The Bacterial Stressosome: A Modular System that Has Been Adapted to Control Secondary Messenger Signaling

Maureen B. Quin, John M. Berrisford, Joseph A. Newman, Arnaud Baslé, Richard J. Lewis, and Jon Marles-Wright

1Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle upon Tyne, NE2 4HH, UK
2Present address: Gortner Laboratory of Biochemistry, University of Minnesota, Saint Paul, MN 55108, USA
3These authors contributed equally to this work

*Correspondence: rick.lewis@ncl.ac.uk (R.J.L.), jon.marles-wright@ncl.ac.uk (J.M.-W.)
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SUMMARY

The stressosome complex regulates downstream effectors in response to environmental signals. In *Bacillus subtilis*, it activates the alternative sigma factor $\sigma^B$, leading to the upregulation of the general stress regulon. Herein, we characterize a stressosome-regulated biochemical pathway in *Moorella thermoacetica*. We show that the presumed sensor, MtR, and the scaffold, MtS, form a pseudo-icosahedral structure like that observed in *B. subtilis*. The N-terminal domain of MtR is structurally homologous to *B. subtilis* RsbR, despite low sequence identity. The affinity of the switch kinase, MtT, for MtS decreases following MtS phosphorylation and not because of structural reorganization. Dephosphorylation of MtS by the PP2C type phosphatase MtX permits the switch kinase to rebind the stressosome to reset the response. We also show that MtT regulates cyclic di-GMP biosynthesis through inhibition of a GG(D/E)EF-type diguanylate cyclase, demonstrating that secondary messenger levels are regulated by the stressosome.

INTRODUCTION

The stressosome signaling system was first identified and characterized in the Gram-positive model bacterium *Bacillus subtilis* (Dufour et al., 1996; Chen et al., 2003). Here, the stressosome appears to act as both the sensor and the initiator of the response to environmental stresses, which is mediated by the alternative RNA polymerase sigma factor $\sigma^B$ (Haldenwang and Losick, 1979; Marles-Wright et al., 2008; Price et al., 2001). Gene clusters encoding stressosome homologs have been found in representatives of most bacterial phyla, from the pathogenic marine bacterium *Vibrio vulnificus*, which can cause fatal septicemia (Horsey and Surani, 2011) to the thermophilic acetogen *Moorella thermoacetica* (Pané-Farré et al., 2005). All species in which stressosome homologs have been identified possess a conserved genetic arrangement, consisting of an upstream module of genes within the operon that encodes proteins equivalent to the Regulator of $\sigma^B$ proteins (Rsb) of *B. subtilis*: RsbR, RsbS, RsbT, and RsbX. In addition, there is a downstream module comprising a series of genes with differing functions (Pané-Farré et al., 2005). In *B. subtilis*, RsbU, RsbV, RsbW, and $\sigma^B$ are inserted between the RsbT and RsbX genes. The RsbR homologs encode a variable N-terminal domain, which may act as a sensor, and a conserved STAS (Sulfate Transport and Anti-anti-Sigma factor) C-terminal domain (Akbär et al., 1997). The gene encoding RsbR is always followed by that for the single STAS domain protein RsbS, which multimerizes with RsbR to form a 1.5 MDa, pseudo-icosahedral complex termed the stressosome (Chen et al., 2003). Consistent with the role of the better conserved STAS domains in protein:protein interactions, it is the C-terminal STAS domain of RsbR that interacts with RsbS to form stressosomes in *B. subtilis* (Marles-Wright et al., 2008). The sequence variability of the N-terminal domains of RsbR is more consistent with a role in sensory perception than as a scaffold (Caffrey et al., 2004). Therefore, the stressosome complex provides a platform for diverse signaling modules to generate a response that is tunable to the magnitude of the signal (Marles-Wright et al., 2008). The subsequent gene in the module encodes a “switch” protein kinase, RsbT, the substrates of which are conserved serine (Ser59 in *B. subtilis*) and threonine (Thr171 and Thr205 in *B. subtilis*) residues in RsbS and RsbR, respectively (Gaidenko et al., 1999). RsbT is also part of the stressosome complex (Chen et al., 2003), and, in a mechanism that is so far not understood, its kinase activity is activated upon the perception of a stress signal. RsbT subsequently phosphorylates both RsbR and RsbS, whereupon RsbT dissociates from the stressosome complex (Chen et al., 2003; Yang et al., 1996). On dissociation from the stressosome, RsbT interacts with, and activates, RsbU, a type 2C protein phosphatase (Yang et al., 1996).

In *B. subtilis*, the N-terminal $\sim$120 amino acids of RsbU is a kinase recruitment domain, which mediates the interaction with RsbT that is critical to the $\sigma^B$ signal transduction cascade (Delumeau et al., 2004), whereas the C-terminal domain provides a catalytic, phosphatase function. The RsbU N-terminal domain is found linked to other effectors in different species. For instance, it is found as a domain within part of hybrid two-component histidine kinase (e.g., in *Paenibacilli*, *Mycobacteria*, and *Oxalobacteraceae*), as a domain in diguanylate cyclases...
from *Methylobacter tundripaludum* and with other conserved domains of unknown function (Pané-Farré et al., 2005).

In *Moorella thermoacetica*, the gene immediately downstream of the homologs of the RsbR-RsbS-RsbT module (orf moth_1475-1473) encodes an RsbX-like PP2C phosphatase (moth_1472), which is conserved in all stressosome loci and is responsible for the dephosphorylation of RsbR and RsbS. A diguanylate cyclase follows (moth_1471), comprising an N-terminal, RsbU-like kinase recruitment domain, a GAF domain and the GG(D/E)EF diguanylate cyclase catalytic domain. GAF domains are often found associated with cyclic di-GMP specific phosphodiesterases (Ho et al., 2000). Diguanylate cyclases have a conserved GG(D/E)EF signature motif near the active site (Hecht and Newton, 1995; Chan et al., 2004) and synthesize the secondary messenger cyclic di-GMP from GTP (Ross et al., 1987). Cyclic di-GMP is used by a wide range of bacteria in a variety of contexts, including various signal transduction processes. For instance, cellulose synthesis is affected by cyclic di-GMP in *Glucanacetobacter xylinus* (Ross et al., 1987), and biofilm formation and motility in *Pseudomonas aeruginosa* are regulated by cyclic di-GMP levels (Hickman et al., 2005). In both *Vibrio cholerae* (Tischler and Camilli, 2004) and *Salmonella enterica* serovar Typhimurium (Hisert et al., 2005), reduction of cyclic di-GMP concentration results in the induction of virulence genes. Cyclic di-GMP has also been shown to interact directly with riboswitch Cd1, where a decrease in cyclic di-GMP concentration increases the level of translation of the downstream flagella operon (Sudarsan et al., 2008). The opposing, degradative activity is performed by phosphodiesterases, which are characterized by conserved amino acid motifs, in this case either functional evidence for the roles of each protein in this widely adopted signaling system, which we show has been adapted by this bacterium to regulate the biosynthesis of a ubiquitous secondary messenger signaling molecule, cyclic di-GMP.

**RESULTS**

*M. thermoacetica* Stressosomes

The first gene in the *M. thermoacetica* stressosome operon, *moth_1475*, encodes a homolog to *B. subtilis* RsbR. However, as $\sigma^A$ is not present in this operon, we have named this protein Mr (Moorella thermoacetica RsbR-like protein) hereafter. The genome of *M. thermoacetica* encodes a single RsbR-like protein; this is in contrast to *B. subtilis*, which encodes four further homologs of RsbR. Mr and *B. subtilis* RsbR share 30% overall sequence identity (12% and 53% for the N- and C-terminal domains, respectively), whereas MtS and *B. subtilis* RsbS share 38% sequence identity. The crystal structure of the N-terminal domain of Mr (MtN-R) was determined to 2.0 Å resolution (Figures 1A and 1B; Table 1). Despite sharing just 12% sequence identity, the MtN-R structure is remarkably similar (root-mean-squared deviation of 2.5 Å over 108 aligned residues) to that of the previously determined equivalent domain of RsbR from *Bacillus subtilis* (PDB ID: 2BNL) (Murray et al., 2005; Figure 1C). The crystallographic MtN-R dimer interface is formed by the long C-terminal $\alpha$ helix, $\alpha_5$, which extends away from the central globin domain and consists of a number of hydrophobic residues (Phe116-Leu141) (Figure 1B). In MtN-R, the C-terminal $\alpha$ helix, $\alpha_5$, is ordered for four helical turns longer than that seen in *B. subtilis* N-RsbR (Figure 1C). When docked into the
**Table 1. Crystallographic Data and Refinement Statistics**

<table>
<thead>
<tr>
<th>Data Collection</th>
<th>N-MtR SeMet</th>
<th>MtS-P</th>
<th>MtS S58E</th>
<th>MtX Halide</th>
<th>MtX SeMet</th>
<th>MtX Native</th>
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<tbody>
<tr>
<td>Wavelength (Å)</td>
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<td>0.9790</td>
<td>1.5400</td>
<td>0.9795</td>
<td>0.9804</td>
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<td>P 2_2,2_1</td>
<td>P 2_2,2_1</td>
<td>P 2_2,2_1</td>
<td>P 2_2,2_1</td>
<td>P 2_2,2_1</td>
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<tr>
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<td>a = 50.7</td>
<td>a = 49.9</td>
<td>a = 42.1</td>
<td>a = 42.1</td>
<td>a = 42.1</td>
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<tr>
<td></td>
<td>b = 56.4</td>
<td>b = 61.2</td>
<td>b = 60.8</td>
<td>b = 47.7</td>
<td>b = 47.7</td>
<td>b = 47.8</td>
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<tr>
<td></td>
<td>c = 148.3</td>
<td>c = 89.5</td>
<td>c = 88.9</td>
<td>c = 87.8</td>
<td>c = 87.8</td>
<td>c = 87.2</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
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<td>61.0–2.87</td>
<td>49.94–1.90</td>
<td>43.76–2.50</td>
<td>47.80–3.00</td>
<td>37.88–1.75</td>
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<td>(2.97–2.70)</td>
<td>(3.50–2.87)</td>
<td>(1.97–1.90)</td>
<td>(2.64–2.50)</td>
<td>(3.16–3.00)</td>
<td>(1.79–1.75)</td>
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<td>78,574</td>
<td>126,207</td>
<td>18,671</td>
<td>121,619</td>
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<td></td>
<td>(55410)</td>
<td>(5,917)</td>
<td>(11,744)</td>
<td>(18,656)</td>
<td>(2,809)</td>
<td>(17,759)</td>
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<td>6.5 (6.2)</td>
<td>3.6 (3.7)</td>
<td>19.7 (20.5)</td>
<td>4.8 (5.1)</td>
<td>6.7 (6.9)</td>
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<td>Anomalous multiplicity</td>
<td>28.4 (29.9)</td>
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<td>—</td>
<td>10.9 (10.9)</td>
<td>2.7 (2.8)</td>
<td>—</td>
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<tr>
<td>Anomalous correlation</td>
<td>87.0 (40.3)</td>
<td>—</td>
<td>—</td>
<td>53.7 (12.4)</td>
<td>6.5 (–4.2)</td>
<td>—</td>
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<tr>
<td>Completeness (%)</td>
<td>100.0 (100.0)</td>
<td>99.7 (99.1)</td>
<td>98.2 (98.9)</td>
<td>99.8 (99.7)</td>
<td>99.8 (100.0)</td>
<td>99.7 (100.0)</td>
</tr>
<tr>
<td>Anomalous completeness (%)</td>
<td>100.0 (100.0)</td>
<td>—</td>
<td>—</td>
<td>100.0 (99.9)</td>
<td>98.6 (99.1)</td>
<td>—</td>
</tr>
<tr>
<td>Average I/sigma I</td>
<td>49.2 (16.8)</td>
<td>20.0 (7.2)</td>
<td>12.2 (2.8)</td>
<td>23.3 (8.9)</td>
<td>9.9 (4.7)</td>
<td>10.5 (3.1)</td>
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<td>Rmerge (%)</td>
<td>7.1 (36.6)</td>
<td>6.7 (24.5)</td>
<td>5.5 (37.0)</td>
<td>9.5 (37.0)</td>
<td>12.5 (32.2)</td>
<td>7.2 (34.1)</td>
</tr>
<tr>
<td>Rfree (%)</td>
<td>2.9 (5.4)</td>
<td>—</td>
<td>3.7 (9.5)</td>
<td>7.0 (2.7)</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

**Refinement**

| Resolution range (Å)     | 52.75–2.70  | 44.52–2.87 | 49.94–1.90 | —          | —          | 29.48–1.75 |
|                          | (2.97–2.70) | (3.03–2.87) | (2.0–1.90) | —          | —          | (1.84–1.75) |
| Reflections              | 12,435 (3,055) | 6,777 (959) | 21,867 (3,164) | —          | —          | 18,067 (2,519) |
| Rwork                    | 0.214 (0.282) | 0.211 (0.256) | 0.192 (0.294) | —          | —          | 0.152 (0.176) |
| Rfree                    | 0.275 (0.384) | 0.273 (0.33) | 0.240 (0.308) | —          | —          | 0.189 (0.191) |
| Number of protein atoms  | 1,113       | 1,763      | 1,824     | —          | —          | 1,516      |
| Number of solvent atoms  | 6           | 25         | 169       | —          | —          | 151        |
| Rmsd                     | —           | 0.016      | —         | —          | —          | —          |
| Bond lengths (Å)         | 1.512       | 0.004      | 0.008     | —          | —          | —          |
| Bond angles (°)          | 0.734       | 0.908      | 1.032     | —          | —          | —          |
| Ramachandran             | Favor (%)    | 97.8       | 92.9      | 97.7       | —          | 98.5       |
| Allowed (%)              | 2.2         | 6.1        | 2.3       | —          | —          | 1.5        |
| Wilson B A²              | 56.7        | 42.9       | 30.5      | —          | 19.3       | —          |
| Average B factor A³      | 68.8        | 48.1       | 38.6      | —          | 29.0       | —          |
| PDB id                   | 3ZTA        | 3ZTB       | 3ZXN      | —          | 3ZT9       | —          |

Values in parentheses are for highest resolution shell.

*B. subtilis* stressosome cryo-EM envelope, this pair of α helices in the MtN-R dimer extend perfectly into the neck region between the peripheral “turrets” and the core of the stressosome (Figure 1D), demonstrating that the crystallographic dimer is the biological form within the stressosome. Based on this docking, helix α5 can be seen to extend into the C-terminal STAS domain, helping to position both the sensory domains, and the switch kinase, above the core of the stressosome (Figure 1E, inset) Structural rearrangements of the stressosome core during the signal transduction process are thus likely to be mediated by this α helix (Marles-Wright et al., 2008). The α5 helix is also structurally equivalent to the “J” helix of the LOV domain of *B. subtilis* YtvA, a paralog of *B. subtilis* RsbR, which regulates the response to blue light (Avila-Pérez et al., 2006; Gaiderenko et al., 2006). In the case of YtvA, it has been suggested that the “J” helix plays an important role in signal transduction (Möglich and Moffat, 2007).

The *M. thermoacetica* RsS equivalent is encoded by *moth_1474* and is referred to herein as MtS (*M. thermoacetica* RsbS-like protein). Coexpression of MtS and full-length MtR leads to the formation of stressosome-like complexes that appear to be morphologically indistinguishable to those of *B. subtilis* (Chen et al., 2003; Marles-Wright et al., 2008 and Figure 1E, inset) when visualized by TEM of negatively stained samples (Figure 1E). *M. thermoacetica* stressosomes display a more marked tendency to aggregate than their *B. subtilis*
counterparts (Chen et al., 2003), limiting their use for high-resolution single particle analysis of frozen, hydrated samples. Nonetheless, only *B. subtilis* stressosomes have been visualized before, and our electron micrographs (Figure 1E) are, to our knowledge, the first demonstration that stressosomes exist in organisms other than *B. subtilis* (Chen et al., 2003; Marles-Wright et al., 2008).

**MtT Is a Kinase toward MtS**

Since *M. thermoacetica* stressosomes proved especially prone to aggregation, and we were unable to produce soluble MtR in isolation, the role of the *M. thermoacetica* RsbT-like kinase (MtT) (encoded by *moth_1473*) was investigated using MtS as the substrate for phosphorylation. Based upon sequence alignments with other characterized STAS domains, the phosphorylation site of MtS was expected to be Ser58. Native gel electrophoresis of MtS following incubation with MtT in the presence of ATP showed that MtS was phosphorylated by MtT, with a distinct band shift observed between MtS and MtS-P as a consequence of the increased negative charge of the phosphorylated protein (Figure 2A). The phosphorylation of MtS was confirmed by mass spectroscopy, which identified a major peak in the MtS-P sample with a mass difference of 80 Da in comparison to the native protein, corresponding to

**Figure 2. Phosphorylation of MtS by MtT**

(A) Nondenaturing PAGE of MtS and MtS-P generated by incubation with MtT and ATP reveals a distinct band shift due to the increased negative charge of MtS-P.

(B) Cartoon of the overall fold of MtS-P with the phosphorylated Serine 58 shown as stick representation, color ramped as in Figure 1.

(C) Experimental 2mFo-DFc electron density for the phosphate accepting region of MtS, map is shown at 1 σ in gray and protein as sticks. The position of residue R62 from the second monomer in the asymmetric unit is depicted with gray carbon atoms.

(D) Interaction of immobilized MtS with various concentrations of MtT as measured by SPR. The duplicate color lines represent the measured response units and the black lines the calculated fit using 1:1 Langmuir binding model, producing calculated constants of $k_a = 2.63 \pm 0.05 \times 10^4 \text{M}^{-1} \text{s}^{-1}$, $k_d = 2.75 \pm 0.03 \times 10^{-2} \text{s}^{-1}$, $K_D = 1 \times 10^{-9} \text{M}$ and $\chi^2 = 3.27$. 
the addition of a single phosphate group. In order to determine the identity of the phosphorylated residue, MtS-P was subjected to LC-MS-MS following trypsin digestion, which revealed that the phosphorylation event occurred between residues 44 and 63 of MtS. This peptide, with the sequence KGKLVIDISALEVVDVFTRV, contains the predicted phosphorylation site, Ser58 (underlined), and single additional serine and threonine residues.

**Structure of MtS-P**

As the phosphopeptide identified by LC-MS-MS contained one threonine and two serine residues, the exact identity of the phosphorylated amino acid was determined by solving the crystal structure of MtS-P to 2.9 Å resolution (Figures 2B and 2C; Table 1), confirming that Ser58 is the recipient of the phosphoryl group from MtT (Figures 2B and 2C). The final model contains residues 6–117 in two chains and has been refined to R_work and R_free values of 0.210 and 0.273, respectively. The overall fold of the MtS-P STAS domain is essentially identical to that of the unphosphorylated MtS (Quin et al., 2008) (PDB ID: 2VY9). The two structures superimpose with an overall root-mean-squared Cα deviation of 0.4 Å with few differences in side-chain geometry. Two α peaks in the electron density adjacent to Leu98-Arg99 in one chain and the side chain of Asn106 in an adjacent chain, and between Leu103-Ala104-Leu105 from two symmetry related chains have been modeled as iodine ions due to the inclusion of sodium iodide in the crystallization conditions of MtS-P.

The electron density maps also revealed that both Ser58 residues in the crystallographic asymmetric unit could be modeled as phosphoserines (Figure 2C) with unit occupancy and B factors comparable to the adjacent amino acids. Ser58 is situated at the N terminus of helix α3, and its side chain points toward the solvent. In the nonphosphorylated form, Ser58 is not involved in any interactions with other residues, leaving it in an accessible position for enzyme-catalyzed phosphorylation and dephosphorylation reactions. In the context of the stressosome, Ser58 is situated on the external face of the complex, in a position accessible to both kinases and phosphatases (Marles-Wright et al., 2008).

To examine the implications of the phosphorylation of MtS, we investigated the interaction between MtT and MtS by surface plasmon resonance (SPR). The K_D of this interaction was determined to be 1.0 μM with a simple 1:1 Langmuir binding model (Figure 2D). It is not known how this interaction may be affected by the absence of MtR, or by the formation of the stressosome, in which MtS would ordinarily be placed. The absence of other interacting components from the stressosome suggests that the measured K_D is a gross underestimate of the interactions between MtT and the stressosome complex. However, this result contrasts with *B. subtilis* in which RsbS and RsbT do not interact in the absence of RsbR (Chen et al., 2003). An MtS S58E mutant was prepared to act as a stable mimic of phosphorylated MtS to analyze the effect of phosphorylation on the interaction between MtS and MtT. SPR analysis of the interaction between the MtS S58E mutant and MtT showed no significant binding, demonstrating that phosphorylation of MtS Ser58 is likely to cause a significant decrease in the affinity of MtT for MtS. To ensure that the MtS S58E mutant had no effect on the structure of MtS, the crystal structure was determined to 1.8 Å resolution, and revealed no significant changes in the overall architecture of the protein (see Supplemental Information available online). We note that MtR has threonine residues (Thr166, Thr200) equivalent to *B. subtilis* RsbR Thr171 and Thr205 in its STAS domain, and conclude that MtR is likely to be subjected to the same phosphorylation by MtT as RsbR is by RsbT. Our data show that rather than structural rearrangements being responsible for the release of MtT from the stressosome, the decrease in affinity of MtT for MtS on phosphorylation leads to the release of MtT to interact with its downstream partners.

**Resetting the Switch: MtX Dephosphorylates MtS-P**

In order to reset this system to the resting state, with MtT bound to the stressosome, the phosphorylated residues on the stressosome must be dephosphorylated. In *B. subtilis*, RsbX catalyzes the dephosphorylation of phospho-amino acids in RsbS-P and in RsbR-P (Kang et al., 1996; Chen et al., 2004; Eymann et al., 2011). The type 2C protein phosphatase RsbX is a strongly conserved part of the stressosome operon and, by analogy to the *B. subtilis* stressosome locus, *M. thermoacetica* RsbX-like protein phosphatase (MtX) (*moth_1472*) is the most likely candidate for this role. Consequently, the activity of MtX as a phosphatase toward MtS-P was tested in the presence of various divalent cations, since the RPM family of phosphatases is metal dependent. Initial experiments using the synthetic substrate pNPP revealed that MtX exhibits divalent cation-dependent phosphatase activity against pNPP (Table 2), with ~200-fold greater activity in the presence of Mn2+ than Mg2+. Phosphatase activity was undetectable in the absence of metal ion, or in the presence of Ca2+, Ni2+, Zn2+, or Fe2+ (Figure 3A). We subsequently assessed the rate of phosphatase activity of MtX toward MtS-P, and found this to also be metal dependent (Figure 3B). Complete dephosphorylation of MtS-P by MtX required the presence of Mn2+, whereas the presence of Mg2+ impaired the rate and final level of dephosphorylation. Little or no dephosphorylation was observed in the presence of Ca2+ or EDTA (Figure 3B). The different level of dephosphorylation of MtS-P by MtX seen in the presence of different cations is a reflection of the chemical requirements of the phosphorolysis reaction. The ability of MtX to dephosphorylate MtS-P in the presence of Mn2+ was confirmed by mass spectrometry and LC-MS-MS, which revealed a mass difference of 80 Da between phosphorylated MtS-P and dephosphorylated MtS. Ions corresponding to the phosphorylated peptide KGKLVIDISALEVVDVFTRV were not

### Table 2. Kinetic Parameters for MtX with pNPP as a Substrate

<table>
<thead>
<tr>
<th>Substrate/Metal Ion</th>
<th>k_cat (s⁻¹)</th>
<th>K_m (mM)</th>
<th>k_cat/K_m (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNPP/Mn²⁺</td>
<td>0.17 ± 0.01</td>
<td>2.56 ± 0.02</td>
<td>65 ± 4.95</td>
</tr>
<tr>
<td>pNPP/Mg²⁺</td>
<td>2.00 × 10⁻³ ± 8.00 × 10⁻⁴</td>
<td>52.63 ± 13.68</td>
<td>0.33 × 10⁻³ ± 7.00 × 10⁻⁴</td>
</tr>
</tbody>
</table>

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observed following dephosphorylation of MtS-P by MtX, indicating that MtX hydrolyses the phosphoryl group on Ser58 of MtS-P.

**Crystal Structure of MtX**

To investigate the molecular mechanism by which the stressosome response is reset, we determined the crystal structure of MtX by single wavelength anomalous dispersion, and the model was refined against data to 1.75 Å resolution (Table 1). The final model comprises residues 2–193 in a single chain (Figure 3C), with crystallographic R_work and R_free values of 0.152, and 0.189, respectively.

The structure of MtX exhibits a PP2C fold (Das et al., 1996), with a central antiparallel β sandwich of nine strands. Each face of the sandwich is flanked by pairs of antiparallel α helices (Figure 3C). Structural homologs of MtX were identified using the DALI web server (Holm and Park, 2000; Table 3). Despite relatively low sequence identity, the PP2C family displays global structural similarity. The defining member of the family, PP2Cα from Homo sapiens (PDB ID: 1A6Q) has an N-terminal, catalytic domain of 290 residues that is a little larger than that of MtX, with six α helices and 11 β strands (Das et al., 1996). This protein superimposes onto MtX with a root mean squared Cα deviation of 2.1 Å over 166 residues. There is a cluster of acidic residues in human PP2Cα, Asp38, Asp60, Asp239, and Asp282, which define the active site. The equivalent residues in MtX are Asp21, Asp38, Asp150, and Asp185. This cluster of acidic residues is highly conserved in prokaryotic and eukaryotic PPM phosphatases (Table 3) and is required for catalysis. Two peaks were observed at levels of 5σ in an anomalous difference map (Figure 3D) calculated from diffraction data collected at a wavelength of 1.54 Å. The two peaks correspond to a pair of octahedrally coordinated metal ions, which were modeled as manganese due to the presence of MnCl2 in the crystallization buffer. The metal ions and the acidic cluster define the active site of MtX, which is described below (see also Supplemental Information for further discussion of the MtX structure).

**MtX Catalytic Site**

The PPM family of phosphatases, of which PP2Cα is a subfamily member, is characterized by the requirement for divalent metal ions for activity. As with other PPM structures, the catalytic
The water molecule that is shared by both Mn\textsuperscript{2+} ions in the same reaction scheme is likely to be adopted by MtX. There-

**Table 3. Structural Homologs of MtX**

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Organism</th>
<th>PDB Accession Code</th>
<th>z Score</th>
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<th>Sequence Identity (%)</th>
<th>Water Coordinating</th>
<th>Metal Coordinating</th>
<th>Water Deprotonating</th>
<th>Substrate Binding</th>
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<tr>
<td>MtX</td>
<td>Moorella thermoacetica</td>
<td>3ZT9</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Asp21</td>
<td>Asp38, Gly39, Asp150</td>
<td>Asp185</td>
<td>Arg13</td>
</tr>
<tr>
<td>Tppha</td>
<td>Thermosynechococcus elongatus</td>
<td>2JB8</td>
<td>21.9</td>
<td>2.5</td>
<td>19</td>
<td>Asp18</td>
<td>Asp34, Gly35, Asp193</td>
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<td>Arg13</td>
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<td>2.6</td>
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Homologs of MtX were identified in the PDB using the DALI program. The resulting z score, rmsd, sequence identities, and the role of the conserved residues, as determined from the crystal structures of a number of PP2C type protein phosphatases following the superimposition of these structures upon MtX are listed below.

**Center of MtX** is located at the apex of the central β sandwich, in an aspartate rich pocket. These conserved residues are involved in the coordination of two metal ions and are also in contact with metal-bound water molecules. The two Mn\textsuperscript{2+} ions in MtX are in close proximity to each other, within 3.8 Å, and are stabilized by metal–oxygen bond distances in the range of 2.1–2.3 Å. The first Mn\textsuperscript{2+} ion is directly coordinated by three oxygen atoms: the carboxylate side chain of Asp38, and the backbone carbonyl oxygens of Gly39 and Gly41. Three water molecules complete the coordination shell (Figure 3D). The second Mn\textsuperscript{2+} ion is coordinated by contacts with the carboxylate side chains of three aspartic acid residues: Asp38, Asp150, and Asp185 and three water molecules (Figure 3D). Asp21 forms hydrogen bonds to two of the metal ion coordinating water molecules. A negatively charged depression on the protein surface around the active site, which project from the surface of the STAS domain.

Both metal ions in the active site of MtX share the carboxylate side chain of Asp38 and one of the water molecules for stabilization (Figure 3D). A metal ion catalyzed dephosphorylation mechanism has already been proposed for PP2C\textalpha (Bellinzoni et al., 2007), whereby a metal ion bridging water molecule acts as the protonating species toward the leaving group. Alternatively, a new water molecule may associate with the departing serine (or threonine) side chain. The structure of SpoIIE from *B. subtilis* reveals an unusual metal coordination with only one manganese ion bound within the active site (Levdikov et al., 2012). The structure of *B. subtilis* SpoIIE superimposes onto MtX with a root-mean-squared Ca deviation of 2.8 Å over 112 residues. Notably SpoIIE lacks the metal ion corresponding to the first manganese in the MtX structure; this may be due to the shortening of the β strand equivalent to β3 in MtX and disorder of the subsequent loop which coordinates the metal ion (residues 39–44 in MtX); however, the SpoIIE structure is a domain-swapped dimer, the formation of which may affect the binding of this metal ion. This suggests that despite a high level of structural conservation in the core of PP2C, the exact reaction mechanism employed by different members of the family will be distinct due to significant differences in the coordination of the catalytically essential metal ions. Indeed, the shortening of this loop in SpoIIE explains why only two metal ions are bound by MtX, whereas in other PP2C structures (Pullen et al., 2004; Bellinzoni et al., 2007; Rantanen et al., 2007; Wehenkel et al., 2007; Schlicker et al., 2008) this loop is longer and three metal ions appear to be required for substrate recognition and/or the dephosphorylation reaction.

**MtG is a Diguanylate Cyclase**

The final gene (*moth_1471*) in the *M. thermoacetica* stressosome operon, which we have termed MtG (*M. thermoacetica* GGEEF), encodes a predicted diguanylate cyclase. MtG comprises an N-terminal RsbU-like kinase recruitment domain, a GAF type small-molecule binding domain, and a domain with a canonical GGEEF sequence motif. Anion exchange chromatography was used to separate the substrates and reaction products produced by MtG after incubation with GTP. It has previously been demonstrated that clear separation of GMP, GDP, GTP, and cyclic di-GMP can be achieved by this procedure using the *Escherichia*
coli diguanylate cyclase YdeH (Zähringer et al., 2011). Following incubation of MtG and GTP, we observed a peak in the anion exchange chromatogram equivalent to the product of the YdeH reaction (Figure 4A). MALDI-TOF mass spectroscopy confirmed this peak to have a mass of 689.08 Da (Figure 4B), which corresponds to cyclic di-GMP (formula mass of 690.09 Da), thus confirming that MtG is indeed a diguanylate cyclase.

An additional peak was observed on the chromatogram that eluted later than cyclic-di-GMP. This species was analyzed by MALDI-TOF mass spectroscopy, revealing a species of mass 867 Da (Figure 4C), which is consistent with that expected for pppGpG (868 Da) and comparative 1D NMR spectra of this compound and cyclic di-GMP was consistent with this assignment. pppGpG is a reaction intermediate which is formed when only one of the two condensation reactions have occurred that are required to convert two molecules of GTP into one molecule of cyclic di-GMP (Ross et al., 1987). Furthermore, the reaction catalyzed by MtG does not proceed to completion. Instead, the reaction rate of MtG decreased with increased substrate concentration, and thus MtG, like most other characterized diguanylate cyclases (Chan et al., 2004; De et al., 2009), is product inhibited. Analysis of this inhibition suggested that it is mixed, represented by a decrease in both $V_{\text{max}}$ and $K_m$ with increasing concentration of cyclic di-GMP, resulting in $K_i$ values of 4 µM for competitive $K_i$ and 0.7 µM for noncompetitive $K_i$ (Figure 4D; Table 4). MtGAF-GGEEF was also product inhibited, with comparable $K_i$ values ($K_{\text{ic}}$ of 1 µM and a $K_{\text{inc}}$ of 0.5 µM), confirming that the absence of the kinase recruitment domain has little impact on the enzymology of MtG. The competitive and noncompetitive inhibition is consistent with MtG having an I site separated spatially from the active site of the enzyme. The presence of a separate I site has been observed in other diguanylate cyclases including PleD from Caulobacter crescentus (Wassmann et al., 2007), and WspR from Pseudomonas aerugi-
nosa (De et al., 2008) and from Pseudomonas syringae (De et al., 2009), the structures of which have been determined with cyclic
di-GMP bound in the I site. Sequence alignment of the GG(D/E) EF domains of C. crescentus PleD and WspR from P. aeruginosa and P. syringae, with MtG reveals sequence identity in this domain of around 40% (38%, 44%, and 42%, respectively) with both the I site and active site conserved between these proteins, suggesting MtG has an I site similar to both PleD and WspR (see Figure S1, related to Figure 4). Analysis of the genome of M. thermoacetica suggests that there are five other putative diacyltransferase present, three of which contain a conserved I site (Figure S1). However, the diacyltransferase activity of these proteins has not been confirmed experimentally.

### DISCUSSION

Herein, we have presented the characterization of the entire M. thermoacetica stressosome signaling system. The data revealed that the proteins of the MrRSST module in M. thermoacetica behave in a similar fashion to those of the RsBRST stressosome of B. subtilis (Chen et al., 2003; Kuo et al., 2004), forming a large macromolecular complex similar to that observed with B. subtilis RsBRST. The M. thermoacetica stressosomes, at least to the level afforded by TEM of negatively stained samples (Figure 1E), have the same construction as those from B. subtilis (Chen et al., 2003; Marles-Wright et al., 2008; Figure 1E, inset). The similarity in architecture of the stressosomes is underlined by the close structural homology of the N-terminal domains of RsBR (Murray et al., 2005) and MrR, which share little meaningful sequence identity (12%), yet both display the same nonhemeglobin fold (root-mean-squared Cα deviation of 2.5 Å over 108 aligned residues) and both form equivalent dimers (Figure 1C). Consequently, the crystal structure of the MrN-R dimer fits as well to the molecular envelope of the B. subtilis stressosome as the equivalent dimer from the cognate RsBR (Marles-Wright et al., 2008; Figure 1D). The structure of the MrN-R domain does not give further insight into the nature of the activating signal for this cascade, just as the molecular mechanism by which the B. subtilis stressosome responds to stress remains unknown.

The nonhemeglobin fold exhibited by N-RsBR and MrN-R has also been observed recently in structural studies of two Bacillus anthracis sporulation inhibitory proteins, pXO1-118 and pXO2-61 (Stranzl et al., 2001), which titrate environmental molecules (fatty acids) that can encourage sporulation. The observed dimer interface is equivalent in all four structures of nonhemeglobin domains, and dimerization appears linked to undecanoic acid binding by the sporulation inhibitory proteins (Stranzl et al., 2011). The same dimer interface is utilized in the globin-coupled aerotaxis sensor HemAT to drive the expected piston-like signal transduction mechanism (Zhang and Phillips, 2003). Hence, the nonhemeglobin domain is an emerging and highly specific molecular sensor, the dimerization of which is likely to have a physiological role in a variety of signaling contexts.
As in the *B. subtilis* system, MtT acts as a kinase toward the STAS domain protein MtS, phosphorylating the conserved Ser58 (Figures 2A–2C). The affinity of MtT for MtS is dramatically decreased following phosphorylation of MtS by MtT, implying phosphorylation of MtS leads to the release of MtT from the MtRS stressosome complex. Our structural analysis has shown that this occurs by a mechanism based on decreased affinity for the phosphorylated form of the protein, rather than structural rearrangements caused by the phosphorylation of MtS. To reset the switch in this signaling pathway, MtS-P is dephosphorylated by MtX, allowing MtT to re-bind *M. thermoacetica* stressosomes. The presence of an MtX homolog in all stressosome operons indicates that there is an absolute necessity for a specific phosphatase to reset this pathway. *M. thermoacetica* encodes two other PP2C type proteins, Moth_0103 and Moth_0392, the former is related to the SpoIIE protein of *B. subtilis*, the structure of the catalytic domain of which has been recently determined (Levdikov et al., 2012). Moth_0103 and Moth_0392 share only modest sequence identity (~20% in the PP2C domain) with MtX and they are thus unlikely to dephosphorylate the stressosome; structural comparisons may reveal the molecular basis of this strong substrate specificity.

The control mechanism that underpins the signal transduction processes that the stressosome regulates is called partner switching. The stressosome in *B. subtilis* regulated one of two partner switching schemes that coordinate the activity of the alternative sigma factor, σ^B^, which governs the response of this bacterium to environmental stress (Boylan et al., 1993). The transition between binding partners occurs as a consequence of the phosphorylation status of the STAS domain in the system. When the STAS domain is not phosphorylated, it binds the switch kinase (Alper et al., 1994). When the STAS domain becomes phosphorylated, the switch kinase disassociates and binds the alternative partner, be that a sigma factor, a phosphatase (Yang et al., 1996), or a diguanylate cyclase as reported here. The implication of this switching behavior is that the affinity of the kinase for the nonphosphorylated STAS domain must be higher than the alternative partner, and this balance is reversed when the STAS domain is phosphorylated. The equilibrium dissociation constants measured here reveal that MtT interacts more strongly with MtG than with MtS. The slow rate of dissociation of MtT from MtG may indicate that the signaling outcome is long lasting, unlike the transient *B. subtilis* σ^B^ response. However, the MtS:mtT K_D is likely to be a significant underestimation as it was measured in isolation, not in the context of the stressosome where MtT will also likely interact with MtR. Analysis of recombinant, core *B. subtilis* stressosomes comprising the STAS domains of RsbR and RsbS, but lacking the N-terminal domains of RsbR shows that they do not sequester RsbT (Marles-Wright et al., 2008), indicating that in the case of *B. subtilis* contact is made between the N-terminal domain of RsbR and the kinase (Murray et al., 2005). Structural similarity between the *B. subtilis* stressosome proteins and those presented here suggest that this may also be the case for *M. thermoacetica*. Furthermore, we have been unable to obtain an estimate of the affinity of MtS-P for MtT using the SSBE phosphorylation mimic of MtS, presumably because the introduction of negative charge at position 58 position reduces the affinity of MtT for MtS by several orders of magnitude, beyond the range measurable by SPR.

The partner switching pathway of *M. thermoacetica* described here is not involved in the regulation of transcription, but instead regulates the biosynthesis of the ubiquitous secondary messenger, cyclic di-GMP (Schirmer and Jenal, 2009) by exerting an inhibitory effect on the GG(D/E)EF-type diguanylate cyclase, MtG. This interaction between MtT and MtG may be stronger in vivo, or could be mediated by some as yet undetermined mechanism involving other factors not present in our analysis. GG(D/E)EF diguanylate cyclases are usually multidomain proteins that are characterized by a great variation in domain architecture beyond the conserved catalytic domain. Five other such diguanylate cyclases exist in *M. thermoacetica* (ORFs moth_0223, moth_0689, moth_2151, moth_2149, and moth_0521, although moth_0521 lacks key aspects of sequence conservation in the GG(D/E)EF domain) these display significant sequence identity to MtG in the catalytic domain only, which include the active site and regulatory I site. Sequence identity in the GAF and kinase recruitment domains is, however, not maintained (see Figure S1A related to Figure 4), providing an opportunity for unique regulatory mechanisms for each diguanylate cyclase. Indeed, we have shown that MtT inhibits MtG specifically through interaction with the N-terminal kinase recruitment domain in MtG. The need for multiple control systems for cyclic di-GMP synthesizing enzymes is necessary because bacteria tend to encode several diguanylate cyclases in their genomes. For instance, sequence analysis suggests there are 3 GG(D/E)EF diguanylate cyclases in *Bacillus subtilis*, 9 in *Caulobacter crescentus*, 10 in *Escherichia coli*, and at least 25 in *Pseudomonas aeruginosa*, and presumably each is involved in a discrete process. Though individual GG(D/E)EF-diguanylate cyclases have been shown to regulate adaptive responses such as biofilm formation, chemotaxis, and virulence in bacteria such as those listed above (Jenal, 2004; Jenal and Malone, 2006), the physiological response that accompanies the stressosome-regulated cyclic di-GMP biosynthesis in *M. thermoacetica* remains unknown, in part because of the absence of an established genetic system for this bacterium and the lack of simple growth conditions for this thermophilic acetogen. No physiological clues come from the genes upstream and downstream of the *M. thermoacetica* stressosome operon to indicate the wider role of these proteins.

The adoption of the stressosome as a signaling module, in both Gram-positive and negative bacteria, shows the utility of this system for mediating the response to various intracellular signals (Pané-Farré et al., 2005). The modular nature of this system allows different input domains to be coupled to various effectors through a biological signaling circuit that is responsive to the level of signal perceived and is resettable through reversible protein phosphorylation. The remarkable array of sensors that can be used in the stressosome system is exemplified by the blue-light responsive, LOV domain-containing YtvA in *B. subtilis* and the aero- or redox-sensitive, true hemoglobin sensory domains in a number of bacteria, including the pathogen, *Vibrio vulnificus* (Pané-Farré et al., 2005). The specific signals and biological responses mediated by these stressosomes remain to be determined. The question of how signaling occurs through the various sensory domains coupled to the
Figure 5. Interaction of MtT with MtG

(A–C) SDS PAGE gels (A–C) of Ni-NTA pull down experiments in which MtT interactions with either (A) full length His6-MtG, (B) control with no other protein (C) the His6-GAF-GGEEF domain of MtG were detected. (A) lane 1 and 2, purified MtT and MtG standards, respectively; lane 3, eluate after applying His6-MtG; lane 4, eluate following subsequent addition of MtT; lane 5, bound protein eluted from the resin containing both MtT and MtG, indicating an interaction between the proteins. (B) control experiment to confirm MtT does not bind to a Ni-NTA column. Lane 1, eluate following addition of MtT; lane 2, bound protein eluted from the resin, demonstrating MtT does not bind to Ni-NTA resin; (C) experiment to determine MtT binding to His6-GAF-GGEEF. Lane 1, eluate following addition of His6-GAF-GGEEF; lane 2, eluate after subsequent application MtT; lane 3, bound protein eluted from the resin. No MtT was observed to coelute with His6-GAF-GGEEF suggesting no interaction.
stressosome will be resolved when activating ligands are identified in individual systems. That the stressosome has been put to use in a wide number of species from different niches shows its general utility as a tunable switching circuit that allows bacteria to respond to varied signals through the implementation of modular sensors and effectors.

**EXPERIMENTAL PROCEDURES**

Additional Experimental Procedures can be found in the Supplemental Information available online.

**Cloning and Protein Expression**

The expression plasmids for use in this study were generated from genomic DNA, amplified and cloned into pET vectors from Novagen, unless otherwise specified. For protein expression, plasmids were transformed into chemically competent *Escherichia coli* B strains and induced with IPTG for either 3–4 hr at 37°C or for 16 hr at 18°C. All cells were harvested by centrifugation and lysed by sonication after suspension in buffers appropriate for the subsequent purification.

**Protein Purification**

The *M. thermoacetica* proteins were purified to electrophoretic homogeneity by either a combination of nickel-NTA and size exclusion chromatography for those proteins that were His₆-tagged, or by anion or cation exchange and size exclusion chromatography for those proteins that were untagged. GST-tagged MtG was also purified by use of glutathione affinity chromatography.

**Phosphorylation of MtS by MtT**

MtS-P was prepared by overnight incubation of MtS with MtT in a reaction buffer containing ATP before purification by ion exchange chromatography.

**Dephosphorylation of MtS-P by MtX**

Dephosphorylation activity analysis was carried out by incubating the two proteins for a defined length of time, before their separation by native gel electrophoresis and analysis by densitometry.

**Surface Plasmon Resonance**

SPR experiments were carried out using a BIAcore 2000 or a BIAcore X-100 (BIAcore, Uppsala, Sweden) and with proteins immobilized on a CMS chip by amine coupling. Target proteins were flowed over the surface of the chip (BIAcore, Uppsala, Sweden) and with proteins immobilized on a CM5 chip (BIAcore, Uppsala, Sweden). The reliability of the fit of the data to the model was measured according to 1:1 Langmuir binding, producing calculated constants *k*₄ and *K*ₕaghetti were calculated from Lineweaver-Burke plots. The malachite green assay was the basis for determining the rate of inorganic phosphate release from reactions including MtT and MtG.

**Identification of MtG Reaction Products and Role of MtT in This Reaction**

A modified version of the method used by Zahringer et al. (2011) was used to separate the nucleotides produced by MtT and MtG by anion exchange.

**X-ray Crystallography**

Data were collected at the Diamond Light Source and the European Synchrotron Radiation Facility, integrated using iMosflm (Leslie, 2006), or XDS (Kabsch, 2010), and scaled and merged using SCALA (Evans, 2006). The structures of MtX and N-MtR were solved by single wavelength anomalous dispersion procedures using iodine and selenium as anomalous scatterers, respectively. Heavy atom positions were located and phases were determined using SHELXC/D/E (Sheldrick, 2010). The structure of MtS-P was solved by molecular replacement using Phaser (McCoy et al., 2007) with MtS (PDB ID: 2VY9) as the search model. All structures were subjected to iterative cycles of manual fitting with COOT (Emsley et al., 2010) and refinement with Phenix-Refine (Adams et al., 2010) until convergence. All data collection and refinement statistics are shown in Table 1.

**ACCESSION NUMBERS**

The atomic coordinates and structure factors have been deposited in the Protein Data Bank (http://www.ebi.ac.uk/pdbe) (PDB ID codes: MtN-R, 3ZTA; MtS-P, 3ZTB; MtX, 3ZT9; MtS S58E, 3ZXN)

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes one figure, Supplemental Results, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.str.2012.01.003.

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