Small Acid-Soluble Proteins with Intrinsic Disorder Are Required for UV Resistance in *Myxococcus xanthus* Spores

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*Bacterial sporulation in Gram-positive bacteria results in small acid-soluble proteins called SASPs that bind to DNA and prevent the damaging effects of UV radiation. Orthologs of Bacillus subtilis genes encoding SASPs can be found in many sporulating and nonsporulating bacteria, but they are noticeably absent from spore-forming, Gram-negative *Myxococcus xanthus*. This is despite the fact that *M. xanthus* can form UV-resistant spores. Here we report evidence that *M. xanthus* produces its own unique group of low-molecular-weight, acid-soluble proteins that facilitate UV resistance in spores. These *M. xanthus*-specific SASPs vary depending upon whether spore formation is induced by starvation inside cell aggregations of fruiting bodies or is induced artificially by glycerol induction. Molecular predictions indicate that *M. xanthus* SASPs may have some association with the cell walls of *M. xanthus* spores, which may signify a different mechanism of UV protection than that seen in Gram-positive spores.

When *Myxococcus xanthus* is starved for nutrients on solid media, hundreds of thousands of cells begin to aggregate and construct a macroscopic fruiting body. Inside this multicellular structure, individual rod-shaped cells begin to differentiate into spherical, dormant spores that are resistant to many types of environmental stress (43, 44). In addition to carbon starvation, it is possible to induce sporulation in liquid-grown *M. xanthus* cultures by adding glycerol (7). When added to log-phase cultures, glycerol induces individual rod-shaped vegetative cells to undergo rapid and synchronous conversion into spores. Glycerol-induced spores (glycerol spores) assume many of the morphological changes and stress-resistance properties associated with fruiting-body spores (44). Both spore types contain protein U (19), and both sporulation processes induce β-lactamase activity (31). Furthermore, mutation and gene expression studies have revealed a number of loci required for both glycerol-induced and starvation-induced sporulation (21, 29). However, multiple differences in the molecular compositions of the two spore types have been found. Glycerol-induced spores have thinner protective layers (15, 20) and lack the spore coat proteins S and C and intracellular protein W, which are seen in fruiting-body spores (16, 26, 32). While fruiting-body spores each contain two copies of their chromosomes, glycerol-induced spores have variable numbers of chromosome copies, which likely reflect the various replication states of vegetatively growing cells when glycerol induction was initiated (35, 47). Glycerol-induced spores have less intracellular trehalose but more ribosomes than their starvation-induced counterparts (25, 51).

In comparison to their vegetative counterparts, mature *M. xanthus* spores (called myxospores or microcysts) are substantially more resistant to environmental factors such as heat, desiccation, and UV light (44). *M. xanthus* spores are structurally complex, with several distinct compartments visible by transmission electron microscopy (TEM) (16, 46). The innermost compartment of a myxospore is the core, which is surrounded by inner and outer membranes, followed by an electron-dense cortex and an outer spore coat.

Studies of spore formation in Gram-positive bacteria can offer only limited clues about how sporulation occurs in Gram-negative bacteria. This is primarily due to two reasons. First, sporulation is an inherently different process between the two groups of bacteria, as a Gram-positive endospore forms inside the protective environment of the mother cells which shields the developing spore from osmotic pressure. Gram-negative spores, however, must maintain the integrity of their cell walls to counter osmotic pressure as they morph from vegetative cells to spherical spores in the absence of a protective mother cell. A second limitation to using Gram-positive sporulation characteristics to formulate hypotheses for Gram-negative sporulation is the paucity of homologs for sporulation genes in myxobacteria. *Bacillus anthracis* is a model organism extensively used for the study of spore formation. Expression studies suggest that over 500 proteins may be involved in endospore formation in *B. anthracis* (22), but scarcely any homologs for these proteins exist in *M. xanthus*. Although it is likely that *M. xanthus* also uses a large variety of proteins to construct a spore, only a few spore-specific proteins have been identified and shown to play roles in spore development within fruiting bodies (10, 14, 16, 26, 32). Furthermore, most of these *M. xanthus* sporulation proteins are not required for stress resistance of the spores. In recent studies, we identified the following four proteins that are important for *M. xanthus* sporulation and stress resistance: CbgA, MspA, MspB, and MspC (4, 46). Using proteome comparisons between vegetative cells and fruiting-body spores, our laboratory recently identified three myxospore proteins, named MspA, MspB, and MspC, that are important for stress resistance (4). Strains lacking *mspA*, *mspB*, or *mspC* formed starvation-induced spores that were more...
Plasmids

**TABLE 1. Bacterial strains and plasmids**

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<thead>
<tr>
<th>Strain or plasmid</th>
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<td>Wild-type motility and development</td>
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<td>AG681</td>
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<td>AG810</td>
<td>Plasmid insert carried by mspC; Kan′</td>
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<tr>
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<td>pJD0619</td>
<td>317-bp fragment extending from bp 74 to 588 of the sapA gene</td>
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**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** Bacterial strains and plasmids used in this study are listed in Table 1. The wild-type strain DK1622 (17) was used as the parental strain from which mutants were generated. The sapA (mxan_7407) gene was inactivated in strain DK1622 by single-crossover recombination of a kanamycin-resistant plasmid containing an internal PCR-generated fragment of sapA, as previously described (2, 46). Briefly, PCR primers 5′-CAACAACTGGGA GAACCTGG-3′ (forward primer) and 5′-GTTCACTCCAGTCTGCGC-3′ (reverse primer) were used to amplify a 317-bp internal region of the gene that was cloned into plasmid pCR2.1-TOPO (Invitrogen) to create plasmid pJD0619. *M. xanthus* strain DK1622 was electroporated with purified pJD0619 before selecting for single-crossover-event cells on CTTYE agar (1% Casitone, 0.5% yeast extract, 10 mM Tris-HCl [pH 8.0], 1 mM KH2PO4, 8 mM MgSO4, 1.5% agar) with kanamycin (40 μg/ml). Insertion of pJD0619 into the sapA gene was confirmed by PCR and Southern blot analysis (data not shown), and the confirmed sapA mutant was named JD0619. Fruiting-body development occurred on TPM agar, as previously described (2, 4). Induction of spore formation using 0.5 M glycerol was performed as previously described (7).

**Preparation of protein lysates and one-dimensional SDS-PAGE analysis.** *M. xanthus* cells were grown in CTTYE to Klett readings of 100 before pelleting cells and lysing them by vortexing with 0.1-mm-diameter glass beads in dithiothreitol (DTT) lysis buffer, as previously described (4). Whole cells and large-cell debris were removed by centrifugation at 12,000 × g for 5 min at 4°C before saving clarified supernatants for precipitation. Trichloroacetic acid (TCA) precipitation of myxococcal proteins was prepared using a modification of the procedure originally described by Natarajan et al. (30). Briefly, TCA was added to clarified lysates to a final concentration of 5% (wt/vol) before incubation on ice for 45 min and then centrifugation for 20 min at 12,000 × g at 4°C. The insoluble pellet was considered the 2.5% TCA-insoluble fraction, and the supernatant was saved and considered the 2.5% TCA-soluble fraction. Further sequential precipitations of 5% and 25% TCA were performed. TCA-precipitated pellets were washed with ice-cold acetone containing 5% HCl, aspirated, dried for 2 min in a 40°C heat block, suspended in 1 × SDS sample buffer (125 mM Tris base, 20% glycerol, 2% SDS, 2% β-mercaptoethanol, 0.001% bromophenol blue), and underwent 12% SDS-polyacrylamide gel electrophoresis (PAGE). Resulting gels were stained with Coomassie brilliant blue, and protein bands were photographed and excised for identification.

**Identification of separated proteins.** Coomassie blue-stained protein bands were excised from 12% polyacrylamide gels and destained for 2 h in a solution of 50% methanol plus 5% glacial acetic acid in distilled water. Gel bands were dehydrated with acetonitrile, followed by reduction and alkylation with 10 mM DTT plus 50 mM iodoacetamide in 100 mM NH4HCO3, dehydrated, rehydrated in 100 mM NH4HCO3, dehydrated again, and digested with trypsin (20 ng/μl) in ice-cold 50 mM NH4HCO3. Samples were incubated overnight at 37°C with 20 μl of 50 mM NH4HCO3. After this incubation, the solutions containing the digested peptides were desalted and concentrated using C18 ZipTip pipette tips (Millipore). Samples were analyzed by matrix-assisted laser desorption/ionization (MALDI) using the Voyager DE RF system (Applied Biosystems). To identify the proteins, the Mascot database (Matrix Science) was searched for monoisotopic peptide masses between 700 and 4,000 Da detected in the samples.

**Electron microscopy.** Scanning electron microscopy (SEM) of fruiting bodies of wild-type *M. xanthus* and the mutant strain JD0619 lacking sapA was performed as previously described (4). Samples were analyzed by a Hitachi S570 SEM, with images being captured with the PCI quartz imaging program.
Stress resistance assays. Vegetatively growing cells and fruiting-body and glycerol-induced spores were assayed for UV resistance at a wavelength of 254 nm and an intensity of 31 μW/cm², as previously described (4). Light microscopy of methylene blue-stained cells was performed to ensure a lack of clumping to cells prior to UV exposure. After cells were subjected to increasing exposure to UV light, they were allowed to recover on CTTYE agar plates with or without kanamycin sulfate (40 μg/ml) before enumeration of survivors by counting the numbers of CFU (numbers of CFU/ml). Spores were also subjected to the stresses of sonication, including heat, SDS, and lysozyme, as previously described (4, 46).

Predictions of protein intrinsic disorder and phosphorylation. Protein disorder for spore proteins was predicted by the algorithms PONDR VSL2 (34) and PONDR VI-XT (13). TMpred (transmembrane prediction) is an algorithm used to predict membrane-spanning regions (12), and PSIPRED (27) is an algorithm used to predict protein structure; both were applied to the myxospore acid-soluble proteins indentified here.

RESULTS

Acid precipitation identifies fruiting-body spore-specific proteins. To investigate whether or not M. xanthus expressed acid-soluble proteins, concentrations of trichloroacetic acid (TCA) precipitations were performed on cell lysates from both vegetatively growing cells and from 5-day-old fruiting-body spores. SDS-PAGE analysis showed that all proteins in the vegetative cell lysates precipitated with 2.5% TCA (Fig. 1, lane 2 and 3). However, two low-molecular-weight proteins in fruiting-body spore lysates were soluble in 2.5% TCA (lanes 2 and 6), 5% TCA (lanes 3 and 7), and 25% TCA (lanes 4 and 8) are shown. Molecular mass standards are shown in lanes 1 and 5.

FIG. 1. SDS-PAGE (12%) analysis of acid-soluble proteins from wild-type M. xanthus strain DK1622. Vegetative, liquid-grown cells (lanes 2 to 4) and 5-day-old fruiting-body spores (lanes 6 to 8) were washed in Tris-buffered saline, lysed by sonication, and subjected to precipitation by increasing concentrations of trichloroacetic acid (TCA). Pelleted proteins were resuspended in running buffer and subjected to SDS-PAGE separation before Commissase blue staining. Protein pellets from 2.5% TCA (lanes 2 and 6), 5% TCA (lanes 3 and 7), and 25% TCA (lanes 4 and 8) are shown. Molecular mass standards are shown.

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Stigmatella aurantiaca, predicted to have 184 amino acids sharing 39% identity with SapA from M. xanthus. The lower-molecular-mass SapB band shown in Fig. 1 (arrowhead) was identified as the gene product of mxan_3885, which is also listed in the M. xanthus annotated genome as a hypothetical protein.

Fruiting body myxospores lacking SapA are more sensitive to UV radiation than WT spores. The sapA gene (mxan_7407) appears to be the last gene in an operon (Fig. 2A). This gene was inactivated by homologous recombination with a plasmid carrying an internal PCR product of mxan_7407, which results in loss of SapA from the TCA-soluble fraction of the fruiting-body spore lysates (Fig. 2B, compare lane 2 to lane 1). Compared to the wild-type strain, the sapA mutant strain JD0619 did not show any alteration in growth rate in liquid CTTYE media (data not shown). However, JD0619 did show moderate alteration in fruiting-body formation on TPM starvation agar. Lane 1, wild-type strain DK1622; lane 2, mutant strain JD0619. The positions of the SapA and SapB proteins are indicated by arrows.

FIG. 2. Inactivation of sapA in M. xanthus. (A) Orientation of the sapA gene (mxan_7407) (dark arrow) in the M. xanthus chromosome relative to that of the surrounding genes (image from NCBI; http://www.ncbi.nlm.nih.gov). (B) Commissase blue-stained 12% SDS-PAGE gel showing 2.5% TCA-soluble proteins from 5-day-old fruiting bodies on TPM starvation agar. Lane 1, wild-type strain DK1622; lane 2, mutant strain JD0619. The positions of the SapA and SapB proteins are indicated by arrows.

The gene encoding this protein (mxan_7407) is also listed in the M. xanthus annotated genome as a hypothetical protein.
important role in UV resistance in *M. xanthus* fruiting-body spores. To determine if SapA enhanced resistance against other forms of stress, fruiting-body spores of DK1622 and JD0619 were further compared for survival. Whereas both spore types showed equal resistance levels to heat (55°C) and sonication (setting of 4.5 with a model 100 Sonic Disembrator [Fisher]), there was a 3-fold decrease in the survival rate of JD0619 spores with regard to SDS resistance and a 2-fold decrease in lysozyme resistance (data not shown). This suggests that fruiting-body spores of the *sapA* mutant may have slightly altered surfaces to cause increased sensitivity to this detergent and enzyme.

Acid-soluble proteins of glycerol-induced spores are different from the acid-soluble proteins of fruiting-body spores. Just as starvation-induced fruiting-body spores were examined for acid-soluble proteins, we also examined glycerol-induced spore lysates. Figure 5A shows ultrastructural comparisons between fruiting-body spores (top) and glycerol-induced spores (bottom) of a wild-type strain. Both spore types are similar in size and shape, with a noticeable difference being that glycerol-induced spores have a reduced electron-dense outer spore coat. Although SapA and SapB were present in acid-soluble fractions from fruiting-body spores (Fig. 5B, lane 1), both proteins are absent from acid-soluble fractions of glycerol-induced spores (Fig. 5B, lane 2). Two different and higher-molecular-weight, acid-soluble proteins were visible in the glycerol spore fraction, and they were identified by peptide mass fingerprinting as MspB and MspC (Fig. 5B, lane 2). These two proteins were recently discovered in our laboratory and characterized for their roles in spore stress resistance (4).

To verify that these two acid-soluble proteins in glycerol-induced spores were MspB and MspC, mutant strains AG710 (*mspB* mutant) and AG810 (*mspC* mutant) were induced by 0.5 M glycerol for spore formation, and cell lysates were subjected to TCA precipitations (Fig. 5C). Glycerol spores of AG810 lack MspC in their acid-soluble protein fractions, and spores of AG710 lack MspB in their acid-soluble fractions (Fig. 5C, lanes 3 and 4, respectively). Because MspA was discovered with MspB and MspC under the same sporulation conditions (4), we examined acid-soluble proteins of the *mspA* mutant strain AG681 but found no difference in protein patterns from those of wild-type cells (Fig. 5C, compare lanes 1 and 2). The absence of *mspB* or *mspC* did not affect the rate of glycerol-induced formation of coccid-shaped spores over a 4-h period, as determined by light microscopy (Fig. 6A). In wild-type DK1622 cells, glycerol induction of MspB and MspC expression is seen early, and these proteins accumulate with prolonged cell exposure to glycerol (Fig. 6B). Therefore, while the MspB and MspC proteins are part of glycerol spores, they are not required for the morphogenesis of glycerol-induced spores.

Glycerol-induced myxospores lacking MspB or MspC are more sensitive to UV radiation. Previously we reported that fruiting-body myxospores lacking MspB or MspC are not altered in UV sensitivity (4). This is not the case, however, with glycerol-induced mutant spores. While vegetatively growing mutant strains show no difference in UV sensitivity from wild-type cells (Fig. 7, dashed lines), the absence of MspB or MspC
from glycerol spores results in an almost 100-fold increase in UV sensitivity compared to that of corresponding wild-type spores (Fig. 7, solid lines).

Predictions of acid-soluble protein structures indicate that they have disordered regions and are membrane associated. Computer programs have been used to predict the level of secondary structure disorder within proteins. Disorder means an absence of predicted secondary structures like α-helices and β-sheets. PONDR (Predictor of Naturally Disordered Regions) is a neural network predictor that was trained with over 1,000 proteins with known intrinsic disorder that has been experimentally confirmed by X-ray crystallography and nuclear magnetic resonance (NMR) analyses (5, 6). The predictor looks at sliding windows of 9 to 21 amino acids, factoring in amino acid composition, hydrophobicity, charge, and other sequence attributes. A PONDR score of 0.0 is an ideal prediction of order, while a score of 1.0 is an ideal prediction of disorder. Any score greater than 0.5 is considered to predict disorder. PONDR analysis predicts regions of order containing α-helices and β strands and large contiguous regions of pre-
dicted disorder (see Fig. S1, S2, S3, and S4 in the supplemental material). SapA (see Fig. S2), MspB (see Fig. S3), and MspC (see Fig. S4) each contain potential transmembrane regions, as predicted by TMpred. Recently, it has been shown that sites of protein phosphorylation reside in disordered regions, and this suggests that intrinsic disorder surrounding phosphorylation sites may be a prerequisite for phosphorylation (18, 34). Depp (Disorder enhanced phosphorylation predictor) analysis uses a neural network predictor trained with over 1,500 known (i.e., experimentally confirmed) phosphorylation sites and takes advantage of the observation that residues adjacent to protein phosphorylation sites have sequence attributes similar to those of residues of intrinsic disorder regions. Depp analysis reveals that MspB has 20 Ser and 14 Thr residues in predicted disordered regions (see Fig. S3 in the supplemental material) and that MspC has 5 Ser and 15 Thr residues in disordered regions (see Fig. S4). SapA has five Ser residues in predicted disordered regions (see Fig. S2).

**DISCUSSION**

Sporulation appears to be such an important phenomenon in the bacterial domain that groups as divergent as Gram-positive and Gram-negative bacteria perform it, albeit through two very different processes. Despite differences in the mechanisms, however, both groups produce spores with UV resistance properties that are appreciably greater than those for vegetatively growing cells. Found in soils around the globe, myxobacteria clearly live in environments exposed to UV radiation. Examination of UV resistance in vegetative *M. xanthus* cells reveals the possibility that the yellow pigment of vegetative *M. xanthus* colonies provides some degree of UV protection (1). *M. xanthus* spores are about 80-fold more resistant to UV light than their vegetative counterparts (44); however, the molecular basis for this enhanced UV resistance in *M. xanthus* spores is unknown. Whereas an extensive series of studies have examined UV resistance in Gram-positive spores, there are no recognizable homologs for α/β-type SASPs in *M. xanthus* (no *M. xanthus* proteins with BLAST E values of <0.38 when the genome was searched with SASP sequences). This is despite the observation that myxospores clearly have enhanced UV resistance compared to that of their vegetative cell counterparts. Evidence is shown here that proteins exist in *M. xanthus* spores that are absent from vegetative cells and that these proteins are linked to sensitivity to UV radiation. Comparisons of mutant and wild-type strains indicate that SapA contributes to UV resistance in fruiting-body spores (Fig. 4), while MspB and MspC facilitate UV resistance in glycerol-induced spores (Fig. 7). These findings raise a series of questions concerning the function of these proteins that potentially play vital roles in myxobacteria. If SapA, MspB, and MspC are found to be associated with the protective layers of myxospores and not with the spore cytoplasm, then they may signify that a completely different mechanism of UV protection exists in Gram-negative spores than that seen in Gram-positive spores. Unlike the SASPs involved in UV protection in *B. subtilis*, acid-soluble proteins from myxospores appear to be membrane associated based upon hydropathy analysis (see Fig. S2, S3, and S4 in the supplemental material). However, any such membrane associations for SapA, MspB, and MspC will have to be empirically determined.

MspB and MspC were first identified by proteomic analysis of fruiting-body spores (4), so it was unexpected that they would be present in the TCA-soluble fraction of glycerol spores but not of fruiting-body spores (Fig. 5B). There are at least three possible hypotheses for the absence of MspB and MspC from the acid-soluble fraction of fruiting-body spores. First, it is possible that the relative levels of *mspB* and *mspC* expression are much greater in glycerol spores than in fruiting-body spores. Recently, it was shown through microarray analysis that *mspC* expression increases more than 200-fold during glycerol-induced sporulation (29). Second, it is possible that in fruiting-body spores, MspB and MspC are complexed with proteins or cell structures that precipitate with TCA, while this is not the case in glycerol spores. Lastly, it is possible that MspB and MspC can exist in two different conformational states, with a structured conformation (i.e., precipitated by TCA) in fruiting-body spores and an intrinsically disordered conformation (i.e., soluble in TCA) in glycerol spores. The second and third hypotheses are not incompatible, since disordered proteins will often acquire tertiary structures upon binding with other molecules. SapA and SapB proteins may be missing from the acid-soluble fraction of glycerol spores because they are not expressed in these spores or because they exist in ordered conformations in glycerol spores that result in their precipitation by TCA.

Traditionally, studies of intracellular signaling cascades in bacteria have focused upon the reversible phosphorylations of histidine and aspartate residues in two-component signaling systems. However, there is a growing appreciation of the prevalence of eukaryotic-like serine/threonine kinases in bacteria (49, 50). The *M. xanthus* genome has revealed upwards of 99 potential protein serine/threonine kinases (PSTKs), which exceeds the number of PSTKs seen in other bacteria (for example, *B. subtilis* has only 1 PSTK). The complexity of PSTKs in *M. xanthus* is likely required for the intricate signaling cascades needed for cell movement, development, sporulation, and germination. Although the MspB and MspC proteins lack any homology to eukaryotic PSTKs, these two proteins may be involved in phosphorylation because their numerous Ser and Thr residues reside in predicted disordered regions. However, the roles of these residues in protein function await analysis by site-directed mutagenesis.

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**REFERENCES**