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1 Analysis of the occurrence and distribution of the Omp25/Omp31 family of surface proteins
2 in the six classical *Brucella* species

3

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1 **Abstract**

2

3 Members of the Omp25/Omp31 family of surface proteins were previously shown to
4 participate in the virulence of some *Brucella* species and a different distribution of the seven
5 proteins of this family among species could be related to the difference in pathogenicity and
6 host preference they exhibit. Accordingly, in this work we have analyzed the expression of
7 the genes coding for the Omp25/Omp31 family in the six classical *Brucella* species and a set
8 of *B. ovis* mutant strains with each *omp* gene inactivated. Immunoblot of whole cell lysates
9 with antibodies raised against the purified recombinant outer membrane proteins (OMPs) did
10 not show the simultaneous presence of the seven OMPs in any of the *Brucella* strains studied,
11 but different Omp25/Omp31 profiles were detected, in our experimental conditions, between
12 the *Brucella* strains representative of the six classical species. Transcripts for *omp31*, *omp25*
13 and *omp25c* were, in general, the most abundant of the family and some hits were found in *B.*
14 *ovis* for a posttranscriptional regulation mechanism and for a compensatory mechanism
15 increasing the synthesis of a protein to compensate for the absence of another one. Finally, the
16 potential interest of Omp25c and Omp31b as subcellular vaccines, considering their
17 occurrence in the *Brucella* strains studied and their antigenic relatedness with other proteins
18 of the family, is discussed.

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21 *Keywords:* *Brucella*; Omp25/Omp31 family; outer membrane protein; vaccine; RNA

22

1 **1. Introduction**

2

3 Brucellosis, an infectious disease caused by bacteria of the genus *Brucella*, can affect
4 many mammal species and is transmissible to humans, thus having an important socio-
5 economic impact. The six classical *Brucella* species (*B. melitensis*, *B. abortus*, *B. suis*, *B.*
6 *canis*, *B. ovis* and *B. neotomae*) (Osterman and Moriyón, 2006) infect terrestrial mammals
7 with a marked host preference and *B. ceti*, *B. pinnipedialis* and *B. microti* have recently been
8 validly published to include *Brucella* strains preferentially infecting cetaceans, seals and
9 common voles respectively (Foster et al., 2007; Scholz et al., 2008).

10 The *Brucella* species show high DNA-DNA relatedness (Verger et al., 1985) but
11 display differences in pathogenicity and host preference which might be related, at least in
12 part, with differences in the composition of the outer membrane (OM), structure involved in
13 the interaction of gram-negative bacteria with the surrounding environment. The *Brucella*
14 Omp25/Omp31 family is constituted by seven homologous OM proteins (OMPs) (Salhi et al.,
15 2003; Vizcaíno et al., 2004) some of which are involved in virulence (Caro-Hernández et al.,
16 2007; Edmonds et al., 2002; Martín-Martín et al., 2008) and whose encoding genes exhibit
17 DNA polymorphism among the brucellae (Vizcaíno et al., 2004). Omp31 and Omp25 are
18 major OMPs with a well-studied distribution in *Brucella* (Cloeckert et al., 2002) but
19 regarding the other members of the family, although they have been detected in at least one
20 *Brucella* species (Al Dahouk et al., 2008; Cloeckert et al., 2002; Connolly et al., 2006;
21 Eschenbrenner et al., 2006; Guzmán-Verri et al., 2002; Salhi et al., 2003; Wagner et al.,
22 2002), little is known about their occurrence among the brucellae.

23 Accordingly, the aim of this work was to analyze the expression of the genes coding
24 for the seven members of the Omp25/Omp31 family in the six classical *Brucella* species.

25

1 2. Materials and methods

2

3 2.1. Plasmids and bacterial strains

4

5 Plasmids (Table 1) were propagated in *Escherichia coli* JM109 or BL21(DE3)
6 cultured in Luria-Bertani medium supplemented with ampicillin (Amp).

7 For construction of pNV31b-com (containing *omp31b* of *B. abortus* 544 under the
8 control of the *lacZ* promoter), *omp31b* was PCR-amplified with primers 31b-F2 and 31b-R2
9 (Table 2) and cloned into pGEM-T Easy in the same direction to *lacZ*. The *SpeI-SacII* insert
10 was finally cloned into *SpeI/SacII*-digested pBBR1MCS-4 (Kovach et al., 1995).

11 *Brucella* strains (Table 1) -propagated in tryptic soy agar supplemented with 0.3%
12 yeast extract and 5% horse serum (Caro-Hernández et al., 2007)- were incubated at 37 °C (44
13 h for *B. melitensis* Rev.1 and *B. ovis* and 20 h for the other strains, in order to reach a growth
14 level starting to be clearly visible and similar for all the strains) and a 10% CO₂ atmosphere
15 was used for *B. ovis* and *B. abortus*. Culture medium for *B. ovis* PA-derived strains was
16 supplemented with Amp, kanamycin (Kan) or both depending on their antibiotic resistance
17 pattern (Table 1).

18 Recombinant plasmid pNV31b-com (see above) was introduced in *B. ovis* PA by
19 electroporation, as previously reported (Vizcaíno et al., 2004), to obtain *B. ovis* PNV31b-com.
20 The other genetically-modified *B. ovis* strains were previously described (Caro-Hernández et
21 al., 2007; Vizcaíno et al., 2004).

22

23 2.2. Primers and nucleic acid techniques

24

1 Primers (Table 2) (Roche, Mannheim, Germany) were chosen according to the
2 published *Brucella* genome sequences. PCR and DNA sequencing were previously detailed
3 (Caro-Hernández et al., 2007).

4 RNA was extracted (from 1 ml of an aqueous bacterial suspension concentrated to
5 obtain an O.D._{600 nm} of 13) following a RNeasy midi kit (Qiagen GmbH, Hilden, Germany)
6 modified protocol (Bellaire et al., 2003) and quantified spectrophotometrically.

7 RNA (1 µg) was treated with RQ1 DNase (Promega) and subjected to cDNA synthesis
8 with the transcriptor first strand cDNA synthesis kit (Roche) using the provided random
9 hexamers as primers for reverse transcriptase (RT). The subsequent PCR reactions were
10 performed using 1 µl of cDNA as template and the specific primers for each target *omp* gene
11 (Table 2). Products amplified by RT-PCR were visualized after electrophoresis in 0.8%
12 agarose gels. Absence of DNA contamination in RNA samples was confirmed with identical
13 reactions but omitting the reverse transcriptase (no amplification products were detected with
14 these reactions). A control RT-PCR reaction with the *IF-1* gene -constitutively expressed in
15 *Brucella* (Eskra et al., 2001)- was also settled.

16

17 2.3. Production of anti-OMP immune sera

18

19 First, the region corresponding to each mature protein of the Omp25/Omp31 family
20 was PCR-amplified, using the pair of primers described in Table 2, from a *Brucella* strain
21 bearing a functional gene (Vizcaíno et al., 2004): *B. ovis* PA (*omp25c*, *omp25d* and *omp22*),
22 *B. melitensis* 16M (*omp25b*) or *B. abortus* 544 (*omp31b*). The primers –except those used for
23 *omp22*- were designed to introduce a 5'-terminal *Bgl*II or *Bam*HI site and a 3'-terminal *Sfu*I
24 site in the amplified gene. The amplicons were ligated into pGEM-T Easy, cleaved with the
25 corresponding restriction enzymes (*Bgl*II/*Sfu*I or *Bam*HI/*Sfu*I) and inserted into pTrcHisB or

1 pTrcHisA (Table 1). For *omp22* higher levels of the recombinant protein were obtained
2 following another cloning strategy. The gene *omp22* of *B. ovis* PA was amplified with primers
3 22MAT-F and 22R, cloned into pGEM-T Easy in the same orientation to *lacZ*, then into
4 *SphI/SalI*-digested pUC19 and finally, into *BamHI/HindIII*-digested pTrcHisA.

5 The resulting plasmids (Table 1) -showing the expected nucleotide sequence- were
6 introduced by transformation into *E. coli* JM109 and BL21(DE3) where the expression of
7 each recombinant gene was induced for 6 h with IPTG (Guilloteau et al., 1999) to evaluate,
8 by SDS-PAGE and Commassie blue staining, the level of synthesis of each recombinant
9 protein. Strain BL21(DE3) provided better results than JM109 except for *omp25b*.

10 The recombinant fusion proteins (having a 5'-histidine tag) were purified (from *E. coli*
11 cells incubated for 6 h in the presence of IPTG) with the ProBond Purification System
12 (Invitrogen) under denaturing conditions. The protein fractions -dialyzed against deionised
13 water- were analyzed by Coomassie blue staining following SDS-PAGE and quantified with
14 the Bio-Rad protein assay (Bio-Rad, Hercules, California, USA).

15 Female New Zealand albino rabbits were inoculated subcutaneously -three times at
16 weekly intervals- with 50 µg of sonicated purified protein in Freund's complete (1st
17 immunization) or incomplete (2nd and 3rd immunization) adjuvant. Seven days after the last
18 injection, blood was taken by heart puncture to obtain the serum.

19

20 2.4. Immunological techniques

21

22 Rabbit sera (1/50 dilution) and monoclonal antibodies (MAbs) A59/05F01/C09 (C09)
23 -specific for the *Brucella* spp. Omp25 protein- and A59/10F09/G10 (G10) and
24 A01/08H06/G02 (G02) -reacting in Western blot with the *B. melitensis* and *B. ovis* Omp31
25 protein, respectively- were used (Cloekaert et al., 1990; Vizcaíno et al., 2001). SDS-PAGE

1 and Western blot with whole-cell lysates (bacterial suspensions concentrated to an O.D._{600 nm}
2 of 20, diluted 1/2 in Laemmli sample buffer and boiled for 10 min) or purified recombinant
3 proteins were previously described (Vizcaíno et al., 2001). The low range SDS-PAGE
4 standard (Bio-Rad) was used to determine, as instructed by the manufacturer, the molecular
5 mass of the proteins of the Omp25/Omp31 family. The proteins transferred to the
6 nitrocellulose membrane following SDS-PAGE were stained with Ponceau S (Sigma, St.
7 Louis, MO, USA) to verify the accuracy of transfer and the total protein charge (which was
8 similar for all the whole cell lysates of the different *Brucella* strains). Goat anti-rabbit and
9 anti-mouse IgG-peroxidase conjugates (Sigma) were used and the blots were never stripped
10 and reprobed. When the nitrocellulose membrane was cut into strips for independent
11 development, their realignment (to reproduce the original nitrocellulose membrane) was
12 performed before analyzing the results.

13 For adsorption of the anti-Omp22 serum, purified Omp22 was submitted to SDS-
14 PAGE and transferred to nitrocellulose. The intense Omp22 band on the membrane was cut,
15 saturated with skim milk and incubated overnight with the serum diluted 1/200 (Vizcaíno et
16 al., 2001). Binding of antibodies to Omp22 was checked as for Western blot. The procedure
17 was repeated until complete removal of the anti-Omp22 antibodies. Unadsorbed control
18 serum was incubated with saturated nitrocellulose under the same conditions. Other anti-OMP
19 sera were similarly adsorbed with the corresponding OMP.

20

21 **3. Results**

22

23 *3.1. Transcription of the genes coding for the Omp25/Omp31 family in the genus Brucella*

24

1 Transcription of *omp25b* in *B. ovis* PA (lacking *omp25b* (Vizcaíno et al., 2004)) was
2 not detected by RT-PCR while transcripts for the other genes of the family were clearly
3 evidenced (Fig. 1). The *B. ovis* PA genetically-modified strains (Fig. 1B) gave the expected
4 RT-PCR pattern when compared to the parental strain: (i) absence of transcript for the
5 corresponding inactivated *omp* gene, with the exception of the unexpected *omp25* faint band
6 in the $\Delta omp25$ mutant (although these transcripts should not have a ribosome binding site and
7 the synthesis of Omp25 was not detected in the $\Delta omp25$ mutant by reactivity with monoclonal
8 or polyclonal antibodies [Fig. 3 and data not shown]), (ii) over-transcription after *in trans*
9 complementation of the mutant with the wild-type gene -only tested for strains PNV25dA-
10 com and PNV22A-com (complemented $\Delta omp25d$ and $\Delta omp22$ mutants respectively)-, (iii)
11 over-transcription of *omp31b* in strain PNV31b-com (parental strain complemented with
12 undefective *omp31b* from *B. abortus*), and (iv) detection of *omp25b* transcripts in strain
13 PNV998-10 (parental strain complemented with a DNA fragment containing *omp25b*).

14 Regarding the other *Brucella* species (Fig. 1A), the *omp31*-specific RT-PCR reaction
15 (lanes 1) was positive in all the strains except in *B. abortus* strains (lacking *omp31* (Vizcaíno
16 et al., 1999; Vizcaíno et al., 1997)). Transcripts for *omp31b* (lanes 2) were detected in all the
17 strains, although in *B. melitensis* strains -having a DNA deletion affecting the 5' end of
18 *omp31b* (García-Yoldi et al., 2005; Vizcaíno et al., 2004)- amplification was exclusively
19 obtained with the pair of primers 31bRT-R/31bMRT-F. Transcripts for *omp25* and *omp25b*
20 (lanes 3 and 4) were evidenced in all the *Brucella* strains tested (except *omp25b* in *B. ovis* as
21 described above). An intense DNA band revealed the presence of *omp25c* transcripts (lanes 5)
22 in all the strains, while the RT-PCR reactions for *omp25d* and *omp22* gave less-intense bands
23 (lanes 6 and 7).

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25 3.2. Immunological detection of the Omp25/Omp31 family proteins in the genus *Brucella*

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3.2.1. *Omp31* and *Omp31b*

Occurrence of *Omp31* in the genus *Brucella* has been previously studied (Caro-Hernández et al., 2007; Vizcaíno et al., 1997) and was included in this study as a reference for detection of *Omp31b*.

The characteristic multiple band pattern for *B. ovis* *Omp31* (Caro-Hernández et al., 2007; Vizcaíno et al., 2001) in Western blot (with two major bands of 28.8 and 30.2 kDa) was revealed with the anti-*Omp31* MAb G02 in all the *B. ovis* PA strains (representative profile in Fig. 2, lane 2), except the $\Delta omp31$ mutant (not shown, (Caro-Hernández et al., 2007)). The other *Brucella* strains, except those belonging to the species *B. abortus* (lacking *omp31* (Vizcaíno et al., 1999; Vizcaíno et al., 1997)), reacted with the anti-*Omp31* MAb G10 (Fig. 2).

With the anti-*Omp31b* polyclonal antibodies (Fig. 2), all the *B. ovis* PA-derived strains, except the $\Delta omp31$ mutant (lane 5), revealed the same band pattern developed by the anti-*Omp31* MAb (lanes 1-9). Accordingly and since the absence of *Omp31b* is expected in *B. ovis* considering DNA data (Vizcaíno et al., 2004), it is inferred that the reactivity of the anti-*Omp31b* serum with *B. ovis* is due to a cross-serological reaction with *Omp31* and not to a specific reaction with *Omp31b*. Additionally, *B. ovis*-PNV31b-com (parental strain complemented *in trans* with undefective *omp31b* from *B. abortus*) displays an additional multiple band profile with a higher molecular mass (starting at about 31 kDa) (lane 3), which should correspond to over-synthesized *Omp31b*.

Regarding the reactivity of the other *Brucella* species with the anti-*Omp31b* serum (Fig. 2), *B. abortus* strains -lacking *Omp31* (Vizcaíno et al., 1999; Vizcaíno et al., 1997) and not reacting with the anti-*Omp31* MAb (see representative results in lane 21)- revealed a

1 smeared protein band of about 31 kDa (lanes 20 and 22-26), also detected in *B. neotomae* and
2 *B. suis* (lanes 16 and 18), probably corresponding to Omp31b. Cross reactivity of the anti-
3 Omp31b serum with Omp31 was also observed with the two latter strains since this serum
4 also developed the protein band revealed with MAb G10 (lanes 16-19), as it also did with *B.*
5 *melitensis* and *B. canis* (lanes 12-15). However, as expected according to the nucleotide
6 sequence of *omp31b* in *B. melitensis* and *B. canis* (Vizcaíno et al., 2004), Omp31b was not
7 detected in these strains.

8

9 3.2.2. *Omp25 and Omp25b*

10

11 Detection by immunoblotting of Omp25 in the genus *Brucella* has been previously
12 performed (Caro-Hernández et al., 2007; Cloeckart et al., 1996) and was included in this
13 study as a reference for the identification of the other homologous proteins. As expected
14 (Caro-Hernández et al., 2007; Cloeckart et al., 1996), detection of Omp25 with MAb C09
15 only failed with the $\Delta omp25$ mutant of *B. ovis* PA (Fig. 3A). In *B. ovis*, two bands
16 corresponding to Omp25 were detected: the major band exhibiting an apparent molecular
17 mass (25.9 kDa) lower than that of Omp25 of the other *Brucella* species (27.5 kDa) and the
18 minor band (more intense in strain PNV31A) of 22.3 kDa.

19 Omp25b was not detected in *B. ovis* (lacking *omp25b* due to a DNA deletion
20 (Vizcaíno et al., 2004)) except in strain PNV998-10 (Fig. 3B, lanes 1-4) (complemented *in*
21 *trans* with the deleted DNA (Vizcaíno et al., 2004)) where Omp25b appeared as a protein
22 band of 32.1 kDa (Fig. 3B, lane 4) also developed with the anti-Omp25c serum (not shown).
23 Regarding the other species, the Omp25b band -disappearing after adsorption of the anti-
24 Omp25b serum with purified recombinant Omp25b (not shown)- was only detected in *B. suis*
25 and *B. melitensis* (Fig. 3B, lanes 6-8, and data not shown). A couple of bands located under

1 Omp25b, revealed with the anti-Omp25b serum in several *Brucella* strains (Fig. 3B), could
2 not be associated to any member of the Omp25/Omp31 family.

3 The anti-Omp25b serum (Fig. 3B) cross-reacted with Omp25 and Omp25c, according
4 to its reactivity with parental *B. ovis* PA and with the $\Delta omp25$ and $\Delta omp25c$ mutant strains
5 (lanes 1-3), the reactivity of the anti-Omp25 MAb C09 (lane 5 and data not shown) and the
6 results obtained with the anti-Omp25c serum (Fig. 4A).

7

8 3.2.3. *Omp25c*, *Omp25d* and *Omp22*

9

10 The anti-Omp25c serum (Fig. 4A) developed two intense bands of 27.5 and 29.5-30.5
11 kDa in *B. ovis* (except in the $\Delta omp25c$ mutant), the smeared upper band being more intensely
12 marked in strains PNV31A and PNV25cA-com (lanes 1-7). This serum also cross-reacted
13 with the *B. ovis* Omp25 protein, since it reacted with a protein developed with all the *B. ovis*
14 strains except with the $\Delta omp25$ mutant (lanes 1-7). A smeared protein band of 29.5-30.5 kDa
15 and a protein of 27.5 kDa were also detected in the other *Brucella* species but since Omp25
16 has in these species the same apparent molecular mass than the lower Omp25c band (see
17 representative reactivity of *B. canis* with MAb C09 in lane 8) it is difficult to elucidate
18 whether the 27.5 kDa band is constituted by Omp25 or by both Omp25 and Omp25c. Two
19 additional bands of lower molecular mass and unknown nature were also revealed in *B.*
20 *abortus* strains (lanes 14-18).

21 Contrarily, Omp25d was only detected in *B. ovis* PNV25dA-com (Fig. 4B and data not
22 shown) -strain where *omp25d* is over-transcribed in comparison with the parental strain (Fig.
23 1B)- as a protein of approximately 27.9 kDa whose reactivity with the anti-Omp25d serum
24 disappeared after adsorption of the serum with purified Omp25d (not shown).

1 The Omp22 protein band –disappearing after adsorption of the anti-Omp22 serum
2 with recombinant Omp22 (not shown)- was detected in all the *B. ovis* strains except in the
3 $\Delta omp22$ mutant (Fig. 4C, lanes 1-8). However, Omp22 in the complemented $\Delta omp22$ mutant
4 (*B. ovis* PNV22A-com) was only detected, as a very faint band, with a more concentrated
5 specific anti-serum (1/10 dilution). Surprisingly, low levels of Omp22 were evidenced in the
6 *B. ovis* PA $\Delta omp25d$ mutant and its corresponding complemented strain (lanes 5-6). A protein
7 band showing a molecular mass similar to Omp22 was observed in *B. canis*, *B. neotomae* and
8 in *B. abortus* strains 544, B19 and 45/20. However, adsorption of the anti-Omp22 serum with
9 purified Omp22 only removed its reactivity with *B. neotomae*.

11 **4. Discussion**

13 Expression in *Brucella* spp. of the seven genes coding for the OMPs of the
14 Omp25/Omp31 family was evaluated in this work by RT-PCR and immunological detection
15 of each protein with specific antibodies. In spite of the fact that direct detection of the proteins
16 has not been performed, this study provides for the first time evidences for the synthesis of
17 Omp31b in *B. neotomae*, of Omp25c in *B. ovis*, *B. canis* and *B. neotomae* and of Omp22 in *B.*
18 *ovis* and *B. neotomae*.

19 The simultaneous presence of the seven members of the family, which according to
20 DNA data would only be feasible in *B. suis* and *B. neotomae* (Vizcaíno et al., 2004), was
21 evidenced in none of the strains analyzed, although the limitation of the immunological
22 detection must be considered. In the experimental conditions used, the smooth or rough
23 phenotype does not seem to influence the OM composition regarding the Omp25/Omp31
24 family (see similar profiles of *B. abortus* 2308 and its derivative rough mutant *B. abortus*
25 RB51) but a different distribution of the surface proteins of the Omp25/Omp31 family was

1 observed between the *Brucella* species (Fig. 2-4), which could contribute to the divergence in
2 pathogenicity and host preference they exhibit. An apparent increase of Omp25c was
3 observed in the $\Delta omp31$ mutant of *B. ovis* PA, when compared to the parental strain and to
4 the other mutants derived from the same strain (Fig. 4A), which could be related to a
5 compensatory mechanism necessary to restore the balance of the OM after removal of major
6 Omp31. A compensatory mechanism has been reported for the orthologous Hbp family of
7 *Bartonella quintana* and for the Omp25/Omp31 family in *B. suis* 1330 (Minnick et al., 2003;
8 Salhi et al., 2003), and a tight balance of its members was revealed essential for the stability
9 of the OM in *B. ovis* PA (Caro-Hernández et al., 2007).

10 Several results described in this work agree with previous DNA polymorphism
11 observations suggesting the absence of some members of the Omp25/Omp31 family in
12 several species (Vizcaíno et al., 2004) and justify why some proteins were not detected even
13 in cases where specific transcripts were evidenced. Thus, defects in or deletion of the
14 encoding-genes (Cloeckert et al., 1996; Vizcaíno et al., 2004; Vizcaíno et al., 1999; Vizcaíno
15 et al., 1997) explain the unsuccessful detection of Omp31 in *B. abortus*, of Omp31b in *B.*
16 *melitensis*, *B. canis* and *B. ovis*, of Omp25b in *B. abortus*, *B. canis* and *B. ovis* and the lower
17 molecular mass exhibited by Omp25 in *B. ovis* (Fig. 2-4 and data not shown).

18 The highly conserved sequence of *omp25c* in the genus (Vizcaíno et al., 2004) and the
19 abundance of its specific transcripts (Fig. 1) justify the detection of Omp25c in all the strains
20 analyzed (Fig. 4A). Moreover, Omp25c seems to be in *B. ovis* one of the most abundant of the
21 family in the conditions of our experiments (Fig. 2-4). On the contrary and in agreement with
22 studies failing in the detection of Omp25d in *B. melitensis*, *B. abortus* or *B. suis* by N-
23 terminal microsequencing or mass spectrometry (Connolly et al., 2006; Eschenbrenner et al.,
24 2006; Guzmán-Verri et al., 2002; Lamontagne et al., 2007; Salhi et al., 2003; Wagner et al.,
25 2002), Omp25d was not detected in any of the wild-type *Brucella* strains. Accordingly, it is

1 probable that the protein co-migrating with Omp25 in *B. suis* and suggested to be Omp25d by
2 Salhi *et al.* (Salhi *et al.*, 2003) does not actually correspond to Omp25d but to the lower
3 molecular mass band of Omp25c -that in *B. suis* would co-migrate with Omp25 (Fig. 4A).
4 The detection of this Omp25c band was possible in *B. ovis* (Fig. 4A) because the apparent
5 molecular mass of Omp25 in *B. ovis* is lower than in the other brucellae (Fig. 3A and 4A)
6 (Cloekaert *et al.*, 1996). A posttranscriptional control mechanism -related to either the
7 translation or the protein turnover rates- could be on the basis of the lack of detection of
8 Omp25d in *Brucella* spp., since *omp25d* is highly conserved in the genus, does not show
9 sequence defects, has a clear ribosome binding site (Vizcaíno *et al.*, 2004), and is transcribed
10 in all the strains analyzed (Fig. 1). Such mechanism could also explain why, in spite of the
11 clear detection of Omp22 in *B. ovis* PA (Fig. 4C), a marked over-transcription of *omp22* in
12 strain PNV22A-com ($\Delta omp22$ mutant complemented with wild-type *omp22*) (Fig. 1B) leads
13 to almost undetectable levels of Omp22 in our conditions (Fig. 4C).

14 Omp22 was only detected in *B. ovis* and *B. neotomae* while in other *Brucella* strains a
15 protein band of the same molecular mass could not be assigned to Omp22 after adsorption of
16 the anti-Omp22 serum with purified Omp22 (Fig. 4C). However, the presence of Omp22 in *B.*
17 *canis* RM6/66, *B. suis* 1330 and *B. abortus* strains 544, B19 and 45/20 can not be completely
18 excluded since the protein/s of molecular mass similar to Omp22 and reacting with the anti-
19 Omp22 serum would prevent the accurate identification of Omp22. Omp22 was not detected
20 in *B. abortus* 2308 (Fig. 4C, lane 16), in contrast to previous works evidencing Omp22 in this
21 strain after mass spectrometry studies with OM fragments or cell envelop extracts (Connolly
22 *et al.*, 2006; Guzmán-Verri *et al.*, 2002). These differences might be related, in addition to the
23 detection techniques, to the different culture conditions or the fact that we have used whole
24 cell extracts.

1 The attenuation in virulence demonstrated for *B. ovis*-PNV25dA (Caro-Hernández et
2 al., 2007) and its defects in invasiveness and intracellular multiplication in professional and
3 non-professional phagocytes (Martín-Martín et al., 2008) would support the synthesis of
4 Omp25d in *B. ovis* PA. However, as described for other homologous genes in other bacteria
5 (Motaleb et al., 2004), this *B. ovis* $\Delta omp25d$ mutant synthesizes lower amounts of Omp22
6 (Fig. 4C) despite transcription of *omp22* seems similar to that of the parental strain (Fig. 1).
7 Since the $\Delta omp22$ mutant is avirulent and unable to survive inside phagocytes while these
8 defects are less prominent in the $\Delta omp25d$ mutant (Caro-Hernández et al., 2007; Martín-
9 Martín et al., 2008), it seems probable that the alterations in the latter mutant be due –at least
10 in part- to the decrease in Omp22. Normal levels of Omp22 were not recovered after
11 complementation of the $\Delta omp25d$ and $\Delta omp22$ mutants with the corresponding wild-type
12 genes (Fig. 4C), which also justify why complementation of both strains did not restore the
13 virulence of the parental strain (Caro-Hernández et al., 2007).

14 Close antigenic relatedness between Omp31 and Omp31b -justified by their 67% of
15 sequence identity (Salhi et al., 2003) and responsible for the strong reactivity of the anti-
16 Omp31b serum with *B. ovis* lysates (Fig. 2)- were evidenced. Since Omp31 protects against
17 rough *B. ovis* and smooth *B. melitensis* infections (Cassataro et al., 2005; Estein et al., 2003),
18 the potential of Omp31b as subcellular vaccine would merit to be evaluated in protecting not
19 only against infection by *Brucella* species synthesizing Omp31b but also against *Brucella*
20 species not having Omp31b but synthesizing the antigenically-related Omp31 protein
21 ((Vizcaíno et al., 2004) and Fig. 1). Similarly, the serological cross-reactivity of Omp25c with
22 Omp25 and Omp25b and the fact that it was detected in all the investigated *Brucella* strains
23 (Fig. 1 and 4A) are also points encouraging its evaluation as subcellular vaccine. A recent
24 report (Al Dahouk et al., 2008) showing that the concentrations of Omp31, Omp31b, Omp25b
25 and Omp25c in *B. suis* (ORFs BRA0423, BR1622, BR0971 and BR0119, respectively)

1 increase inside murine macrophages -when compared to those detected in bacteria growing in
2 culture medium- provides additional data reinforcing this evaluation.

3 This study gives new insights into the characterization of the members of the
4 Omp25/Omp31 family of surface proteins, providing a more detailed map about their
5 distribution in the *Brucella* species and new information supporting the evaluation of some of
6 them as subcellular vaccines against brucellosis.

7

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2

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6

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- 12
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14

1 **Legends of figures**

2

3 Fig. 1. RT-PCR for *omp31* (lanes 1), *omp31b* (lanes 2), *omp25* (lanes 3), *omp25b* (lanes 4),
4 *omp25c* (lanes 5), *omp25d* (lanes 6) and *omp22* (lanes 7) in different *Brucella* species (A) and
5 in the *B. ovis* mutants with these genes impaired (B). RT-PCR of *omp31b* in lanes 2 was
6 performed with primers 31bRT-F and 31bRT-R except in *B. melitensis* strains (*) where
7 transcripts were only detected with the pair of primers 31bMRT-F/31bRT-R. St, DNA
8 molecular size marker VI (Roche). Control reactions with constitutively expressed *IF-1*
9 (Eskra et al., 2001) are shown in panel C, where lane numbers correspond to strain numbers
10 in panels A and B. Control RT-PCR reactions without RT gave no amplification product.

11

12 Fig. 2. Western blot with whole-cell extracts of different *Brucella* strains and anti-Omp31
13 MAbs or polyclonal antibodies obtained against recombinant Omp31b (31b). MAb G02 was
14 used for *B. ovis* strains while MAb G10 was used for the other *Brucella* species. Positions of
15 Omp31 and Omp31b are marked with arrows and strains where Omp31b seems to be detected
16 are identified with an asterisk. The anti-Omp31b serum was used at dilution 1/500 with *B.*
17 *ovis* PNV31b-com (lane 3) instead of dilution 1/50 as employed for the other strains. A line
18 on the left shows the position of the closest protein of the molecular mass standard.

19

20 Fig. 3. Western blot with whole-cell extracts of different *Brucella* strains and anti-Omp25
21 MAb C09 (A) or polyclonal antibodies obtained against recombinant Omp25b (B). Strains not
22 represented in panel B did not reveal the Omp25b band with the anti-Omp25b antiserum.
23 Positions of Omp25, Omp25b (confirmed by adsorption of the serum with purified Omp25b
24 as described in section 2.4) and Omp25c are marked with arrows in panel B. Symbols: #, lane

1 developed with the anti-Omp25 MAb C09; ?, doubtful or non-identified protein bands. A line
2 on the left shows the position of the closest protein of the molecular mass standard.

3

4 Fig. 4. Western blot with whole-cell extracts of different *Brucella* strains and polyclonal
5 antibodies obtained against recombinant Omp25c (A), Omp25d (B) or Omp22 (C). Strains not
6 represented in panel B did not react with the anti-Omp25d antiserum. Positions of the proteins
7 of the Omp25/Omp31 family are marked with arrows. Symbols: #, lane developed with anti-
8 Omp25 MAb C09; ?, doubtful or non-identified protein bands; *, strains where the presence
9 of Omp22 was confirmed. The anti-Omp22 serum was used at dilution 1/10 with *B. ovis*
10 PNV22A-com instead of dilution 1/50 as employed for the other strains (the 1/10 dilution
11 gave with the parental strain and the $\Delta omp22$ mutant the same results shown in panel C, lanes
12 1 and 7, respectively). A line shows in each panel the position of the closest protein of the
13 molecular mass standard.

1 Table 1. *Brucella* strains and plasmids used in this work

<i>Brucella</i> strain or plasmid	Origin ^a	Relevant characteristics
<i>Brucella</i> strains		
<i>B. melitensis</i>		
Rev 1	BCCN V4a	Smooth vaccine
B115	BCCN R19	Smooth strain
<i>B. abortus</i>		
544 ^b	BCCN R4	Smooth strain
2308	BCCN R10	Smooth strain
B19	BCCN V1	Smooth vaccine
RB51	BCCN V5	Rough vaccine
45/20	BCCN V2	Rough strain
<i>B. suis</i> 1330 ^b	BCCN R12	Smooth strain
<i>B. canis</i> RM6/66 ^b	BCCN R18	Rough strain
<i>B. neotomae</i> 5K33 ^b	BCCN R16	Smooth strain
<i>B. ovis</i>		
PA (parental)	BCCN 76-250	Virulent parental rough strain for mutants listed below
PNV31A	(Caro-Hernández et al., 2007)	<i>omp31</i> inactivated. Kan ^r
PNV31A-com	(Caro-Hernández et al., 2007)	complemented PNV31A ^c
PNV25A	(Caro-Hernández et al., 2007)	<i>omp25</i> inactivated. Kan ^r
PNV25A-com	(Caro-Hernández et al., 2007)	complemented PNV25A ^c
PNV25cA	(Caro-Hernández et al., 2007)	<i>omp25c</i> inactivated. Kan ^r
PNV25cA-com	(Caro-Hernández et al., 2007)	complemented PNV25cA ^c
PNV25dA	(Caro-Hernández et al., 2007)	<i>omp25d</i> inactivated. Kan ^r
PNV25dA-com	(Caro-Hernández et al., 2007)	complemented PNV25dA ^c
PNV22A	(Caro-Hernández et al., 2007)	<i>omp22</i> inactivated. Kan ^r
PNV22A-com	(Caro-Hernández et al., 2007)	complemented PNV22A ^c
PNV31b-com	This work	PA complemented with <i>omp31b</i> from <i>B. abortus</i> . Amp ^r
PNV998-10	(Vizcaíno et al., 2004)	PA complemented with pNV998-10 (plasmid with <i>omp25b</i> in a 15 kb DNA fragment of <i>B. melitensis</i> 16M absent in <i>B. ovis</i>). Kan ^r
Plasmids		
pGEM-T Easy	Promega	PCR-cloning vector. Amp ^r
pUC19	Fermentas	Cloning vector. Amp ^r
pBBR1MCS-4	(Kovach et al., 1995)	Cloning vector replicating in <i>Brucella</i> . Amp ^r
pTrcHis A and B	Invitrogen	Cloning vectors for protein synthesis and purification.
pNV31b-com	This work	<i>omp31b</i> of <i>B. abortus</i> 544 cloned in pBBR1MCS-4
pNV31b304	This work	Mature ^d <i>omp31b</i> in pTrcHis B
pNV25b305	This work	Mature ^d <i>omp25b</i> in pTrcHis A
pNV25c306	This work	Mature ^d <i>omp25c</i> in pTrcHis A
pNV25d303	This work	Mature ^d <i>omp25d</i> in pTrcHis A
pNV22303	This work	Mature ^d <i>omp22</i> in pTrcHis A

3
4 ^a BCCN, *Brucella* Culture Collection, Nouzilly, France

5 ^b Reference strains

6 ^c Mutant strains complemented with the corresponding wild-type *omp* gene cloned into pBBR1MCS-4 (Kan and Amp resistant)

7 ^d Gene region corresponding to the mature OMP without the signal peptide

8

1 Table 2. DNA primers used in this work

2

Primer name	Nucleotide sequence ^a	Target gene (size in bp) ^b
Construction of pNV31b-com		
31b-F2	GTC ACA GAC ATT GGA GCC	<i>omp31b</i> (1036)
31b-R2	CTC CAG CAA GCC ATT CGC	<i>omp31b</i>
Construction of plasmids for the purification of the recombinant <i>Brucella</i> spp. OMPs		
31bMAT-BglII	<u>AGA TCT</u> GCC GAC ATC ATC GTT GCT	<i>omp31b</i> (925)
31bR2-SfuI	<u>TTC GAA</u> CTC CAG CAA GCC ATT CGC	<i>omp31b</i>
25bcMAT-BamHI	<u>GGA TCC</u> GCT GAC GCC GTC ATT GAA	<i>omp25b</i> (742) and <i>omp25c</i> (703)
25bR-SfuI	<u>TTC GAA</u> CGC CAT CGC ATT CCG TTG	<i>omp25b</i>
25cR-SfuI	<u>TTC GAA</u> TAT TGG GTG AGG ATT GAC	<i>omp25c</i>
25dMAT-BamHI	<u>GGA TCC</u> GCG GAT GCC ATT GTT GCG	<i>omp25d</i> (749)
25dR-SfuI	<u>TTC GAA</u> CTG AAG GGT AAA TGC GGC	<i>omp25d</i>
22MAT-F	GCC GAC ATG ATG GGA AGG	<i>omp22</i> (710)
22R	GTT TGA ATC CCG GCT GTT	<i>omp22</i>
RT-PCR		
31RT-F	GAC GTG GTT GTT TCT GA	<i>omp31</i> (563)
31RT-R	GTG TAG AGG TAT TCC GA	<i>omp31</i>
31bRT-F	TCC GTC GCA ATG TTG TT	<i>omp31b</i> (633)
31bMRT-F	GTT CAG GCC GGT TAT AA	<i>omp31b</i> (408)
31bRT-R	GTC GGT GTA GAG GTA TT	<i>omp31b</i>
25RT-F	GTC TCT CGT AAT CGT CT	<i>omp25</i> (504)
25RT-R	ATG TTG TCC GTC AGC TT	<i>omp25</i>
25bRT-F	TCG CTT CTT CTT GCA TC	<i>omp25b</i> (677)
25bRT-R	AAT CGG ATG TCG TGT GT	<i>omp25b</i>
25cRT-F	TTC TTT TTG CAT CGA CCG	<i>omp25c</i> (607)
25cRT-R2	TCG TCG AAG CCG TAA TCC	<i>omp25c</i>
25dRT-F	AAT CTA CTT GGC GCT TC	<i>omp25d</i> (595)
25dRT-R2	TGC CGT AAT CGG TGT AGC	<i>omp25d</i>
22RT-F	TCA AGC GTT CTA TCA CC	<i>omp22</i> (588)
22RT-R	TGT TAC GCT GTT CGA TG	<i>omp22</i>
IF1-F	ATG GCG AAA GAA GAA GTC CT	<i>IF-1</i> (214)
IF1-R	TGA AGC GGT AGG TGA TGC GG	<i>IF-1</i>

3
4 ^a Restriction enzymes specific sites in DNA primers are underlined

5 ^b Identification numbers of the genes in the *B. suis* 1330 published genome sequence (GenBank accession numbers AE014291
6 and AE014292 for chromosome I and II, respectively) are: BRA0423 (*omp31*), BR1622 (*omp31b*), BR0701 (*omp25*),
7 BR0971 (*omp25b*), BR0119 (*omp25c*), BR0118 (*omp25d*), BR1284 (*omp22*) and BR0249 (*IF-1*). The size of the PCR-
8 amplified fragments (according to the *B. suis* 1330 genome sequence) is given in the corresponding forward primer line.

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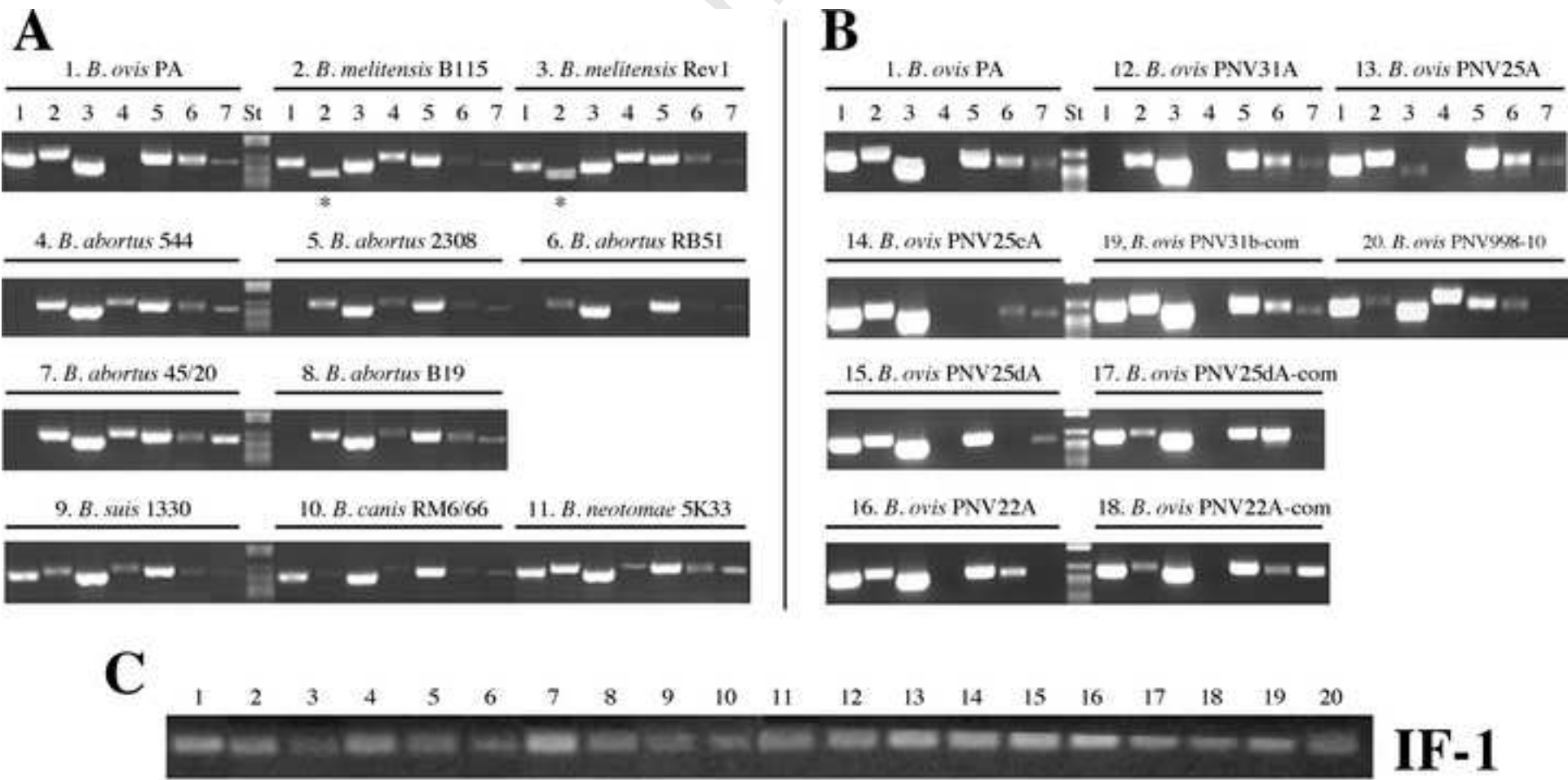
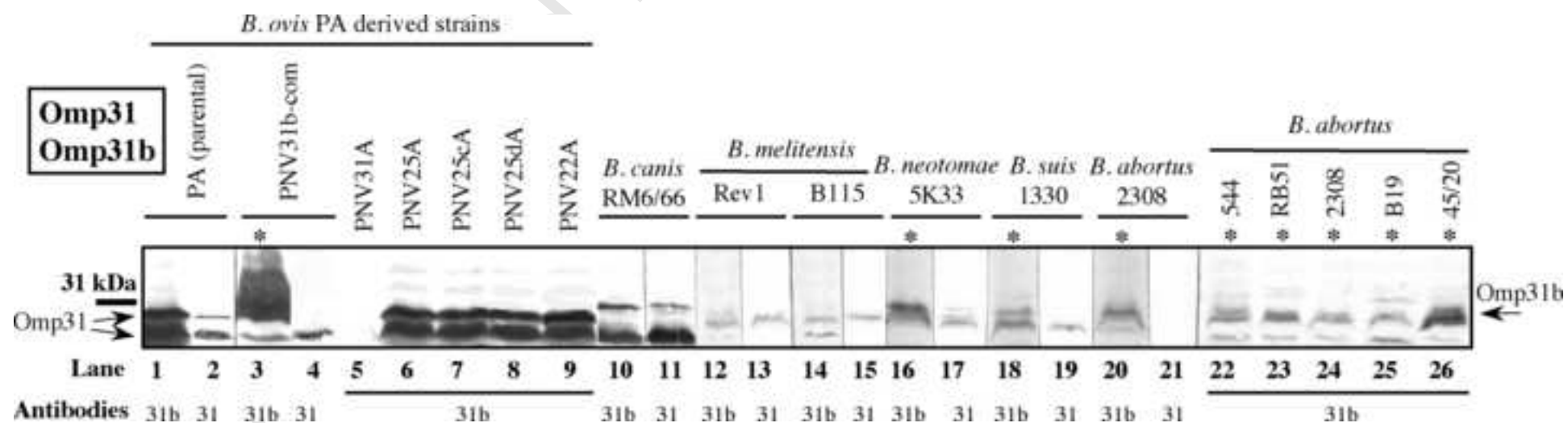


Figure 2

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