Arch Microbiol (2009) 191:571–581
DOI 10.1007/s00203-009-0484-9

ORIGINAL PAPER

Differential composition of culture supernatants from wild-type *Brucella abortus* and its isogenic *virB* mutants

M. Victoria Delpino · Diego J. Comerci · Mary Ann Wagner · Michel Eschenbrenner · Cesar V. Mujer · Rodolfo A. Ugalde · Carlos A. Fossati · Pablo C. Baldi · Vito G. DelVecchio

Received: 28 January 2009 / Revised: 17 April 2009 / Accepted: 30 April 2009 / Published online: 13 May 2009

Abstract The *virB* genes coding type IV secretion system are necessary for the intracellular survival and replication of *Brucella* spp. In this study, extracellular proteins from *B. abortus* 2308 (wild type, WT) and its isogenic *virB10* polar mutant were compared. Culture supernatants harvested in the early stationary phase were concentrated and subjected to 2D electrophoresis. Spots present in the WT strain but absent in the *virB10* mutant (differential spots) were considered extracellular proteins released in a *virB*-related manner, and were identified by MALDI-TOF analysis and matching with *Brucella* genomes. Among the 11 differential proteins identified, DnaK chaperone (Hsp70), choloylglycine hydrolase (CGH) and a peptidyl-prolyl cis–trans isomerase (PPIase) were chosen for further investigation because of their homology with extracellular and/or virulence factors from other bacteria. The three proteins were obtained in recombinant form and specific monoclonal antibodies (mAbs) were prepared. By Western blot with these mAbs, the three proteins were detected in supernatants from the WT but not in those from the *virB10* polar mutant or from strains carrying non-polar mutations in *virB10* or *virB11* genes. These results suggest that the expression of *virB* genes affects the extracellular release of DnaK, PPIase and CGH, and possibly other proteins from *B. abortus*.

Keywords *Brucella* · Extracellular proteins · Type IV secretion system

Communicated by Sebastian Suerbaum.

M. V. Delpino · C. A. Fossati · P. C. Baldi (✉)
IDEHU, Facultad de Farmacia y Bioquímica,
Universidad de Buenos Aires, Junin 956, 4to. Piso,
1113 Buenos Aires, Argentina
e-mail: pablobal@ffyb.uba.ar

D. J. Comerci · R. A. Ugalde
Instituto de Investigaciones Biotecnológicas,
Universidad Nacional de Gral. San Martín,
1650 San Martín, Argentina

M. A. Wagner · M. Eschenbrenner
Institute of Molecular Biology and Medicine,
The University of Scranton, Scranton, PA 18510, USA

C. V. Mujer · V. G. DelVecchio
Vital Probes, Inc., Mayfield, PA 18433, USA

Introduction

To enter and to survive within the cells of their host, many microbial pathogens modify the natural biological processes of the host cell and create a favorable environment. Subversion of eukaryotic processes by bacterial pathogens requires specialized macromolecular secretion systems to deliver virulence factors either into the environment or directly into host cells. Several macromolecular secretion pathways have been identified in Gram negative bacteria (Kostakioti et al. 2005). A group of related export pathways comprising conjugative DNA transfer systems and pathogenicity-related secretion systems have been named type IV secretion systems (T4SS) (Kostakioti et al. 2005; Christie et al. 2005). The best-characterized T4SS machinery is the VirB system that mediates DNA transfer from *Agrobacterium tumefaciens* to the host plant. Examples of T4SS playing a key role in the virulence of medically important pathogens are the pertussis toxin liberation (Ptl) system of *Bordetella pertussis* (Covacci and Rappuoli 1993), the *cag* pathogenicity island-encoded transporter of *Helicobacter pylori* (Censini et al. 1996), the Dot/Icm system of *Legionella pneumophila* (Vogel et al. 1998), and more recently the

VirB system in *Brucella* (O'Callaghan et al. 1999; Sieira et al. 2000).

Brucella is a facultative intracellular bacterium, which can survive inside professional and non-professional phagocytes by evading the endocytic pathway. However, *virB* mutants lose their ability to survive and multiply in mammalian cells (O'Callaghan et al. 1999; Sieira et al. 2000; Foulongne et al. 2000) or in infected mice (Hong et al. 2000). In particular, the *virB10* polar mutants used in the present study were affected in their ability to survive inside HeLa cells, and no viable mutants were recovered at 48 h p.i. (Sieira et al. 2000). Moreover, while wild-type *B. abortus* can escape the endocytic pathway and establish a replication niche in the endoplasmic reticulum, these evasion maneuvers cannot be accomplished by the *virB10* polar mutant (Comerci et al. 2001). A recent study revealed that the cytotoxic effect of rough *Brucella* mutants against macrophages depends on the expression of the T4SS (Pei et al. 2008). These and other findings suggest that *Brucella* secretes virulence factors trough the T4SS encoded by the *virB* operon. The expression in *Brucella* spp. of constituents of the T4SS (VirB proteins) has been confirmed and the assembly of the *Brucella* T4SS has been shown in a heterologous expression system (Rouot et al. 2003; Carle et al. 2006). In addition, proteins whose presence in culture supernatants of *B. abortus* seems to require a full VirB system have been detected using a reporter system (Marchesini et al. 2004). More recently, two proteins of the VjbR regulon of *Brucella* (VceA and VceC) were found to be translocated into macrophages in a T4SS-dependent manner (de Jong et al. 2008). However, other proteins not regulated by VjbR might be also secretion substrates of the T4SS. In *L. pneumophila*, for example, the expression of some T4SS substrates depends on both the CpxR and the PmrA regulators, while that of other substrates depends on CpxR only (Altman and Segal 2008).

Proteomic analyses of culture supernatants from wild type strains and mutants altered in secretion systems have been applied to identify proteins secreted by such systems in different bacteria (Kazemi-Pour et al. 2004; DebRoy et al. 2006; De Buck et al. 2008). This approach was used in the present work to investigate whether extracellular (culture supernatant) proteins from *virB* mutants differ from those present in wild-type *B. abortus*. This could point to potential virulence factors secreted through the T4SS of *B. abortus*.

Materials and methods

Bacterial strains and culture conditions

Brucella abortus 2308 and its isogenic strains with non-polar mutations in *virB10* and *virB11* or with polar mutations in *virB1* and *virB10* (Sieira et al. 2000) were used. To minimize the contribution of exogenous proteins from the medium, bacteria were cultured in a semisynthetic medium consisting of RPMI (Gibco) supplemented with 3.78 mM $(NH_4)_2SO_4$, 6 μM $FeSO_4$, 0.1 mM $MgSO_4$, 6.32 mM $Na_2S_2O_3$, 2 mM L-glutamine, 1% yeast extract and 5% dialyzed tryptose-soy broth (solution outside the dialysis bag, devoid of proteins larger than 8 kDa).

Bacteria were initially grown on tryptic-soy agar and single colonies were transferred to the semisynthetic medium. Cultures were kept at 37°C with constant agitation (150 rpm), and growth was periodically monitored by reading the optical density at 600 nm.

Preparation of culture supernatants for analysis

Cultures harvested at the early stationary phase of growth were centrifuged at 15,000×*g* for 20 min, and supernatants were filter-sterilized and ultracentrifuged (100,000×*g*, 6 h) to eliminate outer membrane blebs. Supernatants were concentrated 1,000× by sequential freeze-drying and dialysis. To estimate the degree of autolysis in *Brucella* cultures, the activity of isocitrate dehydrogenase was measured in culture supernatants by a colorimetric method (Procedure 153-UV, Sigma, St. Louis, MO, USA).

Assessment of VirB9 expression

Previous studies have shown that VirB proteins are expressed in *B. abortus* in the stationary phase of growth in rich medium (tryptic-soy broth), and in the exponential phase of growth in minimal medium (Sieira et al. 2004). To check for the expression of VirB proteins, bacterial pellets harvested in the early stationary phase of growth in the semisynthetic medium, or in the stationary phase in rich medium (positive control) were lysed and analyzed by Western blot with an anti-VirB9 monoclonal antibody.

Infection of murine macrophages

To compare the virulence of *B. abortus* grown in rich medium (tryptic-soy broth) or in semisynthetic medium, these bacteria were used to infect the murine macrophagic cell line J774.A1. Cells were cultured in 24 wells plate in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum. After adding bacteria at a multiplicity of infection of 100 CFU/cell, the plates were incubated for 2 h at 37°C under 5% CO_2 atmosphere. Cells were extensively washed with RPMI to remove extracellular bacteria and incubated for 2, 4, 24 and 48 h in medium supplemented with 100 μg/ml gentamicin and 50 μg/ml streptomycin to kill extracellular bacteria. Samples were washed three times with RPMI before

processing. To monitor *Brucella* intracellular survival, infected cells were lysed with 0.1% (v/v) Triton X-100 in H_2O and serial dilutions of lysates were plated onto tryptic-soy agar plates to enumerate colony forming units (CFU).

MALDI-TOF analysis

Proteins present in concentrated culture supernatants from wild type *B. abortus* (WT) and its isogenic *virB10* polar mutant were separated by 2D-electrophoresis, and the differential spots were analyzed by MALDI-TOF. Proteins were isolated from samples following the protocol of Rafie-Kolpin et al. (1996) with modifications (Wagner et al. 2002). Samples containing 40 µg of proteins were mixed with an equal volume of 10% trichloroacetic acid, incubated for 5 min on ice, and centrifuged. After further washing with 5% trichloroacetic acid and acetone, the pellet was resuspended in 40 µl of sample buffer 1 and 4 µl of sample buffer 2 (Genomic Solutions). The mixture was incubated for 10 min on ice, after which 160 µl of loading buffer and 200 µl of rehydration buffer (Genomic Solutions) were added. IPG strips (18 cm long) of linear pH gradients (4–7 and 6–11) (Amersham Pharmacia Biotech) were rehydrated overnight the sample mixture. IEF was then performed at 20°C for 24 h. After IEF, IPG strips were washed with equilibration buffers and were loaded onto 10% precast Duracryl gels (Genomic Solutions). After electrophoresis (18–19 h at 4°C), the gels were fixed and stained with SYPRO Ruby.

Each gel was analyzed with the Investigator HT Analyzer program (Nonlinear Dynamics). Strong differences in spot intensity between the WT and the isogenic mutant (differential spots) were recorded. Protein spots (1.2-mm diameter) were excised from the SYPRO Ruby-stained 2D gels by a UV box-equipped ProPic robot (Genomic Solutions).

Digestion with trypsin was performed in accordance with the ProGest default long trypsin digestion protocol, modified to include treatment with *o*-methylisourea (Wagner et al. 2002). A 1-µl mixture of tryptic peptides and matrix (α-cyanohydroxycinnamic acid) was spotted onto the stainless steel 384-well Kratos MALDI target plate (Kratos Analytical). All spectra were obtained with the Kratos Axima-CFR running in reflectron mode. The spectra were collected and analyzed with the Kompact software package (Kratos Analytical). Peptide mass fingerprints were searched with the Mascot and Mascot Daemon software (Matrix Science) against all the ORFs of the *B. abortus* genome (Halling et al. 2005).

Recombinant proteins

The open reading frames of DnaK, SurA and CGH were cloned in the Pet17b vector (Novagen) after PCR amplification of *B. abortus* genomic DNA with specific primers. The recombinant plasmids were used to transform *E. coli* JM109 competent cells, and miniprep plasmid DNA was purified from overnight cultures. In each case, the plasmid DNA of a clone containing the insert was used to transform *E. coli* strain BL21(DE3) competent cells (Stratagene). After IPTG induction, recombinant DnaK was successfully expressed in the soluble fraction of *E. coli*, while SurA and CGH were expressed in inclusion bodies. The latter were solubilized in 50 mM Tris–8 M urea (pH 8.0) and refolded by dialysis. Proteins were purified by anion exchange chromatography.

Monoclonal antibodies

Hybridomas specific for DnaK, SurA and CGH were obtained by fusion of splenocytes of immunized mice and NSO myeloma cells (Galfré and Milstein 1981). Hybridomas were screened by indirect ELISA. Monoclonal antibodies were produced from ascites in BALB/c mice injected intraperitoneally with the selected hybridomas.

Western blot analysis of pellets and supernatants

Cultures of the wild type and the mutant strain were performed as described above and were centrifuged to separate the pellet from the culture medium. In addition to the *virB10* polar mutant, *B. abortus* strains carrying non-polar mutations in *virB10* or *virB11,* or a polar mutation in *virB1* were also assayed. The supernatants were concentrated 100× by sequential freeze-drying and dialysis, and the pellets (100 µg, wet weight) were lysed with SDS-PAGE sample buffer. Concentrated culture supernatants (50 µg per lane) and bacterial lysates were analyzed by Western blot with anti-DnaK, anti-CGH or anti-SurA monoclonal antibodies (mouse ascites diluted 1:100).

Results

Development of a semisynthetic culture medium

The exponential growth of *B. abortus* was not supported adequately by the EN + Y medium described by Plommet (1991). Therefore, a semisynthetic medium based on RPMI, inorganic salts, and low percentages of yeast extract and dialyzed TSB was developed. It supported the exponential growth of both WT *B. abortus* and the *virB10* mutant (Fig. 1a), although the duplication time for the WT strain was somewhat longer than in TSB (5 vs. 4 h). Cultures were harvested in the early stationary phase of growth and centrifuged. VirB9 expression at the time of harvest, used as a marker of expression of the *virB* operon, was demonstrated in WT *B. abortus* by Western blot

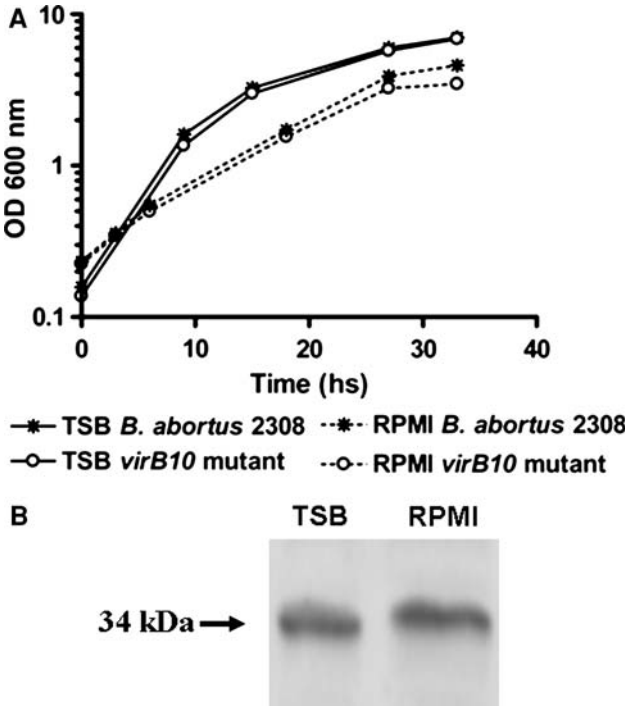

Fig. 1 **a** Growth of *Brucella abortus* (wild type and *virB10* mutant) in a semisynthetic medium (supplemented RPMI) as compared with a rich medium (tryptic-soy broth, TSB). **b** Western blot analysis of VirB9 expression by the wild-type strain at the time of harvest in both media, used as a marker of the expression of the *virB* operon

(Fig. 1b). To assess whether bacteria grown in the semisynthetic medium retain full virulence, the murine macrophagic cell line J774.A1 was infected with *B. abortus* grown in either TSB or the semisynthetic medium. No significant differences were observed between both infections regarding initial invasion ($9.0 \pm 1.0 \times 10^2$ vs. $9.5 \pm 2.5 \times 10^2$ CFU/well at 2 h p.i.) and intracellular replication ($1.25 \pm 0.15 \times 10^5$ vs. $1.41 \pm 0.18 \times 10^5$ CFU/well at 48 h p.i.).

Culture supernatants analysis by 2D-electrophoresis

Culture supernatants were ultracentrifuged, concentrated and subjected to two-dimensional (2D) gel electrophoresis. Proteome maps of WT and the *virB10* mutant grown under similar laboratory conditions were obtained by using IPG strips with pH range of 4.0–7.0 and 6–11 (Fig. 2).

Two-dimensional patterns were compared and differential spots were identified. Only stronger and consistent spots were selected for protein identification. There were 36 protein spots absent in supernatants of the *virB10* polar mutant, but present in the supernatant of WT. We focused on these differential proteins because they are likely to be released to the extracellular medium in a *virB*-related manner. These spots were subjected to MALDI-TOF MS peptide mass fingerprinting.

To ensure that these differences were not due to a differential lysis of the WT strain as compared to the mutant, eight spots having similar expression in both strains were also identified (spots A–H).

Identification of differential and conserved spots

Protein mass and MALDI-TOF data were enough to identify 19 of the differential spots. Mascot data regarding number of matched peaks and percentage of sequence coverage are shown in Table 1. Some spots corresponded to the same protein, with slight differences of pH or MW. Overall, 11 individual proteins were identified (Table 1). Three spots corresponded to the chaperone protein DnaK (Hsp70), and another three spots to aspartate aminotransferase. The remaining spots of interest were identified as polyribonucleotide nucleotidyltransferase, choloylglycine hydrolase (CGH), phosphoserine aminotransferase, 6-phosphoglucolactonase, alkyl hydroperoxide reductase C22 protein, dihydrolipoamide dehydrogenase, cytosol aminopeptidase (leucine aminopeptidase), a peptidyl-prolyl cis–trans isomerase homologous to the SurA protein from *E. coli,* and leucine-, isoleucine-, valine-, threonine-, alanine-binding protein (LIVTA-binding protein).

The spots having similar expression in both strains, used as controls of lysis, were identified as periplasmic ABC transporters of maltose/maltodextrin, D-galactose, D-ribose, and cystine, and an outer membrane protein homologous to TRAP transporters (Table 1).

Differential proteins were analyzed with SignalP 3.0 to detect putative signal peptides and with P-SORTb 2.0 to establish their probable subcellular localization. As shown in Table 1, signal peptides were detected in CGH (probable cleavage site between positions 36 and 37), SurA (positions 30 and 31) and LIVTA-binding protein (see below). Subcellular localizations as predicted by P-SORTb were "unknown" for the first two and periplasmic for the third.

The LIVTA-binding protein, as annotated in the genome of *B. abortus* strain 9-941, does not contain a signal peptide. However, its homolog in *B. melitensis* (AAL53311), which has a shorter sequence, contains a signal peptide with a probable cleavage site between positions 26 and 27. The comparative analysis of these sequences revealed that the putative signal peptide is 100% conserved, but the LIVTA-binding protein from *B. abortus* has been annotated starting from an alternative Met. This determines the addition of 55 amino acid residues as compared to the *B. melitensis* sequence, and makes the signal peptide undetectable by SignalP.

Several studies have pointed to the relevance of the C-terminal amino acid sequence, and specially the positive net charge of this segment, for the recruitment and secretion of proteins substrates by different T4SS (Christie

Table 1 Differential and conserved spots in *Brucella abortus* culture supernatants

Spot[a]	*p*I (gel)	MW (gel) (kDa)	Identification	Matched/ total peaks[b]	Sequence coverage (%)	Accesion number[c]	Signal peptide[d]
Differential spots							
1	5.16	92.9	PNPase[e]	4/4	6	AAX75438	NO
2, 3	4.89 –4.94	71.9–75.6	Chaperone protein DnaK	8/13	15	AAX75397	NO
4, 5	5.87	61.5	Cytosol aminopeptidase	8/21	13–21	AAX74083	NO
6	6.16	46.7	Phosphoserine aminotransferase	8/20	24	AAX74991	NO
7, 8	6.23–6.48	47.3–48.9	Aspartate aminotransferase	5/15	12	AAX74816	NO
9, 10, 11	5.58–6.01	39.8–40.7	Choloylglycine hydrolase	13/28	25–31	AAX74791	YES
12	5.11	26.2	Alkyl hydroperoxide reductase	3/5	16	AAX75941	NO
13	6.18	29.8	6-Phosphogluconolactonase	4/8	19	AAX75876	NO
14	9.30	29.1	Dihydrolipoamide dehydrogenase	6/11	19	AAX76104	NO
15	8.31	17.4	SurA	6/9	15	AAX74078	YES
16	9.05	9.6	LIVTA-binding protein[f]	9/18	28	AAX75476	YES
Conserved spots							
A, B	5.11–5.18	43.9–46.1	Sugar ABC transporter, periplasmic maltose-binding protein	8/17	16–45	AAX73637	YES
C	5.26	37.9	Sugar ABC transporter, periplasmic D-galactose-binding protein	17/31	47–67	AAX76301	YES
D	4.65	35.1	Sugar-binding transcriptional regulator, D-ribose-binding protein	9/26	26–28	AAX74938	YES
E, F	5.51–5.75	34,8–35.1	Outer membrane protein; TRAP transporter solute receptor	11/40	38–56	AAX74537	YES
G	4.74	29.8	Periplasmic D-ribose-binding protein	7/12	22–37	AAX75804	YES
H	8.19	30.0	FliY, periplasmic amino acid-binding protein	11/19	53–58	AAX75964	YES

[a] Spots identification is that used in Fig. 2

[b] For proteins with more than one spot, data for the best hit are shown

[c] Proteins from *B. abortus* strain 9-941 (Halling et al. 2005)

[d] Predicted by SignalP 3.0

[e] PNPase: polyribonucleotide nucleotidyltransferase

[f] LIVTA: leucine- isoleucine- valine- threonine- alanine- binding protein

2004). To assess the presence of positively charged amino acids arranged as (R/K/H)X_3(R/K/H) or (R/K/H)X_4(R/K/H) (Hohlfeld et al. 2006) the last 50 amino acid residues of the C-termini of the 11 proteins were aligned using ClustalW and visually inspected. As shown in Fig. 3, the above mentioned motifs were found in many differential proteins, including choloylglycine hydrolase, 6-phosphogluconolactonse, phosphoserine aminotransferase, dihydrolipoamide dehydrogenase, aspartate aminotransferase, alkyl hydroperoxide reductase, and cytosol aminopeptidase. Notably, the C-terminus of SurA includes three contiguous (R/K)X_5(R/K) motifs, the last two connected by a R–X–K motif.

Confirmation by Western blot of the virB-dependent release of some proteins

Among the 11 differential proteins identified, DnaK chaperone (Hsp70), CGH and SurA were chosen for further investigation because some of their homologs in other bacteria are extracellular and/or are involved in resistance to mammalian lytic mechanisms. Culture supernatants and cells from both WT *B. abortus* and the *virB10* polar mutant were harvested simultaneously and analyzed by Western blot with monoclonal antibodies against these proteins. As shown in Fig. 4a, reactivity bands corresponding to each of the three proteins were observed in the culture supernatant of WT *B. abortus* but not in the supernatant of the *virB10* polar mutant. Conversely, reactivity bands for each protein were observed in the pellet of the mutant but were weaker or absent in the pellet of the WT strain. To further confirm these results, the Western blot analysis was also performed on culture supernatants and pellets of *B. abortus* strains carrying non-polar mutations in *virB10* or *virB11* genes, and a strain carrying a polar mutation in *virB1*. As shown in Fig. 4b, the three proteins were detected in supernatants from the WT strain but not in those from these other mutants, confirming the result obtained with the *virB10* polar mutant.

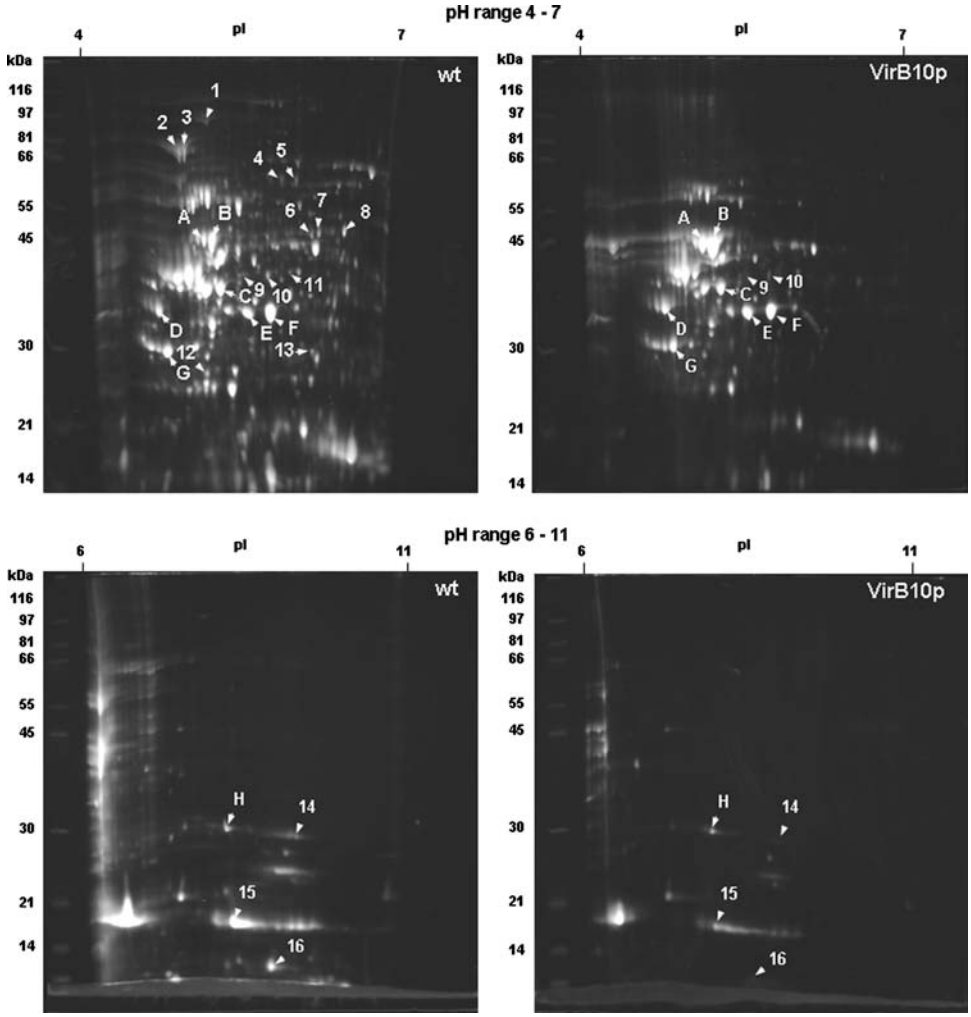

Fig. 2 Two-dimensional analysis of supernatants from *Brucella abortus* 2308 (wild type) and its isogenic *virB10* polar mutant using IPG strips covering *p*Is 4–7 and 6–11. Gels were stained with SYPRO Ruby and visualized under UV light. Differential spots (spots present in wild-type strain but absent in mutants) are identified by *numbers*. Conserved spots are identified by *letters*

```
Pgn    AEAEMLV-LHIEGAARQATLEK--ALAGDDERFMPVRAV-FHHARAPIQLYWAS--
Pho    VAYDIGAYRDAPSGLRIWAGAT---VEASDLEALTEWLN-WAFATQKAALKAAA--
LPD    GISELSA-AFAQAVEMGARLED---IAATIFAHPTLGEG-FAEASMKALGHALHV-
SurA   STRQVSD-DRVAQLVFSMEGAD--SPAGQEKKAEELSKK-YVQELREKATIVNR--
Asp    -VTELLE-TEGVAVVHGSAFGL--GPN-FRISYATSDEL-LEKACIRIQRFCASLR
Lbp    AKFPMVV-QEVSKKARIKADGT--EYGFLPFRTFTGDES-IDPVQESCSMKRPG--
Pnp    --TDVVR-EGQKVWVKLMGFDER-GKVRLSMRVVDQETG-KEIVAEKKKEEVDAE-
AhpC   DPDNVIQ-HVYATNLNVGRAPK---DTLRVLDALQTDEL-CPCNREVGGETLKAA-
Cyt    -LKRFVG-ETPWAHLDVAGTAMG-SPANEYNQSWASGFG-VRLLDRLVRDQFES--
CGH    -FADMKNDVYYIRTYDDQVLRS---FSFKDFDVDSKDILTIKFEPKLDAPSLKK--
DnaK   ---EVSM-KLGQAMYEAAQAAEGAGAEGGEQASSSRDDV-VDADYEEIDDNKKSS-
```

Fig. 3 Analysis of positively charged amino acid residues in the C-termini of differential proteins. Sequences spanning the last 50 residues of each protein were aligned with ClustalW and visually inspected for the presence of arginine (*R*), lysine (*K*) or histidine (*H*), which are shown shaded in *black*. The proteins analyzed were 6-phosphogluconolactonase (*Pgn*), phosphoserine aminotransferase (*Pho*), dihydrolipoamide dehydrogenase (*LPD*), *SurA*, aspartate aminotransferase (*Asp*), LIVTA-binding protein (*Lbp*), PNPase (*Pnp*), alkyl hydroperoxide reductase (*AhpC*), cytosol aminopeptidase (*Cyt*), cholylglycine hydrolase (*CGH*), and the chaperone protein DnaK

Discussion

While the *virB* operon has been implicated in *Brucella* survival inside phagocytic and non-phagocytic cells, the molecular mechanisms underlying such role of *virB* genes have not been clarified. It has been speculated that the T4SS encoded by *virB* genes mediates the extracellular secretion of factors that modulate the intracellular trafficking of *Brucella* (O'Callaghan et al. 1999; Comerci et al. 2001; Boschiroli et al. 2002). Different approaches have been used to attempt the identification of such factors. Marchesini et al. (2004) used a CAT reporter system to identify secreted and surface exposed proteins in *B. abortus*. The reduced secretion of some of the identified proteins

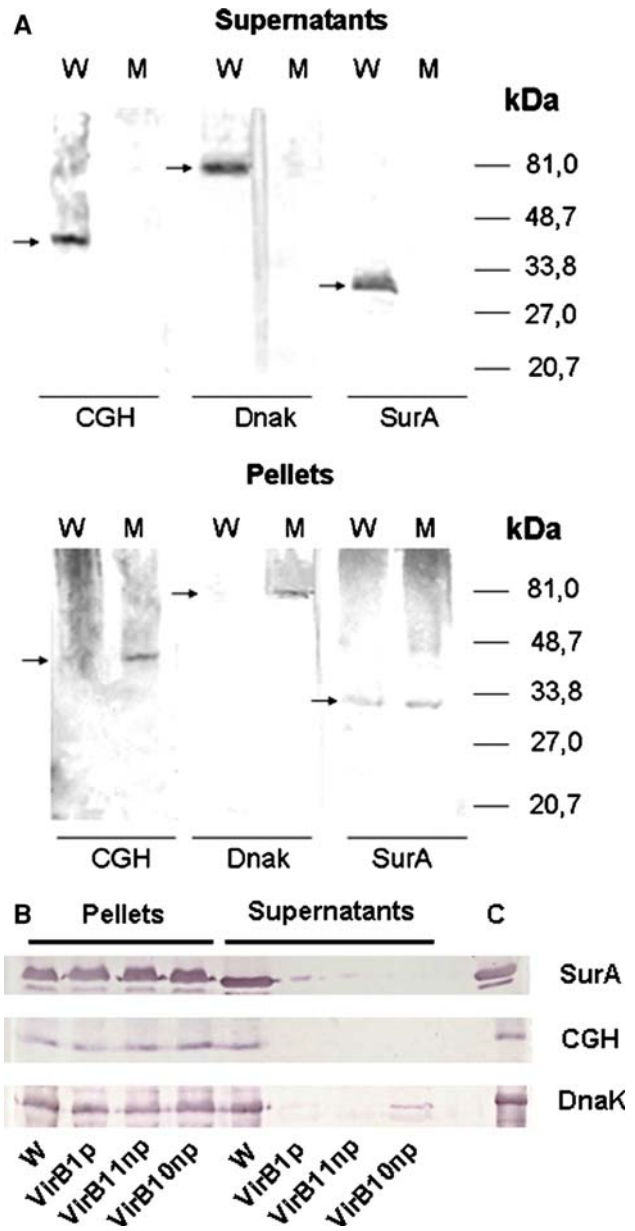

Fig. 4 **a** Western blot analysis of bacterial pellets and culture supernatants from wild-type *B. abortus* (*W*) and its isogenic *virB10* polar mutant (*M*) revealed with monoclonal antibodies to CGH, SurA and DnaK. **b** The same analysis applied to bacterial pellets and culture supernatants from *virB10* and *virB11* non-polar mutants, and a *virB1* polar mutant. Each purified recombinant protein was run in parallel as positive control (**c**)

in a *virB* mutant background suggested that they require a complete T4SS to be secreted. A different approach was used by de Jong et al. (2008). These authors identified a 18 bp box in the promoter region of the *virB* operon that is involved in the activation of this operon by the VjbR regulator. A genomic search allowed the identification of this box in the promoter regions of several *Brucella* genes, which encoded transcriptional regulators, transport and binding proteins, and proteins of hypothetical function, among others. Using a reporter system, two proteins of unknown function (that the authors named VceA and VceC) were found to be translocated into macrophages in a T4SS-dependent manner. While these results are highly valuable, this approach does not allow the identification of T4SS substrates that might be under the control of regulators other than VjbR. At variance with these previous investigations, in the present study, we used a proteomic approach to investigate whether extracellular proteins from a *virB10* mutant differ from those present in wild-type *B. abortus.*

Eleven proteins were detected in the culture supernatant of WT *B. abortus* that were absent in the supernatants of the *virB10* polar mutant. Since this mutant is unable to replicate within HeLa cells and has a reduced virulence in mice (Sieira et al. 2000), some of the differential proteins identified here might be involved in the reduced virulence of the *virB10* mutant. The differences in the extracellular proteome between the WT strain and the *virB* mutants may reflect the involvement of *virB* genes in the extracellular release and/or the regulation of the expression of some proteins identified in the present study.

For several reasons, the differential spots are unlikely to reflect a difference in lysis between WT and mutant strains. Cultures were harvested at the beginning of the stationary phase to minimize the risk of autolysis, and isocitrate dehydrogenase activity, commonly used to check for autolysis in culture supernatants (Andersen et al. 1991), was not detected in concentrated samples. Membrane stability of WT and the *virB* mutant is expected to be similar, since VirB proteins are not known to have a role in membrane structure. In a previous study in *Agrobacterium tumefaciens*, mutations in the different *virB* genes did not result in an increased release of an internal protein (used as autolysis control) as compared to the wild type strain (Lai et al. 2000). Therefore, even if a small degree of lysis took place in the present study, spots corresponding to lysis products should be equally present in supernatants from WT and *virB* mutant, and would not be considered differential spots. Moreover, even if VirB proteins have a role in membrane structure, a greater lysis would be expected for the defective mutant than for the WT strain, which would not explain the occurrence of additional (differential) spots in the supernatants of the WT strain.

In this study, filtered supernatants were extensively ultracentrifuged before 2D-electrophoresis to minimize the contribution of proteins released through membrane blebs (Gamazo et al. 1989). In addition, since we compared the extracellular proteome of the wild-type strain against that of the *virB* mutant, any proteins released by blebbing that might have remained after ultracentrifugation were not taken into account since we just focused on "differential

spots". In support of this approach, no differential spot corresponded to an outer membrane protein.

The differential expression of three proteins (DnaK, SurA and CGH) in culture supernatants was confirmed by Western blot with specific monoclonal antibodies, an approach also used in other studies on differential secretomes (De Buck et al. 2008). This differential expression was further confirmed when the assay was performed using strains carrying non-polar mutations in *virB10* or *virB11* genes, or a polar mutation in *virB1*. Importantly, the detection of DnaK, SurA and CGH in the pellets of the *virB* mutants indicates that the absence of these proteins in the culture supernatants of the mutants is not due to a reduced production of these molecules as compared to the WT strain.

Some of differential proteins identified have been described as extracellular in other bacteria, and some others have been linked to virulence or resistance to lytic mechanisms of the host. These proteins may be of special interest since *virB* genes have been linked to *Brucella* intracellular survival and also have been postulated to mediate the export of extracellular effectors.

One of the differential spots corresponded to the chaperone protein DnaK (Hsp70), which is essential for *Brucella* survival and replication within macrophages (Köhler et al. 2002). DnaK may constitute an exported effector per se or may function as a chaperone for effector proteins secreted through the T4SS. Similar to our findings, DnaK has been found in culture supernatants from *Helicobacter pylori,* which also expresses a T4SS (Cao et al. 1998).

A second differential spot corresponded to choloylglycine hydrolase (CGH, EC 3.5.1.24), which cleaves glycocholic acid to produce glycine and cholic acid. CGH has been well studied in bacteria such as *Listeria monocytogenes* (Dussurget et al. 2002) and *Lactobacillus* spp. (Lundeen and Savage 1990). It is presumed that intestinal bacteria produce CGH to reduce the detergent action of conjugated biliary acids on bacterial membranes (Hofmann and Eckmann 2006). We have recently shown that *Brucella* CGH is active against bile salts and contributes to the success of *Brucella* infection through the oral route in mice (Delpino et al. 2007a).

A third differential spot corresponded to a peptidyl-prolyl cis–trans isomerase (PPIase, EC 5.2.1.8) of the parvulin family, homologous to the SurA protein from *E. coli*. The *Brucella* homolog has a signal sequence, with a probable cleavage site between positions 30 and 31. SurA locates in the periplasm of *E. coli* and constitutes both a folding catalyst and a chaperone for outer membrane proteins (Mogensen and Otzen 2005). However, parvulins can also assist the folding of secreted proteins such as alpha-amylase from *Bacillus subtilis* (Vitikainen et al. 2004). Interestingly, SurA has been recently shown to be involved in the capacity of uropathogenic strains of *E. coli* to suppress epithelial cytokine responses (Hunstad et al. 2005) and to establish intracellular communities (Justice et al. 2006). We have recently shown that immunization with either SurA or DnaK from *Brucella* protects mice from challenge with *B. abortus* (Delpino et al. 2007b).

Another differential spot corresponded to dihydrolipoamide dehydrogenase (LPD, EC 1.8.1.4). LPD constitutes the E3 component of several enzymatic complexes, including pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. It has been shown in *M. tuberculosis* that LPD, together with dihydrolipoamide succinyltransferase (SucB), alkyl hydroperoxide reductase (AhpC), and the adaptor protein AhpD constitute a complex with peroxynitrite reductase and peroxidase activity (Bryk et al. 2002). Notably, AhpC was another differential spot found in *Brucella* supernatants in the present study. In *B. abortus*, the *ahpC* gene is differentially expressed during the stationary phase of growth in culture media, and it has been suggested that AhpC may serve as a detoxifier of H_2O_2 (Roop et al. 2003). Interestingly, a recent study suggests that the *Brucella* homolog of SucB, the third component of the enzymatic complex, is surface exposed or secreted in a *virB*-related manner (Marchesini et al. 2004).

The *virB*-related extracellular proteins identified in the current study were diverse regarding size and function, a situation that has been described for substrates of other T4SS. In the case of the Dot/Icm secretion system from *Legionella pneumophila*, the size of the best-characterized substrates ranges from the 398 amino acid residues of RalF to the 1,294 residues of LepB. Regarding function, Dot/Icm substrates includes guanine nucleotide exchange factors (RalF, LidA, SidM), glucosyltransferases (SetA, Lgt1) and an ubiquitin ligase (LegU2). Moreover, the Dot/Icm system may also mediate the translocation of heterologous T4SS substrates (Ensminger and Isberg 2009).

By using a reporter system, de Jong et al. (2008) showed that VceA and VceC translocate into macrophages by a T4SS-dependent process. The absence of these two proteins within our set of T4SS-related proteins may be due to the fact that VceA and VceC secretion may be stimulated by the phagocytosis of *Brucella* by macrophages but may not occur when the bacterium is cultured in the absence of eukaryotic cells.

We did not find an overlap between the *virB*-related proteins identified in the present study and those identified previously by Marchesini et al. (2004) using a reporter system. However, it must be noted that only two proteins identified in the later work showed a notably reduced secretion in *virB* mutants (hypothetical protein BRA0974 and the flagellar hook protein FlgE). For other four proteins, the reduction was partial or did not occur in all the mutants. We used a prediction tool (Compute *p*I/MW tool,

http://www.expasy.ch/tools/pi_tool.html) to obtain the theoretical *p*I and MW values of the *virB*-related proteins reported in that study, and checked such values against the experimental values of all our differential spots (with or without identification assigned); no matches were found. However, since experimental *p*I and MW values usually differ from theoretical ones, it cannot be ruled out that some differential spots that could not be identified by MALDI-TOF in our study corresponded to some of the *virB*-related proteins reported by Marchesini et al. In addition, some methodological differences may explain the differences in the set of *virB*-related proteins detected in each study. While we focused only on spots present in the wild type strain but absent in the mutant (proteins with minor differences in expression were not considered), most of the *virB*-related proteins identified by Marchesini et al. exhibited only a partial reduction in expression in the culture supernatants.

As mentioned, *Brucella virB* genes code a T4SS, which is postulated to mediate the export of extracellular effectors. The mechanism by which proteins from different bacteria target to the T4SS and cross the different layers of the bacterial outer membrane are just beginning to be unraveled. While translocation of the pertussis toxin is Sec-dependent, signal peptides have not been described for other T4SS substrates, including CagA from *H. pylori,* and Bep proteins from *Bartonella henselae*. In some cases, translocation of the inner membrane seems to be mediated by interaction of the T4SS substrates with a coupling protein (VirD4) associated to the T4SS apparatus (Atmakuri et al. 2003). However, a coupling protein has not been detected in *Brucella* yet, and the translocation mechanism of potential T4SS substrates remains unknown (Christie et al. 2005). Among the differential proteins identified in the present study, CGH, SurA and LIVTA-binding protein contain a signal sequence, while the other proteins do not. As mentioned above, the differential extracellular proteome here described may reflect the involvement of *virB* genes not only in the secretion but also in the regulation of the expression of some proteins. These later proteins may not be secretion substrates of the T4SS but may be instead released through other mechanisms. This may contribute to the diverse nature of the "differential proteins" identified regarding the presence of a signal peptide.

Several studies have pointed to the relevance of the C-terminal amino acid sequence, and specially the positive net charge of this segment, for the recruitment and secretion of proteins substrates by different T4SS. A consensus motif of R–X(7)–R–X–R–X–R–X–X(n) has been described for T4SS substrates in *Agrobacterium tumefaciens* (Vergunst et al. 2005). The C-termini of T4SS substrates from other bacteria do not contain this motif, but still contain one or more motifs with positively charged amino acids arranged as (R/K/H)X_3(R/K/H) or (R/K/H)X_4(R/K/H) (Christie 2004; Hohlfeld et al. 2006). The later motifs were found in most of the differential proteins identified in the present study.

In summary, the differences in the extracellular proteome between the WT strain and the *virB* mutants may reflect the involvement of *virB* genes in the extracellular release and/or the regulation of the expression of some proteins identified in the present study. A possible role of some of these proteins as secreted effectors or as auxiliaries of the secretion process mediated by T4SS should be considered and deserves further investigation.

Acknowledgments We are indebted to the staff of the Centro Nacional de Referencia del Sida, University of Buenos Aires, for their help with the use of the BSL3 facility. This work was supported by grants PICT99 05-06321, 05-14305 and 05-38237 from the Agencia Nacional de Promoción Científica y Tecnológica, Argentina, grant PIP 5212 from Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), and by a Small Grant from the International Society for Infectious Diseases (ISID). M.V.D. was supported by a fellowship from CONICET. C.A.F. and P.C.B. are members of the Research Career of CONICET. C.A.F. is also member of the Facultad de Ciencias Exactas, Universidad Nacional de La Plata.

References

Altman E, Segal G (2008) The response regulator CpxR directly regulates expression of several *Legionella pneumophila* icm/dot components as well as new translocated substrates. J Bacteriol 190:1985–1996

Andersen P, Askgaard D, Ljungqvist L, Bennedsen J, Heron I (1991) Proteins released from *Mycobacterium tuberculosis* during growth. Infect Immun 59:1905–1910

Atmakuri K, Ding Z, Christie PJ (2003) VirE2, a type IV secretion substrate, interacts with the VirD4 transfer protein at cell poles of *Agrobacterium tumefaciens*. Mol Microbiol 49:1699–1713

Boschiroli ML, Ouahrani-Bettache S, Foulongne V, Michaux-Charachon S, Bourg G, Allardet-Servent A, Cazevieille C, Liautard JP, Ramuz M, O'Callaghan D (2002) The *Brucella suis virB* operon is induced intracellularly in macrophages. Proc Natl Acad Sci USA 99:1544–1549

Bryk R, Lima CD, Erdjument-Bromage H, Tempst P, Nathan C (2002) Metabolic enzymes of mycobacteria linked to antioxidant defense by a thioredoxin-like protein. Science 295:1073–1077

Cao P, McClain MS, Forsyth MH, Cover TL (1998) Extracellular release of antigenic proteins by *Helicobacter pylori*. Infect Immun 66:2984–2986

Carle A, Höppner C, Aly KA, Yuan Q, den Dulk-Ras A, Vergunst A, O'Callaghan D, Baron C (2006) The *Brucella suis* type IV secretion system assembles in the cell envelope of the heterologous host *Agrobacterium tumefaciens* and increases IncQ plasmid pLS1 recipient competence. Infect Immun 74:108–117

Censini S, Lange C, Xiang Z, Crabtree JE, Ghiara P, Borodovsky M, Rappuoli R, Covacci A (1996) *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. Proc Natl Acad Sci USA 93:14648–14653

Christie PJ (2004) Type IV secretion: the Agrobacterium VirB/D4 and related conjugation systems. Biochim Biophys Acta 1694:219–234

Christie PJ, Atmakuri K, Krishnamoorthy V, Jakubowski S, Cascales E (2005) Biogenesis, architecture, and function of bacterial type IV secretion systems. Annu Rev Microbiol 59:451–485

Comerci D, Martínez-Lorenzo MJ, Sieira R, Gorvel JP, Ugalde R (2001) Essential role of the VirB machinery in the maturation of the *Brucella abortus*-containing vacuole. Cell Microbiol 3:159–168

Covacci A, Rappuoli R (1993) Pertussis toxin export requires accessory genes located downstream from the pertussis toxin operon. Mol Microbiol 8:429–434

De Buck E, Höper D, Lammertyn E, Hecker M, Anné J (2008) Differential 2-D protein gel electrophoresis analysis of *Legionella pneumophila* wild type and Tat secretion mutants. Int J Med Microbiol 298:449–461

de Jong MF, Sun YH, den Hartigh AB, van Dijl JM, Tsolis RM (2008) Identification of VceA and VceC, two members of the VjbR regulon that are translocated into macrophages by the Brucella type IV secretion system. Mol Microbiol 70:1378–1396

DebRoy S, Dao J, Söderberg M, Rossier O, Cianciotto NP (2006) *Legionella pneumophila* type II secretome reveals unique exoproteins and a chitinase that promotes bacterial persistence in the lung. Proc Natl Acad Sci USA 103:19146–19151

Delpino MV, Marchesini MI, Estein SM, Comerci DJ, Cassataro J, Fossati CA, Baldi PC (2007a) A bile salt hydrolase of *Brucella abortus* contributes to the establishment of a successful infection through the oral route in mice. Infect Immun 75:299–305

Delpino MV, Estein SM, Fossati CA, Baldi PC, Cassataro J (2007b) Vaccination with *Brucella* recombinant DnaK and SurA proteins induces protection against *Brucella abortus* infection in BALB/c mice. Vaccine 25:6721–6729

Dussurget O, Cabanes D, Dehoux P, Lecuit M, Buchrieser C, Glaser P, Cossart P, Consortium EuropeanListeriaGenome (2002) *Listeria monocytogenes* bile salt hydrolase is a PrfA-regulated virulence factor involved in the intestinal and hepatic phases of listeriosis. Mol Microbiol 45:1095–1106

Ensminger AW, Isberg RR (2009) *Legionella pneumophila* Dot/Icm translocated substrates: a sum of parts. Curr Opin Microbiol 12:67–73

Foulongne V, Bourg G, Cazevieille C, Michaux-Charachon S, O'Callaghan D (2000) Identification of *Brucella suis* genes affecting intracellular survival in an in vitro human macrophage infection model by signature-tagged transposon mutagenesis. Infect Immun 68:1297–1303

Galfré GA, Milstein C (1981) Preparation of monoclonal antibodies: strategies and procedures. Methods Enzymol 73:3–46

Gamazo C, Winter AJ, Moriyón I, Riezu-Boj JI (1989) Comparative analyses of proteins extracted by hot saline or released spontaneously into outer membrane blebs from field strains of *Brucella ovis* and *Brucella melitensis*. Infect Immun 57:1419–1426

Halling SM, Peterson-Burch BD, Bricker BJ, Zuerner RL, Qing Z, Li LL, Kapur V, Alt DP, Olsen SC (2005) Completion of the genome sequence of *Brucella abortus* and comparison to the highly similar genomes of *Brucella melitensis* and *Brucella suis*. J Bacteriol 187:2715–2726

Hofmann AF, Eckmann L (2006) How bile acids confer gut mucosal protection against bacteria. Proc Natl Acad Sci USA 103:4333–4334

Hohlfeld S, Pattis I, Püls J, Plano GV, Haas R, Fischer W (2006) A C-terminal translocation signal is necessary, but not sufficient for type IV secretion of the *Helicobacter pylori* CagA protein. Mol Microbiol 59:1624–1637

Hong PC, Tsolis RM, Ficht TA (2000) Identification of genes required for chronic persistence of *Brucella abortus* in mice. Infect Immun 68:4102–4107

Hunstad DA, Justice SS, Hung CS, Lauer SR, Hultgren SJ (2005) Suppression of bladder epithelial cytokine responses by uropathogenic *Escherichia coli*. Infect Immun 73:3999–4006

Justice SS, Lauer SR, Hultgren SJ, Hunstad DA (2006) Maturation of intracellular *Escherichia coli* communities requires SurA. Infect Immun 74:4793–4800

Kazemi-Pour N, Condemine G, Hugouvieux-Cotte-Pattat N (2004) The secretome of the plant pathogenic bacterium *Erwinia chrysanthemi*. Proteomics 4:3177–3186

Köhler S, Ekaza E, Paquet JY, Walravens K, Teyssier J, Godfroid J, Liautard JP (2002) Induction of dnaK through its native heat shock promoter is necessary for intramacrophagic replication of *Brucella suis*. Infect Immun 70:1631–1634

Kostakioti M, Newman CL, Thanassi DG, Stathopoulos C (2005) Mechanisms of protein export across the bacterial outer membrane. J Bacteriol 187:4306–4314

Lai EM, Chesnokova O, Banta LM, Kado CI (2000) Genetic and environmental factors affecting T-pilin export and T-pilus biogenesis in relation to flagellation of *Agrobacterium tumefaciens*. J Bacteriol 182:3705–3716

Lundeen SG, Savage DC (1990) Characterization and purification of bile salt hydrolase from Lactobacillus sp. strain 100–100. J Bacteriol 172:4171–4177

Marchesini MI, Ugalde JE, Czibener C, Comerci DJ, Ugalde RA (2004) N-terminal-capturing screening system for the isolation of *Brucella abortus* genes encoding surface exposed and secreted proteins. Microb Pathog 37:95–105

Mogensen JE, Otzen DE (2005) Interactions between folding factors and bacterial outer membrane proteins. Mol Microbiol 57:326–346

O'Callaghan D, Cazevieille C, Allardet-Servent A, Boschiroli ML, Bourg G, Foulongne V, Frutos P, Kulakov Y, Ramuz M (1999) A homologue of the *Agrobacterium tumefaciens* VirB and *Bordetella pertussis* Ptl type IV secretion systems is essential for intracellular survival of *Brucella suis*. Mol Microbiol 33:1210–1220

Pei J, Wu Q, Kahl-McDonagh M, Ficht TA (2008) Cytotoxicity in macrophages infected with rough *Brucella* mutants is type IV secretion system dependent. Infect Immun 76:30–37

Plommet M (1991) Minimal requirements for growth of *Brucella suis* and other *Brucella* species. Zentralbl Bakteriol 275:436–450

Rafie-Kolpin M, Essenberg RC, Wyckoff JH (1996) Identification and comparison of macrophage-induced proteins and proteins induced under various stress conditions in *Brucella abortus*. Infect Immun 64:5274–5283

Roop RM, Gee JM, Robertson GT, Richardson JM, Ng WL, Winkler ME (2003) *Brucella* stationary-phase gene expression and virulence. Annu Rev Microbiol 57:57–76

Rouot B, Alvarez-Martinez MT, Marius C, Menanteau P, Guilloteau L, Boigegrain RA, Zumbhill R, O'Callaghan D, Domke N, Baron C (2003) Production of type IV secretion system differs among *Brucella* species as revealed with VirB5- and VirB8-specific antisera. Infect Immun 71:1075–1082

Sieira R, Comerci DJ, Sanchez DO, Ugalde RA (2000) A homologue of an operon required for DNA transfer in *Agrobacterium* is required in *Brucella abortus* for virulence and intracellular multiplication. J Bacteriol 182:4849–4855

Sieira R, Comerci DJ, Pietrasanta LI, Ugalde RA (2004) Integration host factor is involved in transcriptional regulation of the *Brucella abortus* virB operon. Mol Microbiol 54:808–822

Vergunst AC, van Lier MC, den Dulk-Ras A, Stüve TA, Ouwehand A, Hooykaas PJ (2005) Positive charge is an important feature of the C-terminal transport signal of the VirB/D4-translocated proteins of *Agrobacterium*. Proc Natl Acad Sci USA 102:832–837

Vitikainen M, Lappalainen I, Seppala R, Antelmann H, Boer H, Taira S, Savilahti H, Hecker M, Vihinen M, Sarvas M, Kontinen VP (2004) Structure-function analysis of PrsA reveals roles for the

parvulin-like and flanking N- and C-terminal domains in protein folding and secretion in *Bacillus subtilis*. J Biol Chem 279:19302–19314

Vogel JP, Andrews HL, Wong SK, Isberg RR (1998) Conjugative transfer by the virulence system of *Legionella pneumophila*. Science 279:873–876

Wagner MA, Eschenbrenner M, Horn TA, Kraycer JA, Mujer CV, Hagius S, Elzer P, DelVecchio VG (2002) Global analysis of the *Brucella melitensis* proteome: identification of proteins expressed in laboratory-grown culture. Proteomics 2:1047–1060