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*.

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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).

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).

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

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 *Eco*RI, ligated together and amplified by PCR. The PCR product was purified and ligated into the *Sma*I 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

Strain or plasmid	Genotype or description	Reference or source
Strains		
<i>M. xanthus</i> DK1622	Wild-type strain	Kaiser (1979)
SW5001	MXAN5092 (<i>clpB</i>) in-frame deletion in DK1622	This study
SW5002	DK1622::pZCY5092 (<i>lacZ</i> fused to MXAN5092)	This study
<i>E. coli</i> XLI-Blue MR	Δ(<i>mcrA</i>)183Δ(<i>mcrCB</i> - <i>hsdSMR</i> - <i>mrr</i>)173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac</i>	Stratagene
<i>E. coli</i> TOP10	Host for expression vector cloning	Invitrogen
<i>E. coli</i> BL21	Host for protein expression	NEB
Plasmids		
pBJ113	Gene replacement vector with KG cassette; Km ^r	Julien <i>et al.</i> (2000)
pBJ-MXAN5092	MXAN5092 in-frame deletion in pBJ113	This study
pZCY11	Plasmid used for <i>lacZ</i> transcriptional fusion	Li <i>et al.</i> (2010)
pZCY5092	Plasmid with MXAN5092 homologous arm for <i>lacZ</i> transcriptional fusion	This study
pET15b	Protein overexpression plasmid	Novagen
pET-MXAN5092	MXAN5092 gene cloned into pET15b	This study

cells were inoculated into liquid CYE medium to a final concentration of $2\text{--}5 \times 10^6$ cells ml^{-1} and cultured at 32°C with shaking (300 r.p.m.). The OD_{600} 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 ($8\ \mu\text{l}$, 5×10^9 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°C , the sizes of the swarming colonies were measured.

Stress survival and thermotolerance acquisition assay. The stress survival ability of the *MXAN5092* mutant SW5001 was analysed as reported by Otani *et al.* (2005). The wild-type strain and mutant were grown exponentially at 32°C (OD_{600} 0.5–0.8). The cells were collected and resuspended to an OD_{600} of 1 and subjected to the following stresses for 90 min: heat stress (37°C , 42°C) and osmotic stress [2% (w/v) NaCl]. After treatment, aliquots of 0.1 ml of the treated cells, containing $2\text{--}5 \times 10^8$ 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°C , 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°C . The cells were collected and resuspended to an OD_{600} of 1. Each culture was divided into two aliquots, one of which was maintained at 32°C while the other was incubated at 40°C for 30 min. Both aliquots were then subjected to heat treatment at 45°C 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 $100\ \mu\text{l}$ MOPS buffer, and submitted to slight sonication for homogenization (Pan *et al.*, 2010). After 2 h of incubation at 50, 55 and 60°C , 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°C , 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 pMinHimarI-*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 5×10^9 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 $100\ \mu\text{l}$ volume of the cell lysate was mixed with $400\ \mu\text{l}$ Z buffer containing $1\ \text{mg}\ \text{ONPG}\ \text{ml}^{-1}$ as substrate. The mixtures were incubated at 37°C until the solution turned sufficiently yellow. The reaction was stopped by the addition of $0.5\ \text{ml}\ 1\ \text{M}\ \text{Na}_2\text{CO}_3$. 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 *XhoI*, 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°C . 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°C in refolding buffer (pH 7.6) containing 30 mM HEPES-KOH, 120 mM KCl, 10 mM MgCl_2 , 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 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.

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.

Fig. 2. Growth curves of *M. xanthus* wild-type strain DK1622 and *MXAN5092* mutant SW5001 cultured at 32 °C. Error bars, SD from two independent experimental replicates.

Next, the involvement of *clpB* (*MXAN5092*) in thermo-tolerance 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 thermotolerance 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

Fig. 3. Heat-resistance ability at different temperatures of spores of *M. xanthus* wild-type strain DK1622 and *MXAN5092* mutant. DK1622 cells formed 4.2×10^6 , 1.2×10^6 and 3.5×10^4 myxospores per colony on MOPS agar after 50, 55 and 60 °C treatment for 2 h, respectively, which were taken as 100 % sporulation. Error bars, SD from two independent experimental replicates.

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 $\pm$ SD are shown.

Strain	Stress condition				
	37 °C	42 °C	2 % NaCl	Shift from 32 to 45 °C directly	Incubate at 40 °C for 30 min before shift to 45 °C
DK1622	66.2 $\pm$ 30.8 %	0.13 $\pm$ 0.03 %	65 $\pm$ 3 %	0.79 $\pm$ 0.11 %	5.3 $\pm$ 0.3 %
SW5001	24.1 $\pm$ 13 %	0.0028 $\pm$ 0.0002 %	41 $\pm$ 4 %	0.032 $\pm$ 0.002 %	0.035 $\pm$ 0.006 %

Fig. 4. β -Galactosidase activity expressed by the *clpB* (*MXAN5092*)-*lacZ* fusion during *M. xanthus* development. β -Galactosidase specific activity is expressed as nmol ONPG formed min⁻¹ (mg protein)⁻¹. The wild-type strain DK1622 was used as the negative control. Error bars, SD from three experimental replicates.

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

Fig. 5. General chaperone activity of *M. xanthus* recombinant protein ClpB (*MXAN5092*). Cell lysate of *E. coli* BL21 carrying expression plasmid pET-*MXAN5092* cultivated under IPTG-induced ($\blacktriangle$) and uninduced ($\blacksquare$) conditions were incubated at 50 °C. Aggregation was monitored for 10 min. Error bars, SD from three independent biological experimental replicates.

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 thermotolerance 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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