

Partial Amino Acid Sequence and Molecular Cloning of the Encoding Gene for the Major Outer Membrane Protein of *Chlamydia trachomatis*

F. E. NANO,^{1*} P. A. BARSTAD,¹ L. W. MAYER,¹ J. E. COLIGAN,² AND H. D. CALDWELL¹

Laboratory of Microbial Structure and Function, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratories, Hamilton, Montana 59840¹ and Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20205²

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The first 25 N-terminal amino acids of the major outer membrane protein of *Chlamydia trachomatis* serovar L2 were determined. The amino acid sequence was used to construct an oligonucleotide probe specific for the major outer membrane protein gene. Using this oligonucleotide as a hybridization probe, we discovered one recombinant clone that produced a 15-kilodalton polypeptide which reacted with a monoclonal antibody directed against the major outer membrane protein type-specific epitope. In a separate set of experiments, we uncovered another recombinant clone that produced a 51-kilodalton polypeptide which was reactive with an anti-major outer membrane protein subspecies-specific monoclonal antibody. The expression of these recombinant DNA plasmids in *Escherichia coli* is discussed.

Members of the genus *Chlamydia* are obligately intracellular procaryotes that have many characteristics resembling those of gram-negative bacteria (for reviews, see references 3 and 31). There are three *Chlamydia trachomatis* biovars, two of which exclusively infect humans. On the other hand, the many types of *Chlamydia psittaci* strains are parasites of a variety of animal species. Although the life cycles of all chlamydiae are similar, it is clear that *C. trachomatis* is not closely related to *C. psittaci*, as determined by DNA relatedness (24, 30). Whether these two species have a common ancestor or whether they are the result of convergent evolution is not clear.

The life cycles of chlamydiae involve the following two forms: metabolically dormant elementary bodies (EBs) and vegetative reticulate bodies. The EBs are able to interact with the surface of a host cell and promote phagocytosis. After engulfment, chlamydial EBs transform into reticulate bodies, grow within the phagosome, and inhibit fusion of the phagosome with the lysosome. After approximately seven rounds of division, the chlamydiae begin to differentiate into EBs prior to release.

It has been postulated that the surface components of *C. trachomatis* play a role in the stimulation of phagocytosis (4) and in the inhibition of phagosome-lysosome fusion (17). Although lipopolysaccharide and a number of outer membrane polypeptides can be separated and identified by polyacrylamide gel electrophoresis (PAGE) (5, 7), functional roles can be assigned to perhaps a few proteins, most notably the major outer membrane protein (MOMP) (2). This protein, which comprises up to 60% by weight of the outer membrane proteins (7), functions both as a structural component (2) and as a porin (2). The MOMP is covalently linked through disulfide cross-bridging to itself and other outer membrane proteins (2, 21, 28). This disulfide cross-bridging is thought to provide the structural stability to the EB outer membrane, which in turn maintains the rigidity of the EB in the absence of a peptidoglycan (2, 21, 28). Recently, reduc-

tion of MOMP disulfide bonds has been proposed as a triggering mechanism that initiates the primary differentiation step of EBs to reticulate bodies (19). In addition, reduction of MOMP seems to regulate its porin function (2). These important biological functions are common to the MOMPs of all chlamydiae, suggesting that these proteins are related evolutionarily despite the apparent lack of genetic relatedness among the chlamydial species. The fact that there is little antigenic (6) or structural relatedness between the MOMPs of *C. trachomatis* and *C. psittaci* indicates that the common functions may reside in small, highly conserved portions of the proteins or that the MOMPs have diverged at the level of primary amino acid sequences while maintaining similar secondary or tertiary structures.

In addition to these biological features, the MOMP is an important chlamydial antigen. It is the predominant serotyping antigen of *C. trachomatis* isolates (9, 33), and polyclonal antibody (8), as well as monoclonal antibody (29) specific for MOMP, is capable of neutralizing in vitro infectivity of the parasite.

Because of the importance of MOMP in both the biology and immunology of the chlamydiae, we used recombinant DNA techniques to isolate the gene encoding the MOMP in order to more thoroughly describe the structure and function of this protein.

MATERIALS AND METHODS

Bacterial strains. The strains and plasmids which we used are listed in Table 1. *Escherichia coli* strains were grown in LB medium (26). *C. trachomatis* LGV-434 (serovar L2) was grown in L-cell suspension culture, and EBs were purified as described previously (7).

Determination of amino acid sequence of MOMP. Outer membranes of *C. trachomatis* EBs were purified by using Sarkosyl solubilization, as described previously (7). The Sarkosyl-insoluble pellet was then extracted with 1% octylglucopyranoside (Calbiochem-Behring, La Jolla, Calif.) in 50 mM sodium phosphate-20 mM EDTA-50 mM dithiothreitol (pH 7.2) at 50°C for 1 h. The mixture was centrifuged at 30,000 × g for 30 min, and the supernatant and pellet were

* Corresponding author.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>C. trachomatis</i> L2/434/Bu		Laboratory strain
<i>E. coli</i> EM24	<i>metB hsdR lacY supE supF galK galT trpR recA rspL</i>	E. Muller; <i>recA</i> derivative of LE392
JM109	<i>recA1 Δ(lac pro) endA1 gyrA96 thi-1 hsdR17 supE44 relA1/F' traD36 proAB⁺ lacI^q-Z M15</i>	J. Messing
Plasmids		
pEMBL8(+)	pUC8::fl replication origin (+)	13
pEMBL9(+)	pUC9::fl replication origin (+)	13
pFEN1	pEMBL9(+): <i>Bam</i> HI- <i>Pst</i> I insertion	This work
pUC8	Ampicillin resistance	33
pFEN6	pUC8:: <i>Sau</i> 3A insertion	This work

analyzed by sodium dodecyl sulfate-PAGE, using the method of Laemmli (25) with a 12.5% acrylamide resolving gel. The octylglucopyranoside supernatant was dialyzed against several 2-liter changes of double-distilled water over a period of 48 h at 4°C. The samples were dried in vacuo and subjected to automated Edman degradation (18). Amino acid sequences were determined by using a model 890P liquid phase sequencer (Beckman Instruments, Inc., Fullerton, Calif.) and a gas phase sequencer (Applied Bio Systems, Inc., Foster City, Calif.). Phenylthiohydantoin derivatives were analyzed by reverse-phase chromatography, using a C-18 column and a Hewlett-Packard model 1084 high-pressure liquid chromatograph (Beckman sequencer) or a Beckman model 890C high-pressure liquid chromatograph (Applied Bio Systems sequencer).

Isolation of DNA and recombinant DNA manipulations. *C. trachomatis* EBs were solubilized with 2% Sarkosyl and treated with 100 µg of proteinase K per ml at 37°C for at least 1 h. The entire cellular extract was subjected to cesium chloride-ethidium bromide buoyant density centrifugation, and the DNA band was recovered. Plasmid vector DNA was isolated by the method of Ish-Horowitz and Burke (23), followed by cesium chloride-ethidium bromide purification. Plasmid DNA was further treated with RNase A and run over a NaCl gradient as previously described (27).

DNA was recovered from agarose gels by using the method of Dretzen et al. (14) and was used in ligation reactions without further treatment.

Southern blots (32) on type BA85 nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) were prehybridized in 5× Denhardt solution (12) for 2 h at 20°C and hybridized with oligonucleotides (5 × 10⁶ cpm) in 6× SSPE (11) for 16 h at 20°C. The unbound probe was removed by washing once in 6× SSPE at 20°C for 30 min and three times at 30°C for 5 min.

DNA restriction digestion and ligation were performed by standard techniques, using enzymes purchased from Bethesda Research Laboratories, Inc., Bethesda, Md., or New England BioLabs, Beverly, Mass. Transformation of *E. coli* to ampicillin resistance (50 µg/ml) was performed by using either a CaCl₂ buffer (10) or a RbCl₂ buffer (20).

Calf intestinal alkaline phosphatase was purchased from

P-L Biochemicals, Inc., Piscataway, N.J., and was used under the conditions specified by the manufacturer.

A mixed oligonucleotide probe was synthesized by using a model SAM-1 instrument (Biosearch, San Rafael, Calif.) according to a protocol supplied by the manufacturer, using the reactions described by Efimov et al. (15, 16).

Oligonucleotides were labeled by using polynucleotide kinase (Bethesda Research Laboratories) and [γ-³²P]ATP (New England Nuclear Corp., Boston, Mass.) according to the protocol recommended by the suppliers.

All recombinant DNA experiments were done under P2 containment conditions according to National Institutes of Health guidelines.

Antibody screening. Monoclonal antibodies directed against the MOMP have been described previously (5). Briefly, monoclonal antibody L2I-45 recognizes a type-specific epitope present only on serovar L2 MOMP, monoclonal antibody L2I-5 recognizes a *C. trachomatis* subspecies epitope (found on MOMP of serovars within the B complex serogroup), and monoclonal antibody L2I-10 recognizes an epitope on MOMP common to all *C. trachomatis* serovars (species reactive). Immunoblots of sodium dodecyl sulfate-polyacrylamide gels were obtained as previously described (5). ¹²⁵I-labeled staphylococcal protein A (New England Nuclear Corp.) was used to label the bound antibody in immunoblots. Colony screening with antibody was done essentially as described by Helfman et al. (22).

Other techniques. All radioactive specimens were exposed to Kodak XAR film. Lightning-Plus intensifying screens (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) were used at -70°C with ³²P- and ¹²⁵I-labeled samples.

RESULTS

Determination of the amino acid sequence of the N terminus of the MOMP. Figure 1 shows the partial amino-terminal amino acid sequence of the MOMP of *C. trachomatis*. Approximately 5 nM MOMP was used in each sequencer analysis, and the sequence shown represents at least duplicate results for each position. In none of the three sequencer trials was the residue at position 10 identified. These samples were not fully reduced and alkylated, and since cysteine-phenylthiohydantoin derivatives are unstable under the conditions used, position 10 may represent a cysteine.

Construction of oligonucleotide probes. Knowledge of the amino acid sequence allowed us to design a family of oligonucleotides, one of which was predicted to hybridize to the 5' end of the antisense DNA strand of the MOMP gene.

1	2	3	4	5	6	7	8	9	10	11			
Leu	Pro	Val	Gly	Asn	Pro	Ala	Glu	Pro	(Cys)	Leu			
12	13	14	15	16	17	18	19	20	21	22	23	24	25
Met	Ile	Asp	Gly	Ile	Leu	Trp	Glu	Gly	Phe	Asp	Pro	Asp	Pro
ATG	AT(T)	GA(T)	GG(T)	AT(T)	TT								
	(G)	(G)	(G)										

FIG. 1. Amino acid sequence of the NH-terminal portion of the MOMP of *C. trachomatis* strain 434, serovar L2. The 17-mer oligonucleotide probe shown was constructed based on the nucleotide sequence predicted by the amino acid sequence that is underlined. Degeneracy positions in the nucleotide sequence are indicated by parentheses. In the third position of certain codons, the full complement of bases was not included in order to minimize the degeneracy of the oligonucleotide mixture (see text).

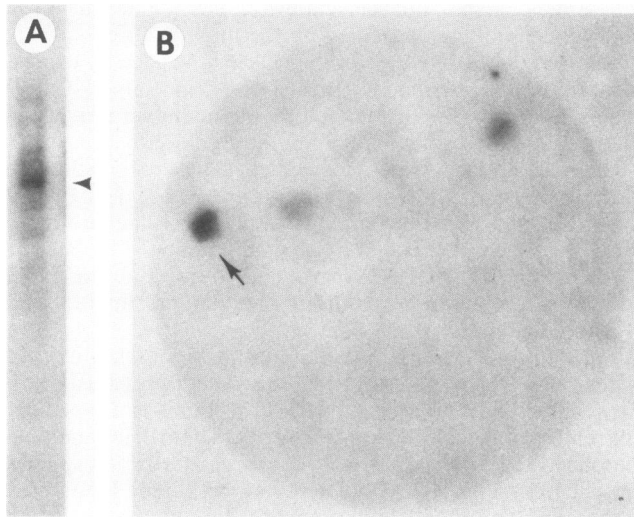


FIG. 2. Screening for recombinant clones containing the MOMP gene. (A) Southern blot analysis of *PstI*-*Bam*HI-digested bulk *C. trachomatis* DNA. The synthetic oligonucleotide shown in Fig. 1 was labeled with 32 P and used as a probe. DNA in the region that reacted with the probe was isolated from an agarose gel and cloned into *PstI*-*Bam*HI-digested pEMBL8(+) and pEMBL9(+). (B) Presumptive recombinants tested for reactivity with a mixture of three monoclonal antibodies directed against different epitopes located on the MOMP. The arrow indicates a recombinant clone that was shown by immunoblots to produce a polypeptide reactive with anti-MOMP antibodies.

The degeneracy of the family of oligonucleotides was kept to a minimum by choosing the stretch of amino acids with the minimum degeneracy in their codons and by incorporating a guanosine residue in the third position of certain codons in place of a cytosine or adenosine residue (Fig. 1). These substitutions allowed a neutral DNA-DNA hybridization reaction at these positions (34) and allowed the use of an 8-fold degenerate probe instead of the 72-fold degenerate probe required if all codons were included. Approximately 2 μ g of this oligonucleotide mixture was labeled with 32 P by using polynucleotide kinase and then used to probe a Southern blot of total genomic DNA isolated from *C. trachomatis* (Fig. 2A).

Isolation of a recombinant clone producing a 15-kd fragment of the chlamydial MOMP. We isolated a recombinant clone which produced a 15-kilodalton (kd) fragment of the chlamydial MOMP. *C. trachomatis* DNA that was doubly digested with *Bam*HI and *Pst*I showed the strongest hybridization signal at about 2 kilobases (kb) in a Southern blot hybridization (Fig. 2A). *Bam*HI-*Pst*I-digested DNA in this size range was recovered from an agarose gel and cloned into plasmids pEMBL8(+) and pEMBL9(+). Directional cloning into these two plasmids ensured that the cloned DNA could potentially be in both orientations with respect to the *lac* promoter on the pEMBL plasmids. We recovered one recombinant clone in pEMBL9(+) that reacted with a mixture of three monoclonal antibodies directed against the chlamydial MOMP (Fig. 2B). The cloned 2.0-kb insertion was subcloned into pEMBL8(+) in order to reverse the insertion orientation. The original recombinant plasmid was

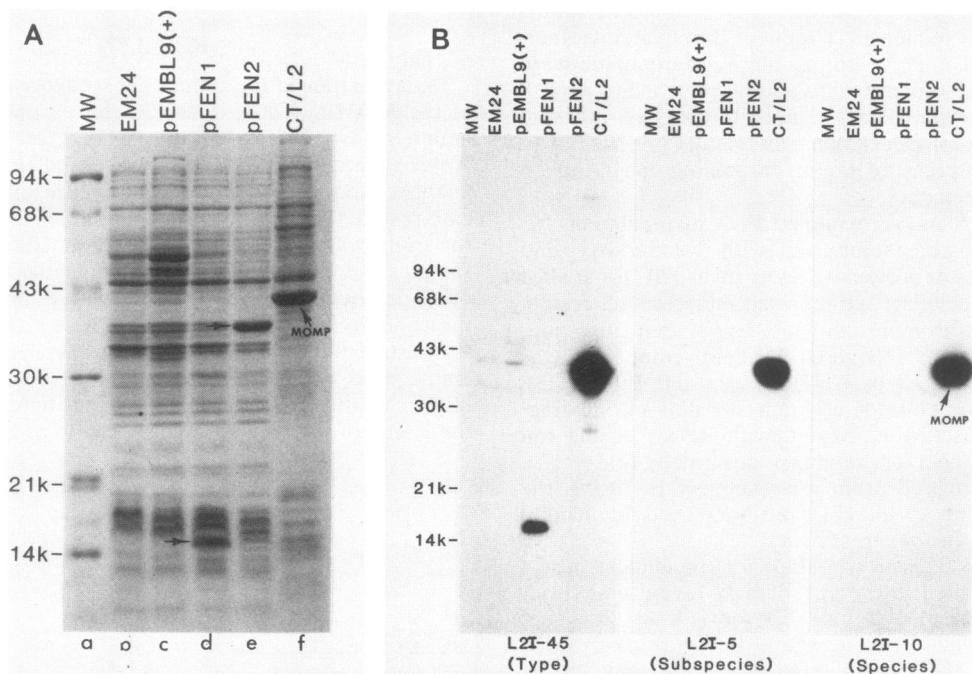


FIG. 3. (A) Sodium dodecyl sulfate-PAGE analysis of whole-cell lysates of recombinant clones. Strain EM24 is a *recA* derivative of *E. coli* LE392. Other *E. coli* strains were derived as transformants of strain EM24. The arrows in lanes d and e indicate protein products of cloned genes. pFEN1 produces a 15-kd polypeptide, and pFEN2 produces a 35-kd polypeptide. Lane f contained a whole-cell lysate of *C. trachomatis*; the arrow indicates the 39.5-kd MOMP. *E. coli* strains were grown in LB medium supplemented with 50 μ g of ampicillin per ml and 10^{-3} M isopropyl- β -D-thiogalactopyranoside. (B) Immunoblot analysis of the recombinant clones shown in (A). Polypeptides were electrophoretically transferred to nitrocellulose paper and reacted with monoclonal antibodies that recognize different epitopes on the chlamydial MOMP. Bound antibody was detected with 125 I-labeled protein A. The 15-kd polypeptide product of clone pFEN1 reacted strongly with type-specific monoclonal antibody L2I-45, but failed to react with antibodies that recognize the subspecies and species epitopes of MOMP. The 35-kd polypeptide expressed from clone pFEN2 did not react with any of the monoclonal antibodies specific for the MOMP. MW, Molecular weight markers; CT/L2, *C. trachomatis* serovar L2.

designated pFEN1, and the subclone was designated pFEN2. The oligonucleotide probe hybridized to the *ClaI-ClaI* fragment of pFEN1 (see Fig. 5), indicating that the amino-terminal end of MOMP is within 800 base pairs of the *PstI* end of the insertion (data not shown).

Expression products of pFEN1 and pFEN2. *E. coli* strains harboring pFEN1 produced a 15-kd polypeptide that reacted with type-specific monoclonal antibody directed against the *Chlamydia* sp. serovar L2 MOMP (Fig. 3). Expression of the 15-kd polypeptide was constitutive and may have resulted from the lack of repression of the *lac* promoter on the multicopy plasmid. Alternatively, chlamydial promoters may have been the dominant transcriptional effectors, at least when the insertion was oriented as it was in pFEN1. *E. coli* strains harboring pFEN2 did not produce any polypeptide products that were reactive with antibody directed against the chlamydial MOMP but did produce a 35-kd peptide. Differential expression of the cloned insertion in the opposite orientation with respect to the *lac* promoter suggests that there is some role for plasmid promoters in expression of the cloned products and also suggests that the 15- and 35-kd products are transcribed in opposite directions.

We thought that the 15-kd immunologically reactive product resulted from a truncated MOMP gene and, therefore, that we could find an intact MOMP gene by using pFEN1 DNA as a hybridization probe. We cloned larger (>2.0-kb) *PstI* and *HindIII* fragments of chlamydial DNA that hybridized to pFEN1 into both plasmid and bacteriophage vectors. In all cases, we detected no cloned protein products either by immunological screening or by Coomassie blue- or silver-stained PAGE (data not shown).

Isolation of a recombinant clone producing a product reactive with subspecies-specific monoclonal antibody. In another approach to obtaining a recombinant clone of the MOMP gene, we constructed a pUC8 bank of *C. trachomatis* DNA. A partial *Sau3A* digest of chlamydial DNA was separated by agarose gel electrophoresis, and 4- to 6-kb DNA was collected and cloned into *Bam*HI-digested, alkaline phosphatase-treated pUC8.

After screening the clone bank with a mixture of monoclonal antibodies, we isolated one recombinant clone that produced a 51-kd peptide which reacted with a subspecies-specific monoclonal antibody (Fig. 4) but not with the type-specific or species-specific monoclonal antibody. The recombinant plasmid, which contained a 5.5-kb insertion, was designated pFEN6. When polyacrylamide gels were heavily loaded, peptides with molecular weights of about 27,000 and 33,000 which were not found in the parent strain were detected in *E. coli* strains harboring pFEN6 (Fig. 4C). The 33-kd peptide was probably a breakdown product of the 51-kd peptide since it reacted with the subspecies-specific monoclonal antibody (Fig. 4D); the nature of the 27-kd polypeptide is unknown. Addition of isopropyl- β -D-thiogalactopyranoside to cultures of strain JM109(pFEN6) increased the production of the 51-kd product.

Restriction maps of plasmids pFEN1 and pFEN6 are shown in Fig. 5.

DISCUSSION

In this work, we used two different approaches to isolate recombinant clones of the *C. trachomatis* MOMP gene. In one approach, we determined the amino acid sequence of a portion of the MOMP in order to construct an oligonucleotide which could be used to probe chlamydial DNA. In order to guarantee a high degree of specificity, a probe needs to be at least 14 bases long for prokaryotic genomes, and the

degeneracy of the oligonucleotide probe is optimally kept low to reduce background noise. The serovar L2 MOMP sequence has two excellent probable sequences within the first 22 residues. The first extends from position 12 to position 16 and is 24-fold degenerate, whereas the second is located at positions 18 through 22 and is 16-fold degenerate. As with most amino acid sequences having low nucleotide degeneracy, each sequence contains either a methionine or a tryptophan.

Our use of the pEMBL plasmid series was designed to allow us to identify recombinant clones in the absence of any gene expression of the clone in *E. coli*. If necessary, we had the option of identifying a MOMP recombinant clone by its reaction with the oligonucleotide probe which we had constructed. Confirmation of a presumptive clone could be accomplished by DNA sequencing and alignment of the DNA sequence with the determined amino acid sequence. The pEMBL plasmids facilitate rapid DNA sequencing since a single strand of the recombinant plasmid can be obtained by infection of the recombinant with a defective single-stranded bacteriophage (13). However, our finding that pFEN1 made an antibody-reactive peptide obviated the need for DNA sequence analysis.

The second approach (use of monoclonal antibodies in the primary screening) also yielded a recombinant clone containing a portion of the MOMP gene. This recombinant clone apparently codes for a different portion of the MOMP, as determined by its reaction with the subspecies-specific mono-

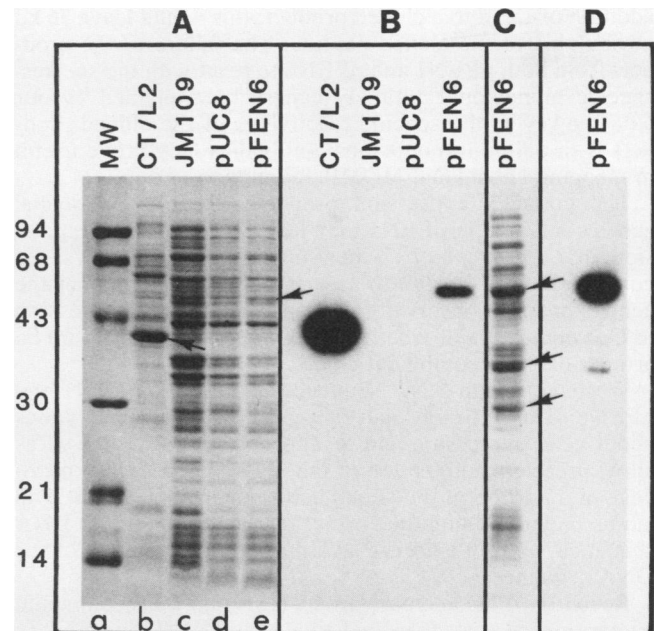


FIG. 4. (A) Sodium dodecyl sulfate-PAGE analysis of whole-cell lysates of strain JM109(pFEN6) and control strains. All *E. coli* strains were derived from strain JM109. The numbers at the left indicate molecular weights ($\times 10^3$). The arrow in lane b indicates the MOMP in the whole-cell extract of *C. trachomatis*. The arrow in lane e indicates the location of the immunologically reactive 51-kd cloned product. (B) Immunoblot of a PAGE gel identical to that shown in (A). Subspecies-specific monoclonal antibody L2I-5 is shown reacting with cloned product. Type-specific and species-specific monoclonal antibodies did not react with any peptides in these samples (data not shown). (C and D) Heavily loaded PAGE gel and immunoblot. The arrows in (C) indicate clone products. MW, Molecular weight markers; CT/L2, *C. trachomatis* serovar L2.

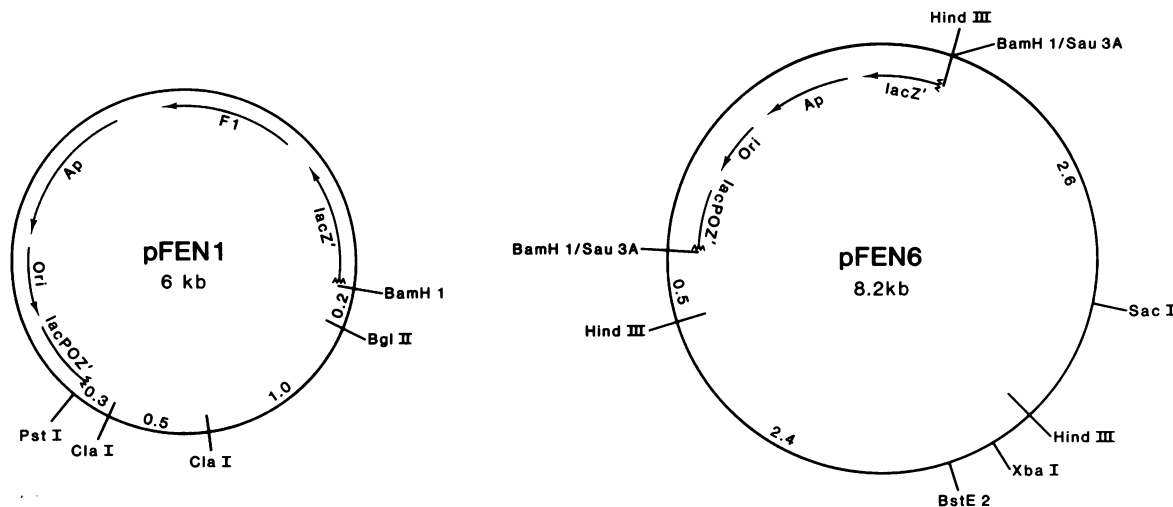


FIG. 5. Restriction maps of recombinant plasmids. Plasmid pFEN1 was derived from pEMBL9(+) and contained a 2.0-kb chlamydial DNA insertion. The sizes of restriction fragments in insertions are indicated (in kilobases). Plasmid pFEN6 was derived from pUC8 and contained an insertion of approximately 5.5 kb. The lengths of restriction fragments (in kilobases) from the ends of the insertion to the *Hind*III restriction sites are indicated. *lacPO*, Lactose operon promoter and operator sequences; *lacZ'*, portion of the *lacZ* gene coding for the α -complementation portion of β -galactosidase; Ap, β -lactamase gene; Ori, origin of replication; F1, origin of replication of bacteriophage f1.

clonal antibody but not the type-specific monoclonal antibody. The size of the peptides made from the recombinant clones suggests that most, if not all, of the MOMP gene is contained within the two recombinant clones. Presumably in pFEN6, a fusion to the *lacZ'* gene would result in the addition of 15 kd to a cloned product; this would leave 36 kd attributable to the cloned peptide. The failure of the products from both pFEN1 and pFEN6 to react with the species-specific monoclonal antibody cannot be explained by our data; however, the specific reactivities of the cloned products with different monoclonal antibodies may prove useful in mapping chlamydial MOMP epitopes.

The complex expression properties of the chlamydial genes contained in pFEN1 may help elucidate gene expression in *C. trachomatis* when further genetic analysis is completed. It is difficult to ascertain at present whether the gene expression observed in *E. coli* reflects gene expression in *C. trachomatis* or whether the expression patterns are an artifact of the recombinant clone.

We have begun DNA sequence analysis of pFEN1 and pFEN6. This type of analysis may help answer questions about gene expression and location of epitopes, as well as allow precise manipulation of the MOMP gene. Our knowledge of a portion of the amino acid sequence of MOMP will aid us in determining the correct reading frame of the DNA sequence, as well as serve as a measure of accuracy of the DNA sequence.

Recently, Allan and co-workers (1) isolated a recombinant bacteriophage that produced two peptides (40 and 41 kd), both of which reacted with serum from a patient with a *C. trachomatis* serovar L1 infection; the target DNA was isolated from *C. trachomatis* serovar L1. The data which these workers present suggest that they cloned an intact MOMP gene. Their use of a bacteriophage vector may have avoided problems of toxic effects of expression of an intact MOMP gene that might be present when a plasmid vector system is used.

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