The clpB gene is involved in the stress response of *Myxococcus xanthus* during vegetative growth and development

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The Clp/HSP100 family of molecular chaperones is ubiquitous in both prokaryotes and eukaryotes. These proteins play important roles in refolding, disaggregating and degrading proteins damaged by stress. As a subclass of the Clp/HSP100 family, ClpB has been shown to be involved in various stress responses as well as other functions in bacteria. In the present study, we investigated the role of a predicted ClpB-encoding gene, MXAN5092, in the stress response during vegetative growth and development of *Myxococcus xanthus*. Transcriptional analysis confirmed induction of this clpB homologue under different stress conditions, and further phenotypic analysis revealed that an in-frame deletion mutant of MXAN5092 was more sensitive to various stress treatments than the wild-type strain during vegetative growth. Moreover, the absence of the MXAN5092 gene resulted in decreased heat tolerance of myxospores, indicating the involvement of this clpB homologue in the stress response during the development of myxospores. The *M. xanthus* recombinant ClpB (MXAN5092) protein also showed a general chaperone activity *in vitro*. Overall, our genetic and phenotypic analysis of the predicted ATP-dependent chaperone protein ClpB (MXAN5092) demonstrated that it functions as a chaperone protein and plays an important role in cellular stress tolerance during both vegetative growth and development of *M. xanthus*.

**INTRODUCTION**

The Clp/Hsp100 family is part of the AAA+ (ATPases associated with various cellular activities) superfamily (Neuwald et al., 1999; Dougan et al., 2002; Shih & Lai, 2007), a widespread group of proteins that participates in the multi-chaperone system to inhibit and reverse protein aggregation (Ben-Zvi & Goloubinoff, 2001; Shih & Lai, 2007). As a member of the Clp/Hsp100 family, ClpB is well conserved among different bacterial species and has been shown to cooperate with other chaperone systems to solubilize and refold aggregated proteins (Motohashi et al., 1999; Zolkiewski, 1999; Kannan et al., 2008). The clpB gene is heat shock-inducible and required for cellular tolerance to a variety of stresses (Simão et al., 2005; Capestany et al., 2008; Kannan et al., 2008; Lourdault et al., 2011), including heat, osmotic, ethanol as well as acidic stress (Squires et al., 1991; Ekaza et al., 2001; Ishikawa et al., 2010). Furthermore, ClpB has been implicated in the expression of virulence factors in different pathogenic bacterial species (Chastanet et al., 2004; Yuan et al., 2007; Capestany et al., 2008; de Oliveira et al., 2011; Lourdault et al., 2011).

Three supplementary figures and a supplementary table are available with the online version of this paper.

*Myxococcus xanthus* is a Gram-negative bacterium, exhibiting remarkably complex social behaviours that are essential for its survival in natural environments (Shimkets, 1990). When subjected to starvation on a solid surface, hundreds of thousands of myxobacterial cells accumulate to form multicellular fruiting bodies, inside which myxospores develop. The myxospores are resistant to adverse conditions and important for myxobacterial survival in stressful environmental conditions (Shimkets, 1990). The genomic sequence of *M. xanthus* revealed multiple copies of chaperone-encoding genes (Goldman et al., 2006), and several of these genes have been shown to be involved in different cellular functions in *M. xanthus* under various stress conditions. Sglk, an hsp70 family protein, was reported to control the production of extracellular fibrils (Weimer et al., 1998; Yang et al., 1998), while a small heat-shock protein, Mx Hsp16.6, was identified and shown to play a critical role in the heat-shock response of *M. xanthus* (Otani et al., 2005). More recently, two copies of groEL genes were reported to be important in the stress response and multicellular social life cycle of *M. xanthus* (Li et al., 2010). The presence of multiple copies of chaperone proteins is proposed to be related to the evolution of the complex social behaviour and environmental adaptation of *Myxococcus* (Goldman et al., 2006).
Among the 12 Clp/Hsp100 homologues that have been identified in the M. xanthus genome, only one gene, clpC (MXAN4832), has been confirmed to be involved in M. xanthus sporulation and self-organization (Yan et al., 2012), while the functions of the remaining Clp/Hsp100 homologues are still unknown. In this study, we investigated the transcriptional expression of other Clp/Hsp100 homologues, including three predicted ClpB chaperone proteins (MXAN4813, MXAN4823, MXAN5092), and demonstrated that only the expression of MXAN5092 was inducible under different stress conditions. The role of the clpB homologue MXAN5092 in the myxobacterial stress response was then further analysed by constructing and characterizing an MXAN5092 null mutant strain under different stress conditions.

METHODS

Strains, plasmids and culture conditions. The bacterial strains, plasmids and PCR primers used in this study are listed in Tables 1 and S1 (available with the online version of this paper). All M. xanthus strains were cultured in CYE liquid medium or on CYE plates supplemented with 1.5% (w/v) agar (Difco) (Campos et al., 1978) and incubated aerobically at 32 °C. For experiments related to developmental conditions, the M. xanthus strains were incubated in MOPS buffer [10 mM MOPS (pH 7.6), 8 mM MgSO4] (Yang et al., 1998) or MOPS agar plates. Escherichia coli strains were cultured in Luria–Bertani (LB) broth and incubated at 37 °C. If necessary, kanamycin (Km) and ampicillin (Amp) were added to the medium at a final concentration of 100 μg ml−1.

Stress conditions. M. xanthus strains were grown at 32 °C to the exponential growth phase (OD600 0.5–0.8). The cells were collected by centrifugation at 9000 g for 5 min, resuspended in CYE medium to OD600 1 and subjected to the following stress treatments for 30 min: heat stress (37 °C, 42 °C) and osmotic stress (2% (w/v) NaCl). The cells were then collected by centrifugation for further analysis.

RNA isolation. Total RNA of the M. xanthus cells subjected to different stresses was isolated using the Promega SV total RNA purification kit according to the manufacturer’s instructions. The RNA concentration, purity and integrity were determined by measuring the absorbance at 260 and 260/280 nm, as well as by evaluation using agarose gel electrophoresis. To eliminate the DNA contamination in the RNA samples, the isolated total RNA was treated with DNase I (Ambion), and a control PCR using the purified total RNA as template was carried out to ensure that there was no DNA contamination prior to the cDNA synthesis and quantitative real-time PCR analysis.

Quantitative real-time PCR. One microgram of the isolated total RNA was reverse-transcribed using the Roche Transcriptor First Strand cDNA Synthesis kit according to the protocol provided by the manufacturer in combination with primed random hexamers. The specific primers (Table S1) for the three putative clpB genes (MXAN5092, MXAN4813, MXAN4823) were then used for the real-time PCR, and the 16S rRNA gene was used to normalize the amounts of the clpB transcript under different stress conditions as reported elsewhere (Li et al., 2010). Quantitative real-time PCR was performed in the iCycler (Bio-Rad) with a total reaction volume of 25 μl containing 500 nM primers, 1 μl SYBR Green (25×), 1.2 μl fluorescein (125 nM), 12.5 μl Phusion Hot Start Flex 2× Master Mix (NEB), 6.8 μl distilled H2O and 0.5 μl cDNA template. Calibration curves were generated for clpB and 16S rRNA from 10-fold dilution of genomic DNA, and the expression of clpB under the different stress conditions was compared.

Construction of the clpB mutant. An in-frame deletion mutant of clpB was constructed following previously reported methods (Ueki et al., 1996; Pan et al., 2009), with some modifications. Upstream and downstream homologous regions flanking MXAN5092 were amplified from the DK1622 genome, digested with EcoRI, ligated together and amplified by PCR. The PCR product was purified and ligated into the Smal site of pBJ113 to generate plasmid pBJ-MXAN5092, which was then transferred into M. xanthus DK1622 by electroporation. Transformants resistant to kanamycin were selected and inoculated onto CYE agar supplemented with 2% (w/v) galactose (Fisher). The putative deletion mutants were identified based on their galactose resistance and Km sensitivity, and further verified by PCR amplification and sequencing. The in-frame deletion mutant of MXAN5092 was designated SW5001.

Growth assay. The growth kinetics of the wild-type strain and the corresponding MXAN5092 mutant SW5001 were compared according to previous protocols (Li et al., 2010). Briefly, mid-exponential

Table 1. Bacterial strains and plasmids

<table>
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<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Reference or source</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>M. xanthus DK1622</td>
<td>Wild-type strain</td>
<td>Kaiser (1979)</td>
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<tr>
<td>SW5001</td>
<td>MXAN5092 (clpB) in-frame deletion in DK1622</td>
<td>This study</td>
</tr>
<tr>
<td>SW5002</td>
<td>DK1622::pZCY5092 (lacZ fused to MXAN5092)</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli XLI-Blue MR</td>
<td>∆(mcrA183∆(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac)</td>
<td>Stratagene</td>
</tr>
<tr>
<td>E. coli TOP10</td>
<td>Host for expression vector cloning</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>E. coli BL21</td>
<td>Host for protein expression</td>
<td>NEB</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pBJ113</td>
<td>Gene replacement vector with KG cassette; Km'</td>
<td>Julien et al. (2000)</td>
</tr>
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<td>pBJ-MXAN5092</td>
<td>MXAN5092 in-frame deletion in pBJ113</td>
<td>This study</td>
</tr>
<tr>
<td>pZCY11</td>
<td>Plasmid used for lacZ transcriptional fusion</td>
<td>Li et al. (2010)</td>
</tr>
<tr>
<td>pZCY5092</td>
<td>Plasmid with MXAN5092 homologous arm for lacZ transcriptional fusion</td>
<td>This study</td>
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<tr>
<td>pET15b</td>
<td>Protein overexpression plasmid</td>
<td>Novagen</td>
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<tr>
<td>pET-MXAN5092</td>
<td>MXAN5092 gene cloned into pET15b</td>
<td>This study</td>
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cells were inoculated into liquid CYE medium to a final concentration of 2–5 × 10⁸ cells ml⁻¹ and cultured at 32 ºC with shaking (300 r.p.m.). The OD₆₀₀ was measured at different time points to evaluate growth.

**Swarming assay.** To assay the swarming capacity of the wild-type strain and MXAN5092 mutant SW5001, aliquots (8 µL, 5 × 10⁸ cells ml⁻¹) were inoculated onto CYE medium containing 1.5 % (w/v) or 0.3 % (w/v) agar (Shi & Zusman, 1993). After 72 h of incubation at 32 ºC, the sizes of the swarming colonies were measured.

**Stress survival and thermotolerance acquisition assay.** The stress survival ability of the MXAN5092 mutant SW5001 was assayed as reported by Otani et al. (2005). The wild-type strain and mutant were grown exponentially at 32 ºC (OD₆₀₀ 0.5–0.8). The cells were collected and resuspended to an OD₆₀₀ of 1 and subjected to the following stresses for 90 min: heat stress (37 ºC, 42 ºC) and osmotic stress [2 % (w/v) NaCl]. After treatment, aliquots of 0.1 ml of the treated cells, containing 2–5 × 10⁶ treated cells ml⁻¹, were subjected to serial dilution, mixed with CYE containing 0.3 % (w/v) agar and plated on CYE agar plates. After 120 h of incubation at 32 ºC, the c.f.u. were determined.

To test the involvement of MXAN5092 in acquiring thermotolerance, the experiments were carried out as reported elsewhere (Otani et al., 2005; Simão et al., 2005). In brief, both the MXAN5092 mutant SW5001 and the wild-type strain DK1622 were grown exponentially in CYE liquid medium at 32 ºC. The cells were collected and resuspended to an OD₆₀₀ of 1. Each culture was divided into two aliquots, one of which was maintained at 32 ºC while the other was incubated at 40 ºC for 30 min. Both aliquots were then subjected to heat treatment at 45 ºC for 60 min. The cells were serially diluted in CYE, mixed with CYE containing 0.3 % (w/v) agar and plated on CYE agar to determine the number of viable cells.

**Development and heat resistance of myxospores.** Development assays were carried out on MOPS agar according to a published protocol (Kearns et al., 2000). After 120 h, the fruiting bodies formed by the wild-type strain and MXAN5092 mutant SW5001 were observed microscopically (Nikon TE200 microscope) and collected to test for sporulation. The cells were resuspended in 100 µl MOPS buffer, and submitted to slight sonication for homogenization (Pan et al., 2010). After 2 h of incubation at 50, 55 and 60 ºC, the cells were serially diluted, mixed with CYE containing 0.3 % (w/v) agar and poured onto CYE plates. After 5–7 days incubation at 32 ºC, c.f.u. were determined to assess spore viability.

**Construction of a M. xanthus strain with an MXAN5092–lacZ transcriptional fusion.** An M. xanthus strain harbouring a lacZ transcriptional fusion to MXAN5092 was constructed using the method reported by Li et al. (2010). Briefly, a 1105 bp DNA fragment containing the stop codon as well as the partial structural gene of MXAN5092 was PCR-amplified from the genomic DNA of M. xanthus DK1622. The PCR fragments were double-digested with KpnI/SpeI and ligated into pZCY11, a plasmid that is a derivative of pMinHimar1-lacZ carrying the replication origin of OriR6K and a promoterless lacZ reporter gene. The resultant plasmid, named pZCY5092, was transformed into M. xanthus DK1622 by electroporation to generate SW5002, a DK1622 derivative that harbours the lacZ reporter gene transcriptionally fused to MXAN5092. The expression level of MXAN5092 in SW5002 during development was analysed by measuring the β-galactosidase activity.

**β-Galactosidase assay.** To analyse the expression of MXAN5092 during development, a β-galactosidase assay was carried out following a published method (Kroos et al., 1986), with some modifications. The SW5002 (MXAN5092-lacZ) and wild-type strain were grown for 24 h, harvested by centrifugation and resuspended to adjust the cell concentration to 5 × 10⁶ cells ml⁻¹. Ten-microlitre aliquots were then spotted on the MOPS agar. The cells were collected carefully at different time points and lysate was prepared by disrupting cells using a bead beater, followed by centrifugation to remove cell debris. A 100 µl volume of the cell lysate was mixed with 400 µl Z buffer containing 1 mg ONPG ml⁻¹ as substrate. The mixtures were incubated at 37 ºC until the solution turned sufficiently yellow. The reaction was stopped by the addition of 0.5 ml 1 M Na₂CO₃. The absorbance of the solution was read at 420 nm. The protein concentration was determined by a bicinchoninic acid (BCA) protein assay (Pierce). β-Galactosidase activity was calculated as reported elsewhere (Kroos et al., 1986).

**Recombinant protein expression of M. xanthus ClpB (MXAN5092).** Plasmid pET-MXAN5092 was constructed to over-express the M. xanthus ClpB (MXAN5092) recombinant protein in E. coli. The DNA sequence encoding MXAN5092 was PCR-amplified from the genomic DNA of M. xanthus DK1622 using MXAN5092-F and MXAN5092-R primers (Table S1). PCR fragments were digested with NdeI and Xhol, and ligated into plasmid PET15b to generate the expression plasmid pET-MXAN5092. pET-MXAN5092 was transformed into E. coli BL21, and the expression of MXAN5092 was induced by adding IPTG (1 mM) and incubating for 4 h at 37 ºC. The bacterial cells were harvested and lysed by sonication. SDS-PAGE was performed following a standard procedure to verify the soluble expression of the M. xanthus ClpB (MXAN5092) recombinant protein in E. coli BL21 (Fig. S3).

**Prevention of the aggregation of substrate proteins.** The prevention of the aggregation of substrate proteins was carried out as reported by Sugimoto et al. (2006), with some modifications. Cell lysate of E. coli BL21 carrying expression plasmid pET-MXAN5092 cultivated under IPTG-induced and uninduced conditions was incubated at 50 ºC in refolding buffer (pH 7.6) containing 30 mM HEPES-KOH, 120 mM KCl, 10 mM MgCl₂, 1 mM EDTA, 1 mM DTT and 5 mM ATP (Barnett et al., 2000), and aggregation was monitored for 10 min as the increase in absorbance at 320 nm.

**RESULTS**

**Bioinformatic analysis of the M. xanthus ClpB homologue.**

In different bacterial species, the size of ClpB varies from 756 to 926 aa. A typical ClpB contains a N-terminal domain, an AAA+ module (AAA-1), a middle domain and a second AAA+ module (AAA-2). Both AAA+ modules contain the consensus Walker A and Walker B motifs as well as two arginines (AAA-1) or one arginine (AAA-2), which are conserved (Squires et al., 1991; Yamasaki et al., 2011). Three copies of the putative ClpB-encoding genes were identified in the genome of M. xanthus DK1622. Sequence alignment of these three putative M. xanthus ClpB proteins with the characterized ClpB proteins from E. coli (Squires et al., 1991), Acetobacter pasteurianus (Ishikawa et al., 2010) and Lactococcus lactis (Chastanet et al., 2004) revealed that the ClpB protein encoded by the genetic locus MXAN5092 was most similar to these ClpB proteins. All of these putative M. xanthus ClpB proteins possess the two highly conserved nucleotide-binding regions, AAA-1 and AAA-2, including the ATPase A and B boxes characteristic of the ClpB protein.
family (Fig. S1). The ClpB protein encoded by MXAN5092 also contains the two characteristic Clp_N domains at the N terminal and a ClpB_D2-small domain at the C terminal, while the other two copies of putative ClpB proteins lack the N-terminal domains (Fig. S1).

**Expression of clpB (MXAN5092) is induced under various stress conditions**

The clpB gene has been shown in other bacteria to be induced under heat shock and other stress conditions. To investigate their involvement in stress responses, the transcriptional regulation of the three putative clpB gene homologues in *M. xanthus* DK1622 subjected to heat and osmotic shock was analysed using quantitative real-time PCR. The results revealed that only the expression of MXAN5092 was induced under both stress conditions, while the other two copies of clpB genes were downregulated under most treatments (Fig. 1). The heat treatment at 37 or 42 °C for 30 min resulted in a four- to fivefold increase in the expression level of MXAN5092, and a similar level of induction (three- to fourfold) of this gene was observed when cells were subjected to osmotic shock [2 % (w/v) NaCl] (Fig. 1a). The increase in MXAN5092 expression under heat shock and osmotic shock indicates its possible role in the stress responses of *M. xanthus*. Thus, the genetic locus MXAN5092 encoding the most typical ClpB protein among the three copies was chosen for further study.

**Lack of clpB (MXAN5092) affects survival under different stress conditions but not vegetative growth**

Based on the results of transcriptional analysis, an in-frame deletion mutant of clpB (MXAN5092) was constructed to further evaluate the role of MXAN5092 in the stress response of *M. xanthus*. The MXAN5092 mutant SW5001 contains a deletion of a 2373 bp fragment (from amino acid 34 to amino acid 824), which encodes most of the functional domains of ClpB (Fig. S2). As clpB had basal-level expression under standard vegetative culture conditions (Fig. 1), the growth curves of the wild-type strain and the MXAN5092 mutant SW5001 were compared to evaluate whether lack of this chaperone protein affects the vegetative growth of *M. xanthus* cells. The growth kinetics of the wild-type strain and MXAN5092 mutant SW5001 showed no significant difference when cultured at 32 °C, indicating that MXAN5092 is not required for vegetative growth of *M. xanthus* at its physiological temperature (Fig. 2). We further analysed the stress survival ability of the mutant cells by exposing them to heat shock (37 °C and 42 °C) and osmotic shock [2 % (w/v) NaCl]. Starting with the same amount of viable cells, the clpB mutant SW5001 exhibited severely reduced viability compared with the wild-type parent strain after 90 min of heat-shock treatment at 37 °C (34.8 ± 3.5 %) or 42 °C (2.2 ± 0.4 %) (Table 2). Similarly, the lack of clpB affected the ability of *M. xanthus* to recover from osmotic shock conditions.

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**Fig. 1.** Quantitative PCR analysis of the expression levels of putative clpB genes MXAN5092 (a), MXAN4813 (b) and MXAN4823 (c) in *M. xanthus* DK1622 in response to heat and osmotic shock. The values are shown as levels relative to the expression level of clpB under standard vegetative culture conditions. Error bars, sd from three independent technical replicates.
Next, the involvement of clpB (MXAN5092) in thermostolerance acquisition in M. xanthus was investigated. For wild-type M. xanthus the pre-incubation at 40 °C for 30 min prior to heat shock at 45 °C resulted in an almost sevenfold increase in the remaining viable cells after treatment compared with cells that were directly shifted from 32 to 45 °C (Table 2). In contrast, deletion of clpB (MXAN5092) completely abolished the thermostolerance acquisition observed in the wild-type strain (Table 2).

ClpB (MXAN5092) is required for heat resistance of myxospores but is not involved in swarming and fruiting body formation

For a comprehensive analysis, we also examined the possible roles of MXAN5092 in the social behaviours of M. xanthus. The absence of MXAN5092 did not affect swarming and fruiting body formation. However, the heat tolerance of the myxospores formed by MXAN5092 mutant SW5001 was drastically reduced (Fig. 3). Specifically, while heat treatment at 50 °C for 2 h did not result in any significant difference in sporulation between MXAN5092 mutant SW5001 and wild-type strain, an increase to 55 °C reduced sporulation of the MXAN5092 mutant SW5001 to less than 15 % of the wild-type. An even more drastic difference was observed when the cells were treated at 60 °C, which decreased the sporulation of the MXAN5092 mutant SW5001 to 6.2 % of the wild-type strain.

MXAN5092 is upregulated during fruiting body development

As the MXAN5092 gene is important for myxospore heat resistance, its transcriptional expression during fruiting body development was analysed using a MXAN5092–lacZ transcriptional fusion via a β-galactosidase assay. As shown in Fig. 4, the transcriptional expression of the clpB (MXAN5092) gene was induced by starvation and upregulated during the fruiting body formation and sporulation process of M. xanthus. The results confirmed the involvement

**Table 2. Viability of cells after stress treatments**

Cell viability was evaluated by plating diluted samples on CYE plates after stress treatments. The initial cell number before treatments was taken as 100 %. DK1622 is the wild-type strain and SW5001 is the in-frame MXAN5092 deletion mutant. Three replicates were performed for each viability assay. Mean values ± SD are shown.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Stress condition</th>
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<th>Infactate at 40 °C for 30 min before shift to 45 °C</th>
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<tr>
<td></td>
<td>37 °C</td>
<td>42 °C</td>
<td>2% NaCl</td>
<td>Shift from</td>
<td>32 to 45 °C directly</td>
</tr>
<tr>
<td>DK1622</td>
<td>66.2 ± 30.8 %</td>
<td>0.13 ± 0.03 %</td>
<td>65 ± 3 %</td>
<td>0.79 ± 0.11 %</td>
<td>5.3 ± 0.3 %</td>
</tr>
<tr>
<td>SW5001</td>
<td>24.1 ± 13 %</td>
<td>0.0028 ± 0.0002 %</td>
<td>41 ± 4 %</td>
<td>0.032 ± 0.002 %</td>
<td>0.035 ± 0.006 %</td>
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of the clpB (MXAN5092) gene in the development of M. xanthus.

Recombinant M. xanthus ClpB (MXAN5092) protein has a general chaperone activity

In order to assess the general chaperone activity of the M. xanthus ClpB (MXAN5092) protein, the effect of recombinant M. xanthus ClpB (MXAN5092) protein on the thermal aggregation of substrate proteins was analysed. Cell lysate was prepared from E. coli carrying an MXAN5092 overexpression plasmid grown under IPTG-induced and uninduced conditions. Our results showed that induction of MXAN5092 expression resulted in a significantly lower maximum absorbance value of the thermal aggregation of substrate proteins compared with that obtained under uninduced conditions. The data indicated that the recombinant M. xanthus ClpB protein can inhibit the thermal aggregation of the E. coli substrate proteins (Fig. 5), thus suggesting a general chaperone activity of this protein in vitro.

DISCUSSION

In their natural environment, myxobacteria such as M. xanthus often encounter a wide range of harsh conditions, including temperature shifts, osmotic shocks, adverse pH conditions and starvation. These diverse stresses can induce damage to cellular components, such as DNA and proteins, resulting in the impairment of a variety of cellular functions and reduced bacterial fitness. To cope with these constant environmental assaults, myxobacteria take advantage of a repertoire of machineries, such as the chaperone systems, to counteract and alleviate these stresses. The M. xanthus genome encodes a large number of chaperones of different families (Goldman et al., 2006). Some of these chaperones have been shown to be induced in response to elevated temperature or other cellular stresses, and their functional roles in helping bacteria to cope with stressful conditions by inhibiting and reversing protein aggregation, as well as their involvement in the complex social behaviours of myxobacteria have been characterized (Weimer et al., 1998; Yang et al., 1998; Otani et al., 2005; Li et al., 2010). In this study we focused on MXAN5092, an M. xanthus ORF encoding a protein with strong sequence homology to ClpB (Fig. S1), a member of the Clp/Hsp100 family. We demonstrated that the clpB gene (MXAN5092) in M. xanthus is involved in stress tolerance, particularly in the heat resistance of spores as well as of vegetative cells.

Our transcriptional data revealed that among the three putative clpB gene homologues identified in M. xanthus, MXAN5092 showed significant induction in its expression when M. xanthus cells were exposed to heat and osmotic stresses (Fig. 1). This was corroborated by the phenotypic analysis, which showed that the MXAN5092 deletion mutant displayed a drastically reduced cellular tolerance of heat treatment, as well as osmotic stress, although to a lesser degree (Table 2). Additionally, the absence of the clpB gene affected thermostolerance acquisition in M. xanthus (Table 2), a phenotype that has been observed in other bacteria, such as Enterococcus faecalis (de Oliveira et al., 2011). Moreover, the recombinant M. xanthus ClpB
(MXAN5092) protein has a general chaperone protein activity (Fig. 5). These genetic and phenotypic data are consistent with a role in M. xanthus for the predicted ClpB encoded by MXAN5092 as a stress-related chaperone protein involved in cellular tolerance of environmental stresses, as reported in other bacteria (Squires et al., 1991; Otani et al., 2005; Simão et al., 2005; Ventura et al., 2005; Meibom et al., 2008).

Interestingly, unlike other characterized chaperone proteins in M. xanthus, whose absence often results in altered swarming and fruiting body formation (Weimer et al., 1998; Yang et al., 1998; Li et al., 2010; Yan et al., 2012), the clpB (MXAN5092) mutation did not affect these social behaviours at the standard physiological temperature of 32 °C, suggesting that clpB in M. xanthus could be more dedicated to heat-related responses. The M. xanthus genome encodes a large number of chaperones of different families, and each family is often represented by multiple homologues (Goldman et al., 2006). These seemingly redundant copies of genes are considered to be related to the evolution of the complex social behaviours and environmental adaptation of myxobacteria (Pan et al., 2010; Goldman et al., 2007). A total of 12 Clp/Hsp100 homologues has been identified in the M. xanthus genome (Yan et al., 2012). One of these homologues, ClpC, was recently characterized by Yan et al. (2012), who found that its main functions were social behaviour-related, although a minor involvement in the heat tolerance of myxospores was apparent. Our results clearly suggested that, unlike clpC, clpB (MXAN5092) was more involved in stress tolerance, particularly the heat resistance of M. xanthus cells.

Furthermore, our data revealed the starvation-inducible expression of clpB (MXAN5092) (Fig. 4) and its involvement in the heat tolerance of myxospores (Fig. 3). The formation of viable myxospores is essential for myxobacteria to survive in adverse environmental conditions, such as starvation. The ability to resist heat is one of the most characteristic features of spores compared with vegetative cells. It is also interesting to note that both Clp/Hsp100 homologues, clpB and clpC, seem to be involved in the heat resistance of myxospores, and it would be informative to further study the relationship between clpB and clpC. Also, the sequence similarities of the AAA domains and divergence between the N- and C-terminal domains of the three copies of putative M. xanthus ClpB proteins (Fig. S1) suggested that the emergence of the multiple copies of Clp/Hsp100 homologues may have resulted from gene duplication and divergence, which is considered to be important in the genome expansion of M. xanthus (Goldman et al., 2006). The different transcriptional expression patterns (Fig. 1) under stress conditions also suggested the functional divergence of these three copies of clpB gene homologues. Further analysis of the functional relationships among these Clp/Hsp100 homologues can better demonstrate the evolutionary role of the emergence of these chaperone proteins in M. xanthus.

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