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SEROLOGICAL DIAGNOSIS

The 18-kDa cytoplasmic protein of *Brucella* species – an antigen useful for diagnosis – is a lumazine synthase

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Previous studies have shown that the detection of antibodies to an 18-kDa cytoplasmic protein of *Brucella* spp. is useful for the diagnosis of human and animal brucellosis. This protein has now been expressed in recombinant form in *Escherichia coli*. The recombinant protein is soluble only under reducing conditions, but alkylation with iodoacetamide renders it soluble in non-reducing media. As shown by gel exclusion chromatography, this soluble form arranges in pentamers of 90 kDa. The reactivity of human and animal sera against the recombinant protein was similar to that found with the native protein present in brucella cytoplasmic fraction, suggesting that the recombinant protein is correctly folded. The protein has low but significant homology (30%) with lumazine synthases involved in bacterial riboflavin biosynthesis, which also arrange as pentamers. Biological tests on the crude extract of the recombinant bacteria and on the purified recombinant protein showed that the biological activity of the *Brucella* spp. 18-kDa protein is that of lumazine synthase. Preliminary crystallographic analysis showed that the *Brucella* spp. lumazine synthase arranges in icosahedric capsids similar to those formed by the lumazine synthases of other bacteria. The high immunogenicity of this protein, potentially useful for the design of acellular vaccines, could be explained by this polymeric arrangement.

Introduction

Information about the sequence, structure and function of protein components of micro-organisms is of increasing interest for both microbiologists and immunologists. The characterisation of proteins from pathogenic bacteria can help understanding of the interaction between the bacterium and the host and facilitate studies of the humoral and cellular immune responses elicited during infection. Furthermore, proteins can be useful as specific antigens for the serological diagnosis of bacterial infections.

The characterisation of proteins from *Brucella* spp., the causative agents of brucellosis, has been the subject of intensive work. Several brucella proteins have been sequenced and cloned [1–5], but only a few have been characterised at the structural and functional level [6–8].

A previous work described an 18-kDa cytoplasmic protein of *Brucella* spp. [9]. A monoclonal antibody specific for this protein was used to develop a capture ELISA that showed the potential usefulness of this protein as an antigen for the serological diagnosis of brucellosis. In man, the determination of anti-18-kDa antibodies made possible the differentiation between active and inactive brucellosis. In addition, detection of anti-18-kDa antibodies proved useful in distinguishing between vaccinated and actively infected cattle [10] and was also useful for the diagnosis of canine brucellosis [11]. The 18-kDa protein was purified by affinity chromatography and the sequence of three internal peptides was determined [9]. More recently, Hemmen *et al.* [12] cloned a gene encoding a 17-kDa brucella protein whose deduced sequence showed

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homology with the internal peptides described previously, suggesting that both proteins may be identical. The antibody response to the 17-kDa protein was useful for diagnosing ovine and bovine brucellosis [12, 13].

DeMot *et al.* [14] identified a gene in *Rhodococcus* spp. encoding a homologue of the 17-kDa brucella protein and noticed a low but significant homology between the sequences of these two proteins and that of lumazine synthases involved in bacterial riboflavin biosynthesis. This study describes the expression and purification of the 18-kDa protein of *Brucella* spp. in a recombinant form.

Materials and methods

Cloning of the gene encoding the 17-kDa protein in a pET vector

The sequence information described previously [12] was used to design specific primers containing either the N-terminal or the C-terminal nucleotide sequences of the 17-kDa protein and incorporating an *Nhe*I restriction site at the 5′-end of each primer. A phenol-inactivated suspension of *B. abortus* 45/20 cells was sonicated and genomic DNA was purified by phenol extraction followed by ethanol precipitation. This DNA was digested with *Nhe*I for 3 h at 37°C. After phenol extraction, digested DNA was used as a template for the PCR with specific primers. The 500-bp PCR product was digested with *Nhe*I and ligated into *Nhe*I-digested and dephosphorylated vector plasmid pET11b (Novagen, Madison, WI, USA). The ligation mix was used to transform *E. coli* Invα competent cells (Invitrogen, Carlsbad, CA, USA). Miniprep plasmid DNA was purified from overnight cultures of 10 recombinant colonies with the Wizard Miniprep kit (Promega, Madison, WI, USA) and the orientation of the insert was checked by restriction with *Nde*I, which cuts within the PCR fragment and *Xba*I, which cuts within the pET11b plasmid. The recombinant clones contain the open reading frame encoding the brucella 18-kDa protein plus an N-terminal addition of the sequence Ala-Ser-Met originating from the incorporation of the *Nhe*I restriction site in the PCR primer sequence.

Expression of the protein

The plasmid DNA of a clone containing the insert in the correct orientation for expression from the *lac* promoter was used to transform *E. coli* BL21(DE3) competent cells (Stratagene, La Jolla, CA, USA). Recombinant clones were grown in LB medium containing ampicillin 100 mg/L, at 37°C with agitation (300 rpm) to an OD_{600} of 1.0; 5 ml of this culture were diluted to 500 ml and grown to reach an OD_{600} of 1.0. At this point the culture was induced by the addition of 1mM IPTG and incubated for 4 h at 37°C with agitation (300 rpm). The bacteria were centrifuged at 15 000 *g* for 20 min at 4°C and stored at −20°C.

Purification and refolding of the protein

Bacterial cells harvested after induction were suspended in a solution of 50 mM Tris, 5 mM EDTA, Triton X-100 1%, pH 8.0, and sonicated three times for 1 min at 4°C. Inclusion bodies were centrifuged at 20 000 *g* for 30 min at 4°C. The precipitate was washed twice with suspension solution lacking Triton X-100. The inclusion bodies were solubilised in 50 mM Tris, 5 mM EDTA, 8 M urea, pH 8.0, at room temperature overnight with agitation. The solubilised material was refolded by dialysis against PBS containing 1 mM dithiotreithol (DTT) for 3 days, with several changes of buffer. The material was centrifuged at 20 000 *g* for 30 min at 4°C and the supernate was dialysed against 50 mM Tris buffer, pH 8.5, containing 1 mM DTT (buffer A). This preparation was purified by FPLC through a MonoQ column (Pharmacia, Uppsala, Sweden) with a linear gradient of buffer B (50 mM Tris, 1 mM DTT, 1 M NaCl, pH 8.5).

Post-translational treatment of the recombinant protein

The recombinant protein solubilised in 10 mM DTT was incubated for 1 h with 25 mM iodo-acetamide at room temperature. The protein was then dialysed against PBS.

Immunoblotting analysis

An extract of total cytoplasmic proteins of *Brucella*, prepared as described previously [15], was boiled in sample buffer and separated by SDS-PAGE in acrylamide 15% gel containing 8 M urea. The iodo-acetamide-treated recombinant brucella 18-kDa protein was electrophoresed in parallel. The proteins were electrotransferred on to nitrocellulose and the membrane was incubated with purified BI24 monoclonal antibody (MAb BI24), specific for the native 18-kDa cytoplasmic protein of *Brucella* spp. After incubation with horseradish peroxidase-conjugated anti-mouse immunoglobulin, the reaction was developed with 4-Cl-α-naphthol and H_2O_2 0.03%.

ELISA assays

Antigenic capture and indirect ELISA were used to assess the reactivity of the same set of sera against the native and recombinant 18-kDa protein of *Brucella* spp., respectively.

The capture ELISA was performed as described previously [9]. Briefly, polystyrene plates were sensitised with MAb BI24 and blocked with PBS containing skim milk 1%. Then, a cytoplasmic fraction of *B. abortus*, depleted of lipopolysaccharide (LPS) and

containing the 18-kDa protein [15], was added. Sera from four patients with acute brucellosis, four dogs naturally infected with *B. canis* and four sheep naturally infected with *B. ovis* were dispensed at 1 in 100 or 1 in 200 dilution. The reactivity of these sera was revealed by incubation with peroxidase-conjugated polyclonal antibodies to human IgG (Dako, Carpinteria, CA, USA), to ovine IgG (Sigma) or to canine IgG (Dako). The reaction was developed by adding 50 μl of a solution containing *o*-phenylenediamine 2 μg/μl and H_2O_2 0.03% in 0.1 M citrate-phosphate buffer, and was stopped with 50 μl of 4 N H_2SO_4. The final colour was read at 490 nm in an ELISA reader (Σ 960 Metertech, Taiwan).

For the indirect ELISA, polystyrene plates were sensitised with the iodo-acetamide-treated recombinant 18-kDa protein 0.2 μg/well. Blocking, addition of sera and conjugates, and development of the reaction were done as indicated for the capture ELISA.

Enzymic activity

Assays to measure 6,7-dimethyl-8-ribityllumazine synthase activity were performed as described previously [16]. The reaction mixtures contained 1 mM 3,4-dihydroxy-2-butanon-4-phosphate, 1 mM 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione and 10 μl of enzyme solution in a total volume of 100 μl. The mixtures also contained 100 mM potassium phosphate (pH 7.0) and 2 mM EDTA. They were incubated at 37°C. Samples were taken at intervals and the reaction was quenched by the addition of trichloro-acetic acid to a final concentration of 0.4 mM. The concentration of 6,7-dimethyl-8-ribityllumazine was determined by high-performance liquid chromatography with a column of Nucleosil RP18 (4 × 250 mm). The effluent was monitored fluorometrically. An eluent containing methanol 10% and 340 mM formic acid was used for the analysis of lumazine (excitation, 408 nm; emission, 487 nm); the retention volume was 3 min. The enzymic activity was expressed as units/mg of total protein (bacterial extracts) or purified protein, as determined by the Bradford method, with bovine serum albumin as standard.

Results

Expression, folding and purification of the recombinant brucella 18-kDa protein

As shown in Fig. 1A, the 18-kDa protein of *Brucella* spp. was successfully expressed in the pET vector as inclusion bodies in BL21(DE3) cells. Attempts to express soluble protein were unsuccessful. The inclusion bodies were solubilised in 8 M urea and the protein was refolded by dialysis against PBS in the presence of a reducing agent (1 mM DTT). No refolded material was obtained in the absence of DTT. The refolded protein was purified by means of anion-exchange chromatography in a MonoQ column under reducing conditions. The protein eluted as a sharp peak at 150 mM NaCl. Pooled material from several runs was analysed by SDS-PAGE, showing an isolated band at 18 kDa (Fig. 1, lane B). The final yield was calculated to be *c.* 10–15 mg of purified protein/L of bacterial culture.

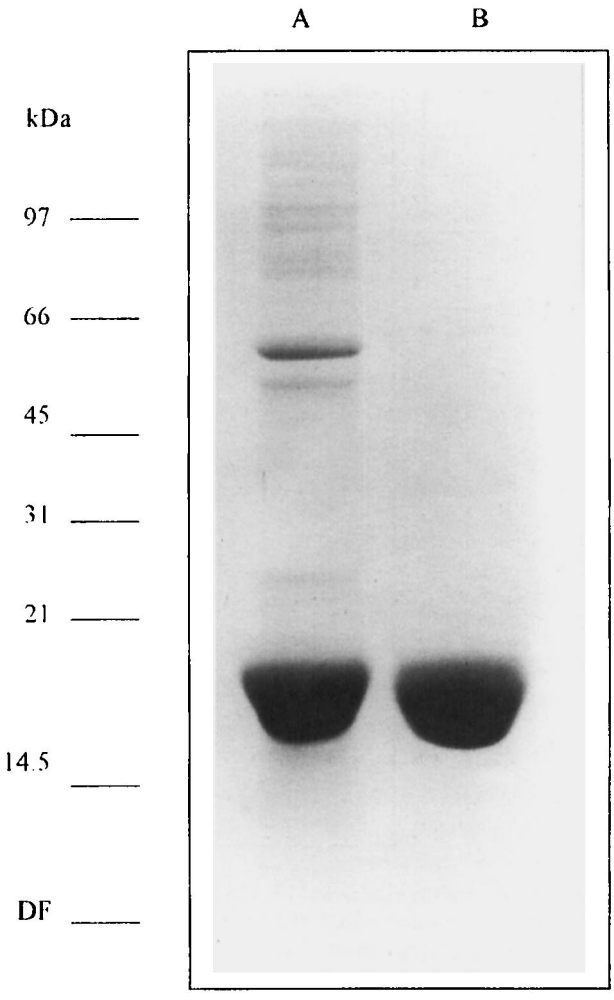

Fig. 1. Expression of the 18-kDa recombinant protein of *Brucella* spp. in *E. coli* as revealed by SDS-PAGE. (**A**) Inclusion bodies from transformed *E.coli* cells boiled in sample buffer. (**B**) Anion-exchange cromatography-purified 18-kDa recombinant protein. DF: dye front.

The recombinant protein was recognised by MAb BI24 (previously used to characterise the 18-kDa brucella protein) (Fig. 2), confirming the common identity of the protein described by us [9] and that cloned by Hemmen *et al.* [12]. To assess if the recombinant protein was appropriately folded, the same set of sera was assayed by indirect and antigen capture ELISA with the recombinant and the native brucella protein, respectively. As shown in Table 1, sera from human patients, dogs and sheep infected with different *Brucella* species showed similar reactivity patterns in both ELISAs, indicating that the recombinant *E. coli* protein folds like the native brucella protein.

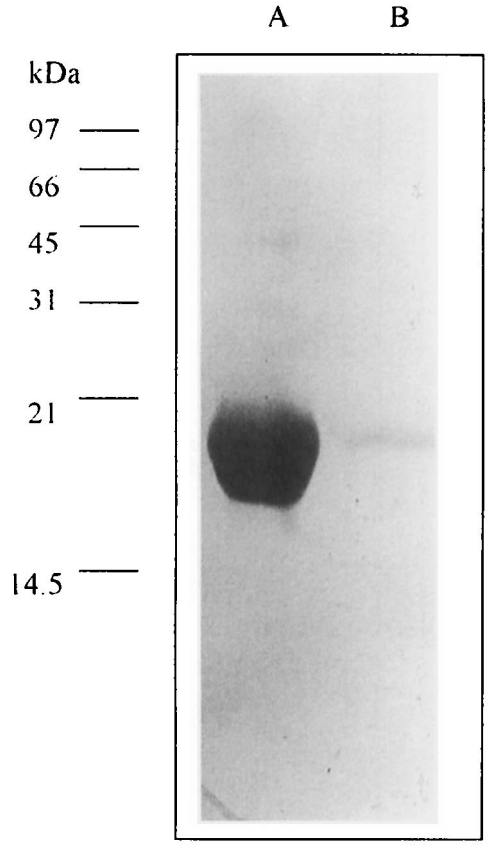

Fig. 2. Immunoblot reactivity of MAb BI24 with the 18-kDa iodo-acetamide-treated recombinant protein (**A**) and the LPS-free cytoplasmic fraction of *B. abortus* (**B**). Proteins were electrophoresed in a polyacrylamide 15% gel, blotted on to nitrocellulose, probed with MAb BI24 and revealed with the appropriate conjugate and substrate.

Table 1. Reactivity of sera from human patients and animals infected with different *Brucella* spp. against the native and the recombinant 18-kDa cytoplasmic protein

Sera*	Reactivity to native protein† (OD_{490})	Reactivity to recombinant protein‡ (OD_{490})
Human 1	0.392	0.785
Human 2	0.916	0.895
Human 3	1.548	1.608
Human 4	2.706	2.990
Canine 1	0.327	0.492
Canine 2	1.423	1.598
Canine 3	2.042	1.981
Canine 4	2.076	2.175
Ovine 1	0.562	0.420
Ovine 2	1.060	0.708
Ovine 3	1.946	1.259
Ovine 4	2.803	2.900

*All sera were assayed at 1 in 100 dilution.
†Antigen capture ELISA with MAb BI24. Cut-off values: 0.280, 0.153 and 0.090 for human, canine and ovine assays, respectively, as determined with 40 normal sera of each species. Specificity (percent of normal samples rendering negative result) 95%, 97% and 95% for human, canine and ovine assays, respectively. Sensitivity (percent of samples from actively infected individuals or animals rendering positive result) 85%, 95% and 93%, respectively.
‡Indirect ELISA. Cut-off values: 0.310, 0.161, 0.110, for human, canine and ovine assays, respectively, determined as stated above. Specificity 94%, 97% and 96% for human, canine and ovine assays, respectively; sensitivity 89%, 98% and 95%, respectively.

Analysis of the structure and function of the brucella 18-kDa protein

The search for amino acid sequence homology showed a low but significant homology with the enzyme lumazine synthase of *Bacillus subtilis* and the putative lumazine synthase of *E. coli* described by Mörtl *et al.* [17]. As shown in Fig. 3, homology with the *Bac. subtilis* enzyme was particularly significant in residues involved in the active site of the enzyme.

E. coli BL21(DE3) containing the plasmid encoding the 18-kDa protein were grown and induced to express recombinant protein. The 6,7-dimethyl-8-ribityllumazine synthase activity found in a cell extract obtained by sonication of recombinant bacteria grown at 37°C was *c.* six-fold higher than the activity displayed by control plasmid-transformed bacteria (1.1 U/mg *versus* 0.2 U/mg, respectively). As this protein tends to aggregate in inclusion bodies, the enzymic activity was also assayed in extracts from bacteria grown at 20°C. The enzymic activity of the purified enzyme was 126 U/mg. These results clearly indicate that the recombinant brucella 18-kDa protein is an enzyme with lumazine synthase activity.

Study of the aggregation of the recombinant brucella 18-kDa protein

The Mono Q-purified 18-kDa recombinant protein was soluble, even at concentrations >10 mg/ml, in solutions containing 1 mM DTT, but aggregated in the absence of a reducing agent. As the aggregation reverted after adding DTT, this behaviour was thought to be related to the presence of an unique cysteine residue in the protein sequence (Fig. 3). To test this hypothesis, the effect of alkylating the free sulphydric group was assessed by treatment with 25 mM iodo-acetamide in the presence of DTT. After this treatment the protein did not aggregate further, even after dialysis against PBS free of DTT. Gel-exclusion chromatography revealed that the iodo-acetamide-treated protein has a mol. wt of *c.* 90 kDa, indicating the presence of a pentamer of the 18-kDa protein.

Discussion

Previous reports have shown the potential usefulness of an 18-kDa brucella cytoplasmic protein in the serological diagnosis of human and animal brucellosis [9, 10, 12, 13]. Interestingly, the determination of the antibody response to this protein yields results equivalent to those obtained with a complex mixture of cytoplasmic proteins of *Brucella* [9–11]. The present study demonstrated that this protein (previously identified by means of a specific MAb [9]), is identical to the 17-kDa brucella cytoplasmic protein cloned and expressed by Hemmen *et al.* [12]. The fact that two different groups, using different methodologies, have concluded that this protein is a serological marker of

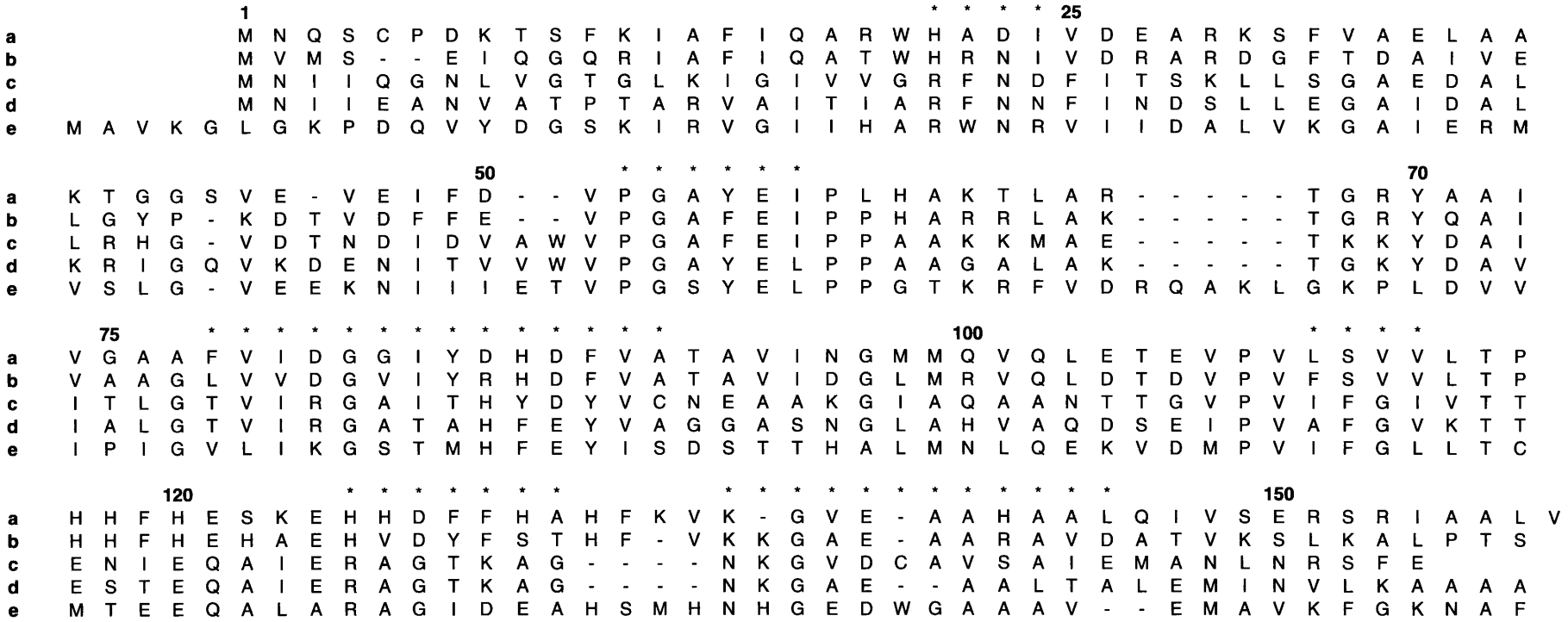

Fig. 3. Comparison of the amino-acid sequence of lumazine synthases from different bacteria and that of the 18-kDa cytoplasmic protein of *Brucella* spp. by the Lipman-Pearson protein alignment algorithm. Sequences correspond to: *Brucella* spp. (**a**); *Rhodococcus* spp. (**b**); *Bac. subtilis* (**c**); *E. coli* (**d**); *Saccharomyces cerevisiae* (**e**). *Amino-acid residues which are part of the active site of the *Bac. subtilis* enzyme, as reported by Ritsert *et al.* [18].

active brucellosis, emphasises the importance of measuring specific antibodies against this antigen.

The present study shows the main characteristics of the recombinant *E. coli*-expressed 18-kDa cytoplasmic protein of *Brucella* spp. The expression system and refolding procedures used resulted in a good yield of the recombinant protein properly folded. Moreover, the post-translational alkylation of the free cysteine residue made it possible to obtain the otherwise aggregated protein as a soluble pentamer with antigenic properties similar to those of the native brucella protein.

The study further analysed the biological activity of this protein. Sequence homology analysis (Fig. 3) showed that this protein has a low but significant homology with the enzyme 6,7-dimethyl-8-ribityllumazine synthase of different bacteria, which catalyses the formation of an intermediate product in the synthesis of riboflavin [17]. The enzymic activity displayed by the purified recombinant 18-kDa brucella protein confirms that the biological activity of this protein is that of lumazine synthase. The biological activity of the brucella enzyme is significantly lower than that of other lumazine synthases described previously (126 U/mg *versus* 10 000 U/mg) [17], but similar to that of the lumazine synthase from *Methanococcus jannaschii* (210 U/mg; S. Mörtl, personal communication). Further studies with different experimental protocols (metal cations, buffers and pH) are needed to determine the optimal conditions for brucella lumazine synthase activity.

Three-dimensional analysis by X-ray crystallography of the *Bac. subtilis* lumazine synthase complexed with an analogue substrate has revealed that the enzyme forms a particle with a mol. wt close to 1000 kDa [18]. This particle comprises 60 β subunit monomers (lumazine synthase) arranged in 12 pentamers, forming an icosahedral capsid. It also contains three α subunits (riboflavin synthase) enclosed inside the capsid. The entire structure is called lumazine synthase-riboflavin synthase complex. In contrast, the lumazine synthase of *E. coli* is not physically associated with another enzyme of the riboflavin pathway [17]. Further studies are needed to assess if, as found in *Bac. subtilis*, the brucella lumazine synthase is physically associated with riboflavin synthase.

As stated by Mörtl *et al.* [17], bacteria are devoid of an uptake system for riboflavin. Therefore, they are dependent on internal synthesis and should be vulnerable to inhibitors of riboflavin synthesis. As this enzyme is not present in mammals, information about its three-dimensional structure could serve as a basis for the rational design of enzyme inhibitors with therapeutic activity. On the other hand, an understanding of the polymerisation mechanisms of this enzyme could help to obtain protein particles of a given size under controlled conditions. Studies with different experimental models have shown that virus-like protein particles are highly immunogenic [19]. It has been suggested that the size of these particles promotes a very efficient uptake by antigen-presenting cells, which in turn produce a more efficient presentation of the derived peptides to specific T and B lymphocytes [20]. The diameter of recombinant lumazine synthase particles of *E. coli* (17 nm) [17] is in the range of typical protein particles of high immunogenicity. For the reasons cited above, structural studies were performed on the 18-kDa protein of *Brucella* spp. [21]. Preliminary X-ray crystallographic analysis showed that, like its *Bac. subtilis* homologue, the brucella lumazine synthase arranges as an icosahedral particle. This fact could explain the high immunogenicity of this protein in infected hosts [9–12]. The similarity in structural arrangement of this 18-kDa protein and the *Bac. subtilis* homologous enzyme further demonstrates that the brucella protein is a lumazine synthase.

In summary, this study shows that the 18-kDa cytoplasmic protein of *Brucella* spp. is an enzyme with lumazine synthase activity. This protein constitutes an interesting candidate for serological diagnosis and for the design of specific chemotherapeutic agents, and its polymeric characteristics could provide the basis for the development of an acellular vaccine.

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