Molecular cloning of a *Coxiella burnetii* gene encoding a macrophage infectivity potentiator (Mip) analogue

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The gene encoding a protein that reacted with antibodies specific for *Legionella pneumophila* macrophage infectivity potentiator (LpMip) was cloned from *Coxiella burnetii*, the obligate intracellular rickettsia that causes Q fever in humans. Nucleotide sequencing analysis revealed an ORF encoding a gene product of 230 amino acids with a molecular mass of 25.5 kDa and a predicted pI of 10.7. The predicted amino acid sequence from the ORF shows similarity with Mip/Mip-like proteins of *Legionella* (46%) and *Chlamydia* (30%). Moreover, like LpMip, the amino acid sequence of the C terminus of this protein has over 35% identity to prokaryotic and eukaryotic FK506-binding proteins (FKBPs) that belong to a superfamily of immunophilins and are peptidyl-prolyl *cis-trans* isomerases (PPlases). When overproduced in *Escherichia coli*, the *C. burnetii* protein also exhibited PPlase activity. Taken together, these results demonstrate that *C. burnetii* encodes a Mip analogue (CbMip). A putative leader peptide at the N terminus of CbMip was detected by computer analysis. Furthermore, *TnphoA* mutagenesis demonstrated that in *E. coli* CbMip was secreted. In view of the role of Mip/Mip-like proteins in the pathogenesis of *Legionella* and *Chlamydia*, CbMip may be a *C. burnetii* virulence factor.

Keywords: *Coxiella burnetii*, Mip, virulence factor

INTRODUCTION

Coxiella burnetii is an obligate intracellular bacterium that causes Q fever in humans (Baca & Paretsky, 1983). During the infectious process, *C. burnetii* is taken up by host cells into phagosomes which then fuse with primary lysosomes to form phagolysosomes. After several cycles of replication, the vacuoles become loaded with bacteria which are released into the extracellular environment upon bursting of the host cell. Formation of the phagolysosome is a critical step during infection because the acidic environment of the phagolysosome appears to activate the metabolism of *C. burnetii* (Hackstadt & Williams, 1981). Therefore, microbial biochemical factors

that support initiation of infection or phagolysosomal survival can be considered to be important virulence factors. While several factors have been proposed to contribute to the virulence of *C. burnetii* (Baca *et al.*, 1993; Heinzen *et al.*, 1992; Hendrix *et al.*, 1993), little is known about its mechanisms of uptake and survival in phagolysosomes.

As a group, intracellular bacteria have evolved strategies for invasion, intracellular survival and growth (Moulder, 1985) and consequently, they may have similar infection or survival mechanisms. Although *C. burnetii* is a phylogenetically distinct organism, a comparison of 16S ribosomal RNA sequences suggests that facultatively intracellular bacteria of the genus *Legionella* are most closely related to *C. burnetii* (Weisburg *et al.*, 1989). Indeed, the organisms share similar intracellular growth compartments at least at the early stages of infection. In searching for virulence factors of *Legionella pneumophila*, Cianciotto *et al.* (1989) identified a gene encoding a 24 kDa surface protein involved in macrophage infection which they designated *mip* (macrophage infectivity potentiator). Since then *mip*-like genes have been cloned from other

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Abbreviations: FKBP, FK506-binding protein; Mip (*mip*), macrophage infectivity potentiator (gene); PPlase, peptidyl-prolyl *cis-trans* isomerase.

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intracellular bacteria, including *Chlamydia trachomatis*, and the gene products shown to possess amino acid sequence similarity to *Legionella pneumophila* Mip (Bangsberg *et al.*, 1991; Cianciotto *et al.*, 1990a; Fischer *et al.*, 1992; Lundemose *et al.*, 1992). Genetic data indicate that Mip plays an important role as a virulence factor in *L. pneumophila*, since mutations of the *mip* gene cause a reduction in intracellular infection (Cianciotto *et al.*, 1990b, 1995a; Cianciotto & Fields, 1992).

Recently Mip/Mip-like proteins have been shown to belong to the FK506-binding protein (FKBP) family within the superfamily of immunophilins (see review by Hacker & Fischer, 1993). Although Mip/Mip-like proteins are large molecules (≥ 24 kDa) compared to eukaryotic FKBP, the proteins share significant amino acid sequence similarity and are peptidyl-prolyl *cis-trans* isomerases (PPIases). PPIase, in free form, catalyses the slow conformational interconversion of peptidyl-prolyl bonds in oligopeptides and proteins (Fischer & Schmid, 1990), suggesting a function in protein folding and trafficking. To date a number of FKBP have been characterized from different sources and all possess PPIase activity which is inhibited by the immunosuppressive drugs FK-506 and rapamycin (Etzkorn *et al.*, 1993). While the actual function of the PPIase is not yet clear, the enzymic activity of Mip/Mip-like proteins appears to be related to the ability of the organisms to initiate infection. For instance, pretreatment of *Cbl. trachomatis* with FK506 or rapamycin significantly reduces the infection of host cells in cell culture (Lundemose *et al.*, 1993).

We have previously reported the possible occurrence of a Mip analogue in *C. burnetii* and other rickettsiae, including the genus *Rochalimea*, based on DNA hybridization and immunoblotting data (Cianciotto *et al.*, 1995b). In this paper we describe the cloning, nucleotide sequence and expression of the *C. burnetii* *mip* gene (*cbmip*). The identified *C. burnetii* Mip analogue (CbMip) represents a new member of the Mip/Mip-like protein family.

METHODS

Bacterial strains, plasmids, media and growth conditions.

Bacterial isolates (*C. burnetii*, *Escherichia coli* and *L. pneumophila*) and plasmids used in this study are listed in Table 1.

E. coli cultures were routinely maintained in Luria-Bertani (LB) medium at 37 °C; SOC medium (l^{-1} : 20 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g NaCl, 20 mM glucose; Ausubel *et al.*, 1989) was used for growing *E. coli* strain Top10 (Invitrogen) carrying the *cbmip* gene on pTrcB expression vectors (Invitrogen). *L. pneumophila* was grown on buffered charcoal-yeast extract agar medium (Cianciotto *et al.*, 1989) at 37 °C. *C. burnetii* was grown in embryonated yolk sacs and purified as previously described (Hendrix & Mallavia, 1984). When needed, antibiotics were incorporated into media at the following concentrations to maintain plasmids in *E. coli*: ampicillin, 100 μ g ml^{-1} ; kanamycin, 50 μ g ml^{-1} and tetracycline, 24 μ g ml^{-1} .

Screening of the *C. burnetii* genomic library. Preparation of *L. pneumophila* Mip (LpMip)-specific polyclonal antibodies from rabbits has been described elsewhere (Cianciotto *et al.*, 1990a). The LpMip-specific antibodies were used to screen a bac-

teriophage λ ZAPII (Stratagene) genomic DNA library of the *C. burnetii* Nine Mile isolate for clones expressing a Mip analogue. The *C. burnetii* genomic DNA library (Mo & Mallavia, 1994) was constructed with *EcoRI*-digested λ ZAPII arms. Bacteriophage λ ZAPII was mixed with *E. coli* strain XL1-blue (Bullock *et al.*, 1987) in LB containing 0.7% agar at ~ 500 viruses per plate and poured over a bottom layer of LB agar (1.5%). Plates were incubated for 4 h at 42 °C when plaques were just visible. Induction was done with IPTG (Gibco) by overlaying the plates with a nitrocellulose membrane (Schleicher & Schuell) impregnated with 10 mM IPTG and incubating for a further 10 h at 37 °C. The membrane was analysed as for Western blotting (Ausubel *et al.*, 1989) using LpMip-specific antibodies (see below). Once positive plaques were identified, secondary screening was conducted to obtain pure, positive clones which were excised *in vivo* in the presence of helper phage R408 (Russell *et al.*, 1986). Positive clones were verified by incubating *E. coli* harbouring an appropriate plasmid in LB broth supplemented with 1 mM IPTG at 37 °C overnight with shaking at 250 r.p.m. and analysing cell lysates by Western blotting using the LpMip-specific antibodies (see below).

Recombinant DNA techniques. A positive clone, pYMA1, isolated from the genomic library, contained a 5.3 kb *EcoRI* DNA fragment in pSK (Fig. 1). Physical mapping and subcloning showed that the putative *cbmip* gene was within a 1.7 kb *EcoRI*-*Clal* fragment of a pSK recombinant, designated pYMA1.1 (Fig. 1). pYMA1.1 was used as a DNA template for sequencing. Digestion of pYMA1.1 with *HindIII* and self-ligation resulted in pYMA1.4 which carried a DNA insert of 187 bp encompassing the upstream region of the *cbmip* gene and the coding region for the first 44 amino acids of the CbMip (Fig. 1). pYMA1.6 was constructed by ligating the cloned 1.7 kb *EcoRI*-*Clal* fragment of pYMA1.1 into pUC19 such that the *cbmip* gene was in the opposite orientation to the *lac* promoter on the vector. Deletion of the 1.7 kb *EcoRI*-*Clal* fragment of pYMA1 and religation resulted in pYMA1.10. To overproduce the CbMip protein, plasmids pYMA1.14-1 and pYMA1.14-2 were constructed. A 694 bp DNA fragment (nucleotides 65–759, see Results, Fig. 3a) was first amplified from pYMA1 by PCR using a primer pair A1-R0 (5' GAAACGATTGATTTTAC) and A1-U1 (5' CTGAGGTTTTATTCTT). The product was separated on a 1.5% agarose gel, purified using GeneClean Kit II (Bio101) and cloned into pCRII (Invitrogen). *E. coli* strain INV α F' (Invitrogen) carrying recombinant plasmids were identified as white colonies on ampicillin-containing LB agar plates supplemented with X-Gal. An insert of the recombinant plasmid was excised by digestion with *EcoRI* and the fragment ligated into *EcoRI*-digested pTrcB previously treated with shrimp alkaline phosphatase (Amersham). Insert orientation was determined by restriction mapping. The plasmid, pYMA1.14-1, had the *cbmip* gene fused in-frame with the sequence encoding the leader peptide of the vector derived from bacteriophage T7 gene 10 and gene expression was controlled by the IPTG-inducible *trc* promoter on pTrcB. pYMA1.14-2 contained the same DNA fragment in pTrcB, but in an opposite orientation. Using the same strategy, a 582 bp DNA fragment amplified using primers A1-R1 (5' CTGGA-AAAGCTTTCAGA) and A1-U1 (see above) was cloned into pTrcC in-frame to construct pYMA1.35-1. This fragment encoded CbMip lacking the 39 N terminus amino acids. The junction regions of the recombinant plasmids and the cloned inserts were verified by nucleotide sequencing. The presence of six histidine residues in tandem in the bacteriophage T7 gene 10 leader sequence allows purification of unprocessed fusion proteins using a nickel-chelating resin (Invitrogen).

TnphoA mutagenesis. Plasmid pYMA1.6 was subjected to

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
XL1-blue	<i>endA1 hsdR17</i> ($r_k^- m_k^+$) <i>supE44 thi-1 recA gyrA96 relA</i> (lac^-) [F' <i>proAB+ lacI^q</i> Δ M15 Tn10(Tc^R)]	Stratagene
INV α F'	<i>endA1 hsdR17</i> ($r_k^- m_k^+$) <i>supE44 thi-1 recA gyrA96 relA</i> ϕ 80 <i>lac</i> Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>deoR</i> F'	Invitrogen
Top10	<i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lac</i> Δ M15 Δ <i>lacX74 deoR recA1 araD139</i> Δ (<i>ara leu</i>)7697 <i>galU galK rpsL endA1 nupG</i>	Invitrogen
CC118	<i>araD139</i> Δ (<i>ara leu</i>)7697 Δ <i>lacX74 phoA</i> Δ 20 <i>galEK thi rpsE rpoB argE recA1</i>	Manoil & Beckwith (1985)
L. pneumophila		
130b	Clinical isolate (Wadsworth strain) <i>mip</i> ⁺	Cianciotto <i>et al.</i> (1989)
C. burnetii		
Nine Mile	Acute disease isolate plasmid QpH1	Samuel <i>et al.</i> (1985)
K	Chronic disease isolate plasmid QpRS	Samuel <i>et al.</i> (1985)
Priscilla	Goat abortion isolate plasmid QpRS	Samuel <i>et al.</i> (1985)
S	Chronic disease isolate, plasmid-less	Samuel <i>et al.</i> (1985)
Plasmid		
pUC19	Ap ^R cloning vector	BRL
pSK	Ap ^R cloning vector	Stratagene
pCRII	Ap ^R Km ^R PCR cloning vector	Invitrogen
pTrcB/pTrcC	Ap ^R expression vector	Invitrogen
pYMA1	pSK carrying <i>cbmip</i> on a 5.3 kb <i>EcoRI</i> DNA fragment	This study
pYMA1.1	pSK carrying <i>cbmip</i> on a 1.7 kb <i>ClaI</i> - <i>EcoRI</i> fragment	This study
pYMA1.4	pSK carrying 187 bp containing the upstream region and the region encoding the first 44 amino acids of the <i>cbmip</i> gene product	This study
pYMA1.6	pUC19 carrying same <i>cbmip</i> as pYMA1.1 but opposite in orientation to <i>lac</i> promoter	This study
pYMA1.10	pSK carrying a 3.6 kb partially digested <i>ClaI</i> - <i>EcoRI</i> fragment from pYMA1	This study
pYMA1.14-1	pTrcB carrying <i>cbmip</i> in frame	This study
pYMA1.14-2	Same as pYMA1.14-1 except opposite orientation	This study
pYMA1.35-1	pTrcC carrying <i>cbmip</i> missing the coding sequence for the 39 amino acids at the N terminus of the gene product	This study
pYMA1.201	<i>cbmip::Tnp_{phoA}</i> in pYMA1.6	This study

Tnp_{phoA} mutagenesis (Manoil & Beckwith, 1985). *E. coli* strain CC118 (PhoA⁻) was first transformed with pYMA1.6 and then transfected with λ ::*Tnp_{phoA}*. Potential *cbmip::Tnp_{phoA}* fusions were selected on ampicillin-containing LB agar plates supplemented with 5-bromo-4-chloro-3-indolyl-phosphate (X-P) and identified by physical mapping of the plasmids (pYMA1.201). When necessary, the fine structure of *Tnp_{phoA}* inserts in the target plasmid was determined by nucleotide sequencing using the primer (5' CCGGTTTTCAGAACAG) complementary to

the DNA sequence of the 5' end of the *phoA* gene (Manoil & Beckwith, 1985).

DNA sequencing, sequence analysis and protein sequencing. Nucleotide sequences were determined by the method of Sanger *et al.* (1977) using Sequenase version 2.0 (Amersham). In addition to M13 universal and reverse primers (Yanisch-Perron *et al.*, 1985), the following customized sequencing primers were used: A1-R0 (see above), A1-R1 (see above), A1-R2 (5' AAGTCACCGTTAATTAC), A1-U1 (see above) and A1-U2

Fig. 1. Physical map of pYMA1 and its subclones, and the reactivity of cloned gene products with LpMip-specific antibodies. A 5.3 kb *EcoRI* DNA fragment in pYMA1 was originally isolated from a *C. burnetii* genomic λ ZAPII library. Abbreviations for restriction enzymes: C, *ClaI*; H, *HindIII*; R, *EcoRI*. The ability of the cloned gene products to react with LpMip-specific antibodies is indicated as +, positive; ±, weakly positive and -, negative.

(5' GCCTGCAAGACTTTGTA) (Ransom Hill Bioscience). The DNA sequence and deduced amino acid sequence data were analysed using the GCG Sequence Analysis package (Devereux *et al.*, 1984). Protein sequencing of the N terminus of CbMip was carried out by the Biochemistry Bioanalysis service at Washington State University.

PCR amplification. All PCR was carried out in a DNA ThermoCycler (Perkin-Elmer) using a GenAmp kit (Perkin-Elmer). The PCR reaction contained 200 μ M of each nucleotide dATP, dGTP, dTTP and dCTP, 1 μ M each of primers, 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 1.75 mM MgCl₂, 1 ng genomic DNA ml⁻¹ or 10 pg plasmid DNA ml⁻¹ as a template and 2.5 units *Taq* DNA polymerase per 100 μ l solution. The amplification protocol consisted of 30 cycles of 0.5 min at 94 °C, 1 min at 42 °C and 0.5–1 min at 72 °C. PCR products were separated in a 1.5 % agarose gel and purified using GeneClean Kit II.

Primers A1-R1 and A1-U1 were used for amplifying the *cbmip* gene from various *C. burnetii* isolates.

Immunological detection of CbMip by LpMip-specific antibodies. *E. coli* strain XL1-blue carrying an appropriate plasmid was incubated in LB broth with ampicillin and 1 mM IPTG at 37 °C overnight, pelleted and lysed in 1 × Laemmli buffer (Laemmli, 1970). For isolation of *L. pneumophila* proteins, strain 130b was grown on buffered charcoal-yeast extract agar medium (Cianciotto *et al.*, 1989) for 3 d at 37 °C. The bacteria were soaked with 4 ml PBS per plate for 2 h at room temperature (RT), collected, centrifuged and suspended in 1 × Laemmli buffer. After separation by SDS-PAGE (15 % separation gel), the proteins were transferred to a nitrocellulose membrane in transfer buffer (Ausubel *et al.*, 1989) for 2 h at 250 mA. Immunological reactions and washes were conducted at 26 °C with gentle shaking. The membrane was soaked in blocking solution [9.5 % (w/v) powdered milk in 20 mM Tris/HCl, pH 7.4, 0.87 % (w/v) NaCl (TBS)] for 1 h and washed in 0.05 % Tween 20 in TBS (TBST). Proteins were reacted with LpMip-specific antibodies (1:1000) in blocking solution for at least 1 h. After three washes with TBST, horseradish-peroxidase-conjugated goat anti-rabbit antibodies (Sigma) diluted 1:1000 were added, incubated for 1 h, washed and signals visualized by adding a 4-chloro-1-naphthol solution containing 0.03 % (w/v) hydrogen peroxide in TBS.

In vitro transcription and translation of the *cbmip* gene.

To express the *cbmip* gene, plasmids were purified by CsCl gradient centrifugation and transcribed and translated *in vitro* using an *E. coli* extract (Promega). Proteins were labelled with [³⁵S]methionine (DuPont NEN) and separated by SDS-PAGE (15 %) using a standard protocol (Ausubel *et al.*, 1989). The gel was dried and exposed to X-ray film.

Overexpression and purification of CbMip protein. Bacterial cells (*E. coli* strain Top10) carrying pYMA1.14-1 were grown in 5 ml SOC containing ampicillin at 37 °C at 300 r.p.m. to an OD of ~0.6, and induced with 1 mM IPTG. At each time point after induction, a 0.5 ml sample was taken, pelleted and suspended in 1 × Laemmli buffer. To examine the solubility of the overproduced protein, 50 ml of the induced cell culture was harvested and resuspended in 10 ml 20 mM sodium phosphate buffer (pH 7.0) containing 500 mM NaCl, and sonicated for a total of 2 min. Bacterial cell debris was separated from soluble proteins by centrifugation at 9000 *g* for 15 min and the pellet resuspended in 10 ml 0.5 % Triton X-100 containing 10 mM EDTA to solubilize membrane proteins. The Triton-containing suspension was further centrifuged under the same conditions to separate Triton-soluble and -insoluble proteins. As a control, the plasmid carrying *cbmip* in the opposite orientation to the *trc* promoter of pTrcB (pYMA1.14-2) was subjected to the same process. Protein samples were resolved by SDS-PAGE (15 %) and stained with Coomassie Brilliant Blue G-250 (Neuhoff *et al.*, 1985).

Attempts to purify the recombinant protein using a nickel-containing column (Invitrogen) were unsuccessful since the recombinant CbMip protein appeared to be processed. Consequently, gel filtration and ion exchange chromatography were employed (Reiland, 1970). Total protein extracts containing CbMip were passed through a Bio-Gel P10 column (Bio-Rad). Major fractions containing CbMip, as determined by SDS-PAGE, were pooled and further purified by loading partially purified CbMip on a CM-Sephadex C25 (Sigma) column equilibrated with 10 mM sodium phosphate buffer (pH 7.0). CbMip was eluted with a linear gradient of 10 mM sodium phosphate buffer containing 0–0.5 M NaCl (pH 7.0) at 4 °C and monitored by absorbance at 280 nm. The major peak containing CbMip eluted at ~0.15 M NaCl.

Enzyme assay. The assay for PPIase activity of CbMip was conducted using the method of Kofron *et al.* (1991). In brief, purified CbMip protein at concentrations of 0, 0.03, 0.06, 0.12, 0.3, 0.6, 0.9 and 1.2 μ M was incubated in 1 ml 35 mM HEPES buffer (pH 7.8) containing 0.25 mg ml⁻¹ α -chymotrypsin at 0 °C; the reaction was initiated by adding 10 μ l succinyl-Ala-Phe-Pro-Phe-*p*-nitroanilide (Bachem). Activity of PPIase was calculated according to Harrison & Stein (1990). Inhibition assays at 100 nM CbMip were carried out under the same conditions in the presence of various concentrations of rapamycin (Wyeth-Ayerst) dissolved in absolute ethanol. The colour change resulting from hydrolysis of the substrate was monitored at 390 nm.

RESULTS

Isolation of clones containing the *cbmip* gene

Approximately 2500 plaques were screened by LpMip-specific antibodies and three were positive. After *in vivo* excision, plasmid DNAs were analysed by physical mapping. All three clones contained the same 5.3 kb *EcoRI* DNA fragment and the plasmid was designated

Fig. 2. Western blotting analysis of Mip/Mip-like proteins using LpMip-specific polyclonal antibodies. Lanes: proteins extracted from 1, *C. burnetii* Nine Mile isolate; 2, *L. pneumophila* strain 130b; 3 and 4, *E. coli* strain XL1-blue carrying pSK and pYMA1, respectively; 5, purified CbMip. Note that the positive protein band from the different sources is about the same molecular mass. Protein size markers are given in kDa on the left.

pYMA1 (Fig. 1). Southern hybridization of *C. burnetii* and *E. coli* genomic DNA using the [α - 32 P]dCTP-labelled 5.3 kb *Eco*RI insert of pYMA1 as a probe verified that pYMA1 was cloned from the *C. burnetii* Nine Mile isolate. Western blotting demonstrated that pYMA1 was capable of encoding a protein that reacted with LpMip-specific antibodies (Fig. 2). Subcloning combined with Western blotting analysis localized the *cbmip* gene within a 1.7 kb *Eco*RI–*Cla*I fragment (pYMA1.1) (Fig. 1).

Sequence analysis

Nucleotide sequencing of 892 bp, starting from the *Eco*RI site on pYMA1.1, revealed an ORF capable of encoding a gene product comprising 230 amino acids with a molecular mass of 25.5 kDa (Fig. 3a). This protein was expected to be highly basic with a pI of 10.7, as predicted by computer analysis. A relatively conserved promoter region for *E. coli* (Hawley & McClure, 1983) and *C. burnetii* (Mallavia, 1991) was detected upstream of the gene. In addition, at 7 bases upstream of the ATG codon was a putative ribosome-binding site (RBS), AGGAGA (Shine & Dalgarno, 1975). At the 3' end of this sequence was a potential termination signal similar to those in the *C. burnetii* *hptAB* operon, the transposase gene and citrate synthase gene (Heinzen & Mallavia, 1987; Hoover *et al.*, 1992; Vodkin & Williams, 1988). Computer analysis indicated it could form a stem-loop structure with a free

energy of -41.5 . Hydrophobicity analysis (Kyte & Doolittle, 1982) suggested that except for the 21 amino acid leader peptide (Fig. 3a), the protein was highly hydrophilic. Codon usage of the *cbmip* gene was biased compared to highly expressed *E. coli* proteins but within the range of most *C. burnetii* proteins (Hoover & Vodkin, 1991).

Amino acid sequence identity and similarity between CbMip and Mip/Mip-like proteins of *Legionella* and *Chlamydia* as well as FKBP

When the deduced amino acid sequence of the ORF was compared to that of the Mip of *L. pneumophila* (LpMip), an overall 46% identity was detected by the GAP program (Fig. 3b). The two proteins exhibit no gap in the alignment of their entire sequences and the molecular masses of the proteins are very close. Sequence identity of CbMip with the larger, 27 kDa, *Chlamydia trachomatis* Mip-like protein (CtMip) was lower (30% identity). Furthermore, a search of protein databases using the FASTA program revealed amino acid sequence conservation of the C terminus of this ORF with respect to those of prokaryotic and eukaryotic FKBP (Mozier *et al.*, 1990; Standaert *et al.*, 1990; Nelson *et al.*, 1991; Pahl & Keller, 1992; Sampson & Gotchlich, 1992). For instance, the 140 amino acids of CbMip at the C-terminal region shared > 35% identity to FKBP from a variety of sources (data not shown). Computer analysis using the MOTIFS program showed that, with very few exceptions, all Mip/Mip-like proteins and FKBP had two signature patterns: (1), 12–13 amino acids at the N terminus of FKBP, (L,I,V,M,C)XYXGX(L,F)X[2,3]GXFD (where amino acids in parentheses means any one of the amino acids may be present and X[2,3] means that any 2 or 3 amino acids may be present); and (2), 17–18 amino acids at the C terminus of FKBP, (A,G)YGX[2](S,A,G,R)X[2,3](G,P)XIX[5]L(L,I,V)F. CbMip had an identical pattern at amino acids 153–166, VNYEGRLLINGTVFD, and amino acids 208–226, AYGEQGAPGVIGPNEALIF (Fig. 3b). Among the amino acids that form the signature patterns, four were shown to be involved in binding to FK506 (Hacker & Fischer, 1993), indicative of functional conservation. In LpMip, Asp-162 was shown by site-directed mutagenesis (Ludwig *et al.*, 1994) to be necessary for full PPIase activity and this amino acid was conserved in CbMip.

Expression of *cbmip* by *E. coli*

To examine whether the *cbmip* promoter was functional in *E. coli*, plasmids containing the *cbmip* gene in either orientation relative to the *lac* promoter of the vector were transcribed *in vitro* and translated (IVTT) using an *E. coli* extract. The IVTT data suggested that the *cbmip* gene had its own promoter within the 1.7 *Eco*RI–*Cla*I fragment because a protein was still produced with the *cbmip* gene in the opposite orientation to the *lac* promoter of pUC19 (pYMA1.6) (data not shown).

The *cbmip* gene was overexpressed in *E. coli* (pYMA1.14-1)

Fig. 3. DNA sequence and deduced amino acid sequence of the *cbmip* gene (a) and sequence comparison (b). (a) The potential promoter region (–10 and –35) is underlined and the potential RBS is bold. The dyad symmetry sequence at the 3' end of the gene is indicated by opposite arrows. The arrow pointing down shows the putative leader sequence

Fig. 4. Overexpression and purification of CbMip protein. Bacterial cultures were harvested 2 h after induction with 1 mM IPTG. Proteins were resolved by SDS-PAGE (15%) and stained with Coomassie Brilliant Blue. Lanes: M, protein markers with molecular mass indicated on the left in kDa; 1 and 2, whole cell lysates of *E. coli* harbouring pYMA1.14-2 and pYMA1.14-1, respectively; 3, 1 µg purified CbMip protein.

(Fig. 4). The expression was detected 1 h after induction and reached maximal levels at 3 h after induction. In contrast, when the *cbmip* gene in pYMA1.14-2 (same as pYMA1.14-1, but in opposite orientation) was overexpressed in *E. coli*, no band corresponding to CbMip was apparent. After separation by successive gel-filtration and ion-exchange chromatography, a protein band with a molecular mass of 24 kDa was obtained (Fig. 4). Western blotting analysis using LpMip-specific antibodies confirmed that this protein was CbMip (Fig. 2, lane 5). The overproduced CbMip protein was soluble as it was purified from the supernatant fraction of cell extracts.

PPIase activity of the recombinant CbMip

The purified CbMip exhibited PPIase activity (Fig. 5a), comparable to that reported for other FKBP's (Sampson & Gotchlich, 1992; Standaert *et al.*, 1990). A consistently higher level of activity was observed using CbMip which

Fig. 5. PPIase activity of CbMip. (a) PPIase activity at various concentrations of CbMip. (b) Inhibition of PPIase activity of CbMip (100 nM) by rapamycin. The relative activity is calculated by comparing the enzymic activity in the absence or presence of rapamycin (Wyeth-Ayerst). One-hundred percent activity (K_{obs}) of 100 nM CbMip is 0.092 s^{-1} . Data shown in the figure are from three experiments with $SE \leq 0.02$.

had been affinity-purified from all extracts containing pYMA1.35-1, providing additional evidence that the PPIase activity was due to CbMip (data not shown). Enzymic activity was inhibited by the immunosuppressive drug, rapamycin. Less than 20% relative activity, compared to that in the absence of rapamycin, remained when the drug concentration was 500 nM (Fig. 5b).

Processing of the CbMip precursor and possible export of CbMip in *E. coli*

A potential N-terminal signal peptide of CbMip was identified by computer analysis of the DNA-deduced amino acid sequence and the cleavage site predicted to be

cleavage site and the arrow pointing up indicates the position of the *TnphoA* insertion in pYMA1.201. (b) The amino acid sequence is compared to that of LpMip (Cianciotto *et al.*, 1989) and CtMip (Lundemose *et al.*, 1992). The vertical bars indicate identical amino acids between two sequences. The essential amino acid (Asp) in LpMip for PPIase activity is conserved in all three proteins and indicated by an open triangle.

Fig. 6. PCR amplification of the *cbmip* fragment from DNA of various *C. burnetii* isolates. The amplified DNA products using primers A1-R1 and A1-U1 were separated in a 1.5% agarose gel. Lanes: 1, *E. coli* DNA; 2, Nine Mile phase I; 3, Priscilla; 4, K; 5, S; M, 100 bp DNA ladder. Note a DNA band of ~600 bp from the different isolates of *C. burnetii*.

between amino acids Ala and Ala at positions 21 and 22 (Fig. 3a). N-terminal amino acid sequencing of CbMip confirmed this prediction. The first seven amino acids of CbMip purified from lysates of cells carrying pYMA1.14-1 was determined to be ATPLKTE. This corresponded to amino acids 22–28 of CbMip (Fig. 3a), demonstrating that the signal peptide was removed in *E. coli*. This explains why recombinant CbMip was not bound to the nickel-containing resin (Invitrogen) in attempts to purify this protein (data not shown). The mature protein has a molecular mass of 23.5 kDa.

The presence of a signal peptide at the N terminus of CbMip suggested secretion of the protein. Furthermore, the size of recombinant CbMip matched with that of LpMip (Fig. 1), suggesting that CbMip was processed to a mature protein. To further examine its possible surface expression in *E. coli*, the *cbmip* gene was subjected to Tn*phoA* mutagenesis. Five inserts were identified by restriction mapping to be within the *cbmip* gene. *phoA* was fused in-frame with the *cbmip* gene, as demonstrated by production of blue pigment on X-P plates. In one of the clones (pYMA1.201), *phoA* was inserted between nucleotides 477 and 478, as determined by nucleotide sequencing (Fig. 3a). In contrast, three randomly picked white colonies had insertions either in the opposite orientation to the *cbmip* gene, or fused to the *cbmip* gene but out-of-frame (data not shown). Thus, the CbMip expressed in *E. coli* and detected by Western blotting was likely an exported, mature protein.

Conservation of the *cbmip* gene

To determine the extent of *cbmip* gene conservation in *C. burnetii*, primer pairs A1-R1 and A1-U1 specific to *cbmip* were used to PCR-amplify DNA from different isolates of *C. burnetii*. The results (Fig. 6) demonstrated the presence of an identical PCR product (582 bp), corresponding to nucleotides 182–764, in the Nine Mile phase I, Priscilla, K and S isolates representing various disease-causing agents and genetic backgrounds (Samuel *et al.*, 1985). An identical band was detected in the Nine Mile phase II isolate (data not shown). Although the Nine Mile phase II isolate is avirulent in animals, it does infect and grow in the phagolysosomes of cultured cells (Baca & Paretsky, 1983).

DISCUSSION

In this study a *mip* analogue was cloned from *C. burnetii* and its nucleotide sequence determined. The predicted amino acid sequence of the gene product was shown to have a high degree of identity and similarity to those of *L. pneumophila* and *Chl. trachomatis* Mip/Mip-like proteins. Mip/Mip-like proteins belong to the prokaryotic and eukaryotic FKBP family based on their amino acid sequence similarities and PPLase activity (Hacker & Fischer, 1993). The prokaryotic FKBP appear to fall into two categories. The larger (≥ 24 kDa) FKBP or Mip/Mip-like proteins of *L. pneumophila* and *Chl. trachomatis* are membrane-associated and/or secreted, while those present in bacteria generally not considered to be intracellular pathogens are smaller (≤ 12 kDa) and appear to be cytosolic proteins (Sampson & Gotchlich, 1992). A possible exception to this general finding is a report that *E. coli* encodes a 28849 Da FKBP-like protein with 28–34% identity to known Mip-like proteins (Horne & Young, 1994). By PCR amplification we also demonstrated that the *cbmip* gene was present in all *C. burnetii* isolates tested, including isolates from cases of acute and chronic Q fever. Mip/Mip-like proteins have been shown to be important for *L. pneumophila* and *Chl. trachomatis* infections (Cianciotto *et al.*, 1990b, 1995a; Cianciotto & Fields, 1992; Lundemose *et al.*, 1992). With its high sequence similarity and similar enzyme activity to Mip/Mip-like proteins, CbMip may be a *C. burnetii* virulence factor.

Mip/Mip-like proteins (≥ 24 kDa) have been identified in at least three intracellular bacterial pathogens, i.e. *L. pneumophila*, *Chl. trachomatis* and *C. burnetii*. Whether these organisms adopt a similar mechanism of resistance to host killing is not clear, but following entry into the host cell, they grow in different cellular compartments (Weiss *et al.*, 1991). For instance, both *L. pneumophila* and *Chl. trachomatis* reside in a phagosome, while acidification of the phagosome by lysosomal fusion is a necessary event that initiates active metabolism and proliferation of *C. burnetii* (Hackstadt & Williams, 1981). In contrast, these organisms have developed similar strategies for the early steps of infection, in which Mip proteins may play a central role. Indeed, LpMip and CtMip have both been

shown to affect some earlier stage of host-parasite interaction of *L. pneumophila* and *Cbl. trachomatis*. Unfortunately, the early steps of *C. burnetii* host interaction are not clear due to a lack of information on cell-surface recognition, attachment and entry processes. For example, it is still not known whether *C. burnetii* is passively phagocytosed into host cells or whether factors from the pathogen are involved (Baca & Paretsky, 1983; Williams *et al.*, 1989).

Because of its exposure to the cell surface, LpMip was shown to be involved in interaction with host cells (Engleberg *et al.*, 1989). CbMip appears to be processed to a mature protein by cleavage of the signal peptide, as demonstrated by N-terminal amino acid sequencing. Moreover, *TnphoA* mutagenesis suggests that CbMip is an exported protein in *E. coli*. Whether CbMip is actually secreted by *C. burnetii* is currently being investigated. Of interest is the recent observation that viable *C. burnetii* cells incubated in host-cell-free medium at low pH rapidly synthesize and export several proteins, one of which is similar in molecular mass to CbMip (Redd & Thompson, 1995). The synthesis and secretion of proteins by *C. burnetii* subjected to low pH (*in vitro*) suggests that this might occur as the organism is being taken in by endocytosis. If CbMip is secreted, one could speculate that it would interact with host components both during uptake and intracellular growth. Identification of CbMip would appear to support the argument that infection of host cells by *C. burnetii* is an active process, in that it requires the presence of specific virulence components.

While the significance of the observation is not known, it is worth noting that CbMip, like LpMip, is a very basic protein. Predicted pIs for LpMip and CbMip are 9.8 (Engleberg *et al.*, 1989) and 10.7, respectively. It will be interesting to see if the basic nature of these secreted proteins is related to a common feature of their different intracellular niches.

Although Mip/Mip-like proteins have been studied in great detail (Hacker & Fischer, 1993), how they function during host-parasite interaction is still unknown. The recent finding of significant homology to FKBP may provide some clues as to the physiological function of Mip-related proteins. FKBP exhibits PPIase activity which may mediate folding of proteins (Fischer & Schmid, 1990; Tropschug *et al.*, 1990). The enzymic activity of both Mip-related proteins and FKBP is inhibited by binding to FK506 or rapamycin (Fretz *et al.*, 1991), but the direct target for FK506 has been shown to be calcineurin (Fruman *et al.*, 1992; O'Keefe *et al.*, 1992), a calcium- and calmodulin-dependent phosphatase that is required for the activation of the cytoplasmic nuclear factor of activated T cells. It will be interesting to see whether impairing host FKBP function leads to facilitated uptake by host macrophages or the intracellular survival of the parasite. Given the potential secretory feature of Mip/Mip-like proteins, one cannot exclude the possibility that such proteins are able to bind to the natural ligand of host FKBP, thus deregulating the host immune system.

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