Evidence that a chaperone–usher-like pathway of *Myxococcus xanthus* functions in spore coat formation

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Many bacteria use the chaperone–usher (CU) secretion pathway to assemble on their surfaces typical or atypical fimbrial organelles. Four consecutive genes of *Myxococcus xanthus* DK1622, MXAN3885–3882, were predicted to constitute an operon encoding a CU-like system involved in the assembly of the spore coat; however, experimental evidence supporting this hypothesis was lacking. In this study, co-transcription of MXAN3885–3883 was verified, and we found that this operon was expressed 12–15 h after initiation of *M. xanthus* development under conditions of stringent starvation. The MXAN3885 protein, which is highly homologous to, but expressed earlier than, the spore coat protein U of another *M. xanthus* strain, DZF1, was present mainly on the outer surface of myxospores. Inactivation of MXAN3883, encoding a putative outer membrane usher, inhibited assembly of MXAN3885 protein on spore surfaces and caused certain morphological alterations in the spore coat. Hence, the CU-like pathway in *M. xanthus* indeed functions in spore coat biogenesis. Based on chaperone amino acid sequence comparisons, our analysis suggests that the structural basis of the *M. xanthus* CU-like pathway for spore coat assembly may be different from that of most surface structures assembled by classical CU systems.

Received 28 November 2010

Revised 27 January 2011

Accepted 31 March 2011

INTRODUCTION

A large number of Gram-negative bacteria depend on the chaperone–usher (CU) secretion pathway to assemble on their surfaces non-flagellar extracellular adhesins called pili or fimbriae. These adhesive fibrous organelles mediate microbial attachment to host cells and are important virulence determinants associated with a wide range of infectious diseases (Sauer *et al.*, 2004). The CU pathway contains two non-structural assembly components: an integral outer membrane, dimeric and pore-forming protein termed the usher; and a periplasmic chaperone. Following translocation across the inner membrane via the general secretory system (Sec), pilus subunits are bound to the chaperone proteins, which facilitates folding of the structural subunits and protects them from aggregation and degradation. Chaperone–subunit complexes are then targeted to the usher protein. Interactions between the

chaperone and usher proteins release the pilus subunits, which are subsequently exported through the usher channel for assembly into pilus fibres and secretion into the cell surface (Saier, 2006; Sauer *et al.*, 2004; Waksman & Hultgren, 2009). Two prototypical members of the CU pathway are the Pap and Fim systems of uropathogenic *Escherichia coli*, which build P and type 1 pili, respectively. Although in most cases the CU system is involved in rod-like pilus biogenesis, this system may assemble proteinaceous fibres on the bacterial surface into non-pilus structures. For example, the capsular F1 antigen from the plague pathogen *Yersinia pestis* consists of linear fibres of the single subunit Caf1. The Caf1-based fibres coil up into a large gel-like capsule on the cell surface, which serves as a prototype for atypical and non-pilus organelles assembled via the CU pathway (Zavialov *et al.*, 2003). Based on phylogenetic analysis of usher sequences, CU systems can be classified into three families: classical, alternate and archaic (Nuccio & Bäuml, 2007). Despite the striking diversity of surface structures assembled by the CU pathway, these architectures are built, at least in the classical and alternate CU families, on the same general principle, termed donor strand complementation or exchange, that mediates chaperone–subunit or subunit–subunit interaction, respectively (Waksman & Hultgren, 2009; Zavialov *et al.*, 2002; Poole *et al.*, 2007).

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Abbreviations: CU, chaperone–usher; OMP, outer membrane protein; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

Myxococcus xanthus is a Gram-negative bacterium characterized by social behaviour and a complex developmental cycle. Under vegetative conditions, *M. xanthus* cells move cooperatively in large swarms; under conditions of high cell density, nutrient starvation and a solid surface, approximately 10^5 cells aggregate in a coordinated fashion to form a fruiting body. Starting 24 h after starvation, the motile, rod-shaped cells inside the fruiting body begin differentiating into dormant ovoid spores that are surrounded by thick protein coats and become stress-resistant (Dworkin, 1996). Fruiting body formation involves temporally ordered change in the expression of genes, which drives multicellular morphogenesis and cell differentiation (Søgaard-Andersen *et al.*, 2003). Examination of the full genome using Tn5 *lac*, a transposable promoter probe, suggests that several hundred genes may increase their expression during *M. xanthus* development (Kroos *et al.*, 1986).

As interfaces to the outside world and as interfaces between cells, *M. xanthus* cell membranes fulfil various functions in cell signalling, transport across cell membranes and cell motility. Using *lacZ* as a reporter, we monitored the expression profiles of certain genes whose products were predicted to be outer membrane-localized. We found that one of these genes, MXAN3883, increased its expression during development. MXAN3883 is predicted to lie in an operon with MXAN3882 and MXAN3885–3884 (Nuccio & Bäumlér, 2007). Based on *in silico* analysis this operon encodes an outer membrane usher protein (MXAN3883), a periplasmic chaperone (MXAN3884) and two spore coat proteins (MXAN3882 and 3885), suggesting that the *M. xanthus* CU-like system might be involved in the assembly of the spore coat, a non-pilus structure on cell surfaces (Nuccio & Bäumlér, 2007). To our knowledge, this was the first indication that a CU pathway might participate in spore coat formation; however, experimental evidence supporting this hypothesis was lacking. Here, we provide biochemical and morphological data that the MXAN3885 product accumulates on the cell surface during the late developmental stage, and that MXAN3885–3883 constitute an operon that does function in spore coat biogenesis, highlighting the structural diversity of proteinaceous fibres assembled by the CU pathway. Interestingly, based on an amino acid sequence comparison, the MXAN3884 chaperone belongs to neither the FGL nor the FGS subfamily of immunoglobulin-like chaperones (Hung *et al.*, 1996).

METHODS

Cell growth, development and measurements of β -galactosidase activity. *E. coli* JM83 was grown in LB broth in the presence of relevant antibiotics. *M. xanthus* strains were grown in CTT medium, as described by Hodgkin & Kaiser (1979). Kanamycin or oxytetracycline was used for selection at concentrations of 40 or $12.5 \mu\text{g ml}^{-1}$, respectively. *M. xanthus* fruiting body development was induced on TPM agar [10 mM Tris/HCl (pH 7.6), 1 mM KH_2PO_4 , 8 mM MgSO_4 , 1.5 % Difco Bacto Agar], unless otherwise specified. Clone-fruiting (CF) agar was prepared as described by Inouye *et al.* (1979a).

The sporulation assay was performed as described by Gorski & Kaiser (1998), except that 5 day-old fruiting bodies were used. Specific β -galactosidase activities were determined according to the protocol of Kroos *et al.* (1986). Protein concentrations were measured by bicinchoninic acid (BCA) protein assay (Pierce).

***M. xanthus* strains.** DK1622 was used as the parent wild-type strain for all *M. xanthus* strains throughout this study. All strains constructed were confirmed by PCR.

Construction of the *lacZ* reporter fusion. The MXAN3883–*lacZ* transcriptional fusion was constructed using the vector pMP220 (Spaink *et al.*, 1987). A 1801 bp DNA fragment containing the putative MXAN3883 promoter region and certain 5' terminal codons was generated by PCR using DK1622 genomic DNA as template. The PCR primers were 5'-GCTCTAGATTCGCCAGAAGTGGGTAGC-AAGGAC-3' and 5'-TTCTGCGAGCGCGGGTTATTGCAAGCCGGG-AGGG-3' (underlined nucleotides indicate restriction enzyme sites), corresponding to nucleotides from –1778 to 31 (+1 indicates translation initiation site of MXAN3883). The amplified product was digested with *Xba*I and *Pst*I; in addition, a 2.9 kb *Eco*RI–*Xba*I myxophage Mx8 *attP* fragment was recovered from pUC18-*attP*, a pUC18 derivative carrying a 2.9 kb Mx8 *attP*. These two fragments were ligated with pMP220 that had been digested with *Xba*I and *Pst*I, resulting in the transcriptional *lacZ* fusion vector pMP-MXAN3883. The plasmid was introduced into wild-type strain DK1622 through electroporation (Kashefi & Hartzell, 1995). Transformants were selected by plating the cells onto CTT plates containing oxytetracycline. The plasmid used in this study could not independently replicate out of the chromosome in *M. xanthus*; thus, all transformants that were resistant to oxytetracycline were the result of integration of the plasmid into the chromosome either by homologous recombination or by site-specific recombination between *attP* on the plasmid and the *attB* site on the chromosome. PCR was performed to screen antibiotic-resistant colonies for proper integration of the plasmid at the *attB* site using the following primer pair: forward, 5'-GAAGGGCCCGGAACCTTGCGATTCCGGG-3'; reverse, 5'-CCATGAGCGGGAAGCGGTGCGGGAGCGT-3'.

Construction of the MXAN3883 mutant. The MXAN3883 mutant was constructed by cloning an internal fragment into the *Eco*RI–*Hind*III sites of pUC18, resulting in pUC18-MXAN3883. The internal fragment was prepared by PCR with the following primer pair (underlined nucleotides indicate added restriction sites): 5'-CGGAATTCCACGGTGCTGGCGTTATCCTTGTCG-3' and 5'-CC-AAGCTTCTCACTGCGGCTGATGGCCTGTGAC-3'. After sequence confirmation, a 1.4 kb *Eco*RI fragment carrying the Tn903 kanamycin-resistance gene from pUC4K (Amersham) was cloned into pUC18-MXAN3883, generating pUCK-MXAN3883. The resulting plasmid was electroporated into DK1622, and cells which had acquired the plasmid were selected by kanamycin resistance. Kanamycin-resistant electroporants should arise from the integration of the vector into host genome at the MXAN3883 locus by homologous recombination. A single crossover event would therefore result in two truncated copies of MXAN3883 in the genome, separated by the kanamycin-resistant vector. The recombination site of the mutant was confirmed by PCR using a primer (5'-GATAGATTGTGCGACCTGATTGCCC-3') located in the kanamycin cassette of pUCK-MXAN3883, and a primer (5'-ACGATGCCCGCCGACGCCCCGTTCC-3') located downstream on the chromosome from the MXAN3883 fragment in the plasmid.

Preparation of samples for scanning electron microscopy (SEM). Fruiting bodies for SEM analysis were prepared by the method of Dahl *et al.* (2007) through a glutaraldehyde fixation step. They were then dehydrated with ethanol and critical-point dried. The

dried samples were coated with gold and examined with an FEI Quanta 200 scanning electron microscope.

Transmission electron microscopy (TEM) analysis of spores.

This was performed according to the protocol of Dahl *et al.* (2007), with modifications. After development on TPM agar for 6 days, cells were harvested and suspended in TPM buffer [10 mM Tris/HCl (pH 7.6), 1 mM KH_2PO_4 , 8 mM MgSO_4]. Pelleted cells were fixed with 2% paraformaldehyde/2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) containing 0.2 M sucrose for 12 h at 4 °C. After being suspended in 0.1 M sodium cacodylate buffer containing 0.2 M sucrose overnight at 4 °C, the cells were rinsed three times with the same buffer before they were suspended in 1.5% molten agar. After solidification, the agar was cut into 1 mm³ cubes. The agar cubes were post-fixed with 1% OsO_4 in 0.1 M cacodylate buffer for 2 h at room temperature, rinsed three times with H_2O , and stained with 1% tannic acid for 1 h at room temperature. Samples were dehydrated in a graded ethanol series and then infiltrated in a stepwise fashion as follows: 100% propylene oxide twice for 10 min at 4 °C each time, followed by propylene oxide/Epon 812 series (1:1, 1:2) for 1 h at room temperature each time, and finally 100% Epon 812 for 1 h at 37 °C twice. Samples were embedded in fresh 100% Epon 812 and allowed to harden at 37, 42 and 60 °C for 24 h. Thin sections were prepared, stained with 2% uranyl acetate for 10 min and lead citrate for 5 min, and viewed with an H-7650 transmission electron microscope (Hitachi).

RNA work and RT-PCR. To analyse the transcriptional organization of the MXAN3883 locus, total RNA was isolated from *M. xanthus* DK1622 cells that had developed for 18 h and treated with DNase I to remove residual DNA. With total RNA as template, the reverse primer from each primer pair was used as the primer to generate cDNA. PCR was performed on the resulting cDNA in the presence of the appropriate pair of primers. To examine whether a PCR product spanning MXAN3886–3885 could be obtained, the forward primer 5'-CGTCTCAAGTCCAATGTCTACCA-3', corresponding to the MXAN3886 ORF, and the reverse primer 5'-GTCTTGATTGCGTTTCATTTGAGT-3', corresponding to the MXAN3885 ORF, were used. The expected size of the PCR product, if any, should be 576 bp. To examine whether a PCR product spanning MXAN3885–3883 could be obtained, the forward primer 5'-CACGCAACTCAAATGAACGCAAT-3', corresponding to the MXAN3885 ORF, and the reverse primer 5'-AACGACAAGGATAACGCCAGCAC-3', corresponding to the MXAN3883 ORF, were used. The expected size of the PCR product, if any, should be 1414 bp. To examine whether a PCR product spanning MXAN3883–3882 could be obtained, the forward primer 5'-TGCGGCTCAACAACCACGAAATC-3', corresponding to the MXAN3883 ORF, and the reverse primer 5'-CCACGTAGAGTTGTATTCCAAG-3', corresponding to the MXAN3882 ORF, were used. The expected size of the PCR product, if any, should be 745 bp. To monitor MXAN3885 mRNA levels in wild-type and MXAN3883 mutant strains, RT-PCR was performed using two primers located within the MXAN3885 ORF: forward, 5'-CGCCAACCTGAACGTCACCGCCAA-3'; and reverse, 5'-AGGTGATGGTGGCGACGACGGTGT-3'.

Biochemical fractionation of spores. *M. xanthus* cells induced to undergo development on TPM agar were harvested 6 days after initiation. Two methods were used to fractionate myxospores. The first method, performed basically as described by Teintze *et al.* (1985), was to boil spores for 5 min in 1% SDS and then centrifuge (5000 g, 3 min) to give soluble (S100) and pellet (P100) fractions. Mature spores in the P100 fraction, which was washed twice with distilled water, were broken by sonication in the presence of glass beads. The beads were removed by dilution with water to allow them to settle down. Subsequently, the supernatant was transferred to a new tube and separated into soluble (P100S) and pellet (P100P) fractions by

centrifugation at 5000 g for 10 min. The three fractions, S100, P100S and P100P, were solubilized in 1 × SDS-PAGE loading buffer [2% SDS, 0.05 M Tris/HCl (pH 6.8), 10% (v/v) glycerol and 0.1 M β -mercaptoethanol] and analysed by SDS-PAGE. For the second method, 6 day-old spores were resuspended in distilled water and sonicated in the absence of glass beads. The pellet fraction thus obtained after centrifugation was then sonicated with glass beads and further fractionated as described above for P100.

Immunoblot analysis. To analyse accumulation of MXAN3885 protein in DK1622 and the MXAN3883 mutant strain, cells were grown in CTT to OD₆₀₀ 0.8. Cells were sedimented and resuspended in a one-tenth volume of TPM buffer. Aliquots (20 µl) of concentrated cells were spotted on TPM or CF agar for various times, and 10 spots harvested at each time point were resuspended in 50 µl water. After addition of 50 µl 2 × SDS-PAGE loading buffer, samples were incubated in a boiling water bath for 5 min and sonicated for 15 s. A 5 µl aliquot of the soluble fraction of each sample was applied, and proteins were resolved by SDS-PAGE. The gel was blotted onto a PVDF membrane and probed with antiserum against MXAN3885 peptide, followed by anti-rabbit IgG conjugated to alkaline phosphatase with chromogenic substrates. To determine the cellular localization of the MXAN3885 protein, spores were fractionated as described above. Then, a 5 µl aliquot of each fraction representative of an equal number of cells was loaded per lane.

Antibody. Polyclonal antiserum against MXAN3885 was made by GenScript (Nanjing, China). To produce the antiserum, the synthetic peptide GSTDAAPQRRMRNT, modified by a cysteine at the carboxyl terminus, was coupled to keyhole limpet haemocyanin (KLH). New Zealand white rabbits were immunized with peptide-KLH conjugates (50 µg per rabbit). The initial injection was followed by three booster injections 2 weeks apart.

RESULTS

Expression analysis of MXAN3883 during *M. xanthus* vegetative growth and development

We were interested in changes in cell surface proteins when *M. xanthus* cells shift their life cycle from expansive vegetative swarming to aggregation into multicellular fruiting bodies. Six computational algorithms were utilized to identify signatures characteristic of outer membrane proteins (OMPs) from the genome of *M. xanthus* DK1622 (GenBank accession no. CP000113): PSORTb, TMB-Hunt, TMBETA-NET, TMBETA-SVM, PSLpred and PA-SUB. Combining the total predictions from the six programs revealed that 11 hits were shared by all predictors. To monitor expression patterns of the *in silico*-identified OMPs, plasmids carrying OMP-*lacZ* transcriptional fusions were introduced separately into *M. xanthus* DK1622 at a chromosomal *attB* site. β -Galactosidase activities of cells carrying various OMP-*lacZ* fusions were measured. Increased expression during development was found in two genes, one of which was MXAN3883. For the MXAN3883-*lacZ* fusion, β -galactosidase activity was undetectable in the first 12 h of development; however, expression of *lacZ* occurred abruptly at 15 h, reached its highest level at 18 h and then gradually decreased after 24 h (Fig. 1a).

Fig. 1. Expression patterns and genomic organization of the MXAN3883 locus. (a) Specific activity of β -galactosidase expressed from the MXAN3883–*lacZ* transcriptional fusion. The *M. xanthus* DK1622 derivative harbouring the MXAN3883–*lacZ* fusion was starved on TPM agar for the indicated periods of time, and samples were harvested and tested for β -galactosidase specific activity. Data are mean $\pm$ SD of four determinations. (b) Physical map of the MXAN3883 locus. ORFs are represented as arrows pointing in the direction of transcription. Coordinates (relative to the translation initiation site of MXAN3883) indicate the start or stop codon of each gene. The dashed line represents the extent of *M. xanthus* DNA present in the MXAN3883–*lacZ* fusion construct. (c) Examination of transcriptional organization by RT-PCR. Lanes: M, DNA markers; 1, 4 and 7, negative controls in which total RNA used for cDNA synthesis was used as the PCR template; 2, 5 and 8, positive controls in which chromosomal DNA was used as the PCR template; 3, 6 and 9, cDNA was used as the PCR template. The location of each primer pair was as follows: lanes 1–3, spanning ORFs MXAN3885–3883; lanes 4–6, spanning ORFs MXAN3886–3885; lanes 7–9, spanning ORFs MXAN3883–3882. (d) Immunoblot analysis of accumulation of the MXAN3885 protein during development. DK1622 cells were starved on TPM agar for the indicated periods of time, and cells were harvested and processed as described in Methods. Preimmune serum was used as a negative control.

A prerequisite for the accuracy of the β -galactosidase activity expressed from the MXAN3883–*lacZ* fusion is that the correct promoter region is included in the fusion construct. Thus, the genomic organization of the MXAN3883 locus was examined. It was found that MXAN3882 and MXAN3883 overlapped by 23 bp, and only 10 bp separated MXAN3883 from MXAN3884 and 16 bp separated MXAN3884 from MXAN3885, yet 439 bp separated MXAN3885 from MXAN3886 (Fig. 1b), suggesting that MXAN3885–3882 are likely to be cotranscribed, while the further upstream gene MXAN3886 might belong to a different operon. To investigate the proposed transcriptional organization of this locus, RT-PCR was performed. Total RNA was isolated from DK1622 that had developed for 18 h. cDNA was made by reverse transcription of mRNA with a specific primer. Then we determined whether a PCR product spanning ORFs MXAN3886–3885 could be obtained from the cDNA generated with a primer complementary to the 3' terminal region of MXAN3885. No PCR products were produced, indicating that MXAN3886–3885 are not cotranscribed (Fig. 1c, lane 6). However, an expected PCR product spanning MXAN3885–3883 could be detected from the cDNA generated with a primer complementary to the 3' terminal region of MXAN3883 (Fig. 1c, lane 3), and as a control the same primer pair did not produce any detectable fragments with total RNA (in the absence of cDNA synthesis) as the template (Fig. 1c, lane 1). Surprisingly, although MXAN3883 overlaps with MXAN3882, an expected PCR product spanning MXAN3883–3882 could not be detected from the cDNA generated with a primer complementary to the 3' terminal region of MXAN3882 (Fig. 1c, lane 9). These observations indicate that MXAN3885–3883 are cotranscribed as one transcript. Hence, the potential promoter region should be included in the MXAN3883–*lacZ* fusion construct.

To further confirm the timing of expression of the MXAN3885–3883 operon measured by the MXAN3883–*lacZ* fusion, developmental cells of DK1622 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 rabbit polyclonal antiserum against an MXAN3885 peptide. A band of approximately 15 kDa, in agreement with the calculated molecular mass of the mature MXAN3885 protein (15 360 Da), appeared at 18 h, after which it seemed to gradually increase throughout the time points examined (Fig. 1d). This band was also detected on day 6 at a level similar to that on day 3 (data not shown). However, the MXAN3885 protein band was not present in extracts of vegetative cells and in extracts of developing cells by 8 h. Therefore, the accumulation profile of the MXAN3885 protein generally followed the expression pattern of the MXAN3883–*lacZ* transcriptional fusion, except that the level of MXAN3885 protein on the immunoblots did not decrease after 18 h of starvation. From these observations we conclude that expression

of the MXAN3885–3883 operon is developmentally regulated.

Localization of the MXAN3885 protein in mature spores

According to BLAST analysis and the TIGR annotation, MXAN3883 of *M. xanthus* DK1622 encodes a protein transporter of the outer membrane fimbrial usher porin (FUP) family, MXAN3884 encodes a protein homologous to *E. coli* P pilus assembly chaperone PapD, and MXAN3885 encodes amino acid sequences containing a spore coat protein U (SCPU) domain. Protein U is a spore coat protein produced during late development of DZF1, an *M. xanthus* strain that differs from strain DK1622 used in this study (Gollop *et al.*, 1991; Inouye *et al.*, 1979a). Based on the inclusion of an ORF encoding the SCPU domain, the operon MXAN3885–3883 may encode a CU pathway involved in spore coat formation. However, sequence similarity does not always imply perfect agreement with respect to expression, localization or even function. For example, the products of *ops* and *tps*, two genes encoding spore-specific proteins, have approximately 90% amino acid sequence similarity, yet they differ in patterns of gene expression and subcellular localization (Downard & Zusman, 1985; Furuichi *et al.*, 1985; Teintze *et al.*, 1985).

To determine whether the MXAN3885 product is mainly assembled on the surface of differentiated DK1622 cells and could be considered to be a spore coat protein, the subcellular localization of this protein at the late developmental stage was analysed. Six-day-old fruiting bodies were collected and protein fractionation was performed as described by Teintze *et al.* (1985). Pelleted cells were boiled for 5 min in 1% SDS to solubilize spore surface proteins and separated into soluble (S100) and pellet (P100) fractions by centrifugation. The pellet fraction, which contains myxospores resistant to boiling in SDS, was then sonicated in the presence of glass beads to release proteins inside the spores. The resulting supernatant was further separated by centrifugation into soluble (P100S) and insoluble (P100P) fractions. The three fractions were resolved by SDS-PAGE followed by Western blotting using polyclonal antibodies against an MXAN3885 peptide. Fig. 2 shows that an MXAN3885 protein band was detected mainly in the S100 fraction (lane 1), while only a minor amount appeared in the P100S fraction (lane 2). This band was completely missing in the P100P fraction (lane 3). We also observed that treatment of 6 day-old spores with sonication in the absence of glass beads released MXAN3885 protein into the supernatant after centrifugation (Fig. 2, lane 4), and the resulting pellet when sonicated in the presence of glass beads was found to contain a very low level of MXAN3885 protein only in the soluble fraction (Fig. 2, lane 5). These observations suggest that the MXAN3885 protein, like protein U in strain DZF1, is present mainly on and not bound tightly to the outer

Fig. 2. Localization of the MXAN3885 protein in 6 day-old DK1622 spores. For lanes 1–3, spores were fractionated into S100 (lane 1), P100S (lane 2) and P100P (lane 3) fractions, as described in Methods. For lanes 4–6, spores were first disrupted by sonication without glass beads. After removing the supernatant (lane 4), the pellet was sonicated with glass beads; then the soluble (lane 5) and insoluble (lane 6) fractions were separated by centrifugation. Five microlitres of each fraction representative of an equal number of cells was resolved by SDS-PAGE and examined by Western blotting using antiserum against an MXAN3885 peptide. The position of the MXAN3885 protein is indicated by an arrow.

surface of myxospores (Gollop *et al.*, 1991; Inouye *et al.*, 1979b).

Developmental production of MXAN3885 protein in strain DK1622 starts earlier than that of protein U in strain DZF1

A BLAST analysis of protein U against the entire DK1622 genome indicated that MXAN3885 is the protein in DK1622 that displays the highest similarity to protein U (86 % identity and 92 % similarity at the amino acid level). As shown in Fig. 1(d), MXAN3885 product was accumulated as early as 18 h after DK1622 cells were induced to develop. However, synthesis of protein U in DZF1 does not begin until 45 h after the initiation of development (Inouye *et al.*, 1979a). The apparent discrepancy in the appearance of MXAN3885 and protein U could not be due to the sensitivity with which the protein was detected. In the work of Inouye *et al.* (1979a), cells were pulse-labelled before protein synthesis was analysed, and this method is sufficiently sensitive because development-specific protein S, for example, was clearly detected as early as 1 h after the onset of development. To determine whether the difference in the timing of these two proteins arose from a difference between the developmental condition (stringent starvation on TPM agar) that we used and that (nutrient-limited starvation on CF agar) used by Inouye and co-workers, expression of β -galactosidase in an MXAN3883–*lacZ* fusion strain and production of MXAN3885 protein in DK1622 on CF agar were assessed. As shown in Fig. 3, β -galactosidase activity was clearly detectable at 18 h, and MXAN3885 protein was detected by Western blotting after 28 h of starvation on CF agar. Therefore, although starvation conditions have an effect on temporal expression of the MXAN3885–3883 operon, under the same starvation conditions, MXAN3885 protein in strain DK1622 is

Fig. 3. Expression patterns of the MXAN3885–3883 operon during development on CF agar. (a) Specific activity of β -galactosidase expressed from the MXAN3883–*lacZ* transcriptional fusion. Data are mean $\pm$ SD of four determinations. (b) Immunoblot analysis of accumulation of the MXAN3885 protein during development. DK1622 cells were starved for the indicated periods of time, and cells were harvested and processed as described in Methods.

synthesized earlier than protein U in strain DZF1 during the developmental cycle of *M. xanthus*. Compared with the data in Fig. 1(a), the MXAN3883–*lacZ* expression shown in Fig. 3(a) did not decrease until 24 h and the maximal level of β -galactosidase activity in Fig. 3(a) was reduced to approximately half of that in Fig. 1(a); this can be explained by the fact that starvation on CF agar is more gradual than that on TPM agar. Also, the *lacZ* expression patterns indicate that it is unlikely that there is an additional promoter responsible for the production of MXAN3885 protein at late developmental times.

The *M. xanthus* CU-like system functions in the assembly of MXAN3885 protein on the spore surface

In an effort to ascertain the predicted function of the MXAN3885–3883 operon, MXAN3883, which encodes a putative outer membrane usher protein, was subjected to inactivation by insertion mutation. To examine the accumulation of MXAN3885 protein in the MXAN3883 mutant strain, developmental cells collected at various time

points were boiled in $1 \times$ SDS-PAGE loading buffer, and soluble fractions were then analysed by Western blotting. As shown in Fig. 4(a), the amount of MXAN3885 protein was significantly reduced in the mutant. The MXAN3885 product appeared as a faint band after 18 h of starvation and stayed at the same level until day 6. In a duplicate Coomassie blue-stained gel, the band corresponding to MXAN3885 was visible with extracts of wild-type cells but not with those of mutant cells. Considering that cells collected at later times may contain differentiated spores

that cannot be lysed by boiling in 1% SDS, there is a possibility that the MXAN3885 protein accumulates inside the cells. To test this, we investigated the subcellular localization of the MXAN3885 product using 6 day-old spores. Spores were fractionated into S100, P100S and P100P fractions as described above. MXAN3885 protein was clearly detected in the S100 fraction of strain DK1622, yet was barely detectable in that of the MXAN3883 mutant strain (Fig. 4b). Note that again a faint MXAN3885 protein band appeared in the P100S fraction of DK1622 cells, yet

Fig. 4. Effect of MXAN3883 mutation on the production and localization of MXAN3885 protein. (a) MXAN3883 mutation significantly decreases the production of MXAN3885 protein and inhibits its assembly on cell surfaces during development. Cells of DK1622 (WT) and the MXAN3883 mutant were harvested at the indicated time points after the initiation of development and processed as described in Methods. Left panel, immunoblot analysis using antiserum against MXAN3885; right panel, duplicate gel stained with Coomassie blue. The position of MXAN3885 is indicated by arrows. (b) MXAN3883 mutation does not result in accumulation of MXAN3885 protein inside the spores. Six-day-old spores of the WT and MXAN3883 mutant were collected, fractionated into S100, P100S and P100P as described in Methods, and examined by immunoblotting using antiserum against an MXAN3885 peptide. (c) Analysis of mRNA abundance. Total RNA was isolated from DK1622 and the MXAN3883 mutant 18 h after the onset of development. RT-PCR was carried out to semi-quantify MXAN3885 transcript using 16S rRNA as a control. DNA contamination was excluded via a control without reverse transcriptase (data not shown).

this band was missing in both the P100S fraction and the P100P fraction in MXAN3883 mutant cells (Fig. 4b). Clearly, MXAN3883 mutation did not result in accumulation of MXAN3885 protein inside the spores. One possible explanation is that MXAN3885 protein is degraded when it fails to be exported; a second possibility is that although MXAN3885 is located upstream of MXAN3883, insertion mutation in MXAN3883 impairs mRNA stability so that the amount of MXAN3885 transcript is greatly reduced. To discriminate between these two possibilities, we analysed MXAN3885 mRNA abundance by semiquantitative RT-PCR (Fig. 4c). The amount of MXAN3885 transcript in the MXAN3883 mutant strain was similar to that in DK1622, suggesting that a proteolytic pathway may be invoked in this mutant. Taken together, inactivation of MXAN3883 greatly inhibits the assembly of MXAN3885 protein on cell surfaces, consistent with the hypothesis that the *M. xanthus* CU-like system functions in spore coat biogenesis.

Effect of MXAN3883 mutation on cell aggregation and differentiation

To determine whether the MXAN3883 mutant has a defect in fruiting body formation, cells were exposed to starvation. The timing of fruiting body formation was similar between the MXAN3883 mutant and the wild-type strain DK1622 (data not shown), and high-resolution images of 5 day-old fruiting bodies obtained by SEM did not reveal a significant phenotype for the MXAN3883-deficient strain, except that in this strain adjacent spores on the outer surfaces of fruiting bodies seemed to be loosely connected to each other (Fig. 5b). To examine any possible alterations in the cellular ultrastructure, thin sections were prepared from MXAN3883 wild-type and mutant spores. As shown in Fig. 5(c), DK1622 spores had well-defined outer layers, including an electron-dense cortex and an outermost coat with a lighter appearance. In contrast, cortex layers of spores of the MXAN3883 mutant were observed not to be well-organized (Fig. 5d–g). They were relatively thin, disconnected into segments, or separated into several cortex layers. Note that besides defects in the cortex, the exterior coat of the spore in Fig. 5(f) is not intact. It is worth mentioning that compared with DK1622, the MXAN3883 mutant did not exhibit lower spore counts after sonication and 2 h of exposure to 50 °C (data not shown).

Structural differences between MXAN3884 protein and chaperones of the classical CU family

Based on structural differences between periplasmic chaperones, the entire chaperone family of classical CU pathways can be grouped into two subfamilies: FGS and FGL (Hung *et al.*, 1996). Members of the FGS subfamily assemble typical rod-like pili, whereas the FGL subfamily consists of chaperones that assemble either non-pilus adhesins or very thin fibres that have an atypical morphology. In this sense, the chaperone of the *M. xanthus* CU pathway that participates in spore coat

Fig. 5. Comparison of fruiting body formation and spore morphology between the wild-type DK1622 and the MXAN3883 mutant. (a, b) High-resolution images of surfaces of fruiting bodies. SEM analysis was performed on fruiting bodies of DK1622 (a) and the MXAN3883 mutant (b) after 5 days of development on TPM agar. Bars, 10 µm. (c–f) Ultrastructure of mature spores. Thin sections of spores prepared from 6 day-old fruiting bodies were analysed using TEM. The spores of the MXAN3883 mutant (d–g) lack the well-organized outer layers seen in spores of the DK1622 strain (c). The electron-dense cortex in the MXAN3883-deficient spores was relatively thin (d, arrows), became disconnected (e, arrow), or separated into several cortex layers (f and g, arrows).

formation should belong to the FGL subfamily. To test our speculation, MXAN3884 protein was aligned with two members in each subfamily (Fig. 6): PapD and FimC, which belong to the FGS subfamily; and Caf1M and SefB, which belong to the FGL subfamily. It has been reported that in all of the FGL chaperones, residues at positions 89 and 110 (following PapD numbering) are invariant cysteine residues which are predicted to make a disulfide bond (Hung *et al.*, 1996); in the case of MXAN3884, however, the corresponding positions are occupied by valine and serine, respectively. Interestingly, the number of amino acids in the F1–G1 loop of the MXAN3884 protein is 13, consistent with a short F1–G1 loop (10–20 aa) in the FGS subfamily; however, position 110, which is always a positively charged residue in the FGS subfamily, is occupied by serine in the MXAN3884 protein. Hence, it seems that the MXAN3884 protein does not fall into either

of the two periplasmic chaperone subfamilies. This view was further supported by the fact that K112, one of the two basic residues (the other one is R8) which play a key role in anchoring pilus subunits in the chaperone cleft and which is invariant among the classical CU chaperone superfamily (Kuehn *et al.*, 1993), is replaced by proline in the MXAN3884 protein (Fig. 6).

DISCUSSION

With *lacZ* as a reporter, our search for developmentally regulated OMP genes in *M. xanthus* DK1622 revealed that MXAN3883 was expressed 12–15 h after starvation initiation. The MXAN3883 product has homology to the non-structural usher protein of the CU pathway responsible for the biogenesis of numerous fimbriae (pili) in Gram-negative

Fig. 6. Amino acid sequence alignment of the MXAN3884 protein (Mx3884) with the N-terminal region of mature chaperones in the FGS (PapD, FimC) and FGL (SefB, Caf1M) subfamilies. For the MXAN3884 protein, a signal peptide of 28 aa based on SignalP prediction (Emanuelsson *et al.*, 2007) is not included in the alignment. Residue numbering refers to PapD. Asterisk, identical residues; colon, conserved residues; period, semi-conservative residues. The locations of the F1 and G1 β -strands are indicated by arrows according to Hung *et al.* (1996). Residues mentioned in the text are highlighted. The alignment was generated using CLUSTAL W2 (Larkin *et al.*, 2007).

bacteria. *M. xanthus* contains pili of type IV whose assembly is mediated by the secretin PilQ rather than the CU pathway (Wall *et al.*, 1999). Moreover, considering that MXAN3883 is not expressed during vegetative growth, and that the MXAN3883 mutant did not reveal any defect with respect to swarming (data not shown) and fruiting body formation, it is unlikely that MXAN3883 participates in type IV pilus biogenesis. Sequence analysis and experimental evidence indicate that MXAN3883 is cotranscribed with MXAN3885–3884. Based on phylogenetic analysis, Nuccio & Bäumlér (2007) have proposed that MXAN3884–3883, which encode the chaperone and usher, respectively, belong to an archaic CU system of a σ -fimbrial clade, and in view of the homology shared by the product of MXAN3885 and the spore coat protein U of *M. xanthus* DZF1, they have suggested that *M. xanthus* uses this putative CU system for spore coat formation. Nevertheless, the morphology and function of the encoded surface structures of most operons in the σ -fimbrial clade are unknown.

The present work provides biochemical and morphological data supporting the view that MXAN3885–3883 encode a CU system involved in spore coat assembly. We have shown that the MXAN3885 protein is found in the soluble fraction of cells at relatively early times in development, and at later times most of the protein is found in the spore coat. Like other spore coat proteins, such as proteins S, C and U (Gollop *et al.*, 1991; McCleary *et al.*, 1991; Teintze *et al.*, 1985), the MXAN3885-encoded product can be released from mature spores by boiling in 1% SDS, indicating that while this product may accumulate inside the cell as a soluble protein before sporulation, it is deposited outside the cell during sporulation and can be considered to be a spore coat protein. However, despite significant amino acid sequence similarity between MXAN3885 (strain DK1622) and protein U (strain DZF1), the timing of production of the two proteins differs. Spore coat protein U and MXAN3885 thus appear to be under different temporal regulation.

The ability of the *M. xanthus* CU system to transport and assemble the spore coat protein was evidenced by a marked reduction of MXAN3885 protein assembly on the spore surface of the MXAN3883 mutant. Although MXAN3885 protein was much less abundant in the coat of MXAN3883-deficient 6-day spores than in that of wild-type spores (Fig. 4a), it did not accumulate inside the MXAN3883[−] spores (Fig. 4b). This could be explained by proteolytic degradation when the MXAN3885 protein was sequestered inside the spore, highlighting a major difference from paradigmatic pilus CU systems, in which in the absence of the usher, chaperone–subunit complexes accumulate in the periplasm (Norgren *et al.*, 1987; Klemm & Christiansen, 1990). Moreover, disruption of MXAN3883 caused certain alterations in the spore coat, especially the cortex regions. The CU system is the most common pathway to assemble pili at the surface of Gram-negative bacteria (Waksman & Hultgren, 2009), and although pili differ in morphology and structure, most pili are adhesive surface organelles

designed for attachment to host cells or the environment (Shi & Sun, 2002). Thus, from the phylogenetic point of view, a CU pathway-dependent spore coat protein can be regarded as a non-pilus extracellular adhesin. This view was supported by the fact that the connections between neighbouring spores on the surfaces of fruiting bodies were loosened in the *M. xanthus* strain deficient in transport of MXAN3885 protein across the outer membrane (Fig. 5b). Inouye *et al.* (1979b) have speculated that the function of protein S on spore surfaces is to act as an adhesive to hold adjacent spores together.

The chaperone component of the CU pathway facilitates folding of pilus subunits, prevents them from aggregation and targets them to the usher (Waksman & Hultgren, 2009). As sequence analysis of the MXAN3884 protein did not reveal the presence of certain residues conserved in FGS or FGL chaperones, we reasoned that the structural basis of chaperone function in *M. xanthus* may not completely follow the general rule of classical CU-mediated pilus biogenesis, consistent with the notion that archaic and classical CU systems belong to different phylogenetic groups (Nuccio & Bäumlér, 2007). For chaperones of the alternate CU family, the amino acids or sequence motifs essential for chaperone function are currently not clear; it is therefore difficult to infer whether the MXAN3884 protein is structurally related to the alternate CU chaperones.

So far it is not known whether myxobacterial spore coat proteins can polymerize. Protein S, a well-characterized protein and the most abundant spore coat protein of *M. xanthus*, is not cross-linked by disulfide bridges as it can be easily extracted as a monomer by NaCl without the addition of mercaptoethanol (Inouye *et al.*, 1979b); however, this does not rule out the possibility that protein S might exist as a polymer in mature spores through noncovalent interactions. Note that in spite of the large variation in appearance, the basic structure of organelles assembled via a CU pathway is linear, even though for instance the F1 antigen of *Y. pestis* does not look like linear fibres but rather an amorphous mass. The capsule-like appearance of the F1 antigen is due to the considerable flexibility of the Caf1 subunit polymers (Knight, 2007), and the basic Caf1 polymer is still built as a linear array of subunits held together by intersubunit donor strand complementation (Zavialov *et al.*, 2002). Examination of the MXAN3885 N-terminal region, ATATANLNVTANVGGGS, revealed the presence of alternating hydrophobic residues (bold type). Alternating hydrophobic residues in the N-terminal extension of the CU pilus subunits form an essential part of the subunit–subunit interaction (Waksman & Hultgren, 2009). Thus, it would be interesting to determine whether the MXAN3885 protein could polymerize and form linear arrays in the cell periplasm or at the cell surface.

In conclusion, we have provided biochemical and morphological evidence showing that the *M. xanthus* CU-like system is involved in spore coat formation, although the structural basis for chaperone–subunit interaction appears to deviate

from the general principle of donor strand complementation in classical CU pathways. In the future, we hope to investigate whether the putative *M. xanthus* CU system is a bona fide CU pathway that mediates spore coat formation.

ACKNOWLEDGEMENTS

We thank Guangming Gan for his assistance in preparing samples for TEM. This work was supported by the Natural Science Foundation of China (30571008).

REFERENCES

- Dahl, J. L., Tengra, F. K., Dutton, D., Yan, J., Andacht, T. M., Coyne, L., Windell, V. & Garza, A. G. (2007). Identification of major sporulation proteins of *Myxococcus xanthus* using a proteomic approach. *J Bacteriol* **189**, 3187–3197.
- Downard, J. S. & Zusman, D. R. (1985). Differential expression of protein S genes during *Myxococcus xanthus* development. *J Bacteriol* **161**, 1146–1155.
- Dworkin, M. (1996). Recent advances in the social and developmental biology of the myxobacteria. *Microbiol Rev* **60**, 70–102.
- Emanuelsson, O., Brunak, S., von Heijne, G. & Nielsen, H. (2007). Locating proteins in the cell using TargetP, SignalP and related tools. *Nat Protoc* **2**, 953–971. doi:10.1038/nprot.2007.131.
- Furuichi, T., Komano, T., Inouye, M. & Inouye, S. (1985). Functional complementation between the two homologous genes, *ops* and *tps*, during differentiation of *Myxococcus xanthus*. *Mol Gen Genet* **199**, 434–439.
- Gollop, R., Inouye, M. & Inouye, S. (1991). Protein U, a late-developmental spore coat protein of *Myxococcus xanthus*, is a secretory protein. *J Bacteriol* **173**, 3597–3600.
- Gorski, L. & Kaiser, D. (1998). Targeted mutagenesis of sigma54 activator proteins in *Myxococcus xanthus*. *J Bacteriol* **180**, 5896–5905.
- Hodgkin, J. & Kaiser, D. (1979). Genetics of gliding motility in *Myxococcus xanthus* (Myxobacterales): two gene systems control movement. *Mol Gen Genet* **171**, 177–191.
- Hung, D. L., Knight, S. D., Woods, R. M., Pinkner, J. S. & Hultgren, S. J. (1996). Molecular basis of two subfamilies of immunoglobulin-like chaperones. *EMBO J* **15**, 3792–3805.
- Inouye, M., Inouye, S. & Zusman, D. R. (1979a). Gene expression during development of *Myxococcus xanthus*: pattern of protein synthesis. *Dev Biol* **68**, 579–591.
- Inouye, M., Inouye, S. & Zusman, D. R. (1979b). Biosynthesis and self-assembly of protein S, a development-specific protein of *Myxococcus xanthus*. *Proc Natl Acad Sci U S A* **76**, 209–213.
- Kashefi, K. & Hartzell, P. L. (1995). Genetic suppression and phenotypic masking of a *Myxococcus xanthus* *frzF*[−] defect. *Mol Microbiol* **15**, 483–494.
- Klemm, P. & Christiansen, G. (1990). The *fimD* gene required for cell surface localization of *Escherichia coli* type 1 fimbriae. *Mol Gen Genet* **220**, 334–338.
- Knight, S. D. (2007). Structure and assembly of *Yersinia pestis* F1 antigen. In *The Genus Yersinia: from Genomics to Function*, pp. 74–87. Edited by R. D. Perry & J. D. Fetherston. New York: Springer.
- Kroos, L., Kuspa, A. & Kaiser, D. A. (1986). A global analysis of developmentally regulated genes in *Myxococcus xanthus*. *Dev Biol* **117**, 252–266.
- Kuehn, M. J., Ogg, D. J., Kihlberg, J., Slonim, L. N., Flemmer, K., Bergfors, T. & Hultgren, S. J. (1993). Structural basis of pilus subunit recognition by the PapD chaperone. *Science* **262**, 1234–1241.
- Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A. & other authors (2007). CLUSTAL W and CLUSTAL_X version 2. *Bioinformatics* **23**, 2947–2948.
- McCleary, W. R., Esmon, B. & Zusman, D. R. (1991). *Myxococcus xanthus* protein C is a major spore surface protein. *J Bacteriol* **173**, 2141–2145.
- Norgren, M., Båga, M., Tennent, J. M. & Normark, S. (1987). Nucleotide sequence, regulation and functional analysis of the *papC* gene required for cell surface localization of Pap pili of uropathogenic *Escherichia coli*. *Mol Microbiol* **1**, 169–178.
- Nuccio, S. P. & Bäuml, A. J. (2007). Evolution of the chaperone/usher assembly pathway: fimbrial classification goes Greek. *Microbiol Mol Biol Rev* **71**, 551–575.
- Poole, S. T., McVeigh, A. L., Anantha, R. P., Lee, L. H., Akay, Y. M., Pontzer, E. A., Scott, D. A., Bullitt, E. & Savarino, S. J. (2007). Donor strand complementation governs intersubunit interaction of fimbriae of the alternate chaperone pathway. *Mol Microbiol* **63**, 1372–1384.
- Saier, M. H., Jr (2006). Protein secretion and membrane insertion systems in Gram-negative bacteria. *J Membr Biol* **214**, 75–90.
- Sauer, F. G., Remaut, H., Hultgren, S. J. & Waksman, G. (2004). Fiber assembly by the chaperone–usher pathway. *Biochim Biophys Acta* **1694**, 259–267.
- Shi, W. & Sun, H. (2002). Type IV pilus-dependent motility and its possible role in bacterial pathogenesis. *Infect Immun* **70**, 1–4.
- Søgaard-Andersen, L., Overgaard, M., Lobedanz, S., Ellehaug, E., Jelsbak, L. & Rasmussen, A. A. (2003). Coupling gene expression and multicellular morphogenesis during fruiting body formation in *Myxococcus xanthus*. *Mol Microbiol* **48**, 1–8.
- Spaink, H. P., Okker, R. J. H., Wijffelman, C. A., Pees, E. & Lugtenberg, B. J. J. (1987). Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1J1. *Plant Mol Biol* **9**, 27–39.
- Teintze, M., Thomas, R., Furuichi, T., Inouye, M. & Inouye, S. (1985). Two homologous genes coding for spore-specific proteins are expressed at different times during development of *Myxococcus xanthus*. *J Bacteriol* **163**, 121–125.
- Waksman, G. & Hultgren, S. J. (2009). Structural biology of the chaperone–usher pathway of pilus biogenesis. *Nat Rev Microbiol* **7**, 765–774.
- Wall, D., Kolenbrander, P. E. & Kaiser, D. (1999). The *Myxococcus xanthus* *pilQ* (*sglA*) gene encodes a secretin homolog required for type IV pilus biogenesis, social motility, and development. *J Bacteriol* **181**, 24–33.
- Zavialov, A. V., Kersley, J., Korpela, T., Zav'yalov, V. P., MacIntyre, S. & Knight, S. D. (2002). Donor strand complementation mechanism in the biogenesis of non-pilus systems. *Mol Microbiol* **45**, 983–995.
- Zavialov, A. V., Berglund, J., Pudney, A. F., Fooks, L. J., Ibrahim, T. M., MacIntyre, S. & Knight, S. D. (2003). Structure and biogenesis of the capsular F1 antigen from *Yersinia pestis*: preserved folding energy drives fiber formation. *Cell* **113**, 587–596.

Edited by: W. J. J. Meijer