Characterization of McuB, a Periplasmic Chaperone-Like Protein Involved in the Assembly of *Myxococcus* Spore Coat

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The MXAN3885 to -3882 gene locus cluster (designated here *mcuABCD*) of *Myxococcus xanthus* encodes a member of the archaic chaperone-usher (CU) systems that functions in spore coat formation. We show here that McuD, a putative spore coat protein, affects cellular accumulation and cell surface localization of the spore coat protein McuA. We previously reported that genetic disruption of the putative usher McuC nearly eliminates surface display of McuA and show here that lack of the periplasmic chaperone-like protein McuB, which forms a complex with McuA, has a similar effect. Deletion mutation confirms that the G1 β strand of McuB is absolutely essential for the stability and secretion of McuA. Site-directed mutagenesis identified two additional alternating hydrophobic residues Ile113 and Val115, together with the highly conserved proline within the G1 strand, as critical residues for chaperone function. These findings suggest that the assembly proteins McuB and McuC mediate the transport of McuA onto the cell surface and that McuA may interact with another spore coat protein, McuD, for its secretion. Importantly, although our data argue that the *M. xanthus* CU system is likely to use the basic principle of donor strand complementation (DSC), as in the cases of classical CU pathways, to promote folding and stabilization of the structural subunit(s), the periplasmic chaperone McuB appears to exhibit structural variation in mediating chaperone-subunit interaction.

Many Gram-negative bacteria display nonflagellar proteinaceous organelles on their outer surfaces. These adhesive extracellular structures, called pili or fimbriae, mediate bacterial attachment to host cells and play a key role in the pathogenicity of a wide range of infectious diseases. It has now become clear that pili can be classified into five major groups based on their biosynthetic pathways and that the chaperone-usher (CU) pili form the most abundant group of bacterial cell surface appendages (1). The CU biosynthetic pathway involves two nonstructural assembly components: a specialized periplasmic chaperone and an outer membrane protein called the usher. The chaperone binds and facilitates folding of pilus structural subunits, prevents them from aggregation or degradation in the periplasm, and targets them to the usher. Interactions between the chaperone-subunit complex and the usher release pilus subunits, which are subsequently exported through the usher channel for assembly into pilus fibers and secretion into the cell surface (2, 3, 4). Based on phylogenetic analysis of usher sequences, CU pili are divided into six major clades designated α , β , γ , κ , π , and σ (5). The analysis also reveals that the σ -fimbriae comprise an archaic CU family whose members share limited or no sequence homology with members of the alternate (α -fimbriae) or the classical (β -, γ -, κ -, and π -fimbriae) CU families. Within the CU assembly class, the archaic CU system is the most widely distributed, with representatives being present not only in *Alpha*-, *Beta*-, *Gamma*-, and *Deltaproteobacteria* but also in the phyla *Cyanobacteria* and *Deinococcus-Thermus*. In contrast, representatives of the classical and alternate CU systems are restricted only to *Beta*- and *Gamma*proteobacteria (5).

Current knowledge of the pilus assembly process has been largely derived from the uropathogenic *Escherichia coli* type I and P pili, both of which are members of the classical CU family. In addition, the capsular F1 antigen from the plague pathogen *Yersinia pestis* serves as a prototype for atypical and nonpilus organelles assembled by the classical CU pathway (6, 7). The subunits of these pilus and nonpilus protein fibers are characterized by an

incomplete immunoglobulin (Ig)-like fold that lacks the C-terminal β strand. As a result, all subunits possess a solvent-exposed hydrophobic groove. The chaperone stabilizes the subunit by inserting its G1 β strand into this groove, a process termed donor strand complementation (DSC) (4, 8). In the chaperone-subunit interactions, the chaperone G1 strand runs parallel to strand F in the subunit, resulting in an atypical Ig fold that maintains the subunit in a polymerization-competent state. Pilus subunits polymerize using the same interaction groove as DSC. During polymerization, the complementing G1 strand donated by the chaperone is replaced by the N-terminal extension (Nte) of 10 to 20 residues on the incoming subunit, a process termed donor strand exchange (DSE) (4, 8). This mechanism has been implicated in the assembly of a number of Gram-negative surface organelles of various morphologies, all belonging to pili formed either by the classical CU pathway or by the alternate CU pathway (9, 10, 11, 12, 13). Given the wide phylogenetic distribution of gene clusters belonging to the σ -fimbriae (i.e., the archaic CU family), surprisingly little is known about the morphology or function of the encoded surface structures, let alone the mechanisms of pilus biogenesis mediated by the archaic CU pathway (5).

Myxococcus xanthus is a Gram-negative soil bacterium which, in response to nutrient deprivation, initiates a developmental program to form multicellular structures called fruiting bodies. Around 24 h after starvation, rod-shaped cells inside the nascent fruiting body begin differentiating into spherical spores which mature over the next 48 h (14, 15). Mature spores are surrounded

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FIG 1 Homology modeling of McuB (A), McuA (B), and McuD (C). Six templates (refer to Materials and Methods) were used to generate a Phyre-threaded final model of McuB. The predicted structure consists of two Ig-like domains arranged in an L-shape, as in the cases of classical CU chaperones PapD and FimC. Both McuA and McuD were modeled based on a single highest scoring template, the type 1 pilus subunit FimG of *E. coli*.

by thick protein coats and become stress resistant. Based on *in silico* analysis, Nuccio and Bäumlér proposed that the genes at loci MXAN3885 to -3882 (named here *mcuABCD* for *Myxococcus* chaperone-usher-like) of *M. xanthus* encode an archaic CU-like system that might be involved in assembly of the spore coat, a nonpilus structure on cell surfaces (5). Later, our experimental data supported the notion that the operon *mcuABC* functions in spore coat biogenesis, highlighting the structural diversity of proteinaceous fibers assembled by the CU pathway (16). This allows us to use *M. xanthus* as a tractable system for further elucidation of the mechanisms of pilus assembly by members of the archaic CU family.

The archaic, alternate, and classical CU assembly pathways are distinguished by the absence of significant primary sequence similarities in their assembly and structural components. Secondary-structure predictions demonstrated that both the spore coat protein McuA and the putative periplasmic chaperone McuB have an Ig-like fold (Fig. 1), implying that McuB might bind McuA in a manner that is basically similar to that of the DSC of the PapD-PapK and FimC-FimH chaperone-subunit interactions (17, 18). In this work, we show that McuD, a predicted spore coat protein-encoding gene located immediately downstream of the *mcuABC* operon, plays a role in cell surface display of McuA. We also show that McuB interacts with McuA. Moreover, a large part of this study addresses the significance of certain amino acid residues of McuB for chaperone function. Our studies indicate that the structural basis of McuA-McuB interaction is similar, but not identical, to that of the classical periplasmic chaperone-subunit interactions.

MATERIALS AND METHODS

Cell growth and development. *Escherichia coli* JM83 was grown in LB broth in the presence of relevant antibiotics. *M. xanthus* strains were grown in CTT media (1% Casitone, 8 mM MgSO₄, 10 mM Tris-HCl [pH 7.6], 1 mM potassium phosphate, pH 7.6) as previously described (19). Kanamycin (Km) or oxytetracycline (Tc) was used for selection at a concentration of 40 µg ml⁻¹ or 12.5 µg ml⁻¹, respectively. *M. xanthus* fruiting body development was induced on TPM agar (10 mM Tris-HCl [pH 7.6], 1 mM KH₂PO₄, 8 mM MgSO₄, 1.5% Difco Bacto agar).

***M. xanthus* strains.** *Myxococcus xanthus* strains used in this work are listed in Table 1. DK1622 (20) was used as the parent wild-type (wt) strain for all *M. xanthus* strains throughout this study. All strains constructed were confirmed by PCR.

Secondary- and tertiary-structure predictions. Sequence-based structural relatives of McuA, McuB, and McuD were searched with the Protein Homology/analogy Recognition Engine Phyre (21). For McuB, the following six templates were returned as the best hits: F1 capsule assembly chaperone Caf1M of *Yersinia pestis* (Protein Data Bank code 1Z9S), Saf pilus assembly chaperone SafB of *Salmonella enterica* (2CO7), type 1 pilus assembly chaperone FimC of uropathogenic *Escherichia coli* (1QUN), CupB pilus assembly chaperone CupB2 of *Pseudomonas aeruginosa* (3Q48), S pilus chaperone SfaE of *E. coli* (1L4I), and P pilus assembly chaperone PapD of *E. coli* (1QPX). The homology match between McuB and each of the six templates is 100%. The final three-dimensional (3D) structure of McuB shown in Fig. 1 was generated based on these templates, with 95% of the residues modeled at >90% confidence. For both McuA and McuD, the type 1 pilus subunit FimG (code 3BFW) of *E. coli* was returned as the highest scoring template. Based on this template, McuD was modeled with 97.5% confidence and 94% coverage whereas McuA was modeled with 97.7% confidence and 84% coverage. The Phyre consensus secondary structure of McuB is based on Psi-pred, JNet, and SSPro.

Construction and expression of McuB regional deletion and point mutations. Regional deletion mutation and site-specific substitutions within McuB were prepared by the method of two-step PCR (22). A plasmid, pMP-*mcuABC* (Table 1), which contains a 1.8-kb insertion carrying some 5'-terminal codons of *mcuC* and the entire open reading frames (ORFs) of *mcuAB* together with the putative promoter region, was used as the template for PCR. Two overlapping PCR fragments were amplified in separate reactions with the external forward primer P1 and a deletion or point mutation-containing internal reverse primer and with the external reverse primer P2 and a deletion or point mutation-containing forward internal primer (Table 2). The two overlapping products from these two PCRs were mixed and used as the templates for a second PCR mixture containing only the external primers P1 and P2. All final PCR products were gel purified, digested with EcoRI and HindIII, and ligated to pBlue-script cut with the same enzymes followed by transformation into *E. coli* JM83. All deletion and point mutations thus obtained were confirmed by DNA sequencing, and the resulting proteins were designated by the region or the residue(s) of mutation. The 1.8-kb fragment in pMP-*mcuABC* was then replaced by an XbaI-PstI fragment of an *mcuA* or *mcuB* mutant version in pBlue-script to create pMP-X, where X denotes a mutant gene. For expression of each mutant protein, the resulting plasmids were separately integrated at the phage Mx8 *attB* gene after electroporation (23) into the Δ *mcuB* strain.

Construction of in-frame deletion Δ *mcuB* and Δ *mcuD* strains. Plasmids pBJ113- Δ *mcuB* and pBJ113- Δ *mcuD* are pBJ113 derivatives (24) generated to create in-frame deletions of the *M. xanthus* *mcuB* and *mcuD* genes, respectively. To construct pBJ113- Δ *mcuB*, a 1-kb fragment upstream of the *mcuB* ORF and a 1-kb fragment downstream of the *mcuB*

TABLE 1 Plasmids and strains

Plasmid or strain	Relevant characteristic(s)	Reference
Plasmids		
pBJ113	Vector for constructing in-frame deletion; Gal ^s , Km ^r	24
pBJ113- Δ <i>mcuB</i>	pBJ113 carrying <i>mcuB</i> in-frame deletion	This study
pBJ113- Δ <i>mcuD</i>	pBJ113 carrying <i>mcuD</i> in-frame deletion	This study
pMP- <i>mcuAB</i> _{his6}	pMP220-based vector for expression of <i>mcuB</i> _{his6} from its native promoter; Tc ^r	This study
pMP- <i>mcuAB</i>	pMP220-based vector for expression of untagged <i>mcuB</i> from its native promoter; Tc ^r	This study
pMP- <i>mcuABC</i>	<i>mcuABC-lacZ</i> transcriptional fusion containing the entire <i>mcuA</i> and <i>mcuB</i> sequences and some of the N-terminal coding sequence of <i>mcuC</i> ; used here as <i>mcuB</i> wt expression vector; Tc ^r	Identical to pMP-MXAN3883 in reference 16
pMP- <i>mcuB</i> Δ G1	pMP- <i>mcuABC</i> with <i>mcuB</i> coding sequence from L107 to V115 deleted	This study
pMP- <i>mcuB</i> -L107TV109T	pMP- <i>mcuABC</i> carrying <i>mcuB</i> L107TV109T mutation	This study
pMP- <i>mcuB</i> -T111L	pMP- <i>mcuABC</i> carrying <i>mcuB</i> T111L mutation	This study
pMP- <i>mcuB</i> -I113T	pMP- <i>mcuABC</i> carrying <i>mcuB</i> I113T mutation	This study
pMP- <i>mcuB</i> -I113TV115T	pMP- <i>mcuABC</i> carrying <i>mcuB</i> I113TV115T mutation	This study
pMP- <i>mcuB</i> -P116K	pMP- <i>mcuABC</i> carrying <i>mcuB</i> P116K mutation	This study
pMP- <i>mcuB</i> -P116A	pMP- <i>mcuABC</i> carrying <i>mcuB</i> P116A mutation	This study
pMP- <i>mcuB</i> -R9G	pMP- <i>mcuABC</i> carrying <i>mcuB</i> R9G mutation	This study
pMP- <i>mcuB</i> -R9 M	pMP- <i>mcuABC</i> carrying <i>mcuB</i> R9 M mutation	This study
<i>M. xanthus</i> strains		
DK1622	Wild type	20
Δ <i>mcuB</i> strain	In-frame deletion of <i>mcuB</i>	This study
Δ <i>mcuD</i> strain	In-frame deletion of <i>mcuD</i>	This study
Δ <i>mcuB</i> /pMP- <i>mcuABC</i> strain	Δ <i>mcuB</i> attB::pMP- <i>mcuABC</i>	This study
Δ <i>mcuB</i> /pMP- <i>mcuB</i> Δ G1 strain	Δ <i>mcuB</i> attB::pMP- <i>mcuB</i> Δ G1	This study
Δ <i>mcuB</i> /pMP- <i>mcuB</i> -L107TV109T strain	Δ <i>mcuB</i> attB::pMP- <i>mcuB</i> -L107TV109T	This study
Δ <i>mcuB</i> /pMP- <i>mcuB</i> -T111L strain	Δ <i>mcuB</i> attB::pMP- <i>mcuB</i> -T111L	This study
Δ <i>mcuB</i> /pMP- <i>mcuB</i> -I113T strain	Δ <i>mcuB</i> attB::pMP- <i>mcuB</i> -I113T	This study
Δ <i>mcuB</i> /pMP- <i>mcuB</i> -I113TV115T strain	Δ <i>mcuB</i> attB::pMP- <i>mcuB</i> -I113TV115T	This study
Δ <i>mcuB</i> /pMP- <i>mcuB</i> -P116K strain	Δ <i>mcuB</i> attB::pMP- <i>mcuB</i> -P116K	This study
Δ <i>mcuB</i> /pMP- <i>mcuB</i> -P116A strain	Δ <i>mcuB</i> attB::pMP- <i>mcuB</i> -P116A	This study
Δ <i>mcuB</i> /pMP- <i>mcuB</i> -R9G strain	Δ <i>mcuB</i> attB::pMP- <i>mcuB</i> -R9G	This study
Δ <i>mcuB</i> /pMP- <i>mcuB</i> -R9 M strain	Δ <i>mcuB</i> attB::pMP- <i>mcuB</i> -R9 M	This study
Δ <i>mcuB</i> /pMP- <i>mcuAB</i> _{his6} strain	Δ <i>mcuB</i> attB::pMP- <i>mcuAB</i> _{his6}	This study
Δ <i>mcuB</i> /pMP- <i>mcuAB</i> strain	Δ <i>mcuB</i> attB::pMP- <i>mcuAB</i>	This study

ORF were PCR amplified using primer pair McuBH1HindIII and McuBH1BamHI and primer pair McuBH2BamHI and McuBH2EcoRI, respectively (Table 2). The two amplified products flanking the *mcuB* ORF were digested, ligated, and cloned into pBJ113 to obtain plasmid pBJ113- Δ *mcuB*. pBJ113- Δ *mcuD* was constructed in a similar way. After being verified by DNA sequencing, plasmids were introduced into *M. xanthus* DK1622 by electroporation and transformants were selected on CTT plates containing 40 μ g ml⁻¹ kanamycin. Individual Km^r transformants were then grown in CTT broth in the absence of kanamycin and plated onto CTT plates supplemented with 1% galactose (Gal) for negative selection. PCRs were used to screen Gal^r and Km^s colonies for proper excision of the wild-type copy as described previously (25).

Construction of the strains expressing His-tagged or untagged McuB. pMP-*mcuAB*_{his6}, a plasmid expressing C-terminal His-tagged McuB (McuB_{his6}), was constructed by replacing the 1.8-kb XbaI-PstI fragment in pMP-*mcuABC* with a PCR-generated fragment containing the putative promoter region of *mcuABC*, the entire *mcuA* ORF, and the entire coding sequence of *mcuB* with the addition of six histidine codons immediately upstream of the *mcuB* stop codon. The Δ *mcuB*/pMP-*mcuAB*_{his6} strain, which is a Δ *mcuB* derivative carrying pMP-*mcuAB*_{his6} at attB, was used for the expression of McuB_{his6}. The Δ *mcuB*/pMP-*mcuAB*

strain was constructed in a similar way except that McuB was expressed without a His tag.

Immunoblot analysis. Accumulation of McuA protein in *M. xanthus* strains was analyzed by immunoblotting. Developmental cells were harvested at the indicated times, and protein samples were prepared as previously described (16). For each sample, 5- μ l aliquots representative of equal numbers of cells were applied to the lanes. Blots were probed with rabbit anti-McuA serum followed either by anti-rabbit IgG conjugated to alkaline phosphatase with chromogenic substrates or by peroxidase-conjugated anti-rabbit IgG with chemiluminescence reagent.

Pulldown assays. McuB-McuA interaction was tested by nickel bead pulldown. Cells of the Δ *mcuB*/pMP-*mcuAB*_{his6} strain or the Δ *mcuB*/pMP-*mcuAB* strain (as a control) were grown to a density of approximately 5×10^8 cells ml⁻¹ in CTT liquid broth with oxytetracycline, harvested, resuspended in TPM buffer to a density of 5×10^9 cells ml⁻¹, and spotted on TPM agar (20 μ l/spot). Cells from 20 spots harvested at 21 h poststarvation were washed in wash buffer (50 mM NaH₂PO₄, 100 mM NaCl, 5 mM MgCl₂, pH 8.0) before being resuspended in 500 μ l of lysis buffer (50 mM NaH₂PO₄, 100 mM NaCl, 5 mM MgCl₂, 20 mM imidazole, 5 μ l protease inhibitor cocktail [Calbiochem], pH 8.0). The cells were lysed by sonication, and cell debris was removed by centrifugation at 8,000 rpm at 4°C for

TABLE 2 Primers used in this study

Name	Sequence (5'–3') ^a	Description
Primers for generating regional deletion and point mutations		
P1	GCTCTAGATTTCGCCAGAAGTGGGTAGCAAGGAC	External primer with XbaI site, forward
P2	TTCTGCAGCGCGGGTTATTGCAAGCCGGGAGGG	External primer with PstI site, reverse
ΔG1	AAGACAGGCCCGTTGTCGGGGTG CAACGGGGCCTGTCTTTGTCGCGCC	Internal primer for ΔG1, reverse Internal primer for ΔG1, forward
L107TV109T	GATTCGGGTGAGCGTATTGGTCCCGTTGTCGGGGTG CACCCGACAACGGGGGaccAATacgCTCACCCGAATC	Internal primer for L107TV109T, reverse Internal primer for L107TV109T, forward
T111L	CAGGCACTGAGATTCGGAGGAGGACATTCAACCCCG CGGGGTTGAATGTCCTCctCGGAATCTCAGTGCCTG	Internal primer for T111L, reverse Internal primer for T111L, forward
I113T	ACAAAGACAGGCACTGAGGTTTCGGGTGAGGACATTC GAATGTCTCTACCCGAAcCTCAGTGCCTGTCTTTGT	Internal primer for I113T, reverse Internal primer for I113T, forward
I113TV115T	ACAAAGACAGGGGTTGAGGTTTCGGGTGAGGACATTC GAATGTCTCTACCCGAAcCTCAaccCCTGTCTTTGT	Internal primer for I113TV115T, reverse Internal primer for I113TV115T, forward
P116K	TGGGCGCGACAAAGACCTTCACTGAGATTTCGGGTGA TCACCCGAATCTCAGTgaagGTCTTTGTCGCGCCCA	Internal primer for P116K, forward Internal primer for P116K, reverse
P116A	TGGGCGCGACAAAGACGGCCACTGAGATTTCGGGTGA TCACCCGAATCTCAGTgGcGTCTTTGTCGCGCCCA	Internal primer for P116A, forward Internal primer for P116A, reverse
R9G	TTGAATCCAATTCAAGgCcAACCGGACTGACATCGA TCGATGTCAGTCCGGTTgGcCTTGAATTGGATTCAA	Internal primer for R9G, reverse Internal primer for R9G, forward
R9 M	TTGAATCCAATTCAAGCatAACCGGACTGACATCGA TCGATGTCAGTCCGGTTatGCTTGAATTGGATTCAA	Internal primer for R9 M, reverse Internal primer for R9 M, forward
Primers for generating in-frame deletions		
McuBH1HindIII	CCAAGCTTTAGGACGTGTTCACCTCGCTCGC	Downstream amplicon for ΔmcuB, reverse
McuBH1BamHI	TTGGATCCGCTCGCTGACCGACGCACCATG	Downstream amplicon for ΔmcuB, forward
McuBH2BamHI	TTGGATCCACGGTTACGCATGTGTTATCGCC	Upstream amplicon for ΔmcuB, reverse
McuBH2EcoRI	CGGAATTCAGAAAGTGGGTAGCAAGGACGG	Upstream amplicon for ΔmcuB, forward
McuDH1HindIII	CCAAGCTTCTCAAGAGACACCCAGCGAGGT	Downstream amplicon for ΔmcuD, reverse
McuDH1BamHI	TTGGATCC ACGGTGACCTTCTGAGGGGCCG	Downstream amplicon for ΔmcuD, forward
McuDH2BamHI	TTGGATCCCGTCATGGACACACCTCACGGTT	Upstream amplicon for ΔmcuD, reverse
McuDH2EcoRI	CGGAATTTCTGGATGACGACCACCCGACCCT	Upstream amplicon for ΔmcuD, forward
Primers for constructing plasmids expressing his-tagged or untagged McuB		
PmcuB _{his6} -5	Identical to P1	Upstream primer
PmcuB _{his6} -3	TTCTGCAGTCAGTGGTGGTGGTGGTGGCGAG CGCAGGGAGGCGGGAGACTC	Downstream primer
PmcuB-5	Identical to P1	Upstream primer
PmcuB-3	TTCTGCAGTCAGCGAGCGCAGGGAGGGGAGACTC	Downstream primer
Primers for measuring mcuA transcript		
PmcuA-5	CGCCAACCTGAACGTCACCGCCAA	Upstream primer
PmcuA-3	AGGTGATGGTGGCGACGACGGTGT	Downstream primer

^a Restriction sites are underlined. Lowercase letters indicate substituted nucleotides. Altered codons are shown in bold in the forward strands.

10 min. The resulting supernatant was mixed with 150 μl Ni²⁺-nitrilotriacetic acid (Ni²⁺-NTA) agarose beads (Qiagen) which had been pre-equilibrated with wash buffer. Beads and lysates were subsequently incubated at 4°C on a rotating platform. After 2 h, the beads were collected by centrifugation at 2,500 rpm for 1 min and washed three times in lysis buffer. Elution was performed by adding 40 μl of 1× SDS-PAGE loading buffer directly to the beads and boiling for 10 min. Pulled-down proteins were analyzed by immunoblotting using anti-McuA serum.

RNA work. To analyze *mcuA* mRNA abundance in the wt and Δ*mcuB* strains, total RNA was isolated from the cells that had developed for 22 h and treated with DNase I to remove residual DNA. With total RNA as the template, primer PmcuA-3 (Table 2) was used to generate cDNA. PCR was performed on the resulting cDNA with primer pair PmcuA-5 and PmcuA-3.

RESULTS

McuD is necessary for secretion of McuA. A BLAST search of the entire *M. xanthus* DK1622 genome indicated that *mcuABCD* is the only CU-like cluster in this bacterium, with *mcuABC* expressed as a single transcriptional unit (5, 16). We have previously shown that McuA protein is present mainly on the surface of myxospores and that inactivation of the putative usher McuC inhibits assem-

bly of McuA protein on spore surfaces, indicating that the *M. xanthus* CU-like system functions in spore coat formation (16). It is noteworthy that the *mcuD* downstream gene may code for another spore coat protein (NCBI description) and that the ORF of *mcuD* overlaps with that of *mcuC*. Furthermore, McuD is predicted to exhibit an Ig-like fold (Fig. 1C). Therefore, there is a possibility that McuA and McuD proteins are exported together through the putative usher pore McuC and interact with each other. If this is the case, McuD may play a role in the surface display of McuA protein. To test this, an in-frame deletion strain of *mcuD* (the Δ*mcuD* strain) was constructed. As shown in Fig. 2, deletion of *mcuD* nearly abolished the accumulation of McuA (the band of approximately 15 kDa) at all examined developmental time points. It is worth mentioning that at 24 h after starvation, *M. xanthus* cells just begin differentiation and cells collected at this time can be broken by boiling in 1× SDS-PAGE loading buffer. At a later time (e.g., 72 h after starvation), mature spores form, and when the spores are boiled in 1× SDS-PAGE loading buffer, McuA is released from the spore coat but the spores cannot be disintegrated. Hence, although *mcuD* and *mcuA* are not cotrans-

FIG 2 Analysis of McuA accumulation in *M. xanthus* wild-type (DK1622) and *mcuD*-deficient ($\Delta mcuD$) cells. Cells were starved on TPM for the indicated periods of time, harvested, and processed as described in Materials and Methods. M, protein markers.

cribed, McuD is important for surface localization of the spore coat protein McuA.

McuB is necessary for the accumulation of McuA. The Phyre prediction algorithm reported structure similarity between McuB and well-known CU chaperones such as Caf1M, FimC, and PapD (Fig. 1A). Phyre also predicted an architecture of McuA similar to that of the pilus subunit FimG of *E. coli* (Fig. 1B). Therefore, in the *M. xanthus* CU-like system, McuB may serve a chaperone-like function in the assembly of McuA on spore surfaces. We previously showed that the McuA protein appears at 18 h poststarvation, after which the level of the protein gradually increases throughout *M. xanthus* development (16). To investigate whether McuB plays a role in McuA accumulation, we attempted to examine the amount and surface localization of McuA in isogenic strains expressing or lacking McuB. For this purpose, a mutant strain (the $\Delta mcuB$ strain) carrying an in-frame deletion in the *mcuB* gene was constructed. Developmental cells of DK1622 and the $\Delta mcuB$ strain were collected at different time points and boiled in 1× SDS-PAGE loading buffer, and soluble cell lysates were resolved by SDS-PAGE followed by Western blotting using McuA antiserum. Accumulation of McuA was observed in the wt strain at 30 h and 6 days but was almost undetectable in the $\Delta mcuB$ strain at all time points examined (Fig. 3A). With respect to the level of McuA, strain $\Delta mcuB$ was clearly complemented by introducing a plasmid (pMP-*mcuABC*) expressing *mcuB* under the control of the wild-type promoter (Fig. 3B). Considering that cells collected at later times (>24 h) contain differentiated spores that cannot be lysed by boiling in 1% SDS, it is possible that at a late developmental stage McuA accumulates inside the $\Delta mcuB$ cells. However, biochemical fractionation of 6-day-old spores of $\Delta mcuB$ strain did not reveal the presence of McuA inside the cell (data not shown). One possible explanation for the lack of McuA accumulation in the $\Delta mcuB$ mutant is that McuA is degraded without McuB; a second possibility is that mutation in *mcuB* impairs *mcuA* transcription or mRNA stability such that McuA is not synthesized. Semiquantitative reverse transcriptase PCR (RT-PCR) analysis demonstrated similar amounts of *mcuA* transcript from wt and $\Delta mcuB$ cells (Fig. 3C). From these observations, we conclude that McuB might function as a chaperone and is required for accumulation and cell surface display of McuA.

McuB interacts with McuA. In classical CU systems, a chaperone binds to its cognate pilus subunits to stabilize them. To determine whether McuB associates with McuA, we complemented the $\Delta mcuB$ mutant with a construct allowing expression of McuB

FIG 3 McuB is necessary to stabilize McuA. (A) McuA was not detected by immunoblot analysis in the absence of McuB. Cells of DK1622 and the *mcuB* in-frame deletion strain ($\Delta mcuB$) were harvested at the indicated time points after initiation of development and processed as described in Materials and Methods. (B) Expression of wild-type (wt) McuB from a plasmid integrated at the $\Delta mcuB$ chromosome *attB* site ($\Delta mcuB$ /pMP-*mcuABC*) resulted in the rescue of McuA accumulation in this strain. (C) The *mcuA* transcript could be detected in the $\Delta mcuB$ strain. Cells of the DK1622 and $\Delta mcuB$ strains were exposed to starvation on TPM agar for 22 h. Total RNA was isolated, and *mcuA* transcript levels were determined by semiquantitative RT-PCR using 16S rRNA as a control. DNA contamination was excluded via the use of a control without reverse transcriptase (not shown).

fused to a His tag at its C terminus (McuB_{his6}) under the control of the endogenous *mcuABC* promoter. Interaction between McuB and McuA was tested by affinity pull-down using nickel beads. As shown in Fig. 4, McuA was readily pulled down by McuB_{his6} in the soluble extract of $\Delta mcuB$ /pMP-*mcuAB*_{his6} cells that had developed for 21 h, suggesting that McuA can form a complex with McuB. Therefore, McuB stabilizes McuA by interacting with it.

The putative G1 strand of McuB is required for McuA binding. In terms of DSC in the classical CU machinery, the McuB G1 strand is predicted to complete the Ig fold of McuA in a noncanonical fashion by interacting with its C-terminal β strand. To assess the significance of the McuB G1 strand, a deletion mutant (the McuB Δ G1 mutant) was created in which a large portion (from L107 to V105) of the putative donor strand of McuB was deleted. A plasmid expressing McuB Δ G1 (pMP-*mcuB* Δ G1) in the context of the wild-type promoter region was integrated by site-specific recombination into the chromosome of the strain $\Delta mcuB$, generating a strain called $\Delta mcuB$ /pMP-*mcuB* Δ G1. The McuA-binding ability of this mutant chaperone was assayed by monitoring its ability to protect McuA from degradation in the *M. xanthus* strain $\Delta mcuB$ /pMP-*mcuB* Δ G1. Developmental cells of the DK1622 and $\Delta mcuB$ /pMP-*mcuB* Δ G1 strains were collected after starvation on TPM agar for the indicated periods of time, and McuA accumulation in total cell lysates was examined by immunoblotting using McuA antiserum. As shown in Fig. 5, McuA was present in wt strain at days 1, 3, and 5 whereas it was no longer detectable in the $\Delta mcuB$ /pMP-*mcuB* Δ G1 strain at the same time points. These results provide evidence that the McuB G1 strand is essential for McuA binding and stabilization.

Experimental design for site-directed mutagenesis in the McuB G1 strand. The G1 strand of classical CU chaperones is characterized by a conserved motif of at least three alternating

FIG 4 McuB interacts with McuA. McuB_{his6} was expressed from its native promoter in the $\Delta mcuB$ strain carrying the plasmid pMP-*mcuAB*_{his6} at the *attB* site ($\Delta mcuB$ /pMP-*mcuAB*_{his6}). The Ni bead pulldown products of the whole-cell extract from $\Delta mcuB$ /pMP-*mcuAB*_{his6} were blotted with McuA antiserum (lane 1). An isogenic strain ($\Delta mcuB$ /pMP-*mcuAB*; lane 2) expressing untagged McuB was used as a specificity control to show that McuA was recovered only when McuB_{his6} was present.

bulky hydrophobic residues (P1 to P3) which is inserted inside the corresponding subunit groove pockets (8, 13). Sequence alignment illustrated that in spite of the existence of three conserved bulky hydrophobic residues at the putative P1, P2, and P3 positions (except T111 of McuB), the G1 region of archaic CU chaperones possesses certain amino acid alterations (Fig. 6). For example, in classical CU chaperones, the residue at position 110 (following PapD numbering) is either a positively charged residue (FGS chaperones) or an invariant cysteine (FGL chaperones) (26). In the case of McuB, however, this position is occupied by a serine which is highly conserved in archaic CU chaperones. At position 116, the lysine residue which plays a key role in anchoring pilus subunits and is invariant among classical chaperones is replaced by proline in McuB. On the basis of these and other observations, we reasoned that the structural basis of chaperone function in *M. xanthus* may deviate from that of its counterparts in classical CU systems. To better understand the structural basis of McuB G1 in DSC reactions, three classes of residues in the McuB G1 region were selected for mutagenesis: (i) alternating hydrophobic residues (L107, V109, I113, and V115) that are conserved in archaic CU chaperones, (ii) a nonconserved hydrophilic residue (T111) that is present in McuB but absent at the corresponding position in other members of the archaic chaperones, and (iii) invariant residues (R112, S114, and P116) that are present in every member of the archaic CU chaperone family (Fig. 6A). The residues were altered either singly or in pairs by site-directed mutagenesis of McuB using the plasmid pMP-*mcuABC* as a template. Plasmids containing the mutant alleles were separately integrated at the *attB* gene in the *mcuB*-deficient $\Delta mcuB$ strain. Because the McuB G1 strand is essential for McuA binding and stabilization, the significance of specific residues in the McuB G1 region was investigated by comparing the amount of McuA in isogenic strains expressing wild-type *mcuB* or its mutant alleles.

Role of conserved hydrophobic residues and a nonconserved hydrophilic residue of the McuB G1 strand in McuB-McuA interaction. In classical CU systems, a motif of 3 to 5 alternating hydrophobic residues on the chaperone G1 strand is implicated in subunit binding (4, 8, 13). These residues are designated P1 to P5. Sequence alignment and Phyre secondary-structure prediction suggested that the P2 and P3 residues in the G1 strand of McuB are hydrophobic V109 and L107, respectively; however, the residue positioned at P1 is a hydrophilic T111 (Fig. 6B). Residues at these

FIG 5 Effect of G1 strand deletion on the ability of McuB to bind McuA. Cells of the $\Delta mcuB$ strain expressing either wild-type McuB ($\Delta mcuB$ /pMP-*mcuABC*, positive control) or an McuB G1 deletion mutant ($\Delta mcuB$ /pMP-*mcuB* Δ G1) were harvested at the indicated time points after the initiation of development and processed as described in Materials and Methods. McuA was detected by immunoblot analysis using McuA antiserum.

positions were changed, and the ability of mutant McuB to bind to McuA was assayed by testing for the amount of McuA in total cell lysates. Immunoblot experiments demonstrated that the L107TV109T mutation abolished or significantly decreased the amount of McuA inside the cell and on the cell surface (Fig. 7A), confirming the importance of P2 (V109) and P3 (L107) hydrophobic residues in the McuB G1 region in interactions with McuA. When the hydrophilic P1 residue was changed to a hydrophobic one, the T111L McuB mutant could not stabilize McuA inside the cell, as shown by the absence of an immunoreactive band of approximately 15 kDa in cell extract from the $\Delta mcuB$ /pMP-*mcuB*-T111L strain that had developed for 1 day (Fig. 7A); this mutant, however, was able to stabilize and had no effect on the surface localization of McuA when sporulation is complete (e.g., at days 3 and 5).

In contrast to classical CU chaperones, P1 in McuB G1 is followed by two additional alternating nonpolar residues (I113 and V115), a conserved signature that is commonly found in members of the archaic CU family (Fig. 6A). To assess the significance of the additional alternating nonpolar residues after P1, I113 was changed to a threonine. While the I113T mutant chaperone was ineffective in protecting McuA from degradation in $\Delta mcuB$ /pMP-*mcuB*-I113T cells that had been starved for 1 day, the amount of McuA in the cells starved for 3 days and 5 days was similar to that in the *mcuB* wt-complemented $\Delta mcuB$ cells (Fig. 7B), implying that after sporulation began, the McuB-I113T mutant was capable of stabilizing McuA and this mutation did not block the translocation of McuA across the outer membrane. Importantly, when I113 and V115 were simultaneously mutated to threonine residues, the resulting mutant McuB-I113TV115T protein clearly failed to protect McuA from degradation at all time points examined (Fig. 7B). Thus, we conclude that in the *M. xanthus* CU-like chaperone, the two alternating hydrophobic residues immediately after the P1 position are required for McuB-McuA intermolecular interaction, with V115 being the more important of the two.

Role of McuB residues R112, S114, and P116 in McuB-McuA interaction. Within the McuB G1 strand, three residues, R112, S114, and P116, are present in all of the six randomly selected archaic CU chaperones (Fig. 6A). The three individual residues were targeted for point mutation in order to investigate their role

FIG 6 Amino acid sequence comparison. Numbers in parentheses indicate the coordinates of the amino acid sequence of each mature protein. (A) McuB was aligned with the N-terminal region of six randomly selected archaic CU chaperones. Underlined sequence corresponds to the G1 strand of PapD-like chaperones based on Phyre2 secondary-structure prediction. The potential P1 to P3 residues and two additional alternating hydrophobic residues after P1 are shaded. An asterisk indicates identical residues, a colon indicates conserved residues, and a single dot indicates semiconserved residues. (B) The McuB G1 region was aligned with those of classical CU chaperones in the FGS (PapD, FimC) and FGL (SefB, Caf1M) subfamilies (adapted and modified from reference 16). P1 to P3 represent the conserved hydrophobic residues that interact with subunits by the principle of donor strand complementation as demonstrated in classical CU pathways.

in the mechanism of McuB action. The R112 residue was changed to a methionine. The R112M mutation reasonably maintains side-chain packing while it removes the charged guanidinium group at the same time (27), thereby abolishing the ability of this side chain to form, if any, hydrogen bonds or salt bridges with McuA. S114 was changed to a cysteine because serine and cysteine are stereochemically very similar. The S114C mutation was created to test the importance of hydroxyl side chain group in, for instance, forming a hydrogen bond with McuA. P116 was changed to an alanine or a lysine. Alanine is a hydrophobic residue like proline but lacks the ring structure. P116K was made because lysine at this position is highly conserved in classical CU chaperones (13, 26).

Mutation of P116 of McuB to either alanine or lysine completely abolished accumulation of McuA. In contrast, amounts of McuA were higher in the strain carrying an R112M or S114C McuB mutation than in the wild-type strain (Fig. 7C and D). We conclude that, of the three invariant residues on the donor strand of McuB, R112 and S114 are not important whereas P116 is critical in mediating McuB-McuA intermolecular interaction.

R9 of McuB is not critical for binding McuA. Besides the G1 strand, classical CU chaperones apply a pair of conserved positively charged residues located between the two Ig-like domains (e.g., R8 and K112 in PapD) to bind subunits by anchoring their C-terminal carboxyl groups (4, 13). As the residue in McuB which

FIG 7 Effect of point mutations in the G1 strand on the ability of McuB to bind McuA. Immunoblot analysis was performed to examine McuA accumulation in the $\Delta mcuB$ strain expressing either wild-type McuB ($\Delta mcuB$ /pMP-*mcuABC* strain, positive control) or mutant McuB ($\Delta mcuB$ /pMP-*mcuABC* derivatives with mutations on G1 strand) proteins at days 1, 3, and 5 after the onset of development. (A) McuA accumulation in the presence of L107TV109T or T111L McuB in which residues at positions P1, P2, and P3 (see Fig. 5B) were changed. (B) McuA accumulation in the presence of I113T and I113TV115T McuB in which additional alternating hydrophobic residues after P1 were changed. (C and D) McuA accumulation in the presence of P116A, P116K, R112M, and S114C McuB in which invariant residues in the G1 region among archaic chaperones were changed.

corresponds to R8 in PapD is also an arginine, this residue (R9) was changed to a glycine and a methionine to examine the effect of a point mutation at this position on McuB activity. Compared with R9M, which is largely isosteric, R9G is a radical mutation which completely removes the side chain and thus any interaction it may make. The $\Delta mcuB$ strain was complemented with plasmids containing the wild-type *mcuB* gene (pMP-*mcuABC*) or the mutant *mcuB* genes (pMP-*mcuB*-R9G and pMP-*mcuB*-R9M). In $\Delta mcuB$ /pMP-*mcuB*-R9G cells that had been starved for 1 day, McuA was degraded, as determined by immunoblotting (Fig. 8). However, on days 3 and 5, a McuA-specific band could still be seen in the strain harboring the McuB-R9G mutant, although at a level slightly lower than that seen with the wild type. The differential effect of the R9G mutation on the ability of McuB to bind to McuA during *M. xanthus* development is reminiscent of that of the I113T mutation in the McuB G1 strand. In contrast, expression of the

FIG 8 Effect of a point mutation at R9 on the ability of McuB to bind McuA. R9 of McuB, which corresponds to the conserved subunit anchoring arginine in the classical periplasmic chaperone family, was changed separately to glycine and methionine, which differ in the side chain volume. McuA accumulation was analyzed as described in the legend to Fig. 7.

TABLE 3 Summary of McuB point mutations

Residue(s)	β strand	Class	Mutation(s)	Significance of residue(s) for chaperone function ^a
R112	G1	Invariant	R112M	—
S114	G1	Invariant	S114C	—
P116	G1	Invariant	P116K, P116A	++
L107 + V109	G1	Conserved	L107TV109T	++
I113	G1	Conserved	I113T	+/-
I113 + V115	G1	Conserved	I113TV115T	++
T111	G1	Variable	T111L	+/-
R9	A1	Variable	R9M, R9G	—

^a Data assessed by the ability of the indicated mutation(s) to protect McuA from degradation. ++, high significance; +/-, moderate significance; —, no significance.

McuB-R9M mutant did not cause proteolytic degradation of McuA; rather, this mutant McuB even enhanced stabilization of McuB at all time points examined. These results suggest that, in contrast to the importance of R8 of PapD in subunit binding, the corresponding R9 of McuB is not critical for McuA binding, a finding consistent with the observation that R9 is not conserved among archaic periplasmic chaperones.

DISCUSSION

Many adhesive fibrous structures found on the Gram-negative cell surface are assembled via CU pathways; among these, the sigma-fimbriae (archaic CU family) represent a large and phylogenetically diverse clade whose members are present among important environmental and pathogenic microbes (5). Based on *in silico* analysis, McuA, McuB, McuC, and McuD of *M. xanthus*, a genetically tractable and important model organism for studying prokaryotic development, may serve as a prototype of the archaic CU system (5). We have previously shown that genetic disruption of McuC, a putative outer membrane usher, nearly eliminates accumulation of McuA on spore surfaces (16). In this study, we also demonstrated that McuB, a periplasmic chaperone-like protein, stabilizes McuA by forming a complex with it. The requirement for a chaperone and an usher for surface display of McuA and the Phyre-generated Ig-like topology of both McuB and McuA suggest that in spite of being a nonpilus surface structure, the spore coat protein McuA is exported to the cell surface via a pathway similar to that used in the assembly of classical and alternate CU pili. In addition, since deletion of *mcuD* interferes with McuA stability/surface display, it can be inferred that the *M. xanthus* CU system is encoded by the gene cluster *mcuABCD*, with *mcuABC* expressed as a single transcriptional unit (16) and *mcuAD* encoding the structural components.

During the morphogenesis of both classical and alternate CU pili, the interaction of periplasmic chaperones with subunits is critical for stabilization and progression of these proteins along the productive assembly pathway. In the present work, we investigated the significance of certain amino acids for McuB function (Table 3). In classical CU chaperones, two conserved basic residues (e.g., R8 and K112 of PapD) are involved in binding pilus subunits by anchoring their C-terminal carboxyl groups, while the G1 β strand makes main-chain/main-chain hydrogen bonding interactions with the subunits via a mechanism termed DSC (18, 27). In the case of McuB, a R9M mutation (corresponding to R8 of

PapD) caused an apparent increase in the accumulation of McuA, demonstrating that this basic residue is dispensable for chaperone function. Because the N-terminal β strand of classical CU chaperones also contributes to the formation of interactive surfaces by contacting the subunit A1 β strand (4, 6), and in view of the preference of amino acids for the β strand, the increase of McuA accumulation in the presence of the McuB-R9M mutant could have been due to generation of a more pronounced β -strand conformation whereas the decrease of the McuA amount in the presence of the McuB-R9G mutant could be ascribed to weakening of the local β strand. When the P116 residue of McuB (corresponding to K112 of PapD) was changed to a lysine, McuA was not detectable at any of the time points examined. The demonstration that R9 does not play a role in stabilizing McuA, together with the observation that positively charged residues corresponding to PapD R8 and K112 are not conserved among archaic CU chaperones, provides strong evidence that classical and archaic CU chaperones differ in the architecture of interactive surfaces that contact structural subunits.

Genetic and structural studies have revealed that DSC is common to both classical and alternate CU pathways (9, 12, 18, 28, 29). As shown by studies on the *E. coli* Pap and *Y. pestis* Caf systems, which assemble pilus and nonpilus surface organelles, respectively, in complex with the structural subunit, the chaperone G1 strand inserts a conserved motif of 3 to 5 alternating hydrophobic residues (P1 to P5) into binding pockets in the hydrophobic groove of the subunit (13). A large internal deletion and a P3/P2 double mutation (L107TV109T) within the G1 strand resulted in McuB mutants that were no longer functional as a chaperone. The absolute requirement of the G1 strand and the necessity of two bulky hydrophobic residues at positions P3 and P2 for McuB function imply that McuB G1 is likely to bind to McuA via DSC, analogous to classical periplasmic chaperone-subunit interactions. However, McuB clearly belongs to a family different from that of the classical CU chaperones. For instance, one distinguishing feature of McuB is a variable hydrophilic residue at the P1 (T111) position followed by two alternating hydrophobic residues that are conserved in archaic but not in classical CU chaperones. On the basis of site-directed mutagenesis performed in this study, L107, V109, I113, and V115 were identified as crucial residues for McuB chaperone function. When L107 and V109 or I113 and V115 of McuB were mutated in pairs, McuA was almost completely degraded. Compared with I113TV115T double mutation, the single I113T mutation of McuB did not affect McuA accumulation after 24 h, indicating that the hydrophobic V115 residue is more important than I113 in chaperoning McuA. The importance of I113 and V115 for McuB function and the very frequent presence of two additional alternating hydrophobic residues after P1 in other archaic CU chaperones argue that McuB seems to use a variation of the paradigmatic PapD-PapK DSC mechanism in which a longer stretch of alternating hydrophobic residues in the McuB G1 region participates in interacting with McuA. In this respect, McuB is similar to Caf1M-like FGL chaperones. For *Y. pestis* Caf1M, the G1 donor strand contributes a run of five rather than three alternating hydrophobic residues (as with the PapD-like FGS chaperones) to complex with the Caf1 subunit (6, 13, 30).

A unique feature of archaic CU chaperones is the presence of three highly conserved residues (R112, S114, and P116) in the G1 β strand. Two McuB mutants, P116A and P116K, were identified as nonfunctional, underscoring the importance of P116 for McuB

function. Proline is a residue most commonly found in the flanking segments of protein-protein interaction sites (31, 32); its occurrence in β sheets is rare, as its side chain has a rigid pyrrolidine ring that is unable to participate in H-bonding. Considering that G1 is an edge strand of a periplasmic chaperone, a proline may be introduced into an edge strand without compromising β -sheet stability if this position is not involved in the H-bonding network (33). Note that the conserved subunit anchoring lysine is also changed to proline in the G1 strand of three classical CU chaperones: FasB, CswB, and FotB (13). We do not currently know the structural significance of this functionally important McuB G1 strand-bearing proline. The function of McuB was not affected by an R112M or S114C point mutation, indicating that either the two residues are not important for chaperone function or the amino acid alteration at that position was sterically a conservative substitution.

In summary, the present paper and our previous work demonstrate that in *M. xanthus*, McuB and McuC mediate the transportation of the spore coat protein McuA onto the cell surface. McuB functions as a molecular chaperone to stabilize its interactive partner McuA. In addition, McuA may interact with another putative spore coat protein, McuD, when it is exported through the McuC usher pore. The necessity of the McuB G1 strand and the importance of the hydrophobic residues at positions P3 and P2 for its chaperone function, together with the overall conservation of the Ig-like fold of McuB, McuA, and McuD, have provided evidence that the *M. xanthus* CU system may use the basic principle of DSC to promote proper folding and stabilization of the structural subunits. However, the two alternating hydrophobic residues immediately after the P1 position and the absolutely conserved proline residue within the G1 strand probably provide additional structural features for McuB chaperone function. Being a representative of the archaic CU family, the *M. xanthus* CU pathway can be studied further to dissect fine details of the architecture of this class of surface structures and the molecular mechanism of their assembly. These efforts will shed light on the creation of rationally designed compounds targeting the biogenesis of archaic CU pili that are present among many important pathogenic microbes.

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