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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*Chlamydia psittaci* strain guinea-pig inclusion conjunctivitis (GPIC) produces a self-limiting ocular infection of guinea-pigs, and this condition is a representative animal model of ocular chlamydial disease. Convalescent guinea-pigs, which are resistant to reinfection, produce antibodies to several elementary-body proteins, including an uncharacterized antigen of 28 kDa. Convalescent guinea-pig sera were used to identify, from a lambda expression library, two overlapping GPIC genomic clones that produced the 28 kDa antigenic protein. Nucleotide sequence analysis revealed that the gene coding for the 28 kDa protein was similar to the *mip* (macrophage infectivity potentiator) genes from *Legionella pneumophila* and *Chlamydia trachomatis*. The GPIC gene and its product were accordingly designated *mip* and Mip, respectively. Analysis of the regions flanking *mip* identified three tightly linked open reading frames coding for predicted products with sequence similarity to asparagine tRNA ligase (Asps), rRNA methylase (SpoU), and thioredoxin (TrxA). The arrangement of these genes in GPIC was *asps-mip-spoU-trxA*. Sequence analysis of PCR products produced using genomic DNA from an ovine abortion strain of *C. psittaci* and from *C. trachomatis* strain LGV-434 demonstrated that the arrangement of *mip*, *spoU* and *trxA* is common among these chlamydiae.

**Keywords:** *Chlamydia*, Mip-like protein, chlamydial antigen, intracellular pathogen, intracellular survival

### 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.
Fig. 7. Preparative immunoblot of GPIC EB probed with sera from individual convalescent guinea-pigs. Lanes: 1 and 2, sera from two naive guinea-pigs; 3–11, sera from nine individual guinea-pigs that recovered from two serial challenges with GPIC. The positions of chlamydial lipopolysaccharide (LPS) and major outer-membrane protein (MOMP) are shown, and molecular masses are indicated in kDa.

**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). Deduced 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 EBs or DH5α(pGP14.1) were loaded 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). Deduced 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).

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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 *mip* gene (Lundemose et al., 1992), or from the nucleotide sequences generated in these experiments. Amplifications were conducted with AmpliTag 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 pCRII 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* mip 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)
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pGP14.1 and pGP14.2, had inserts of 2.8 and 2.9 kbp, respectively, and were shown to be overlapping by restriction endonuclease analyses (not shown). The rescued pBluescript plasmid clones, designated DHScc(pGP14.1) and DHScc(pGP14.2) comigrated precisely with the 28 kDa antigen in the EB lysates (Fig. 2). There were also lower molecular mass antigenic bands in lysates of both GPIC and the recombinant E. coli, which may represent common processing products of the 28 kDa protein (Fig. 2, Fig. 3). Additionally, all convalescent sera that recognized the 28 kDa product coded by plasmids (pGP14.2) comigrated with it. We have recently described (Rockey et al., 1995). The other two clones coded for a 28 kDa protein recognized by both convalescent antisera and sera from guinea-pigs immunized with formalin-fixed EBs (Fig. 2). There were also lower molecular mass antigenic bands in lysates of both GPIC and the recombinant E. coli, which may represent common processing products of the 28 kDa protein (Fig. 2). These results support the conclusion that the 28 kDa product coded by plasmids

**Fig. 4.** Nucleotide sequence and the derived amino acid sequence of the GPIC asps, mip, spoU and trxA genes, encoded by pGP14.1. The asps sequence is a partial reading frame. The PstI and HindIII sites that flank the mip coding sequence are indicated, and a possible ribosome-binding site upstream of mip is underlined. Amino acid sequences are indicated in boldface, below the first nucleotide in the codon. The trxA gene is coded by the complementary strand, and is indicated with italics.
Chlamydia psittaci 28 kDa antigen

<table>
<thead>
<tr>
<th>GPIC Mip</th>
<th>CtMip</th>
<th>LMip</th>
<th>Consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28 kDa antigen</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 fragment identified an ORF that coded for a predicted 28 kDa protein (Fig. 4). The deduced amino acid sequence of this protein was similar to the sequences of LMP and CtMip (25 and 58% identity respectively; Fig. 5). Sequence identity was highest in regions of the protein that are involved in the interaction between FKBP and FK506 (Hacker & Fischer, 1993). A 4 base sequence 9 bases upstream of the ATG ATG (Fig. 4, position 377) resembles a consensus ribosome-binding site (Gold et al., 1981). The predicted Mip polypeptide has a putative signal peptidase II leader sequence similar to the amino terminus of unprocessed CtMip (Figs 4 and 5; Lundemose et al., 1992, 1993b). This suggests that C. psittaci Mip, like CtMip, is a lipoprotein.

Nucleotide sequence analysis of the genomic DNA flanking mip

The regions 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).

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 spoU exhibited 52% identity with the last 98 amino acids of aspartate tRNA synthase from E. coli (Sharples & Lloyd, 1991), the predicted product of C. psittaci spoU exhibited 39% identity with the SpoU homologue from E. coli (Koonin & Rudd, 1993; Mallinder et al., 1992), and the predicted product of C. psittaci trxA exhibited 47% identity with thioredoxin from Bacillus subtilis (Chen et al., 1989). The partial C. psittaci AspS is directly upstream of mpc while the spoU and trxA homologues are downstream. The trxA gene is oriented with opposite polarity to asps, mpc and spoU. The intergenic region between asps and mpc is 74 bp, and the junctions between mpc/spoU and spoU/trxA overlap. The sequence ATGA at position 1141–1144 contains the mpc and spoU stop and start codons, while the sequence TTA at position 1608–1610 contains the stop codons for both spoU and trxA (Figs 4 and 6).

Arrangement of asps, mpc, 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
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 aspS/mip</td>
<td>CAACGTATTCATAGTTCTCTTTATTAGCTTCTG</td>
<td>CTGTTATAGCTTTCTG</td>
<td>+ + -</td>
</tr>
<tr>
<td>Pair B mip/spoU</td>
<td>GAATCCGAATATGAGG</td>
<td>CCATAGGAAGAGCCAGTCC</td>
<td>ND + -</td>
</tr>
<tr>
<td>Pair C mip/trxA</td>
<td>GGAACAGCTGGGACAATTACC</td>
<td>TATGGGGGAACCGGTAG</td>
<td>ND ND +</td>
</tr>
<tr>
<td>Pair D spoU/trxA</td>
<td>AGGGATGGATTACTG</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 gene...
juncti ons in C. trachomatis; the LGV-434 mip and spoU
genes are separated by 15 bp, while the spoU and trxA
genes are separated by 53 bp (Fig. 6; Lundemose et al.,

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
(Cianciootto et al., 1995), Pseudomonas spp. (Isaki et al.,
1990; Konyecsni & Deretic, 1990), and E. coli (Horne &
Young, 1995). Functionally, LMP is associated with
increased infection proficiency by Legionella spp. in several
cell types (Cianciootto et al., 1989; Cianciootto & 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 sequenc-
ing 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
(Piulakkainen 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 manu-
script 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 trxA 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;

While there is no known function in vivo for any
prokaryotic FKBP, the presence and possible surface
localization of these proteins in several different intra-
cellular 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 follow-
ing chlamydial infection.

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Chlamydia psittaci 28 kDa antigen


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