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MlpA, a Lipoprotein Required for Normal Development of *Myxococcus xanthus*

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The *mlpA* gene encoding a 236-residue polypeptide has been identified immediately downstream of the *oar* gene of *Myxococcus xanthus* (M. Martinez-Canamero, J. Munoz-Dorado, E. Farez-Vidal, M. Inouye, and S. Inouye, J. Bacteriol. 175:4756–4763, 1993). The amino-terminal 21 residues of MlpA encode a typical prokaryotic signal sequence with a putative lipoprotein cleavage site. When expressed in *Escherichia coli* in the presence of [2-^{3}H]glycerol, ^{3}H-labeled MlpA had a molecular mass of 33 kDa and was found to be associated with the membrane fraction. Globomycin, an inhibitor of signal peptidase II, caused a shift in the mobility of *E. coli*-expressed MlpA to 35 kDa. Subsequently, a *mlpA* disruption strain (*oar*$^+$) was constructed and found to have delayed fruiting body formation (by approximately 36 h), with significantly larger fruiting bodies being produced compared with those of the wild-type strain. Nevertheless, spore yields for the two strains were identical after 120 h of development. These data indicate that MlpA, the lipoprotein identified in *M. xanthus*, is required for normal fruiting body formation.

Myxococcus xanthus is a gram-negative, soil-dwelling bacterium which migrates by gliding on semisolid surfaces in a complex social pattern (6, 32). Upon deprivation of nutrients, cells migrate towards aggregation centers, forming large spiral patterns (27) which further develop into mounds called fruiting bodies (16, 19). Within the fruiting bodies, cells differentiate into myxospores. The aggregation process results in multicellular morphological changes, progressing from low mounds to elevated mounds within 24 to 36 h (3, 6, 22). *M. xanthus* has been extensively used as a model system to study cell-cell interaction during developmental morphogenesis. A number of development-specific proteins, protein S (12), myxobacterial hemagglutinin (28), SigB (2), protein U (7), and eukaryotic-like protein Ser-Thr kinases (25, 34, 38), have been identified and characterized.

In an attempt to isolate membrane-associated, motility-linked proteins, we identified a 110-kDa protein, designated Oar, associated with the inner membrane (21). The gene for Oar was cloned, and its DNA sequence was determined and found to encode a polypeptide of 1,061 amino acid residues. Downstream of *oar*, a second open reading frame (ORF) consisting of 236 residues was found and designated *mlpA* because of its structural similarity with a major outer membrane lipoprotein of *Escherichia coli* (Fig. 1). We demonstrated that deletion of the *oar* gene did not affect motility but resulted in a severe defect in the development of fruiting bodies.

The initial *oar* deletion construct was constructed prior to identification of the entire ORF and was later determined to cause a disruption of not only the *oar* ORF but the *mlpA* ORF as well (21). This construct was designated DZF1(Δ*oar-mlpA*) (Fig. 1). Phenotypic studies of the *M. xanthus* mutant showed the inability to form fruiting bodies (21). A second construct that disrupted only the ORF of the *oar* gene was created and designated DZF1(Δ*oar*) (Fig. 1). Phenotypic studies of this *M. xanthus* mutant showed its phenotype to be identical to that of DZF1(Δ*oar-mlpA*) (21).

In this report, we demonstrate that the *mlpA* gene is not expressed in the DZF1(Δ*oar*) strain described above, even though the *mlpA* ORF is intact. Subsequently, an *mlpA* disruption mutant that expressed Oar at a normal level was constructed in order to determine the function of MlpA in the *M. xanthus* life cycle. MlpA was found to be required for normal fruiting body formation in *M. xanthus*. Disruption of this gene caused a 36-h delay in development and spore formation. The fruiting bodies produced were twice the size of those of the wild-type strain. We further demonstrate that MlpA is a lipoprotein associated with the membrane of *M. xanthus.*

MATERIALS AND METHODS

Materials. α-^{35}S-dATP and [α-^{32}P]dCTP were purchased from Amersham. A Sequenase kit was purchased from U.S. Biochemicals. Restriction enzymes were purchased from New England Biolabs or Boehringer Mannheim. T4 DNA ligase was obtained from Gibco, Bethesda Research Laboratories. Polyvinylidene difluoride membrane was obtained from Millipore. *Taq* DNA polymerase for PCR was obtained from Boehringer Mannheim.

Bacterial strains, media, and plasmids. *M. xanthus* DZF1 was grown in CYE medium (4) and on clone fruiting agar (CF agar) (8). *E. coli* JM83 (35), CL83 (20), DH5α (10), and LE392(DE3) (31), which encodes T7 RNA polymerase (33), were used as recipient strains for transformations. Cells were grown in LB medium (23) supplemented with 50 μg of ampicillin per ml or 25 μg of streptomycin per ml when necessary. For the expression of MlpA with T7 RNA polymerase, M9 medium (23) was used. pUC9 (35) was used for cloning, subcloning, and sequencing experiments. pUC7Str was derived from pUC7 and contained the 2.3-kb streptomycin resistance gene. pET11a was used for the expression of *mlpA* (33). CTT medium was used for electroporation and was prepared as described previously (18).

Construction of *mlpA* expression vectors. An *Nde*I site was created at the initiation codon of *mlpA* by PCR. Synthetic oligonucleotide 5′-AACAGAATTCATATGACCAAGAACATCGTC-3′ (3498) along with the reverse sequencing primer were used to amplify a 600-bp fragment from plasmid pEFJ9 and to clone it into pUC9. Oligonucleotide 3498 also contained an encrypted *Eco*RI site at the 5′ end. After the DNA sequence was confirmed, the PCR fragment was released from pUC9 by *Eco*RI and cloned in the correct orientation into a pUC9 plasmid already containing the *Eco*RI-*Bam*HI(b) fragment from phage 23A7

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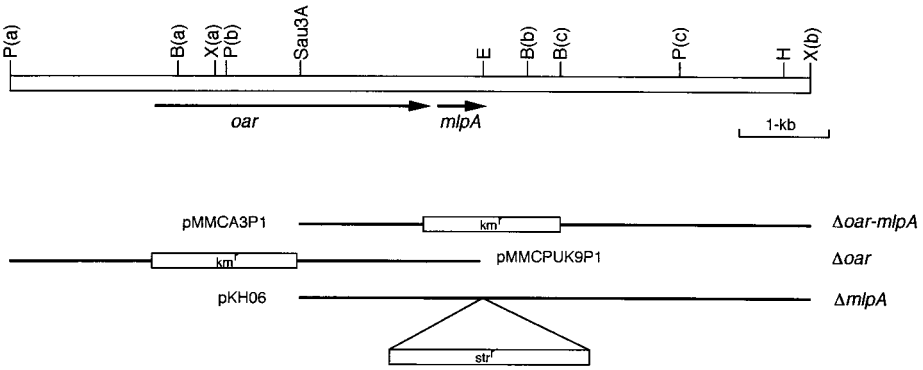

FIG. 1. Restriction and disruption construct maps for *oar* and *mlpA*. The kanamycin resistance gene is denoted by km^r, and str^r denotes the streptomycin resistance gene. The bold arrows indicate the ORFs of *oar* and *mlpA*. Restriction sites are indicated as follows: P, *Pst*I; B, *Bam*HI; X, *Xho*I; E, *Eco*RI; and H, *Hin*dIII. The only *Sau*3A site used for the construction of the deletion is shown.

(Fig. 1). The final construct was designated pET*mlpA*. DNA sequencing was carried out by the chain termination method (30), and PCR was performed according to the method described previously (29).

Labeling MlpA with [2-^{3}H]glycerol. *E. coli* LE392(DE3) cells were transformed with pET11a as a control or pET*mlpA*. Transformants were grown in M9 medium at 37°C to 80 Klett units. Cultures (10 ml) were treated with or without 100 μg of globomycin per ml, an inhibitor of signal peptidase II, for 10 min. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM. After the culture was incubated for another 10 min, [2-^{3}H]glycerol (10 μCi/ml) was added. After a 1-h incubation, the cells were harvested by centrifugation and the membrane fractions were prepared as described previously (37). Isolated membrane fractions were resuspended in 200 μl of water by sonication and precipitated by adding 1.2 ml of cold acetone. The precipitate was extracted two times with 0.5 ml of $CHCl_3$-methanol (2:1), evaporated to dryness, and resuspended in 50 μl of water. Samples were diluted with 50 μl of 2× solubilization buffer (80 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 0.2 M β-mercaptoethanol, and 0.02% bromophenol blue), boiled for 5 min, and separated on an SDS–10% polyacrylamide gel electrophoresis (PAGE) gel. The gel was treated with En^{3}Hance as recommended by the manufacturer, dried, and exposed to film for 1 week.

Antiserum production. Because MlpA is a lipoprotein and would most likely be localized to the membrane, we expressed MlpA without its first 66 amino acids and started with Met-67 to help ensure that it would localize in the cytoplasm during expression. For the induction of *mlpA* expression, *E. coli* harboring modified pET*mlpA*(Met-67) was grown in M9 medium to 40 Klett units, and IPTG was added to the culture to a final concentration of 1 mM and incubated for 2 h at 37°C. MlpA(Met-67) was produced as inclusion bodies which were solubilized in 1× solubilization buffer and purified on a preparative SDS–12.5% PAGE gel. MlpA(Met-67) extracted from the gel was used for preparation of polyclonal antisera.

Construction of *mlpA* disruption mutant. The *mlpA* disruption mutant was constructed with the DNA fragments indicated in Fig. 1. The 2.3-kb streptomycin resistance gene was inserted in the ORF of *mlpA* at the *Eco*RI site. The 2.3-kb *Sau*3A-*Eco*RI and the 3.8-kb *Eco*RI-*Hin*dIII fragments were used, respectively, as upstream and downstream regions flanking the streptomycin resistance gene. This produced plasmid pKH06 (11.0 kb) (Fig. 1). Plasmid pKH06 was introduced into *M. xanthus* DZF1 by electroporation (17). Cells were plated with 0.7% soft agar on CTT plates containing 500 μg of streptomycin sulfate per ml (Sigma). Colony hybridization was carried out as described previously (14).

Fruiting body formation. Fruiting body formation was carried out by concentrating exponentially growing cells from CYE at 100 Klett units to 4,000 Klett units in TM buffer (10 mM Tris-HCl [pH 7.5] and 8 mM $MgSO_4$) and by plating them on CF agar plates as described previously (8).

Developmental myxospores harvested from CF agar plates were suspended in TM buffer and sonicated at 80 W for 2 min in an ice bath to disrupt vegetative cells. The remaining spores were counted in a Petroff-Hausser bacterium counting chamber (Arthur H. Thomas Co., Philadelphia, Pa.).

Western blot (immunoblot) analysis. Cell cultures were grown in CYE to 100 Klett units. Cultures were harvested by centrifugation and washed in 20 mM Tris-HCl (pH 7.6) buffer containing 10 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, 0.1 mM aprotinin, and 0.1 mM pepstatin (W buffer). Cells were resuspended to 1/100 of the original volume in the same buffer and sonicated to lyse cells. Samples were microcentrifuged for 5 min at 5,000 × *g* to remove any large cell debris and then microcentrifuged at 100,000 × *g* for 30 min to pellet membranes. The supernatant was removed, and the membranes were resuspended to 1/200 of the original volume in the same buffer. Samples of equal cell numbers were separated on SDS–12.5% PAGE gels. Electrophoresed proteins were transferred to polyvinylidene difluoride membrane with a Sartblot semidry transfer apparatus as recommended by the manufacturer (Sartorious, Göttingen, Germany).

RESULTS

Western analysis of MlpA of strain DZF1(Δ*oar*). Since the *mlpA* gene is located immediately downstream of the *oar* gene (21), it is quite possible that these two genes are in the same operon. Therefore, we first examined if *mlpA* in the DZF1 (Δ*oar*) strain was expressed. For this purpose, the soluble and membrane fractions from DZF1 and DZF1(Δ*oar*) cells grown in CYE medium as described in Materials and Methods were prepared.

Western analysis was then carried out with these samples, with a polyclonal rabbit antiserum to MlpA being used. As can be seen in Fig. 2, a 33-kDa band localized predominantly to the membrane fraction in DZF1 (lanes 1 and 2). In contrast, in DZF1(Δ*oar*), MlpA was not detected in either the membrane or the soluble fraction (Fig. 2, lanes 3 and 4). These data indicate that both genes are likely to be in the same operon and that the disruption of the *oar* gene prevents *mlpA* expression. Therefore, the phenotypic characterization of DZF1(Δ*oar*) (21) reflects a result of the absence of both gene products, indicating that the role of each protein in the observed phenotype is unknown.

Construction of *mlpA* disruption mutant DZF1(Δ*mlpA*). In order to determine what role MlpA plays in the development of *M. xanthus*, we created a disruption of the *mlpA* gene in DZF1 but kept *oar* intact. Plasmid pKH06 was constructed for the disruption of *mlpA* by inserting a 2.3-kb streptomycin resistance gene at the *Eco*RI site in the *mlpA* gene as described in Materials and Methods (Fig. 1). The plasmid was introduced into *M. xanthus* DZF1 by electroporation (17). Mutants were

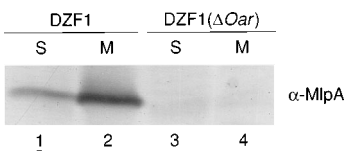

FIG. 2. Western blot analysis of MlpA in DZF1 and DZF1(Δ*oar*). Samples were prepared as described in the text, and equal cell equivalents per lane were loaded on an SDS–12.5% PAGE gel. S, supernatant; M, membrane. The gel was transferred to polyvinylidene difluoride membrane and blotted with a rabbit polyclonal antiserum, α-MlpA. The molecular mass of the band was estimated to be 33 kDa by comparing it with the positions of molecular weight markers (data not shown).

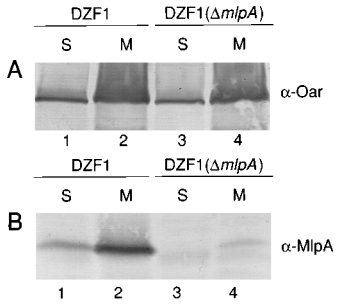

FIG. 3. Western blot analysis of DZF1 and DZF1(Δ*mlpA*). Samples were prepared as described in the text. Protein was transferred to polyvinylidene difluoride membrane as described in the text. (A) Membrane blotted with a rabbit polyclonal antiserum against Oar (α-Oar). (B) Membrane blotted with a rabbit polyclonal antiserum against MlpA (α-MlpA). S, supernatant; M, membrane.

selected by resistance to streptomycin at 500 μg/ml. Approximately 43% of the streptomycin-resistant colonies were disruption mutants resulting from double homologous recombination events, as determined by Southern hybridization with pUC9 being used as a probe (data not shown). The disruption of *mlpA* was designated DZF1(Δ*mlpA*).

DZF1 and DZF1(Δ*mlpA*) cultures were grown to 100 Klett units, and membranes and soluble fractions were isolated as described in Materials and Methods. Samples with equal cell numbers were separated on SDS–12.5% PAGE gels. Western analysis with a polyclonal rabbit antiserum to MlpA showed that a 33-kDa band localized predominantly to the membrane fraction in DZF1 (Fig. 3B, lanes 1 and 2) and was not present in DZF1(Δ*mlpA*) (lanes 3 and 4). To confirm the presence of Oar in DZF1(Δ*mlpA*), a second Western analysis was performed with the same samples, with antiserum raised to Oar being used (Fig. 3A). Oar was found at equal quantities in both DZF1 (Fig. 3A, lanes 1 and 2) and *mlpA* disruption mutant DZF1(Δ*mlpA*) (lanes 3 and 4).

Developmental analysis of DZF1(Δ*mlpA*). When the vegetative growth of DZF1(Δ*mlpA*) in CYE medium was compared with that of DZF1, no difference in the rates of growth was observed (data not shown). This was also found to be the case with DZF1(Δ*oar*) under similar conditions. When DZF1 (Δ*mlpA*) and DZF1 were examined for development on CF agar plates at 30°C, DZF1 developed to mature fruiting bodies within 36 to 48 h (Fig. 4). However, DZF1(Δ*mlpA*) developed dramatically slower than DZF1 by approximately 36 to 40 h (Fig. 4). DZF1(Δ*mlpA*) produced fruiting bodies with approximately twice the diameter of those of DZF1 (Fig. 4). To compare spore formation during development, DZF1 and DZF1 (Δ*mlpA*) were incubated on CF agar plates and samples were harvested at different time intervals. The number of spores produced in DZF1(Δ*mlpA*) during early development was lower than that produced by DZF1, but the numbers were equal after prolonged incubation (Fig. 5). The morphology of the spores produced by DZF1(Δ*mlpA*) appeared to be identical to that of the wild-type strain. Germination of the spores from DZF1(Δ*mlpA*) also appeared to be identical to that of DZF1. Previously, it has been shown that the DZF1(Δ*oar*) mutant, which was unable to produce both Oar and MlpA, failed to make fruiting bodies (21). Therefore, the present results suggest that Oar may be primarily responsible for fruiting body formation.

[2-^{3}H]glycerol labeling of MlpA in *E. coli*. The predicted amino acid sequence of *mlpA* was used as the basis for a search of the GenBank data base, and it was discovered that the amino-terminal 21 amino acids of MlpA shows features typical of a prokaryotic signal sequence. Within the signal sequence, there is a putative lipoprotein lipid attachment site, SLLTGC, called a lipo-box (36). This suggests that a signal peptidase (signal peptidase II) cleaves the signal sequence in front of the Cys residue to which a glyceride acylated by fatty acid residues is attached (36). In order to further characterize MlpA as a lipoprotein, we attempted to label it with [2-^{3}H]glycerol in *E. coli* by using a T7 expression system (33) as described in Materials and Methods. As can be seen in Fig. 6, one major band with a size of approximately 33 kDa in the membrane fractions was labeled in the presence of [2-^{3}H]glycerol (lane 2). This band shifted to 35 kDa when the cells were treated with 100 μg

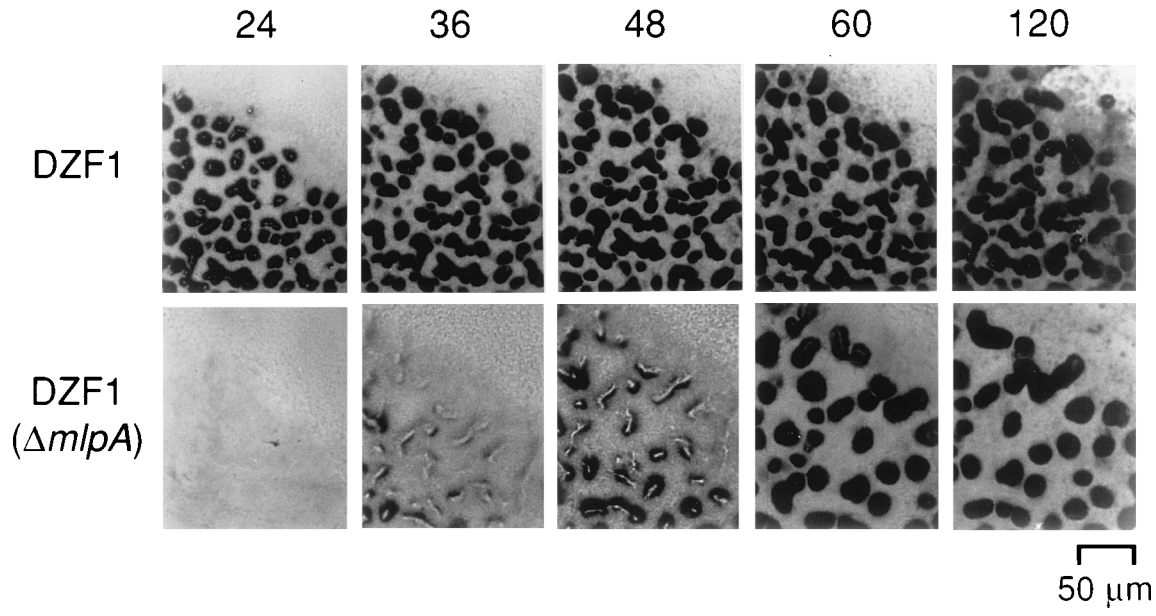

FIG. 4. Morphogenesis of fruiting body formation by strains DZF1 and DZF1(Δ*mlpA*). Numbers indicate the hours of development at 30°C.

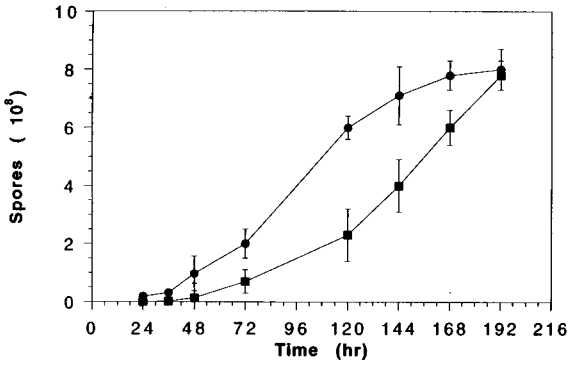

FIG. 5. Time course of spore production by DZF1 and DZF1(Δ*mlpA*). Spores were harvested as described in Materials and Methods. The points represent the means of three experiments, and the vertical bars indicate the range within the same three experiments. Solid circles and squares indicate spore numbers for DZF1 and DZF1(Δ*mlpA*), respectively.

of globomycin per ml, an antibiotic that inhibits signal peptidase II (Fig. 6, lane 3). Induction of cells harboring control plasmid pET11a did not result in any labeled bands under the condition used (Fig. 6, lane 1). It should be noted that MlpA produced in *M. xanthus* existed predominantly in the membrane fraction and that it also migrated as a 33-kDa protein in an SDS-PAGE gel, as can be seen in Fig. 3B. These results indicate that MlpA is indeed a lipoprotein of *M. xanthus* which exists in the membrane fraction.

DISCUSSION

The membrane protein, Oar, was previously identified during a search for organelles involved in the motility of *M. xanthus* (9). When the gene for Oar was sequenced, a second ORF located 37 bases downstream was identified. This ORF encodes a protein with a typical prokaryotic signal sequence containing a so-called lipoprotein box at the cleavage site. Because of these attributes, the gene was designated, *M. xanthus* lipoprotein A (*mlpA*) (21). Deletion of either the *oar* gene or both the *oar* and *mlpA* genes created developmentally deficient mutants with identical phenotypes. We show in this report that the mutant created by deletion of only the *oar* gene loses the ability to produce the gene product of the *mlpA* gene, suggesting that these two genes are in the same operon. As was previously described (21), this operon is considered to be terminated at the stable secondary structure immediately downstream of *mlpA*. Such stable secondary structures have been found for a number of *M. xanthus* genes, including *mbhA* (28), *ndk* (24), *rpoD* (13), and *recA* (26). Therefore, an *mlpA* deletion mutant was subsequently constructed. The deletion mutation has no effect on vegetative growth or the localization of Oar in the membrane. However, the *mlpA* deletion mutation caused a long delay in fruiting body formation. On the other hand, no fruiting body formation occurred with cells which were unable to produce both Oar and MlpA (21), suggesting that the two membrane proteins are coordinately playing a role during development.

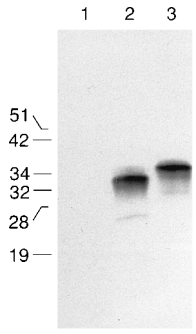

FIG. 6. Autoradiogram of [2-^{3}H]glycerol-labeled MlpA. MlpA was labeled with [2-^{3}H]glycerol during expression in *E. coli*, with the T7 promoter of the pET11a vector being used. MlpA was labeled and samples were prepared as described in the text. Lane 1, pET11a (control plasmid); lane 2, pET11a(*mlpA*); lane 3, pET11a(*mlpA*) with globomycin (100 μg/ml). Molecular mass markers are indicated in kilodaltons.

Because of the conserved (lipo-box) sequence at the putative cleavage site of the signal sequence, MlpA was assumed to be a lipoprotein. This notion was supported by the facts that (i) MlpA could indeed be labeled with [2-^{3}H]glycerol when expressed in *E. coli*, (ii) MlpA was detected as a 33-kDa membrane protein in both *E. coli* and *M. xanthus*, and (iii) the cleavage of the signal peptide from pro-MlpA in *E. coli* was inhibited with globomycin, a specific inhibitor for signal peptidase II. MlpA is the first lipoprotein to be identified in *M. xanthus.*

It is interesting to note that the secretion machinery which is required for the translocation of proteins across the outer membrane of gram-negative bacteria contains lipoproteins (1, 5, 11). During development and vegetative growth, *M. xanthus* is known to secrete proteases and other lytic enzymes into the medium (15). It is therefore intriguing to examine whether Oar and MlpA are associated with protein secretion across the outer membrane.

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