

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

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The soil bacterium *Bacillus subtilis* possesses a fine-tuned and complex heat stress response system. The repressor CtsR, whose activity is regulated by its modulators McsA and McsB, controls the expression of the cellular protein quality control genes *clpC*, *clpE* and *clpP*. Here, we show that the interaction of McsA and McsB with CtsR results in the formation of a ternary complex that not only prevents the binding of CtsR to its target DNA, but also results in a subsequent phosphorylation of McsB, McsA and CtsR. We further demonstrate that McsB is a tyrosine kinase that needs McsA to become activated. ClpC inhibits the kinase activity of McsB, indicating a direct role in initiating CtsR-controlled heat shock response. Interestingly, the kinase domain of McsB is homologous to guanidino phosphotransferase domains originating from eukaryotic arginine and creatine kinases. Mutational analysis of key residues of the guanidino kinase domain demonstrated that McsB utilizes this domain to catalyze the tyrosine phosphorylation. McsB represents therefore a new kind of tyrosine kinase, driven by a guanidino phosphotransferase domain. *The EMBO Journal* (2005) **24**, 3435–3445. doi:10.1038/sj.emboj.7600780; Published online 15 September 2005
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Introduction

The Gram-positive soil bacterium *Bacillus subtilis* can respond to various and multiple changes of its natural environment. The various cellular stress response systems of *B. subtilis* enabling this fast adaptation serve as a general model system for regulatory circuits, which include, for example, two-component systems, alternative sigma factors and regulated proteolysis. For one of these systems, the heat shock response of *B. subtilis*, it could be demonstrated that it is regulated by at least five different mechanisms. The major

regulatory proteins of four of these classes were identified and characterized. Class I genes are regulated primarily by the repressor HrcA, the alternative sigma factor σ^B controls the class II genes, the repressor CtsR controls the class III genes and the two-component system CssRS controls the class V genes (Darmon *et al.*, 2002; Schumann *et al.*, 2002).

The proteins we investigated in this study, McsB and McsA, encoded by class III heat shock genes are directly involved in the regulation of class III heat shock response. The major regulator CtsR is a dimeric repressor, which binds to a highly conserved heptanucleotide direct repeat, located upstream of *clpP*, *clpE* and the *clpC* operon (Derre *et al.*, 1999a, b; Krüger and Hecker, 1998). The regulation of the CtsR regulon is thought to be based on maintaining a basal steady-state level of CtsR at 37°C and that at elevated temperatures a rapid degradation of the repressor by ClpCP occurs (Krüger *et al.*, 2001). All the genes of the *clpC* operon, which consist of *ctsR*, *mcsA*, *mcsB* and *clpC*, are involved in the regulation of the activity of CtsR, because McsA and McsB act as modulators of CtsR (Krüger *et al.*, 2001). McsB, a putative kinase was shown to repress the DNA-binding ability of CtsR and it was further proposed that McsB could modify CtsR to target it for degradation by ClpCP. Such an McsB-dependent modification of CtsR was detected by an *in vivo* approach, suggesting a possible phosphorylation of the repressor. However, a kinase activity of McsB was not demonstrated yet. Interestingly, McsB contains a domain that is highly conserved among ATP:guanidino phosphotransferases (referred to as guanidino kinases) (Krüger *et al.*, 2001). This domain is used by the phosphagen kinase family and catalyzes the phosphorylation of guanidino molecules such as arginine or creatine, serving as ‘energy-storage’ in maintaining energy homeostasis by buffering cellular ATP concentrations of cells of higher eukaryotes, which have to utilize high amounts of ATP (Ellington, 2001).

In this study, the precise role of both modulators of CtsR was investigated. Therefore, we established an *in vitro* phosphorylation assay and could demonstrate a kinase activity of McsB. Surprisingly, the characterization of the phosphoamino acids and mutational analysis of McsA and McsB revealed phosphorylation on tyrosines. Tyrosine phosphorylation was considered to be restricted to eukaryotes until phosphotyrosine kinase (PTK) activity could be demonstrated in *Escherichia coli* (Manai and Cozzone, 1982). So far, bacterial PTKs were found to be involved in the regulation of the synthesis of exopolysaccharides in *B. subtilis* and other bacteria (Morona *et al.*, 2000; Mijakovic *et al.*, 2003) and possibly in the regulation of the heat shock response in *E. coli* (Klein *et al.*, 2003).

The tyrosine kinase activity of McsB required the activation by McsA and resulted in phosphorylation of both McsA and McsB. We could further show that CtsR is a bona fide phosphorylation substrate of McsB and that the kinase

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activity of McsB is antagonized by ClpC, indicating a direct role of this phosphorylation cascade in initiating CtsR-controlled heat shock response.

Results and discussion

McsB is a protein kinase that is stimulated by McsA and phosphorylates CtsR

The amino-acid (aa) sequence of McsB contains a highly conserved domain with similarity to guanidino kinases (kinase domain aa 119–253). Furthermore, the heat shock-induced and McsB-dependent occurrence of an additional acidic charged subspecies of CtsR, detected by two-dimensional gel analysis, suggested modification of CtsR by phosphorylation (Krüger *et al*, 2001).

In order to experimentally examine a possible kinase activity of McsB, we used the purified components to establish an *in vitro* phosphorylation assay, using radioactively labeled [γ - 32 P]ATP. The results of this assay depicted in Figure 1 demonstrate that McsB alone, unlike McsA or

CtsR, appeared to be phosphorylated at a very low level (Figure 1A, lane 2) when incubated with the labeled ATP, suggesting a low-level autophosphorylation activity of McsB. Addition of equal amounts of McsA stimulated this activity by several orders of magnitude and led to the concurrent phosphorylation of McsA (Figure 1A, lane 6). Analysis of the time course of this reaction revealed that a maximal level of phosphorylation for both proteins was achieved after 20 min and remained stable for at least an additional 60 min (Figure 1B, and data not shown). The addition of equimolar amounts of CtsR to this *in vitro* phosphorylation assay resulted in the immediate phosphorylation of CtsR, but only in the presence of both McsA and McsB (Figure 1A, lanes 7 and 3–5). We titrated the amount of CtsR and observed that the phosphorylation of CtsR became saturated at a ratio of two CtsR per McsA/McsB (data not shown). It was previously observed that a dimer of CtsR is the active species (Derre *et al*, 1999a, b, 2000; Krüger and Hecker, 1998).

We conclude from these results that McsA acts as a specific activator necessary for the full kinase activity of McsB, resulting in the phosphorylation of McsB, McsA and subsequently CtsR.

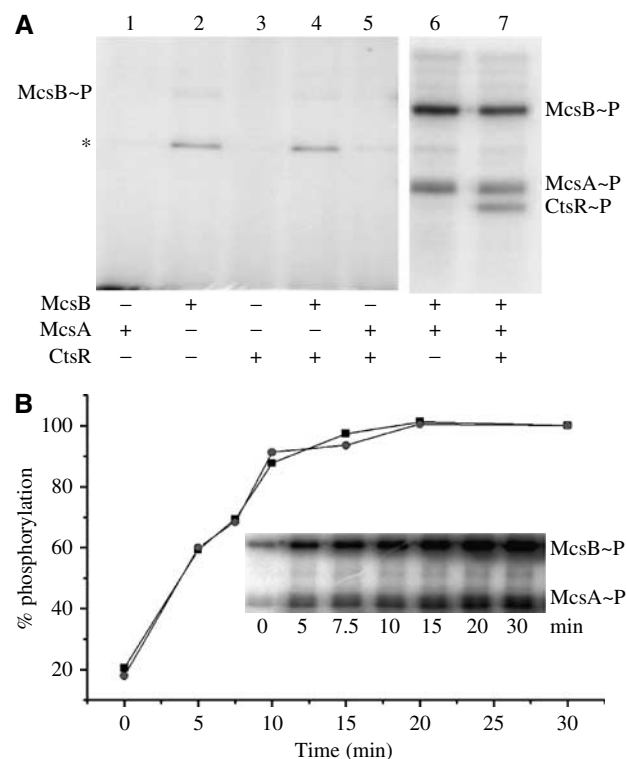


Figure 1 Characterization of the phosphorylation activity of McsB. (A) McsB exhibits a weak autophosphorylation activity, which is strongly stimulated by McsA, and phosphorylates McsA and CtsR. McsB, McsA and CtsR was incubated with [γ - 32 P]ATP for 20 min and subsequently analyzed by SDS-PAGE and autoradiography (as indicated below the autoradiogram). The position of McsB, McsA and CtsR is indicated on the right side. The star indicates a low abundant contaminant of the McsB purification from *E. coli*, which possesses an autophosphorylation activity independent of McsB. (B) Quantitative analysis of the time course of the phosphorylation of McsB and McsA. Filled squares represent the relative phosphorylation of McsB and filled circles McsA. The inset depicts the autoradiogram of the time course of the phosphorylation level of McsA and McsB. McsA (1 μ M) and McsB (1 μ M) were incubated with [γ - 32 P]ATP and samples were withdrawn at the indicated time points, analyzed and quantified with a phosphorimager.

Interaction of McsB with McsA and CtsR

To allow phosphorylation of McsA, McsB and CtsR, an interaction between McsA, McsB and CtsR must occur. To investigate this complex in more detail, we used two approaches. First, as an *in vivo* approach, co-immunoprecipitation experiments were performed with either McsA, McsB or CtsR antibodies immobilized on protein A-coated magnetic beads and lysates were prepared from wild-type (wt), Δ mcsA, Δ mcsB or Δ ctsR mutant cells, which were grown at 37°C or heat shocked at 50°C. Subsequently, a Western blot was performed to analyze whether McsA, McsB or CtsR was co-immunoprecipitated from the lysates.

The experiment shown in Figure 2A demonstrates that using McsA antibodies, capture of CtsR by McsA was possible only when wt lysate and not lysate prepared from Δ mcsB cells was used (Figure 2A). This indicated that the presence of McsB is necessary for an interaction of McsA with CtsR. In a pull-down experiment using McsB antibodies, CtsR and McsA co-precipitated together with McsB. CtsR could also be detected in the same experiment using a lysate prepared from Δ mcsA cells (Figure 2B), which demonstrated that the interaction of McsB with CtsR was independent of McsA. Using CtsR antibodies, McsB co-precipitated in a wt as well as in a Δ mcsA extract, whereas McsA co-precipitated only in lysates of wt but not of Δ mcsB cells (Figure 2C). In summary, these experiments suggested that McsA and CtsR were able to bind simultaneously to McsB and that the interaction of CtsR with McsA proceeded via McsB.

The ability of McsB to interact directly with either McsA or CtsR was confirmed *in vitro* using surface plasmon resonance (SPR) with a BIAcore instrument. As depicted in Figure 2D, both McsA and CtsR showed interaction with McsB. CtsR, whose binding to McsB was about five times stronger, also appeared to have a very low off-rate compared to McsA binding to McsB.

These protein interaction experiments demonstrated the ability of McsB to bind directly to CtsR and to McsA, and the co-immunoprecipitation (co-IP) experiments strongly

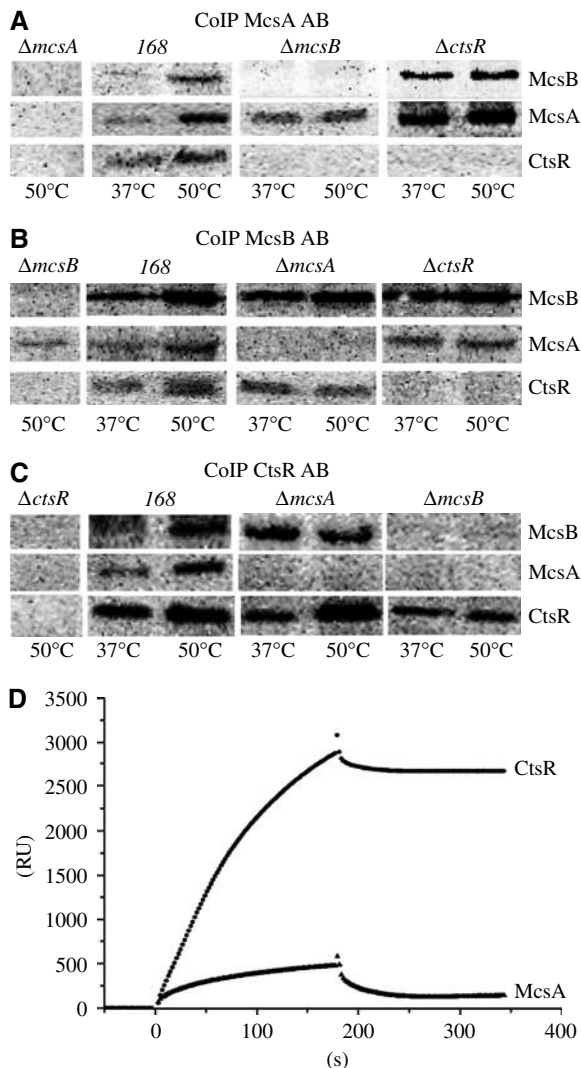


Figure 2 Analysis of the interaction between McsA, McsB and CtsR. (A–C) Pull-down experiments using protein A-coupled McsA (A), McsB (B) and CtsR antibodies (C), with lysates prepared from *B. subtilis* 168 (wt), $\Delta ctsR$, $\Delta mcsA$ or $\Delta mcsB$ strains (as indicated above). Co-precipitated proteins were analyzed by SDS-PAGE and subsequent Western blotting with the respective antisera (indicated on the right). (D) Interaction of McsB with McsA and CtsR by SPR. The binding response is measured in resonance units (RU). McsB was immobilized on a CM5 chip and McsA (0.8 μ M) and CtsR (0.8 μ M) (as indicated in the sensorgram) were passed over the chip surface as analytes.

suggested that McsB was located at the center of a ternary complex of McsA–McsB–CtsR. This implies that the observed induction of the McsB kinase activity by McsA, which resulted in the concurrent phosphorylation of CtsR (Figure 1A), takes place in this ternary complex.

Phosphorylation occurs on tyrosine residues

To gain insight into the nature of the phosphorylation events, catalyzed by McsB and McsA, we wanted to determine what kind of amino acid was phosphorylated. First, we examined the stability of the phosphorylation of McsA, McsB and CtsR at high temperature (95°C), acidic (HCl, 1 M) and basic (NaOH, 1 M) conditions. Our results demonstrated that the phosphorylation of McsA, McsB and CtsR was stable under

all these conditions (Figure 3A). The observed stability under acidic and heat conditions is consistent with hydroxyamino acid phosphorylation and since only phosphotyrosine residues resist high pH, these experiments suggested tyrosines as phosphorylation sites (Duclos *et al*, 1991).

To verify this assumption, we used two-dimensional thin-layer chromatography to analyze the phosphoamino acid (Mijakovic *et al*, 2003). The migration pattern of the radio-labeled hydrolysis products was compared with phosphoamino acid standards P~Ser, P~Thr and P~Tyr. As shown in Figure 3B, radioactive products of hydrolyzed McsA and McsB comigrated with the P~Tyr standard.

The chemical stability and the phosphoamino acid analysis by two-dimensional thin-layer chromatography demonstrated that McsB and McsA became phosphorylated at tyrosines.

YwIE, a tyrosine phosphatase, dephosphorylates McsA, McsB and CtsR

It was previously proposed by Kobayashi and colleagues that YwIE, a protein with homology to low molecular weight tyrosine phosphatases, carrying the conserved active site signature motif, CTGNTCRS/T (Zhang *et al*, 1995), could act as counterpart to McsB (Schumann *et al*, 2002). In addition, Mijakovic *et al* (2003) reported that YwIE could dephosphorylate the autophosphorylated tyrosine kinase, YwqD, and two proteins phosphorylated at tyrosines, [P-Tyr]-YwqF and [P-Tyr]-TuaD. We cloned, expressed and purified YwIE and tested its effect on the *in vitro* phosphorylation of McsA and McsB. Both proteins were preincubated for 20 min with [γ - 32 P]ATP to gain the maximal phosphorylation state (Figure 1B), YwIE was added and samples were withdrawn subsequently following the indicated time course (Figure 3C). Compared to the control reaction in the absence of YwIE, an immediate loss of the phosphorylation signals of McsA and McsB was observed (Figure 3C). The phosphatase activity of YwIE could also be demonstrated for CtsR (Figure 3D). Furthermore, the presence of YwIE did not alter the Ser56 phosphorylation of RsbV by RsbW, which served as a control and demonstrated the P~Tyr specificity of YwIE (Figure 3C). We also purified YwqE, which is the cognate tyrosine phosphatase of YwqD, YwqF and TuaE (Mijakovic *et al*, 2003), and YfkJ, the closest paralog (30% identity) of YwIE. Unlike YwIE, neither of these two phosphatases interfered with the phosphorylation state of McsA and McsB (data not shown).

The dephosphorylation of McsA, McsB and CtsR by the tyrosine phosphatase YwIE further supported our previous findings (Figure 3A and B), that McsB, its activator protein McsA and their substrate-protein CtsR are phosphorylated at tyrosine residues.

Mapping of the phosphorylation sites within McsB and McsA

The most conserved tyrosine residues of McsB, among those Gram-positive bacteria with a low GC content that encode for an McsB ortholog, are located within the kinase domain (aa 119–253; Y155, Y163 and Y210) (Krüger *et al*, 2001). Nevertheless, we substituted all eight tyrosine residues of McsB against its closest structural homolog, phenylalanine. Figure 4A depicts the result of the kinase assay of McsA with wt McsB as well as all tyrosine point mutants of McsB. No phosphorylation of either McsA or McsB in the assay could be

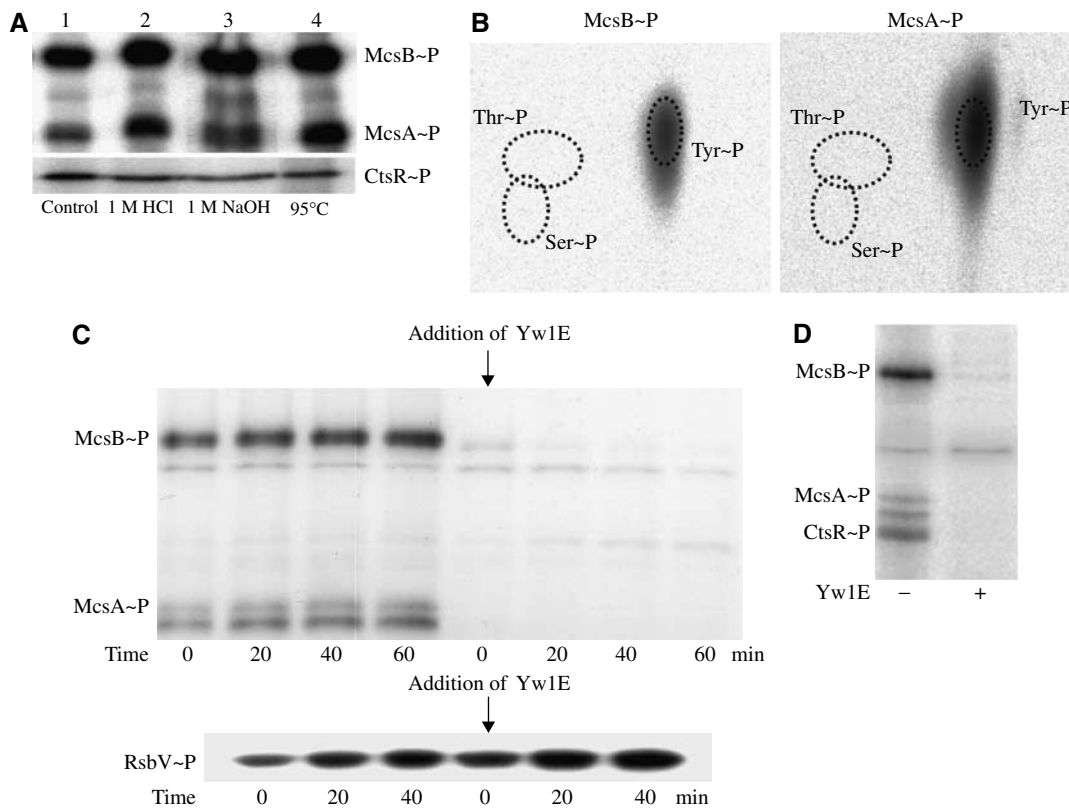


Figure 3 Analysis of the phosphoamino acid. (A) Autoradiogram of the chemical stability of McsA, McsB and CtsR. All three proteins (1 μ M) were incubated with [γ - 32 P]ATP for 20 min and subsequently treated with HCl, NaOH or boiled at 95°C (as indicated below) for an additional 10 min. The position of McsA, McsB and CtsR is depicted on the right. (B) Autoradiogram of the analysis of the phosphoamino acid by two-dimensional thin-layer chromatography of hydrolyzed McsA~P and McsB~P. An overlay with the position of the standard phosphoamino acids, P~Ser, P~Thr and P~Tyr (~20 μ g), which was analyzed by ninhydrin staining, is indicated by dotted circles. (C) Dephosphorylation of McsA~P and McsB~P by YwIE. McsA and McsB were preincubated with [γ - 32 P]ATP for 20 min. Then, 1 μ M YwIE was added at time point 0 (lanes 5–8) or not added (lanes 1–4) and the reaction was followed for another 60 min. Samples were withdrawn at the indicated time points and analyzed by SDS-PAGE and autoradiography. The relative position of McsB~P and McsA~P is depicted on the left. YwIE did not alter the serine phosphorylation of RsbV by RsbW. RsbW and RsbV were incubated with [γ - 32 P]ATP as described above and the phosphorylation was followed in the presence and absence of YwIE (lanes 9–14). (D) Dephosphorylation of CtsR by YwIE. McsA, McsB and CtsR (1 μ M) were incubated with [γ - 32 P]ATP for 20 min, followed by the addition of YwIE (as indicated below), and analyzed as described above. The relative position of McsB~P, McsA~P and CtsR~P is depicted on the left.

detected for McsBY155F and only a very weak signal could be detected for McsBY210F. This demonstrated that both tyrosine residues necessary for the kinase activity of McsB are possible targets for phosphorylation.

These results implicated that an intramolecular phosphate transfer, as recently discussed for the KaiC phosphorylation (Xu *et al*, 2004), could occur in McsB, although it cannot be excluded that the protein kinase gains its activity in a two-step mechanism as shown for the *E. coli* PTK Wzc (Grangeasse *et al*, 2002). To test such a two-step process, which requires an initial phosphorylation to activate the kinase, we created Y155E and Y210E point mutants of McsB to mimic phosphorylation by an acidic charge of glutamate (Gryz and Meakin, 2003). As shown in Figure 4C, no kinase activity could be detected for these mutants as well as for the YF substitutions of Y155 and Y210 (Figure 4A). Since no phosphorylation appeared even in the YE variants of McsB, we conclude that McsB does not employ an intrinsic two-step mechanism as described for Wzc (Grangeasse *et al*, 2002).

In gel filtration experiments, McsB appeared to run as a monomer (data not shown), but nevertheless we also

tested a 1:1 mixture of both single mutant proteins, YF as well as YE substitutions of Y155 and Y210, in the kinase assay. But no phosphorylation signal appeared, suggesting that a missing tyrosine could not be complemented in *trans* by a tyrosine in another McsB molecule (Figure 4B and C). These results support our first assumption of an intramolecular phosphate shuttle between the two tyrosines in McsB.

We were interested in whether the McsA phosphorylation is achieved by an intermolecular phosphate transfer from McsB~P and is thereby a part of the putative phosphate shuttle. We addressed this issue by incubation of McsA with purified [32 P]Tyr-McsB, where free [γ - 32 P]ATP was removed. As depicted in Figure 4D, no McsA phosphorylation signal could be detected in an autoradiogram, suggesting that the McsA phosphorylation did not result from a phosphate transfer from McsB~P but rather utilized the γ -phosphate of a new ATP molecule.

McsA is characterized by its two Zn-binding motifs in the N-terminus and a C-terminal *uvr* domain (amino-acid residues 139–174). Deletion of this C-terminal domain did not abolish the phosphorylation signal, demonstrating that

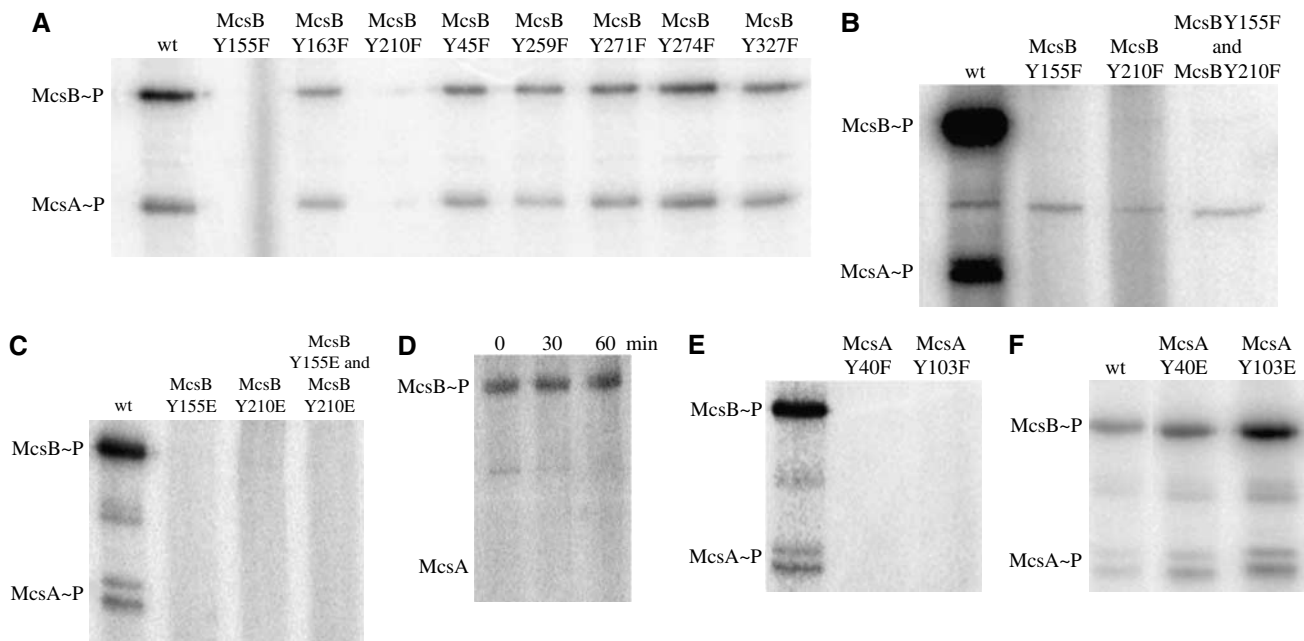


Figure 4 Mapping of the phosphorylation sites within McsB and McsA. (A) Autoradiogram of a phosphorylation assay of McsA with McsB and all YF point mutants of McsB (as indicated above). The position of McsB~P and McsA~P is depicted on the left. (B, C) The phosphorylation activity of McsB could not be complemented in *trans*. McsBY155F or McsBY210F (B) and McsBY155E or McsBY210E (C), respectively, either alone or together (as indicated above the autoradiograms), were incubated with McsA and [γ - 32 P]ATP for 20 min and analyzed by SDS-PAGE and autoradiography. The position of McsB~P and McsA~P is depicted on the left. (D) The McsA phosphorylation did not result from a P_i transfer from McsB~P. McsB (10 μ M) was incubated with [γ - 32 P]ATP for 30 min, purified from free [γ - 32 P]ATP and subsequently incubated with McsA (10 μ M). Samples were withdrawn immediately after the addition of McsA (0 min) and 30 min as well as 60 min later (as indicated above the autoradiogram) and analyzed by SDS-PAGE and autoradiography. The position of McsB~P and McsA~P is depicted on the left. (E) McsA is phosphorylated at the tyrosines Y40 and Y103. Autoradiogram of the phosphorylation assay of McsB with McsA and both YF point mutants of McsA (as indicated above) is depicted. The position of McsB~P and McsA~P is depicted on the left. (F) The acidic charge of the McsA phosphorylation could be mimicked by replacement of Y against E and thereby recovering the kinase activity of McsB. McsB was incubated with each of McsA, McsAY40E and McsAY103E (as indicated above) in the presence of [γ - 32 P]ATP for 20 min and analyzed by SDS-PAGE and autoradiography. The position of McsB~P and McsA~P is depicted on the left.

phosphorylation takes place in the N-terminus (data not shown). Indeed, using single phenylalanine substitutions of the N-terminal-localized two tyrosines of McsA (McsAY40F and McsAY103F), no phosphorylation of McsB or McsA was observed in the kinase assay (Figure 4E). This raised the question whether only the phosphorylated state of McsA stimulates the kinase activity of McsB. To test this hypothesis, we substituted the tyrosine residues of McsA against glutamate to mimic the acidic charge of phosphorylation. As depicted in Figure 4F, both single tyrosine to glutamate (McsAY40E, McsAY103E) substitutions, unlike the previously described tyrosine to phenylalanine substitutions (McsAY40F, McsAY103F), were able to activate McsB, resulting in the phosphorylation of McsB as well as the second, not altered, tyrosine residue of McsA. This suggested that both tyrosines of McsA are not only required for phosphorylation of McsA and the activation of McsB but were both themselves phosphorylated.

In summary, these experiments demonstrate that the tyrosines 155 and 210 of McsB and the tyrosines 40 and 103 of McsA could be phosphorylation targets and that they are essential for the tyrosine kinase activity of McsB and its activation by McsA. Moreover, our experiments indicate that the phosphorylation of McsB resulted from an intramolecular phosphate transfer between both phosphorylation sites, Y155 and Y210, whereas no intermolecular phosphate transfer from McsB~P to McsA was detected.

McsB-mediated release of CtsR from DNA is enhanced in the presence of McsA and ATP

Our results indicated that McsA could form a ternary complex with McsB and CtsR and concurrently activated the kinase activity of McsB. Therefore, we investigated the influence of McsA and McsB on the DNA-binding activity of CtsR in more detail. The repression of the class III heat shock genes is based on the binding of the dimeric CtsR to a heptanucleotide direct repeat, which is located in varying copies within the promoter regions of *clpC*, *clpE* and *clpP*. To monitor the DNA-binding ability of CtsR, we amplified the promoter region of *clpC*, which contains two CtsR-binding sites. As already shown, McsB antagonizes the CtsR–DNA interaction (Krüger *et al*, 2001). However, titration of both modulators allowed a more precise analysis. Increasing amounts of McsB prevented DNA binding by CtsR (Figure 5A). This McsB-dependent inhibition of CtsR binding was enhanced by McsA, in the presence of ATP, reducing the McsB level, which is required for its inhibiting effect, by half (Figure 5A). ATP depletion or replacement by the slow hydrolyzable analog, ATP γ S, weakens the influence of McsA (data not shown). This McsA activation appears to be coupled to the presence of ATP, indicating phosphorylation of McsB.

To verify the assumption that phosphorylated McsB exhibits a higher affinity toward CtsR, we tested the phosphorylation mimicking McsB mutant, McsBY155E, in the gel retardation assay. As depicted in Figure 5B, McsBY155E could

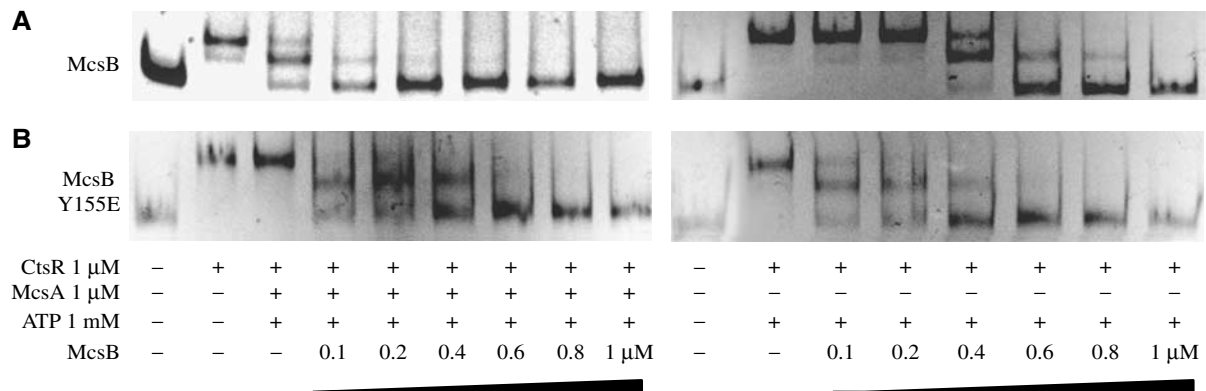


Figure 5 Modulation of the CtsR–DNA interaction by McsA and McsB measured by DNA gel retardation. **(A)** Influence of McsA, McsB and ATP on the DNA-binding ability of CtsR. CtsR (1 μM) was incubated with McsA (1 μM), 1 mM ATP and increasing concentrations of McsB (0–1 μM) (as indicated below the gel). DNA-binding analysis was initiated by addition of the promoter fragment. The DNA retardation was analyzed by ethidium bromide staining of the native gel. **(B)** Influence of the McsBY155E mutation on the CtsR–DNA interaction. McsBY155E, a variant of McsB, which carries an acidic charge at one phosphorylation site, was tested for its influence on the CtsR activity as described above.

diminish the CtsR–DNA interaction alone as efficient as wt McsB in the presence of McsA and ATP.

However, since McsB is able to inhibit DNA binding of CtsR on its own, phosphorylation of CtsR cannot be a prerequisite for its release. It seems more likely to assume a higher affinity of McsB for CtsR in a phosphorylated state, either alone or together with McsA.

In summary, these results demonstrate that phosphorylated McsB, activated by McsA, is a stronger inhibitor of the CtsR repressor than unphosphorylated McsB.

A structural model of the guanidino kinase domain of McsB

A structural model of the McsB kinase domain would be valuable to understand the role of the guanidino kinase domain and the tyrosines in this new tyrosine kinase. Two of the eight tyrosines of McsB are necessary for the kinase activity of McsB. They are conserved in all McsB homologs and located within the kinase domain, but they are not conserved in AK/CK (Figure 6A). This suggests that these tyrosines, which are possible phosphorylation targets, are not necessarily key residues of the catalytic mechanism of guanidino kinase domains. A number of creatine and arginine kinases bearing the guanidino kinase domain were crystallized and their structures solved (Fritz-Wolf *et al*, 1996; Zhou *et al*, 1998). Based on this, SwissModel (<http://swissmodel.expasy.org/SWISS-MODEL.html>) a fully automated protein structure homology modeling server, could successfully be used to obtain a structural model of the kinase domain of McsB (Figure 6A). The program based that model of the McsB kinase domain (aa 119–253) on the homologous domain of different arginine and creatine kinase structures deposited in PDB (1bg0, 1m15, 1p50, 1p52, 1qk1, 1rl9). Figure 6A shows an alignment of the McsB kinase domain with the guanidino kinase domain of the AK from *Limulus polyphemus* (PDB code 1bg0), whose previously determined crystal structure served also as model template (Zhou *et al*, 1998). The activity of AK is controlled by a conformational switch, upon binding of substrate and ATP ('induced fit'), and relies on several key residues and structural elements. These include (in the AK nomenclature) the essential C271, the highly conserved NEED segment (residues 223–227), the flexible loop (aa

309–320) with E314 and the arginines R229, R280 and R309. As depicted in the alignment (Figure 6A), all these key residues are conserved in McsB. Y210 of McsB is located within the flexible loop region (aa 309–320). For the glutamate (E314) of this flexible loop region, it was demonstrated that it interacts together with a cysteine (C271) and a glutamate (E225 of the NEED motif) with the substrate arginine upon binding of ATP. All three amino acids thereby stabilize and orient the substrate arginine in the transition state of the phosphate transfer reaction of AK. The second tyrosine of McsB, Y155, is located on the other side of the ATP-binding domain with C271 and the NEED loop in between (Figure 6A). The positive charges of the conserved arginines R229, R280 and R309 are thought to stabilize the bound nucleotide (Zhou *et al*, 1998; Yousef *et al*, 2002).

Mutation of essential residues within the guanidino kinase domain abolishes the kinase activity of McsB

We were interested in whether the catalytic mechanism of ATP binding and the phosphorylation reaction of McsB proceeds analogous to the mechanism described for AK/CK. Guided by the alignment and the structural model, we decided to substitute Cys167 (C271 in AK), which is highly conserved and thought to enhance the catalytic rate through electrostatic stabilization of the transition state, with a serine (Gattis *et al*, 2004). The kinase activity of this mutant McsB C167S was completely abolished (Figure 6B). In the gel retardation assay, McsB C167S could still inhibit the DNA binding of CtsR but McsA had no influence on the CtsR inhibiting activity of McsB C167S anymore (Figure 6D), indicating that McsB C167S is active and behaved like non-phosphorylated McsB.

Next, we also substituted the conserved E121 (E225 in AK) of the NEED motif and the adjacent E120 (E224 in AK) to alanine and created also a double mutated variant EE120/121AA (EE224/225AA in AK). All three McsB variants with glutamate to alanine replacements revealed no kinase activity (Figure 6C). To test the influence of the flexible loop region (aa 309–320), we decided to fix this loop by substitution of I209 against a proline, which resulted also in the inactivation of the kinase of McsBI209P (data not shown). The kinase-deficient McsB variants demonstrate that all the mutated

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E224/E225 R229                                     C271   R280
KARG 223 NEEDHLRIISMQKGGDLKTVYKRLVTAVDNIESKLPFSHDDRFGLTFCPTNLGTTMRAS 282
McsB 119 NEEDHIRIQCLFPFGQLLEAMKAANQVDDWIEEKVDYAFNEQRGYLTSCPTNVGTGLRAS 178
*****:* . : * : * . * . * * * . * : : : : : * : * * * : * : * * *
                                     R309 E314 Y155
KARG 283 VHIQLPKLAKDRKVLEDIAS--KFNLQVRGTRGEHTESEGGVYDISNKRRLGLTEYQAVR 340
McsB 179 VMMHLPALVLTRQINRIIPAINQLGLVVRGIYEGSEAVGNIFQISNQITLGKSEQDIVE 238
* : : * * . * : : . * : : : : * * * * : * : * : : * * * : * : * : * .
                                     Y210
KARG 341 EMQDGILEMIKMEKAAA 358
McsB 239 DLNSVAAQLIEQERS- 253
: : : . : : * : * : :
    
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Flexible loop region

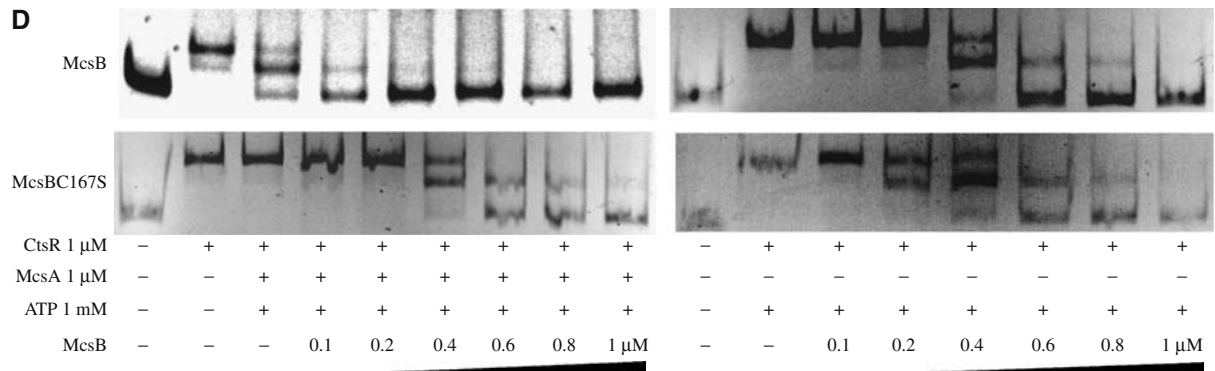
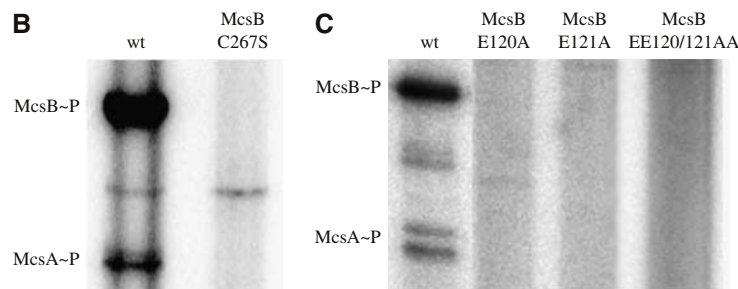
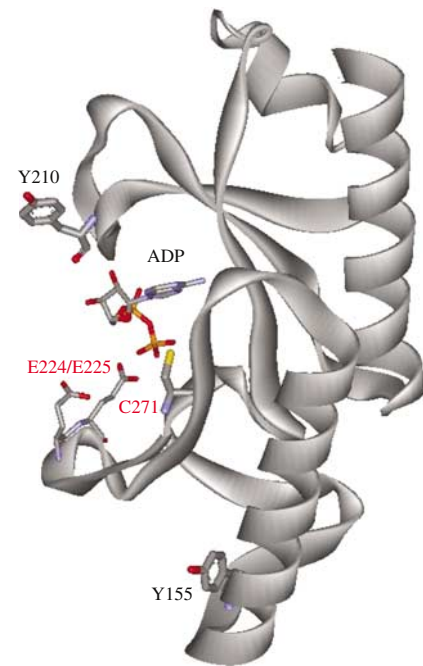


Figure 6 Structural model of the McsB kinase domain and mutational analysis of key catalytic residues of AK within McsB. (A) Structural model of the McsB kinase domain (aa 119–253) derived by SwissModel based on homologous guanidino kinase domains of several AKs/CKs (PDB code 1bg0, 1m15, 1p50, 1p52 1qk1 and 1r119) is depicted on the right. The positions and stick models of ADP, Y155, Y210, E224, E225 and C271 are shown. An alignment of the guanidino kinase domain of *L. polyphemus* (KARG) with the McsB kinase domain (McsB) is displayed on the left of the figure. The key residues of the catalytic AK kinase mechanism as well as the corresponding residues in McsB are indicated above and below the sequences (see text for discussion). (B, C) Autoradiogram of the phosphorylation assay of McsA with McsBC167S (B) and McsBE120A, McsBE121A and McsBEE120/121AA (C) (as indicated above the autoradiograms). The relative position of McsB~P and McsA~P is depicted on the left. (D) Influence of McsBC167S on the DNA-binding activity of CtsR. CtsR (1 μ M) was incubated with McsA (1 μ M), 1 mM ATP and increasing concentrations of McsBC167S (0–1 μ M) compared to McsB (as indicated on the left and below the gels) and analyzed as described above.

McsB residues, which are conserved key residues of guanidino kinases, are also essential for the kinase activity of McsB. This strongly suggests that McsB utilizes the phosphorylation mechanism of the phosphagen kinase family for its tyrosine kinase activity.

Arginine/creatine kinase domain as part of a tyrosine kinase

The kinase domain of McsB is homologous to a guanidino kinase domain originating from phosphagen kinases, which are found only in eukaryotes. These homologous enzymes

bind ATP and transfer the gamma phosphate of ATP to a specific amino group of their small molecule substrates arginine or creatine. This reversible formation of a high-energy bond is used in cells with fluctuating energy requirements, like muscle or brain cells, to buffer the amount of ATP (Ellington, 2001).

The kinase domains of the so far identified and characterized prokaryotic and eukaryotic tyrosine kinases are not related to one another nor to the guanidino kinase domain. The ATP-binding domains of prokaryotic tyrosine kinases are homologous to P-loop Walker type ATPase domains and thereby clearly differ from eukaryotic tyrosine kinases. This demonstrates that evolutionarily different ATP-binding and hydrolyzing domains can be part of tyrosine kinases. Our results indicate that the ATP-binding and gamma phosphate transfer activity of the guanidino kinase domain of arginine and creatine kinases must have been utilized by McsB/McsA to transfer the γ -phosphate onto tyrosines. This adds a new, third kind of ATP-binding domains to tyrosine kinase function.

McsB alone exhibits only a very low autokinase activity and the interaction with McsA is necessary to fully activate the McsB kinase activity. This activation could result from a conformational switch from the open to the closed state upon McsA and ATP binding, resulting in relocalization of either Y155 and/or Y210 of McsB into a position where they become a substrate for phosphorylation. During the activation of McsB, the activator McsA is concurrently phosphorylated. The tight regulation of eukaryotic tyrosine or related Ser/Thr kinases is often mediated by interacting activating proteins, which themselves become phosphorylated, to ensure a regulated signal transduction (Huse and Kuriyan, 2002).

Negative influence of ClpC on the kinase activity of McsB and McsA

One remarkable feature of the *clpC* operon is the translational coupling of *mcsA-mcsB* and *mcsB-clpC*, suggesting an interaction between McsA, McsB and ClpC directly after its synthesis. Therefore, we tested the influence of ClpC on the kinase activity of McsB (Figure 7A). Surprisingly, the addition of ClpC led to a weak phosphorylation of ClpC and using increased amounts of ClpC, it also diminished the kinase activity of McsB. A six-fold excess of ClpC, which is active as a hexamer, reduced the phosphorylation level of McsA and McsB to ~20%. Similar results were obtained when we tested the double Walker B mutant of ClpC, DWB-ClpC, which is able to bind to but not hydrolyze ATP and lacks therefore chaperone activity (Figure 7A). This excludes a chaperone-mediated decrease of the kinase activity of McsB, for example, by unfolding of McsB. In addition, an influence by the ClpC ATPase on the ATP level can be eliminated.

The inhibition of the McsB kinase activity by ClpC supports a titration model where this interaction would serve as sensor for heat shock or general stress. It has been previously demonstrated that ClpC localized upon heat shock *in vivo* to protein aggregates (Krüger *et al*, 2000) and it was demonstrated *in vitro* that ClpC in the presence of the adaptor protein MecA interacted and could even disaggregate and refold protein aggregates (Schlothauer *et al*, 2003). The accumulation of misfolded proteins, for example, upon heat stress could result in a competition of McsA/McsB and

misfolded proteins for interaction with ClpC. This would result in a relief of the inhibitory effect of ClpC on the McsB/McsA kinase activity, enabling the subsequent phosphorylation of McsA and McsB followed by the derepression of the CtsR regulon.

We tried to examine this model experimentally *in vitro* (Figure 7B) and used the phosphorylation intensity of McsB, induced by added McsA, as a read-out for interaction with ClpC. The intensity of McsB phosphorylation in the presence of McsA served as control and was set to 100% (lane 1). In the presence of a hexamer of ClpC or ClpC-DWB, the phosphorylation of McsB was inhibited (Figure 7B, lane 2, and Figure 7A). We performed order of addition experiments to test the influence of potential competitors on ClpC. We used both ClpC and ClpC-DWB, which gave similar results. To eliminate a possible interference of the ATPase activity of ClpC, we present the results performed with the ClpC-DWB variant.

First, we tested the ability of McsA to reactivate the kinase activity of McsB, which was preincubated with ClpC-DWB and 1 mM of ATP (Figure 7B, lane 3 versus 2). Then, we tested whether the addition of MecA as well as MecA and casein, a model substrate for misfolded protein, to the preincubated ClpC-DWB and McsB (lanes 4 and 5) influenced the inhibitory effect of ClpC. As an additional control, ClpC-DWB with MecA and casein was preincubated and McsB together with McsA was added afterwards (lane 6). After the various incubations, the kinase activity was monitored for all reactions by the final addition of [γ - 32 P]ATP.

The result of one representative experiment depicted in Figure 7B demonstrates that in the reaction where McsA, MecA and casein were added afterwards, more McsB became phosphorylated (lane 5, 51%) compared to the experiment where only McsA was added later (lane 3, 18%), but less compared to the sample where McsB and McsA were added to a preformed complex of ClpC-DWB with MecA and casein (lane 6, 61%). This suggested that the addition of unfolded protein with its cognate adaptor MecA could indeed result in a release of McsB from ClpC. In addition, the phosphorylation signal of McsB in lane 6 was significantly lower (61%) than that in the absence of any ClpC (lane 1, set to 100%), suggesting that ClpC preincubated with MecA/casein could still inhibit the subsequently added McsB and McsA. This indicated that under our experimental conditions, the ClpC populations interacting with McsB or MecA/casein were in an equilibrium, which could be shifted in both directions. These experiments demonstrated that an unfolded protein-mediated release of McsB and McsA, with a subsequent activation of the phosphorylation cascade, can be observed *in vitro*.

The heat shock-induced degradation of CtsR depends on the presence of McsA and McsB

We could demonstrate that McsA activated the kinase activity of McsB and that phosphorylated McsB had, compared to unphosphorylated McsB, a stronger CtsR DNA-binding inhibiting activity. It was also previously suggested that following heat shock, CtsR was inactivated by modification and even degraded (Krüger *et al*, 2001). We were interested to further validate this assumption and to examine the possible involvement of McsA and McsB by probing the stability of CtsR under normal growth and heat shock conditions. Therefore, we performed pulse-chase and Co-IP experiments with

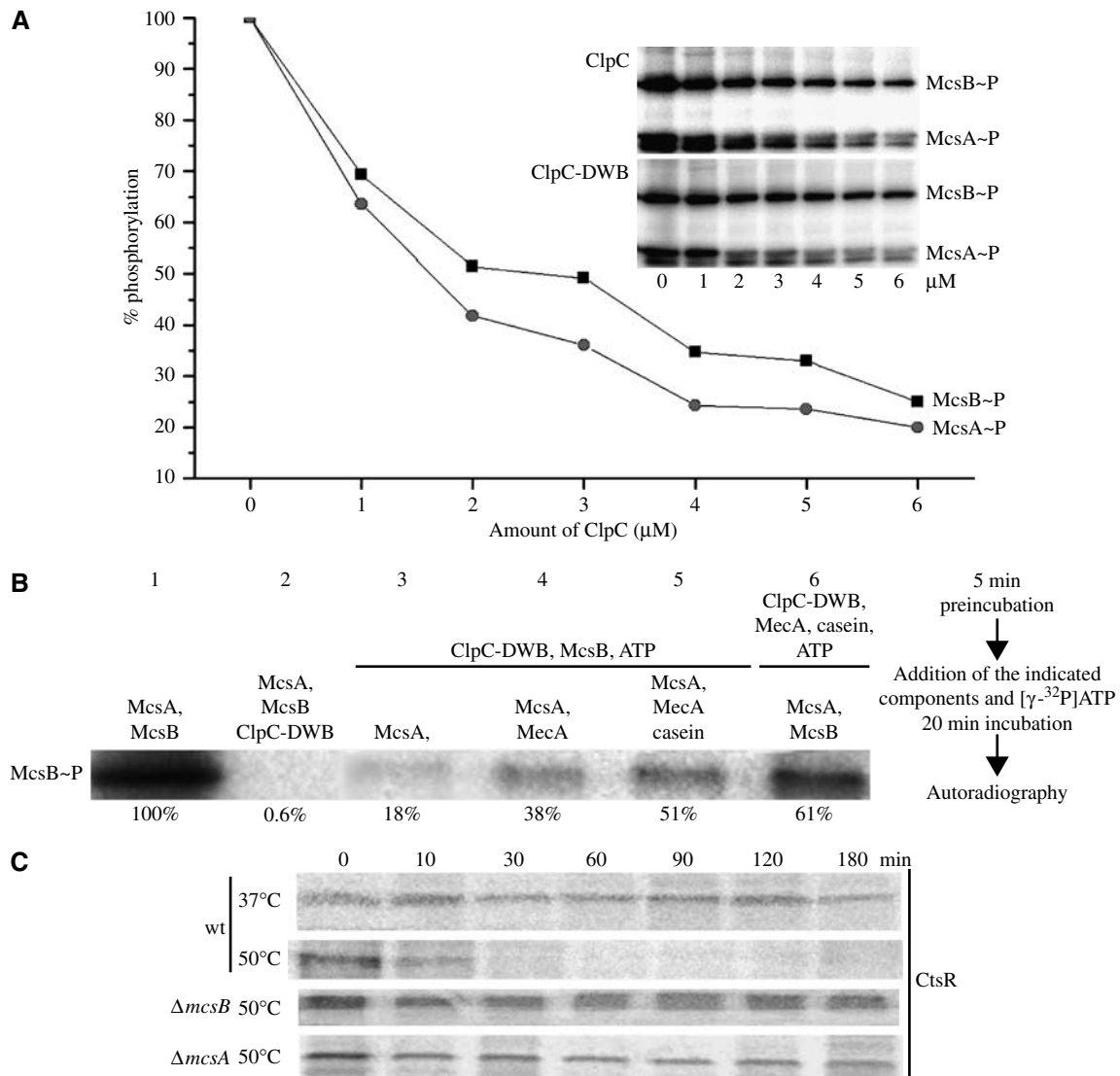


Figure 7 Regulation of the McsB kinase activity and its effect on the CtsR stability. **(A)** Influence of varying amounts of ClpC and ClpC-DWB on the McsB kinase activity. The relative phosphorylation of McsA (circles) and McsB (squares) in the presence of increasing amounts of ClpC is depicted in the graph. The inset shows the corresponding autoradiogram of the phosphorylation assay of McsA, McsB and ClpC or DWB-ClpC. McsA (1 μM), McsB (1 μM) and ClpC/DWB-ClpC (0–6 μM; as indicated in the panel below) were incubated with [γ - 32 P]ATP for 20 min and subsequently analyzed by SDS-PAGE and autoradiography. The positions of McsA and McsB are depicted on the right. **(B)** Negative influence of ClpC could partially be reversed in the presence of competing ClpC substrates. ClpC-DWB (6 μM), McsB (1 μM) (lanes 3–5), or ClpC-DWB (6 μM), MecsA (6 μM) and casein (1 μM) (lane 6) were preincubated with ATP (1 mM) for 5 min. Subsequently, McsB (1 μM) and McsA (1 μM) (lane 1) or McsB (1 μM), McsA (1 μM) and ClpC-DWB (6 μM) (lane 2) were incubated. At the same time, McsA, MecsA and casein as well as McsA with MecsA and casein (as indicated) were added to the preincubated ClpC-DWB and McsB (lanes 3–5) or McsA and McsB were added to the preincubated ClpC-DWB, MecsA and casein (lane 6) and the kinase assay was started by addition of [γ - 32 P]ATP followed by a 20 min incubation (as described in the scheme on the right). The effect of ClpC-DWB, MecsA and casein on the kinase activity of McsB was measured by the McsB~P level. The phosphorylation signals were quantified with a phosphorimager and are depicted as percentage relative to the control reaction of McsA and McsB (lane 1) below the lanes. **(C)** *In vivo* stability of CtsR by pulse-chase analysis. Cells of wt, Δ mcsA and Δ mcsB were grown at 37°C and upon an $OD_{500\text{nm}} = 0.5$, the cultures were divided and one half was exposed to 50°C and the other half was kept at 37°C. The cells were pulse-labeled simultaneously with [35 S]methionine for 10 min as described in Materials and methods. Samples were withdrawn at the indicated time points, and immunoprecipitated CtsR was detected by autoradiography.

Anti-CtsR antibody serum to determine the stability of CtsR. The experiment depicted in Figure 7C demonstrates that CtsR is degraded only upon heat shock and that this heat-induced degradation is abolished in *mcsA* and *mcsB* mutant strains. McsA, unlike McsB, is not directly interacting with CtsR (Figure 2). Therefore, McsA could influence the stability of CtsR only via McsB, most possibly via its McsB kinase activating ability. These results strongly suggest that the McsA-mediated phosphorylation of CtsR is necessary for

its degradation. In addition, the temperature dependence together with the important role of McsA argues for the involvement of the McsA/McsB kinase activity in the discussed titration model.

Conclusion

By investigating the function of McsA and McsB in regulating the activity of the repressor CtsR, we revealed a complex

interplay of CtsR, McsA, McsB and ClpC, encoded in this order by the *clpC* operon of *B. subtilis*. We could demonstrate that McsB is at the center of a network interacting with the three other proteins (Figures 1, 2 and 7). We identified and biochemically characterized McsB as a new kind of tyrosine kinase, which utilizes a guanidino kinase domain, known only from eukaryotic arginine and creatine kinases. We could demonstrate that McsB needs to be activated by McsA, which becomes concurrently phosphorylated by McsB. This activation of McsB resulted in a stronger inhibition of the DNA-binding activity of CtsR and in the immediate and concurrent phosphorylation of CtsR (Figures 3–6). ClpC on the other hand is inhibiting the kinase activity of McsB (Figure 7A). In addition, CtsR is degraded *in vivo* upon heat shock and this heat shock-induced degradation depends on the presence of McsA (Figure 7C).

This constellation suggested a new kind of enhanced titration model to regulate the activity of CtsR, which we tested *in vitro* (Figure 7B). The relief of the inhibition of the McsB kinase activity by competition of unfolded proteins for ClpC will cause a fast phosphorylation of CtsR by McsB and McsA, finally resulting in the degradation of CtsR (Figure 7C).

The induced phosphorylation cascade of McsB and McsA appears to act like an amplification module for the release of the chaperone by competition, for example, with unfolded proteins. This additional module might result in a more sensitive and responsive signal transduction system compared to a signal transduction system acting by a direct 'relief-of-interaction' titration model proposed for other heat shock response systems.

We demonstrated *in vitro* and *in vivo* that the protein phosphorylation by the tyrosine kinase McsB and its activator McsA is important for the inhibition and degradation of CtsR. We could also demonstrate *in vitro* that YwIE is a cognate tyrosine phosphatase that could antagonize the kinase activity of McsB. Further experiments are necessary to elucidate the role of this tyrosine phosphatase in this complex network. In addition, we are currently investigating the role of the tyrosine kinase activity in the mechanism by which CtsR is targeted for degradation after heat shock.

Materials and methods

General methods

DNA manipulations, protein separation and detection were carried out according to standard protocols (Sambrook and Russell, 2001).

For the preparation of the soluble cell extract of *B. subtilis*, 168, $\Delta mcsA$, $\Delta mcsB$ or $\Delta ctsR$ cells were incubated in LB media under vigorous agitation at 37°C. During exponential growth, cultures were divided and one half was exposed to 50°C for 10 min and the other half was kept at 37°C. Cells were harvested by centrifugation, resuspended in TE buffer and lysed with a FrenchPress. Cell debris was removed by centrifugation at 4°C and 10 000g. The supernatant, containing the soluble protein fraction, was used for the co-IP experiment. Protein concentrations were determined using the Bio-Rad protein assay (Bradford, 1976).

Phosphorylation assay

Each protein tested for phosphorylation was incubated at a concentration of 1 μ M in phosphorylation assay buffer (25 mM Tris-HCl pH 8, 300 mM NaCl, 5 mM MgCl₂ and 1 mM DTT) at 30°C in the presence of 10 μ Ci of [γ -³²P]ATP in a final volume of 15 μ l in the indicated combinations of McsA, McsB, CtsR and ClpC. If not stated otherwise, samples of 10 μ l were withdrawn after 20 min and mixed with 4 μ l of 4 \times SDS sample buffer and resolved by SDS-PAGE. Phosphorylation signals were detected by autoradiography

using the phosphorimager MD STORM 860 (Amersham Biosciences).

The chemical stability of phosphorylation was examined by adding the appropriate amount of HCl or NaOH (final concentration of 1 M) after 20 min to the phosphorylation assay. After a further incubation for 15 min, the samples were analyzed as described above. The heat stability was examined by an additional incubation at 95°C for 15 min after the 20 min incubation period of the phosphorylation assay and then analyzed as described above.

The phosphoamino acid analysis by two-dimensional thin-layer chromatography was carried out as described previously (Mijakovic *et al.*, 2003).

To test a P_i transfer reaction from McsB ~ P to McsA, 10 μ M McsB was incubated with [γ -³²P]ATP for 30 min followed by a buffer exchange to remove free [γ -³²P]ATP using Bio-Spin P-6 columns (Bio-Rad). Autophosphorylated McsB was further incubated with 10 μ M McsA and samples were withdrawn immediately after the addition of McsA (0 min) and 30 min as well as 60 min later and analyzed by SDS-PAGE and autoradiography.

To test the influence of unfolded proteins on the kinase inhibitory effect of ClpC, proteins were preincubated in various mixtures as indicated for 5 min in the presence of 1 mM unlabeled ATP followed by addition of further protein components and [γ -³²P]ATP to start the reaction. After 20 min, the kinase reaction was stopped by addition of 4 \times sample buffer and analyzed by SDS-PAGE and autoradiography. The reactions were carried out in a final volume of 20 μ l, and McsA, McsB and casein were used at a concentration of 1 μ M and MecA and ClpC-DWB at a concentration of 6 μ M.

Co-immunoprecipitation

Protein A-coated magnetic beads were incubated with 25 μ l of antisera (directed against McsA, McsB and CtsR) at 4°C for 30 min according to the manufacturer's (Dyna) instructions. This protein A-antibody complex was further incubated with the soluble cell extract, representing 500 μ g protein, from 168, $\Delta mcsA$, $\Delta mcsB$ and $\Delta ctsR$ cultivated at 37°C as well as heat-shocked cells. After the formation of the protein A-antibody-antigen complex for 1 h at 4°C, three wash steps using 0.1% Triton X-100 in PBS were performed to exclude nonspecific binding of abundant proteins. Finally, precipitated proteins were treated with SDS sample buffer at 95°C and then analyzed by SDS-PAGE and subsequent Western blotting.

Pulse-chase analysis

Pulse labeling and subsequent immunoprecipitation of CtsR in the wt, $\Delta mcsA$ and $\Delta mcsB$ background was carried out as described earlier (Pan *et al.*, 2001), with the following modifications. Cells were incubated in Belitsky minimal medium under vigorous agitation at 37°C. At OD_{500 nm} = 0.5, cells were exposed to 50°C or kept at 37°C and concurrently labeled by addition of 25 μ l/ml [³⁵S]methionine and chased after 10 min with 400 μ l of 0.3 M unlabeled methionine. Samples of 1.5 ml were withdrawn at the indicated time points. For immunoprecipitation, protein A-coated magnetic beads and 10 μ l of polyclonal CtsR antibodies were used.

BIAcore analysis

Using a BIAcore 3000 instrument, McsB was covalently attached to a CM5 chip surface according to the manufacturer's instructions (Biacore) yielding 3000 RU of immobilized McsB. McsA (0.8 μ M) or CtsR (0.8 μ M) in HBS buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) surfactant P20) was passed over the chip surface with immobilized McsB and a reference surface at a flow rate of 20 μ l/min. The subsequent injection of HBS buffer allowed monitoring of the dissociation.

Gel shift assays

Gel retardation analysis was carried out as described earlier (Krüger and Hecker, 1998) with the following modification. The DNA-binding analysis was initiated by addition of 0.5 μ g of the *clpC* promoter fragment (171 bp), which carries two CtsR-binding sites, to the preincubated protein mixture (each 1 μ M). The DNA retardation was then analyzed by ethidium bromide stain of the native gel.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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References

- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254
- Darmon E, Noone D, Masson A, Bron S, Kuipers OP, Devine KM, van Dijl JM (2002) A novel class of heat and secretion stress-responsive genes is controlled by the autoregulated CsrRS two-component system of *Bacillus subtilis*. *J Bacteriol* **184**: 5661–5671
- Derre I, Rapoport G, Devine K, Rose M, Msadek T (1999a) ClpE, a novel type of HSP100 ATPase, is part of the CtsR heat shock regulon of *Bacillus subtilis*. *Mol Microbiol* **32**: 581–593
- Derre I, Rapoport G, Msadek T (1999b) CtsR, a novel regulator of stress and heat shock response, controls clp and molecular chaperone gene expression in gram-positive bacteria. *Mol Microbiol* **31**: 117–131
- Derre I, Rapoport G, Msadek T (2000) The CtsR regulator of stress response is active as a dimer and specifically degraded *in vivo* at 37 degrees C. *Mol Microbiol* **38**: 335–347
- Duclos B, Marcandier S, Cozzzone AJ (1991) Chemical properties and separation of phosphoamino acids by thin-layer chromatography and/or electrophoresis. *Methods Enzymol* **201**: 10–21
- Ellington WR (2001) Evolution and physiological roles of phosphagen systems. *Annu Rev Physiol* **63**: 289–325
- Fritz-Wolf K, Schnyder T, Wallimann T, Kabsch W (1996) Structure of mitochondrial creatine kinase. *Nature* **381**: 341–345
- Gattis JL, Ruben E, Fenley MO, Ellington WR, Chapman MS (2004) The active site cysteine of arginine kinase: structural and functional analysis of partially active mutants. *Biochemistry* **43**: 8680–8689
- Grangeasse C, Doublet P, Cozzzone AJ (2002) Tyrosine phosphorylation of protein kinase Wzc from *Escherichia coli* K12 occurs through a two-step process. *J Biol Chem* **277**: 7127–7135
- Gryz EA, Meakin SO (2003) Acidic substitution of the activation loop tyrosines in TrkA supports nerve growth factor-dependent, but not nerve growth factor-independent, differentiation and cell cycle arrest in the human neuroblastoma cell line, SY5Y. *Oncogene* **22**: 8774–8785
- Huse M, Kuriyan J (2002) The conformational plasticity of protein kinases. *Cell* **109**: 275–282
- Klein G, Dartigalongue C, Raina S (2003) Phosphorylation-mediated regulation of heat shock response in *Escherichia coli*. *Mol Microbiol* **48**: 269–285
- Krüger E, Hecker M (1998) The first gene of the *Bacillus subtilis* clpC operon, ctsR, encodes a negative regulator of its own operon and other class III heat shock genes. *J Bacteriol* **180**: 6681–6688
- Krüger E, Witt E, Ohlmeier S, Hanschke R, Hecker M (2000) The clp proteases of *Bacillus subtilis* are directly involved in degradation of misfolded proteins. *J Bacteriol* **182**: 3259–3265
- Krüger E, Zühlke D, Witt E, Ludwig H, Hecker M (2001) Clp-mediated proteolysis in Gram-positive bacteria is autoregulated by the stability of a repressor. *EMBO J* **20**: 852–863
- Manai M, Cozzzone AJ (1982) Endogenous protein phosphorylation in *Escherichia coli* extracts. *Biochem Biophys Res Commun* **107**: 981–988
- Mijakovic I, Poncet S, Boel G, Maze A, Gillet S, Jamet E, Decottignies P, Grangeasse C, Doublet P, Le Marechal P, Deutscher J (2003) Transmembrane modulator-dependent bacterial tyrosine kinase activates UDP-glucose dehydrogenases. *EMBO J* **22**: 4709–4718
- Morona JK, Paton JC, Miller DC, Morona R (2000) Tyrosine phosphorylation of CpsD negatively regulates capsular polysaccharide biosynthesis in *Streptococcus pneumoniae*. *Mol Microbiol* **35**: 1431–1442
- Pan Q, Garsin DA, Losick R (2001) Self-reinforcing activation of a cell-specific transcription factor by proteolysis of an anti-sigma factor in *B. subtilis*. *Mol Cell* **8**: 873–883
- Sambrook J, Russell D (2001) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press
- Schlothauer T, Mogk A, Dougan DA, Bukau B, Turgay K (2003) MecA, an adaptor protein necessary for ClpC chaperone activity. *Proc Natl Acad Sci USA* **100**: 2306
- Schumann W, Hecker M, Msadek T (2002) Regulation and function of heat-inducible genes in *Bacillus subtilis*. In: Sonenshein AL, Hoch JA, Losick R (eds) *Bacillus subtilis and Its Closest Relatives: From Genes to Cells*. Washington, DC: ASM Press
- Xu Y, Mori T, Pattanayek R, Pattanayek S, Egli M, Johnson CH (2004) Identification of key phosphorylation sites in the circadian clock protein KaiC by crystallographic and mutagenetic analyses. *Proc Natl Acad Sci USA* **101**: 13933–13938
- Yousef MS, Fabiola F, Gattis JL, Somasundaram T, Chapman MS (2002) Refinement of the arginine kinase transition-state analogue complex at 1.2 Å resolution: mechanistic insights. *Acta Crystallogr D* **58**: 2009–2017
- Zhang ZY, Palfey BA, Wu L, Zhao Y (1995) Catalytic function of the conserved hydroxyl group in the protein tyrosine phosphatase signature motif. *Biochemistry* **34**: 16389–16396
- Zhou G, Somasundaram T, Blanc E, Parthasarathy G, Ellington WR, Chapman MS (1998) Transition state structure of arginine kinase: implications for catalysis of bimolecular reactions. *Proc Natl Acad Sci USA* **95**: 8449–8454