CtsR, the Gram-positive master regulator of protein quality control, feels the heat

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Protein quality networks are required for the maintenance of proper protein homeostasis and essential for viability and growth of all living organisms. Hence, regulation and coordination of these networks are critical for survival during stress as well as for virulence of pathogenic species. In low GC, Gram-positive bacteria central protein quality networks are under the control of the global repressor CtsR. Here, we provide evidence that CtsR activity during heat stress is mediated by intrinsic heat sensing through a glycine-rich loop, probably in all Gram-positive species. Moreover, a function for the recently identified arginine kinase McsB is confirmed, however, not for initial inactivation and dissociation of CtsR from the DNA, but for heat-dependent auto-activation of McsB as an adaptor for ClpCP-mediated degradation of CtsR.

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Introduction

A number of environmental stresses cause damage to protein structure leading to protein aggregation, which affects survivability of all living organisms. Refolding or degradation of misfolded proteins is a major task of protein quality networks. Accordingly, these networks, though constitutively expressed, are strongly induced under a variety of stress conditions that alter protein conformation (Wickner *et al*, 1999; Sauer *et al*, 2004; Hartl and Hayer-Hartl, 2009).

Bacterial cells have the capability to respond rapidly to a wide range of environmental stresses. This adaptation to an altered milieu is mediated by complex regulatory networks. Regulation of the bacterial responses to heat stress serves as model for understanding the general mechanism of protein quality control, which is important for the survival during stress (Frees *et al*, 2007) as well as for virulence of pathogens (Ingmer and Brøndsted, 2009). In low GC, Gram-positive

bacteria central protein quality networks are controlled by the highly conserved CtsR heat-shock regulator. CtsR is a classical winged helix-turn-helix (HTH) dimeric DNA-binding protein that recognizes a hepta-nucleotide direct repeat sequence within the promoter of regulated genes (Derré *et al*, 1999, 2000), thereby suppressing their expression. It has been shown previously that the modulator of CtsR activity, McsB, is necessary to inactivate CtsR *in vitro* (Kirstein *et al*, 2005). This process is thought to be performed by the recently discovered *in vitro* arginine kinase activity of *Geobacillus stearothermophilus* McsB, which phosphorylates CtsR on various arginine residues and thus inactivates it (Fuhrmann *et al*, 2009). In addition, YwlE was shown to be the cognate phosphatase for the McsB kinase *in vitro* (Kirstein *et al*, 2005). Furthermore, McsB was reported to be essential for regulated degradation of CtsR during heat stress by the ClpCP protease (Kirstein *et al*, 2007). Degradation of CtsR relies on the ability of McsB to function as an adaptor protein for ClpC. Adaptor proteins are needed for efficient oligomerization and activity of *Bacillus subtilis* ClpC (Kirstein *et al*, 2006).

Here, we report that McsB is not involved in CtsR inactivation *in vivo* during heat stress, which contradicts conclusions reported previously that were based on *in vitro* experiments (Fuhrmann *et al*, 2009). Instead, McsB is only necessary as a kinase with adaptor function during heat stress mediating efficient degradation of non-functional CtsR. Remarkably, CtsR is an intrinsic heat sensor and acts as protein thermometer to protect bacterial cells in a fast and efficient manner.

Results

McsB does not participate in regulation of CtsR activity during heat stress *in vivo*

The starting point for this study was the observation that McsB, which has been considered to be crucial for heat stress response in all previous studies in low GC, Gram-positive bacteria (Krüger *et al*, 2001; Kirstein *et al*, 2005; Fuhrmann *et al*, 2009), is absent from the genome of some low GC, Gram-positive bacteria (Supplementary Figure S1) (Varmanen *et al*, 2000; Fiocco *et al*, 2010). However, CtsR-regulated genes are induced during heat stress in these species as well (Varmanen *et al*, 2000; Chastanet *et al*, 2001), suggesting either a second regulatory pathway or a general McsB-independent mechanism.

On basis of this knowledge, we analysed a *B. subtilis* *mcsB* mutant for CtsR-dependent transcription *in vivo*. We found that *clpE*, solely regulated by CtsR, was fully inducible in the *mcsB* mutant upon heat exposure (Figure 1A), indicating that McsB is not required for CtsR inactivation during heat stress *in vivo*, as would have been expected from previous *in vitro* data (Krüger *et al*, 2001; Fuhrmann *et al*, 2009). This observation is supported by the fact that a *ywlE* mutant, which was described to be the cognate phosphatase for McsB kinase *in vitro* (Kirstein *et al*, 2005), shows no impact on CtsR activity neither at 37°C nor during heat stress (Supplementary

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Figure 1 Impact of the McsB kinase on CtsR-dependent transcription. (A) Northern blot analyses of *clpE* transcription in response to a shift to 50°C of exponentially growing cultures in wild-type *B. subtilis* and the *mcsB* mutant. (B) Schematic representation of the modified *clpC* operon, which was inserted at its original chromosomal locus in *B. subtilis* with critical amino-acid substitutions (stars) in CtsR and McsB. (C) Induction profile of the *clpE* mRNA in the wild-type *B. subtilis*, in cells harbouring the *in vivo* expression system integrated at the chromosomal site of the *clpC* operon and in an McsB point mutant with a defective kinase.

Figure S2). On the basis of these results, we propose that a second, McsB-independent pathway for CtsR inactivation must exist.

Establishment of an *in vivo* system to investigate critical point mutations

To gain a more detailed understanding of regulatory mechanisms for CtsR inactivation and degradation, we engineered a genetic system that allowed expression of an altered *clpC* operon from wild-type promoters *in vivo* (Figure 1B). We substituted the original *clpC* operon with a modified version, which was generated by introducing a resistance marker cassette in the intergenic region between *ctsR* and *mcsA* using a modified two-step fusion PCR protocol (Wach, 1996). The regulation of expression of the entire *clpC* operon was not altered, and CtsR activity was comparable with the wild type (Figure 1C). This gave us the opportunity to substitute critical residues of the proteins encoded by the *clpC* operon, monitoring the impact of these mutations on CtsR activity *in vivo*. We investigated an McsB point mutant that possesses no kinase activity *in vitro* (Kirstein *et al*, 2005), and CtsR activity of this mutant was identical to the wild-type *in vivo* (Figure 1C). This result underlines our hypothesis that McsB is not involved in CtsR de-repression during heat stress.

B. subtilis CtsR is inactivated in a temperature-dependent manner *in vitro*

CtsR activity is regulated independently of McsB kinase during heat stress in *B. subtilis*. Therefore, it seems likely that a common, McsB-independent mechanism exists in all low GC, Gram-positive bacteria. However, a recent publication showed that *B. subtilis* McsB is the only direct interaction partner of CtsR (Kirstein *et al*, 2005). In addition, a previous study suggested that CtsR itself may act as an intrinsic heat sensor (Derré *et al*, 1999). To test whether the DNA-binding activity of CtsR is temperature dependent, we first examined the DNA binding of *B. subtilis* CtsR at different temperatures. DNA binding of CtsR was dramatically reduced at heat-shock temperatures (50°C) as compared with 37°C *in vitro*. Strikingly, more CtsR was needed to bind to same DNA amount at heat stress temperatures than under control temperatures (Figure 2A and B).

Figure 2 Temperature-dependent DNA-binding activity of CtsR. Electrophoretic mobility shift assay analysis with *clpC* promoter fragment and increasing amounts of *B. subtilis* CtsR at 37°C (A) or 50°C (B). *B. subtilis* CtsR dissociated in greater portion from the DNA after a shift to 50°C, when DNA binding had been allowed at 37°C for 30 min. Then the mix was divided and one half remained at 37°C and the other was shifted to 50°C for 5 min (C). DNA was visualized by ethidium bromide staining of the native polyacrylamide gel. Quantification of *B. subtilis* CtsR binding to the *clpC* promoter fragment at different temperatures for *B. subtilis* CtsR averaged from three independent experiments (D).

We also determined the apparent relative dissociation constants and found that *B. subtilis* CtsR displays a substantially lower binding affinity to the *clpC* operator at 50°C ($K_d = 8.76 \pm 0.49 \mu\text{M}$) than at 37°C ($K_d = 0.93 \pm 0.091 \mu\text{M}$) (Figure 2C). More importantly, CtsR massively dissociated from the *clpC* operator when shifted to heat stress after DNA binding had been allowed at control conditions (Figure 2C). Interestingly, CtsR was able to regain DNA-binding ability when it was shifted back from heat stress to 37°C (Figure 2D). This implies that CtsR is able to sense the temperature shifts by intrinsic features that either allow binding to the DNA at standard conditions or suppress it during heat stress so that the promoter becomes accessible for transcription. Thermo-induced reduction of CtsR activity was also observed for the *clpE* promoter with multiple CtsR-binding sites (Supplementary Figure S3A–C), emphasizing that CtsR property is independent from the promoter. CtsR dissociation was not accompanied with loss of CtsR dimerization (Supplementary Figure S4), indicating that elevated temperatures probably only affect correct positioning of DNA-binding segments.

CtsR from other Gram-positive bacteria is also inactivated in a temperature-dependent manner

If CtsR indeed functions as a specific protein thermometer in all low GC, Gram-positive bacteria, different CtsR proteins should be inactivated in the same way, but at species-specific temperatures. We also investigated the DNA-binding properties of CtsR from *Lactococcus lactis* and *G. stearothermophilus* at normal and heat-shock temperatures. These two species possess other temperature optima than *B. subtilis* with regard to their ecological niche. *L. lactis* exhibits a heat-shock response already at 42°C, whereas *G. stearothermophilus* does so at around 70°C. CtsR from *L. lactis* displayed a considerably higher DNA affinity under control condition ($K_{d30^\circ\text{C}} = 1.47 \pm 0.047 \mu\text{M}$) compared with heat stress ($K_{d42^\circ\text{C}} = 16.34 \pm 1.49 \mu\text{M}$) (Figure 3A). Thermo-induced reduction of CtsR activity was also observed for *G. stearothermophilus* CtsR, whereas CtsR binds DNA more efficiently under control conditions ($K_{d55^\circ\text{C}} = 1.05 \pm 0.087 \mu\text{M}$) than at elevated temperatures ($K_{d70^\circ\text{C}} = 17.32 \pm 1.05 \mu\text{M}$) (Figure 3B).

CtsR is a specific heat sensor in vivo

To verify that CtsR is an intrinsic heat sensor *in vivo* with distinct temperature thresholds for each bacterial species, we complemented an isogenic *B. subtilis* *ctsR* mutant with either *L. lactis* or *G. stearothermophilus* *ctsR* *in trans*. According to our model, CtsR-dependent gene expression should be de-repressed already at 42°C by *L. lactis* CtsR and still be repressed at 50°C by *G. stearothermophilus* CtsR. Precisely, these results were obtained (Figure 3C), indicating that CtsR activity is regulated in a temperature-dependent manner with species-specific set points probably in all low GC, Gram-positive bacteria.

A glycine-rich loop is the molecular thermometer in vitro

Thermosensing is well known for a variety of macromolecules, including proteins (Schumann, 2009). Some bacterial transcriptional regulators have already been described to display thermo-dependent DNA-binding activity (Hurme *et al*, 1997; Servant *et al*, 2000; Herbst *et al*, 2009).

Figure 3 CtsR is a specific thermosensor for all Gram-positive *in vitro* and *in vivo*. Quantification of CtsR binding with the respective *clpC* promoter fragment at different temperatures for *L. lactis* CtsR (A) or *G. stearothermophilus* CtsR (B) averaged from three independent experiments. (C) Either *L. lactis* or *G. stearothermophilus* *ctsR* was ectopically over-expressed in a *B. subtilis* *ctsR* mutant and complementation of heterologous *ctsR* expressed *in trans* was monitored at different temperatures by blue/white screening on X-gal containing LB-agar plates using a thermostable *clpE*-BgaB reporter gene fusion. White colonies show that *clpE* transcription was repressed by *trans* CtsR, whereas blue colonies indicate *clpE* expression with inactive *trans* CtsR. *L. lactis* CtsR is active at 30°C under the favoured growth conditions and is inactivated already at 42°C. However, *B. subtilis* CtsR is still active at 42°C repressing the transcription of the corresponding genes. Finally, *G. stearothermophilus* CtsR is still active at 50°C in *B. subtilis*, at a temperature in which *B. subtilis* CtsR is inactivated.

However, in contrast to CtsR, these regulators are less conserved across a major phylogenetic group of bacteria. In addition, they are structurally unrelated to CtsR and, therefore, presumably are regulated by different modes of action. We wanted to identify the CtsR domain that is critical for heat sensing. A first indication came from a sequence alignment of CtsR proteins from different low, GC Gram-positive bacteria, which showed that CtsR contains three highly conserved regions (Derré *et al*, 1999). A second clue was provided by error-prone PCR experiment that identified CtsR residues critical for *in vivo* activity (Supplementary Figure S5; Derré *et al*, 2000). Two regions, the HTH and the winged HTH domains, are both conserved and crucial for CtsR activity. Derré *et al* (2000) described two point mutants for *B. subtilis* CtsR (V16M and G65S), suppressing CtsR inactivation during

heat stress. When we transferred these mutations into our *in vivo* expression system, no effect on CtsR activity was observed (Supplementary Figure S6), which showed that these mutations had no influence on CtsR activity *in vivo*.

We substituted a glycine residue with a more rigid proline at the position 64 in *B. subtilis* CtsR, which is located at the tip of the hairpin in a conserved glycine-rich loop within the highly conserved winged HTH domain (Fuhrmann *et al*, 2009). This substitution was designed to create CtsR with enhanced thermostability (Matthews *et al*, 1987) and is no longer able to display thermo-induced reduction of DNA binding. No structural changes relative to the wild-type protein, except the substituted amino acid, were detected with a theoretically modelled G64P mutant CtsR structure, suggesting that any effect of the substitution depends on the decrease of conformational entropy of the denatured state (Supplementary Figure S7).

The mutated CtsR protein shows similar DNA-binding activity under control conditions such as the wild-type protein *in vitro* (Figure 4A) as well as *in vivo* (Figure 4E). To test whether this substitution has an impact on CtsR activity at elevated temperatures, DNA-binding experiments were performed. DNA binding of mutated CtsR^{G64P} was nearly identical under control conditions ($K_{d37^{\circ}\text{C}} = 0.87 \pm 0.096 \mu\text{M}$) and heat stress ($K_{d50^{\circ}\text{C}} = 0.91 \pm 0.088 \mu\text{M}$) (Figure 4B–D), showing that CtsR^{G64P} could not be heat-inactivated unlike wild-type CtsR. Moreover, CtsR^{G64P} did not easily dissociate from the DNA upon heat shock (Supplementary Figure S8). Consequently, CtsR^{G64P} lacks the strictly temperature-dependent regulation, which was observed for wild-type CtsR and cannot respond to heat stress.

Glycine residue 64 is essential for *B. subtilis* CtsR activity *in vivo*

The *in vivo* effect of the G64P point mutation was also assessed in our aforementioned expression system (Figure 1B). Transcription of CtsR target genes was only slightly inducible during heat stress (approximately 5% of wild-type induction) in this mutant (Figure 4E), showing a severe impact of this amino-acid substitution on CtsR activity *in vivo*. The mutated CtsR protein could not be inactivated upon heat exposure and was still binding to the DNA operator sequences preventing induction of target genes. The very small residual rate of *clpE* transcription clearly depended on heat-activated McsB-P, which targets free CtsR for degradation (see below), because *clpE* transcription was not detectable in an *mcsB*/CtsR^{G64P} double mutant (Figure 4E).

The critical glycine residue 64 is responsible for thermosensing in several low GC, Gram-positive CtsR

To confirm that different low GC, Gram-positive CtsR species also sense temperature shifts by the winged HTH domain, we substituted the corresponding glycine with a proline residue in *L. lactis* (G65P) and *G. stearothermophilus* (G64P) CtsR as well and monitored their activity *in vitro*. Both mutated CtsR proteins exhibit nearly identical DNA-binding affinities at their control and heat stress conditions ($K_{d30^{\circ}\text{C}} = 1.83 \pm 0.18 \mu\text{M}$ and $K_{d42^{\circ}\text{C}} = 2.03 \pm 0.19 \mu\text{M}$ for *L. lactis* and $K_{d55^{\circ}\text{C}} = 1.42 \pm 0.12 \mu\text{M}$ and $K_{d70^{\circ}\text{C}} = 1.73 \pm 0.19 \mu\text{M}$ for *G. stearothermophilus* CtsR) (Figure 4F and G), which stands in sharp contrast to the results obtained with wild-type proteins (Figure 3A and B). These results suggest that

Figure 4 CtsR^{G64P} mutant protein is no longer susceptible to thermo-induced inactivation. Electrophoretic mobility shift assay analysis with *clpC* promoter fragment and increasing amounts of *B. subtilis* CtsR^{G64P} at 37°C (A) or 50°C (B). Quantification of CtsR binding at the *clpC* promoter fragment for wild-type and mutant CtsR at 37°C (C) or at different temperatures for *B. subtilis* CtsR^{G64P} (D). *In vivo* transcriptional analysis of *clpE* during heat stress using our expression system with wild-type CtsR, a G64P substitution and a CtsR^{G64P} point mutant in a $\Delta mcsB$ background (E). Quantification of CtsR binding with the *clpC* promoter fragment at different temperatures for *L. lactis* CtsR^{G65P} (F) or *B. stearothermophilus* CtsR^{G64P} (G).

probably all low GC, Gram-positive bacteria sense heat stress by the glycine-rich loop.

CtsR stability depends on kinase activity of McsB *in vivo*

As shown above, CtsR activity during heat stress is regulated independently of McsB by the intrinsic heat-sensor function of CtsR. Previous studies affirmed that McsB is necessary for effective CtsR degradation, both *in vivo* and *in vitro* (Kirstein *et al*, 2005, 2007). The wild-type like expression in an *mcsB* knock-out (Figure 1A) emphasizes that CtsR degradation is not critical for CtsR activity as had been suggested by previous studies (Derré *et al*, 2000; Krüger *et al*, 2001). Nevertheless, controlled proteolysis of non-functional CtsR during heat stress is an important feature of the specific heat-shock response, preventing excessive accumulation and/or aggregation of the regulator. The essential function of the McsB kinase for CtsR degradation during heat stress was verified in our *in vivo* expression system containing an McsB^{C167S} point mutation abrogating the kinase activity (Figure 1B). We found that McsB kinase is indispensable for effective degradation of CtsR during heat stress (Figure 5).

CtsR phosphorylation is not required for CtsR degradation *in vivo*

Demonstrating that McsB kinase and accordingly CtsR phosphorylation is not involved in CtsR inactivation during heat stress, we were interested whether the recently described phosphorylation of CtsR (Fuhrmann *et al*, 2009) is at least essential for CtsR degradation. Fuhrmann *et al*. reported that *G. stearothermophilus* CtsR was phosphorylated *in vitro* on at least one arginine residue within the highly conserved winged HTH region (amino acids 18–82). We substituted all five arginine residues to lysine for *B. subtilis* CtsR in this region (R28, R50, R55, R63, R70) and used the aforementioned *in vivo* expression system. However, these CtsR-mutated species were degraded at the same rate as wild-type CtsR during heat stress (Figure 5). These results imply that CtsR phosphorylation is not required for CtsR degradation *in vivo*. This is consistent with a previous finding that phosphorylation of *B. subtilis* CtsR is not sufficient for degradation *in vitro* (Kirstein *et al*, 2007).

McsB displays heat-specific modifications

Although the McsB kinase is essential for CtsR degradation upon heat exposure, but direct CtsR phosphorylation by McsB is not, McsB kinase must phosphorylate other sites to initiate CtsR targeting and degradation. It was recently described that auto-phosphorylation of McsB is a pre-requisite to target CtsR *in vitro* (Kirstein *et al*, 2007). We used 2D gel electrophoresis to analyse whether McsB exhibits heat-specific modifications. We found that McsB is only present with a main spot and an additional minor spot under control conditions (Figure 6A). In contrast, 10 min upon heat exposure McsB displayed elongated spot chains with two additional spots in the acidic area (Figure 6A). These two additional spots may represent auto-phosphorylated forms of McsB; 60 min after heat exposure, these two additional McsB spots disappeared, and McsB displayed the same pattern as under control conditions (Figure 6A).

To test whether McsB auto-phosphorylation is a pre-requisite for CtsR targeting, we analysed an McsB kinase-deficient mutant for its ability to bind CtsR during heat stress *in vivo*.

Figure 5 Stability of CtsR in different mutants. Pulse-chase labeling and immunoprecipitation of CtsR after heat stress in *B. subtilis* wild-type, McsB kinase-deficient mutant cells (McsB^{C167S}) or CtsR^{R63K}, CtsR^{R63K/R70K} and CtsR^{R28K/R50K/R55K/R63K/R70K} mutant cells.

A previous study revealed that McsB–CtsR interaction is strongly increased during heat stress (Kirstein *et al*, 2007). However, an McsB kinase-deficient point mutant cannot properly bind to CtsR during heat stress and McsB–CtsR interaction was nearly completely abolished (Supplementary Figure S9). This observation shows that McsB kinase activity is essential for efficient CtsR binding upon heat exposure.

These results suggest that only heat-activated McsB is able to bind CtsR. However, McsB is not involved in de-repression of CtsR; therefore, McsB most likely targets non-functional CtsR proteins dissociated already from the DNA. We also investigated the thermal stability of CtsR^{G64P} *in vivo*. Under control conditions stability of CtsR^{G64P} was comparable with wild-type protein (data not shown). But during heat stress, CtsR^{G64P} appeared more stable than wild-type CtsR (Figure 6B), reflecting that only free CtsR can be bound by activated McsB. However, CtsR^{G64P} was very slowly degraded (Figure 6C). This weak degradation also indicates that heat-induced McsB-P is able to bind and target free CtsR, which takes place over time for all CtsR molecules.

YwIe regulates McsB stability during heat stress

In the course of regulated proteolysis, adaptor proteins such as McsB are able to sense and integrate environmental signals, thus affecting the stability of their substrates (Kirstein *et al*, 2009). McsB kinase is activated during heat stress leading to regulated degradation of CtsR. However, during permanent heat stress, CtsR is also re-activated as a DNA-binding repressor leading again to repression 30 min after heat exposure (Krüger *et al*, 1994). We were interested of how the activated McsB adaptor is shutdown to prevent degradation of functional CtsR. Therefore, we turned our attention to regulation of McsB adaptor activity.

We focussed on the stability of McsB, which is very stable in *B. subtilis* wild type under control and heat stress conditions (Figure 6D). However, McsB was rapidly degraded upon heat exposure in a *ywIe* mutant (Figure 6D), a small phosphatase, which has recently been described as the cognate phosphatase for McsB kinase *in vitro* (Kirstein *et al*, 2005). Interestingly, McsB degradation during heat stress strictly depends on its kinase activity, because a kinase-deficient mutant protein (McsB^{C167S}) was stabilized in a *ywIe* mutant during heat stress (Figure 6D). These results show that McsB

Figure 6 McsB adaptor ability during heat stress. (A) Western-blot analysis of McsB after 2D separation. (B) Pulse-chase labelling and immunoprecipitation of CtsR^{G64P} after heat stress. (C) Quantification of CtsR^{G64P} *in vivo* stability after heat stress, WT and McsB^{C167S} mutant cells (Figure 2A). (D) Pulse-chase labelling and immunoprecipitation of McsB in wild-type, *ywlE* mutant or McsB^{C167S}/*ywlE* mutant cells either under control conditions or during heat stress. (E) Western-blot analysis of McsB in a *ywlE* or *ywlE/clpC* mutant strain after 2D separation.

is stable when de-phosphorylated by YwlE, but gets rapidly degraded when phosphorylated.

To confirm these findings, we analysed *ywlE* and *ywlE/clpC* mutants with regard to putative McsB modifications by 2D gel electrophoresis and western blot. In a *ywlE* knock-out, McsB displays wild-type-like pattern (Figure 6E), because phosphorylated McsB, which is not de-phosphorylated by YwlE, was rapidly degraded. However, in a *ywlE/clpC* double mutant, McsB-P should be stabilized. McsB exhibits a heat-shock-like pattern with two more spots in the acidic part of the gel even 60 min after heat stress (Figure 6E), indicating that McsB-P is neither de-phosphorylated nor degraded. These results strongly suggest that auto-phosphorylation and activity of McsB as an adaptor is counteracted by YwlE, showing for the first time that YwlE is the corresponding phosphatase of McsB also *in vivo*. When de-phosphorylation by YwlE failed, McsB-P is rapidly degraded ensuring an appropriate shutdown of McsB adaptor function.

Proteolysis of CtsR in Gram-positive bacteria lacking McsB

As mentioned above, McsB is absent in several groups of low GC, Gram-positive bacteria, all of which encode the *ctsR* gene. To test whether CtsR gets degraded in these bacteria, we investigated stability of CtsR exemplarily in *L. lactis*, a species that lacks McsB. CtsR was degraded immediately upon heat exposure (Supplementary Figure S10). Consequently, a CtsR degradation mechanism that is independent of McsB kinase must exist in these bacteria. As ClpEP was previously shown to be partially involved in degradation of CtsR in *B. subtilis* (Miethke *et al*, 2006), it is likely that ClpEP also does so in *L. lactis*. CtsR was stabilized during heat stress in a *L. lactis clpE* mutant (Supplementary Figure S10). In these low GC, Gram-positive bacteria, CtsR degradation is neither essential for CtsR activity as it

was shown previously that a *L. lactis clpE* mutant is fully inducible during heat stress (Varmanen *et al*, 2003).

Discussion

In this study, we showed that global transcriptional regulator CtsR acts as protein thermosensor in all low GC, Gram-positive bacteria. CtsR activity was shown to be strictly temperature dependent both *in vivo* and *in vitro*, whereas CtsR binds to DNA with higher affinity at lower temperatures. However, the temperature sensitivity of CtsR is adapted to the specific living conditions of different low GC, Gram-positive bacteria.

Moreover, we were able to determine the precise functional site for thermosensing within the CtsR molecule. Substitution of a flexible glycine at position 64 in *B. subtilis* CtsR or corresponding residues in other Gram-positive CtsR species with a more rigid proline residue decreases conformational entropy of the denatured state and consequently stabilizes the winged HTH region during heat stress. We conclude that the specific intrinsic heat-sensor ability of CtsR depends on a highly conserved tetraglycine loop within the winged HTH domain. This conserved region possesses high conformational entropy and, therefore, displays decreased thermostability, senses specific temperature shifts and regulates gene activity, whereas the more flexible regions of CtsR are responsible for adaptation to host-specific temperatures.

In contrast to previous studies, we showed that McsB kinase does not participate in initial inactivation of CtsR during heat stress. However, McsB kinase is absolutely required for effective CtsR targeting during heat stress. Auto-phosphorylation of McsB activates McsB as an adaptor protein for ClpC and targets non-functional CtsR. The adaptor

function of McsB is inhibited by YwlE, showing for the first time that YwlE is the cognate McsB phosphatase *in vivo*.

Our results stand in conflict with a recent publication (Fuhrmann *et al*, 2009), who used proteins from the thermophile *G. stearothermophilus* for *in vitro* experiments to establish a general CtsR model for Gram-positive bacteria. We showed by *in vivo* and *in vitro* experiments that *G. stearothermophilus* CtsR activity is regulated in a temperature-dependent manner, similar to other mesophilic Gram-positive bacteria and does not depend on an McsB-mediated phospho-switch, as was postulated by Fuhrmann *et al*. Finally, we cannot completely rule out that phosphorylation of CtsR occurs *in vivo*, but if it would occur, it should not result in any regulatory effect *in vivo*, as we have shown. We suggest that the discrepancies regarding the function of McsB between our results and former *in vitro* studies (Krüger *et al*, 2001; Kirstein *et al*, 2005; Fuhrmann *et al*, 2009) depend on the fact that McsB is responsible for CtsR inactivation during disulphide stress (Supplementary Figure S11). Consequently, McsB is able to inactivate CtsR *in vitro*, but is not involved in heat-shock inactivation of CtsR *in vivo*. We are currently investigating the specific mechanism by which CtsR becomes inactivated during disulphide stress.

In Gram-positive bacteria that lack the McsB kinase, our findings suggest that ClpEP instead of ClpCP has taken over the regulated degradation of CtsR during heat stress. Consistent with this observation is the previous discovery

that ClpE unlike ClpC is able to perform ATPase activity in the absence of an adaptor such as McsB (Kirstein *et al*, 2006; Miethke *et al*, 2006). Probably, ClpEP degrades CtsR, whereas ClpCP lacking the corresponding heat-shock adaptor McsB cannot do so. This conclusion is further supported by the observation that, in contrast to *B. subtilis*, a *L. lactis* *clpE* mutant exhibits a severely thermosensitive phenotype, whereas a *L. lactis* *clpC* mutant is largely unaffected during heat stress (Ingmer *et al*, 1999), which suggests a more prominent function of ClpE for heat adaptation in *L. lactis*.

We propose the following model for CtsR inactivation in low GC, Gram-positive bacteria (Figure 7). Under control conditions, CtsR is active as a repressor and binds to its cognate DNA operator sequences. Furthermore, McsB kinase is kept largely inactive by binding to ClpC (Kirstein *et al*, 2005). Upon heat exposure, CtsR loses ability of DNA binding because of conformational changes in the winged HTH domain, leading to an induced transcription of the target genes. Heat-activated McsB-P is only able to capture non-functional CtsR species, which is a first pre-requisite for efficient CtsR degradation. Temperature-dependent activation of McsB as an adaptor protein by McsA is a second, indispensable requirement for CtsR degradation. As a result, controlled CtsR degradation is regulated by a sophisticated two-step mechanism. Within 30 min of heat stress, CtsR is re-activated as DNA-binding repressor, inhibiting transcription of the target genes. To prevent inadequate degradation of

Figure 7 Model for heat-dependent regulation of CtsR activity. During control conditions, depicted by the green thermometer, CtsR is active as a DNA-binding protein and binds to its operator sequences within the promoter of the regulated genes. McsB binds to ClpC and is thereby repressed in its kinase activity. Upon heat exposure, depicted by the red thermometer, CtsR undergoes a temperature-induced alteration of the winged HTH region that leads to dissociation of CtsR from the DNA (1). Consequently the transcription of the regulated genes is induced. In addition, McsB is titrated away from ClpC and is activated in its adaptor function by auto-phosphorylation. McsB kinase is thereby activated by McsA. Heat-activated McsB~P targets free CtsR (2) for ClpCP-dependent degradation (3) preventing CtsR aggregation. After 30 min, CtsR is re-activated and represses transcription. Activated McsB is shutdown either by phosphorylation of YwlE or ClpCP-dependent degradation (4).

functional CtsR repressor, heat-activated McsB adaptor is down-regulated by either de-phosphorylation of McsB by YwlE or rapid degradation of phosphorylated McsB adaptor. The presented model improves our understanding of mechanisms for the rapid cellular adaptation of Gram-positive bacteria to life-threatening heat stress conditions.

Materials and methods

General methods

Strains and primers used in this study are listed in Supplementary Tables S1 and S2. *B. subtilis* was grown in liquid media or on LB-agar plates with tetracycline (17 µg/ml), spectinomycin (200 µg/ml), kanamycin (5 µg/ml) or chloramphenicol (5 µg/ml). *L. lactis* was grown with tetracycline (2 µg/ml). *Escherichia coli* DH5α (Invitrogen) was used for cloning experiments.

DNA manipulation and other molecular biological procedures were carried out according to standard protocols. Transformation of *B. subtilis* cells was performed by a two-step protocol (Hoch, 1991). Site-directed mutagenesis was conducted using a plasmid as template containing either the modified *clpC* operon or *ctsR* itself. PCR was performed using primers with the desired nucleotide substitution after the instructions of the manufacturer (Gene Tailor System, Invitrogen).

Strain construction

The *B. subtilis* strains containing *ywlE*, *mcsA/mcsB* and the modified *clpC* operon were constructed using a modified two-step fusion PCR protocol (Wach, 1996). For this purpose, a linear DNA fragment carrying a central resistance marker flanked by homologous sequences representing the chromosomal up- and downstream regions of the desired integration site was generated in a standard PCR using a proofreading DNA polymerase with chromosomal DNA of *B. subtilis* 168 and different resistance markers as a template, respectively. The resistance markers are composed only of the gene product together with a corresponding Shine-Dalgarno sequence, but without a promoter or terminator to exclude any impact of the inserted marker on the expression of the downstream genes. The PCR products were separated from template and primer DNA by electrophoresis in a 0.8% agarose gel. The fragments were cut out of the agarose gels in the absence of UV irradiation and purified with the Qiaquick Gel Extraction kit (Qiagen). The purified fragments were fused in a second PCR reaction through their complementary ends. The linear fusion product was also purified after electrophoresis from a 0.8% agarose gel and directly used for transformation or cloned into the pTOPO vector (Invitrogen) for respective nucleotide substitutions. Mutants that had inserted the fragment by a double-cross-over event were selected on LB-agar plates containing the corresponding amount of selective antibiotics. Chromosomal DNA from the mutants was used to amplify the mutated site, which was sequenced to verify the correct insertion or nucleotide substitution and to exclude undesired point mutations in the up- and downstream regions.

See Supplementary Table S1 for the full genotypes of strains and plasmids and Supplementary Table S2 for a list of primers used in this study.

Culture conditions

B. subtilis 168 and the corresponding mutants were inoculated from an exponentially growing overnight culture to an OD₅₀₀ of 0.08 and routinely grown in synthetic medium (Stülke *et al*, 1993) in 500 ml Erlenmeyer flasks in a shaking water bath at 180 r.p.m. and 37°C until mid-exponential phase (OD at 500 nm 0.5) and then shifted to a pre-warmed Erlenmeyer flask in a water bath with 50°C for heat stress experiments.

Protein purification

B. subtilis, *B. stearothermophilus* and *L. lactis* CtsR and CtsR^{G65P} were purified after over-expression from *E. coli* BL21(DE3)pLysS as follows. Overnight cultures were grown at 37°C in Luria-Bertani (LB) supplemented with 100 µg/ml ampicillin for plasmid maintenance, inoculated to an starting OD₅₅₀ of 0.08 in 1 l fresh SB medium and grown at 37°C until OD₅₅₀ of 0.5. IPTG was added to a final concentration of 1 mM to induce expression of the recombi-

nant His-tagged protein. The culture was grown with IPTG for 2 h at 37°C. Harvested cells were resuspended in ice-cold buffer W (100 mM Tris/HCl, 150 mM NaCl) and disrupted using a French Press. Phenylmethyl sulfonyl fluoride was added to a final concentration of 1 mM to prevent protein degradation. Unbroken cells and cell debris were removed by centrifugation at 20.000 × g for 30 min. The proteins were purified using an Ni-NTA Superflow cartridge H-PR according to standard procedures of the manufacturer (IBA GmbH, Germany).

Experiments on the stability of proteins in vivo

Experiments for *B. subtilis* were conducted as described previously (Gerth *et al*, 2008) with the following modifications. Cells were grown in minimal medium until an OD₅₀₀ of 0.5, transferred to an Erlenmeyer flask at 50°C if stated, immediately radioactively labelled with S³⁵ methionine for 5' and then chased with a more thousand-fold excess of L-methionine. For *L. lactis*, experiments were carried out according to Savijoki *et al* (2006) with the exception that cells were pulse-chase labelled after the shift to 42°C. For experiments with both bacterial species, a polyclonal *B. subtilis* CtsR antibody was used for immunoprecipitation.

RNA isolation and northern blots

Samples were taken from unstressed/non-stressed cultures immediately before the shift to 50°C and at different time points during heat exposure. For RNA extraction, cell pellets were resuspended with 0.5 volumes of ice-cold killing buffer (20 mM Tris-HCl, 5 mM MgCl₂, 20 mM NaN₃). All samples were immediately cooled with liquid nitrogen, spun down at 10 000 × g for 8 min at 4°C and stored in liquid nitrogen until further preparation. RNA isolation and northern blot analysis were performed as described previously (Reder *et al*, 2008). Each RNA blot was stained with methylene blue before hybridization in order to check RNA quality and ensure that equal amounts of RNA were loaded and blotted for each lane. Digoxigenin-labelled anti-sense RNA probes were used for *clpE* and *clpP* mRNAs (Gerth *et al*, 2004).

Electrophoretic mobility shift assays

Gel retardation analysis was carried out as described earlier (Kirstein *et al*, 2005) with the following modifications. The CtsR/DNA mix was incubated at specific temperatures for 30 min. For temperature shift, the mix was divided and one half remained at 37°C and the other was transferred for 5 min to 50°C. DNA band shifts were visualized by ethidium bromide staining of the native 6% polyacrylamide gel, which allowed differentiation of the different DNA species. DNA binding was quantified from three independent experiments by using ImageJ software.

2D-PAGE and western-blot analysis

2D-PAGE was performed as described previously (Büttner *et al*, 2001). In the first dimension, proteins were separated according to their pI. For that, 70 µg of cytoplasmic protein extract were loaded onto an IPG strip (18 cm, pH 4–7; GE-Healthcare). In the second dimension, proteins were separated according to their molecular mass. This was accomplished by a polyacrylamide gel (12%) electrophoresis. Western-blot analysis of McsB and CtsR was performed as described previously (Kirstein *et al*, 2005).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Authors contribution: AKWE, UG and MH created the experiments and wrote the paper. AKWE and UG analysed the results. AKWE

created all mutants except for BDZ12, which were created by DZ. AKWE performed all experimental work except for 2D analysis of McsB, which was performed under the guidance of DZ.

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Conflict of interest

The authors declare that they have no conflict of interest.