned surface (e.g. cleaned glass plate). Next the membrane was completely covered with ECF (5μL/cm$^2$) solution.. Then the blot was covered with a foil for avoiding any air bubbles between foil and blot. After that the blot was incubated at room temperature for 5 to 10 min.
The incubation time for optimal sensitivity depends on the target concentration and should be experimentally determined for each new Western blot application.
Blots can be air dried in the dark before imaging. The blot was positioned, protein-side down, onto a clean glass plate.
The optimal instrumental settings for the fluoroimager FLA-3000 have to be determined experimentally. The detected fluorescence is quantified using the MacBAS software (Fuji Film).

## 3.5 *In vitro* Activity Assays

*3.5.1 ATPase assay*

The ATPase assay was performed according to procedures described by Liberek et al. (Liberek et al., 1991). This approach takes into account that ATP can be separated by thin layer chromatographically from ADP. The ATP provided carries a $^{32}$P-labelled phosphate residue at the α position, which allows to monitor and determine the relative amounts of ATP as well as ADP, using ATP hydrolysis rates under steady-state conditions were determined by

incubating 400 nM ClpC (wild type and variants) in ATPase buffer containing 2 mM ATP and [$\alpha$-$^{32}$P] ATP (0.1 µCi, Amersham) at 30°C.

Hydrolysis of [$\alpha$-$^{32}$P] ATP was monitored after separation of ATP and ADP by thin layer chromatography in chromatography buffer. The amount of radioactive ATP or ADP was quantitated on a phosphoimager (FLA3000, Fuji) by using the MACBAS version 2.5 (Fuji). Rates of ATP hydrolysis were determined by using the programme GRAFIT version 3.0 (Erithacus software). The resulting specific ATPase rates are given as molecules ATP hydrolyzed per second per molecule of the HSP100/Clp protein.

Buffers:

ATPase (MDH-refolding) buffer:

100 mM Tris/HCl pH 7.5
150 mM KCl
20 mM $Mg(OAc)_2$
1mM DTT

Chromatography buffer:

400 mM LiCl
10% (v/v) acetic acid

*3.5.2 Prevention of aggregation and refolding of heat denatured Luciferase*

Luciferase was diluted to a final concentration of 100 nM in refolding buffer containing additional 4 mM ATP supplemented with various chaperones (ClpC 0.6 µM, MecA 0.6 µM or KJE (DnaK 1 µM, DnaJ 0.2 µM, GrpE 0.1 µM)) in refolding buffer. The reaction mixture was incubated for 15 minutes at 43°C. After this, either KJE or the buffer was added and luciferase activity was determined at different time points by diluting 1 µl of the reaction mixture into 124 µl of assay buffer. After addition of 100 µl of D-luciferin (Sigma) (44 µM final concentration), light emission at 560 nm was measured over a period of 5s in a Berthold Lumat 9501. To determine the prevention of aggregation properties of Luciferase, Luciferase (100 nM) was heat-denatured in the presence of chaperones (ClpC 0.6 µM, MecA 0.6 µM, DnaJ 0.6 µM in refolding buffer with ATP regeneration mix (50 µl)) for 15 minutes at 43°C. After this, the total luciferase activity was determined as described above. The remaining

reaction mixture (30 µl) of each experiment was centrifuged at 16.000 g for 20 min at 4°C. The supernatant was carefully removed and the pellet was resuspended in the appropriate amount of refolding buffer (max. 50 µl). Pellet and supernatant were analyzed by SDS-PAGE followed by a Western-blot analysis with anti-Luciferase antibodies and subsequently quantified using the computer program MacBAS.

Buffers:

Refolding buffer:

25 mM HEPES/KOH pH 7.6
5 mM $MgCl_2$
50 mM KCl
2 mM ATP

Assay Buffer:

25 mM glycylglycine
5 mM ATP
15 mM $MgSO_4$

*3.5.3 Luciferase disaggregation and refolding*

The Luciferase (200 nM) was heat denatured in the absence of chaperones for 15 minutes at 43°C, after which the Luciferase was diluted 1:1 with the reaction mixture containing the chaperones (ClpC 0.6 µM, MecA 1.8 µM ClpB 0.4 µM, KJE (DnaK 1 µM, DnaJ 0.2 µM, GrpE 0.1 µM)) and 2 mM ATP in a regeneration mixture. The Luciferase activity was determined as described above.

*3.5.4 MDH disaggregation and refolding*

MDH (2 µM) was denatured at 47°C for 30 min in MDH-refolding buffer and diluted to a final concentration of 0.72 µM in the presence of chaperones (ClpC 0.72 µM, MecA 0.72 µM, ClpB 0.72 µM, KJE (DnaK 1µM, DnaJ 0.2 µM, GrpE 0.1 µM)) and 2 mM ATP in the presence of an ATP-regenerating system. MDH disaggregation was determined by monitoring

light scattering. MDH (2 µM) was denatured at 47°C for 30 min in MDH-folding buffer and diluted to a final concentration of 0.72 µM in the presence or absence of indicated chaperones (ClpC 0.72 µM, MecA 0.72 µM or 0.24 µM, ClpB 0.72 µM, KJE (DnaK 1µM, DnaJ 0.2 µM, GrpE 0.1 µM)). The reaction mixture was incubated for two minutes at 30°C. The disaggregation/refolding reaction was started by adding 2mM ATP together with an ATP-regenerating system. Right angle light scattering was determined in a thermostat-controlled cuvette (30°C) at 550nm excitation and emission wavelength in a Perkin Elmer LS50B spectrofluorometer. The activity of MDH was measured at 30°C in assay buffer. The time-dependent oxidation of NADH by MDH was monitored at 340 nm in a Shimadzu UV-1601 spectrophotometer. Diluting the reaction mixture 1:70 into the assay buffer started the reaction: L-malate + $NAD^+$ ⇔ oxaloacetic acid + NADH + $H^+$.

Buffers:

ATP regenerating system:

2mM ATP
4mM phosphoenol pyruvate
20 ng/ml pyruvate kinase

MDH-refolding buffer:

100 mM Tris/HCl pH 7.5
150 mM KCl
20 mM $Mg(OAc)_2$
1mM DTT

MDH assay Buffer:

150 mM potassium phosphate buffer, pH 7.6
1 mM DTT
0.5 mM oxaloacetate
0.28 mM NADH (Sigma)

### *3.5.5 Interaction of chaperones with MDH aggregates*

MDH (2 µM) was denatured in MDH-refolding buffer as described above and diluted to a final concentration of 0.72 µM supplemented with the chaperones (ClpC and MecA in different combinations with aggregated or native MDH molecules) at the same concentration. After the addition of 4.5 mM ATP in the presence of a ATP-regenerating system the reaction mixture was incubated at room temperature for 5 min and subsequently centrifuged at 16000 g for 30 min at 4 °C. The supernatant and the pellet fractions were analyzed by SDS-PAGE with subsequent Coomassie-stain.

### *3.5.6 MDH/α-Casein degradation*

MDH (4 µM) was denatured at 47 °C for 30 min in MDH-folding buffer and then diluted to a final concentration of 1.44 µM, which contained the reaction mix supplemented with ClpC, ClpP and MecA (all 0.88 µM). To start the reaction ATP (5 mM) with the ATP-regenerating system was added to the chaperone-mixture. The reaction was stopped at different time points by mixing an aliquot of the reaction mixture with SDS-sample-buffer (on ice). α-Casein (5.8 µM) degradation was also performed in the MDH-refolding Buffer with the same concentration of chaperones.

### *3.5.7 Analytical Size Exclusion Chromatography*

Size-Exclusion Chromatography uses porous particles to separate molecules of different sizes. It is generally used to separate biological molecules and to determine molecular weights and molecular weight distributions of polymers. Molecules that are smaller than the pore size can enter the particles and therefore have a longer path and longer transits time than larger molecules that cannot enter the particles. All molecules larger than the pore size are unretained and elute together. Molecules that can enter the pores will have an average residence time in the particles that depends on the molecules size and shape. Different molecules therefore have different total transit times through the column.

BufferA:

100 mM Tris/HCl pH 7,5
150 mM KCl
20 mM $MgAc_2$
1mM DTT
5% Glycerin

A S200 HR10-30 (Amersham) column, connected to a Pharmacia ÄKTA FPLC apparatus, was equilibrated with buffer A and 50 µl of ClpC (6µM) or the ClpC mutant proteins ClpC-DWB (6µM), ClpC-Wb1 (6µM), ClpC-Wb2 (6µM), ClpC-ΔN (6µM), ClpC-ΔLi (6µM) was injected. In separate experiments, MecA (6µM/1µM) or the CTD of MecA (6µM) was applied to the column in different combinations with the ClpC-WT or Walker B mutants with or without $^{3}$H-α-Casein (3µM/1µM). The molecular weights were calculated using a calibration curve, which was previously determined by running a set of protein standards (Biorad 670 kDa, 158 kDa, 44 kDa, 17 kDa, 1.35 kDa)
The proteins have been diluted to their final concentration and have been incubated in the presence or absence of 2mM ATP for 10 minutes. Chromatographic steps were performed with a flow rate of 1.0 ml/min at RT.
Fractions of 0.3 ml have been collected and concentrated by Methanol precipitation (this was done by: adding 3x Methanol, centrifugation for 10 min, discarding the supernatant, drying pellets in a spin vac and taking it up with 40 µl 1x SDS Sample Buffer, 95°C for 5 min.).
These samples were further analysed by silver stain or western blot analysis.

### *3.5.8 $^{3}$H labeling of α-casein*

α-Casein were labeled with *N*-succinimidyl-[2,3-3H]propionate (Amersham) as described previously (Gamer et al., 1996), except that free *N*-succinimidyl-[2,3-3H]propionate was removed by dialysis against gelfiltration buffer A
Tritium labelled α-casein was used to follow substrate interaction of α-casein with the ClpC/MecA chaperone system. $^{3}$H-α-Casein (3µM/1µM) (77000cpm/25666cpm) was incubated with the proteins as described above and applied to the S200 column. The fractions (0.3ml) were collected into scintillation vials and mixed with scintillation-coverfluid (Packard Ultima Gold). Radioactivity of the samples was detected by the radioimager (FLA 3000)

BufferA:

100 mM Tris/HCl pH 7,5
150 mM KCl
20 mM $MgAc_2$
1mM DTT
5% Glycerin

*3.5.9 Cross-linking Assays*

All ClpC variants were diluted into the cross-linking buffer (MDH assay buffer) to a final concentration of 1 µM. ClpC was incubated at 30 °C in the presence or absence of nucleotides (2 mM ATP or ADP) and MecA (1µM) for 10 min. The cross-linking reactions were started by adding of 0.1% glutaraldehyde and stopped within 10 min. Reactions were stopped upon addition of Sample Buffer. Cross-linking products were separated by a gradient SDS-PAGE (4–15%) followed by silver staining.

Buffers:
Cross-linking Buffer:

100 mM Tris/HCl pH 7.5
150mM KCl
20mM $Mg(OAc)_2$
1 mM DTT

Sample buffer (4x)

40 % glycerol
500 mM Tris/HCl (pH 6.8)
8 % (w/v) SDS
0.6% (w/v) bromphenol blue
20 % (v/v) β-mercaptoethanol

*3.5.10 BIAcore analysis*

Real-time bio molecular interaction analysis (BIA) uses SPR (surface plasmon resonance) to monitor bio molecular interactions. One protein is immobilized on the ligand sensor surface (e.g. with a nickel bound His-tag) and solution containing the other protein is passed over the sensor surface. As molecules from the solution bind to the immobilized protein, changes in the refractive index are registered (Schuck, 1997). In all of the SPR experiments described in this study only qualitative conclusions were drawn. This is because large amounts of protein were immobilized on the chip surfaces which makes it likely that mass transport limitations and rebinding artefacts would obscure any quantitative kinetic analysis (Schuck, 1997)
These assays were performed with a BIAcore 2000 machine. All Buffers and solutions were filtered (0,22 µm) and degassed. The amount of the CTD of MecA being anchored on the surface of a Ni-NTA-chip was adjusted so that about 800 resonance units of each protein were bound. ClpC (200 nM) was passed over the sensor in Eluent buffer. ATP was added as described in the text.

Buffers:

Eluent buffer:

0.01 M HEPES
0.15 M NaCl
50µM EDTA
0.005% Surfactant P20, pH 7.4

Nickel-solution:

500µM $NiCl_4$ in eluent buffer.

Regeneration solution:

0.01 M HEPES,
0.15 M NaCl,
0.35 M EDTA,
0.005 % Surfactant P20, pH 8.3

*3.5.11 Circular dichroism spectroscopy*

Circular dichroism (CD) is very sensitive method to determine the secondary structure of polypeptides and proteins. Circular dichroism spectroscopy is a form of light absorption spectroscopy that measures the difference in absorbance of right- and left circularly polarized light (rather than the commonly used absorbance of isotropic light). The differential absorption of radiation polarized in two directions as function of frequency is called dichroism. When applied to plane polarized light, this is called linear dichroism; for circularly polarized light, circular dichroism. Ellipticity is the unit of circular dichroism and is defined as the tangent of the ratio of the minor to major elliptical axis. It has been demonstrated that CD spectra between 260 and approximately 180 nm can be used to analyze the different secondary structures of a proteins such as: alpha helices, parallel and antiparallel beta sheets, turns and others. Like the other forms of absorption spectroscopy (UV/Vis, IR, etc.), CD is particularly powerful in monitoring conformational changes. In the region of 230-178 nm, one expects to observe effects of backbone conformational changes while CD effects at longer wavelengths (>230 nm) should isolate aromatic chromophore contributions and being environment dependent should reflect more global, three-dimensional properties of the protein.

Measuring Circular dichroism:
Far-UV circular dichroism (CD) spectra were recorded in a Jasco J715 spectropolarimeter at protein concentrations of 5µM in phosphate puffer ($Na_xH_yPO_4$) using 0,2 cm cuvettes.
The residue ellipticity [Θ] MRW (degrees x cm2 x dmol-1) was calculated from the measured Θ (in degrees) by using following equation:

$$[\Theta]MRW = \frac{\Theta \times MRW \times 0{,}1}{d \times c}$$

| | |
|---|---|
| Θ | = measured ellipticity |
| MRW | = mean residue weight |
| d | = path length in cm |
| c | = protein concentration in mg/ml |

## 3.6 *In vivo* activity assays

### *3.6.1 Gel filtration of cell extracts*

Cells (*clpC-WT/ clpC-DWB/ clpC-ΔLinker*) were grown in 200 ml 2YT Medium (inoculated from an overnight-culture with Kanamycin) at 37°C up to an OD600 ≈ 0,6-0,7. 100 ml of the culture were pelleted for the analysis of the logarithmic growth phase. The other half was treated with a heat shock (54°C for 30 min) and then spun down at 4500 rpm 15 min (Heraeus multifuge 3 S-R). The Pellets were resuspended in 0.6 ml STM buffer and washed once again in the same buffer. 20 µl Lysozyme were added and incubated for half an hour at 37°C to obtain protoplasts. Cells were centrifuged again at 10000 rpm at 4°C for 10 min. The supernatant (cell wall fraction) and the pellet was collected and then washed again in STM buffer. To lyses the cells the pellet was resuspended in 0.5 ml TM buffer and 10 µl DNaseI (1mg/ml, 0.15 M NaCl, 50 % glycerol) and 5 µl PMSF (0.1M in ETOH) were added. After an incubation of 10 min. on ice the cells were sonificated 5 times (5cycles at 50 % with the Sonifier (Branson)), then left on ice for 3 min. This procedure was repeated three times.
The protein concentration of the cell extracts was determined by using the Bio-Rad Bradford assay with BSA as a standard protein.
The same amount of proteins from each strain was loaded onto a size exclusion column (S300, 120 ml, pharmacia). The GF-runs were performed at 0.5 ml/min in the gelfiltration buffer. The fractions (1.5 ml) of interest were precipitated, using the TCA precipitation protocol (described below). The already prepared samples were separated with SDS-PAGE and were further analyzed by western blotting using antibodies against ClpC and MecA.

Buffers:
STM:

50 mM NaCl
25% Sucrose
50 mM Tris/HCl pH 8.0
5 mM $MgCl_2$

TM:

50 mM Tris/HCl pH 8.0
5 mM $MgCl_2$

Gelfiltration Buffer:

200 mM KCl
50 mM Tris/HCl pH 8.0
5 mM $MgCl_2$
5% Glycerol
0.5 mM DTT

*3.5.9.1 TCA precipitation*

The samples (1 ml of the GF-run fractions) were mixed with 100µl $Na^+$desoxycholat and 100µl TCA-solution. The samples were then incubated on ice for 20 min and centrifuged for 15 min at 13000 rpm. Afterwards the supernatants were discarded and the pellets were resuspended in ice-cold acetone. The suspension was centrifuged again for 5 min at 13000 rpm. The acetone was removed by vacuum and the pellets were dissolved in 1x protein sample buffer. To adjust the pH a few µl of a 1M Tricine solution were added until the colour of the samples switched back to blue.

Buffer:

72% TCA
0,15M $Na^+$desoxycholat
ice cold acetone (-20°C)

*3.6.2 Thermal resistance*

Thermal resistance of the strains of interest were determined by spot tests as well as following growth curves after heat shock.

*3.6.2.1 Spot-tests*

Spot tests were performed using overnight cultures grown in LB medium. The cultures were diluted to the same $OD_{600}$ (=1). Aliquots from these dilutions were further diluted from $10^{-1}$ to $10^{-6}$ in 2YT medium. 10 µL of the dilutions $10^{-3}$, $10^{-4}$, $10^{-5}$ and $10^{-6}$ were spotted onto LB plates. Plates were incubated at the indicated temperatures for 24 h.

*3.6.2.2 Growth curves*

Overnight cultures of the strains to be tested were diluted into 2YT-medium with Kanamycin (20 µl/ml) and incubated again at 37°C for 2h in a shaker. The cultures were diluted to the same $OD_{600}$ (=1). 1ml of this dilution was used to inoculate 50 ml of fresh 2YT-medium without an antibiotic. Growth was followed by the measurement of the optical density in a spectrophotometer. A heat shock was given by moving the cultures to a 54°C water bath, after an optical density of each strain of $OD_{600}$ (=0.6). At each time point 1 ml of cells was removed, and prepared for Western blot analysis as described in chapter 3.6.1

*3.6.3 β-Galactosidase determinations*

Cells were grown in competence medium (SS-II-medium) and extracts, buffers and ONPG solutions were prepared as described previously (Albano et al., 1987). The Activity was measured in microtitre plates with a Rainbow Shell microplate reader (Tecan). The extract (100 µl) was mixed with 400µl assay buffer, and the reactions were started upon addition of 200 µl of ONPG. 100µl were pipetted into a well of a 96-well-plate and the absorbance at 420 and 550 nm was measured

The frozen stocks were spread out onto TBAB-plates for growth over night at 30°C. The cells were resuspended in Spiz II medium, grown again to the early log Phase and used to inoculate a 50 mL Culture of spizII-medium. The time points were taken close enough to determine $t_0$.

From each time point three different samples of 1 ml were taken and pelleted at 13000 rpm for 10 min. The pellets were resupended in 0.5 ml Assay buffer. 100 µl of the samples were diluted with 400 µl assay buffer, 10µl Toluol were added and the samples were vortexed for 15 sec. and stored at room temperature for 30 min.

200 µL ONPG (4mg/ml disolved in assay buffer) were added subsequentially to each sample. Then the sample was vortexed and the incubation time for each sample was noted. When a yellow colour developed the reaction was stopped with 0.5 mL $Na_2CO_3$ (1M).

The cells were pelleted again in a tabletop centrifuge for 5 min at 13000 rpm. 100 µl were pipetted into a 96 well plate and the absorbance at 420nm and at 550 nm were determined

The β-Galactosidase activity was calculated as followed:

Miller units = $$1000 \times \frac{OD_{420} - 1.75 \times OD_{550}}{t \times v \times OD_{600}}$$

$OD_{420}$, $OD_{550}$ = absorption (at 420 and 550 nm) of the sample after the reaction

$OD_{600}$ = optical density of the culture before it is pelleted for the β- Galactosidase assay

t = reaction time

v = volume of the culture used for the assay

Buffers:

SS-II-Medium:

44 mM $KH_2PO_4$ anh.
80 mM $K_2HPO_4$ anh.
15 mM $(NH_4)_2SO_4$
0.7 mM Na citrate x $2H_2O$

Added to 500 ml 1x SS:

5 ml 50% glucose
5 ml 2% casein hydrolysate
5 ml 10% yeast extract
2.5 ml histidine 10 mg/mL
2.5 ml leucine 10 mg/mL
2.5 ml methionin 10 mg/mL
5 ml $CaCl_2$ 0.05 M
12.5 ml $MgCl_2$ 0.1M

Assay buffer:

0.1 M $Na_xH_yPO_4$ pH 7.0
(to get pH 7.0 mix 0.5 M Stock of $NaH_2PO_4$ ≈ 400ml
and 0,5 M Stock of $Na_2HPO_4$ ≈ 850 ml)
0.001 M MgSO4
0.001 M KCl
0,1 M β-Mercaptoethanol (add just before use)

# 4 RESULTS

## 4.1 Chaperone activity of ClpC

### *4.1.1 Chaperone assays using Luciferase and Malate Dehydrogenase*

A first step to understand the role of ClpC in protein quality control was to test the activity of ClpC and its adaptor protein MecA in several in vitro chaperone assays. These assays include the ability to prevent aggregation, refold denatured proteins or disaggregate previously aggregated proteins. Chaperone activities are tested *in vitro* with well-characterized protein substrates, like Luciferase (Schröder et al., 1993) from the North American Firefly (Photinus Pyralis) or Malate Dehydrogenase (MDH) (Goloubinoff et al., 1999) from pig heart.

The activity of refolded Luciferase, a monomeric 61-kDa enzyme, can be monitored by the activation of D-luciferin. Measuring the bioluminescence of D-luciferin (in Light Units LU) monitors the refolding of chemically denatured Luciferase or disaggregation with subsequent refolding after heat denaturing.

Thermally denatured MDH is another well-characterized chaperone substrate, which can be used to study the effects of chaperones on protein aggregates (Goloubinoff et al., 1999). Heat treatment at 47°C results in the irreversible loss of more than 99% of MDH activity in 30 min. After an initial delay of 5–8 min, the light scattering (turbidity) of the solution increased rapidly as a consequence of formation of large aggregates. The turbidity and residual enzymatic activity remained constant after a temperature downshift to 25°C for at least 5 hours, demonstrating that heat-inactivated MDH forms stable and irreversible aggregates. Under a phase-contrast microscope, aggregated MDH (2 μM) appeared as loose, reticulated 5- to 50-μm-long filaments. Gel filtration and SDS-gel electrophoresis of sedimentation-soluble and insoluble fractions (4 min at 14,000 *g*) showed less than 3% of soluble inactive monomers in the heat-treated MDH sample. In the absence of chaperones, less than 3% of the MDH activity was spontaneously recovered during 24 hours at 25°C (Goloubinoff et al., 1999).

To follow the chaperone dependent disaggregation of MDH, turbidity was measured in a spectrofluorometer. The light scattering decrease in direct correlation to the decrease of MDH aggregates. Malate Dehydrogenase (MDH) catalyzes the following enzymatic reaction:

L-malate + $NAD^+$ ⇔ oxaloacetic acid + NADH + $H^+$.

This enzymatic activity can be followed by the decrease in absorbance at 340 nm resulting from the oxidation of NADH. The rate of the decrease in absorbance is used to measure the refolding of MDH (in % of the total activity per second).

*4.1.1.1 Luciferase*

*4.1.1.1.1 Interaction of ClpC and MecA with Luciferase aggregates*

In order to test the ability of ClpC and MecA to prevent Luciferase aggregation during heat shock, Luciferase was heat denatured 15 minutes at 43°C together with MecA, ClpC, and ClpC with MecA. As a positive control the holder chaperone DnaJ was added. Bovine serum albumin (BSA) was used as a control for unspecific protein-protein interactions.
After five minutes incubation at 30°C, the amount of soluble or aggregated Luciferase was determined by centrifugation of the sample. In addition to that, the Luciferase activity was measured to determine the total activity after the heat treatment. This allowed the comparison of the total Luciferase activity with the relative amount of soluble Luciferase.
The resulting supernatant and pellet fractions were analyzed by SDS-PAGE with subsequent Western blot (Fig. 1). The determination of the amount of soluble Luciferase, from multiple experiments represented in Fig. 1, demonstrated that ClpC, together with MecA, was able to maintain 40-50% of the Luciferase in the soluble fraction. Interestingly, 30-40% of Luciferase remained soluble in the presence of MecA or ClpC alone.

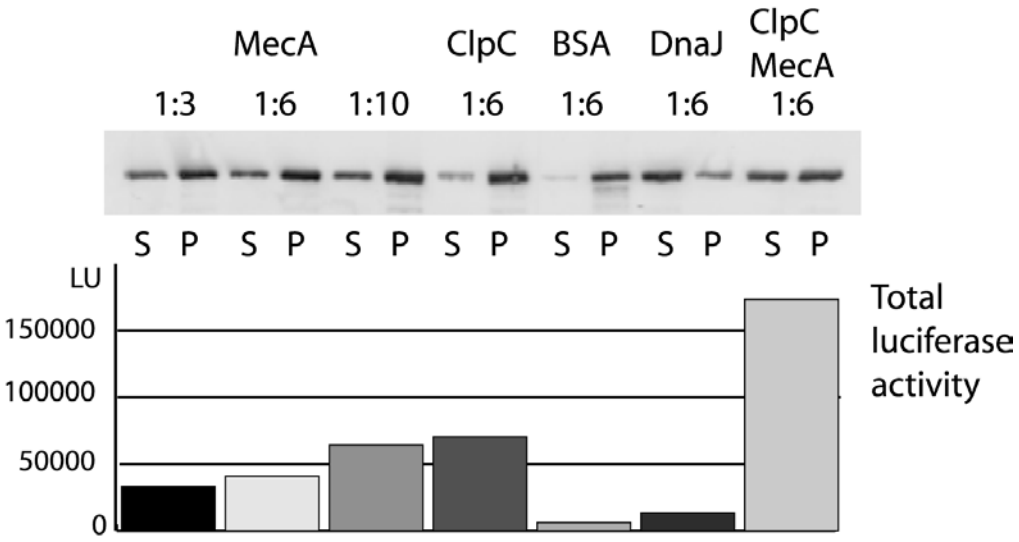



**Fig.1)** Luciferase was heat denatured 15' at 43°C together with MecA (in different molar ratios), ClpC, BSA, DnaJ or ClpC & MecA (in a 1:1 ratio to each other). Subsequently aliquots were centrifuged. The western-blot with anti-Luciferase antibodies of the resulting supernatant and pellet fraction is depicted. The concomitantly measured total luciferase activities before the centrifugation for the different experiments are shown in the graph below the western-blot.

60-70% of Luciferase remained in the soluble fraction in presence of DnaJ while in contrast only 10% remained soluble in the presence of BSA. The Luciferase activity of these samples

revealed that although either MecA or ClpC were able to partially maintain Luciferase in a soluble state, only when both proteins were present significantly higher activity could be rescued. Furthermore, as a control, BSA or DnaJ rescued almost no activity, although luciferase solubility was greatest in the presence of DnaJ (Fig. 1).

The result from this experiment suggested, that ClpC together with MecA exhibited some, albeit weak, ability to prevent Luciferase aggregation, and that this level is comparable to that of MecA or ClpC alone. The slightly higher prevention of aggregation ability of ClpC together with MecA (40-50%) compared to MecA or ClpC alone (30-40%) could result from an additive effect of the two components. The level of Luciferase activity after 5 minutes, rescued by ClpC and MecA indicates that MecA together with ClpC could have disaggregation and refolding abilities.

*4.1.1.1.2 Prevention of aggregation and reactivation of Luciferase*

To examine prevention of aggregation with additional disaggregation and refolding activities of ClpC and MecA, the activity of heat-denatured Luciferase was followed with time.

The Luciferase was heat denatured in the presence of ClpC or MecA, ClpC and MecA and as a control with KJE. The Luciferase activity was determined at the indicated time points after the samples were returned to 30°C.

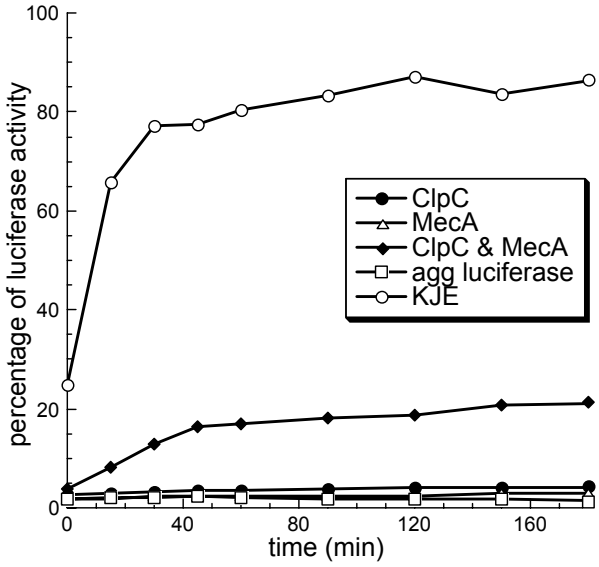


**Fig. 2)** Prevention of aggregation and refolding assays with heat aggregated luciferase. Luciferase was heat denatured 15' at 43°C with ClpC, MecA, ClpC and MecA or KJE. Subsequently luciferase activity was determined at the indicated time points.

Only ClpC together with MecA could slowly recover Luciferase activity up to 20%, after 3h. The initial Luciferase activity was below 5% in the presence of ClpC or MecA, as shown in the first experiment and none of these proteins alone could recover Luciferase activity with time. The high initial luciferase activity (25%) in the presence of the KJE system reflects the prevention of aggregation abilities of this chaperone system. The addition of KJE during heat denaturation resulted in the recovery of approximately 80% Luciferase activity.
The result from these experiments demonstrated, that the ClpC/MecA system was not efficient in preventing aggregation compared to KJE, but was able to rescue activity after heat treatment. This activity can also be tested by Luciferase refolding experiments. An experimental setup to elucidate Luciferase-refolding activity was therefore used

*4.1.1.1.3 Refolding of chemically denatured Luciferase*

In this assay Luciferase was denatured with 6M-guanidinium-hydrochloride and subsequently diluted into refolding buffer. Under these conditions Luciferase molecules are unstructured and tend to aggregate with time if no chaperone is present. This experimental system can be utilized to examine prevention of aggregation and refolding activities. In the experiment shown in Fig. 3 the chaperones were added immediately after denaturation, to ensure that aggregation can be prevented (holding activity) and refolding starts directly.

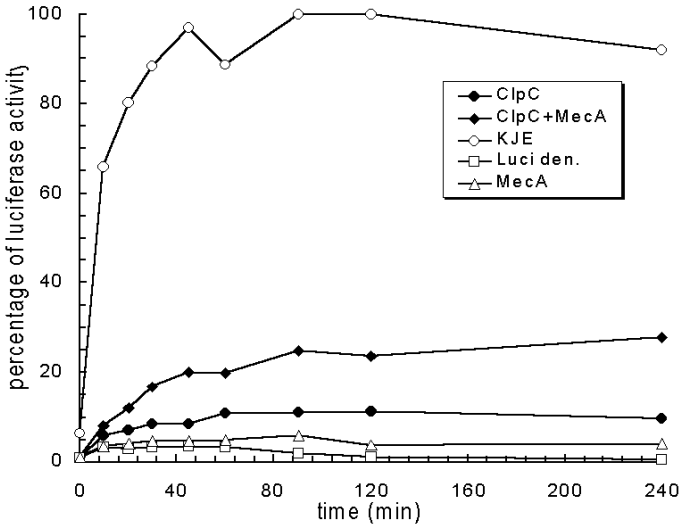


**Fig. 3)** Refolding of guanidinium hydrochloride denatured luciferase with ClpC, MecA, ClpC and MecA or KJE as shown in the legend. Luciferase activity was determined at the indicated time points.

This experiment demonstrated, that the ClpC/MecA system was not as efficient as the KJE-system in Luciferase refolding, although the percentage of rescued Luciferase was slightly higher in comparison to the activity of ClpC/MecA in the previous experiment. The KJE-system was able to rescue 100% of the Luciferase to the native state. ClpC alone regained 10 % of Luciferase activity. MecA was not able to recover Luciferase activity, in comparison with the control of denatured Luciferase without chaperones.
In summary ClpC together with MecA can refold denatured Luciferase, but at a low level compared to the KJE system. These results favor the testable hypothesis that ClpC together with MecA may act to disaggregate and assist subsequent refolding, instead of preventing aggregation and assisting refolding of Luciferase.

#### *4.1.1.2 MDH*

##### *4.1.1.2.1 Physical association with MDH aggregates*

To dissect further the possible chaperone activities of MecA and/or ClpC, the influence of ClpC and MecA on preformed protein aggregates were examined. Heat aggregated MDH was used as a model system, because it is known to form large protein aggregates. These aggregates can be centrifuged to separate aggregated MDH from native proteins.
First the physical association of ClpC and MecA with aggregated proteins was tested with a supernatant pellet experiment. Heat aggregated MDH and native MDH were incubated with the various chaperones for 5 minutes. The pelletable fraction was separated from the soluble fraction by centrifugation. Only in the presence of ATP, ClpC and MecA could be recovered in the pellet fraction (Fig. 4). In the absence of ATP, the recovery of MecA and ClpC in the pellet was greatly reduced. If MecA was omitted, then ClpC did not associate with the aggregated MDH. If ClpC was absent no association of MecA with the aggregate was visible. This indicated, that in presence of ATP a tertiary complex consisting of ClpC and MecA interacting with protein aggregates could form. Such a tertiary complex has been previously observed for ClpC, MecA and ComK (Turgay et al., 1997). Native MDH was always

recovered in the supernatant fraction and heat denatured MDH was always localized to the pellet fraction in the absence of chaperones (Fig. 4).

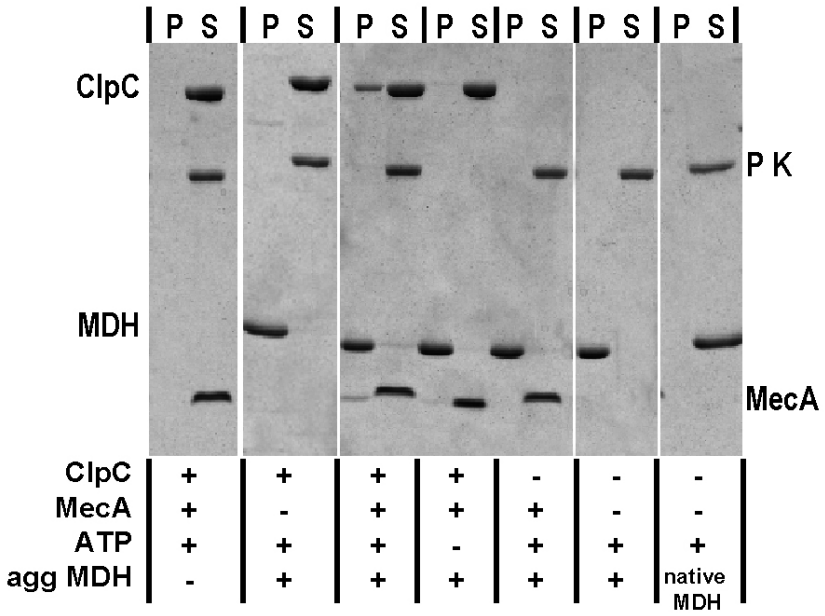



**Fig. 4)** ClpC together with MecA and ATP associates with aggregated protein. Heat aggregated MDH was incubated for 5 min at room temperature with ClpC in the presence or absence of MecA with or without ATP (with pyruvate kinase (PK) and phosphoenolpyruvate as ATP regenerating system), as indicated below the gel. In addition a control without aggregated MDH (agg. MDH) is shown. A subsequent centrifugation for 30' at 16000 g separated supernatant and pellet fractions. Supernatant (S) and pellet (P) fractions were analyzed by Coomassie-stained SDS PAGE. Positions of the different proteins on the gel are marked. Denatured MDH is located in the pellet fraction and native MDH is always recovered in the supernatant.

*4.1.1.2.2 MDH disaggregation activity*

Next, the ability of ClpC and MecA to disaggregate MDH aggregates was investigated.
MDH was denatured at 47°C for 30 minutes. MDH disaggregation was monitored by the changes in light scattering after addition of the respective chaperones.
The results of these experiments demonstrate that only ClpC together with MecA was able to disaggregate MDH aggregates at rates, comparable to the ClpB/KJE system (Fig. 5). Neither MecA nor ClpC alone could disaggregate these MDH aggregates. The heat aggregated MDH molecules scatters light over 60 minutes without a significant decrease of the signal.

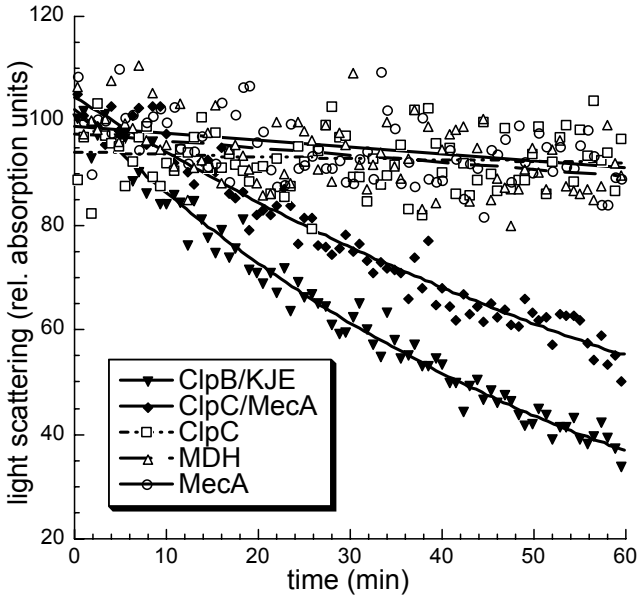



**Fig. 5)** ClpC together with MecA could disaggregate heat aggregated MDH. Previously heat aggregated MDH was incubated with the indicated proteins. The disaggregation reaction was subsequently followed in time by light scattering measurements. In addition curve fits of the data points are shown.

After it was shown that ClpC/MecA bind and disaggregates MDH aggregates, it remained to be elucidated whether ClpC/MecA can also subsequently refold the disaggregated MDH molecules.

### *4.1.2 Disaggregation and refolding activity of ClpC and MecA*

To determine the fate of the disaggregated MDH, MDH activity was followed upon addition of the chaperones. The enzyme activity of MDH can be determined by the decrease in absorbance at 340 nm resulting from the oxidation of NADH (see chaperone assays).
The results of this experiment are depicted in Fig. 6. Only ClpC together with MecA was able to disaggregate and refold heat aggregated MDH, comparable to the ClpB/KJE control (Fig. 6A).
Interestingly the ClpC and MecA mediated disaggregation of MDH was observed in the first 30 minutes (Fig.5). Whereas a significant increase of the activity of refolded MDH started always after half an hour.

Neither ClpC nor MecA alone was able to recover MDH activity from the protein aggregates. To ensure that these results were not specific for heat aggregated MDH the disaggregation and refolding of heat aggregated Luciferase was also monitored (Fig. 6B). Just like aggregated MDH, only ClpC together with MecA could rescue Luciferase activity from the aggregated state.

After 120 minutes the percentage of Luciferase rescued by ClpB/KJE was just 10 % higher than the percentage rescued by ClpC/MecA. As a control the chaperone system KJE was added without ClpB.

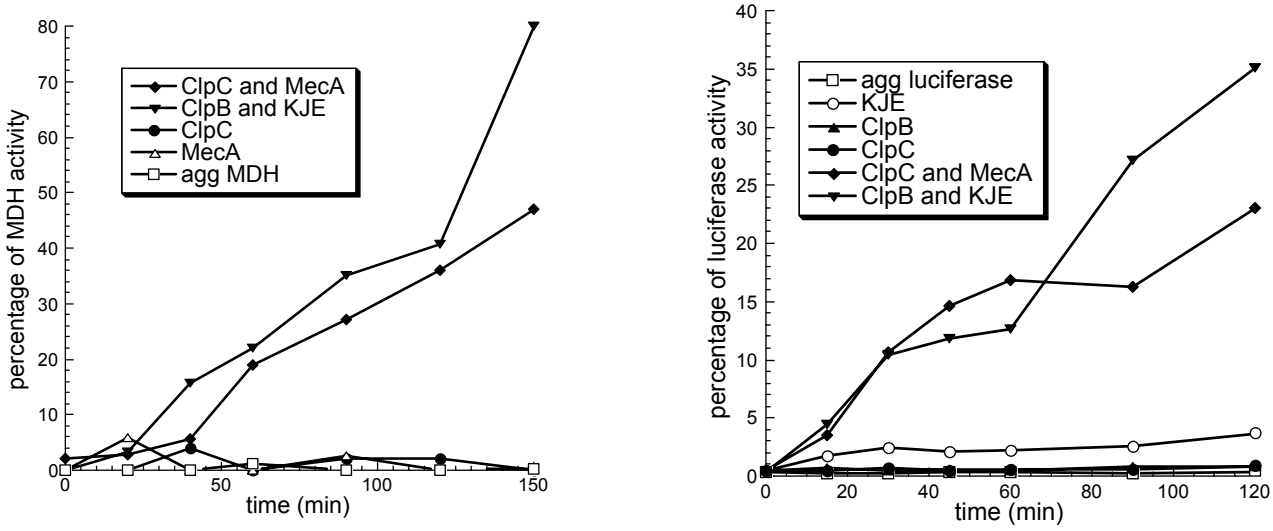



**Fig. 6A)** ClpC together with MecA can disaggregate and refold previously heat aggregated MDH
Previously heat aggregated MDH was incubated with the indicated proteins and MDH activity was determined.
6B) ClpC together with MecA can disaggregate and refold previously heat-aggregated Luciferase

Previously heat aggregated luciferase was incubated with the indicated proteins and Luciferase activity was determined at the indicated time points.

These experiments suggested that ClpC, together with the adaptor protein MecA, forms a chaperone system, which binds in presence of ATP to protein aggregates and is able to disaggregate and refold these aggregated proteins.

## 4.2 ClpCP mediated degradation

Previously it was shown that ClpCP degrades ComK and ComS, but only in the presence of MecA. Interestingly, MecA itself was also degraded by ClpCP *in vivo* and *in vitro,* but much slower than the presented substrates (Turgay et al., 1998).
The preceding experiments demonstrated, that ClpC together with MecA was able to disaggregate and refold substrates like MDH and Luciferase. Therefore it was interesting to test whether ClpCP in the presence of MecA is able to degrade misfolded and aggregated proteins. α-Casein which is a mostly unstructured protein (Herskovits, 1966) was used as substrate representing an unfolded protein. As implicated by the name of the Clp-proteins (casein lysing proteins) α-casein was one of the first identified substrates of this protein class.

### *4.2.1 Casein is a substrate for the ClpCP/MecA system*

To test the MecA-dependent degradation of α-casein by ClpCP (Fig 7), α-casein was incubated with ClpC and ClpP together with MecA. In the presence of MecA, α-casein was rapidly degraded following which MecA itself was degraded (Fig. 7C). If the amount of MecA was reduced to 0,1µM the band of MecA was not visible anymore after 30 min. while α-casein degradation was still observable (Fig. 7D)
As a control the degradation of MecA without α-casein and the degradation of α-casein without MecA was also tested (Fig. 7A+B). MecA as a natural substrate for ClpCP targeted itself and was degraded very rapidly. Degradation of α-casein by ClpCP in the absence of MecA was not detectable (Fig. 7B). These results demonstrated that the presence of MecA is essential for the efficient ClpCP-mediated degradation of α-casein.
The MecA degradation was slowed down in the presence of α-casein, as it was previously demonstrated for the substrates ComK or ComS.

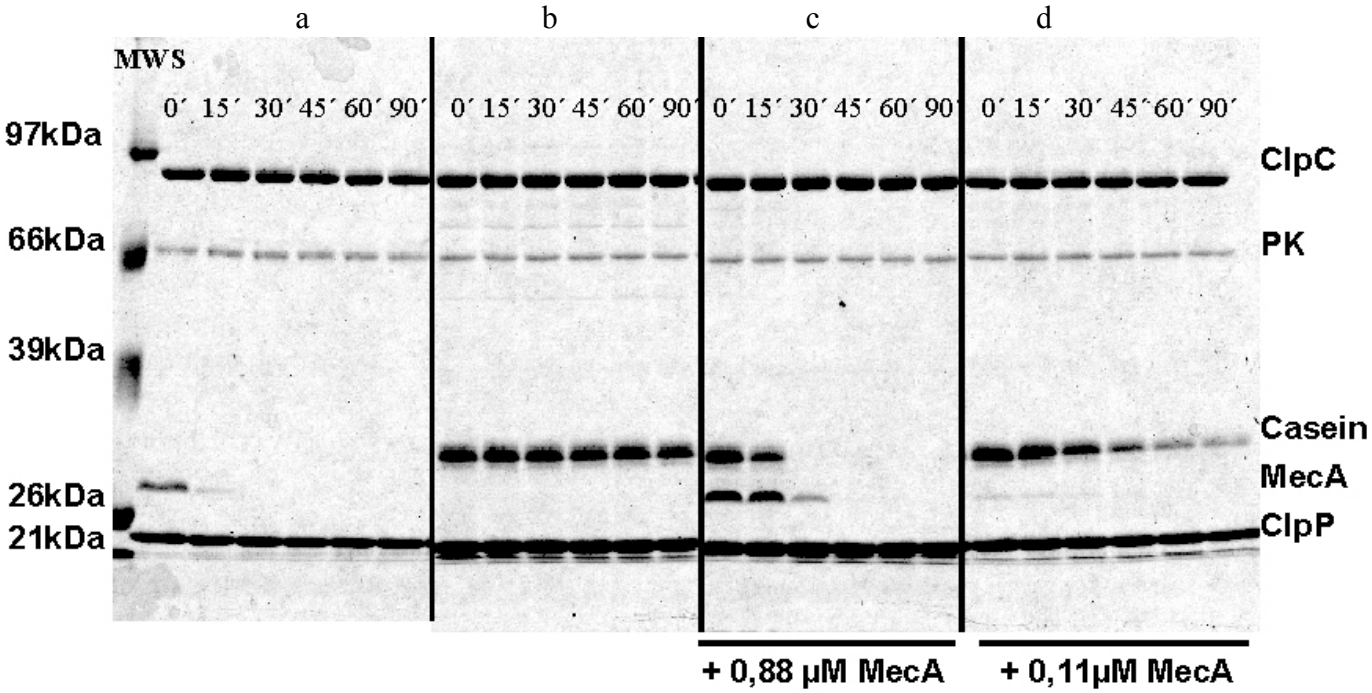



**Fig. 7)** Degradation of α-Casein by ClpCP depends on the presence of MecA **a**) MecA itself is degraded within 15 min. **b**) α-Casein is not a substrate for ClpCP **c**) α-Casein is degraded by ClpCP and MecA **d**) A minimal amount of MecA is sufficient to degrade α-Casein. ClpCP with ATP and PK/PEP regenerating system was incubated with α-casein with or without MecA (as indicated above). Samples were taken at indicated time points and analyzed by coomassie-stained-SDS-PAGE. Sizes of the molecular weight standard (MWS) were depicted on the left and the proteins were marked on the right side of the gel.

### *4.2.2 Aggregated MDH can be degraded by the ClpCP/MecA system*

To test degradation of heat denatured and thereby aggregated MDH, ClpC, ClpP and MecA were incubated with previous aggregated MDH and native MDH. The degradation assay was started by the addition of 2mM ATP. The experiment demonstrated (Fig. 8) that after an hour up to 50% of the aggregated MDH was degraded. Native MDH was stable in the presence of ClpCP, MecA and ATP. In addition the ClpCP mediated degradation of aggregated MDH was depending on the presence of both MecA and ATP (Fig. 8).

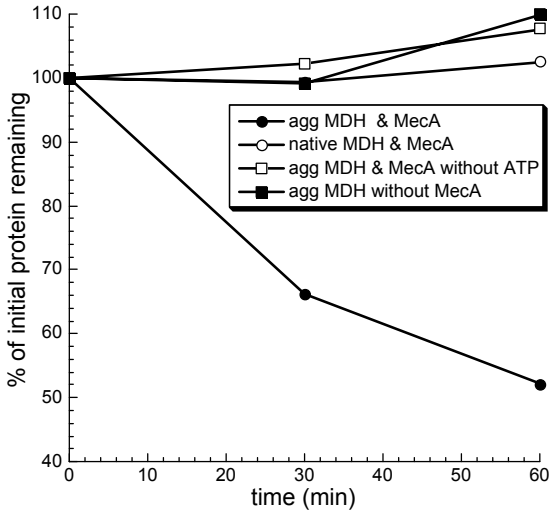



**Fig. 8)** Previously heat aggregated MDH (1µM) was degraded by ClpCP (1+1µM) only when MecA (1µM) is present. Heat aggregated (agg. MDH) or native MDH was incubated with ClpCP with the indicated components. Samples were taken at indicated time points and analyzed by coomassie stained SDS-PAGE. The gels were scanned and the relative amount of MDH was determined by image analysis and plotted.

However the degradation of aggregated MDH was much slower and less efficient compared to the degradation of α-casein (Fig. 8). A concurrent degradation of MecA could explain the lower rate. To test the MecA dependency of MDH degradation the amount of MecA was titrated in this assay (data not shown). 2µM MecA was needed for the complete degradation of the aggregated MDH within 60 minutes. With only 0,5 µM MecA the degradation of the aggregates stopped at 70% of the total MDH amount.

α-Casein degradation continued even at a very low MecA (0.1 µM) concentration (Fig. 7d). In contrast only in the presence of a twofold overshoot of MecA (2µM), MDH could get degraded almost completely.

These experiments demonstrate that aggregated MDH is a substrate for MecA mediated degradation by ClpCP, whereas native MDH molecules are not degraded. In contrast to α-casein degradation more MecA was needed for MDH degradation activity. Heat aggregated MDH is forming big aggregates, and α-casein is an unstructured protein, that does not form aggregates. An explanation for this difference could be a different recognition of these different kinds of substrates.

### 4.3 The ATPase activity of ClpC is necessary but not sufficient for chaperone activity

In order to further determine the mechanism by which MecA activates ClpC, the MecA dependent ATPase stimulation of ClpC was determined (Table 1 and Fig. 11). The basal ATP hydrolysis rate of ClpC was 0.004 $s^{-1}$. MecA itself has no ATPase activity and it stimulates the ClpC rate up to a 360 fold (1,5 $s^{-1}$) in a ratio of one ClpC to six MecA molecules. In the standard disaggregation and refolding assays a ratio of ClpC to MecA of 1:1 was used. This molecular ratio induces the ATP hydrolysis of ClpC up to 270 fold (1.1 $s^{-1}$). The substrates used in the assays before (α-casein, luciferase and MDH) were also tested for ClpC ATPase stimulation. These substrates did not stimulate the ATPase rate of ClpC (see Table 1). In combination with MecA, neither α-casein nor luciferase or MDH stimulated the MecA induced ATPase rate as it was shown for the natural substrates of ClpCP/MecA ComK and ComS (Turgay et al., 1997). This was also tested with different ratios of MecA to ClpC (data not shown).

The basal rate of ClpC was compared with the basal rates of ClpA (0.54 $s^{-1}$) and ClpB (0.02 $s^{-1}$). The basal ClpC-ATPase rate was about a hundred fold lower than the basal rate of ClpA. The addition of MecA to ClpC induced the ATPase activity of ClpC to a level similar and higher compared to the ClpA basal rate (Table 1).

These experiments were consistent with the hypothesis that MecA is needed for ClpC function, because it induces the ATPase and thereby the general activity of ClpC.

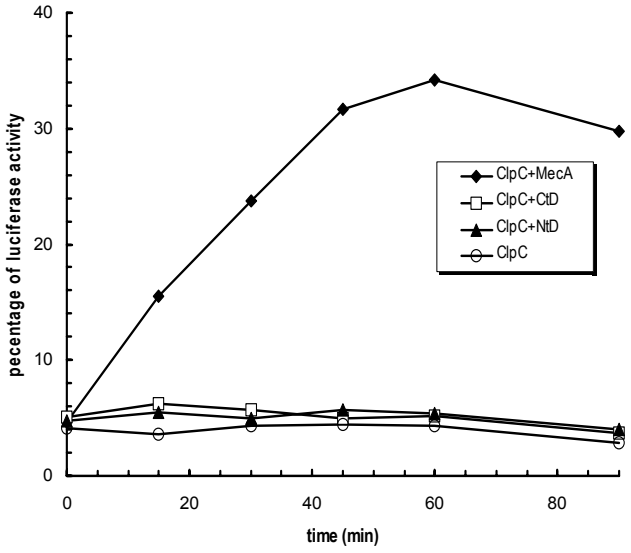
| Protein | ATPase | fold stimulation | comment |
|---|---|---|---|
| ClpC | -0.0041 $s^{-1}$ | | basal rate |
| ClpC + MecA (1:1) | -1.108 $s^{-1}$ | 270x | |
| ClpC+ MecA (1:6) | -1.475 $s^{-1}$ | 360x | |
| ClpC + Casein | -0.007 $s^{-1}$ | 2x | |
| ClpC + den. Luci | -0.0041 $s^{-1}$ | no stimulation | |
| ClpC + den. MdH | -0.0041 $s^{-1}$ | no stimulation | |
| ClpA | -0.54 $s^{-1}$ | | 131x ClpC basal |
| ClpB | -0.015 $s^{-1}$ | | 3.6x ClpC basal |
| ClpC +CtD (1:1) | 0.42 | 100x | Stimulation as with full length MecA |

| | | | |
|---|---|---|---|
| | | (As much as MecA in the same experiment) | (Persuh et al 1999) |
| ClpC + NtD | -0.0041 $s^{-1}$ | no stimulation | |

**Table 1:** ATPase stimulation of different ClpC substrates and a comparison with the ATPase rate of the homologue Clp protein of *E.coli*. The ATPase rate is given in hydrolysis of an ATP molecule per second per ClpC molecule ($s^{-1}$)

Previous experiments demonstrated that MecA consists of two domains connected by a flexible loop. The N-terminal domain interacts with ComK and ComS, while the C-terminal domain interacts with ClpC and is responsible for the induction of the ClpC ATPase reaction (Persuh et al., 1999) These fragments of MecA were tested for the stimulation of the ClpC ATPase and whether they are sufficient to activate the disaggregation and refolding capacity of ClpC. The experiments were carried out with luciferase and MDH as aggregated protein substrates. Neither the addition of the N-terminal, or the C-terminal domain of MecA, nor the simultaneous addition of both domains (data not shown) to ClpC, resulted in detectable disaggregation and refolding activity (Fig.10).

10A




10B

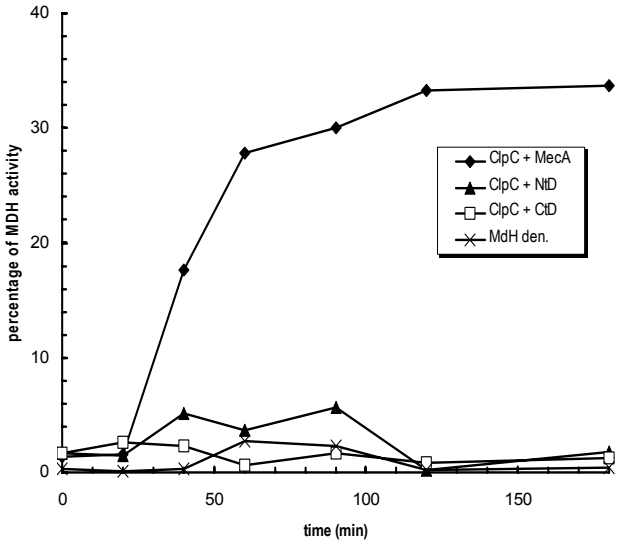



**Fig 10)** disaggregation and refolding activities of the C-terminal and the N-terminal domain of MecA with the substrates Luciferase **(A)** and MDH **(B)**.

Nevertheless the C-terminal MecA domain by itself induced the ClpC ATPase to the same extent as full length MecA (Table 1). Consistently, addition of the C-terminal MecA domain alone was unable to mediate degradation of α-casein or aggregated MDH by ClpCP (data not shown and Figure 10 B). The result of these experiments suggested, that the induction of the ClpC ATPase is not sufficient for the disaggregation and refolding activity of ClpC together with MecA or the degradation by ClpCP.

To get more insight into the correlation of the ATPase and the disaggregation and refolding activity, we followed both the ClpC ATPase rates and MDH refolding rates at several different ratios of MecA/ClpC (Fig. 14). A clear correlation between the MecA concentration and the yields and rates of refolding and ClpC ATPase rates was apparent (Fig.11 and data not shown). With increasing amounts of MecA up to a protomer ratio of 1:1 (MecA: ClpC) both the ATPase rate and the refolding rate correlated (Fig. 11).

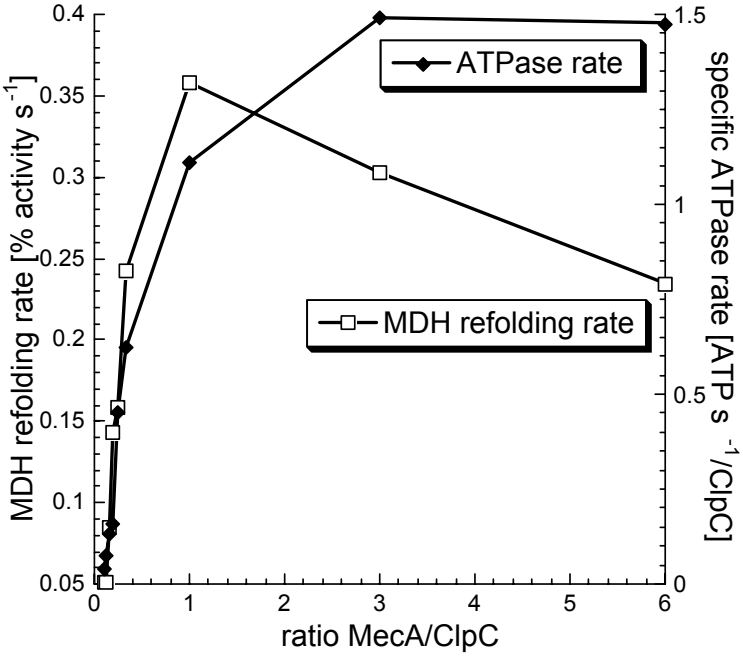



**Fig. 11)** Comparison of the MDH refolding rate with the ATPase rate MDH refolding rates (left y-axis) and specific ATPase rates (right y-axis) were determined as described at different ratios of MecA to ClpC monomers (depicted on x-axis) and plotted as indicated in the legend.

Above the 1:1 ratio, the disaggregation and refolding reaction of ClpC/MecA was inhibited, while the ClpC ATPase rate continued to increase. Since MecA also acts as a substrate for ClpC this could represent a competitive inhibition by MecA. Similar experiments with heat-aggregated luciferase instead of MDH gave essentially the same results (data not shown).

These results suggest that the concomitant targeting of substrate by MecA together with the ATPase induction of ClpC is necessary for the disaggregation and refolding activity of ClpC with MecA.

## 4.4 YpbH represents another adaptor protein of ClpC

### *4.4.1 Comparison of MecA with YpbH*

YpbH is a paralog of MecA. The search of sequence databases with *mecA* as a query revealed the presence of a gene, *ypbH*, the product of which shows high similarity to MecA. The *B. subtilis* MecA paralog shares 26% sequence identity and 52% sequence similarity with the *B. subtilis* MecA on the amino acid level. MecA consists of two domains, the N- and C-terminal domains, with a linker region between them (Persuh et al., 1999). The similarity of YpbH to MecA extends over both domains, but YpbH is 24 amino acids shorter than MecA. *ypbH* is present in two other sequenced *Bacillus* species: *Bacillus anthracis* and *Bacillus halodurans*. It seems that *ypbH* is present only in the genus *Bacillus* while MecA, with a much broader distribution, is found in essentially all low-GC gram-positive bacteria (*Bacillus*, *Listeria*, *Staphylococcus*, *Streptococcus*, *Lactococcus*, and *Enterococcus*) The strong similarity of YpbH to MecA further implied that YpbH, like MecA, might bind to ClpC. YpbH is able to stimulate the ATPase activity of ClpC like MecA (data not shown). A ClpC YpbH interaction was shown by Persuh et al.2002 with surface plasmon resonance. The experiment depicted in Fig. 12 demonstrated that YpbH enables chaperone activity of ClpC. Both MecA and/or YpbH are without any preference, simultaneously recognized and degraded by ClpCP. And both can simultaneously target α-casein for degradation by ClpCP (see also Fig. 14).
The ability of YpbH to refold chemically denatured luciferase and to disaggregate and refold heat denatured luciferase has been tested (Fig. 12)

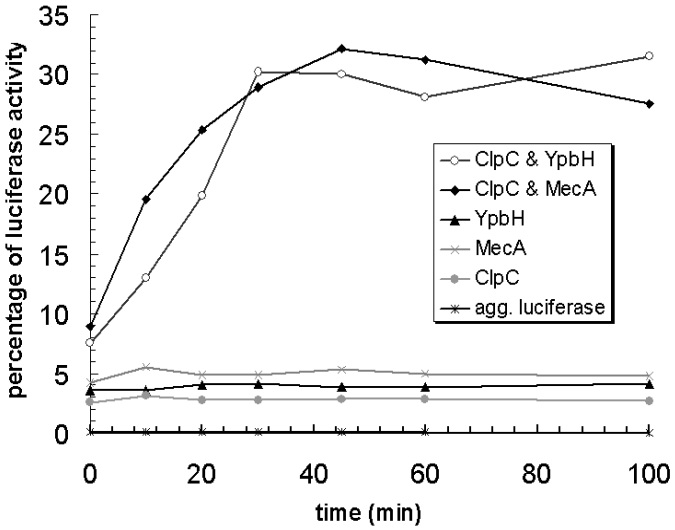



**Figure 12 A**) refolding of heat denatured luciferase. ClpC together with MecA such as ClpC with YpbH can disaggregate and refold previously heat-aggregated luciferase**.** Previously heat aggregated luciferase was incubated with the indicated proteins and luciferase activity was determined at the indicated time points.

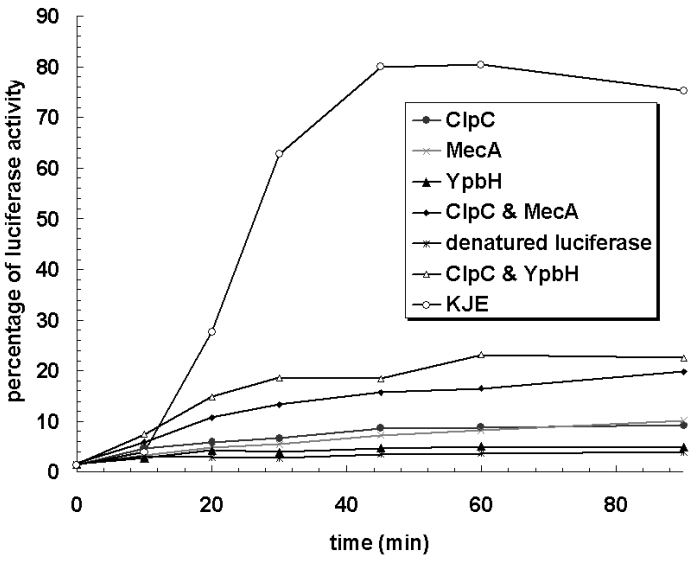



**Figure 12 B**) Refolding of guanidinium hydrochloride denatured luciferase with KJE, ClpC, MecA, YpbH, ClpC and MecA or YpbH as shown in the legend. Luciferase activity was determined at the indicated time points.

YpbH together with ClpC is able to disaggregate and refold luciferase, as MecA/ClpC does (Figure 12A). In addition the YpbH mediated activity to refold chemical denatured luciferase was comparable to ClpC/MecA

These results demonstrate, that MecA and YpbH display identical chaperone activities. A deletion of *ypbH* affects competence development and sporulation. But unlike MecA, YpbH

does not target ComK for degradation. Both proteins have an influence on sporulation when overproduced, and in both cases this effect is dependent on ClpC (Persuh et al., 2002).

### *4.4.2 Adaptor preferences of ClpC*

Both MecA and YpbH target themselves for degradation. To test which adaptor is favored, competition for degradation by ClpCP was examined. Equimolar amounts of MecA, YpbH and ClpCP were used in this assay. The results of these experiments depicted in Figure 13 demonstrated that MecA as well as YpbH were degraded in less than 20 min (lane a +b). If both proteins are incubated together, no decrease in the degradation rate, of one of the adaptor proteins was observed (lane c). This result was also observed with higher concentrations of MecA (2µM) and YpbH (2µM) incubated together with ClpCP (1/1µM) (data not shown). Only the CTD of MecA is preferentially degraded, because in its presence the degradation rate of MecA and YpbH is slower (lane d + f).The degradation of the smaller CTD of MecA seems to be favored.

As a control the effect of the presence of the N-terminal domain of MecA was tested for competition in the degradation of full length MecA and no influence was observed (lane e).

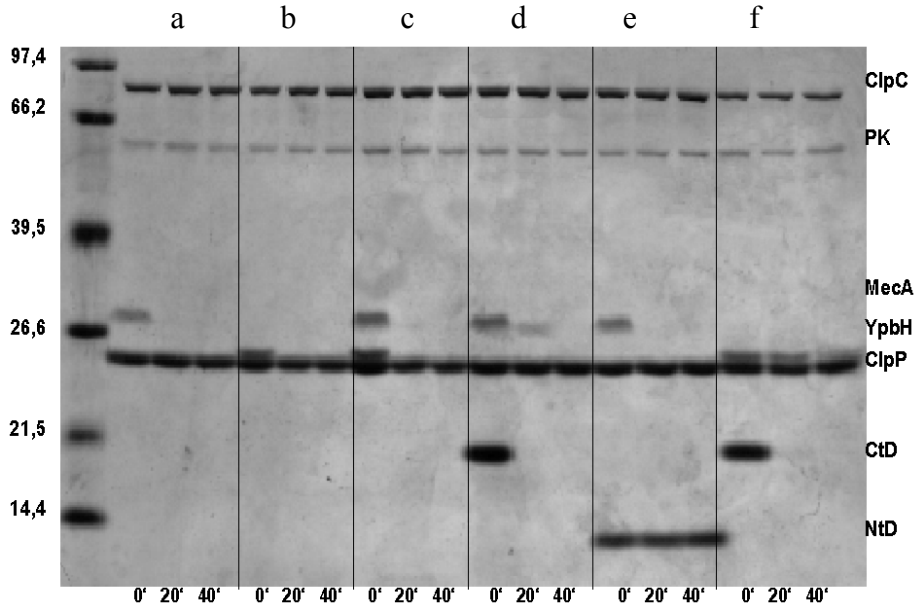



**Fig. 13)** Coomassie stained gel of ClpC adaptor degradation. MecA (1µM) and YpbH (1µM) are degraded in less than 20 minutes (lane a+b). None of the adaptors are preferentially degraded if both are present (lane c) and equimolar amounts of ClpC/P are used. The C-terminal domain of MecA decelerates the degradation of full

length MecA as it does for YpbH (lane d and f). The N-terminal domain of MecA is not degraded and does not interfere with the MecA degradation (lane e).

The presence of α-Casein lowers the MecA degradation rate, while it is presented as a substrate for ClpCP (Fig 14 b). YpbH mediates α-casein degradation (Fig 14 lane d) and the YpbH degradation rate was also lowered in presence of α-casein. To test whether MecA or YpbH is the preferred adaptor for substrate degradation both were incubated together with α-casein (Fig 14 lane e). This experiment could demonstrate that one of these adaptors is more important for α-casein degradation and therefore different degradation rates of the adaptors should be observed.But in the experiment shown in Figure 14e no change of the degradation rates of MecA and YpbH has been observed in comparison to the degradation of these adaptors alone shown in Figure 14 lane b and d. The same results were obtained when the concentrations of MecA and YpbH were doubled (Fig.14 lane f).

Two possible explanations for this observation exist. First, both adaptors bind at the same time on a ClpC hexamer (e.g. a ClpC hexamer bound with three molecules of MecA such as three YpbH molecules), second the complex out of ClpCP/MecA such as ClpCP/YpbH degrades free YpbH and MecA without any preference

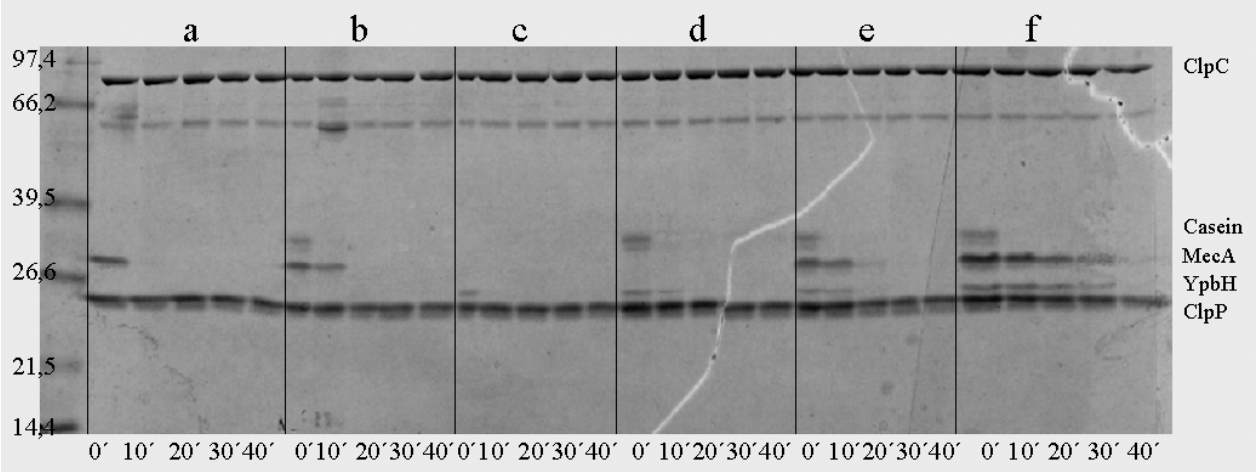



**Fig. 14)** Coomassie stained gel of adaptor mediated α-casein degradation. YpbH is able to degrade α-casein within 10 minutes (lane d, all proteins in a concentration of the 1μM). MecA and YpbH in equimolar amounts degrade α-casein in less than 10 minutes, but none of the proteins is degraded preferentially (lane e, all proteins 1μM), also not if MecA (2μM) and YpbH (2μM) are higher concentrated (lane f).

It remains to be elucidated if these adaptors share the same binding site of ClpC {??}, or if it is possible that both adaptors caninteract at the same time with one ClpC hexamer.

These results indicate, that MecA and YpbH are comparable in their chaperone activities, but *in vivo* different substrate specificity must exist because different substrates utilizes different adaptors (ComK and ComS are not degraded by YpbH)

## 4.5 Oligomerisation of ClpC

### *4.5.1 ClpC alone cannot form stable hexamers*

At this stage it was known that ClpC needed MecA for all observed activities such as ATPase hydrolysis, substrate targeting for degradation and chaperone activity.
Experiments with different AAA+ proteins, like ClpA, ClpB or HSP104 already indicated, that the hexameric ring structure of HSP100/Clp proteins appeared to be a crucial prerequisite for their chaperone and ATPase activity (Hattendorf and Lindquist, 2002; Mogk et al., 2003a; Schirmer et al., 2001; Schlee et al., 2001). Therefore the oligomerisation abilities of ClpC and the influence of MecA on ClpC oligomerisation were investigated.
Size exclusion chromatography and as a second method chemical cross linking with glutaraldehyde (GAXL) was used to analyze the oligomerisation status of ClpC *in vitro*.

### *4.5.2 ClpC forms a monomer in Gelfiltration runs*

Unlike for most studied HSP100/Clp proteins, a hexamer of ClpC was not detectable by size exclusion chromatography.
Gelfiltration runs performed with ClpC with or without ATP preincubation depicted only a peak consistent with the size of a monomer of ClpC (Figure 15)

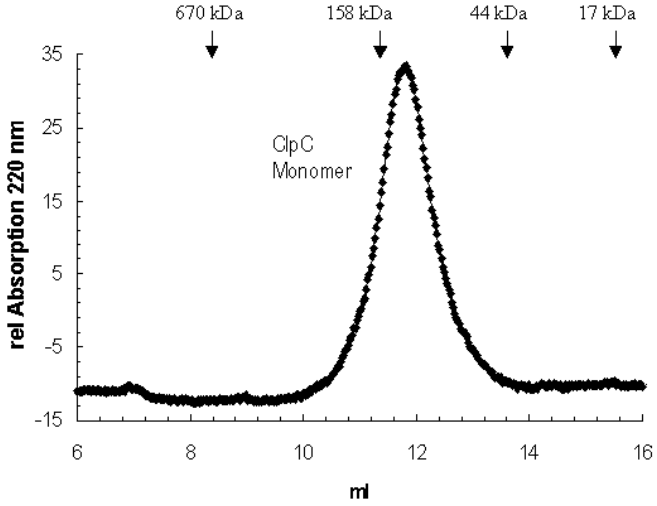



**Fig. 15)** A monomer of ClpC but no higher oligomer was detected by size exclusion chromatography. ClpC (6 µM) (preincubated with ATP (2mM)) were run on a S200 gelfiltration column. The elution profile of the protein detected at 220 nm is depicted in the graph. Arrows indicate positions of standard proteins with different molecular weights, separated on the same column. The fractions were analyzed by SDS-PAGE with subsequent silverstain and westernblot analysis

This result was also obtained by a gelfiltration run with ATP in the running buffer (data not shown). Additionally the size of ClpC under these conditions has been determined using static light scattering measurements with a detector connected to a Äkta purifier with a S200 gelfiltration column (Fig. 15). The molecular weight of the observed monomer of ClpC could be determined with this method to 96 kDa without ATP and to 100 kDa with ATP. In addition no peak consistent with a hexamer or higher molecular weight was observed with this very sensitive detection (data not shown).

The oligomerisation behavior of ClpC has also been tested with chemical cross-linking with glutaraldehyde (GA). Since transient protein interactions are trapped by the chemical cross link, less stable oligomeric states of ClpC, which cannot be detected with size exclusion chromatography, could be detected with this method (Mogk et al., 2003b).

ClpC was incubated with or without ATP; the cross link was initiated by the addition of GA and stopped by adding sample buffer. The samples were analyzed by gradient SDS-PAGE (4% - 15%) and visualized by silver stain. The results shown in Fig. 16 (lane 1-3) with or without ATP demonstrated that most of the total ClpC migrated as a monomer, but also a small amount of ClpC became detectable running at a size consistent with higher oligomeric forms of ClpC.

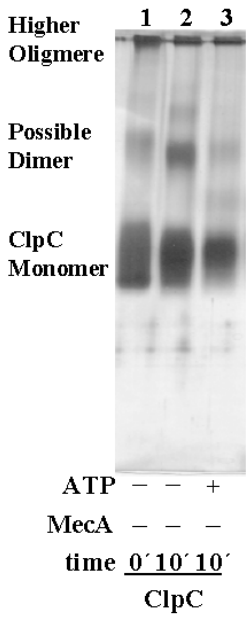



**Fig. 16)** In the presence of ATP, a small amount of ClpC, becomes detectable by chemical cross linking at the size of higher oligomeres. ClpC (1µM) was incubated with or without ATP (2mM) as indicated for the lanes in the panel on the bottom. Chemical cross linking was initiated by the addition of glutaraldehyde (GAXL) and stopped immediately (0') or after 10 min incubation (10') and analyzed on a SDS-PAGE gradient gel (4-15% acrylamide) with subsequent silver stain (Mogk et al., 2003b). For size determination see also Figure 18.

### *4.5.3 MecA and ClpC are forming a heterodimer*

To test the influence of the Adaptor protein on the oligomerisation behaviour of ClpC, MecA was added in equimolar amounts to ClpC in the size exclusion chromatography experiments Fig. 17 shows the result of a typical gelfiltration run of ClpC together with MecA. Only two peaks became visible in the elution profile. The first peak appeared at a size corresponding to a heterodimer of ClpC and MecA and a second peak was detected running at the size observed previously for MecA (Persuh et al., 1999). Analysis of the fractions by SDS-PAGE and silver stain or Western-blot with Anti-MecA antibodies confirmed the suggested composition of the peaks. Similar results were obtained in experiments with or without ATP preincubation or ATP presence in the running buffer (data not shown).

This suggests that ClpC and MecA can form a stable heterodimeric complex also in the absence of ATP

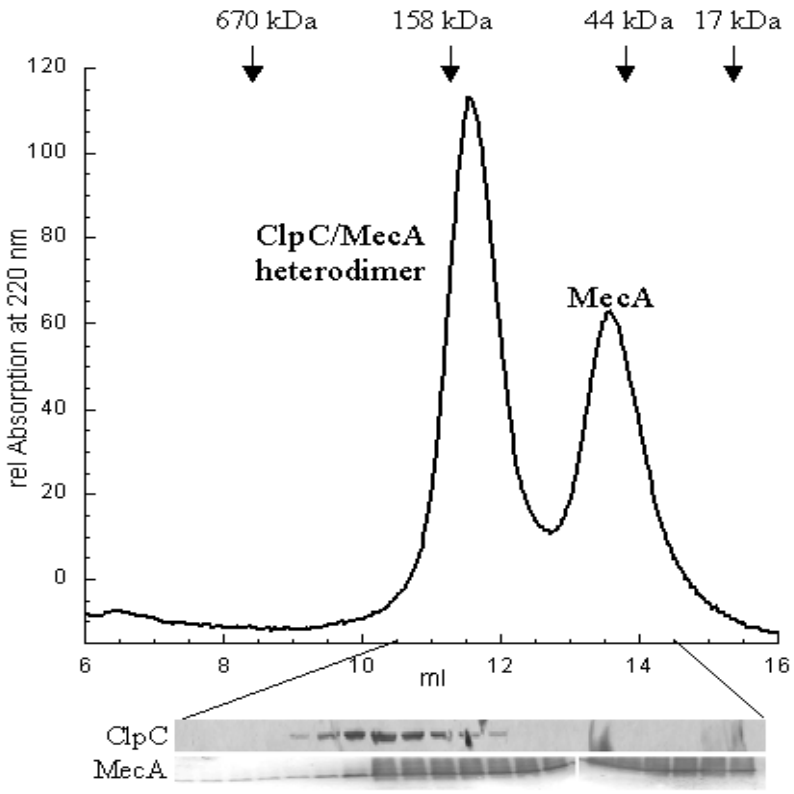



**Fig. 17)** a stable heterodimer of ClpC and MecA but no higher oligomer was detected by size exclusion chromatography. ClpC (6 µM) and MecA (6µM) were run on a S200 gelfiltration column. The elution profile of the protein detected at 220 nm is depicted in the graph. Arrows indicate positions of standard proteins with different molecular weights, separated on the same column. The fractions were analyzed by SDS-PAGE with subsequent silverstain and westernblot analysis

### *4.5.4 MecA and ATP are necessary for the formation of ClpC oligomers*

The influence of MecA on ClpC oligomerisation with using the GA cross-link assay was examined. ClpC incubated with or without MecA or ATP. Chemical cross-linking was initiated by the addition of glutaraldehyde and stopped by adding sample buffer. In the presence of MecA, without the addition of ATP a band was noticeable, which could correspond to the ClpC-MecA heterodimer detected in the gelfiltration experiment (Fig. 18, lane 4). When MecA was added in the presence of ATP a strong effect on the size distribution of ClpC and MecA became visible. Under these conditions the majority of protein migrated at a size consistent with higher oligomeric forms of a ClpC/MecA complex and concurrently the monomer bands of MecA and ClpC were becoming less visible (Fig. 18, lane 5).

These findings suggested that in the presence of ATP a higher oligomeric complex containing both ClpC and MecA was formed. Interestingly this complex appears to be transient as it could only be detected by the GA cross-link experiment and not by size exclusion chromatography. As a size reference Fig. 18 also includes an additional GA cross-link experiment where the oligomerisation of ClpC with MecA and ClpC + MecA and ATP was directly compared with ClpB oligomerisation without ATP. This experiment also demonstrates, that in the absence of ATP most of ClpB migrated as higher oligomer compared to ClpC.

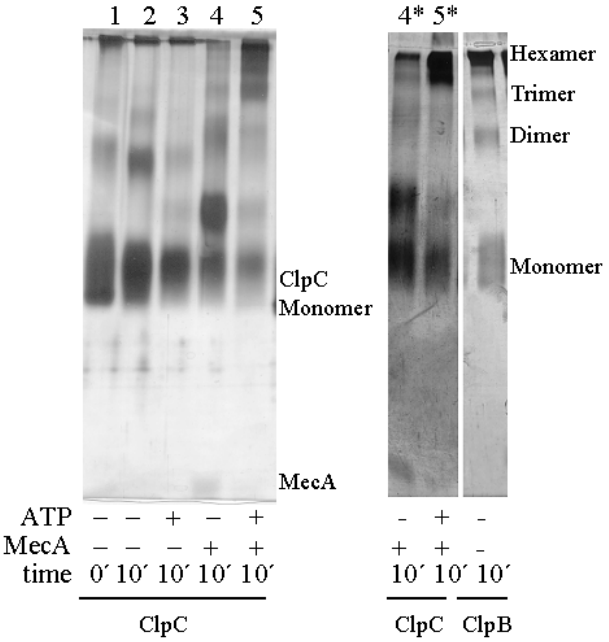



**Fig. 18)** In the presence of MecA and ATP, a higher oligomeric complex, not containing all the available ClpC, becomes detectable by chemical cross-linking. ClpC (1µM) was incubated with or without ATP (2mM) or MecA (1µM) as indicated for the lanes in the panel on the bottom. ClpB (1µM) oligomerisation was shown as a control for the size determination of intermediates. Chemical cross linking was initiated by the addition of glutaraldehyde (GAXL) and stopped immediately (0') or after 10 min incubation (10') and analyzed on a SDS-PAGE gradient gel (4-15% acryl amide) with subsequent silver stain (Mogk et al., 2003b).

### *4.5.5 ClpC-DoubleWalkerB(DWB) forms together with MecA a higher oligomer*

It has been demonstrated for some other AAA+ proteins that a mutation of the Walker B motif, which prevents the hydrolysis of ATP but not its binding, can help to stabilize the higher oligomer form of these proteins(Babst et al., 1998; Hartman and Vale, 1999).
Hartman et al. showed that the AAA+ protein p60 of Katanin is only stable enough to be detected in a gelfiltration experiment in presence of ATP if the Walker B motif is modified

(Hartman and Vale, 1999). To examine whether similar changes in the walker B motif of ClpC could result in the stable formation of hexameric ClpC, similar mutants forms of ClpC were constructed by K. Turgay with the help of C. Escher, where the conserved Glu of the Walker B motif in one or both ATPase domains were changed to an Ala (ClpC E280A (ClpC-WB1), ClpC E618A (ClpC-WB2), ClpC E280A/E618A (ClpC-DWB)). The respective *B. subtilis* mutant strains (*clpC-WB1; clpC-WB2 and clpC-DWB*) have been constructed as described previously (Turgay et al., 2001).

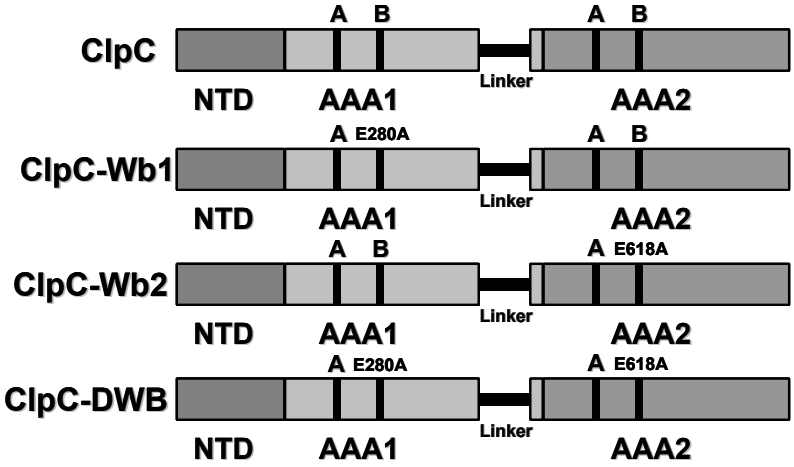



**Fig. 19)** schematic view of the domain organization of the ClpC-WB1, WB2 and ClpC-DWB-mutants

GAXL experiments depicted in Figure 20 showed that without MecA only a small proportion of the total amount of ClpC-DWB could be detected at a size consistent with the formation of a higher oligomer, the rest stayed as a monomer. In contrast to other AAA+ proteins like ClpB a stable hexamer of ClpC or ClpC-DWB without MecA could not be detected.

Unlike for the experiment performed with ClpC (Fig 18), all the ClpC-DWB was shifted without detectable intermediates exclusively to the highest oligomeric state, in the presence of MecA and ATP (Lane 5). But in the presence of MecA without ATP (lane 4), similar to the experiment performed with ClpC; a major band appeared, which could represent the ClpC-DWB MecA heterodimer (this was also detected by size exclusion chromatography (Fig 20)).

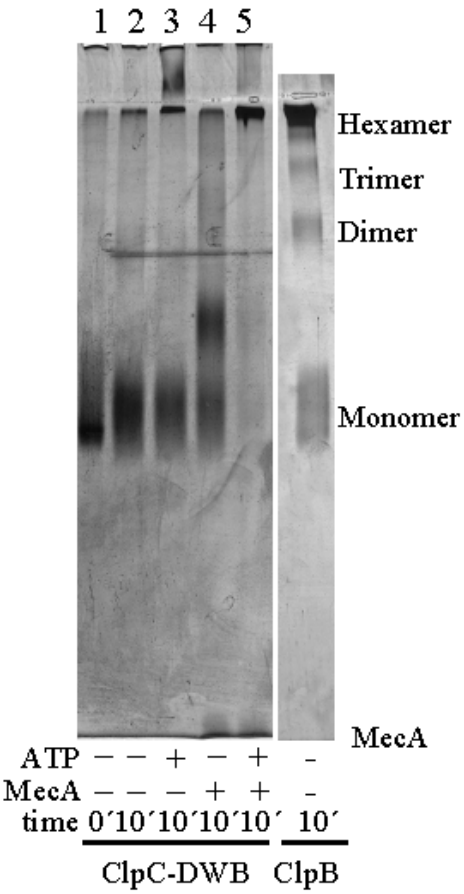



**Fig. 20)** In the presence of MecA and ATP the ClpC-DWB is detected by chemical cross linking almost exclusively in the higher oligomeric complex. GAXL experiments of ClpC-DWB (1 µM) with and without ATP (2 mM) and/or MecA (1 µM) as indicated, carried out like the experiment shown in Fig 18. ClpB (1µM) oligomerisation was shown as a control for the size determination of intermediates.

Judged by the GA cross-link experiment the mutation in both Walker B motifs enabled the *in vitro* oligomerisation abilities of ClpC together with MecA but not in the absence of MecA. Therefore also size exclusion chromatography was used to analyze this higher oligomeric complex of ClpC-DWB and MecA, which appeared more uniform and stable compared to wildtype ClpC and MecA. Figure 21A depicts the gelfiltration run performed with DWB-ClpC and MecA after preincubation of these proteins with ATP, but without ATP in the running buffer. A distinct complex consisting of DWB-ClpC, MecA was clearly detectable in the elution profile. The size of this complex (approximately 700 kDa) would be consistent with the size of a ClpC-DWB hexamer with up to six MecA molecules bound. The silverstain

in Fig. 21A supports the composition of the peaks, of ClpC and MecA as suggested for the protein peaks visible in the gelfiltration run.

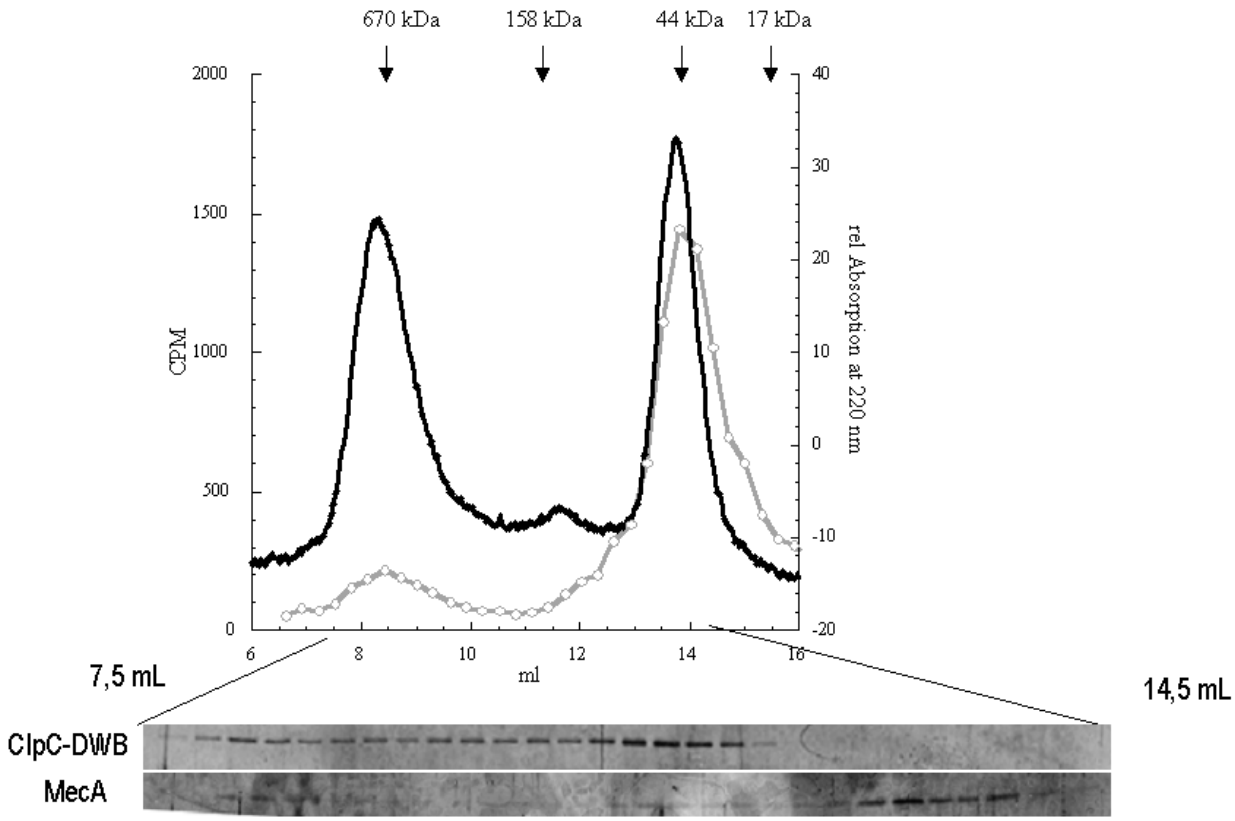



**Fig. 21A)** ClpC-DWB and MecA can form a stable higher oligomeric complex, which can bind substrate protein. The elution profile of size exclusion chromatography experiments with ClpC-DWB (6µM), MecA (6 µM) and α-casein ($^3$H-labelled) (1 µM) (preincubated with ATP (2 mM) but without ATP in the running buffer) and the amount of radioactivity in the fractions are depicted in the graphs. Arrows indicate positions of standard proteins with different molecular weights, separated on the same column. The fractions were analyzed by SDS-PAGE with subsequent silverstain analysis

The pre-incubation with ATP was essential for the formation of the higher oligomeric complex of ClpC and MecA. Using size exclusion chromatography in the absence of ATP and without preincubation of ClpC-DWB and MecA with ATP, only a ClpC-DWB MecA heterodimer, as demonstrated for MecA and ClpC was detected (data not shown).

Fig. 21B depicts the silver stained gel of an SDS-PAGE of the fractions of a size exclusion chromatography of ClpC-DWB and MecA performed in the presence of ATP in the running buffer. It is evident that the higher oligomeric complex consisted of ClpC and MecA and that under these conditions all the ClpC was shifted to this higher oligomer, whose size is consistent with a ClpC-DWB hexamer and six MecA molecules.

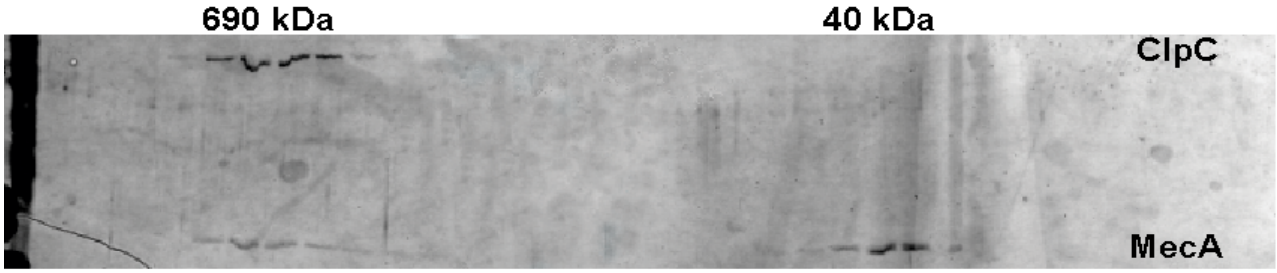



**Fig. 21B)** The fractions of a gelfiltration run of DWB-ClpC with 2 mM ATP in the running buffer were analyzed by SDS-PAGE with subsequent silverstain analysis The position of MecA and ClpC-DWB and the estimated molecular weights are indicated.

### *4.5.6 Isolation of a ClpC/MecA/substrate complex*

In addition to the MecA mediated formation of a ClpC-DWB hexamer the substrate interaction with α-casein ($^3$H labeled) was tested. α-Casein, an intrinsically unfolded protein, was degraded by ClpCP and MecA, so it was interesting to examine whether α-casein could associate with the higher oligomer complex of ClpC and MecA.

Fig. 21A depicts that a distinct complex consisting of ClpC-DWB, MecA and α-casein was clearly detectable in the elution profile. This indicated that α-casein interacted directly with the higher oligomeric complex of ClpC-DWB and MecA. No substrate, coeluting with the transient heterodimer of MecA and ClpC was detected (data not shown). The suggested composition of the peaks, were confirmed by SDS-PAGE with subsequent analysis by Westernblot and silverstain. These experiments suggested that the higher oligomer complex of hexameric ClpC-DWB with MecA represents the active substrate-binding species of this chaperone system.

The size of the ClpC-DWB hexamer with MecA does not change significantly in the presence of α-casein, suggesting that α-casein encompassed in this complex.

The ratio of MecA/ClpC was also lowered to six ClpC to one MecA molecules to test the MecA dependency of the ClpC-DWB oligomerisation and binding of α-casein Substrate interaction with the higher oligomer of ClpC-DWB and MecA was almost not detectable anymore with this amount of MecA (Fig. 22).

Lowering the amount of MecA resulted also in shifting the equilibrium from the higher oligomeric form of the hexamer ClpC with MecA to the intermediate ClpC-MecA

heterodimer fraction (Fig. 22). Without MecA no formation of the ClpC-DWB hexamer was detectable by size exclusion chromatography (data not shown).

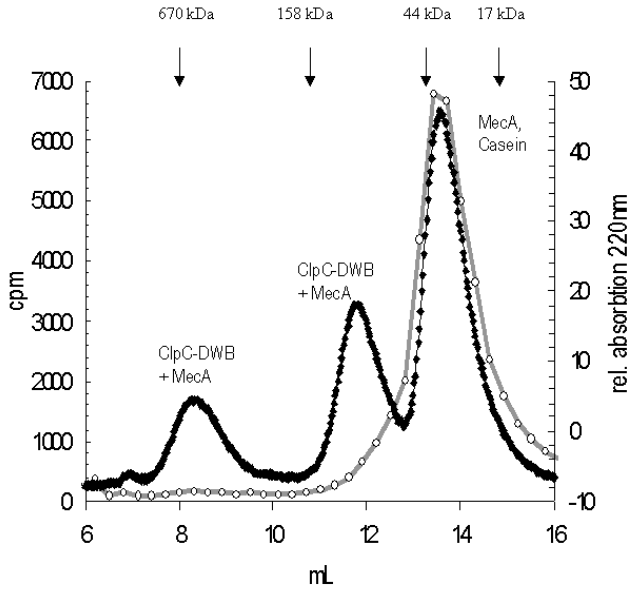



**Fig. 22)** The elution profile of size exclusion chromatography experiments with ClpC-DWB (6µM), MecA (1 µM) and α-casein ($^3$H-labeled) 3µM (preincubated with ATP (2 mM) but without ATP in the running buffer) and the amount of radioactivity in the fractions are depicted in the graphs. Arrows indicate positions of standard proteins with different molecular weights, separated on the same column.

These results suggest that the interaction of MecA with ClpC in the ATP bound state leads to the formation of a higher oligomeric complex, via a heterodimer intermediate.
The complex of ClpC and MecA, which hydrolyze ATP, is not stable enough to be detected in a gelfiltration run. This complex can only be detected by gelfiltration, if ATP binding, but no ATP hydrolysis by ClpC is possible, like in the ClpC-DWB variant

### *4.5.7 Further characterization of ClpC-DWB*

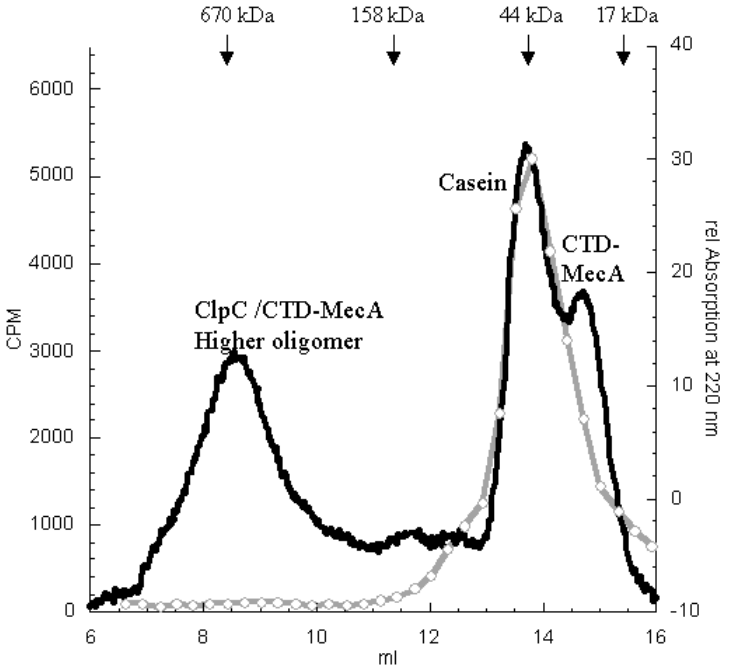
The ClpC-DWB mutant protein has been tested in MDH disaggregation/refolding and degradation of α-casein, but ClpC-DWB displayed no detectable activity in all these assays (data not shown). As a control also the MecA mediated ATPase stimulation of ClpC-DWB has been determined, but no ATP hydrolysis activity was measurable. To test the importance of the ATP hydrolysis activity of the ClpC monomers within the ClpC hexamer the ClpC-WT protein was mixed with ClpC-DWB protein in a ratio of 5:1 (WT: DWB)

The addition of ClpC-DWB to ClpC-WT at this stoichiometry inhibited all the MecA mediated ATPase activity of the WT protein and in addition all the ClpC-WT MDH disaggregation, refolding activity was completely blocked. This was also observed in α-Casein degradation experiments (data not shown).
On the other hand a negative influence of ClpC-WT on ClpC-DWB oligomerisation was also observed, because in a gelfiltration experiment no ClpC-DWB hexamers have been observed if ClpC-WT and ClpC-DWB were mixed in a 1:1 ratio; demonstrating that the formation of the ClpC-DWB- hexamers in presence of MecA was inhibited by ClpC-WT (data not shown).
The result of these experiments are consistent with a mixed oligomer formation of ClpC-WT and ClpC-DWB, because ClpC-DWB inhibits the MecA mediated ATPase and chaperone activities of ClpC-WT and ClpC-WT inhibited the MecA mediated oligomerisation of ClpC-DWB.
In addition these experiments suggests that a cooperative ATP hydrolysis within the ClpC hexamer is essential for ClpC activity.

### *4.5.8 The CTD of MecA is sufficient for assisting the oligomerisation of ClpC.*

To test whether full length MecA was required to assist the oligomerisation, or the C-terminal domain was sufficient the same experiments, as with full length MecA, were performed with the C-terminal domain (CTD, 16,8 kDa) of MecA
The CTD of MecA is able to bind ClpC like full length MecA. This could be demonstrated with plasmon resonance experiments (see also Fig. 30). A heterodimer formation as observed for ClpC and MecA (Fig 15) can be detected with size exclusion chromatography (data not shown). The gelfiltration run shown in Figure 23A demonstrate that the CTD of MecA is sufficient to assist the oligomer formation of ClpC. This could also be detected with GAXL-experiments (Fig 23B).
In contrast to the higher oligomer of ClpC-DWB stabilized by MecA the CTD of MecA bound to the ClpC-DWB oligomer is not able to mediate substrate interaction, because α-casein was not detected running with the higher oligomeric complex of ClpC-DWB and the CTD of MecA (Fig 23A). Although the CTD of MecA stimulate the ATPase activity of ClpC up to the same rates as full length MecA, no CTD of MecA mediated refolding or degradation activity have been detected as discussed in chapter 3.5.

23A




23B

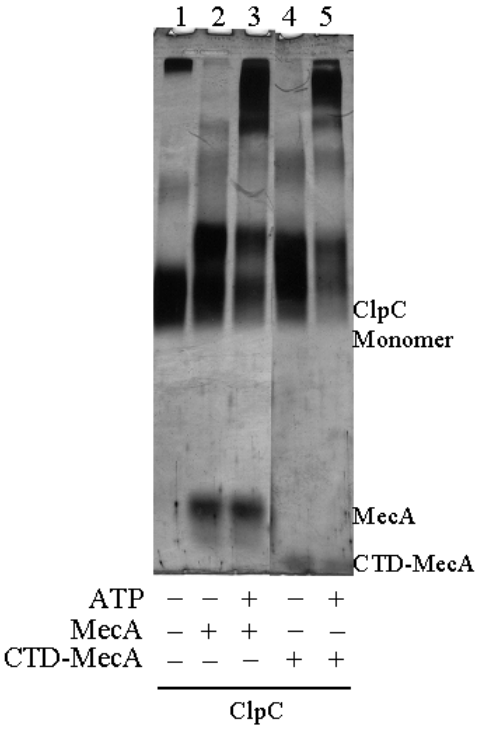



**Fig. 23)** The CTD of MecA is sufficient to assist the oligomer formation of ClpC. The CTD of MecA forms a higher oligomeric complex with ClpC-DWB. The elution profile of size exclusion chromatography experiments with ClpC-DWB (6µM), CTD-MecA (6 µM) and α-casein ($^3$H-labeled) (3µM) (preincubated with ATP (2 mM) but without ATP in the running buffer) and the amount of radioactivity in the fractions are depicted in the graphs. Arrows indicate positions of standard proteins with different molecular weights, separated on the same column.

**(B)** CTD-MecA supports the oligomerisation of ClpC like MecA. ClpC (1µM) was incubated with or without ATP (2mM), MecA or CTD-MecA (1µM) as indicated for the lanes in the panel on the right. Chemical cross linking was initiated by the addition of glutaraldehyde (GAXL) and stopped after 10 min incubation and analyzed on a SDS-PAGE gradient gel (4-15% acrylamide) with subsequent silver stain.

It has been observed that the CTD of MecA is able to target itself for degradation (Fig. 25); therefore it can be assumed that ClpC-WT possesses full activity. But without the substrate binding sites of the NTD of MecA, although higher oligomer formation of ClpC is possible (Fig.23), the CTD of MecA is not able to bind and subsequently to degrade α-casein (data not shown), which is consistent with the finding, that the NTD of MecA is necessary to target all other known substrates to ClpC (Persuh et al., 1999; Schlothauer et al., 2003).

### *4.5.9 Oligomerisation of ClpC by other adaptor proteins*

MecA is an adaptor for ClpC and enables the recognition and targeting of substrate proteins and simultaneously induces the ATPase activity of ClpC. The oligomerisation of ClpC mediated by MecA has most likely an important role in ClpC activity. The second adaptor protein YpbH displays the same activities needed for ClpC chaperone activity. For that reason also the YpbH mediated ClpC oligomerisation was investigated. Recently another possible ClpC adaptor McsB has been identified and tested for ClpC oligomerisation such as ATPase stimulation (J. Kirstein, K. Turgay unpublished data).

McsB is a kinase that modulates the repressor of Class III heat shock genes (CtsR). CtsR, the protein encoded by the first gene of the four genes *clpC* operon, is the repressor of the class III heatshock genes, including *clpC*, *clpP* and *clpE* (see introduction). The second and third gene of this operon encodes McsA and McsB. A Δ*mcsA* strain behaves like a Δ*ctsR* strain and in a Δ*mcsB* strain the relief from repression by CtsR is inhibited. ClpCP was also shown to be responsible for the degradation of CtsR at high temperatures. Both ClpC and McsB have a negative and McsA a positive effect on CtsR activity or stability. (Krüger et al., 2001).

McsB is similar to kinases and the N-terminal domain of McsA has Zn binding motifs and shows also similarity to the N-terminal domain of ClpX and ClpE. With purified components, it was tested whether McsA or McsB could have influence on ClpC activity *in vitro*. McsB but not McsA or CtsR induced specifically the ATPase activity of ClpC comparable to MecA (data not shown and personal communication, J. Kirstein). *In vitro* McsB could get degraded and could target McsA or CtsR for degradation by ClpCP (personal communication, J. Kirstein)

To elucidate the role of the three known adaptors of ClpC in ClpC oligomerisation, MecA, YpbH and McsB were directly compared. The GAXL experiments shown in Fig 24 with ClpC and the different adaptors depicts, that MecA enables the oligomerisation as shown before (lane 1-3). YpbH forms the possible heterodimer with ClpC (lane 4) and stimulates the oligomerisation of ClpC (lane 5). McsB forms oligomeres of ClpC without visible intermediates in the absence of ATP (lane 6). With ATP and McsB the yield of ClpC hexamers is increased (lane 7).

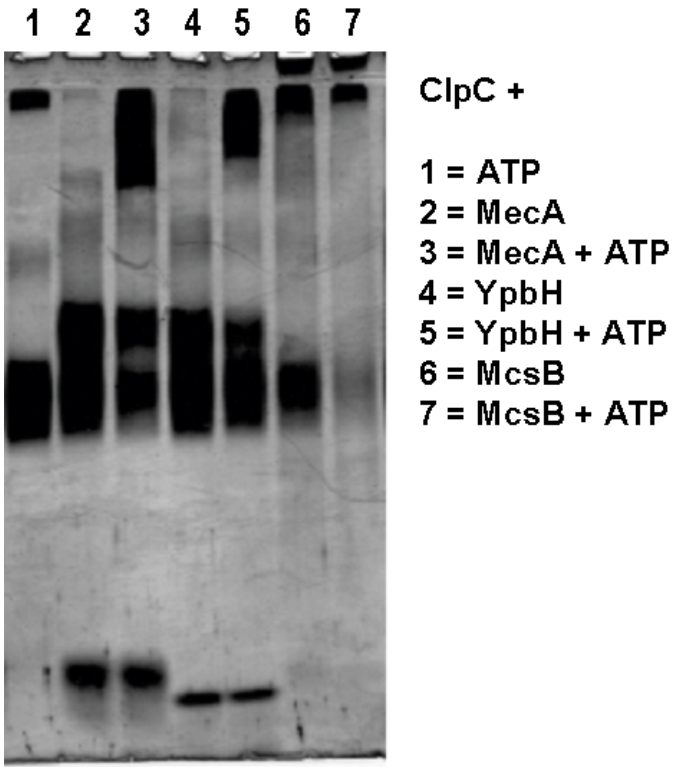



**Fig. 24)** ClpC with and without ATP and different adaptor proteins (indicated at the lanes) were chemically cross linked with glutaraldehyde and analyzed by gradient SDS-PAGE with subsequent silverstain.

The oligomerisation of ClpC by McsB was much more efficient compared to MecA and YpbH. McsB already mediates ClpC oligomerisation in the absence of ATP, and in presence of ATP the oligomerisation was as efficient as the MecA mediated ClpC-DWB oligomerisation (see figure 17B). Another difference to MecA and YpbH, visible in the GAXL, was that no McsB-ClpC heterodimer was detected. This result indicates that McsB is very important for ClpC activity *in vivo*.

### *4.5.10 ClpC oligomers in vivo.*

To get an insight about the size distribution of ClpC, closer related to the *in vivo* situation, lysates from *B. subtilis* strains with and without carrying the *clpC-DWB* mutation were prepared. These lysates were separated by size exclusion chromatography and the fractions of the elution profiles were precipitated by MeOH and analyzed by SDS-PAGE with a subsequent western blot with anti-ClpC and anti-MecA antibodies. This analysis for all the fractions of the lysate of the wildtype cells is depicted in Fig. 25A. Higher oligomer forms of

ClpC were detected in these extracts, although the heterodimer form is more prominent. MecA was found to coelute with the higher-oligomer and heterodimer forms of ClpC. The direct comparison of fractions (Fraction 1 (void volume), Fraction 7 (higher oligomer) Fraction 21 (heterodimer) of the same experiment performed with wildtype strain compared to the *clpC-DWB* strain (Fig. 25B) revealed, that more higher-oligomer formed with ClpC-DWB could be detected. A stronger interaction of MecA with ClpC-DWB could be the reason for the observation that more MecA seemed to coelute with ClpC-DWB compared to ClpC (Fig. 25B).

Interestingly a rather strong signal was detected for both ClpC variants and MecA at the size where the protein complexes of apparent higher molecular weight run. This fraction could represent aggregated protein. This co-localization of ClpC and MecA to the aggregated protein fraction is consistent with previous studies, demonstrating that ClpC localizes *in vivo* to protein aggregates (Krüger et al., 2000) and that MecA can target ClpC to protein aggregates *in vitro*

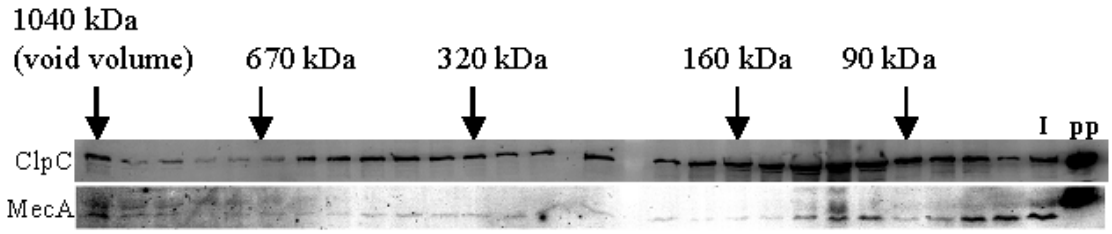



**Fig. 25 A)** Size distribution of ClpC and MecA in a lysate of a WT *B.subtilis* strain. The soluble fractions of the lysate were separated by size exclusion chromatography on a S300 column. The subsequent analysis by SDS-PAGE and westernblot with Anti ClpC and Anti MecA antibodies of the eluted fractions from the chromatography is depicted. The detected proteins are indicated on the left and the estimated approximate molecular weight of the fractions, is indicated on top. On the right the pure proteins (pp) are loaded as a direct comparison. (I = Input, a dilution (1:8) of the lysate loaded on the column).

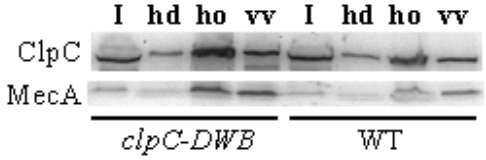



**Fig. 25B)** Comparison of the distribution and relative amount of ClpC and MecA of lysates prepared from WT with clpC-DWB *B.subtilis* strains. The same amount of total protein was loaded on the S300 size exclusion column. The figure depicts the westernblot with anti-ClpC and anti-MecA antibodies, from one SDS-PAGE gel loaded with samples of the same volume and the same fraction from the two different runs. The lanes are named

according to the ClpC MecA species whose size they represent: vv (void volume, (Fraction 1)), ho (higher oligomer (Fraction 7)), hd (heterodimer, (Fraction 21)) and I (Input, a dilution (1:8) of the lysate loaded on the column). The strain the lysate originated from is depicted below and the detected proteins are indicated on the left.

Taken together a size distribution for ClpC and MecA, including the heterodimer and higher-oligomer forms can also be detected in lysates prepared from *B. subtilis* cells. This indicated that the *in vitro* observations of variable ClpC and MecA distribution could be applied to describe the *in vivo* situation.

## 4.6 Interaction of ClpC and MecA.

### *4.6.1 Role of the Walker B motif in AAA-1 and AAA-2*

The mutation in the Walker B motifs one and two were used as a tool to elucidate the importance of the AAA1 and AAA2 in ClpC activity. With these mutants the activity of one Domain could be investigated, while the other is blocked in the ATP bound state.
First the ATPase activity of ClpC-WB1 and ClpC-WB2 was measured, which displayed the same basal rate as ClpC (0.004 $s^{-1}$). The induction of the ATPase of ClpC-WB1 by MecA (in a 1:1 ratio) ((0.03 $s^{-1}$+/- 0,01 $s^{-1}$) 7x the basal rate) was in the same range as the ATPase induction of ClpC-WB2 ((0.03 $s^{-1}$+/- 0,02 $s^{-1}$) 7x the basal rate). Both mutants were tested in the degradation of the C-terminal domain of MecA (Fig. 26).

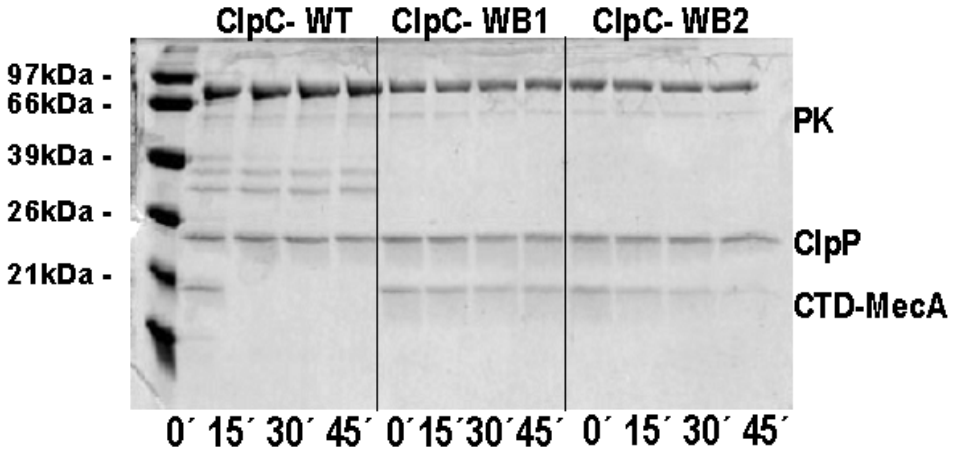



**Fig. 26)** Degradation of C-terminal MecA, ClpC-WT, ClpC-WB1 and ClpC-WB2 were incubated with ClpP, ATP and PK/PEP regenerating system. Samples were taken at indicated time points and analyzed by coomassie-stained-SDS-PAGE. Proteins are marked on the right side of the gel.

In comparison to WT-ClpC (degradation completed after 10 min.), ClpC-WB1 and ClpC-WB2 degraded the C-terminal domain of MecA much slower. Thus the low ATPase induction was sufficient for ClpC-WB1 and ClpC-WB2 to degrade CTD-MecA. The ClpC-WB2 mutant is able to degrade the CTD of MecA faster than the ClpC-WB1 mutant, suggesting that the hydrolysis activity of the first Walker A domain is more important for degradation of the CTD of MecA. In an additional experiment ClpC-WB1 and ClpC-WB2 has been tested to degrade α–casein in presence of MecA, but no degradation of α–casein has been observed. The low ATPase activity of the ClpC-Walker B mutants seems not to be sufficient for any degradation of α–casein (data not shown).

MecA dependent oligomerisation of ClpC-WB1 and ClpC-WB2 were also examined by size exclusion chromatography and GAXL,

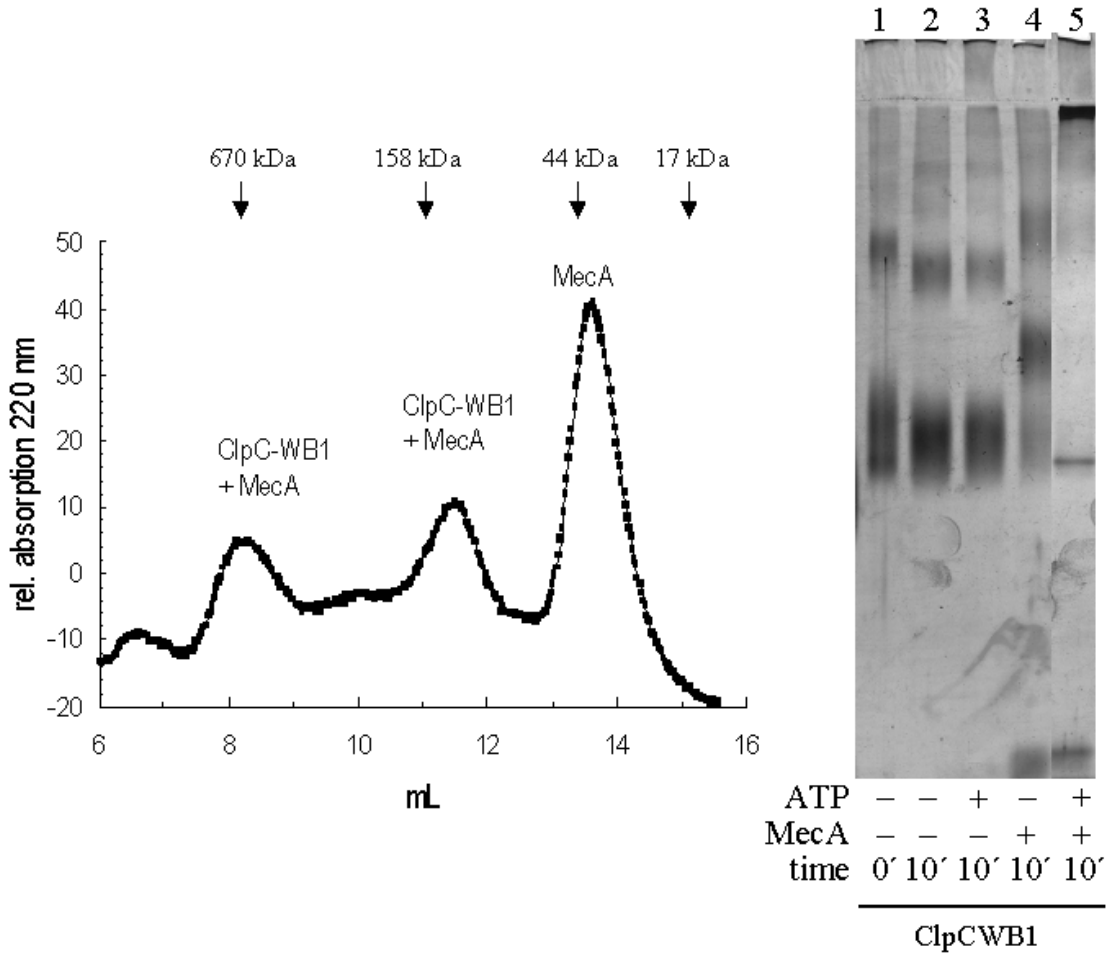



**Fig. 27 A+B)** The elution profile of size exclusion chromatography experiments with ClpC-WB1 (6μM) MecA (6 μM) (preincubated with ATP (2 mM) but without ATP in the running buffer) are depicted in the graph **(A)**. Arrows indicate positions of standard proteins with different molecular weights, separated on the same column. **(B)** GAXL experiments carried out with ClpC-WB1 (1 μM), with and without ATP (2 mM) and/or MecA (1 μM) as indicated and as described for the experiment shown in Fig 17.

The experiments depicted in Figure 27A and 28A demonstrated that ClpC-WB1 and ClpC-WB2 could form the heterodimer with MecA and both could form the higher oligomers induced by MecA and ATP determined by the GAXL experiment (Fig 27B and 28B), but only ClpC-WB1 and not ClpC-WB2 could form a stable higher oligomeric complex with MecA, which could be detected by size exclusion chromatography (Fig 27A).

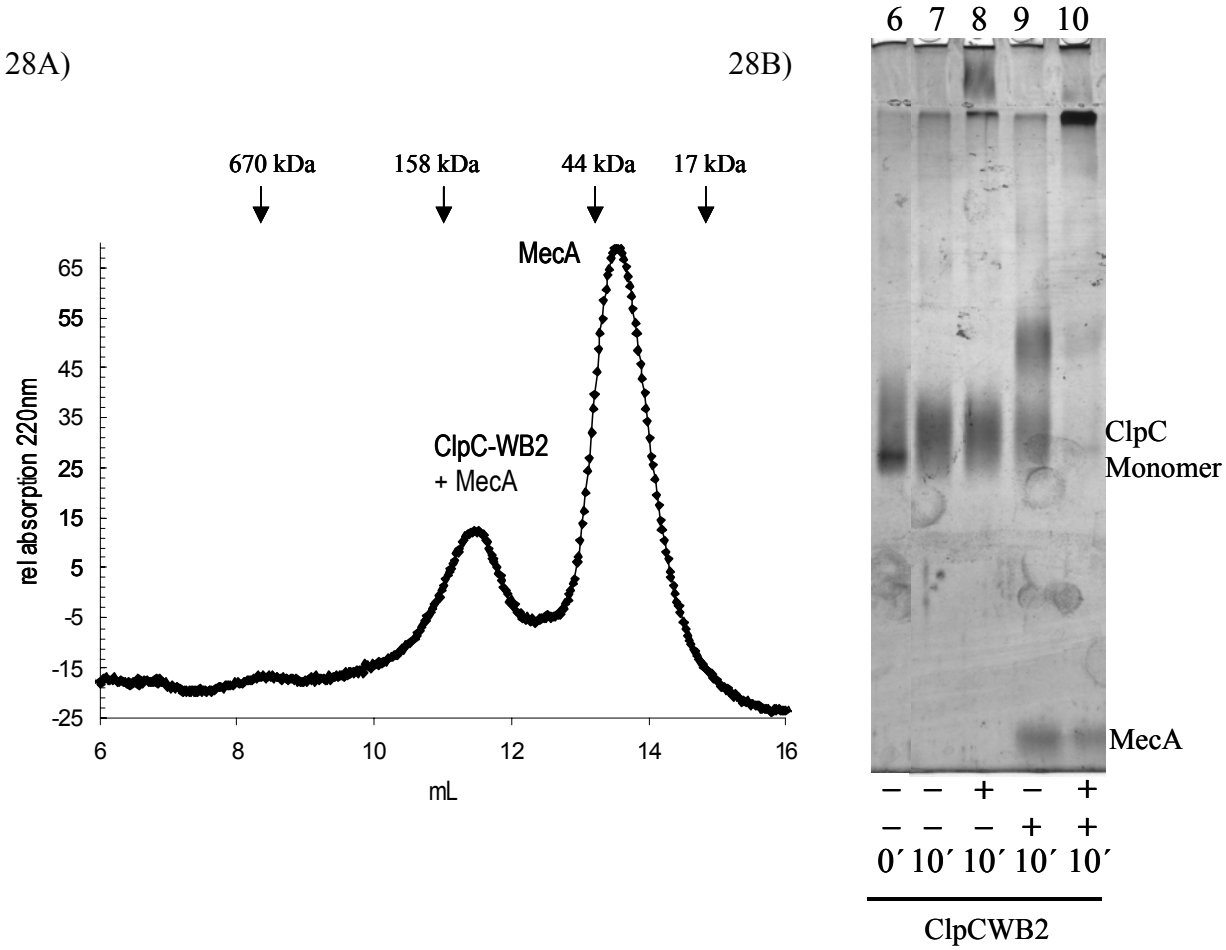



**Fig. 28 A+B)** The elution profile of size exclusion chromatography experiments with ClpC-WB2 (6µM) MecA (6 µM) (preincubated with ATP (2 mM) but without ATP in the running buffer) is depicted in the graph **(A).** Arrows indicate positions of standard proteins with different molecular weights, separated on the same column. **(B)** GAXL experiments carried out with ClpC-WB2 (1 µM), with and without ATP (2 mM) and/or MecA (1 µM) as indicated and as described for the experiment shown in Fig 17.

To test the binding of ClpC-DWB, ClpC-WB1 and ClpC-WB2 to the CTD of MecA surface plasmon resonance analysis were performed. These experiments demonstrated that all the Walker B mutants are able to bind the CTD of MecA as ClpC-WT (data not shown).

In summary the ClpC-WB1 behaves more like the DWB-mutant. The abilities of both Walker B mutants to form a heterodimer with MecA are similar as shown by the biacore analysis,

gelfiltration and GA cross-linking, but the experiment in Fig. 27A demonstrated that the ClpC-WB1 is able to form higher oligomers in presence of MecA stable enough to be detected by gelfiltration experiments. Also a stronger defect in degradation of the CTD of MecA has been shown for the ClpC-WB1 mutant suggesting that the ability of the translocation of the substrate (as shown by (Reid et al., 2001))is inhibited more than for ClpC-WB2, or a more stable interaction of the CTD of MecA with ClpC-WB1 inhibit the degradation of this substrate.

The results shown for the ClpC-WB1 mutation and the ClpC-WA1 (K214Q) (Turgay et al., 2001) imply that the determinants for MecA interaction could be located in or close to the AAA1 of ClpC.

### *4.6.2 The N domain and the Linker domain of ClpC are crucial for MecA association*

An extra domain in AAA-1 unique for the ClpC subfamily (Schirmer et al., 1996) is the attached Linker domain. The Linker domain of ClpC is homologous to the Linker from ClpB from *Thermus thermophilus*, which forms a coiled coil structure (Lee 2003). This Linker is likely to play a role in protein-protein interactions. Another domain connected directly to AAA1 is the N-terminal domain (NTD) of ClpC (Fig 29). The NTD of the Hsp100 is known to be important in substrate, or adaptor interaction (Dougan et al., 2002a) as it was shown for the NTD of ClpA and its adaptor protein ClpS (Zeth et al., 2002b).

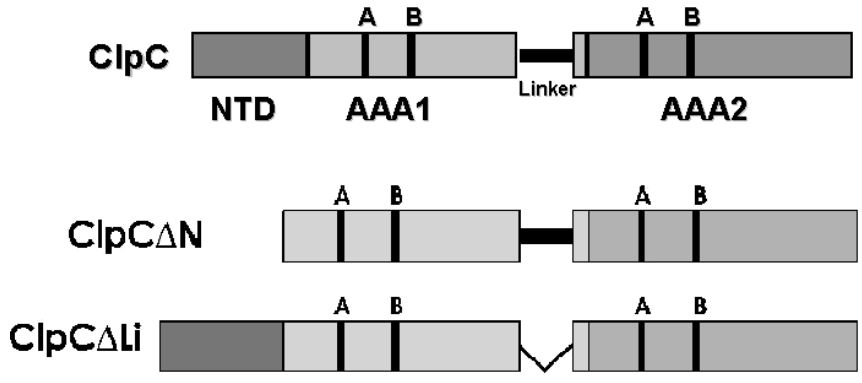



**Fig. 29)** Schematic view of the domain organisation of the ClpCΔN and ClpCΔLi-mutants

To examine the hypothesis that these domains could be responsible for the MecA and ClpC interaction, variants of ClpC missing the NTD (ClpC-ΔN) or the Linker domain (ClpC-ΔLi) have been constructed. To investigate whether ClpC-ΔN and ClpC-ΔLi have folding defects,

due to the deletion of whole domains in the size of the NTD (141aa) and the Linker (58aa), the mutant proteins were analyzed in comparison to WT-ClpC by circular dichroism (CD) measurements. For that CD measurements were performed at different temperatures (Fig 26). From the CD-spectra performed at 20°C/30°C/37°C/40°C/50°C and 60°C no major differences in the secondary structure were observed and the peak between 190 and 200 nm showed that the absorbtion of the α-helical parts of all proteins was in the same range.

Additionally all proteins had a minima at 208 nm and 222 nm which is characteristic of a α-helical structure. Minor differences can be ascribed to the absence of the α-helices from the NTD and the Linker in the ClpC-ΔN and ClpC-ΔLi proteins. As an example the wavelength scan at 30 °C is shown in figure 30A. To visualize the transition states during unfolding the ellipticity at 220 nm was measured in a temperature scan between 10°C and 85°C (Fig. 30B)

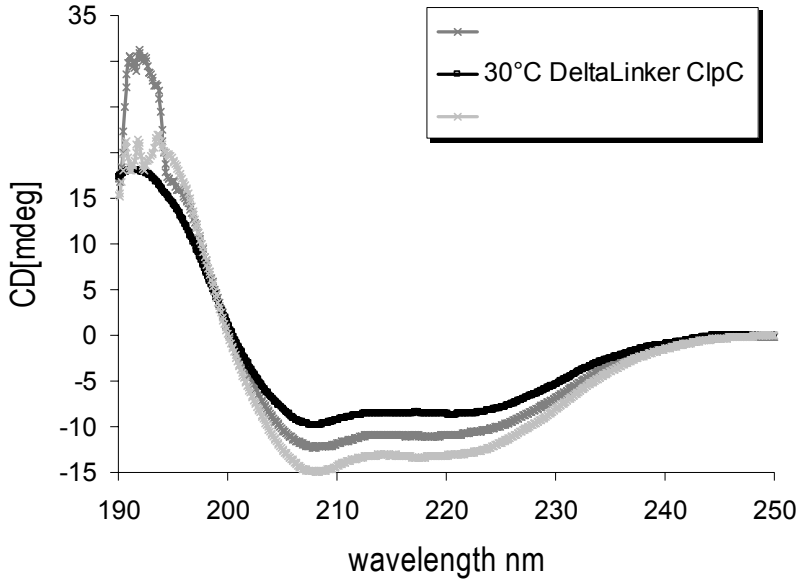



**Fig. 30A**) Six different wavelength scans with ClpC-WT, ClpC-ΔN and ClpC-ΔLi have been performed with circular dichroism measurements at 20°C/30°C/37°C/40°C/50°C and 60°C. In all measurements the part of α-helices visible in the peak between 190 and 200 nm, was basically the same in WT and mutants. As an example the wavelength scan at 30 °C is shown in this figure.

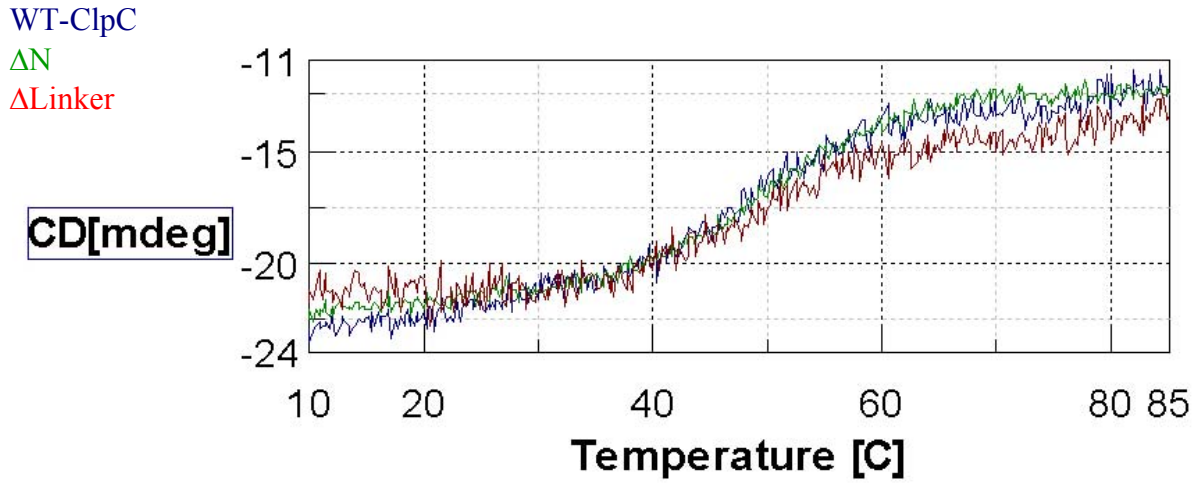



**Fig. 30B**) Overlay of the temperature scan at 220 nm of ClpC-WT (blue), ClpC-ΔN (green) and ClpC-ΔLi (red). The temperature transition states are at the same temperatures for the WT and ClpC- ΔN and ClpC-ΔLi.

The comparison of the temperature transition states reveals that around 40°C the temperature depended unfolding of ClpC-WT, ClpC-ΔN and ClpC-ΔLi take place. The second transitions to total unstructured proteins are around 60°C. The resulting melting temperature were 48,51°C for ClpC-WT, 50,79°C for ClpC-ΔN and 45,66°C for ClpC-ΔLi.

ClpC-ΔN and ClpC-ΔLi were tested in several assays for their basal and MecA induced ATP hydrolysis rate and chaperone activity, but beside a basal rate comparable to ClpC-WT no MecA stimulated ATPase rate and no chaperone activity has been observed (data not shown)

Additionally binding to MecA, heterodimerformation in gelfiltration runs and GAXL-experiments has been analyzed (see Figure 32-34).

First the ability of ClpC-ΔN and ClpC-ΔLi to bind to the C-terminal domain of MecA in comparison to ClpC-WT was tested by surface plasmon resonance.

No interaction of ClpC-ΔN with the CTD of MecA was detectable and ClpC-ΔLi displayed weak interaction with the CTD of MecA under these conditions (Fig 31). In another biacore experiment J.Kirstein and K.Turgay showed some interaction of ClpC-ΔN and also some more binding of ClpC-ΔLi with the CTD of MecA. But these interactions were still weak in comparison to the signal of ClpC-WT.

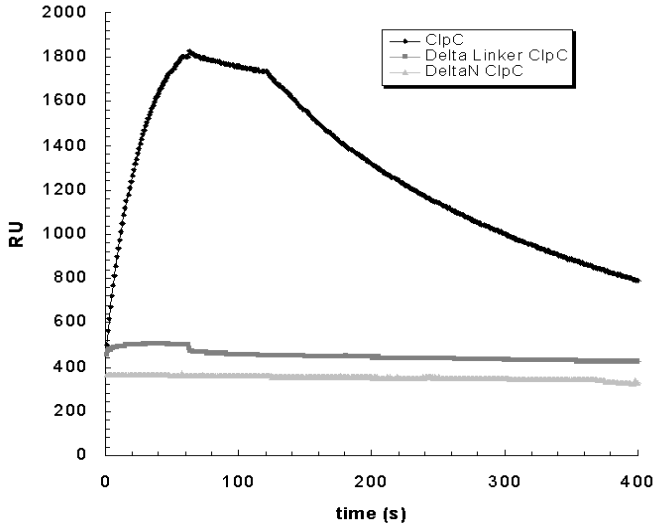



**Fig. 31)** Comparison of ClpC, ClpC-ΔN and ClpC-ΔLi binding to MecA, by surface plasmon resonance. The binding response is measured in resonance units (RU). About 500 RU of the N-terminally his-tagged CTD-MecA was bound on a Ni-NTA affinity chip and ClpC, ClpC-ΔN or ClpC-ΔLi proteins (200 nM) (with 2 mM ATP) were passed over the chip surface. After 60 s buffer with 2.0 mM ATP was introduced and buffer lacking ATP was passed over each chip after 120 s.

This low response of the mutants indicates that NTD and the Linker play a role in MecA interaction, but only MecA binding and no other activity could have been detected with this experiment.

For both ClpC variants no ClpC MecA heterodimer was detectable by gelfiltration with and without ATP preincubation (Fig.32 and data not shown).

Subsequent westernblot and silverstain analysis of the fractions of the gelfiltration run with ATP preincubation depicts that most of the MecA was detectable at the size of a MecA monomer in the western blot at the position of ClpC (Fig. 33A+B).

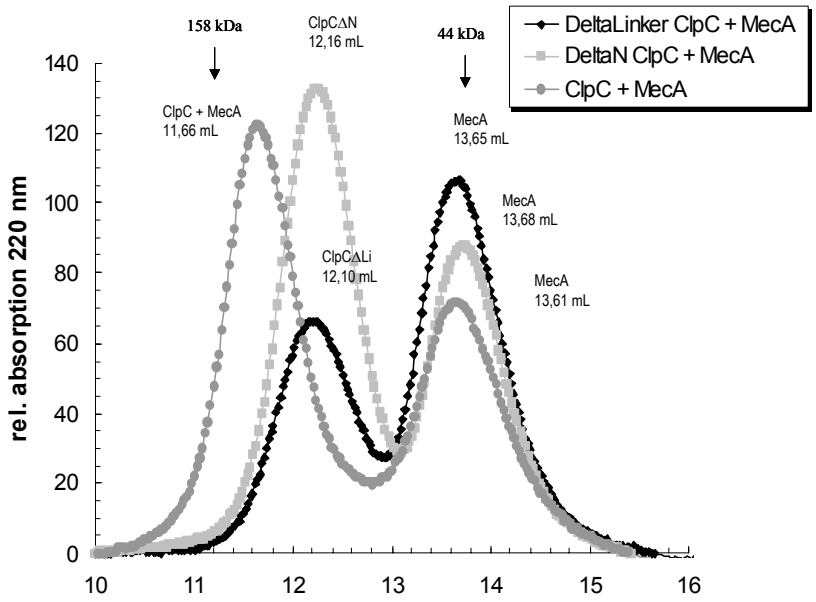



**Fig 32)** The elution profiles of size exclusion chromatography experiments with ClpC (6µM), ClpC-ΔN (6µM) and ClpC-ΔLi (6µM), all with MecA (6 µM) with ATP (2 mM) Arrows indicate positions of standard proteins with different molecular weights, separated on the same column.

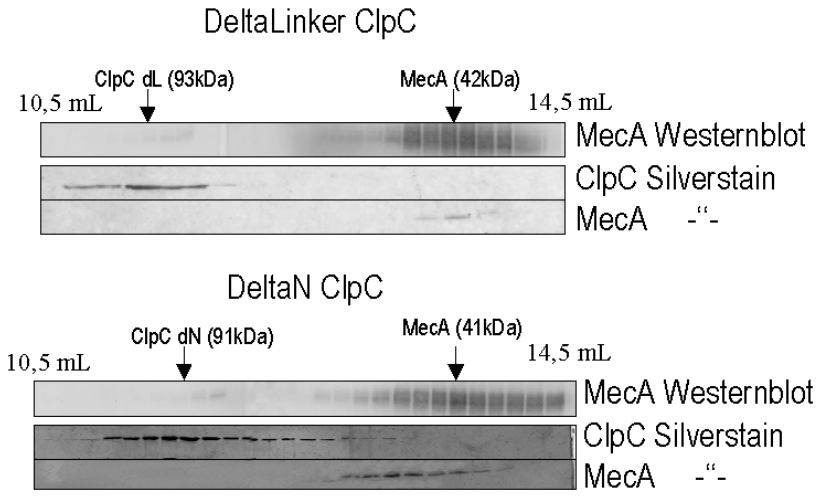



**Fig. 33 A+B)** The fractions from the gelfiltration run shown in Fig. 31 were collected and analyzed by SDS-PAGE with subsequent silverstain and westernblot analysis

GA cross-link experiments demonstrated, that due to the deletion of the NTD, MecA could neither interact nor form the heterodimer with ClpC anymore. The deletion of the linker showed the same behavior, although a weak interaction of MecA with ClpC-ΔLi was still detectable, indicating that the NTD of ClpC is the primary interaction site, which together with the linker domain could constitute the MecA receptor. Consequently MecA could not

assist the oligomerisation of ClpC-ΔN or ClpC-ΔLi as demonstrated by GAXL experiments (Fig 34). The deletion of the NTD did not interfere with the ability of ClpC to form transient oligomers, because a minor fraction of transient higher oligomers, comparable to ClpC (Fig 16), was detected by GAXL for ClpC-ΔN (Fig 34 lane 6). But MecA could not stabilize or assist the formation of this higher oligomer (lane 7 and 8 Fig 34). A higher oligomer form of ClpC-ΔLi could not be detected with or without MecA (Fig 34 lane 1-4), even at higher concentrations (data not shown). The presence of the linker domain might be important for the oligomerisation ability of ClpC.

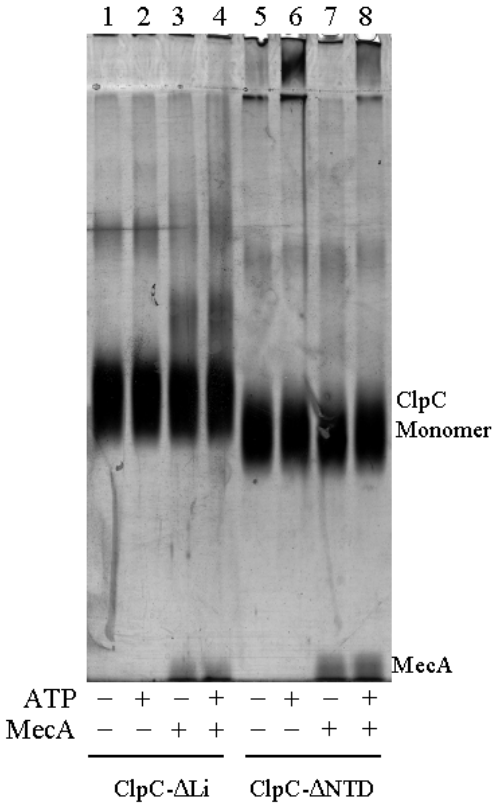



**Fig. 34)** GA cross-link experiments carried out with ClpC-ΔN (1 µM) and ClpC-ΔLi (1 µM) with and without ATP (2 mM) and/or MecA (1 µM) as indicated and as described for the experiment shown in Fig 16.

The GAXL experiments with ClpC-ΔN and ClpC-ΔLi depicted that both proteins do not form oligomers in presence of MecA.

### 4.7 *In vivo* phenotype of ClpC mutants

To elucidate the in vivo Phenotypes of ClpC-DWB, ClpC-ΔLinker, ClpC-WB1 and ClpC-WB2 the mutants were tested under heat shock conditions shown in growth curves and spot tests.

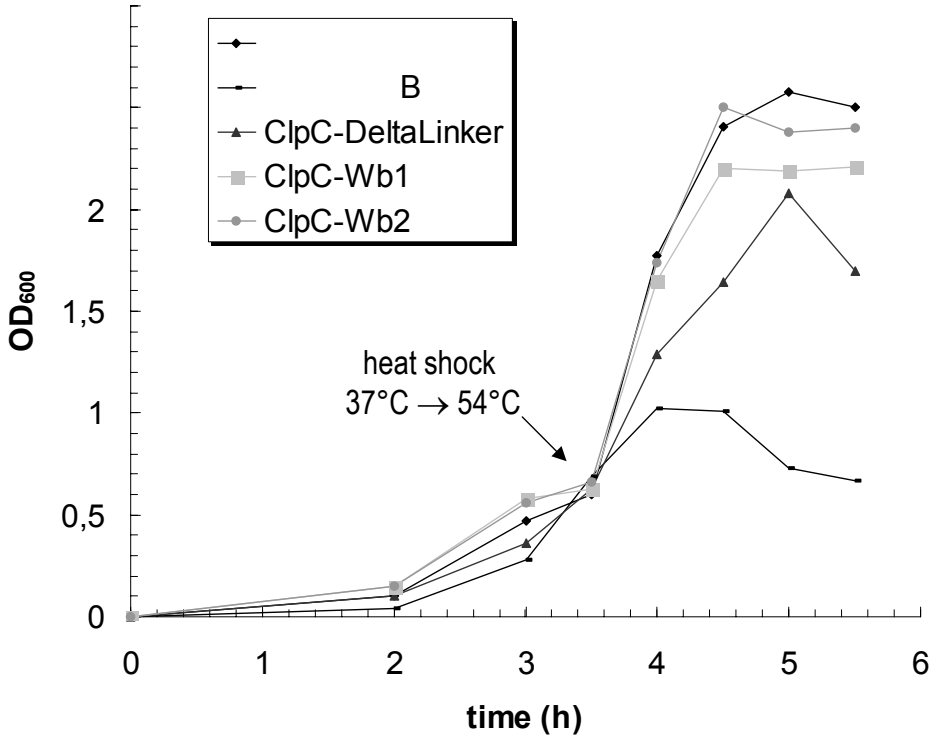



**Fig 35)** Growth curve of ClpC-WT, ClpC-DWB, ClpC-ΔLinker, ClpC-WB1 and ClpC-WB2. Cells were inoculated in 2YT medium from fresh grown cultures with the same number of cells ($OD_{600}$ =1.0). At an $OD_{600}$ of 0.6 the cells were shifted from a 37°C to a 54°C water bath and samples were taken at the indicated time points.

The graph shown in Fig. 35) indicates that the *clpC-DWB* mutant has the strongest growth defect under these conditions, followed by ClpC-ΔLinker. The single Walker B1 and Walker B2 mutants have only minor defects, coherent with the observation that they are able to fulfill minor ClpC-WT activities *in vitro*. At 37°C no growth differences of the mutant strains in comparison to the WT were observed (data not shown)

Spot tests were performed in addition to elucidate the differences in growth under constant heat shock conditions. The fresh inoculated cultures of the strains *clpC-WT*, *clpC-DWB*, *clpC-ΔLinker*, *clpC-WB1* and *clpC-Wb2* were diluted to the indicated $OD_{600}$ = 1.

An aliquot of these dilutions were spotted onto LB-plates and incubated at different temperatures (37°C, 52°C and 53°C). At 53°C a strong difference of the phenotype was observable (Fig. 36).

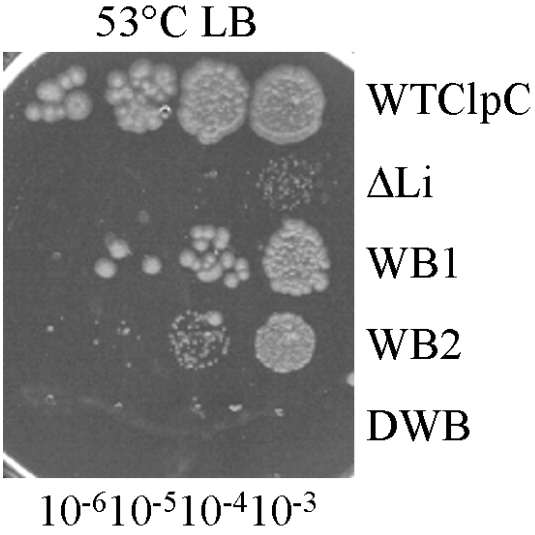



**Fig. 36)** Spot test of clpC-WT, clpC-DWB, clpC-ΔLinker, clpC-WB1 and clpC-WB2. Cells were inoculated with the same number of cells from fresh grown cultures. Dilutions of the cultures were spotted onto LB plates and placed in incubators with different temperatures. The Plates placed at a 53°C-incubator was shown in this figure (53°C LB kindly provided by A.Mogk).

On TBAB or LB plates no colonies of the *clpC-DWB* strain were observed at 53°C. The cells of the *clpC-ΔLinker* strain showed growth of some colonies in a dilution of $10^{-3}$. The strains *clpC-WB1* and *clpC-Wb2* grow in 2YT medium after a heat shock like WT (Fig. 35), but in the spot tests differences between *clpC-WB1* and *clpC-WB2* in comparison to the WT were observed (Fig. 36). The *clpC-WB1* and *WB2* mutant strains displayed under these conditions clear growth limitations. But opposite to the *in vitro* data, the *clpC-WB2* displays stronger growth limitation under high temperatures. At normal temperatures no differences in growth are visible in comparison to the *clpC-WT* strains.

In another *in vivo* experiment the ability of these mutants to inhibit ComK activity in comparison to *clpC-WT* was tested. To determine the competence phenotypes of the clpC mutant strains, the β-galactosidase activities of $P_{comK}$–lacZ reporter fusions, integrated at the ectopic *amyE* locus of these strains and the growth of the strains in competence medium were followed (Fig. 37). The comK–lacZ fusion actually reports the cellular content of active ComK, as this protein is needed for the expression of its own gene (van Sinderen and Venema, 1994). As expected, the control strain with a wildtype clpC gene exhibited a minor increase in β-galactosidase activity after the transition to stationary phase (4h).

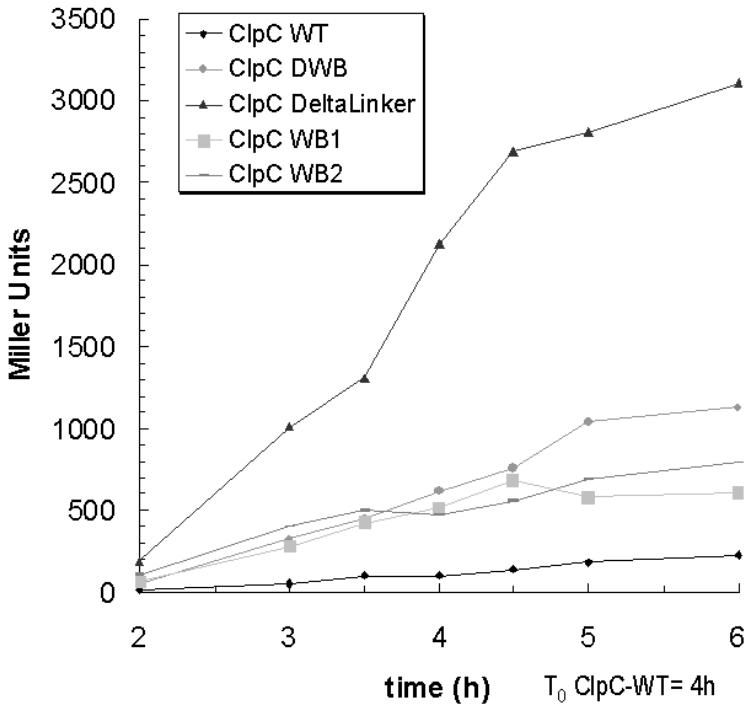



**Fig. 36)** Effects of clpC mutations on comK–lacZ expression. Cells were grown in competence medium (Spiz II-medium) and samples were taken at the indicated time points to be tested for β-galactosidase activity. Miller Units are calculated from the average of two experiments.

The Δ-Linker mutation showed a dramatic effect. This mutant strain behaved like other *B.subtilis* cells with a *clpC* knockout (data not shown). This was another indication that ClpC-Δ-Linker was unable to bind MecA and thereby inhibit ComK activity. β-Galactosidase activity was also detected in the strains with the mutation in the walker B motifs. This suggest that ComK activity is also not inhibited by ClpC-DWB, ClpC-WB1 and ClpC-WB2 but due to the fact that MecA can be bound even tighter than ClpC-WT to this mutant proteins (as shown in Chapter 4.6) this effect is not as strong as for ClpC-Δ-Linker.

The findings shown in chapter 4.7 corroborate the previous *in vitro* data shown for the ClpC-Δ-Linker and the importance of ATP hydrolysis activity of Domain 1 and 2 (AAA1 & AAA2), because also the *in vivo* activity of these proteins is affected.

# 5 DISCUSSION

## 5.1 ClpC and MecA - a bi-chaperone system

The results presented in this work demonstrated that adaptor proteins like MecA or YpbH are necessary for the *in vitro* disaggregation and refolding of protein aggregates by ClpC, a chaperone of the HSP 100 subfamily. MecA is also necessary for the *in vitro* proteolysis of unfolded or aggregated proteins by ClpCP. These adaptors enables the recognition and targeting of substrate proteins to ClpC and simultaneously induces the ATP hydrolysis of ClpC. ClpC is activated by MecA, which acts as an assembly factor for ClpC, by interacting with the N-domain and the Linker of the ClpC monomer. This activity of MecA is observed for the first time for adaptor proteins of AAA+ proteins.

### *5.1.1 Adaptor controlled oligomerisation of ClpC*

A monomeric state is unusual for HSP100 proteins, because the hexamer of HSP100/Clp proteins appear to be a crucial prerequisite for their activity. It should also be kept in mind that the mechanism by which HSP100/Clp proteins recognize, unfold and translocate substrate protein, preparing them for the peptidase ring, is mostly consistent with ring forming HSP100/Clp proteins as the active species. That a hexamer of an HSP100/Clp protein interacts and processes substrate was in fact directly observed for e.g. ClpA and ClpX (Ishikawa et al., 2001; Kim et al., 2001; Ortega et al., 2000; Reid et al., 2001). A clear correlation of ATPase and chaperone activity and oligomeric state was also observed for ClpB (Mogk et al., 2003b; Schlee et al., 2001) and Hsp104 (Hattendorf and Lindquist, 2002; Schirmer et al., 2001), but how are the ATPase and the chaperone activity and the oligomeric state of the HSP100 protein ClpC controlled ?

This study demonstrated, that in the absence of an adaptor protein, most of the ClpC population resides in a monomer form and only a minor fraction of the total ClpC population, reaches a higher oligomer, presumably hexamer form in the presence of ATP.

In the presence of MecA, ClpC or the ClpC Walker B mutants and the adaptor protein form an intermediate heterodimer independent of the presence of ATP. This heterodimer is shifted towards a higher oligomeric complex of ClpC together with the adaptor protein only in the presence of ATP or ADP (data not shown). It could be demonstrated that this higher oligomeric complex of ClpC and MecA is the active complex of this chaperone system,

because a complex of ClpC-DWB and MecA can interact with substrate proteins (Fig.21A). The estimated size of this complex is consistent with a hexamer of ClpC interacting with up to six molecules of MecA. This composition is consistent with the observation that the chaperone activity of ClpC and MecA reached a maximum at a ratio of six MecA molecules to one ClpC hexamer (Fig.11).

Persuh et al. demonstrated that the adaptor protein MecA possesses an N-terminal (NTD) substrate interaction domain and a C-terminal (CTD) domain necessary for ClpC binding and stimulation of the ATP hydrolysis rate of ClpC (Persuh et al., 1999). The CTD of MecA was sufficient to initiate the assembly of the active complex of ClpC and MecA, but only in the presence of full length MecA, can a substrate protein, α-casein, also co-migrate with the higher oligomeric complex (Fig. 18). This demonstrated that the NTD of MecA is an indispensable prerequisite for substrate recognition and full length MecA for the chaperone activity of ClpC-MecA system (Fig 10).

### *5.1.2 Adaptor mediated substrate recognition*

Beside the MecA mediated oligomerisation of ClpC the MecA mediated substrate recognition is very important for the ClpC chaperone activity. Adaptor proteins, like MecA, bound to their cognate HSP100/Clp proteins, could enable, diversify, and expand substrate recognition and thereby raise the overall affinity for complex substrates like aggregated proteins. Studies on *E. coli* ClpX showed that the simultaneous multiple recognition of substrates like λO is an important prerequisite for the disaggregation and refolding activity by HSP100/Clp proteins (Gonciarz-Swiatek et al., 1999; Wawrzynow et al., 1995).

MecA was considered a specific regulatory adaptor for the recognition of ComK and ComS (Persuh et al., 1999; Turgay et al., 1998). However, as this study demonstrates, MecA is also able to target parts of unfolded proteins in protein-aggregates to ClpC. But MecA is not only important for the recognition and disaggregation of protein aggregates, it is also necessary for efficient recognition of unfolded proteins, like α-casein, which are recognized and degraded by other AAA+ proteins (e.g., ClpAP or Lon) without any adaptor protein.

The MecA-dependent ATPase of ClpC is significantly induced by ComK and ComS (Turgay et al., 1997) but for yet unknown reasons not significantly induced by α-casein, distinguishing these substrate classes. Little is known about the substrate specificity of MecA, besides the interaction of ComK and ComS with the N-terminal domain of MecA (Persuh et al., 1999).

The N-terminal domain of MecA is also necessary for the recognition of heat-aggregated proteins. The recognition sequences located in ComK and ComS may be similar to those recognized by MecA in unfolded or aggregated proteins. Consistent with this idea, NMR studies suggest for ComS an unfolded state (J.Cavanagh).
Taken together, these findings strongly suggest that, in contrast to *E.coli* ClpA, ClpC alone cannot select and recognize substrates and that adaptor proteins like MecA are necessary for all of the functions of ClpC, including substrate recognition.

### *5.1.3 Other ClpC adaptors*

Interestingly, in *B.subtilis* YpbH a paralog of MecA exists. This protein, however, is not involved in the targeting and degradation of ComK in competence development (Persuh et al., 2002). This study demonstrates that YpbH also induces the ATPase activity of ClpC and enables ClpCP to degrade α-casein and to disaggregate and refold Luciferase aggregates (Fig. 34). YpbH also assists the oligomerisation of ClpC judged by the GA crosslink assay (Fig. 37). A third adaptor protein for ClpC McsB, which is no paralog of MecA was recently identified and is currently analyzed. McsB displayed similar adaptor and activator abilities, including the assistance of ClpC oligomerisation (J. Kirstein, T.S., Ulf Gerth, Michael Hecker & K.T., in preparation and Fig.37).
Since MecA is known to be a specific regulatory adaptor it has been tested whether ClpC favor YpbH to target more general substrates e.g. α-casein but no preferences between MecA and YpbH have been observed (Fig 14).

### *5.1.4 Comparison to adaptors in E.coli*

The activity control of ClpC by the adaptor protein MecA is also new in comparison to the functions of the known adaptor proteins in *E.coli*. Until now adaptor proteins of *E. coli* was only known as modulators of HSP100/Clp activity. The function of *E. coli* adaptor-like co-factors of Clp proteins differs from the ClpC-MecA system, because they are not necessary for the chaperone function of their cognate HSP100/Clp or Lon homologues and they do not induce their ATPase activity or function as substrates. RssB takes part in specific and regulated recognition of the σ Factor $\sigma^S$ by ClpXP (Becker et al., 1999; Zhou et al., 2001).

SspB a ribosome associated protein helps recognizing and targeting SsrA tagged proteins for degradation by ClpXP (Levchenko et al., 2000). Recently even a simple non-proteinaceous adaptor namely inorganic polyphosphate, targeting free ribosomal proteins for degradation by Lon, was identified in *E. coli*. (Kuroda et al., 2001).

### *5.1.5 Role of MecA and YpbH in vivo*

This work demonstrated that an adaptor protein is an important prerequisite for ClpC oligomerisation and chaperone activity *in vitro*, due to this fact the role of MecA and YpbH should be also very important for ClpC activity *in vivo*.

The presented results indicate that MecA and YpbH may have a general and complementary function in protein quality control. MecA homologs are found in all sequenced genomes of low GC Gram-positive bacteria, which also always encode ClpC homologs but not necessarily ComK homologs. Other known ClpCP substrates from *B. subtilis*, like Spx (Nakano et al., 2002b) or CtsR (Krüger et al., 2001), need MecA for the *in vitro* degradation by ClpCP (Nakano et al., 2002b)(and data not shown). Studies on the role of ClpC in sporulation suggested that more adaptor proteins for ClpC could exist (Pan and Losick, 2003).

A *mecA ypbH* double-mutant strain is not defective in thermosensitivity, as measured by growth on plates at high temperature. However, when additional MecA is supplied on a plasmid in *trans* (Persuh et al., 1999), *B. subtilis* cells are rendered more thermosensitive (Schlothauer et al., 2003), suggesting that MecA may function in the protein quality control system *in vivo*. Nevertheless, because of the involvement of MecA and ClpC in multiple regulatory processes, considerably more effort will be required to dissect these functions *in vivo*.

It has been shown before that unlike the synthesis of *clpC* or *clpP* mRNA, neither *mecA* nor *ypbH* transcription is heat shock-regulated (Helmann et al., 2001; Petersohn et al., 2001; Price et al., 2001), although recent studies demonstrated that *mecA* transcription levels are induced 14,6 fold after 30 minutes of disulfide stress (Leichert et al., 2003) and 3,3 fold after an oxidative stress (Mostertz et al., 2004).

It was already observed *in vivo* and *in vitro* that MecA is also a ClpCP substrate (Msadek et al., 1998; Turgay et al., 1998). We observed *in vitro* that the presence of model substrates like α-casein also stabilizes MecA (Fig. 7). Therefore, it is tempting to speculate that the

availability of MecA substrates could regulate the cellular level of MecA, as proposed by Msadek *et al.* (Msadek et al., 1998) based on their *in vivo* observations.

### *5.1.6 Competition of substrates for ClpC binding in vivo*

The proteolytic system ClpCP/MecA is focused on the degradation of the first identified substrates ComK and ComS. Is there a competition between this substrates and aggregated proteins *in vivo*? Under normal growth conditions the ComK turnover can be handled by a fraction of the available MecA and ClpCP (Turgay et al., 1998). ComK is not synthesized in heat-shocked cells (Turgay et al., 2001), and competence develops only under very specific conditions where protein aggregation does not play a role. This indicates that a direct competition between ComK and misfolded or aggregated proteins should not pose a problem *in vivo*, but it is also not elucidated if ClpC and MecA refold aggregated proteins or this proteins are degraded by ClpCP and MecA.

### *5.1.7 Is there disaggregation or degradation of ClpCP substrates?*

As shown in this work, ClpC is able to disaggregate and refold protein aggregates *in vitro*, together with MecA, but in presence of ClpP, ClpC associates with the peptidase subunit and form a proteolytic complex. Comparing the degradation rate of aggregated MDH with the rate of disaggregation with subsequent refolding showed that the degradation is faster than the refolding of proteins (Fig. 8 and Fig.6A)

However, is the disaggregation and refolding activity of ClpC and MecA also required *in vivo*?

It has been demonstrated that *B. subtilis* ClpC, ClpX, and ClpP associate *in vivo* with protein aggregates and inclusion bodies and in addition, their involvement in the *in vivo* degradation of misfolded proteins has been shown (Jürgen et al., 2001; Krüger et al., 2000). The results presented in this work suggest that ClpCP may involve MecA also *in vivo* in the degradation of misfolded proteins. On the other hand ClpC and MecA should be able to display chaperone activity *in vivo* if not all ClpC oligomeres are occupied by the proteolytic subunit ClpP.

However Gerth and co-workers (Gerth et al., 2004) showed, that there is obviously no competition between the different Clp-ATPases (ClpC, ClpE and ClpX) for ClpP in *B.subtilis*,

either under standard growth conditions or in the presence of heat shock. According to their calculation, a maximum of approximately 75% of the total Clp ATPase-binding sites on ClpP can be occupied by Clp ATPase subunits during standard growth (37°C), and only about 60% can be occupied after heat shock even if all Clp proteins are present at their maximal level at the same time.

But it has been shown that HSP100/Clp proteins and also ClpC can have dual functions as a chaperone and as part of a protease complex (Burton et al., 2001; Turgay et al., 1998; Turgay et al., 1997; Wickner et al., 1994). In addition the *B. subtilis* genome does not encode an ortholog of ClpB of *E. coli*. Therefore ClpC and MecA could fulfill this disaggregation function in *B. subtilis*, as suggested by the presented *in vitro* results.

The involvement of the possibly redundant functions of ClpE, ClpX, the MecA paralog YpbH, and their functional interplay with other chaperone systems, like KJE and GroEL/ES of *B.subtilis*, in disaggregation and refolding or degradation remains to be elucidated.

## 5.2 Mechanism of MecA mediated oligomerisation of ClpC

### *5.2.1 Adaptor proteins control ClpC oligomerisation*

To activate ClpC by assisting its oligomerisation via different adaptor proteins with e.g. different substrate affinities or targeting abilities is a very effective and new control mechanism of an HSP100/Clp protein. The pre-existing monomeric ClpC will be rendered inactive, unless activated by an adaptor protein after formation of a heterodimer. Thereby ClpC is immediately available on demand for different physiological or regulatory purposes at various times and cellular locations. This process is fully reversible, because MecA can also become a substrate for ClpC in the absence of other substrates (Fig. 7), thus the assembly factor is degraded and the ClpC complex disassembles.

### *5.2.2 Heterodimer formation between ClpC and MecA*

The heterodimer of ClpC and MecA is an intermediate step and a prerequisite, ultimately resulting in the formation of the active higher oligomer of ClpC and MecA. No interaction of the substrate protein with the ClpC-MecA heterodimer was observed. Interestingly the heterodimer formation detected by size exclusion chromatography appeared to be independent of ATP, which was confirmed by protein interaction studies demonstrating that MecA interacted with ClpC, ClpC-DWB, ClpC-WB1 and ClpC-WB2 independent of ATP (data not shown and (Turgay et al., 2001)).

### *5.2.3 Role of the individual AAA-domains in MecA binding*

To identify the role of both AAA domains for ClpC activity, ClpC-WB1 and ClpC-WB2 were tested for ATPase induction the remaining degradation activity and especially for the MecA mediated oligomerisation.

The observed MecA induced ATPase of ClpC-WB1 and ClpC-WB2 and its correlation to their activity could reflect, at least partially, the activity of the ClpC-WB1 and ClpC-WB2 hexamers, because of the correlation of the MecA dependent ATPase and the chaperone activity. The MecA induced ATPase of ClpC at a 1:1 ratio, where the chaperone activity was at a maximum, could be as high as 1.1 $s^{-1}$ (Table 1), which was 35 times more than the MecA induced ATPase of ClpC-WB1 and ClpC-WB2. The remaining ATPase activity of ClpC-WB1 and ClpC-WB2 appeared to be sufficient for some remaining activity. Both ClpC single WB variants were still able to degrade the CTD of MecA *in vitro*, albeit at a much lower rate (Fig. 22). (Whereas the degradation rate of ClpC-WB1 is slower than that of ClpC-WB2). This indicated that both ATP binding sites are necessary for a full ATPase induction of the ClpC hexamer by MecA and it also strongly suggests that a synergistic cooperation, between the ATPase of two AAA+ domains must exist (Turgay et al., 2001).

A coordinated ATP hydrolysis within the ClpC hexamer seems to be also important for ClpC activity. Experiments with the ClpC-DWB mutant showed, that ClpC-DWB inhibits at a ratio of 1:5 the MecA mediated ATPase and chaperone activities of ClpC-WT (see chapter 4.8.6).

The *in vivo* phenotypes of ClpC-WB1 and ClpC-WB2 showed a less strong effect than the in vitro observations. Competence measured by β-Galactosidase activity of a $P_{comK}$-lacZ reporter fusion and heat resistance measured by growth at high temperature (Turgay et al.,

2001) of the respective *clpC-WB1* and *clpC-WB2* strains displayed only partial phenotypes. The heat resistance of *clpC-WB2* was partially more affected then *clpC-WB1* as observed previously for other mutants in the first and second AAA+ domain of ClpC,(Turgay et al., 2001), but clpC-DWB was unable to grow under these conditions (Fig32).

However this work demonstrates that MecA could stably interact with ClpC, when the first AAA+ domain was altered in such a way that it could bind but not hydrolyze ATP (ClpC-WB1) (Fig. 23). A similar mutation in the second AAA+ domain (ClpC-WB2) showed no stabilization by MecA in gelfiltration experiments. Only GA crosslink experiments demonstrated that ClpC-WB1 and ClpC-WB2 could form MecA dependent higher oligomers comparable to ClpC-DWB (compare Fig. 23B and 24B).

The importance of the first AAA+ domain of ClpC for a stable MecA interaction in gelfiltration runs suggested that the binding partner(s) for MecA are located in or next to AAA-1.

### *5.2.4 N-domain and Linker mediate MecA association*

The ClpC domains located in AAA-1 necessary and sufficient for the heterodimer formation are the N-domain and the linker domain. The N-domain interacts stronger with MecA compared to the Linker domain of ClpC. Since the presence of one of the two domains is not sufficient for the heterodimer formation a concurrent interaction of MecA with both domains is likely to occur.

The absence of one of the two domains in ClpC, which can interact on their own with MecA, has a negative effect on the binding ability of these ClpC-deletion variants to MecA. This negative cooperative effect suggests that for ClpC the presence of both domains, possibly interacting with one another, is necessary to allow a productive interaction with MecA.

To test the importance of the N-domain and the Linker D. Dougan and J. Kirstein constructed hybrid proteins of ClpA combined with the N-domain and the Linker of ClpC (CNTD-ClpA and ClpA-CLi) and analyzed the MecA interaction of these proteins with surface plasmon resonance. Both hybrid proteins showed MecA interaction proving that these ClpC domains are responsible for MecA interaction. Compared to the interaction of the single domain ClpA hybrid proteins or the single N-domain of ClpC the strength of the interaction with MecA of the single domain variants of ClpC, resulting from the deletion of the N-domain or the Linker domain (ClpC-ΔLi and ClpC-ΔNTD), is one order of magnitude lower (T.S., D. Dougan, J. Kirstein, A.Mogk, B.Bukau & K.Turgay in preparation).

The interaction of MecA with the NTD of ClpC could be important for the delivery of the substrate, preparing ClpC to accept substrate protein, as discussed e.g. for ClpS and the NTD of ClpA (Zeth et al., 2002b). Other N-domain's of HSP100/Clp proteins, not homologous to the N-domain of ClpC, were also identified as interacting partners for adaptor proteins like e.g. SspB and the N-domain of ClpX (Dougan et al., 2002c; Dougan et al., 2003; Wojtyra et al., 2003; Zeth et al., 2002b) or the N-domain of p97, which is also the interacting site for p97 specific adaptor proteins (Yuan et al., 2001). The structure of ClpB from *Thermus thermophilus* has recently been determined. The Linker domain of ClpB comprises an independently folded mobile domain consisting of two coiled coil structures, whose mobility is important for the chaperone function of ClpB. In addition the analysis of the three obtained structures demonstrated that the N-domain is very mobile (Lee et al., 2003). Analyzing the homologous linker domain from ClpC revealed that it lacks the second coiled coil structure (Motif 2 or M3 and M4 in (Lee et al., 2003)). The investigation of a structural model of ClpC built upon the ClpB structure, which took the shortened Linker domain of ClpC into account, demonstrated that the NTD of ClpC could be positioned proximal to the linker domain (data not shown). A concurrent contact of MecA with the mobile ClpC Linker domain and the N-terminal domain would be feasible and would position the flexible NTD in a position which may allow better access of the substrate to the pore of the hexameric ClpC.

The interaction of different adaptor proteins with the NTD's of ClpA or ClpX was shown to be necessary for the targeting activity of this adaptor. The additional interaction of MecA with the linker could therefore be necessary for the assistance of oligomerisation of ClpC. Consistent with this hypothesis no higher oligomer form of ClpC was detected for ClpC-ΔLi, with or without MecA in the GA crosslink experiment, even at higher concentrations of ClpC-ΔLi (Fig. 30, data not shown).

In vivo *clpC*-ΔLi displayed a defect comparable to a *clpC* knock out, because competence measured by β-Galactosidase activity also indicated that no ClpC-ΔLi- MecA interaction is possible (Fig. 36).

### *5.2.5 Model of MecA mediated oligomerisation*

The results presented in this work lead to the following model of ClpC oligomerisation mediated by MecA or other adaptor proteins (Fig 38).

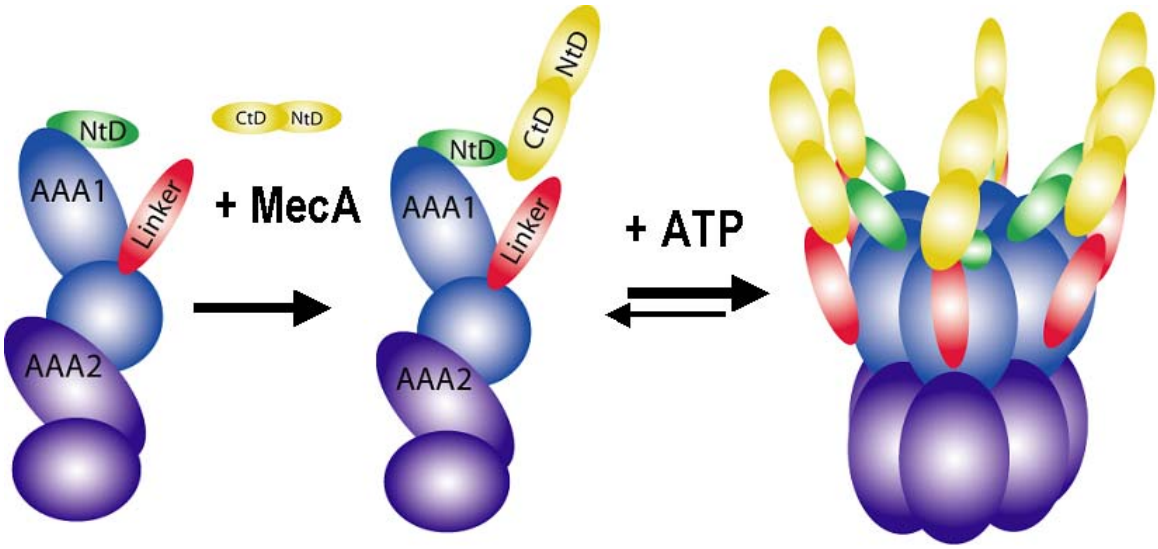



**Figure 38** A model summarising the mechanism of the activation by oligomerisation of the AAA+ protein

MecA binds independently of ATP to the N-terminal domain and the Linker of ClpC; a heterodimer between these two proteins is formed, stable enough to be detected by gelfiltration experiments, if the walker B motif of the AAA-1 domain is mutated in a way that ATP hydrolysis is not possible. In presence of ATP and MecA conformational changes of ClpC are likely to occur which enables the formation of a stable oligomere of ClpC with up to six MecA molecules bound. This complex is able to interact with substrates like α-casein and fulfill the chaperone activities as described for the ClpC/MecA system, or to degrade substrate proteins in connection with the peptidase subunit ClpP.

### *5.2.6 Oligomerisation of other AAA+ proteins*

For at least one other member of the AAA+ protein family, it has been suggested but not demonstrated, that an adaptor protein negatively regulated its activity by controlling its oligomeric state. PspF is a bacterial enhancer protein of the AAA+ family, which, as a hexamer, activates $\sigma^{54}$ dependent transcription (Chaney et al., 2001; Zhang et al., 2002). PspA is an inhibitor of PspF and because it inhibits the ATPase activity of PspF, it has been suggested that it could act by inhibiting the oligomer formation of this AAA+ protein (Dworkin et al., 2000; Elderkin et al., 2002)

A dynamic oligomerisation of an AAA+ protein has also been observed for other AAA+ proteins. The specialized microtubule severing ATPase katanin is a heterodimer consisting of p60, containing a single AAA+ domain and p80 whose WD40 domain allows targeting of the

complex to the centrosome of eukaryotic cells (Hartman et al., 1998; McNally and Vale, 1993). Experiments with the isolated p60 demonstrated, that a hexamer of p60 could only be detected in a stable manner, when the Walker B motif was altered, as described above for ClpC. This oligomeric p60 could interact via its N-terminus with multimers of tubulin but not with tubulin monomers and the ATPase induction of p60 by tubulin correlated with its oligomer formation and tubulin severing activity (Hartman and Vale, 1999). The p80 subunit, which possesses no ATPase activity, can also induce the ATPase and the tubulin induced ATPase of p60 at least twofold (Hartman et al., 1998). The influence of p80 on the oligomerisation of p60 was not examined in these experiments. It would be tempting to speculate that, because of its p60 ATPase induction activity, p80 might also assist the oligomerisation of p60.

## 5.3 Conclusions

The results for ClpC and MecA demonstrate that controlling the ability of an AAA+ protein to form an active hexameric ring is an important functional aspect and a mechanism by which the recognition and targeting of misfolded/aggregated proteins or specific proteins like ComK to ClpC can be regulated. The adaptor protein MecA activates the ATPase activity and coordinates substrate availability with the activation of ClpC, adding a new layer of regulation of HSP100/Clp proteins and enabling the involvement of ClpC in a wide variety of different aspects of the *B. subtilis* cellular physiology, by presenting specific substrates like ComK. This notion of controllable dynamic oligomerisation is a concept, which also applies to the understanding of the function and regulation of seemingly distant AAA+ proteins of eukaryotic origin, involved in various and diverse fundamental cellular functions.

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# 7 ABBREVIATIONS

| | |
|---|---|
| aa | amino acid(s) |
| ADP | adenosine diphosphate |
| APS | ammonium peroxodisulfate |
| agg. | aggregated |
| ATP | adenosine triphosphate |
| bp | base pairs |
| *B. subtilis* | *Bacillus subtilis* |
| CD | Circular dichroism |
| µCi | micro Curie |
| CTD | C-terminal domain |
| cm | centimeter |
| CV | column volume |
| Da | Dalton (atomic mass unit) |
| den. | denatured |
| DMSO | dimethyl sulfoxide |
| DNA | desoxyribonucleic acid |
| DnaJ | Hsp40 chaperone in *E. coli* |
| DnaK | Hsp70 chaperone in *E. coli* |
| DTT | 1,4-dithiothreitol |
| DWB | double walker B- mutant |
| *E. coli* | *Escherichia coli* |
| EDTA | ethylenediamine-N,N,N',N'-tetraacetic acid |
| EtOH | ethanol |
| FPLC | fast-pressure liquid chromatography |
| g | gram |
| *g* | gravitation |
| GAXL | Glutaraldehyde crosslink |
| GroEL | Hsp60 chaperone in *E. coli* |
| GroES | Hsp10 cochaperone in *E. coli* |
| GrpE | nucleotide exchange factor for DnaK |
| h | hour |
| $^{3}H$ | tritium |
| hd | heterodimer |

| | |
|---|---|
| ho | higher oligomer |
| HEPES | 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid |
| HPLC | high-performance liquid chromatography |
| HSP | heat shock protein |
| I | input |
| IPTG | Isopropyl-β-D-thiogalactopyranoside |
| kDa | kilodalton |
| KJE | chaperone-system DnaK/DnaJ/DnaJ |
| LB | Luria Bertani |
| LU | Light Units |
| M | Molar |
| µM | micromolar |
| MDH | malate dehydrogenase |
| MeOH | methanol |
| min | minute |
| ml | millilitre |
| NADH | Nicotinamide dinucleotide hydrate |
| nm | nanometer |
| NTD | N-terminal domain |
| NMR | nuclear magnetic resonance |
| OD | optical density |
| ONK | over night culture |
| ONPG | o-nitrophenyl-β-D-galactopyranoside |
| pp | pure protein |
| P | pellet |
| PAGE | polyacrylamide gel electrophoresis |
| PEP | Phoshoenolpyruvate |
| PMSF | phenylmethanesulfonyl fluoride |
| PK | pyruvate kinase |
| PVDF | poly(vinylidene difluoride) |
| RNA | ribonucleic acid |
| Rpm | revolutions per minute |
| RT | room temperature |
| RU | resonance Units |

| | |
|---|---|
| s | seconds |
| S | supernatant |
| SDS | sodium dodecyl sulphate |
| SPR | surface plasmon resonance |
| sHsp | small heat shock protein |
| TEMED | N, N, N', N'-tetramethylethylendiamine |
| TF | Trigger Factor |
| TFA | trifluoroacetic acid |
| Tris | 2-Amino-2-(hydroxymethyl)-1,3-propanediol |
| V | volt |
| vv | void volume |
| v/v | volume / volume |
| WB-1/2 | walker B-mutant 1/2 |
| WT | wildtype |
| w/v | weight / volume |
| YT | Yeast extract |

# 8 PUBLICATIONS

*Adaptor protein controlled oligomerisation activates the AAA+ protein ClpC*
T. Schlothauer, D.A. Dougan, J. Kirstein, A. Mogk, B. Bukau & K.Turgay
Manuscript submitted

*MecA, an adaptor protein necessary for ClpC chaperone activity*
T. Schlothauer, A. Mogk, D.A. Dougan, B. Bukau &K. Turgay
*Proc Natl Acad Sci U S A*, **100**, 2306.