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Expression of the Major Outer Membrane Protein of *Chlamydia trachomatis* in *Escherichia coli*

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The major outer membrane protein (MOMP) of *Chlamydia trachomatis* was expressed in *Escherichia coli*. To assess whether it assembled into a conformationally correct structure at the cell surface, we characterized the recombinant MOMP (rMOMP) by Western immunoblot analysis, indirect immunofluorescence, and immunoprecipitation with monoclonal antibodies (MAbs) that recognize contiguous and conformational MOMP epitopes. Western blot analysis showed that most of the rMOMP comigrated with authentic monomer MOMP, indicating that its signal peptide was recognized and cleaved by *E. coli*. The rMOMP could not be detected on the cell surface of viable or formalin-killed *E. coli* organisms by indirect immunofluorescence staining with a MAb specific for a MOMP contiguous epitope. In contrast, the same MAb readily stained rMOMP-expressing *E. coli* cells that had been permeablized by methanol fixation. A MAb that recognizes a conformational MOMP epitope and reacted strongly with formalin- or methanol-fixed elementary bodies failed to stain formalin- or methanol-fixed *E. coli* expressing rMOMP. Moreover, this MAb did not immunoprecipitate rMOMP from expressing *E. coli* cells even though it precipitated the authentic protein from lysates of *C. trachomatis* elementary bodies. Therefore we concluded that rMOMP was not localized to the *E. coli* cell surface and was not recognizable by a conformation-dependent antibody. These results indicate that rMOMP expressed by *E. coli* is unlikely to serve as an accurate model of MOMP structure and function. They also question the utility of rMOMP as a source of immunogen for eliciting neutralizing antibodies against conformational antigenic sites of the protein.

Chlamydia trachomatis, an obligate intracellular bacterium, is an important human pathogen that causes infections which are generally restricted to mucosal epithelial cells of the conjunctiva or urogenital tract. These infections usually resolve without complications but, under some circumstances, may provoke severe local inflammatory responses that can lead to blindness or infertility. *C. trachomatis* is the leading cause of preventable blindness in individuals in developing countries (7) and is the most common agent of sexually transmitted disease in industrialized nations (16, 18). Because diseases caused by *C. trachomatis* represent significant public health problems throughout the world, a considerable effort is being expended to produce an effective chlamydial vaccine.

The chlamydial major outer membrane protein (MOMP) is the most promising candidate antigen for the development of a vaccine. The MOMP is the predominant constituent of the outer membrane and has been proposed to function as an adhesin that promotes the interaction of infectious chlamydial elementary bodies (EBs) with their eucaryotic host cells (16, 18). MOMP-specific monoclonal antibodies (MAbs) can neutralize infectivity in vitro by preventing chlamydial attachment (13) and are capable of passively neutralizing chlamydial infectivity in a subhuman primate model of chlamydial ocular infection (19). Thus, a recombinant MOMP (rMOMP) immunogen, capable of evoking neutralizing antibodies, would be an important first step toward the development of a vaccine against trachoma and chlamydial sexually transmitted disease.

The native conformation of the MOMP appears to be important both for its function as an adhesin and in the generation of protective neutralizing antibodies. Conformational changes in the MOMP, produced by mild heat treatment of infectious EBs, are associated with an inability of the EBs to attach to host cells (13). Similarly, in situ treatment of EBs with trypsin selectively cleaves the MOMP; this cleavage promotes conformational changes in the molecule that are associated with a significant decrease in attachment and infectivity (14). MAbs which are capable of neutralizing chlamydial infectivity recognize both conformational and linear MOMP epitopes (1, 13, 19). Most if not all of these MAbs have, however, been generated by immunization with intact chlamydial EBs and thus with conformationally correct MOMP. Moreover, vaccination experiments conducted in a guinea pig model of chlamydial genital tract infection suggest that MOMP conformation is important for the development of protective immunity (3, 4). Thus, the efficient induction of neutralizing Ab responses, even those to surface-exposed linear epitopes, may be dependent on the conformation in which the MOMP is presented.

The goal of the work described here was to determine whether the rMOMP expressed by *Escherichia coli* assembles into a native conformation on the cell surface. Expression of rMOMP in a native conformation would provide a convenient source of immunogen for evaluating whether MOMP alone is capable of inducing protective immunity and, if so, whether native structure is required. Additionally, it would provide the opportunity to use genetic approaches, such as site-directed mutagenesis, to more precisely study the structural and functional relationships of the protein.

The expression of rMOMP in *E. coli* has previously been described by Kaul et al. (9). More recently, Koehler et al. (10) reported the overexpression of rMOMP in *E. coli*. However, they did not attempt to discern whether rMOMP

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assembles into a native conformation. Since native conformation is critical to both the functional and immunological properties of MOMP, we have focused on determining whether rMOMP assumes a native conformation on the surface of *E. coli*. Our results indicate that when MOMP is expressed in *E. coli*, it does not localize to the cell surface and fails to take on a conformationally correct structure. These findings question the utility of rMOMP expressed by *E. coli* for studies of structure and function or as a source of immunogen for eliciting neutralizing antibodies against conformational antigenic sites of the protein.

MATERIALS AND METHODS

Bacterial strains and plasmid construction. *C. trachomatis* B/TW-5/OT was used as source of genomic DNA. Chlamydiae were grown and purified as described previously (6). Genomic DNA was purified from 10^9 inclusion-forming units of purified B serovar EBs. Briefly, EBs were pelleted by centrifugation and resuspended in 0.5 ml of TE (10 mM Tris, 1 mM EDTA [pH 7.4]) containing 1% sodium dodecyl sulfate (SDS), 10 mM dithiothreitol, and 100 μg of proteinase K (Boehringer Mannehim Biochemicals). The suspension was incubated for 1 h at 56°C. An additional 100 μg of proteinase K was added, and the incubation was continued for an additional 1 h. The suspension was then extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1). DNA was precipitated by addition of 0.1 volume of 3 M sodium acetate and 0.6 volume of isopropanol. The DNA was rinsed in 70% ethanol, dried, and then suspended in TE.

The MOMP gene, *ompl*, was obtained by polymerase chain reaction amplification from *Kpn*I-digested genomic DNA. The amplification primers flanked either end of the *ompl* gene open reading frame and included restriction sites for subsequent cloning (Fig. 1). To limit the potential accumulation of errors in the amplified gene, relatively large amounts of template were amplified for a limited number of cycles with a DNA polymerase (Vent; New England Biolabs Inc.) which possessed a 3′-5′ "proofreading" exonuclease activity. The amplification reaction was carried out in a 100-μl volume containing 5 μg of genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 200 μm each deoxynucleoside triphosphate, 1 μM primer, and 0.5 U of Vent polymerase. A DNA Thermal Cycler (Perkin-Elmer Cetus) was used with 1 min of melting at 94°C, 1 min of annealing at 37°C, and 1 min of polymerization at 72°C for 15 cycles. An amplification product of the correct size (~1,200 bp) was isolated from a 1% agarose gel and purified with a Geneclean Kit (Bio 101, Inc.).

The amplified *ompl* DNA was digested with *Bam*HI and *Sal*I. The plasmid vector pTTQ-18 (11) was also digested with *Bam*HI and *Sal*I and then treated with calf intestinal alkaline phosphatase. Both the *ompl* and vector DNAs were excised from a 1% agarose gel. The *ompl* DNA was ligated into the vector, "in-gel" (12), and transformed into *E. coli* DH5α. Several of the resulting clones were shown by restriction enzyme analysis to contain a plasmid with *ompl* downstream from the *tac* promoter, as shown in Fig. 1. The expression of MOMP from these plasmids was confirmed by Western immunoblot analysis as described below. One of the plasmids was designated pTTQ-MOMP and was used in all subsequent analyses. pTTQ-18 was used as a control in all analyses.

Western blot analysis of rMOMP. The expression of MOMP by DH5α/pTTQ-MOMP cells was evaluated by Western blot analysis. Cultures of DH5α/pTTQ-MOMP and DH5α/pTTQ-18 were grown overnight in LB broth plus 0.2% glucose and 120 μg of ampicillin per ml. Overnight cultures were diluted 1/10 in fresh LB-glucose and grown to mid-log phase (optical density at 600 nm, ~0.5), and rMOMP expression was induced by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Samples were removed for analysis at 10-min intervals. The cells were separated from culture supernatants by centrifugation and then solubilized by boiling in sample buffer. Polypeptides were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto a nitrocellulose membrane (15). The membrane was blocked with 3% bovine serum albumin and then probed for MOMP with MAb DIII-A3 (1). Antibody was detected by using ^{125}I-labeled protein A and autoradiography (5).

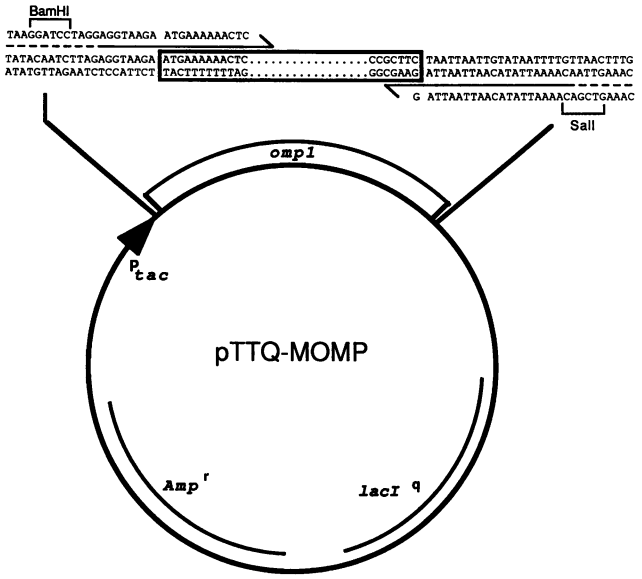

FIG. 1. Construction of pTTQ-MOMP. The B serovar MOMP gene, *ompl*, was amplified from genomic DNA with primers (arrows) which flanked either end of the *ompl* open reading frame (boxed). The primers included restriction sites (*Bam*HI and *Sal*I) which were used for cloning into the corresponding sites in the vector pTTQ-18, resulting in pTTQ-MOMP. Expression of rMOMP from pTTQ-MOMP is under the regulation of the *tac* promoter. The gene encoding the promoter's repressor (*lacI*) is also encoded on the plasmid and prevents detectable levels of expression in uninduced cultures (Fig. 2).

Immunofluorescence of rMOMP-expressing cells. The localization and conformation of the rMOMP expressed by DH5α/pTTQ-MOMP were analyzed by indirect immunofluorescence. DH5α/pTTQ-18 and purified B serovar EBs were included in the analyses as negative and positive controls, respectively.

The properties of the MOMP-specific MAbs used for characterizing rMOMP expressed by *E. coli* are summarized in Table 1. The MAbs DIII-A3 and B-A2 react with viable unfixed serovar B EBs by dot immunoblot, and both are neutralizing antibodies (1, 19). DIII-A3 reacts with a linear MOMP epitope as defined by the ability of the antibody to react with denatured MOMP by Western blotting (1, 19). In contrast, MAb B-A2 does not react with denatured MOMP by Western blotting or with EBs that have been subjected to mild heat treatment (56°C for 30 min) (19). We have interpreted the thermolability of the B-A2 epitope to mean that it

TABLE 1. Summary of MAb reactivities to serovar B MOMP

Method	Antibody reactivity		Reference
	DIII-A3	BA-2	
Immunofluorescence			
Methanol-fixed EBs	+	+	This study
Formalin-fixed EBs	+	+	This study
Unfixed EBs	+	+	This study
Dot blot			
Viable EBs	+	+	1, 19
Heated EBs	ND[a]	−	19
Neutralization	+	+	1, 19
Immunoprecipitation	+	+	19; this study
Western blot	+	−	1, 19

[a] ND, not done.

is conformational in nature (i.e., thermally induced conformational changes in MOMP destroy the antigenicity of the epitope). MAb A-20, a serovar A MOMP-specific antibody (19), was used as a negative control antibody in these studies.

DH5α/pTTQ-MOMP and DH5α/pTTQ-18 were grown and induced with IPTG as described above. For immunofluorescence analysis, cells were fixed with either methanol or formalin or stained without fixation.

(i) **Methanol fixation.** Cells were air dried on microscope slides, and the slides were submerged in absolute methanol for 10 min at room temperature. The fixed cells were washed three times with phosphate buffered saline (PBS), air dried, and reacted for 30 min at 37°C with MOMP-specific MAbs diluted in PBS to a final antibody concentration of 15 μg of protein per ml. The cells were washed three times in PBS and incubated with a 1:200 dilution of fluorescein-conjugated anti-mouse immunoglobulin antibody (Cappel, Organon Tecknika Corp.) for 30 min at 37°C. The cells were washed three times in PBS, counterstained with 0.005% Evans Blue for 1 min, rinsed with water, air dried and overlaid with glycerol-PBS and a no. 1 coverglass (Corning).

(ii) **Formalin fixation.** Cells were fixed with formalin in situ by addition of 2 ml of formalin to 100-ml cultures (2% [vol/vol] formalin). The cells were maintained in 2% formalin overnight at 4°C, washed three times in PBS, and then resuspended in 0.2% formalin in PBS. The formalin-fixed cells were applied to slides, air dried, and then reacted with MAbs to MOMP followed by fluorescein-conjugated anti-mouse antibodies as described above.

(iii) **Unfixed cells.** We followed the procedure of Koehler et al. as described in their studies on MOMP expression in *E. coli* (10). Briefly, cells were washed three times in PBS and then incubated for 30 min at 4°C in PBS containing diluted MAbs (15 μg of antibody per ml). The cells were then washed three times in PBS, spotted onto microscope slides, air dried, fixed in methanol, washed three times with PBS, and air dried. The fixed cells were then reacted with fluorescein-conjugated anti-mouse antibodies, washed three times with PBS, and counterstained with 0.005% Evans Blue. Fluorescence was visualized with a Zeiss fluorescence microscope.

Immunoprecipitation of rMOMP. DH5α/pTTQ-MOMP and DH5α/pTTQ-18 cultures were grown and induced for 40 min as described above. The induced *E. coli* cells and B serovar EBs were collected by centrifugation and resuspended in lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 10 mM EDTA, 0.5% deoxycholate, 0.1% SDS, 0.5% Triton X-100) to a final protein concentration of 1 mg/ml. The suspensions were incubated for 30 min at 37°C and then sonicated twice for 10 s at 100 W with a Braun-Sonic 2000 microprobe (B. Braun Co.). The sonicate was clarified by centrifugation at 10,000 × *g* for 10 min at 4°C. Each of the MAbs was added to a 50-μl aliquot of each of the sonicates to obtain a final antibody concentration of 0.2 mg/ml. The solutions were incubated overnight at 4°C. To each tube, 100 μl of a 5% solution of protein A CL-4B beads (Sigma Chemical Co.) was added, and incubation was continued at 4°C for 1 h. The protein A beads were then collected by centrifugation and washed five times in 500 μl of cold lysis buffer. After the final wash, the beads were resuspended in SDS-PAGE loading buffer and boiled for 10 min. Solubilized protein samples were analyzed by Western blot with the MAb DIII-A3 as described above.

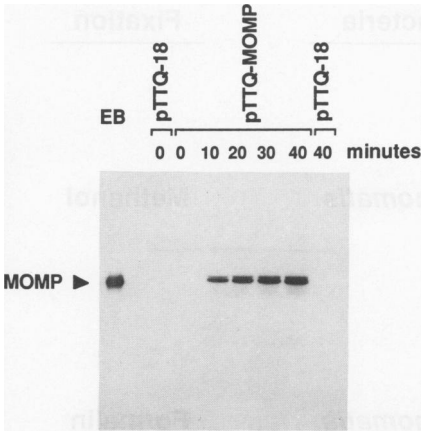

FIG. 2. Induction of MOMP expression in DH5α/pTTQ-MOMP cells. Mid-log-phase cultures were induced with IPTG. Samples were removed at 10-min intervals and assayed for rMOMP expression by Western blot. Authentic MOMP from *C. trachomatis* (lane EB) and rMOMP expressed by DH5α/pTTQ-MOMP were detected with MAb DIII-A3. Uninduced cells (0 min) did not express detectable levels of rMOMP.

RESULTS AND DISCUSSION

The MOMP gene, *omp1*, was obtained by polymerase chain reaction amplification from *C. trachomatis* serovar B genomic DNA and was cloned into the plasmid vector pTTQ-18, resulting in the plasmid pTTQ-MOMP (Fig. 1). The expression of rMOMP from this plasmid was induced by the addition of IPTG. The accumulation of rMOMP in induced cultures was analyzed by Western blot (Fig. 2). The rMOMP was not detectable in uninduced cells but was readily detectable by 10 min after induction and continued to accumulate for at least 40 min. Two forms of rMOMP were detected. The lower-molecular-mass band (approximately 40 kDa) comigrated with authentic mature MOMP from EBs and was presumably a form from which the signal peptide had been cleaved. The second form of rMOMP was present at much lower concentrations and was approximately 2 kDa larger. This higher-molecular-mass form was presumably a precursor from which the signal peptide had not been cleaved. The observation of MOMP signal peptide cleavage

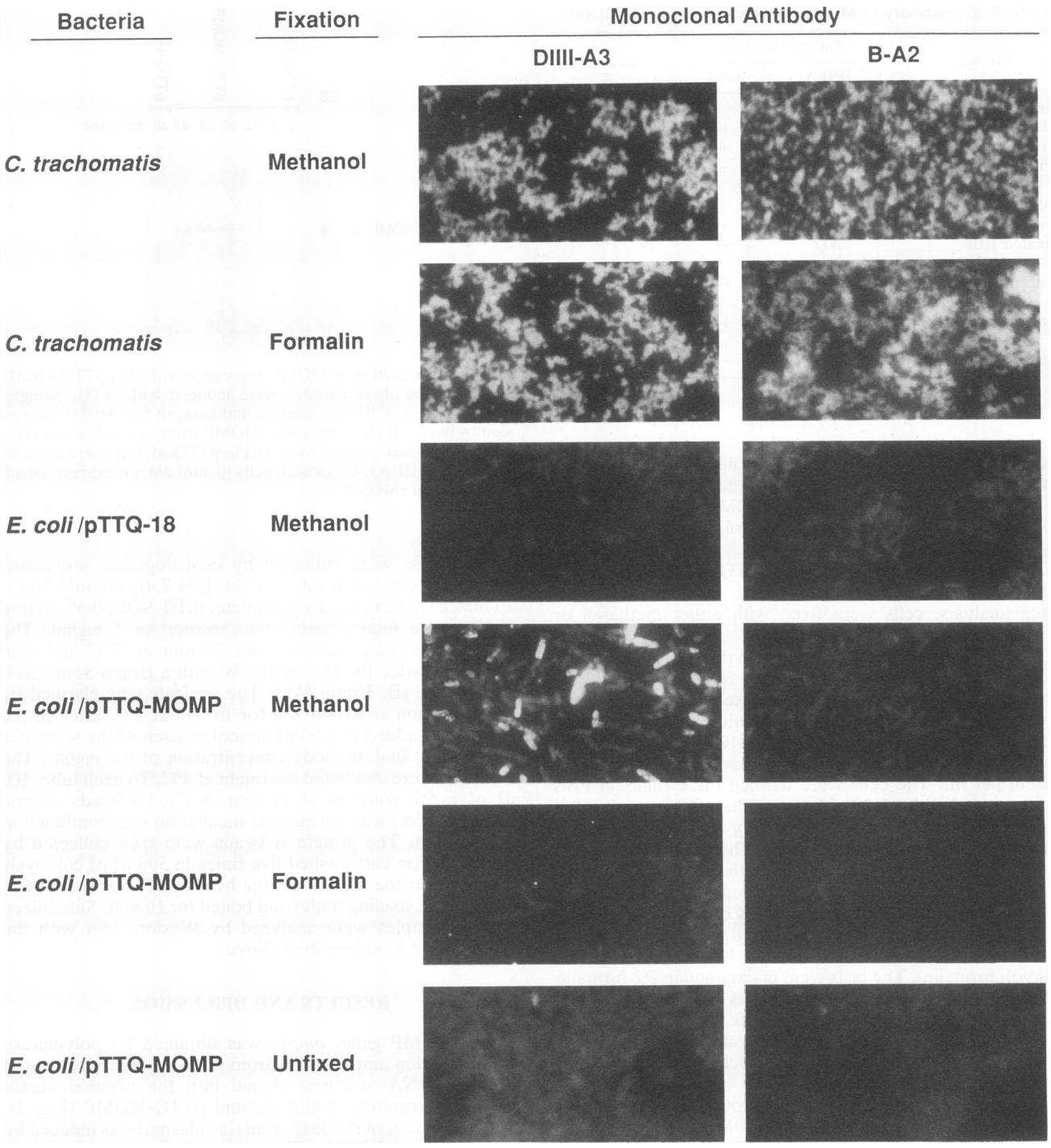

FIG. 3. Immunofluorescence of MOMP-expressing cells. *C. trachomatis* EBs or induced *E. coli* cells fixed with methanol or formalin or left unfixed were reacted with MAbs which recognized either a contiguous epitope (DIII-A3) or a conformational epitope (B-A2). For methanol fixation, cells were dried on slides, methanol fixed, and reacted with MAbs. Unfixed cells were reacted with MAbs in solution and then methanol fixed to slides. Cells were fixed with formalin in solution, air dried on slides, and reacted with MAbs. Antibody bound to cells was detected with fluorescein-conjugated secondary antibody. All cells were counterstained with Evans Blue, which photographs as a light grey.

by *E. coli* is in agreement with the earlier reports of Kaul et al. (9) and Koehler et al. (10).

The usefulness of rMOMP expressed by *E. coli* as an accurate model for study of the structural, functional, and immunological properties of MOMP depends on its ability to assemble into a structure which closely resembles the conformation of the authentic molecule as it occurs on the surface of the chlamydial EBs. Therefore it was important to

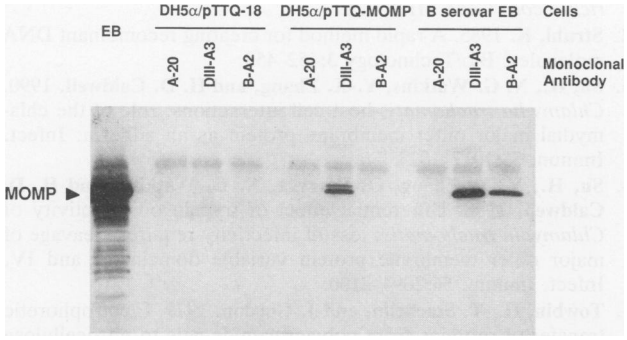

FIG. 4. Immunoprecipitation of authentic and recombinant MOMP from cell lysates. *C. trachomatis* EBs and induced DH5α/pTTQ-18 and DH5α/pTTQ-MOMP cells were suspended in lysis buffer and sonicated. Clarified sonicates were reacted with MAbs A-20, DIII-A3, and B-A2 and then with protein A-agarose beads. Immunoprecipitated MOMP was analyzed by Western blot with MAb DIII-A3. Purified EBs were included as a control antigen on the Western blot.

critically assess whether the rMOMP expressed by *E. coli* was localized to the surface and assembled into a native conformation. The MOMP from *C. trachomatis* serovar B was chosen for use in these studies because MAbs which recognized either the native or denatured forms of the protein were available as probes. MAb DIII-A3 recognizes native MOMP on the surface of EBs or denatured MOMP in Western blots. MAb BA-2 recognizes MOMP in its native conformation on EBs but not denatured MOMP in Western blots. Characterization of these MAbs is summarized in Table 1.

These MAbs were used to assess the localization and conformation of rMOMP in induced cells by immunofluorescence at 20 min (data not shown) and 40 min after induction, with similar results at each time point. Induced *E. coli* cells and B serovar EBs were prepared for immunofluorescence analysis by three different methods. In the first method, cells were fixed with methanol. Under these conditions DIII-A3 readily detected rMOMP but the BA-2 was not immunoreactive (Fig. 3). Therefore, rMOMP was accessible to antibodies but did not possess the conformational epitope recognized by BA-2. The reactivity of BA-2 with authentic MOMP on the surface of EBs was unaffected by methanol fixation (Fig. 3; Table 1); therefore it was unlikely that the lack of BA-2 immunoreactivity to rMOMP in methanol-fixed *E. coli* cells was due to the destruction of the epitope by fixation. In preliminary experiments, however, it was found that methanol fixation allowed specific antisera to react with maltose-binding protein, a normal periplasmic protein, indicating that methanol could permeabilize membranes and consequentially expose proteins which were not actually surface localized. Therefore, it was not possible to determine by methanol fixation whether rMOMP was surface localized. As more valid methods of determining whether rMOMP was surface localized, antibodies were reacted with unfixed cells or with formalin-fixed cells (Fig. 3). Neither DIII-A3 nor BA-2 was reactive with the surface of expressing cells under these conditions, indicating that the rMOMP was not surface localized.

As an additional approach to test for the presence of rMOMP in a native conformation, expressing cells were solubilized under mild conditions and immunoprecipitations were conducted (Fig. 4). Although MAb BA-2 could precipitate the authentic MOMP from EBs, it failed to recognize rMOMP expressed by *E. coli*. Under the same conditions, MAb DIII-A3 precipitated both authentic MOMP from EBs and rMOMP from *E. coli*. Therefore the absence of the conformational epitope recognized by BA-2 was confirmed and the conclusion that rMOMP was not expressed in a native conformation was verified.

The inability of B serovar rMOMP expressed in *E. coli* to surface localize and fold into a native conformation raises doubts about the validity of this system as a model for the authentic molecule. Koehler et al. (10) used a somewhat different system for the expression of *C. trachomatis* serovar L2 MOMP in *E. coli*. They concluded that some of the L2 rMOMP expressed by *E. coli* was localized to the cell surface. They also reported a very marked and rapid loss in cell viability after induction of rMOMP expression. Therefore it was possible that the rMOMP which they reported on the surface of these cells had been exposed or released by cell lysis. Moreover, these investigators did not attempt to demonstrate the presence of native structure in the rMOMP expressed by these cells. Therefore the utility of *E. coli* as a surrogate system for the study of the structural and functional properties of MOMP remains in doubt. The ability of rMOMP to induce protective immune responses requires direct testing. Unfortunately, it does not appear that rMOMP will serve as a useful reagent for determining the relative importance of native conformation in the induction of protective immune responses.

In some respects, the failure of rMOMP to successfully assemble into a native conformation on the surface of *E. coli* is not surprising. Although chlamydial cell walls appear at first glance to be similar in structure to those of gram-negative organisms, phylogenetic studies based on 16S rRNA sequence comparisons indicate that chlamydiae are only distantly related to *E. coli* and other gram-negative bacteria (17). It is known that there are significant differences between important cell wall structures of these organisms, most notably the absence of peptidoglycan in the chlamydial cell wall (2, 8). Therefore there may well be a fundamental incompatibility of chlamydial MOMP with later stages of export and assembly in *E. coli*.

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