Characterization of a Small Heat Shock Protein, Mx Hsp16.6, of
Myxococcus xanthus

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A number of heat shock proteins in Myxococcus xanthus were previously identified by two-dimensional (2D) gel electrophoresis. One of these proteins was termed Mx Hsp16.6, and the gene encoding Mx Hsp16.6 was isolated. Mx Hsp16.6 consists of 147 amino acid residues and has an estimated molecular weight of 16,642, in accordance with the apparent molecular mass in the 2D gel. An α-crystallin domain, typically conserved in small heat shock proteins, was found in Mx Hsp16.6. Mx Hsp16.6 was not detected during normal vegetative growth but was immediately induced after heat shock. Expression of the hsp16.6 gene was not induced by other stresses, such as starvation, oxidation, and high osmolality. Mx Hsp16.6 was mostly localized in particles formed after heat shock and precipitated by low-speed centrifugation. Furthermore, Mx Hsp16.6 was detected in highly electron-dense particles in heat-shocked cells by immunoelectron microscopy, suggesting that it forms large complexes with heat-denatured proteins. An insertion mutation in the hsp16.6 gene resulted in lower viability during heat shock and lower acquired thermostolerance. Therefore, it is likely that Mx Hsp16.6 plays critical roles in the heat shock response in M. xanthus.

Cells respond and adapt to heat shock by inducing heat shock proteins (HSPs) (1, 28). HSPs are classified into five groups, namely, HSP100, HSP90, HSP70, HSP60, and low-molecular-weight HSPs (sHSPs). Chaperones, such as HSP70 (also known as DnaK) and HSP60 (also known as GroEL), are conserved in all organisms (3, 7). They are also expressed under normal growth conditions and are essential for proper folding of newly synthesized proteins and renaturation of denatured proteins. Moreover, they mediate cellular functions, such as proper transport of newly synthesized proteins to intracellular organelles (1, 28). sHSPs represent a family of 12- to 40-kDa proteins and are also present in all organisms (6, 9, 17, 23). They share a conserved sequence with the α-crystallin proteins of the vertebrate eye lens in their C-terminal 90 amino acids. The α-crystallin domain of sHSPs is preceded by an N-terminal region that varies in size and sequence. sHSPs are known to form multimers consisting of 9 to >30 monomers and to function as ATP-independent chaperones by interacting with heat-denatured proteins to prevent irreversible protein denaturation (13). The proteins interacting with the sHSPs are subsequently facilitated to be restored from inactive proteins to functional proteins in cooperation with ATP-dependent chaperones, such as DnaK and GroEL/GroES. Furthermore, an sHSP from Synechocystis sp. was found not only to have protein-protective activity but also to stabilize lipid membranes (22).

Myxococcus xanthus is a gram-negative soil bacterium that is capable of differentiation into heat-resistant spores under starvation conditions. This differentiation is coordinated by exchange of intercellular signals involving the formation of multicellular fruiting bodies within which the spores are formed. Therefore, M. xanthus has been studied as a simple model system for cellular differentiation.

We previously investigated the heat shock response of M. xanthus by two-dimensional (2D) gel electrophoresis, and 18 HSPs, including DnaK, GroEL1, GroEL2 and GroES, were identified (19). One of these HSPs, a protein with a molecular mass of 16.6 kDa that was not detected under normal growth conditions, was highly induced by heat shock. This protein was designated Mx Hsp16.6. In this study, we isolated the gene encoding Mx Hsp16.6 in order to examine the function of Mx Hsp16.6 in the heat shock response. Analysis of hsp16.6 mRNA expression showed that the hsp16.6 gene was specifically induced by heat shock but not by other stresses, such as starvation, oxidation, and high osmolality. Western blot analysis revealed that Mx Hsp16.6 was produced immediately after heat shock and was localized predominantly in aggregates that were precipitated by low-speed centrifugation. Furthermore, Mx Hsp16.6 was detected in highly electron-dense particles in heat-shocked cells by immunoelectron microscopy. Characterization of an hsp16.6 gene insertion mutant indicated that the hsp16.6 gene was essential for viability in the heat shock response. These results suggest that Mx Hsp16.6 plays a critical role in the heat shock response by forming large complexes with heat-denatured proteins and preventing irreversible aggregation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. M. xanthus DZF1 (10) was used as the parent strain and grown in CYE medium (4). Escherichia coli JM83 (27) was used for plasmid isolation and was grown in LB medium (15). Ampicillin (50
The coding region of the hsp16.6 gene was amplified by PCR with primers 5′CTCTCATATGCAGACT/H11032 and 5′/H11032CTCTGGATCCTCAGCCCTGGACCTTGACTT3, which flank an 88-bp HindIII-BamHI fragment that was amplified from genomic DNA of M. xanthus DZF1. Proper double-crossover recombinants were identified on plates containing kanamycin and hygromycin (8). Amino acids that are identical in more than 75% of the sequences and homologous substitutions are indicated black and gray backgrounds, respectively. The secondary structures of Mj Hsp16.5 that have been determined are indicated above the sequences.

Preparation of total RNA. Total RNA was prepared from M. xanthus DZF1 cells at the mid-log and stationary phases in CYE liquid medium, after heat shock at 42°C for 10 min, after oxidative stress due to 10 mM H2O2 treatment for 10 min, and after osmotic shock due to 0.3 M NaCl treatment for 10 min, respectively.

Examination of viability and acquired thermotolerance. Cells grown to the mid-log phase at 30°C in CYE liquid medium were shifted to 40 or 42°C and incubated for 10 min. After heat shock, the cells were harvested before and after heat shock and at 42°C for 10 min, after oxidative stress due to 10 mM H2O2 treatment for 10 min, and after osmotic shock due to 0.3 M NaCl treatment for 10 min, and at 6 h after the initiation of fruiting body development.

Construction of an insertion mutant, hsp16.6::A marker. A kanamycin resistance cassette was cloned at the Smal site located 175 bp downstream of the translation initiation site in the hsp16.6 gene of pPH2.0. The resultant plasmid was linearized and electroporated into M. xanthus DZF1. Proper double-crossover recombinants were identified on plates containing kanamycin and hygromycin (8).

Preparation of an antibody against Mx Hsp16.6. A 147-amino acid protein consisting of Mx Hsp16.6 was purified from E. coli expressing the His6-tagged protein and affinity-purified using a nickel column. The antibody was raised as described previously (18).

Fractionation of cellular proteins. Cells grown in CYE liquid medium were harvested before and after heat shock at 42°C. The cells were suspended in sonication buffer (20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and disrupted by sonication. Unbroken cells were removed by centrifugation at 2,000 × g for 20 min at 4°C, and the supernatant was then centrifuged at 12,000 × g for 20 min at 4°C. The precipitate was designated the pellet fraction. The supernatant was then centrifuged at 105,000 × g for 30 min at 4°C. The resulting supernatant and precipitate were designated the cytoplasmic and membrane fractions, respectively. The membrane fraction was washed with sonication buffer once.

Analysis of Western blot hybridization and immunoelectron microscopy. Western blot hybridization and immunoelectron microscopy were carried out as described previously (18).

RESULTS

Isolation of the hsp16.6 gene. On the basis of 2D gel analysis of M. xanthus heat shock proteins, Mx Hsp16.6 was previously identified as an HSP (19). To elucidate the function of Mx Hsp16.6, the gene encoding Mx Hsp16.6 was isolated. The hsp16.6 gene encodes a protein consisting of 147 amino acid residues with an estimated molecular weight of 16,612 (data not shown). As shown in Fig. 1, the protein contains the 29-residue N-terminal sequence MQTRNPFNSAWVNPLMRD and one putative Shine-Dalgarno sequence predicted to precede the initiation codon of another putative ORF in the genome sequence.

When the amino acid sequence of Mx Hsp16.6 was com-
pared with the sequences in the database, it was found to contain an α-crystallin domain (Fig. 1). Therefore, Mx Hsp16.6 belongs to the α-crystallin-type sHSP family. The amino acid sequences of members of the sHSP family do not exhibit high levels of homology, even in their α-crystallin domains, as high as the levels of homology of amino acid sequences of other HSPs, such as DnaK and GroEL. The α-crystallin domain from residues 33 to 105 of Mx Hsp16.6 exhibits 61.1, 51.4, and 34.7% identity to SP21 of Stigmatella aurantiaca, Hsp16.6 from Synechocystis sp. strain PCC6803, and HSP16.5 of Methanococcus jannaschii, respectively. The highly conserved motif AXXT XGXL is well conserved in the C terminus of the α-crystallin domains (Fig. 1). It is interesting that nine residues (TLTLT RREE) downstream of AXXXXGXL are also highly conserved among these proteins. However, the N-terminal region preceding the α-crystallin domain of Mx Hsp16.6 shows little similarity to regions of other sHSPs. It has been demonstrated that HSP16.5 from M. jannaschii forms multimers consisting of 24 monomers and that deletion of 12 residues at the N terminus causes failure of multimer assembly, suggesting that the N-terminal region plays a role in the assembly process (13).

Induction of the hsp16.6 gene in various stress conditions and fruiting body development. To examine expression of the hsp16.6 gene in various stress conditions at the transcription level, primer extension analysis was performed. Total RNA was prepared from cells at the mid-log and stationary phases, after heat shock, oxidative stress, and osmotic shock, and during fruiting body development. As shown in Fig. 2A, the hsp16.6 mRNA was detected only in heat-shocked cells and not in other stress conditions. Two 5' ends were identified, and one of these was induced more than the other. From these results, −35 and −10 promoter elements were assigned, as shown in Fig. 2B. One of the hsp16.6 promoter sequences (sequence b), which induced weaker expression than the other sequence (sequence a), exhibited high levels of similarity with the sequences of the lonD promoter and the vegA promoter (Fig. 2B), but no sequence similar to the lonD cis element was present in the hsp16.6 promoter. In addition, the stronger promoter (sequence a) of the hsp16.6 gene exhibited little similarity to other known promoters, including the heat shock promoters in other bacteria.

The induction pattern of Mx Hsp16.6 in the early stage after heat shock was examined by Western blot analysis using an anti-Mx Hsp16.6 IgG prepared as described in Materials and Methods. Although Mx Hsp16.6 was not detected under normal growth conditions, it was present at 5 min and the maximum level was observed at 30 min after a heat shock (Fig. 2C), suggesting that Mx Hsp16.6 was induced immediately after cells entered the heat shock conditions. Moreover, Mx Hsp16.6 was found to be present even 60 min after a temperature shift from 42°C to 30°C (data not shown). These results revealed that the expression pattern of Mx Hsp16.6 is distinctly different from the expression patterns of other HSPs, such as DnaK, GroEL1, GroEL2, and GroES, which are present at low levels under normal growth conditions and are induced at 30 min after heat shock (data not shown). Mx Hsp16.6 expression during fruiting body development was also examined by Western blot analysis using an anti-Mx Hsp16.6 IgG. Mx Hsp16.6 was not detected at 0, 2, 4, 6, 8, 12, and 24 h during development (data not shown).

Characterization of the hsp16.6::km mutant. Analysis of the M. xanthus genome sequence showed that M. xanthus contains no homologs of Mx Hsp16.6, as is the case for the cyanobacterium Synechocystis sp. strain PCC6803 (11), although some other organisms have several sHSPs (5, 16). To examine the function of Mx Hsp16.6 during the heat shock response, an insertion mutant with a mutation in the hsp16.6 gene was constructed by inserting the kanamycin resistance gene at the SmaI site in the opposite orientation, as described in Materials and Methods. Although Mx Hsp16.6 was not detected under normal growth conditions and are induced at high levels of homology, even in their α-crystallin domains, as high as the levels of homology of amino acid sequences of other HSPs, such as DnaK and GroEL. The α-crystallin domain from residues 33 to 105 of Mx Hsp16.6 exhibits 61.1, 51.4, and 34.7% identity to SP21 of Stigmatella aurantiaca, Hsp16.6 from Synechocystis sp. strain PCC6803, and HSP16.5 of Methanococcus jannaschii, respectively. The highly conserved motif AXXT XGXL is well conserved in the C terminus of the α-crystallin domains (Fig. 1). It is interesting that nine residues (TLTLT RREE) downstream of AXXXXGXL are also highly conserved among these proteins. However, the N-terminal region preceding the α-crystallin domain of Mx Hsp16.6 shows little similarity to regions of other sHSPs. It has been demonstrated that HSP16.5 from M. jannaschii forms multimers consisting of 24 monomers and that deletion of 12 residues at the N terminus causes failure of multimer assembly, suggesting that the N-terminal region plays a role in the assembly process (13).

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cells were shifted back to 30°C, and cell viability was measured. The cells were then shifted to 43°C and incubated for 60 min. Finally, the viability of the mutant cells was 53% at 60 min after a heat shock, whereas the expression of other HSPs, such as DnaK, GroEL1, GroEL2, and GroES, was not affected by a temperature shift from 30°C to 40°C and then incubated for 60 min, since a heat shock at 40°C had less effect on the viability than a heat shock at 42°C (Fig. 3A and B). These results suggest that Mx Hsp16.6 is critical for viability during a heat shock.

It is known that a nonlethal mild heat shock increases the thermotolerance of cells, and this is known as acquired thermotolerance (12, 21). We examined whether Mx Hsp16.6 is involved in acquired thermotolerance. Cells were treated with a temperature shift from 30°C to 40°C and then incubated for 60 min, since a heat shock at 40°C had less effect on the viability than a heat shock at 42°C (Fig. 3A and B). The cells were then shifted to 43°C and incubated for 60 min. Finally, the cells were shifted back to 30°C, and cell viability was measured (Fig. 3C and D). A heat shock at 43°C was found to severely reduce the cell viability of the parent and the mutant cells without pretreatment at 40°C (Fig. 3D). However, with pretreatment at 40°C for 60 min, the parent cells started to grow 2 h after the temperature shift to 30°C, and normal growth was observed at 6 h after the temperature shift to 30°C. On the other hand, the mutant cells were notably slower to recover from a heat shock at 43°C, even with the pretreatment (Fig. 3C).

Localization of Mx Hsp16.6. It is known that sHSPs form large oligomers consisting of 9 to >32 monomers that interact with denatured proteins. In addition, the sHSPs are localized in the membrane and stabilize it during heat shock. We fractionated the cellular proteins and examined the localization of Mx Hsp16.6 (Fig. 4). The size of the pellet fraction obtained after low-speed centrifugation increased dramatically after a heat shock (data not shown), indicating that the proteins denatured by the heat shock formed large aggregates. Nearly 90% of the Mx Hsp16.6 was detected in the pellet fraction after the heat shock, and about 10% was in the membrane fraction. Mx Hsp16.6 was not detected in the supernatant fraction. These results suggest that most of the Mx Hsp16.6 was localized in aggregates with the heat-denatured proteins, while some was present in the membrane.

To further examine the localization of Mx Hsp16.6, we observed Mx Hsp16.6 in the parent and mutant cells by immunoelectron microscopy (Fig. 5). There were no differences in the cellular shape before and after heat shock, although the sizes of small vacuoles increased after the heat shock in both the parent and mutant cells. Large electron-dense particles appeared in the parent cells after the heat shock, and gold particles conjugated with anti-Mx Hsp16.6 IgG were localized within the electron-dense particles. On the other hand, a larger number of smaller denser black particles appeared in the mutant cells. It is possible that these particles in the mutant cells were aggregates of proteins irreversibly denatured by the heat shock. Mx Hsp16.6 immediately induced by a heat shock appeared to form complexes with denatured proteins, which could then be renatured after the temperature returned to the temperature under normal conditions.

**DISCUSSION**

sHSPs are 12- to 42-kDa proteins that form multimers containing 9 to >32 monomers to mediate their chaperone activities. In the case of the sHSP of *M. jannaschii*, it has been reported that Mj HSP16.5 forms a homomeric complex of 24
monomers with a hollow spherical structure (13). The N-terminal 32 residues of Mj HSP16.5 are disordered and not involved in the spherical structure. However, deletion of the first 12 residues at the N terminus results in failure of sphere assembly, suggesting that the N-terminal region plays a role in the assembly process. As shown in Fig. 1, Mx Hsp16.6 has an α-crystallin domain, in which the highly conserved motif AXXXXGXL and hydrophobic residues involved in maintaining the tertiary structure of the folding monomer are conserved. Mx Hsp16.6 was detected in the pellet fraction obtained by low-speed centrifugation and was observed in large particles by immunoelectron microscopy, suggesting that Mx Hsp16.6 forms large complexes with heat-denatured proteins and prevents irreversible denaturation of proteins caused by heat shock.

Furthermore, the mechanism of the chaperone activity of Mj HSP16.5 was investigated using the single-chain molecule monellin, which was found to bind and prevent the formation of insoluble aggregates (13). Since Mx Hsp16.6 was induced by heat shock but not by other stresses, such as starvation, oxidation, and high osmolarity, and was essential for viability during heat shock, it appears that Mx Hsp16.6 plays critical roles under heat stress conditions. It is proposed that sHSPs function as ATP-independent chaperones to prevent irreversible protein aggregation of heat-denatured proteins and facilitate subsequent protein renaturation in cooperation with ATP-dependent chaperones. It has been shown that substrates bound to Mj HSP16.5 on the outside surface of the sphere at high temperatures are prevented from forming insoluble aggregates in vitro (13). Recently, the proteins associated with Hsp16.6 from Synechocystis sp. strain PCC6803 during heat stress were identified, and they could be released from Hsp16.6 by the ATP-dependent activity of DnaK and cochaperones (2). These proteins are involved in transcription, translation, signal transduction, and secondary metabolism, which are essential for cellular viability. Since such versatile proteins form complexes with sHSPs during heat shock, it is reasonable to speculate that the hsp16.6 mutant cells lost viability during heat shock due to irreversible aggregation of a set of proteins essential for cellular viability in the mutant cells.

It has been reported that SP21 of S. aurantiaca, which is induced by heat shock, by oxidative stress, and during development, was detected at the cell periphery in heat-shocked cells and cells induced by indole for 6 h by immunoelectron microscopy. Furthermore, SP21 was predominantly located at the cell wall of spores produced during fruiting body formation (14). Even though M. xanthus and S. aurantiaca are closely related myxobacterial species and the α-crystallin domains of Mx Hsp16.6 and Sa SP21 exhibit 61.1% identity, they appear to behave differently. Since the sHSP from Synechocystis sp. was found not only to have protein-protective activity but also to stabilize lipid membranes (22), the small amount of Mx Hsp16.6 localized in the membrane may be involved in stabilization of the membrane during heat shock.

Primer extension analysis showed that the hsp16.6 gene was expressed during heat shock but not during other stress responses, such as the responses to starvation, oxidation, osmotic shock, and differentiation. Many HSPs are known to be ex-
pressed under normal conditions, whereas Mx Hsp16.6 is not detectable during normal vegetative growth. Based on characterization of the insertion mutant, Mx Hsp16.6, is neither essential for housekeeping nor necessary for the responses to starvation, oxidative stress, high osmolality, and differentiation. However, Mx Hsp16.6 is essential for viability during heat shock.

It is likely that induction of the hsp16.6 gene is regulated at the transcriptional level. M. xanthus contains three homologs of heat shock sigma factors found in bacteria (24). However, they are neither heat shock inducible nor necessary for the production of HSPs. Recently, we identified a sigma factor that shows some similarity to heat shock sigma factors, but we found that it was not necessary for the production of HSPs (26). In addition, no other homologs of heat shock sigma factors were found in the M. xanthus genome (data not shown). Thus, it is likely that heat shock response transcription in M. xanthus is driven by unique systems. In M. xanthus, only one heat shock gene, lonD, has been characterized for its heat shock response transcription (25). The ~35 and ~10 promoter elements of lonD show some similarity with those of a non-heat shock gene, vegA, while the transcription of lonD is induced via a cis element activated by a two-component system, HsfA and HsfB. Although one of the hsp16.6 promoter sequences (sequence b), which induced weaker expression than the other promoter sequence (sequence a), exhibits higher levels of similarity with the sequences of the lonD promoter and the vegA promoter (Fig. 2B), no sequence similar to the lonD cis element is present in the hsp16.6 promoter. In addition, the stronger promoter (sequence a) of the hsp16.6 gene exhibits little similarity with other known promoters, including the heat shock promoters in other bacteria. Furthermore, some heat shock genes are known to be repressed via inverted repeat sequences bound by repressor proteins during normal growth (20). Such sequences are not found in the hsp16.6 promoter. Since the expression of the hsp16.6 gene seems to be regulated by unknown mechanisms, further studies are necessary to elucidate hsp16.6 expression.

The results of the current study indicate that large quantities of Mx Hsp16.6 are induced immediately after heat shock and that Mx Hsp16.6 prevents irreversible aggregation by forming large complexes with denatured proteins to acquire thermotolerance. We are currently investigating the chaperone activity of Mx Hsp16.6 and proteins that associate with Mx Hsp16.6 during heat shock to elucidate the roles of Mx Hsp16.6 in M. xanthus.

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