Cloning, Nucleotide Sequence, and Expression of the Gene Coding for a Ribosome Releasing Factor-Homologous Protein of *Brucella melitensis*

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The gene coding for a *Brucella melitensis* cytosoluble protein (CP24) that is immunogenic in infected sheep and a major component of brucellin INRA was cloned and sequenced. As in *Brucella* cells, CP24 was located in the cytoplasm of recombinant *Escherichia coli*. The amino acid sequence predicted from the cloned gene revealed 48 and 46% identity with the ribosome releasing factor, a protein factor required for release of the 70S ribosome from the mRNA, of *E. coli* and *Haemophilus influenzae* Rd, respectively. Sera from naturally infected sheep and sheep experimentally infected with *B. melitensis* H38 showed antibody reactivity against recombinant CP24.

Microorganisms belonging to the genus *Brucella* are the etiological agents of brucellosis, an infectious disease affecting animals and humans that causes high economic losses and important risks for human health. *Brucella melitensis* may cause abortions in sheep and goats and is the most pathogenic species for humans. One of the principal aims in brucellosis research is the identification of *Brucella* antigens, eliciting humoral and/or cell-mediated immune responses, that might be of interest for the development of subcellular vaccines or diagnostic tests that avoid the drawbacks of those currently used.

The usefulness of *Brucella* smooth lipopolysaccharide for diagnostic and protection purposes has been widely demonstrated (2, 5, 10, 13, 19, 20), but interest in protein antigens has not been carefully analyzed. Several proteins from the cytosoluble protein extract of *B. melitensis* have been found to induce an antibody response in infected sheep (4). Among them, a protein of 24 kDa (CP24), detected in all *Brucella* species and biovars (3), has also been identified as a major component of brucellin INRA (18), an allergen used in the skin test for detection of delayed-type hypersensitivity reactions in infected animals (6) and whose antigenic composition has been poorly studied. The present report describes the cloning and nucleotide sequence of the gene coding for *B. melitensis* CP24. Expression of the gene in *Escherichia coli* was also analyzed by using a monoclonal antibody (MAb) with specificity for CP24.

**Cloning and expression in *E. coli* of the *B. melitensis* cp24 gene.** A *B. melitensis* 16M genomic library was constructed in lambdaGEM-12 *Xho*I half-site arms (Promega, Madison, Wis.) by following the instructions of the manufacturer. Briefly, *B. melitensis* 16M DNA, extracted and purified as described previously (17), was partially digested for 30 min at 37°C with *Sau*3AI (Promega) at 0.014 U/μg of DNA, the enzyme concentration giving the highest percentage of fragments ranging from 15 to 23 kb. DNA fragments were partially filled in with dGTP and dCTP, by using Klenow DNA polymerase (Promega), and ligated with T4 DNA ligase (Promega) to lambdaGEM-12 digested with *Xho*I and partially filled in with dTTP and dCTP. Recombinant phage DNA was packaged in vitro with the Packagene System (Promega), and the library was titrated by determination of the number of PFU that appeared after infection of *E. coli* KW251 cells (Promega). Recombinant phages were transferred to nitrocellulose filters, and phages expressing cp24 were identified by reactivity with anti-Cp24 MAb V78/04C12/A12 (3). DNA of a positive phage was extracted from culture supernatants of *E. coli* KW251 cells infected with the phage and cultured until lysis was observed, as described previously (12). Phage DNA was then cut with *Not*I, *Bam*HI, *Eco*RI, or *Sac*I, and restriction fragments were ligated into pGEM-5Zf+ (Promega) cut with *Not*I or into pGEM-7Zf+ (Promega) cut with *Bam*HI, *Eco*RI, or *Sac*I, respectively. Competent *E. coli* JM109 cells (Promega) were transformed with recombinant plasmid DNA as described previously (12), and bacteria were spread on LB-ampicillin (50 μg/ml) plates containing isopropyl-1-thio-β-D-galactopyranoside (IPTG) and kDa

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**FIG. 1.** Western blotting, with anti-Cp24 MAb V78/04C12/A12, of *B. melitensis* H115 cp24 protein extract (lane 1) and *E. coli* cells bearing pNV122407 (lane 2). Samples were heated for 10 min at 100°C in Laemmli sample buffer before electrophoresis. The positions of protein molecular size markers are indicated on the left.
5-bromo-4-chloro-3-indolyl-\(\beta\)-d-galactopyranoside (X-Gal). 

*E. coli* JM109 colonies bearing recombinant plasmids were transferred to nitrocellulose, lysed with 10% sodium dodecyl sulfate, and screened with anti-CP24 MAb V78/04C12/A12 in a colony blotting technique. Positive colonies were found bearing plasmids with *Bam*HI or *Sac*I inserts. These plasmids were named pNV122405 and pNV122407, respectively. Expression of *cp24* in *E. coli* cells bearing plasmid pNV122405 or pNV122407 was analyzed by Western blotting (immunoblotting) with the anti-CP24 MAb, and both of them showed one band with an apparent molecular mass of 24 kDa according to protein molecular weight standards (see the representative pNV122407 profile in Fig. 1, lane 2). A band with the same apparent molecular mass was also revealed with the cytosoluble protein extract from *B. melitensis* cells (Fig. 1, lane 1), suggesting that the entire gene was present in recombinant plasmids pNV122405 and pNV122407. Control *E. coli* cells bearing nonrecombinant pGEM-7Zf showed no reaction with the anti-CP24 MAb (data not shown). Insert length analysis of pNV122405 and pNV122407 on 1% agarose gels showed that the smallest *Brucella* DNA fragment was that subcloned in plasmid pNV122407 (*Sac*I fragment of approximately 4.4 kb), which was therefore selected for subsequent experiments. The orientation of the *Brucella* insert subcloned in pNV122407 was inverted, resulting in plasmid pNV122408, which was selected

FIG. 2. Binding, visualized by electron microscopy, of anti-CP24 MAb V78/04C12/A12 to thin sections of *E. coli* cells bearing pNV122407. Magnification, \(\times34,400\).
according to the enzyme restriction profile. *E. coli* cells containing plasmid pNV122408 showed a band in a Western blot with the same apparent molecular mass as that observed for *E. coli* cells bearing plasmid pNV122407 and for the cytosolic protein extract from *B. melitensis* cells (data not shown), suggesting that the *cp24* gene in pNV122407 and pNV122408 was transcribed from its own promoter. The localization of CP24 in recombinant *E. coli* was determined by electron microscopy after reaction of the anti-CP24 MAb with whole cells or thin sections (3) of *E. coli* bearing pNV122407 or pGEM-7Zf, followed by immunogold labeling (3). *E. coli* cells bearing pGEM-7Zf or pNV122407 showed no surface labeling (data not shown). Thin sections of *E. coli* bearing pGEM-7Zf showed no reaction with the MAb (data not shown), while thin sections of bacteria bearing pNV122407 were labeled in the cytoplasm (Fig. 2), suggesting that CP24 has the same localization in recombinant *E. coli* as in *Brucella* cells (3).

**Nucleotide sequence of *cp24***. Recombinant plasmids pNV122407 and pNV122408, bearing the *cp24* gene in opposite orientations, were unidirectionally digested with exonuclease III by using the Erase-a-bases system (Promega) in accordance with the instructions of the manufacturer. A series of plasmids containing inserts differing in approximately 200 bp was obtained and used for sequencing by the primer-directed dideoxy method (16) with the SequiTTherm Cycle Sequencing Kit (Epicentre Technologies, Madison, Wis.) and the pGEM-7Zf T7-promoter primer (Promega). Expression of the *cp24* gene in plasmids bearing the deleted inserts was analyzed by using anti-CP24 MAb V78/04C12/A12 by the Western blot technique, allowing localization of the *cp24* gene within a region of about 1 kb of the 4.4-kb *SacI* cloned *Brucella* insert. Sequence information about this insert fraction was obtained from both DNA strands, revealing an open reading frame of 558 bp with the ATG codon starting at position 215 (Fig. 3). A nucleotide sequence (AAGGAG), homologous to the 3′-terminal sequence of the *E. coli* 16S rRNA, which probably constitutes the ribosome-binding site was found 8 bp upstream the initiation codon. Inverted repeats typical for rho-independent transcription termination were not found downstream of the termination codon. However, an ATG was found 45 bp downstream from the TAG termination codon with an AAGGAG sequence located 10 bp upstream that might constitute the ribosome-binding site for an adjacent gene. The open reading frame coded for a protein of 186 amino acids with a predicted molecular mass of 20,709 Da. No sequence with features typical of signal sequences for protein export was found, indicating that the cloned gene likely codes for a cytoplasmic protein.

**Sequence homologies**. A search for amino acid sequence homologies (FASTA program [15]) revealed that *B. melitensis* CP24 exhibits 48 and 46% identity with the *E. coli* ribosome releasing factor (RRF) (9) and an RRF-homologous protein of *Haemophilus influenzae* Rd (PIR accession number D64095), respectively. Alignment of the three protein sequences is shown in Fig. 4. RRF, originally found in the soluble fraction of *E. coli* (7), is a protein factor required for the protein synthesis process and essential for bacterial growth (11). Working together with GTP and elongation factor G, RRF releases the 70S ribosome from the mRNA (8) after the synthesized protein has been released from tRNA, allowing recycling of ribosomes for the next round of protein synthesis.

**Serum reactivity**. The antibody reactivity of naturally infected sheep or sheep experimentally infected with *B. melitensis* H38 (4) against recombinant *E. coli* cells bearing pNV122407 was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blotting. All sera revealed the same protein band (Fig. 5, lanes 2 to 8) as that observed with anti-CP24 MAb V78/04C12/A12 (Fig. 5, lane 1), which was not detected with sera from *Brucella*-free sheep (data not shown).

No information regarding the immunogenic properties of
RRF has been previously stated. This is the first report of an RRF-homologous protein for which an antibody response has been observed in infected sheep (4) (Fig. 5) and for which specific MAbs are available (3).

*B. melitensis* CP24 has been identified as one of the proteins of brucellin INRA (18), an allergen used to detect delayed-type hypersensitivity reactions in infected animals (6) and of brucellin INRA (18), an allergen used to detect delayed-type hypersensitivity reactions in infected animals (6) and whose antigenic composition has not been clearly identified. Only the L7/L12 ribosomal protein of *B. abortus* (14), which, like the RRF, is a component of the protein synthesis machinery, has been shown to be involved in the development of delayed-type hypersensitivity reactions by brucellin (1). The availability of recombinant CP24 will provide a means to determine its contribution to the delayed-type hypersensitivity reactions induced by the brucellin INRA in infected animals and to analyze its usefulness as a diagnostic or protective antigen.

**Nucleotide sequence accession number.** The nucleotide sequence of the cp24 gene has been deposited in GenBank under accession number U53133.

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**REFERENCES**


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