A 28 kDa major immunogen of *Chlamydia psittaci* shares identity with Mip proteins of *Legionella* spp. and *Chlamydia trachomatis*—cloning and characterization of the *C. psittaci* mip-like gene

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**INTRODUCTION**

The *mip* (macrophage infectivity potentiator) locus was first identified in *Legionella pneumophila*; it is associated with enhanced intracellular survival of legionellae in human macrophages and other cells (Cianciotto *et al.*, 1989; Cianciotto & Fields, 1992; Engleberg *et al.*, 1989; O’Connell *et al.*, 1995). The *L. pneumophila* *mip* gene codes for a 24 kDa protein (LMip) which is localized to the bacterial cell surface (Engleberg & Eisenstein, 1991). Sequence comparisons and *in vitro* enzymic analyses have shown that LMip is structurally and functionally related to a family of peptidyl-prolyl cis/trans isomerases that bind the macrolide antibiotics FK506 and rapamycin, compounds that are therapeutically important as suppressors of T cell activation (Hacker & Fischer, 1993). Although the Mip protein is associated with enhanced infectivity by the legionellae, its role in the infectious process is not understood.

A *mip* homologue from *Chlamydia trachomatis* was recently cloned and expressed in *Escherichia coli* (Lundemose *et al.*, 1991, 1992). Subsequent reports have shown that the *C.*
trachomatis Mip protein (CtMip) is a lipoprotein (Lundemose et al. 1993b), has enzymic activity characteristic of other FK506-binding proteins (FKBP) (Lundemose et al., 1993a), and may be exposed at the bacterial cell surface (Lundemose et al., 1992). While no direct role of CtMip in the chlamydial infectious process has been demonstrated, FK506 added between 0 and 16 h post-infection has been shown to interfere with the developmental cycle of C. trachomatis (Lundemose et al., 1993b).

Recent studies in our laboratory have focused on the immune response of guinea-pigs following ocular infection with Chlamydia psittaci (strain GPIC). The resulting clinical condition, called guinea-pig inclusion conjunctivitis, is used as an animal model for ocular chlamydial disease in humans (Monnickendam et al., 1980; Morrison et al., 1989; Watkins et al., 1986). Guinea-pigs that resolve a primary infection are resistant to reinfection (Rank et al., 1988) and these animals produce antibodies against a variety of chlamydial antigens (Batteiger & Rank, 1987; Rank et al., 1990; Rockey et al., 1995; Rockey & Rosquist, 1994; Treharne & Shallal, 1991). One of the chlamydial antigens consistently recognized by convalescent guinea-pig sera is an uncharacterized 28 kDa protein. In this study, these convalescent sera were used to identify the gene coding for the 28 kDa protein from an expression library of GPIC DNA. The predicted amino acid sequence encoded by this gene shared identity with the Mip-like family of FKBP. The nucleotide sequence of the GPIC mip-like gene and three surrounding open reading frames (ORFs) is presented, and a similar gene arrangement is shown to be present in another strain of C. psittaci and in C. trachomatis serovar L2.

**METHODS**

**Bacterial strains, genomic library manipulation, and nucleotide sequence analysis.** Chlamydia psittaci strain GPIC, ovine abortion strain B577, and C. trachomatis strain LGV-434 (serovar L2) were cultured in HeLa cells. Original stocks of chlamydiae were purchased from the American Type Culture Collection. Chlamydial culture and elementary body (EB) purification were performed using described methods (Rockey & Rosquist, 1994; Caldwell et al., 1981). Construction, screening and manipulation of a GPIC genomic library were conducted using methods described by Rockey et al. (1995). Phagemid rescue of pBlue-script plasmids from purified bacteriophage was performed using the Exassist helper bacteriophage and SolR strain of Escherichia coli, with methods provided by the supplier (Stratagene Cloning Systems). E. coli DH5α was used for routine plasmid propagation and immunological analysis of expressed Mip. Nucleotide sequencing was done using the Sequenase system (US Biochemical) with the M13 forward and reverse primers, and internal primers synthesized on a Milligen/Biosearch Cyclone Plus DNA synthesizer. ORFs were identified and translated using the MacVector Sequence Analysis Software (International Biotechnologies). Deducible amino acid sequences were compared with those in the computer databases using the BlastP program available from the National Center for Biotechnology Information (e-mail address: blast@ncbi.nlm.nih.gov). Amino acid sequences were aligned using the 'lineup' program contained in the University of Wisconsin Genetics Computer Group sequence analysis package (Devereux et al., 1984).

**Polyacrylamide gel electrophoresis and immunoblotting.** Electrophoresis and immunoblotting were performed using described procedures (Rockey & Rosquist, 1994), with the following modifications. Comparisons of the reactivity of a collection of antisera with a single antigen were conducted using a slot-blotting device (Miniblotter-25, Immunetics). Lysates of GPIC E Bs or DH5α(pGP14.1) were loaded and electrophoresed through preparative 12% (w/v) polyacrylamide gels and transferred to nitrocellulose. These filters were placed into a blocking solution consisting of phosphate-buffered saline (PBS, 150 mM NaCl, 10 mM sodium phosphate, pH 7.2) plus 0.1% Tween-20 (TPBS) and 2% (w/v) bovine serum albumin (BSA-TPBS). After blocking, the filters were placed into the slot-blot device. Individual antisera were diluted 1:10 in BSA-TPBS and added to independent slots. After a 2 h incubation with gentle rocking, blots were washed five times with TPBS using the MF-2 washing device (Immunetics), and incubated in 125I-protein A (3700–5550 Bq ml−1; New England Nuclear) for 1 h. Blots were washed three times in TPBS, dried, and placed in contact with autoradiography film at room temperature for 16 h. To reduce background reactivity in immunoblots using E. coli lysates as
antigen, the antisera were first incubated for 30 min at room temperature in an equal volume of an E. coli extract (Promega) prior to dilution in BSA-TPBS and incubation with the blot.

Polymerase chain reaction (PCR). PCR was used to amplify relevant regions from genomic DNA of C. psittaci strain B577 and C. trachomatis strain LGV-434. Deoxyribonucleotides employed in PCR (Table 1) were derived from the nucleotide sequence of the GPIC genomic DNA insert of pGP14.1, from the published sequence of C. trachomatis LGV-434 mlp gene (Lundemose et al., 1992), or from the nucleotide sequences generated in these experiments. Amplifications were conducted with AmpliTaq DNA polymerase and the 'Hot Start' procedure, using AmpliWax beads, as described by the manufacturer (Perkin-Elmer Cetus). All PCR was performed with a denaturing temperature of 94 °C, an annealing temperature of 50 °C, and a 2 min extension at 72 °C. Following amplification, reaction products were directly cloned into the pCR11 vector (Invitrogen). Plasmids were purified and the ends of the PCR products were sequenced with M13 forward and reverse primers. If needed, internal primers were generated to extend and confirm the resulting sequences. The sequences were translated where appropriate, and compared to the respective GPIC sequences and to those within the computer databases.

RESULTS

Recognition of a 28 kDa EB protein by sera from convalescent guinea-pigs

Immunoblotting with sera from 10 guinea-pigs that had recovered from two serial ocular challenges with GPIC demonstrated that each produced antibodies to several chlamydial antigens, including the previously characterized 39 kDa major outer-membrane protein (MOMP), lipopolysaccharide, and the genus-common 60 kDa antigens (Batteiger & Rank, 1987; Morrison et al., 1989). Other antigens that were consistently recognized by convalescent animals were a protein of 28 kDa and a group of proteins at approximately 90 kDa (Fig. 1). Convalescent sera from animals that had recovered from a single experimental infection also produced antibodies against this collection of antigens (not shown).

Cloning and sequencing of the C. psittaci mlp gene

To identify the genes coding for the uncharacterized antigens detected in these immunoblots, a genomic expression library of GPIC DNA was constructed in bacteriophage lambda. Screening of this library with convalescent guinea-pig antiserum identified four clones which coded for two uncharacterized C. psittaci proteins. Two clones coded for a 39 kDa chlamydial protein (IncA
28 kDa protein expressed by DHScc(pGP14.1) and respectively, and were shown to be overlapping by which we have recently described (Rockey et al., 1995).

pGP14.1 and pGP14.2 is the same protein as the 28 kDa antigen in GPIC EBs.

Subcloning experiments demonstrated that the coding capacity for the complete 28 kDa protein was present on a 1100 bp HindIII–PstI fragment of insert DNA from pGP14.1 (not shown). Nucleotide sequencing of this protein was similar to the sequences of LMip and 28 kDa protein (Fig. 4). The deduced amino acid sequence of pGP14.1 and pGP14.2 is the same protein as the 28 kDa protein (Fig. 4). The deduced amino acid sequence of this protein was similar to the sequences of LMip and 28 kDa protein (Fig. 4). The deduced amino acid sequence of this protein was similar to the sequences of LMip and 28 kDa protein (Fig. 4).

These products include the carboxy terminus of asparagine tRNA ligase (AspS), a family of rRNA methylases (SpoU), and thioredoxin (TrxA). Each of the ORFs that flank mip has been named on the basis of their similarity to known E. coli gene products (Bachmann, 1990). The predicted product of the partial C. psittaci micP (GPIC Mip), C. trachomatis Mip-like protein (CtMip), and L. pneumophila Mip (LMip). A consensus sequence of 3 of 3 matches (capital letters) and 2 of 3 matches (lowercase letters) is included. The boxed sequences at the amino terminus of the chlamydiae proteins are predicted signal sequences. The boxed amino acids in the consensus sequence are the locations of amino acids which are predicted to be important in the binding of FK506.

**Fig. 5. Comparisons of the predicted amino acid sequences of the C. psittaci Mip (GPIC Mip), C. trachomatis Mip-like protein (CtMip), and L. pneumophila Mip (LMip). A consensus sequence of 3 of 3 matches (capital letters) and 2 of 3 matches (lowercase letters) is included. The boxed sequences at the amino terminus of the chlamydiae proteins are predicted signal sequences. The boxed amino acids in the consensus sequence are the locations of amino acids which are predicted to be important in the binding of FK506.**

**Arrangement of aspS, mip, spoU and trxA in other chlamydiae**

PCR was used to examine if a similar arrangement of this gene cluster was present in another strain of C. psittaci (B577) and in C. trachomatis LGV-434. Different sets of genes flanking C. psittaci mip on pGP14.1 were sequenced to determine if mip was adjacent to other genes coding for proteins with known function. These analyses demonstrated that mip was linked to three ORFs whose deduced amino acid sequences shared identity with a diverse collection of protein products (Figs 4 and 6).
Fig. 6. Schematic representation of the gene organization of *aspS*, *mip*, *spoU*, and *trxA*, and analyses of the homologous loci in *C. psittaci* B577 and *C. trachomatis* LGV-434 (serovar L2). Arrows indicate the direction of transcription/translation. The sequences presented at the bottom of the figure show the junctions of *mip/spoU* and *spoU/trxA* in the genomic sequences of strain GPIC and the amplified products from B577 and LGV-434. Predicted amino acid sequences surrounding the junctions are presented in boldface both above and below the nucleic acid sequences.

Table 1. PCR oligonucleotides used for the amplification of *mip* and linked genes from other chlamydiae

<table>
<thead>
<tr>
<th>Region amplified</th>
<th>5' oligonucleotide</th>
<th>3' oligonucleotide</th>
<th>Homologous product*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pair A <em>aspS/mip</em></td>
<td>CAACGTATTCATAACG</td>
<td>CTGTTTATTAGCTTTCTG</td>
<td>+ + –</td>
</tr>
<tr>
<td>Pair B <em>mip/spoU</em></td>
<td>GAAATCCGAATATGGG</td>
<td>CCATAGGAAAGCCAGCTC</td>
<td>ND + –</td>
</tr>
<tr>
<td>Pair C <em>mip/trxA</em></td>
<td>GGAAACGCTGGACAAAATTACC</td>
<td>TATGGGGGAACCGGTAG</td>
<td>ND ND +</td>
</tr>
<tr>
<td>Pair D <em>spoU/trxA</em></td>
<td>AGGGATGAGTACTG</td>
<td>TATGGGGGAACCGGTAG</td>
<td>+ + +</td>
</tr>
</tbody>
</table>

* An amplification was considered positive if sequence analysis of the cloned PCR product demonstrated that the predicted ORF is contained within the fragment. A negative result indicates that the predicted product was not amplified using these oligonucleotide pairs and the indicated genomic DNA as template. ND, Not determined.

oligonucleotide pairs (Table 1) were used to amplify homologous sequences from genomic DNAs of B577 and LGV-434. The products resulting from these PCR reactions were cloned and sequenced, and the predicted products of ORFs present in these sequences were compared to proteins in the computer databases. The primers used in these experiments and the results of the amplifications are presented in Table 1. PCR reactions were scored as positive if nucleotide sequence analysis of the products identified ORFs with deduced amino acid sequences homologous to those identified in GPIC. These studies demonstrated that the gene organization in B577 was identical to that in GPIC, and that a similar arrangement of *mip*, *spoU* and *trxA* was present in *C. trachomatis* LGV-434. We were unable to examine the sequences upstream of *mip* in LGV-434 because no combination of our primers amplified a product spanning that region. The sequencing also revealed that in the B577 genome both the junction between *mip* and *spoU*, and the junction between *spoU* and *trxA*, have an arrangement of overlapping codons identical to that seen in GPIC (Fig. 6). This is distinct from that seen for each of these genes.
junctions in *C. trachomatis*; the LGV-434 *mip* and *spoU* genes are separated by 15 bp, while the *spoU* and *trx.A* genes are separated by 53 bp (Fig. 6; Lundemose et al., 1992).

**DISCUSSION**

Proteins which bind the immunosuppressant FK506 are present in several different prokaryotic species (reviewed by Hacker & Fischer, 1993). FKBP are either cytosolic, as with the *Neisseria* spp., or membrane-associated, as with *Legionella* spp. and *C. trachomatis*. Mip-like proteins or *mip*-like ORFs have also been identified in *Coxiella burnetii* (Cianciotto et al., 1995), *Pseudomonas* spp. (Isaki et al., 1990; Konyecsni & Deretic, 1990), and *E. coli* (Horne & Young, 1995). Functionally, LMip is associated with increased infection proficiency by *Legionella* spp. in several cell types (Cianciotto et al., 1989; Cianciotto & Fields, 1992), and CtMip may have a role in the early stages of the *C. trachomatis* infectious process (Lundemose et al., 1993a).

We and others (Batteiger & Rank, 1987) have previously shown that sera from convalescent guinea-pigs recognize a 28 kDa GPIC protein. Because this protein was highly immunogenic in the context of infection, we pursued its identification and characterization. In this report we have described the cloning of a GPIC DNA fragment coding for a 28 kDa antigenic GPIC protein. Nucleotide sequencing of the insert in plasmid pGP14.1 identified four ORFs, one of which encoded a predicted 28 kDa protein that shared sequence identity with CtMip. The cloned product comigrated with the 28 kDa antigen in lysates of GPIC EB, and all sera that recognized the 28 kDa protein in EB recognized Mip expressed in *E. coli*. While other authors have discussed antigenic 28 kDa chlamydial proteins that are not Mip-like proteins (Comanducci et al., 1994), our results support the conclusion that the *mip* gene described in this work codes for the 28 kDa EB protein recognized by convalescent guinea-pigs.

These experiments demonstrate that the Mip protein is one of several immunodominant proteins in the guinea-pig model of chlamydial disease. However, the immune response to chlamydial Mip-like proteins is not likely to be limited to this model system. There are reports in the literature that chlamydial infection of turkeys, mice and sheep stimulates the production of antibodies against a 28–30 kDa protein (Griffiths et al., 1992; Jensen et al., 1990; Pal et al., 1993; Tan et al., 1990). Additionally, humans that have recovered from pneumonia caused by *C. pneumoniae* produce antibodies against a 30 kDa protein (Puolakkainen et al., 1993; Wilson et al., 1994). While it is not known if each of these species is producing antibodies against Mip-like proteins, it is possible that Mip is a common immunodominant antigen following chlamydial infection.

The *mip* gene from *C. trachomatis* has been cloned and sequenced from three different serovars. Within this species the sequence is 99% identical, with only two amino acid differences present in the predicted sequences (Lundemose et al., 1992). The predicted Mip product from *C. psittaci* is 58% identical to CtMip and 25% identical to LMip. During the preparation of this manuscript the sequence of a *mip*-like gene from *Coxiella burnetii*, another obligate intracellular bacterial pathogen, was submitted to the GenBank (accession number U14170). The deduced amino acid sequence of *C. burnetii* Mip is 27% identical to GPIC Mip. Through the region of the protein that encompasses the FK506 binding function (amino acids 169–241) Mip from GPIC and CtMip are 80% identical, and the GPIC Mip protein is 37% and 39% identical to LMip and *C. burnetii* Mip, respectively.

The *mip* gene of *C. psittaci* is placed within a cluster of previously unidentified chlamydial genes coding for proteins with diverse functions, including asparagine tRNA ligase (AspS), rRNA methylase (SpoU), and thioredoxin (TrxA). The arrangement was conserved among *C. psittaci* strains GPIC and B577, and *mip*, *spoU* and *trx.A* were shown to be linked in *C. trachomatis* LGV-434. It is difficult to propose any functional association among these apparently unrelated gene products or any reason why their genes are juxtaposed in the genome. None of these genes are closely linked in *E. coli*, although homologues to each have been mapped (Bachmann, 1990; Horne & Young, 1995; Sharples & Lloyd, 1991).

While there is no known function in *vivo* for any prokaryotic FKBP, the presence and possible surface localization of these proteins in several different intracellular pathogens suggests some role for the protein in the intracellular stage of the life cycle. In the eukaryotic cell, cytosolic FK506/FKBP exert their suppressive effects by interfering with calcineurin-mediated regulation of gene activation (Flanagan et al., 1991; Liu et al., 1991). Membrane-associated FKBP of intracellular bacteria may mimic host FKBP and subsequently influence intracellular signalling pathways in order to direct cellular events in favour of the pathogen. Additionally, because this protein is potentially surface-associated and is recognized by infected animals, it may be an important determinant in the immune response against the chlamydiae. Future analyses investigating the immune response to Mip or its function within infected cells are likely to further our understanding of the host–pathogen interaction following chlamydial infection.

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**REFERENCES**


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