

Oar, a 115-Kilodalton Membrane Protein Required for Development of *Myxococcus xanthus*

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Myxococcus xanthus is a developmental gram-negative bacterium which forms multicellular fruiting bodies upon nutrient starvation. This bacterium was found to contain a 115-kDa membrane protein which separated with the inner membrane fraction by sucrose density gradient centrifugation. The gene for this protein was cloned, and its DNA sequence was determined. The deduced amino acid sequence consists of 1,061 residues. This protein contains a putative signal sequence and many short segments, found scattered throughout the entire protein, that have sequence similarities with OmpA, a major outer membrane protein of *Escherichia coli*. Thus, the gene was designated *oar* (OmpA-related protein). A second open reading frame was found 36 bases downstream of the *oar* termination codon. This open reading frame encodes a protein of 236 residues and contains a putative lipoprotein signal sequence. An *oar* disruption mutation (Δoar) showed no effect on vegetative growth but caused abnormal morphogenesis during development and reduced myxospore formation. When examined with a light microscope, Δoar cells were unable to aggregate on developmental agar, indicating that Oar is required for cellular adhesiveness during development.

Myxococcus xanthus has been used as a model system for the study of cell-cell interactions during developmental morphogenesis. It is a gram-negative bacterium living in soil and migrates by gliding on semisolid surfaces. Upon nutritional starvation, cells aggregate and form fruiting bodies, within which cells differentiate into myxospores (for reviews, see references 24 and 28). A number of development-specific proteins (proteins S and S1 [12], myxobacterial hemagglutinin [4, 23]; sigma factor, SigB [1]; protein U [6]; and protein serine/threonine kinase, Pkn1 [21]) have been identified and characterized.

Nevertheless, proteins associated with cellular adhesion during developmental morphogenesis and organelles retained for gliding motility have not yet been identified. In the present study, we have identified a protein with a very high molecular weight, fractionated with the inner membrane, which plays an important role in developmental morphogenesis. This protein, designated Oar, was found during the course of experiments to isolate possible organelles involved in gliding motility. The gene for Oar was cloned, its amino acid sequence was deduced, and it was found to encode a polypeptide of 1,061 residues. The disruption of *oar* resulted in a severe defect in the development of fruiting bodies. The results presented here indicate that Oar may be required for cellular adhesion during development.

MATERIALS AND METHODS

Bacterial strains, phage, and growth conditions. *M. xanthus* DZF1 (*sglA1*; an FB strain derived from DK101) (11) was used and grown in Casitone-yeast extract (CYE) (3) at 30°C. To select kanamycin-resistant transductants, cells were first grown on Casitone-yeast extract plates containing 25 μ g of kanamycin sulfate per ml for 1 day, and subsequently the plates were overlaid with 3 ml of soft agar containing 500 μ g of kanamycin sulfate per ml as described

by Kroos and Kaiser (16). A half-concentration CTT medium containing 1.5% agar (9) was used to examine cell motility. Development was induced on a clone fruiting (CF) agar plate (7) as previously described (11).

Escherichia coli JM83 (31) and KE94 (HB101 *pcnB* Tn10) (18) were used and grown in L-broth medium (19) supplemented with ampicillin (50 μ g/ml), kanamycin sulfate (50 μ g/ml), or chloramphenicol (25 μ g/ml), when necessary.

Phage P1clr100cm (25) was used to transduce cloned DNA from *E. coli* to *M. xanthus* by the method described by Shimkets et al. (29).

Plasmids. pUC9 (31) was used to clone and subclone chromosomal DNA fragments. pREG411 was a pBR322 derivative carrying a 6.6-kb *EcoRI* fragment encoding P1-specific incompatibility (29). pPIEK (15) was constructed by inserting a 5.4-kb *EcoRI-KpnI* fragment from pREG411 into *EcoRI-KpnI* sites of pUC19 (33). pUCKan5 and pUC9Km (*PstI*⁻) were derivatives of pUC9 carrying 2.5- and 1.3-kb fragments of the kanamycin resistance gene from Tn5, respectively. The *PstI* site in the 1.3-kb fragment was eliminated by mutagenesis.

DNA manipulation and sequencing. Chromosomal DNA was prepared by the method previously described by Avery and Kaiser (2). Southern blot analysis was carried out by the method of Southern (30). The DNA sequence was determined in both strands by the dideoxy-chain termination method (27) with double-stranded DNA and synthetic primer oligonucleotides. Other DNA manipulations were carried out by methods described previously (17).

Construction of *oar* and *oar-mlpA* deletion mutants. *oar* and *oar-mlpA* deletion mutants were constructed by using the DNA fragments indicated in Fig. 5. For the *oar-mlpA* deletion mutant, pMMCA3P1 (Fig. 1, see Fig. 5) was first constructed by replacing the 1.6-kb *Sall*(e)-*Bam*HI(c) fragment with the 2.5-kb kanamycin resistance gene from Tn5. To isolate the 1.5-kb *Sau*3A-*Sall*(e) left-hand fragment for pMMCA3P1 (see Fig. 5), the *Sall*(e) site was changed to an *Xho*I site by oligonucleotide-directed mutagenesis (14). The

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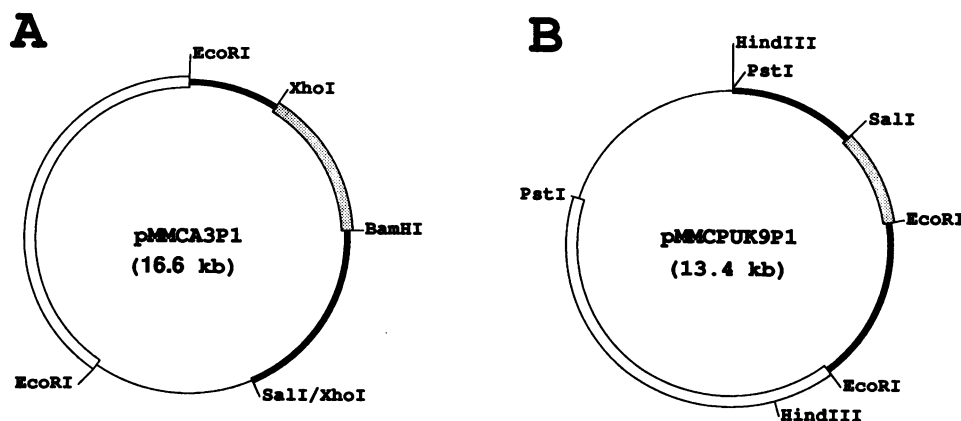


FIG. 1. Circular maps of pMMCA3P1(A) and pMMCPUK9P1(B), which were used to create the $\Delta oar-mlpA1$ and $\Delta oar1$ deletion mutants. Fragments are drawn approximately to scale. Shaded regions, kanamycin resistance gene of Tn5; open boxes, 6.6-kb (A) and 5.4-kb (B) incompatibility regions of phage P1. Thin and thick lines represent vector and *M. xanthus* DNAs, respectively.

1.5-kb *Sau3A-XhoI* and the 3.2-kb *BamHI(c)-XhoI(b)* fragments were then used, respectively, for the upstream and downstream regions flanking the kanamycin resistance gene (see Fig. 5). The addition of this construct to the 6.6-kb *EcoRI* fragment carrying the P1 incompatibility region from pREG411 (5) results in the P1-transducible plasmid pMMCA3P1, and its circular map is shown in Fig. 1A.

To construct the *oar* deletion mutant, the 1.6-kb fragment between *SalI(a)*, which exists 130 bases upstream of putative initiation codon ATG (bases 235 to 237 in Fig. 6), and *Sau3A* (base 1802 in Fig. 6) was replaced with the 1.3-kb kanamycin resistance gene of Tn5 from pUC9Km(*PstI*⁻). The 1.7-kb *PstI(a)-SalI(a)* and the 2.3-kb *Sau3A-EcoRI* fragments were used, respectively, as the upstream and the downstream regions flanking the kanamycin resistance gene. To this construct, the 5.4-kb *PstI-EcoRI* fragment carrying the P1 incompatibility region from pP1EK (15) was added, resulting in the P1-transducible plasmid pMMCPUK9P1, and its circular map is shown in Fig. 1B.

These plasmids were then transduced into *M. xanthus* DZF1 by P1 transduction (29). The deletion mutants that resulted from double crossover events were screened by using nick-translated pUC9 DNA as a probe, and the mutations were verified by Southern blot analysis using *PstI*-digested chromosomal DNA (data not shown).

Isolation and partial sequence of Oar. Cells from a 1-liter culture were washed with 10 mM Tris HCl-8 mM MgSO₄ and suspended in 20 ml of 20% sucrose-10 mM EDTA-0.1 M Tris HCl (pH 7.6). The suspension was incubated on ice for 30 min. After addition of Triton X-100 to a final concentration of 0.5%, 0.2 ml of 1 M MgCl₂ and 60 μ l of DNase I (10 mg/ml) were added to the solution, and the mixture was shaken gently at 30°C for 30 min. After centrifugation to remove cell debris at 6,000 \times g for 10 min, the supernatant was further centrifuged at 60,000 \times g for 45 min at 4°C. The pellet was resuspended in 0.5 ml of 0.1 M Tris HCl (pH 7.8) containing 1 mM EDTA and 0.1% Triton X-100 (TTE buffer), loaded on a stepwise sucrose gradient (10, 25, 40, 55, and 70%), and centrifuged at 100,000 \times g for 90 min at 4°C. Eight 1.5-ml fractions were collected from the top of the gradient, and 20 μ l from each fraction was analyzed by sodium dodecyl sulfate-17.5% polyacrylamide gel electrophoresis (SDS-PAGE). The fractions containing Oar were combined and loaded for preparative SDS-PAGE. After staining to visualize bands, the 115-kDa region correspond-

ing to Oar was cut out and electroeluted with Little Blue Tank (ISCO, Inc., Lincoln, Nebr.). The protein was precipitated by adding trichloroacetic acid to 10%. After approximately 100 μ g of protein was partially digested with 5 μ g of protease V8 for 30 min, the digest was loaded for SDS-PAGE and transferred to a polyvinylidene difluoride membrane by using a semidry blotter apparatus (Sartorius, Göttingen, Germany). The membrane was stained with Coomassie blue, and the band corresponding to 32 kDa was cut out. Approximately 600 pmol was used to determine the amino-terminal amino acid sequence. The amino acid sequence was obtained with an Applied Biosystems, Inc., 470A protein sequencer.

Inner and outer membranes were isolated by the method described previously (22).

Nucleotide sequence accession number. The nucleotide sequence data presented in this paper has been deposited in the GenBank data base under accession number L12992.

RESULTS

Purification of the 115-kDa protein fractionated with the inner membrane. *M. xanthus* cells are known to move by gliding on semisolid surfaces, but the molecular mechanism for the gliding motility is not well understood. We have attempted to isolate possible organelles which might be involved in gliding motility by employing the method for isolation of flagellar basal bodies of *Caulobacter crescentus* (8) to *M. xanthus* DZF1 with some modifications. Vegetatively growing cells were treated with lysozyme and Triton X-100 as described in Materials and Methods. The insoluble fractions were fractionated by a five-step sucrose gradient centrifugation. Each fraction was analyzed on SDS-PAGE, and the results are shown in Fig. 2. One protein, indicated by an arrow with Oar, that migrated at a size greater than that of the 92.5-kDa marker was clearly enriched in three fractions (fractions 3, 4, and 5). The molecular mass of this protein was estimated to be approximately 115 kDa by using β' and β subunits of RNA polymerase as markers (data not shown).

To determine the cellular localization of Oar, the inner and outer membranes were separated by sucrose density gradient centrifugation (22), and their protein patterns were analyzed by SDS-PAGE as shown in Fig. 3. It has been shown that, in contrast to that of *E. coli*, the inner membrane

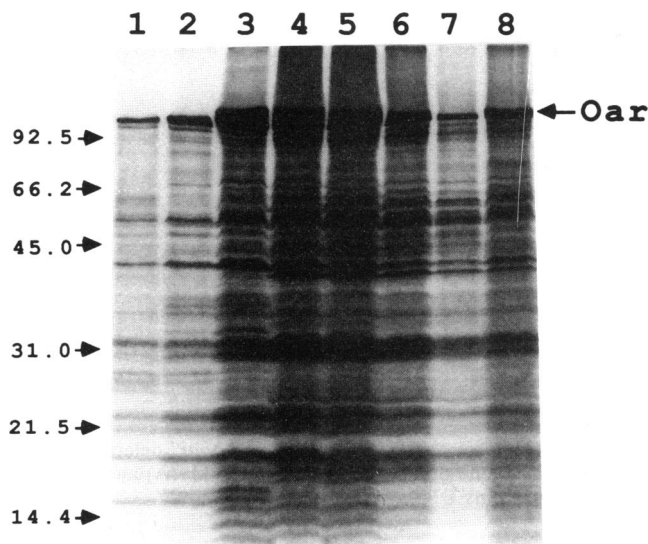


FIG. 2. Protein pattern of the fraction separated by sucrose density gradient centrifugation. The insoluble fraction treated with lysozyme and Triton X-100 was resuspended in TTE buffer, and after sucrose gradient centrifugation (see Materials and Methods), the fraction was analyzed on an SDS-polyacrylamide gel. A sample from the top of the gradient was in lane 1, and a sample from the bottom of the gradient was in lane 8. The position corresponding to Oar is indicated by an arrow. Numbers at the left are molecular weights in thousands.

fraction of *M. xanthus* has a higher density ($\rho = 1.221$ g/ml) than the outer membrane (22). The outer membrane fraction thus was located in fractions 1 to 3, while the inner membrane fraction was sedimented in fractions 5 to 12 (Fig. 3). The Oar protein, indicated by an arrow with Oar, was found in the inner membrane fraction.

Cloning and sequencing of the gene that encodes the 115-kDa protein (Oar). In order to clone the gene that encodes

the Oar protein, the protein was separated by SDS-PAGE and partially digested with protease V8 as described in Materials and Methods. The region corresponding to a major band at 32 kDa was cut out, and its amino-terminal sequence was determined. The sequence obtained was V-L-A-N-T-X-10

L-G-A-S-Y-T-X-K-D-M-N-S-V-I-E, where X represents an unidentified amino acid. On the basis of this sequence, 24-mer degenerate oligonucleotides 5'CTCGATGACGGAG C C

TTCATGTCCTT3' (oligo 911), which correspond to the complementary sequence from Lys (residue 14) to Glu (residue 21), were synthesized. Because of the high G and C usage at the third base of codons in *M. xanthus* (13), only G and/or C was used for the third position of each codon. Figure 4 shows a Southern blot analysis of a chromosomal DNA digest using several restriction enzymes and probed with oligo 911. One major band and a few minor bands could be seen in each lane.

To clone the gene, a λ phage genomic library, constructed by ligating partially *Sau3A*-digested chromosomal DNA into the *Bam*HI sites of EMBL4 (Progenia) (34), was screened by using oligo 911 as a probe. One (phage no. 23 A-7) of 960 phages was found to hybridize and contained an insertion of approximately 20 kb. The insertion contained part of the *oar* gene consisting of the 6-kb *Sau3A*-*Xho*I(b) fragment and a 14-kb fragment downstream of *Xho*I(b) site (Fig. 5). However, the screening of the same phage library for the upstream coding region by using the 300-bp *Sau3A*-*Sal*I(b) fragment (Fig. 5) as a probe was unsuccessful. Therefore, the 2.1-kb *Sma*I(a)-*Sma*I(b) fragment (Fig. 5) was cloned from the chromosomal digest into the *Sma*I site of pUC9 by using the 300-bp *Sau3A*-*Sal*I(b) fragment as a probe. Subsequently, the 2.8-kb *Pst*I fragment [*Pst*I(a)-*Pst*I(b) in Fig. 5] was cloned into pUC9 by using a 0.8-kb *Sma*I(a)-*Pst*I(b) fragment as a probe. In both cases, positive colonies could not be found unless *E. coli* KE94 (18) was used as a recipient cell. This *E. coli* strain is known to maintain a very low plasmid copy number in the cell. Subsequently, the nucle-

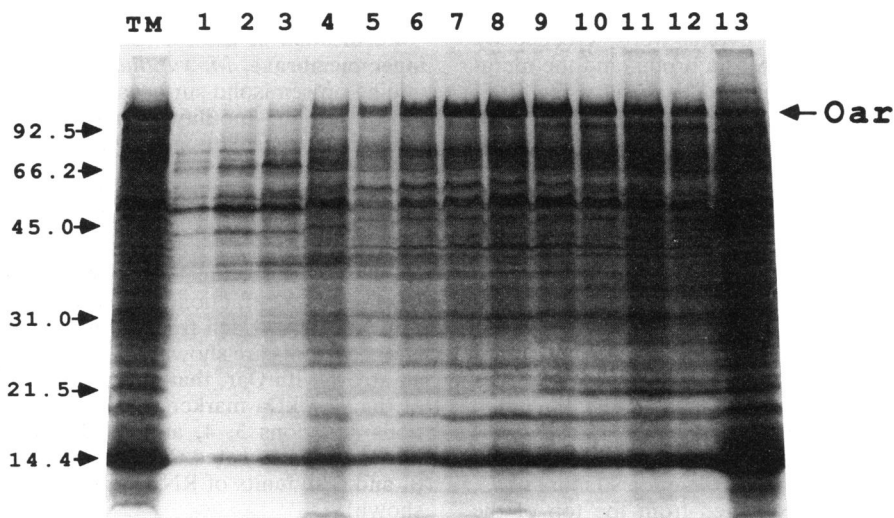


FIG. 3. Protein pattern of inner and outer membranes separated by sucrose density gradient centrifugation. Inner and outer membranes were prepared as described previously (22) and separated by sucrose density gradient centrifugation. TM, total membrane fraction. Samples applied in lanes 1 to 13 are the fractions from the top of the gradient to the bottom; 10- and 5- μ l aliquots were applied in lanes 1 to 4 and in lanes 5 to 13, respectively. The position of Oar is indicated with an arrow. The band that migrates at 14.4 kDa is lysozyme, which was used to lyse the cells. Numbers at the left are molecular weights in thousands.

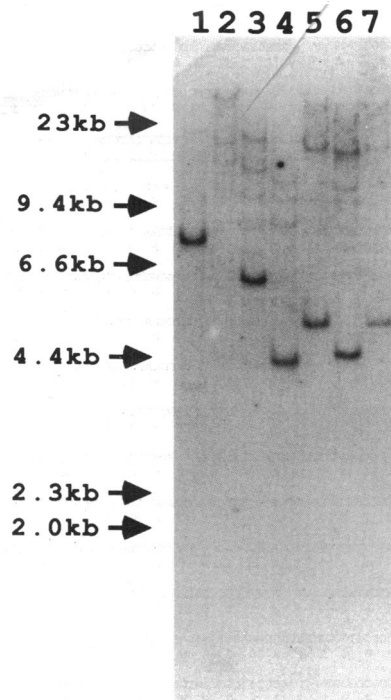


FIG. 4. Southern blot analysis of chromosomal DNA digested with various restriction enzymes. The 24-mer degenerate oligonucleotides (oligo 911) were used as probes. Lanes: 1, *Xho*I; 2, *Sal*I; 3, *Pst*I; 4, *Bam*HI-*Pst*I; 5, *Bam*HI-*Hind*III; 6, *Bam*HI-*Eco*RI; 7, *Bam*HI.

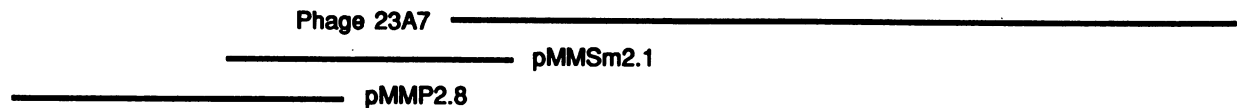
otide sequence of 4,710 bases encompassing two genes, *oar* (for OmpA related) and *mlpA* (for *M. xanthus* lipoprotein A), was determined (Fig. 6).

Analysis of the ORFs for *oar* and *mlpA*. In the nucleotide sequence of 4,710 bases and its deduced amino acid sequence shown in Fig. 6, the region corresponding to the

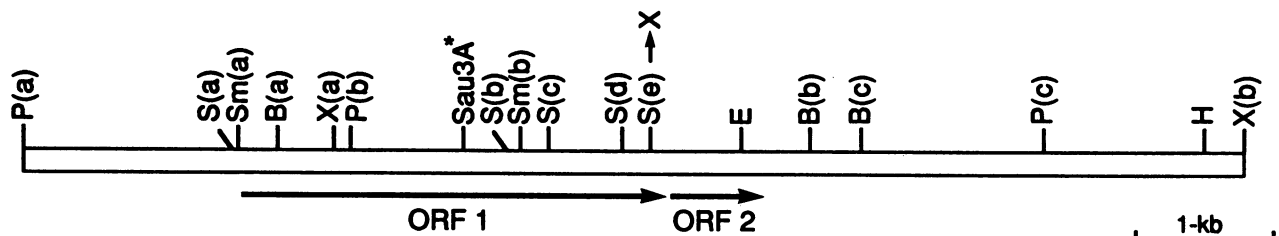
amino acid sequence V-L-A-N-T-X-L-G-A-S-Y-T-X-K-D-¹⁰

M-N-S-V-I-E is found from bases 2560 to 2622 (underlined in Fig. 6), indicating that the first open reading frame (ORF) indeed encodes Oar. From the amino-terminal Val residue (bases 2560 to 2562) of this sequence to the termination codon, TAA (bases 3418 to 3420), there are 286 amino acid residues, which agrees well with the size of the 32-kDa V8 fragment used for the determination of the partial amino acid sequence. This result indicates that the 32-kDa V8 fragment was derived from the C-terminal portion of the Oar protein. The initiation codon of *oar* was assigned ATG at bases 235 to 237 for the following reasons. (i) The molecular weight of Oar was estimated to be 115,000 by SDS-PAGE. The ORF encodes 1,061 amino acid residues with a calculated molecular weight of 114,448 (with the signal peptide). (ii) Oar was isolated as an inner membrane protein. The ORF product contained a typical signal peptide of 26 amino acid residues at the amino-terminal end (boxed in Fig. 6). The prokaryotic signal peptides are known to consist of a basic amino-terminal end followed by a long hydrophobic sequence (10).

A



B



C

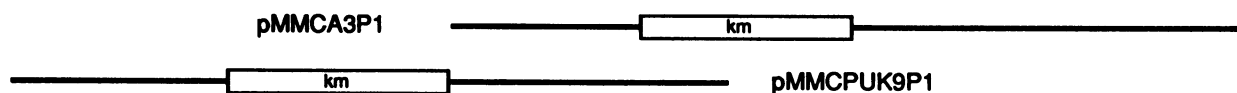


FIG. 5. Schematic diagram of the DNA fragment encompassing the *oar* and *mlpA* genes. (A) Regions that were cloned in phage 23A7 and plasmids pMMSm2.1 and pMMP2.8. (B) Restriction map of the 10.5-kb fragment. Thick lines with arrows indicate locations and orientations of ORF1 (*oar*) and ORF2 (*mlpA*). P, *Pst*I; S, *Sal*I; B, *Bam*HI; X, *Xho*I; E, *Eco*RI; H, *Hind*III; Sm, *Sma*I. Only the *Sma*I sites used to clone the *Sma*I fragment in pMMSm2.1 are shown. The *Sau*3A site at the 5' end of the *M. xanthus* chromosomal DNA which was cloned into the *Bam*HI site of EMBL4 is shown with an asterisk, and other *Sau*3A sites are not shown. S(e) with an arrow indicates that the *Sal*I site was mutated to an *Xho*I site. (C) Upstream and downstream regions used to construct the deletion mutants by flanking the kanamycin resistance gene.

1 GCCTAGACTCTCTTACCAAGTGGACACCAATTGGCCAGCTCCGCCGGATGAAGGG

61 CGCGCTACCAATACAGAAACCGCTCCAGGTTCAAGCAAGCAAGCTGACGGACGGGGCGT

121 GATCCCGCAATTCGCCACTGGTACCAAAAAGCAAGCTGATGACAACTGCTGAC

181 TCGATATTTTCCGGGTCAITTTACATTCGACGCCCACTTGGAGGGTCTGGTATGCA

Oar M H

241 TTGAACCGAGTCCCGGAAACCGAGTGTGTGTCGCCAGGTTCTCTGACGGATCC

22 L N R V L R E T G V V A A G L L Y G S

301 CGCGCTTTCGGCAGTCGACGACATCATGGTACGGTATGACGCTGACAGTCCGAG

42 A A F A Q S S T I I G T V I D A Q S R Q

361 CCTGCCGCTGAGTCTGTGTCAGCCGACCTGCCCACTTCAGGTTGACGAGCGGCT

62 P A A D V V V T A T S P N L Q G E Q T V

421 GTCACCGAGCGAGGTAACCTACCGCTCCCGAGTTCGCCCGGACCTACACCTG

82 V T D A Q G N Y R I P Q L P P G D Y T L

481 CGGTTGAGAAGGAGCAGTTCAGCGTACGCTGCTTCCGCCATCCAGCTGCGTCAAC

102 R F E K E Q F K P Y A R S A I Q L R L N

541 CGCAACATCCGCTCAACGTCGAGTCTCCCGAGCGCTCGGTGAGTGGTGGATC

122 R T I R V N V E L L P E A L G E V V E I

601 GTCGCCGCCCCCGACCTGACGCTGGTTCACGAGATGGCGTGAACGTGATCAG

142 V G A P P T I D V G S T T M G V N V D Q

661 GAGTTCATCAAGCCATCCCGTTCGGGCTCCGGTGGCAAGCGCGCGACCCGCTCC

162 E F I K R I A V A R P G G K G G A T R S

721 TTAGGTCCTCCGCGAGTCCGCTGCGGCTCCCGGCAAGCAAGCTACGCGGTGTCATC

182 F E S L A E L A P G A Q N D N Y G V S I

781 AACGGCTGCACTCCGCTGAGAAAGCTGAGTGGTGGACGGCTTCGACGAAAGCGCG

202 N G S T S P E N G Y V V D G L S T N D P

841 GCCTCCGGCTGAAAGCCACCGCTGAGCATGAGTTCGTCGAGGCTGAACATCATC

222 A F G V N A S P L S I E F V Q D V N I I

901 ACGCGGTTACATGCGGAGTTCGGCGGTCACCGCGGCTCATCAAGCGGTCCAC

242 T G G Y M P E F G R S T G G V I N A V T

961 CGGTGGGCTCCAGGAGTTCAGGCTCCGTTGTCGCACTGACGCGCGGCAACCTC

262 R S G S N E F H G S V P A N M T P G T L

1021 GAGGTTACCGAGCAGATTTGTAAGAGGCGAGGTCATCAGCGCCAGAACAGCTG

282 E G T R K Q I R E E G T V I T G Q N Q L

1081 CAGAACCTGGGAGCTTCGGCGCCAGCTCCGATCTCAAGGCAAGCTGTGG

302 Q N L G D F G A T L G G P I L K D K L W

1141 TTCTCCGGCTCCGCGGCTGCTTCCCGCTTACAGCAGCAGCGCTCAATCGC

322 F F A G F A P S F T R Y Q H T R T L N A

1201 CTGCGCTGATGACGAGGCAACAGATCAAGGAGAGAGCTTACCGTCCGCGAC

342 L R V D D E G N T I K D E T D F T V A D

1261 CGCATCCCGGTTCCCGCAGGATGACGCGGCTCCGCTACCACTTCAATCATAGGT

362 A I P G S A R K Y I A D S R T I Q Y M G

1321 AAGTTGACGACTTACACAGCAGCACAAGCTGCTTCCCGTGAAGGTTAGCGG

382 K L T Y L I N Q D H N V S P A L N G T P

1381 ACCTGACGGGTGCTTGGAGCTGAGGCTCAATCCCGAGTCCGAGGCGCTCCCGGC

402 T S T G G L G K L S V N P Q S G G L P G

1441 GTCTGCGTACCGCTCCGGTACTTCGCTTACGGAGAGAGGCAACAGCAGCT

422 V L A T R P G D F L G P D G S N L G D R

1501 CTGCGCTGAGTACCGCGTCCCTCCGCGCAAGAGTCTCGTTGATCGCAACTC

442 L A L K Y A G A F A D K K V L V D A N L

1561 GGCTGTTCCAGCAGCGGCTCCACCTCCCGGCTGACGAGCAACCTGGCGATCGC

462 G W F H Q T A S T L P G D G S N L G D R

1621 ACGCGCTGCTGCTACTCGGAGTGTGACACCAACCGCGCGCTGACCTGTTC

482 T G L A G Y S R M V Y T T P R A L T L F

1681 GAAGCCCTCCCGAAGCGAGGAGTCCCTCCGCGAGCAGCGGAGGAGCAACTGCT

502 E A L P E G Q E G A C G S T P E E Q L V

1741 CGCTCCCGCTGACGCGCTACGGGCTGGGTCGCCCTTCAAGGAGCAGCAGCTG

522 R S P V T G Y G V G G P G F M S D Q T L

1801 GATCGTACCAAGGCAAGCGGCACTACCTGCAAGCGCTGCGCACCCACGCT

542 D R Y Q A N A K A T Y L L N A L G T H V

1861 TTCAAGCAGCGGTTGAGCTGAGTTCGCTGCTGTCGACAGGTTGAAGGCTACGGCGC

562 F K A G V D V E L L S F D Q V K A Y G G

1921 GCGTGTCTTCCAGGAGGCTTCAACGAGCGGCTTCCGCGAGGCGCCCGCTGAC

582 G V F F Q E G S N Y G V A G Q G P A V H

1981 GATGCGGCTGCTACGGGATCAGACCGGCTCTGACTTCCGGTACGAGTTCACCCAG

602 D A R R Y G Y Q T G F D S A V T Q F T Q

2041 GTTCCAGCAGCAGCAGCAGCAGGTCGCTGCTTCCAGGACTCTGCTTCAATCCG

622 V A K T T S T T V G G F L Q D S W S I A

2101 AACCGGCTGACTTGAACCTGGCGCTCCGCTACGAGTTCAGCGGCTTACGGGCAAT

642 N R V T L N L G V R Y D V Q A L Y G G N

2161 GGCGACTCTGCTGCTGCTCCGCACTAGTGGTCCGCGGTA TCGGCGCACTGCTGAC

662 G D L S L L L G N Q W S P R I G A I V D

2221 CGGTTCCCAAGCGCGCGAGGTTGCTGAGACTTCCGTTACTACGAGGAGCT

682 P F A N G R A K V F V N P A R Y Y E Q V

2281 CCCTCAACGTTGAGCCGCGCTCCCGGTTGAGAACGCACTCCCGCGCTCCCTC

702 P L N L M D R A F P G E N R I S A R R S

2341 CTGGCGAGCGCGCCAGGCGACCGGACCTGTCGACCGCTCCAGCTTCCGAGCCAG

722 L A E P G Q G T A T S C D P S S F E S Q

2401 CAGGCTACGTAACACCGACTGCAACCTGCTGCCATTCGGAGGAGCGCAACGCTG

742 Q A T C N T D S N L L A I P E S S R N V

2461 AACCGCTTACACCGCGGACCGTTCGGCGGACCGCGTACCGGACATCAAGGCC

762 N R F Y T G G T V G G T P V D P D I K A

2521 CAGTCTTACAGAAATGCTGGTCCGCGCGAGTACGAGTCTGCGGTAACCGGCTG

782 Q S S D E I V V G A E Y E Y L A N T R L

2581 GGTGGAGCTACAGCAGAGGACATGAACTCGCTCATCGAGGACATGAGCGCGAGC

802 G A S Y T H K D M N S V I E D M S R D D

2641 GGCACACGACTTCTCGGTAACCGCGGCGGCTTCGCGGTAAGTTCGCGAGCGCG

822 G N T Y P L G N P G S G F A G E F P T P

2701 GTTCTAATACGACAGCTACCGCTTACGTAACCGTACGTTCCGCGCGCTGCTC

842 V R N Y D N V T V Y L N R T F A D G W L

2761 CGCCAGCCAACTACACTGCTCCGCGCTTACGCTTACGTAATCTGCTGTTCCGCTC

862 A Q A N Y T M S R L Y G N Y P G L F R P

2821 GAGAGCGGCGACTGCAACCGAATCTCTCGGACTTCCGCTTACGAGCTCTGGAG

882 E T G Q L D P N I L S D P D L I E L L E

2881 AACCGACGGCTTCTCGGTTCCGCGGCGGCTTCGCGGTAAGTTCGCGAGCGCG

902 N R T G L L P F D R T H Q I K V F G A K

2941 GAGTCAACACTCGAAGCGCTTCCGCGGCGGCTTCCGCGGTAAGTTCGCGAGCT

922 E F N I S N A L S A S V G V S Y R G S S

3001 GGTACCGGATCACTACTGGGTAGCCACTGGGCTTCCGCTTACGAGGAGTCTCTGCT

942 G T P I N Y M G S H W A Y L Q D E S F V

3061 CTCCCGCGTGGCGCTGCGCGCGTACCGCGTGAATCAACCACTGACCTCAACATGGC

962 L P R G A G G R T P M I N T I D S N I G

3121 GTGAATACCGCTCAGCAAGGACAGCGTGTGCTTCCGCTTCCGCTTCCGCTTCC

982 V N Y R V S K D S V V S F T L D V F N L

3181 TTCACTTCCAGGCGTGAACCGCTTCCGCGGAGTCAACCTTCGCGGATCAAGGCC

1002 F N F Q G V N T V D Q T Y T L R D I K P

3241 ATCCCGGCTGGCAGCGCTGCGGAGCTGGAGAACCTCCGCGGCGGCTGAGGTTCCAGG

1022 I P G G T P A D L E N L P G R V E F Q D

3301 CAGGCGCGGCTGAGGAGCGCTTCCGCGGCTGCGGAGGAGTCAAGCAAGAACTCAAG

1042 Q A P R D E P P F G S V D G D V N K N P K

3361 AACCGCTCTCGTCCAGCGCGCGCTCAGGTCGCTTCCGCTTCCGCTTCCGCTTCC

1061 N P L S Y Q A P R Q V F R I R Y T F *

3421 TCCAGCGGCAAGCAAGTCAAGCACTGACGAGCAAGCACTGCAATACT

MlpA M T K N I V N T

3481 CGGTTGGTCTCGTGGCGCGGAACTCCGTCGACGCTTCCGCTTCCGCTTCCGCTTCC

28 A L V L V G A G S L L T G C N F E Q P E

3541 ACCAATGCTTGTGAGGAGTCCGCGGCTGGCGGTTGAAGTACGAGCTTGGGATCC

48 T N C F V Q E S P S W A V K Y D V V D S

3601 CCCAAGGAGCGGATGCGGAGGAGTCCGCGGAGTCCGCGGAGTCCGCGGAGTCCGCG

68 P K D A N G D E C T T T A P L V E L M G

3661 GTCTAAGTACGTAAGCGGAGAGCGGCGGCTGAGCTGCACTTCCGCGGAGT

88 V Y K Y V N P E T G A A Q L A L R P A T

3721 CTGGGAGCGCGGAGTCTGACAGGAGGAGTCCGCGGAGTCCGCGGAGTCCGCGG

108 L A S R A I A D T T T S A D Q T S L G

3781 TCTTGGACCGAAGCAAGGACCTGCTTCCGCGGAGGAGTCCGCGGAGTCCGCGG

128 S L D T E P K D H G F C H A N T F C P

3841 TTGTTCAAGTGGCGGCTCGGACTCCGCGGAGGAGTCCGCGGAGTCCGCGGAGT

148 F V N V A A S D T A A A N T I R Y E F T

3901 AATGTTCCGCTGACTCCGCGGCTTCCGCGGAGTCCGCGGAGTCCGCGGAGTCCG

168 N V R V Y S A A V A P G T Q F T E L K

3961 TACCAAGCAAGTCCGCTTCCGCGGAGTCCGCGGAGTCCGCGGAGTCCGCGGAGT

188 Y T S N G C T S S Y V M R A V W P P A P

4021 TCGATACCGCTTCCGCGGAGGAGTCCGCGGAGTCCGCGGAGTCCGCGGAGTCCG

108 C D T A S T E P A E N C G V G S G L N P

4081 GAATCCGCGTGGTTCGCGGAGGAGTCCGCGGAGTCCGCGGAGTCCGCGGAGT

228 E F A V V C Q P T S A T G T Y G V C V P

4141 CGCGTGACATCCGCTGCTCAAGTAGTGAACAGTCTGCTGCAACCGCGGACCC

236 A G D I P S F K *

4201 ACCTGGTGGCGCACTCCGCGGCGGCTTCCGCGGAGTCCGCGGAGTCCGCGGAGT

4261 CCOCGCTTTAAAAGCTGAGCTGACTTGGAGACATGAGGCGGCTTACTACTCA

4321 TCGAGCGGCTTCCGCGGAGTCCGCGGAGTCCGCGGAGTCCGCGGAGTCCGCGG

4381 ACGCGGCTTCCGCGGAGTCCGCGGAGTCCGCGGAGTCCGCGGAGTCCGCGGAGT

4441 AGCCATCTACGTAAGATCCAGGAGTCCGCGGAGTCCGCGGAGTCCGCGGAGT

4501 CGGATGAGTGGAGGCTTCAAGCGGAGTCCGCGGAGTCCGCGGAGTCCGCGGAGT

4561 TCCGCTGCGGAGTCCGCGGAGTCCGCGGAGTCCGCGGAGTCCGCGGAGTCCGCGG

4621 GGCTCTTCCATGTTGAGGCTGACGGGATCCCGCTTCCGCGGAGGAGCGGAGT

4681 GCGCTATCCGCTGCTGCGGCGGCGGCGG

FIG. 6. Nucleotide sequence of 4,710 bases encompassing the *oar* and *mlpA* genes and their deduced amino acid sequences. The amino acid sequence determined from the 32-kDa V8 fragment is underlined. The putative signal sequences of Oar and MlpA are boxed. The inverted repeat sequences at the 3' end of the *mlpA* gene are shown by arrows. Numbers at left and right indicate the nucleotide bases and amino acid residues, respectively.

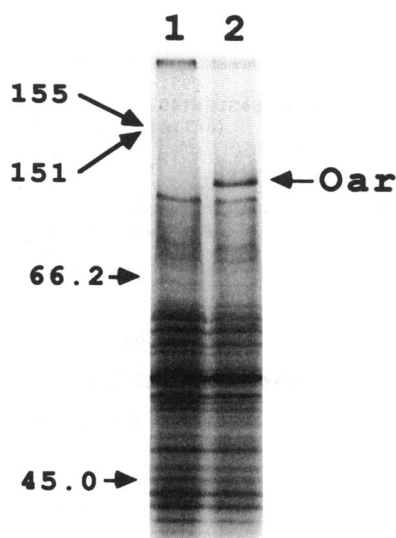


FIG. 7. Patterns of membrane proteins of DZF1 and $\Delta oar-mlpA1$. Total membrane fractions isolated from DZF1 (lane 1) and $\Delta oar-mlpA1$ (lane 2) were analyzed by SDS-10% PAGE. The position of Oar is indicated with an arrow. Numbers at the left are molecular weights in thousands; 155 and 151 correspond to the positions of β' and β subunits of RNA polymerase, respectively.

The cleavage site of the signal peptide is tentatively assigned to the alanine residue at position 26. The cleavage site could be the residue at either position 23 or 24. Oar is thus considered to be secreted across the inner membrane, using the signal peptide. (iii) The putative initiation codon is preceded by eight bases with a purine-rich sequence, GAGG, which may serve as a ribosome-binding site. (iv) Except for the signal peptide-coding region (65%), the third positions of codons in the ORF use G or C at a very high frequency (89%) throughout the entire ORF. In contrast, upstream codons used in the same reading frame as the ORF use G or C at the third position with a much lower frequency.

Another ORF was found 37 bases downstream of the termination codon for *oar*. This ORF, from the initiation codon at bases 3457 to 3459 to the termination codon at bases 4165 to 4167, encodes a putative polypeptide of 236 amino acid residues. The high usage of G or C (81%) at the third positions of codons was similar to that in *oar* and other *M. xanthus* genes (13). The amino-terminal 21-amino-acid sequence also showed features typical of a prokaryotic signal sequence, as in the case of *oar*. It is interesting that the signal peptide has a so-called lipoprotein box, SLLTGC, at the cleavage site (32), suggesting that the cleavage of the signal peptide occurs between Gly (residue 21) and Cys (residue 22) after the cysteine residue may be modified with a lipid. Therefore, the gene was designated *mlpA*, the *M. xanthus* gene for lipoprotein A. There is a nine-base inverted repeat with a ΔG of -17 kcal (ca. -71 kJ) (26) 23 bases downstream of the termination codon. This inverted repeat probably serves as a transcription termination signal for the *oar* operon.

Characterization of *oar-mlpA* and *oar* deletion mutants. In order to examine the functions of *oar* and *mlpA*, *oar-mlpA* and *oar* deletion mutants were constructed as described in Materials and Methods. pMCA3P1 for the construction of an *oar-mlpA* deletion contained the deletion between the *SalI*(e) site 87 bp upstream of the termination codon of the

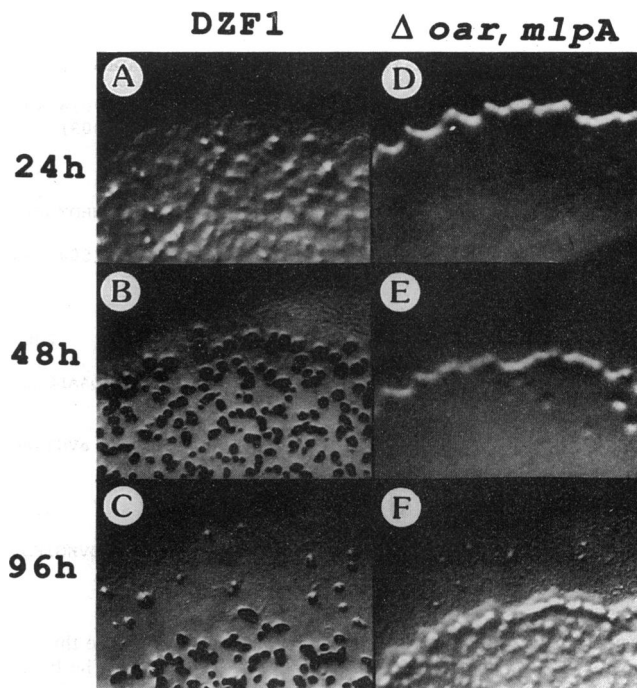


FIG. 8. Sequence of morphological changes of DZF1 and $\Delta oar-mlpA1$ cells during development. Cells were spotted on CF agar plates as described in Materials and Methods. Photographs were taken under a dissecting microscope with a Polaroid camera at the indicated times after spotting.

oar gene and the *Bam*HI(c) site 750 bp downstream of the *mlpA* gene, as shown in Fig. 5 (also see Fig. 1A). In order to delete only the *oar* gene, pMCPUK9P1 was constructed by replacing the 2.0-kb *SalI*(a)-*Sau*3A fragment with the 1.3-kb kanamycin resistance gene, as shown in Fig. 5 (also see Fig. 1B). Both plasmids were introduced into DZF1 by P1 transduction (29), and colonies were selected for kanamycin resistance. Approximately 50 and 25% of kanamycin-resistant colonies were *oar-mlpA* and *oar* deletion mutants, respectively, which resulted from double crossover events. The deletion mutations, designated $\Delta oar-mlpA1$ and $\Delta oar1$, were verified by Southern blot analysis of *PstI* chromosomal digests of the deletion strains (data not shown). The protein patterns of the membrane fractions of $\Delta oar-mlpA1$ and $\Delta oar1$ strains were examined. Figure 7 shows the absence of Oar in the membrane fraction of the $\Delta oar-mlpA1$ strain, indicating that the 27-amino-acid deletion from the carboxyl-terminal end of Oar is sufficient to eliminate Oar in the membrane fraction. Besides the Oar band, no specific band missing from the membrane fraction of the $\Delta oar-mlpA1$ strain was detected, probably because of the very low MlpA production (not shown). Oar was also completely deleted in the membrane fraction of the $\Delta oar1$ strain (not shown).

When the growth of both deletion mutants in Casitone-yeast extract liquid medium was examined, both the $\Delta oar-mlpA1$ and $\Delta oar1$ strains normally grew with the same doubling time as the wild-type strain. However, most interestingly, they adhered much less to the culture flask during the late exponential growth phase and the stationary phase than the DZF1 strain, indicating that Oar may be associated with cellular cohesion. When these mutants were examined for development on CF agar plates, their fruiting body formation was much delayed compared with DZF1 cells. As

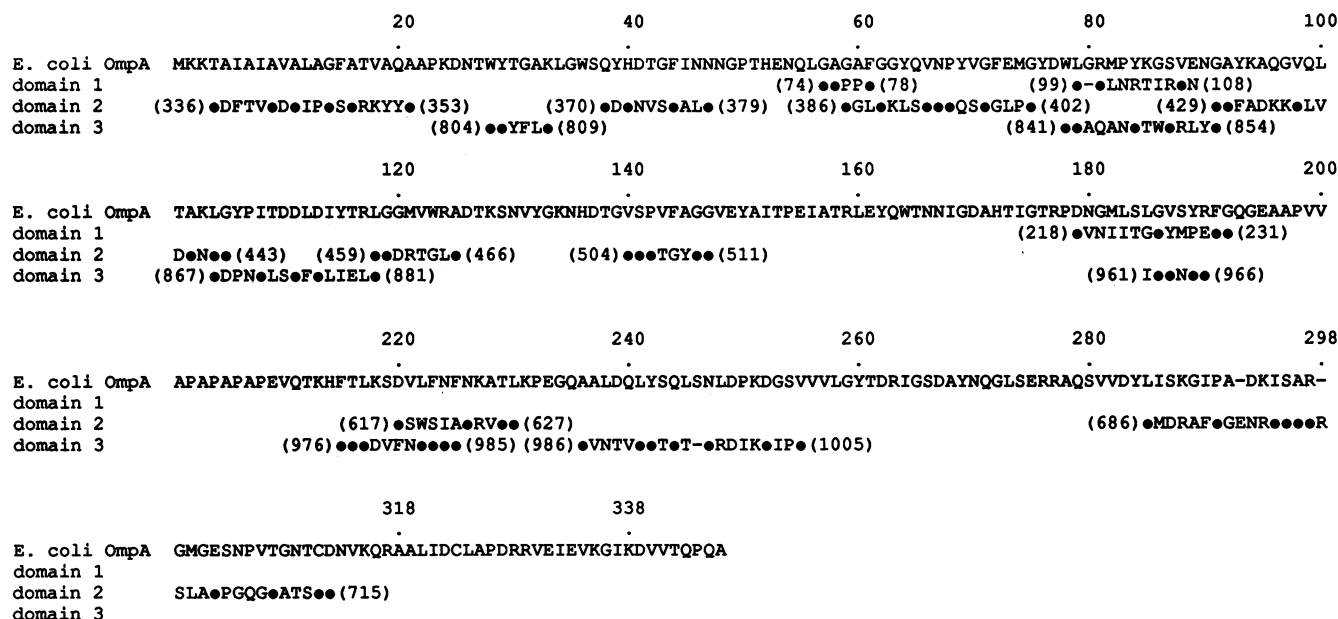


FIG. 9. Alignment of amino acid sequences between the three domains of Oar (domain 1 from residues 27 to 350, domain 2 from 351 to 790, and domain 3 from 791 to 1061) and *E. coli* OmpA. The homology of Oar with other proteins was explored by using the FASTA and BLAST programs. Amino acid residues identical to those in OmpA are represented by dots. Bars represent gaps. Numbers in parentheses indicate positions of amino acid residues in the Oar protein (Fig. 6).

shown in Fig. 8, DZF1 cells clearly aggregated, forming mounds after 24 h (A) and distinct fruiting bodies at 48 h (B). In contrast, no clear signs of cellular aggregation were seen for mutant cells even after 48 h (Fig. 8D and E). However, after 96 h (Fig. 8F), mutant cells showed some tendency to aggregate. The yield of myxospores was significantly reduced (as much as 30% after 6 days) for the mutant strains. Further characterization of these mutants during differentiation will be described elsewhere.

DISCUSSION

In the present paper, we identified a protein of 115 kDa which fractionates with the inner membrane of *M. xanthus*. This protein, designated Oar, was originally isolated by a method used for the isolation of the flagellar basal body of *C. crescentus*. However, we were not able to detect any basal body-like structures in this fraction by electron microscopy. Fractionation analysis of the sucrose density gradient centrifugation of the membrane fraction by SDS-PAGE revealed that Oar is a major inner membrane-associated protein.

When the gene for Oar was cloned and sequenced, it was deduced to encode a 1,061-amino-acid protein. The *oar* gene was found to be nonessential for vegetative growth of *M. xanthus*, while its deletion caused a serious defect in developmental morphogenesis, with reduced aggregate and fruiting body formation. As a result, the spore yields in *oar* deletion strains were substantially reduced. However, the deletion mutants were found to be as mobile as the wild-type cells on CF agar plates (not shown), indicating that Oar is not associated with the gliding motility of *M. xanthus*. These results indicate that Oar is associated with cellular adhesion required for fruiting body formation.

Oar was found to contain scattered amino acid segments with sequence similarities with OmpA, a major *E. coli* outer membrane protein (20). It appears that the OmpA motifs are repeated three times throughout the entire protein: the first

domain is from residues 27 to 350, the second domain is from residues 351 to 790, and the third domain is from residues 791 to 1061. The sequences with high similarities are shown along with the OmpA sequence in Fig. 9; the second domain revealed the highest homology. Since the function of OmpA has not been elucidated, it is not known at present how the similarity between Oar and OmpA is functionally significant.

We are currently investigating further the role of Oar during development. Preliminary results, together with the present study, indicate that Oar is associated with cellular adhesion which is essential for fruiting body formation. The *mlpA* gene appears to be in the same operon as the *oar* gene. We were not able to detect the gene product in *M. xanthus*, and therefore it remains unclear whether the gene product is a lipoprotein or whether it plays an essential role during development, as does Oar.

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