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A two-component regulatory system playing a critical role in plant pathogens and endosymbionts is present in *Brucella abortus* and controls cell invasion and virulence

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Summary

Two mutants showing increased sensitivity to polycations and surfactants were obtained by transposon mutagenesis of virulent *Brucella abortus* 2308 Nal^r. These mutants showed no obvious *in vitro* growth defects and produced smooth-type lipopolysaccharides. However, they hardly multiplied or persisted in mouse spleens, displayed reduced invasiveness in macrophages and HeLa cells, lost the ability to inhibit lysosome fusion and were unable to replicate intracellularly. Subsequent DNA analyses identified a two-component regulatory system [*Brucella* virulence related (Bvr)] with a regulatory (BvrR) and sensory (BvrS) protein. Cloning of *bvrR* in the BvrR-deficient mutant restored the resistance to polycations and, in part, the invasiveness and the ability to multiply intracellularly. BvrR and BvrS were highly similar (87–89% and 70–80% respectively) to the regulatory and sensory proteins of the chromosomally encoded *Rhizobium meliloti* ChvI–ExoS and *Agrobacterium tumefaciens* ChvI–ChvG systems previously shown to be critical for endosymbiosis and pathogenicity in plants. Divergence among the three sensory proteins was located mostly within a periplasmic domain probably involved in stimulus sensing. As *B. abortus*, *R. meliloti* and *A. tumefaciens* are phylogenetically related, these observations suggest that these systems have a common ancestor that has evolved to sense stimuli in plant and animal microbial environments.

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Introduction

Brucellosis is one of the five most common bacterial zoonosis in the world and an important cause of human suffering and economical losses. This disease is caused by the species of the genus *Brucella*, a homogeneous group of Gram-negative bacteria that are able to multiply within professional and non-professional phagocytes (Corbel and Brinley-Morgan, 1984; Riley and Robertson, 1984; Anderson and Cheville, 1986; Detilleux *et al*., 1990a,b). Sequence analysis of the 16S RNA gene places *Brucella* in the α-2 subdivision of the proteobacteria along with bacteria that are associated pericellularly or intracellularly with animals and plants either as pathogens or as endosymbionts. Accordingly, it has been hypothesized that intracellular association with eukaryotic cells is an evolutionary trend in the α-2 subdivision (Moreno *et al*., 1990; Rasool *et al*., 1992), but up to now no homologous structural, physiological or genetic features have been shown to be critical for the invasiveness and intracellular behaviour.

Despite intense research, the mechanisms allowing *Brucella* to behave as an intracellular parasite are not defined. In contrast to other intracellular pathogens (Finlay and Falkow, 1997), *Brucella* species do not produce exotoxins, antiphagocytic capsules or thick cell walls, resistance forms or fimbriae and do not display antigenic variation. In addition, plasmids or lysogenic phages that could transfer virulence genes have not been found despite extensive search (for review see Moreno, 1997). Although *B. abortus* has been observed to invade cells, to replicate within intracellular compartments (Detilleux *et al*., 1990a,b) and to modulate protein expression during intracellular growth (Rafie-Kolpin *et al*., 1996), the mechanisms behind these activities have not been resolved.

Attempts to identify *Brucella* genes critical for virulence have been made. For many years it was believed that genes necessary for the use of host erythritol were

necessary for *B. abortus* virulence (Pearce *et al*., 1962), but mutants unable to use it remain virulent (Meyer, 1990; Sangari *et al*., 1998). Likewise, mutants not expressing stress proteins such as RecA, HtrA or SodC show reduced multiplication during the first days of infection, but they finally establish chronic infections and, accordingly, they are virulent (Tatum *et al*., 1992; 1993; Phillips *et al*., 1995). Finally, mutants defective in urease or catalase also remain virulent (Grilló, 1997).

Up to now, the only known defect rendering *B. abortus* avirulent has been the loss of the O-chain of the lipopolysaccharide (LPS) molecule occurring when the bacterium mutates from smooth to rough phenotype. Even although the properties of some rough mutants have been partially studied (Kreutzer *et al*., 1979; Riley and Robertson, 1984; Detilleux *et al*., 1990a,b; Schurig *et al*., 1991; Stevens *et al*., 1994; Martínez de Tejada *et al*., 1995; Freer *et al*., 1996), there is no published information on their specific genetic defects. Moreover, there are both naturally occurring rough *Brucella* species that are fully pathogenic and *B. abortus* smooth strains that display reduced virulence (Corbel and Brinley-Morgan, 1984), implying that the O-chain of LPS is not the only factor necessary for virulence (Rasool *et al*., 1992).

Based on structural and biological assays, we have proposed (Rasool *et al*., 1992; Martínez de Tejada *et al*., 1995; Freer *et al*., 1996) that the virulence of *B. abortus* depends in part on a set of outer membrane (OM) properties seldom present simultaneously in other Gram-negative bacteria. First, the *B. abortus* OM is markedly resistant to bactericidal cationic peptides present in lysosomes and body fluids (lysozyme, lactoferrin and lactoferricin B, defensin NP-2, CAP18 and bactenecin) and to other bactericidal polycations, such as polymyxin B, magainins, melittin, poly-L-lysine and poly-L-ornithine (Martínez de Tejada *et al*., 1995; Freer *et al*., 1996), that disrupt the OM of most Gram-negative bacteria (for review see Vaara, 1992; Nicolas and Mor, 1995). Second, *B. abortus* OM is permeable to hydrophobic agents (Martínez de Tejada and Moriyón, 1993; Martínez de Tejada *et al*., 1995; Freer *et al*., 1996) and resistant to the OM disturbing action of divalent cation chelators (Moriyón and Berman, 1982). Finally, in contrast to other LPS, *Brucella* LPS is a poor activator of the bactericidal mechanisms in phagocytes (Rasool *et al*., 1992).

To gain insight into the genetics of *Brucella* virulence, which according to our hypothesis may be related to the distinctive properties of its OM, we generated *B. abortus* mutants sensitive for the cationic antibiotic polymyxin B. This approach led to the identification of a two-component regulatory system critical for the control of polycation resistance, virulence, cell invasion and intracellular replication. This system shows only low similarity with the systems described in animal pathogens, including those implicated in polycation resistance and virulence in *Salmonella*, a characteristic intracellular pathogen (Fields *et al*., 1989; Roland *et al*., 1993). On the other hand, the *B. abortus* system is highly similar to a system previously shown to play a critical role in the behaviour of some α-2 subdivision plant pathogens and endosymbionts.

Results

Isolation and characterization of polymyxin B-sensitive mutants of B. abortus *2308 Nal^r*

By mating *B. abortus* 2308 Nal^r with *Escherichia coli* SM10 (λ pir) [pUT/kanamycin (Km)] (see Table 1 for bacterial strains and plasmids), 5064 Tn*5* insertion mutants were obtained from which 16 were counter-selected by screening for viability loss after a controlled exposure to polymyxin B. As it is well known that rough *B. abortus* is avirulent (Corbel and Brinley-Morgan, 1984; Alton *et al*., 1988) and that the loss of the O-chain partially reduces the resistance to polymyxin B (Martínez de Tejada *et al*., 1995; Freer *et al*., 1996), rough mutants were screened out by the absence of co-agglutination with IgG to *B. abortus* smooth LPS and a positive acriflavine agglutination. Using these tests, only five mutants were smooth, and further screening for maximal polymyxin B sensitivity identified two mutants (designated 2.13 and 65.21) with an eightfold sensitivity increase (minimal inhibitory concentrations of 16 μg ml^{-1} for the parental strain versus 2 μg ml^{-1} for both mutants). The increase in sensitivity was not restricted to polymyxin B as it was also observed for melittin and poly-L-lysine in survival assays (Fig. 1). These last assays also revealed that the sensitivity of 2.13 to melittin and polymyxin B appears to be qualitatively different from that of 65.21: for polymyxin B, it appears to be two-phase, with a more sensitive region starting before 65.21; and for melittin, inhibition appears to plateau at about 50% for 65.21 but to be complete for 2.13 (Fig. 1). In addition, minimal inhibitory concentration assays showed that both mutants were two times more sensitive to surfactants [*N*-lauroyl-*N*-methylglycine (Sarkosyl), sodium dodecyl sulphate (SDS) and sodium deoxycholate] than the parental strain suggesting altered OM properties. However, SDS–PAGE of LPS extracts using *B. abortus* 2308 Nal^r and *B. abortus* RB51 LPS as smooth and rough references, respectively, confirmed the presence of a smooth-type LPS in 2.13 and 65.21, and densitometric analyses (not shown) demonstrated that there were no quantitative differences in the LPS profiles of the two mutants and 2308 Nal^r. The mutants showed no obvious defects with regard to the ability to grow on standard media.

*Cloning of the genetic determinants affected by Tn*5 *insertion*

When the corresponding *Eco*RI chromosomal DNA digests

Table 1. Bacterial strains and plasmids.

Strain/plasmid	Characteristics	Source/reference
Brucella abortus		
2308 Nal^r	Wild-type, virulent, biotype 1, smooth-LPS, Nal^r spontaneous mutant of strain 2308	Sangari and Agüero (1991)
2.13	2308 Nal^r *bvrS*::Tn*5*, smooth LPS	This work
65.21	2308 Nal^r *bvrR*::Tn*5*, smooth LPS	This work
65.21-*bvr*R	65.21 harbouring the plasmid pBBR2.13	This work
RB51	Rough mutant derived from strain 2308	Schurig *et al*. (1991)
Escherichia coli		
SM10 (λ pir)	*recA*::RP4–2-Tet::Mu, Km^r, *thi leu thr supE* λ pir	Simons *et al*. (1983)
CC118 (λ pir)	Δ(*ara-leu*) *araD* Δ*lac*X74 *galE galK phoA*20 *thi*-1 *rpsE rpoB argE*(Am) *recA*1 λ pir	Herrero *et al*. (1990)
XL1-Blue	Tet^r, *recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 *lac* [F′ *proAB lacI*^{q}ZΔM15 Tn*10* (Tet^r)]	Stratagene
Plasmids		
pUT/Km	Amp^r, Tn*5*-based delivery plasmid with Km^r	Herrero *et al*. (1990)
pBluescript-II KS+	Cloning vector, Col E1 origen, Amp^r	Stratagene
pBBR1MCS-4	Amp^r, broad host range cloning plasmid	Kovach *et al*. (1994)
pBPS2.13	6.0 kb *Eco*RI fragment carrying *bvrR-bvrS* with Tn*5* insertion in *bvrS* cloned into pBluescript-II KS+	This work
pBPS2.13p	8.0 kb *Eco*RI fragment (partial digestion) carrying *bvrR–bvrS* with Tn*5* insertion in *bvrS* cloned into pBluescript-II KS+	This work
pBBR2.13	6.0 kb *Eco*RI fragment carrying *bvrR-bvrS* with Tn*5* insertion in *bvrS* cloned into pBBR1MCS-4	This work
pBPS65.21	As pBPS2.13 with the Tn*5* insertion in *bvrR*	This work

were probed with the labelled internal *Not*I fragment of Tn*5*, it was observed that 2.13 and 65.21 carried a single Tn*5* insertion located in a similar 6 kb chromosomal *Eco*RI fragment (not shown). Thus, the DNAs from 2.13 and 65.21 were digested with *Eco*RI, cloned using the pBluescript-II KS+, and the kanamycin-resistant characteristic of the transposon was used to select recombinants containing Tn*5* and the flanking DNA. The resulting recombinant plasmids were designated pBPS2.13 and pBPS65.21. In addition, DNA from 2.13 was partially digested with *Eco*RI and cloned into pBluescript-II KS+. The resulting recombinant plasmid was designated pBPS2.13p. A restriction map of the DNA inserts in pBPS2.13, pBPS2.13p and pBPS65.21 is shown in Fig. 2. Using Southern hybridization with the appropriate probes, it was observed that plasmids pBPS2.13 and pBPS65.21 carried the same 6 kb *Eco*RI fragment but with the transposon inserted into two different positions 1.8 kb apart (Fig. 2).

The mutated region corresponds to a two-component regulatory system

The DNA sequences for both strands of the ends of the inserts from plasmids pBPS65.21 and pBPS2.13 were determined and complemented with partial sequences of the two strands of the pBPS2.13p insert (see Fig. 2 for details). The whole sequence (not shown, GenBank accession numbers AF005157 and AF012539) contained two open reading frames (ORFs), which were designated *bvrR* and *bvrS* (for *Brucella* virulence related, see below). It also contained two potential promoter −10 and −35 sequences located 50 bp upstream *bvrR* ORF, and a potential ribosome-binding site centred 9 bp upstream of the initiation codon. The *bvrR* ORF 27 kDa encoded a 237-amino-acid protein, and the *bvrS* ORF 66.7 kDa encoded a 601-amino-acid protein. The insertion sites of the mini-Tn*5* within the *bvrR* and the *bvrS* were the amino acid positions 69 and 380 respectively. The G+C content of the whole sequence was similar to that observed for *B. abortus* chromosomal DNA (58%) (Hoyer and McCullough, 1968).

A database search of the deduced amino acid sequence for the BvrR and BvrS proteins revealed a high level of identity with a two-component regulatory system of *Rhizobium (Sinorhizobium) meliloti* (ChvI-ExoS; Osteras *et al*., 1995; Cheng and Walker, 1998) and *A. tumefaciens* (ChvI-ChvG; Charles and Nester, 1993; Mantis and Winans, 1993). BvrR had 85% identity (89% similarity) and 83% identity (87% similarity) to the *R. meliloti* and *A. tumefaciens* ChvI respectively. BvrS had 60% identity (80% similarity) to *R. meliloti* ExoS and 57% identity (70% similarity) to *A. tumefaciens* ChvG.

BvrR showed significant similarity to response regulators proteins (Stock *et al*., 1989) and, accordingly, its N-terminal domain had highly conserved aspartic (positions 14, 15 and 58) and lysine (position 107) residues. Based on sequence similarities found at the C-terminus, BvrR would belong to the OmpR subfamily of response regulators

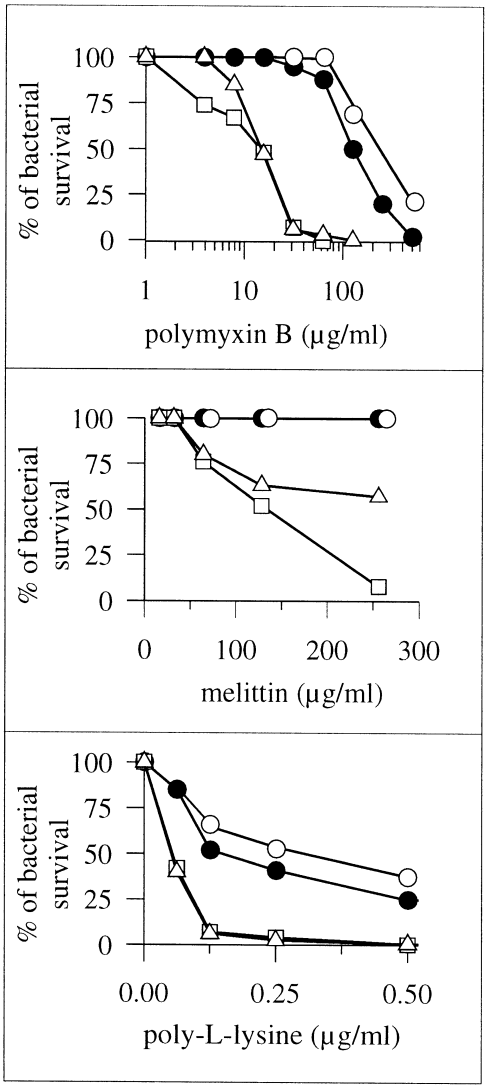


Fig. 1. Effect of polycations on the viability of *B. abortus* 2308 Nal^r (●), 2.13 (□), 65.21 (△) and 65.21-*bvrR* (○). Bacteria were exposed to increasing amounts of polycations for 60 min at 37°C and the viability then assessed by plating on tryptic soy agar plates. Each point is the average of three independent determinations each made in triplicate. The standard error representations are within the size of the symbols.

(Mizuno and Tanaka, 1997), which includes FeuP of *B. melitensis* and PhoP and PmrA of *S. typhimurium*.

BvrS appeared homologous to sensor proteins of the histidine protein kinase family, with four highly conserved regions in the C-terminus termed boxes H, N, D/F and G (Stock *et al*., 1995). Box H (amino acid positions 368–385) included a conserved histidine residue at position 374, which would be the presumed site of autophosphorylation, and box N (positions 479–500) had a conserved asparagine residue at position 496. The characteristic glycine-rich boxes D/F and G extended from positions 519–546 and 555–574 respectively. The Kyte–Doolittle hydrophobicity plot (Kyte and Doolittle, 1982; http://expasy.hcuge.ch/cgi-bin/protscale.pl) of BvrS (not shown) revealed two main hydrophobic regions, and additional analyses (http://ulrec3.unil.ch/software/TMPRED_form.html) predicted two transmembrane domains corresponding to amino acid positions 49–67 and 291–311. These hydrophobic regions delimited a hydrophilic region predicted to contain the periplasmic domain between positions 68 and 290. More detailed comparison of BvrS with ChvG revealed that most of the divergence between the two proteins was concentrated in this periplasmic domain and between positions 153–247 (11% identity and 36% similarity). Both this localized divergence and the presence of 111 amino acid residues in the C-terminus of ChvG lessened the overall similarity between BvrS and ChvG. For BvrS and ExoS, however, the identity and similarity of the same periplasmic region were 47% and 73%, respectively, and both proteins were close in size.

The sequence (not shown) of the 440 bp region starting from the *Eco*RI site upstream of *bvrR* in pBPS65.21 (Fig. 2) showed a 77% identity to the *pckA* gene of *Rhizobium* sp. strain NGR234, which encodes a phosphoenolpyruvate carboxykinase (Osteras *et al*., 1991).

The mutants are avirulent in mice

The recovery times 50 (RT_{50}, time in weeks at which half of the subcutaneously inoculated mice recover from spleen infection) of 2.13 and 65.21 were similar ($P = 0.99$), but both were significantly ($P < 0.0001$) shorter than that of the virulent 2308 Nal^r parental strain (Fig. 3A). Moreover, whereas 2308 Nal^r quickly grew in spleens and reached high numbers that persisted for at least 36 days, both mutants hardly multiplied, and even at the highest point of the multiplication curve the spleens contained significantly lower ($P < 0.0001$) numbers of bacteria than the virulent 2308 Nal^r parental strain (Fig. 3B). Consistent with its slightly lower polycation susceptibility, 65.21 was recovered from spleens in slightly higher numbers than 2.13 during the first 6 days of infection. Both mutants were cleared out by day 12 post inoculation.

The maintenance of the phenotypic and genetic markers was checked throughout these experiments. All the 2.13 and 65.21 isolates showed the same polymyxin B sensitivity, Tn*5* location and smooth LPS as the inoculum, indicating that both were stable and that no smooth to rough dissociation had taken place during infection.

The mutants show very limited invasiveness and fail to replicate in cells

To study the invasiveness and ability to replicate intracellularly as separate phenomena, the behaviour of parental and mutant strains in professional and non-professional

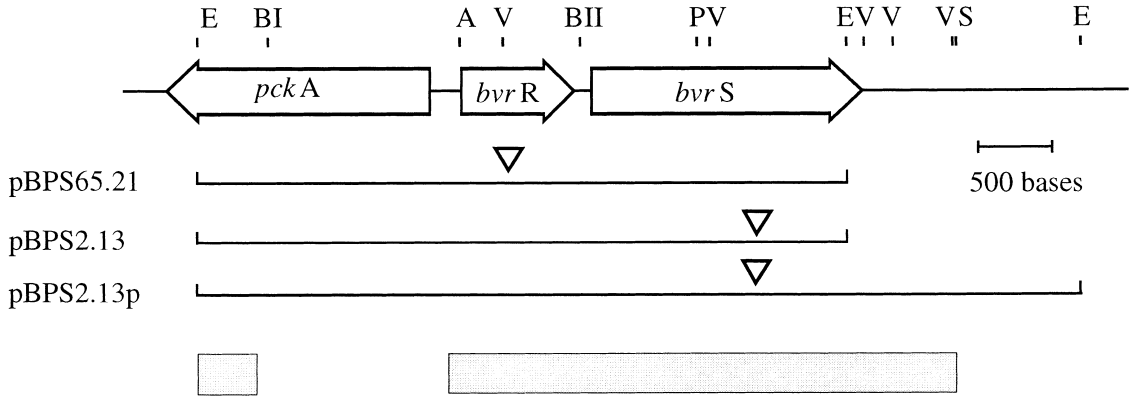


Fig. 2. Restriction map of the region encoding the two-component regulatory system *bvrR–bvrS* in *B. abortus*. The location of the Tn*5* insertions in the mutants are denoted by open triangles. The inserts in plasmids pBPS65.21, pBPS2.13 and pBPS2.13p are shown below. The location of *bvrR* and *bvrS* and the ORF with high identity to *pckA* are indicated by open regions on the restriction map. The arrows indicate the predicted direction of transcription. The fragments that were sequenced are shadowed. Restriction site abbreviations: A, *Ase*I; BI, *Bgl*I; BII, *Bgl*II; E, *Eco*RI; V, *Eco*RV; P, *Pvu*II; S, *Sac*II.

phagocytes was examined. Although with different invasion rates and replication kinetics that relate to the inherent functions of these cells, the virulent strain replicated within mouse peritoneal macrophages and HeLa cells (Fig. 4). In contrast, none of the mutants multiplied in either type of cell (Fig. 4). As this deficient replication could be due to a failure in bacterial adherence, to a low penetration rate or to a loss of the ability for intracellular survival, the distribution

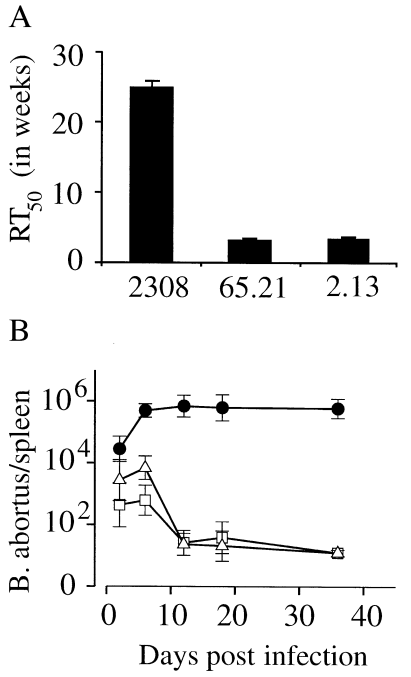


Fig. 3. Virulence assays of *B. abortus* 2.13 and 65.21 in mice.
A. Recovery times 50 (RT_{50}, time in weeks at which half of the subcutaneously inoculated mice recover from spleen infection) were measured by detecting bacterial CFUs per murine spleen at regular time intervals after inoculation. *P*-values of both mutants versus the virulent 2308 Nal^r strain were less than 0.0001 for all points.
B. Time-course of bacteria replication in spleens of mice infected with *B. abortus* 2308 Nal^r (●) and with mutants 2.13 (□) and 65.21 (△). Values are the mean CFU/spleen ± SD (when not shown, SD values are included in the size of the symbol).

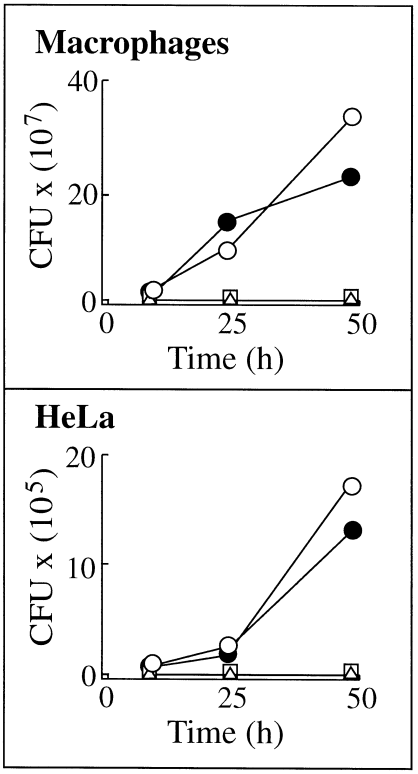


Fig. 4. Intracellular replication of *B. abortus* 2308 Nal^r (●), 2.13 (△), 65.21 (□) and 65.21-*bvrR* (○) in murine macrophages or HeLa cells. Each point is the average of three independent determinations made in triplicate. In all cases, the standard error for each point was less than 5%.

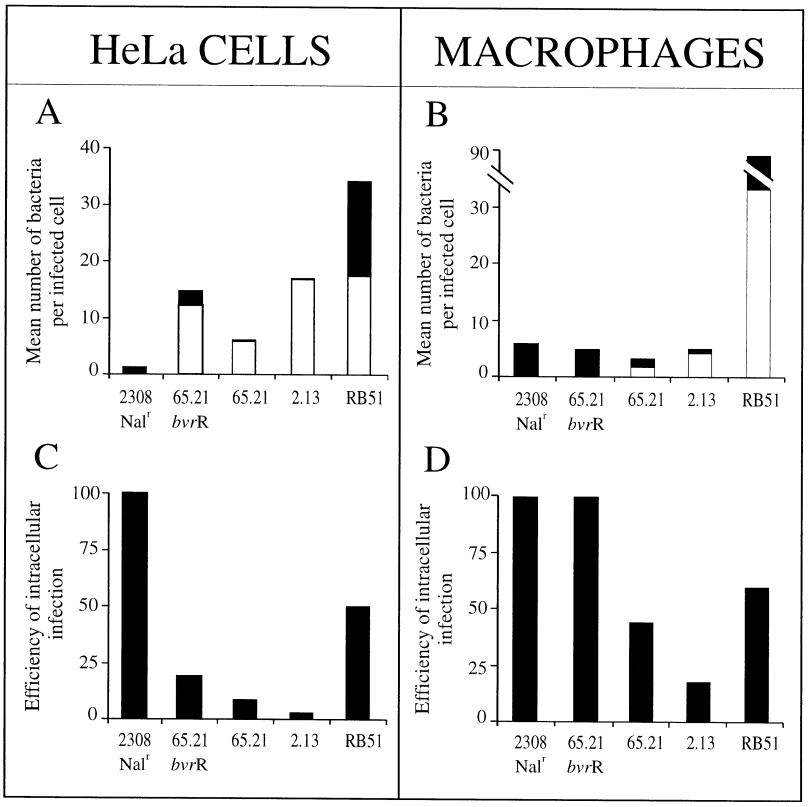


Fig. 5. Numbers of extracellular and intracellular *B. abortus* per infected HeLa cells and macrophages and efficiency of intracellular infection. After infection with smooth virulent (2308 Nal^r), mutated (2.13 and 65.21), transgenic (65.21-*bvrR*) or rough avirulent (RB51) *B. abortus*, cells were washed and the number of extracellular (white bars) and intracellular (black bars) bacteria per host cell were recorded by confocal laser scanning microscopy (A and B). The corresponding efficiencies of intracellular infection [(intracellular bacteria per infected cell/extracellular + intracellular bacteria per infected cell) × 100] are presented in C and D. Each value is the average of three independent determinations each made in triplicate. The standard error for each bar was in all cases less than 10%.

of bacteria was analysed using a double immunofluorescence assay (Figs 5 and 6). Whereas the virulent parental 2308 Nal^r strain was seldom found extracellularly on infected cells, a relatively large number of 65.21 and 2.13 bacteria were found on the cell surface (mean number of 6.5 and 18 bacteria per HeLa and of three and five bacteria per macrophage; Figs 5A and B, and 6). Conversely, the virulent strain was in most cases intracellular and the mutants were rarely intracellular, in particular in non-professional phagocytes (mean number of 0.5 and 0.4 bacteria per HeLa cell and of 1.6 and 1 bacteria per macrophage; Fig. 5A and B). Thus, when the results were analysed in terms of efficiency of intracellular invasion, the invasiveness of 65.21 and 2.13 was much lower than that of 2308 Nal^r (Fig. 5C and D). Significantly, 2.13 and 65.21 also behaved differently from the virulence-attenuated RB51 rough strain. As reported before (Detilleux *et al*., 1990a), RB51 also showed a high adherence to the cell surface, of macrophages in particular (Fig. 5A and B), but was more efficient than either mutant for intracellular invasion (Fig. 5C and D).

The fate of the few 65.21 and 2.13 bacteria that penetrated into cells was examined by analysing the distribution of bacteria and bacterial debris by double immunofluorescence. Phagosomes containing the parental virulent strain did not collocalize with compartments containing the lysosome marker cathepsin D (results in HeLa cells after 1 h of infection are shown in Fig. 6A, and after 24 h of infection are shown in Fig. 7A–C). In contrast, phagosomes containing mutant bacteria collocalized with cathepsin D 1 h after infection (Fig. 6B), and bacterial fragments were subsequently detected (Fig. 7D–F). Immunofluorescence experiments performed with macrophages gave similar results (not shown). In spite of the relatively high numbers of intracellular rough RB51, once within cells these bacteria were destroyed within lysosomes in a similar fashion to the 65.21 and 2.13 mutants (not shown). All these data show that both 65.21 and 2.13, once inside the cells, could not replicate and were targeted to lysosomes for degradation.

Expression of bvrR *in* bvrR*-deficient 65.21 mutant partially rescues the virulent behaviour*

The transgenic *B. abortus* 65.21-*bvrR* strain harbouring a plasmid construct that allows the expression of *bvrR* (see Table 1) manifested the same minimal inhibitory concentration (16 μg ml^{-1}) and resistance to polymyxin B and the same resistance to melittin and poly-L-lysine (Fig. 1) as the virulent strain 2308 Nal^r. Similarly, 65.21-*bvrR* recovered the ability to replicate within professional and non-professional phagocytes to levels close to those of 2308 Nal^r (Fig. 4). Moreover, quantitative analyses in macrophages showed almost no extracellular 65.21-*bvrR* bacteria, and both the mean number of intracellular versus extracellular

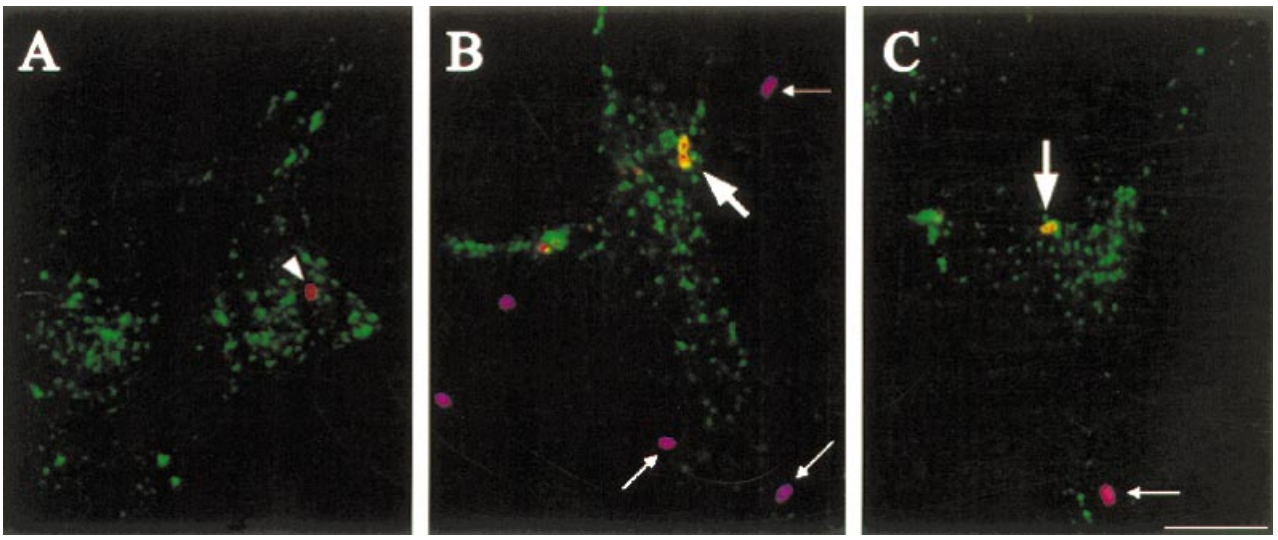


Fig. 6. Analysis by confocal immunofluorescence microscopy of the extracellular and intracellular distribution of *B. abortus* 2308 Nal^r (A), 65.21 (B) and 65.21-*bvrR* (C) in HeLa cells 1 h after infection. Extracellular bacteria (shown in purple, pointed by small arrows) were detected using mouse monoclonal antibodies against *B. abortus* LPS revealed by cyanin-5 conjugate. Intracellular bacteria (shown in red, pointed by arrowheads) were detected, after permeabilization of the cells, using cow anti-*Brucella* antibodies revealed by Texas red conjugate. The presence of cathepsin D-positive compartments (shown in green) was detected with rabbit anti-cathepsin D antibodies revealed by FITC conjugate. Collocalization of intracellular bacteria with cathepsin D is shown in yellow and a large arrow. Purple colour assigned to extracellular bacteria was obtained by computer manipulation of the original colour. Bar = 10 μm. Notice the high number of extracellular *B. abortus* 65.21 in comparison with 2308 Nal^r and 65.21-*bvrR* brucellae.

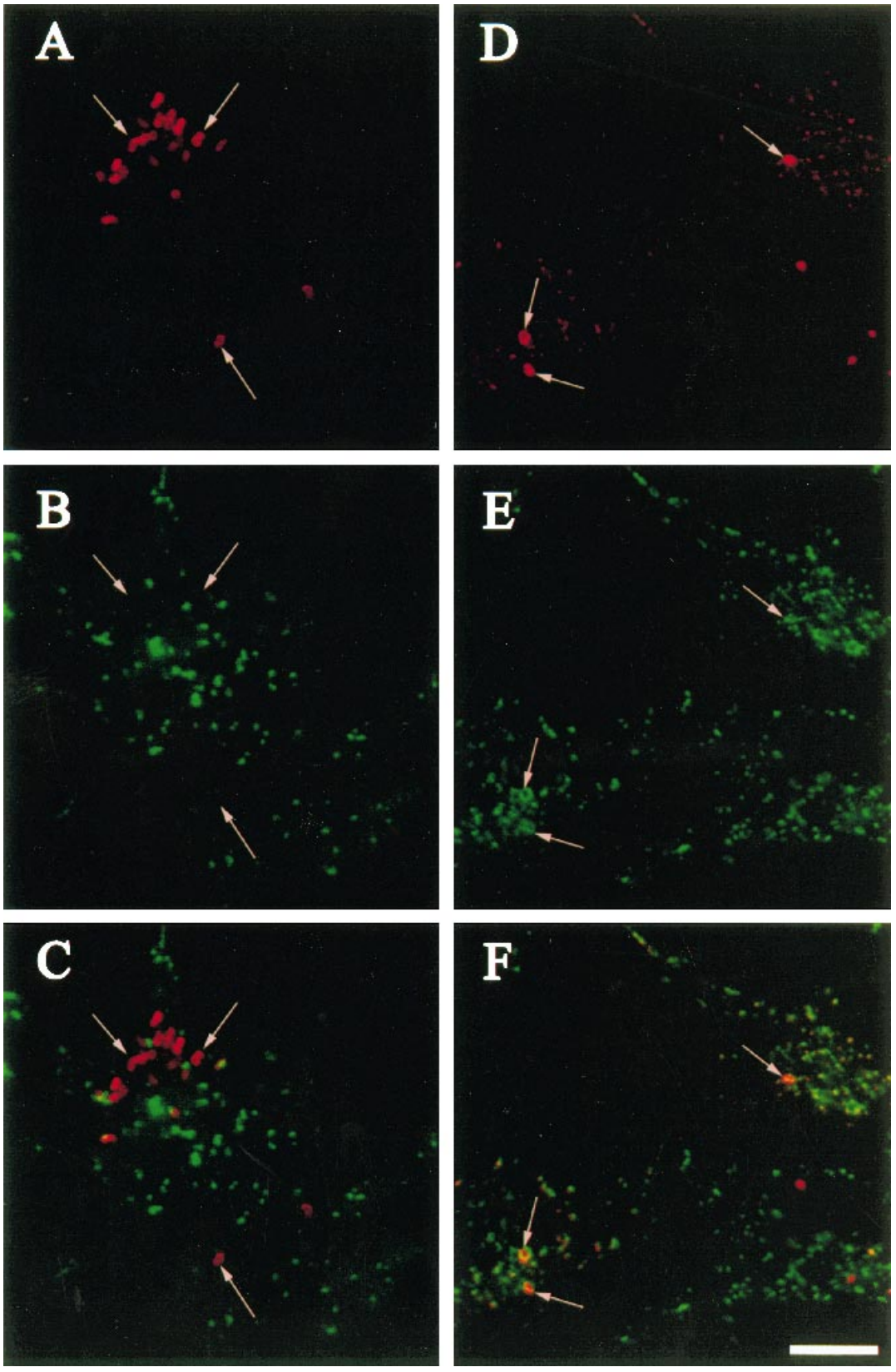
65.21-*bvrR* bacteria per infected cell and the efficiency of intracellular invasion were similar to those observed for 2308 Nal^r (Fig. 5B and D). On the other hand, the analyses in HeLa cells revealed extracellular 65.21-*bvrR* bacteria (Fig. 6C) and increased numbers of both intracellular and extracellular 65.21-*bvrR* bacteria per infected cell (Fig. 5A). Thus, although the efficiency of invasion of 65.21-*bvrR* was higher than that of 65.21 or 2.13, it was not restored to the levels of the virulent strain (Fig. 5C). Moreover, although 65.21-*bvrR* was found mostly in phagosomes devoid of cathepsin D after 1 h of chase, a few intracellular bacteria already collocalized with this marker, revealing that bacterial degradation was initiated at this early time of infection (Fig. 6C). After 24 h, practically all cells had relatively high numbers of both bacteria within phagosomes devoid of cathepsin D and of debris collocalizing with this marker (not shown). This shows that, in contrast to either virulent 2308 Nal^r or to 65.21, both bacterial replication and destruction occurred. The fate of intracellular 65.21-*bvrR* bacteria within macrophages was similar to that observed for HeLa cells (not shown).

Discussion

Bacterial pathogens modulate their gene expression to adapt to the various environments to which they are exposed during the course of infection. This adaptive response is often under the control of two-component regulatory systems, generally consisting of a sensor protein kinase and a response regulator (for review, see Finlay and Falkow, 1997). We demonstrated here that *B. abortus* is endowed with one of such systems and that this system is highly relevant for the virulence of this bacterium.

Dysfunction of the BvrR–BvrS system alters OM properties that relate to the susceptibility to bactericidal polycationic substances and surfactants. As bactericidal cationic peptides are important defences against pathogenic bacteria (Vaara, 1992), this alteration is relevant in the light of the unusually high resistance of *Brucella* to these agents (Martínez de Tejada *et al*., 1995; Freer *et al*., 1996). Concomitant with this OM alteration, the *bvrR* and *bvrS* mutants also showed impaired invasiveness: both accumulated on the surface of cells, penetrating only in reduced numbers. Furthermore, the BvrR–BvrS system is also relevant for intracellular survival and replication: in contrast to the virulent parental strain, mutant bacteria (in early times) and bacterial debris (in later times) collocalized with the lysosomal marker and, consistent with this, they were recovered in very low numbers from cultured cells and mouse spleens.

The predicted importance of the two actions (sensory and regulatory) of BvrR–BvrS as a system is stressed by the observations made with the transgenic 65.21-*bvrR* strain. Expression of *bvrR* in 65.21 rescued both the resistance to bactericidal cationic peptides and the overall pattern of entry and replication within cells. However,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transgenic 65.21-*bvrR* was less efficient than virulent 2308 Nal^r in invading cells and in preventing lysosomal fusion and subsequent destruction, two observations made by using non-professional phagocytes (HeLa cells). Thus, although animal experiments (e. g. RT_{50}) with the 65.21-*bvrR* strain and more specific DNA manipulations (e.g. chromosomal constitutive expression of BvrR–BvrS) are precluded (natural brucellae do not harbour plasmids and are A-3/L-3 level pathogens), these results demonstrate that the mere expression of *bvrR* is not enough for full virulence. Accordingly, these results also suggest that for restoration of full virulence the bacteria requires the expression of both components (*bvrS* and *bvrR*) of the system. In this sense, the BvrR–BvrS system is similar to other two-component regulatory systems described in Gram-negative intracellular bacteria (Gunn and Miller, 1996).

As demonstrated for other two-component regulatory systems, multiple genes should be under the control of BvrR–BvrS. It has been shown recently that, in addition to variations in the level of expression of 73 proteins, repression of 50 *in vitro* expressed proteins and induction of 24 new proteins occurs during growth of *B. abortus* within macrophages (Rafie-Kolpin *et al*., 1996). Interestingly, these authors also showed that, although acid, oxidative, nutritional and heat stresses all induce new proteins (including GroEL, HtrA, SodC and DnaK), the sum of these *in vitro* induced proteins is not equivalent to those expressed in macrophages. Taken together, these observations indicate a complex regulation similar to that of the PhoP–PhoQ system of *S. typhimurium*, with both induced and repressed genes, and a strong candidate to take part in such a regulation is the BvrR–BvrS system. Although it has to be stressed that BvrR–BvrS had low homology with PhoP–PhoQ, it is interesting that, among other characteristics, PhoP–PhoQ activates the PmrA–PmrB system (Gunn and Miller, 1996; Soncini and Groismann, 1996), which, in turn, regulates the lipid A structure at levels known to induce resistance to polymyxin B (Guo *et al*., 1997). As the LPS core and/or lipid A of *Brucella* is also involved in polycation resistance (Martínez de Tejada *et al*., 1995; Freer *et al*., 1996), and the *bvrR* and *bvrS* mutants had this property diminished, it is likely that the *B. abortus* LPS structural features relevant in polycation resistance are under BvrR–BvrS control. Further studies on the structure and genetics of *Brucella* LPS are necessary to elucidate this point.

A search in the data banks revealed that the *B. abortus* BvrR–BvrS system showed a high similarity with chromosomally encoded systems present in some plant endosymbionts and pathogens (*R. meliloti* and *A. tumefaciens*) that are closely related to *B. abortus*. This similarity is much higher than that existing with other two-component regulatory systems of bacterial animal parasites, and the similarity of the genetic region is accentuated further by the contiguous presence of a phosphoenolpyruvate carboxykinase gene in these three species (Mantis and Winans, 1993; Osteras *et al*., 1995). This group of highly related systems seems to be of critical importance for the establishment of a relationship between these bacteria and their hosts, no mater whether the latter are animal or plant cells. *R. meliloti exoS* is involved in regulating the production of succinoglycan, which plays a crucial role in the establishment of the symbiosis between *Rhizobium* and its host plant (Cheng and Walker, 1998). Also, insertion mutations in either *chvG* (the sensor histidine protein kinase) and *chvI* (the response regulator) render *A. tumefaciens* unable to elicit tumour formation in susceptible plants (Charles and Nester, 1993). Furthermore, although the genes regulated by ChvI–ChvG are unknown, it is significant that the *chvI* and *chvG* mutants show the increased sensitivity to surfactants (Charles and Nester, 1993; Mantis and Winans, 1993) reported here for the *B. abortus bvrR* and *bvrS* mutants. Thus, as hypothesized by Charles and Nester (1993) for *A. tumefaciens*, it is likely that these regulatory elements control the synthesis and/or assembly of OM components essential in the interaction with eukaryotic cells. This does not mean that the sensor proteins (ChvG, ExoS and BvrS) should respond to the same environmental stimulus. In fact, the predicted periplasmic amino acid sequences of ChvG, ExoS and BvrS (presumably involved in environmental sensing) showed less similarity than other protein domains, and this should reflect the sensing of a different stimulus. However, it is remarkable that the two intracellular bacteria (*R. meliloti* and *B. abortus*) were more homologous in this region than the pericellular one (*A. tumefaciens*).

The above similarities have a phylogenetic meaning supporting the notion that the establishment of pericellular and intracellular relationships with eukaryotic cells, no matter whether they are animal or plant, is an evolutionary trend in the α-2 subdivision of the proteobacteria (Moreno *et al*., 1990). Characterization of the protein export systems of Gram-negative bacteria has shown that animal and plant pathogens use remarkably similar machinery (the

Fig. 7. Analysis using confocal immunofluorescence microscopy of the distribution of intracellular bacteria and bacterial debris in cathepsin D-positive compartments of HeLa cells 24 h after infection. Cells were infected with *B. abortus* 2308 Nal^r (left) or 65.21 (right). Intracellular bacteria were detected, after permeabilization of the cells, with serum from a *B. abortus*-infected cow revealed by a Texas red conjugate (shown in red, A and D), and cathepsin D was detected with rabbit anti-cathepsin D antibodies revealed by a FITC conjugate (shown in green, B and E). C and F are superimposed images of intracellular *B. abortus* and cathepsin D. *B. abortus* 2308 Nal^r does not collocalize with lysosomal cathepsin D (C). *B. abortus* 65.21 mutant collocalizes with lysosomal cathepsin D (shown in yellow, F). Arrows display the position of intracellular bacteria. Bar = 10 μm. Similar results to those observed with 65.21 were obtained with 2.13.

type III secretion pathway) to deliver proteins into host cells (Lee, 1997). However, these type III secretion genes appear in phylogenetically unrelated bacteria, in a variety of genomic locations, display protein identities seldom higher than 45% and often show G+C contents significantly different from the remaining chromosomal DNA (Van Gijsegem *et al*., 1995; Bogdonave *et al*., 1996). This evidence suggests that these sequences are transmitted horizontally as 'pathogenicity islands', a situation different from the phylogenetic relationship that can be deduced for the ChvI–ChvG, ChvI–ExoS and BvrR–BvrS systems on the basis of the analyses of the 16S RNA sequences and other phenotypic features of the corresponding bacteria and of the similar G+C contents of the region and of the overall chromosome. As BvrS, ExoS and ChvG differed mostly in the periplasmic domain, it seems likely that the *B. abortus*, *R. meliloti* and *A. tumefaciens* systems share a common ancestor that has evolved to sense different stimuli in plant and animal hosts.

Experimental procedures

Bacterial strains and growth conditions

The bacterial strains and plasmids used are listed in Table 1. Bacteria were routinely grown in standard tryptic soy broth or tryptic soy agar (TSA) either plain or with Nal, Km and/or ampicillin (Amp) at 25, 50 and 50 $\mu g\,ml^{-1}$ respectively. For survival assays, broth cultures were performed in 300 ml side-arm flasks in a rotatory water bath at 37°C, and exponentially growing bacteria were harvested by centrifugation (7000 × *g*, 15 min, 4°C) and resuspended immediately in the appropriate buffer. For LPS extraction (Moreno *et al*., 1990), bacteria were propagated in 2 l flasks (500 ml of broth per flask) on an orbital shaker (200 r.p.m.) at 37°C. All strains were stored in skim milk at −80°C.

Transposon mutagenesis

Mini-Tn*5* mutagenesis of *B. abortus* 2308 Nal^r was performed by mating with *E. coli* SM10 (λ pir) harbouring the suicidal plasmid pUT/Km (Herrero *et al*., 1990) under the conditions described previously (Sangari and Agüero, 1991). *B. abortus* Tn*5* mutants were selected by plating appropriate dilutions of the mating mixtures onto TSA–Nal–Km plates, which were incubated at 37°C for 4 days. The observed insertion frequency was 10^{-6}.

Screening method for polymyxin B sensitivity

Colonies of Km^r *B. abortus* exconjugants were picked directly from the TSA–Nal–Km master plate and individually transferred to the wells of microtitre-type polystyrene plates containing 200 μl per well of a polymyxin B sulphate (8000 units mg^{-1}; Sigma) solution (125 $\mu g\,ml^{-1}$) in sterile 0.133 M NaCl–0.1 M NaH_2PO_4 (pH 4.6) (Martínez de Tejada *et al*., 1995). After incubation for 1 h at 37°C, cell viability was tested by transferring an aliquot to Petri dishes containing TSA–Nal–Km and incubation at 37°C for 2 days. *B. abortus* Tn*5* mutants rendered non-viable by this exposure to polymyxin B were selected from the master plate for subsequent studies.

Susceptibility assays

Bacterial survival after a controlled exposure to polymyxin B, poly-L-lysine (molecular weight 7000–10 000), and bee venom melittin (all from Sigma) was assayed as follows. Stock solutions of polymyxin B in sterile 0.133 M NaCl–0.1 M NaH_2PO_4 (pH 4.6) or in sterile 1 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES) (pH 8.0) were prepared, and serial dilutions made in 96-well microtitre-type plates (100 μl per well) using the corresponding buffer. Melittin was tested only in 0.133 M NaCl–0.1 M NaH_2PO_4 (pH 4.6) and poly-L-lysine in HEPES buffer. Mid-exponential phase cells were suspended at $\approx 2 \times 10^4$ colony forming units (CFU) ml^{-1} in the corresponding buffer solution, 100 μl aliquots were dispensed into duplicate rows and plates were incubated for 1 h at 37°C. Viable counts were performed by spreading 5 μl from each well onto TSA or TSA–Km plates. All assays were performed in triplicate, and the results were expressed as the percentage of survival with respect to that of controls incubated without the peptide. Minimal inhibitory concentrations of polymyxin B and surfactants (Sarkosyl, SDS and sodium deoxycholate) were determined in Mueller–Hinton plates as described elsewhere (Nikaido, 1976).

Typing and characterization of B. abortus

The absence of smooth to rough dissociation was checked by testing the sensitivity to the smooth specific Tb phage (Alton *et al*., 1988) by a positive co-agglutination (Kronvall, 1973) with *Staphylococcus aureus* coated with IgG to *B. abortus* smooth LPS [obtained from the sera of infected rabbits (Díaz *et al*., 1968)], and by the absence of agglutination in acriflavine solutions (Alton *et al*., 1988). In addition, LPS extracts obtained using the SDS–proteinase K method (Garin-Bastuji *et al*., 1990) were examined by SDS–PAGE (Tsai and Frasch, 1982). For this purpose, duplicate LPS extracts were obtained from identical amounts (on a dry-weigh basis) of two independently grown batches of mutant bacteria, 2308 Nal^r (smooth) and RB51 (rough), and aliquots were electrophoresed on the same gels and comparisons were made using densitometry (IMAGEMASTER version 1.10, Pharmacia Biotech).

Virulence assays

The mouse model for brucellosis was used (Montaraz and Winter, 1986; Bosseray and Plommet, 1990; Winter *et al*., 1996). For each strain, the inoculum was prepared from 48 h cultures on blood agar base (Difco Laboratories) at 37°C, under a 10% CO_2 atmosphere. Bacteria were harvested in 20 mM phosphate-buffered saline (PBS) (pH 6.8) and suspended in this diluent to the appropriate concentration (see below). Before the assays, each strain was inoculated intraperitoneally into mice and recovered from the spleen 7 days later to assess the particular phenotypic characteristics under the experimental conditions (Winter *et al*., 1996). The colonial size on blood agar base was assessed after 5 days

of incubation at 37°C in 10% CO_2 atmosphere, and the smooth–rough dissociation by using the Tb phage and the acriflavine agglutination test. These characteristics, plus the presence of the Tn*5* insert in the corresponding restriction fragment, were routinely examined for the isolates at each of the sampling intervals (see below). RT_{50} was calculated according to the method of Bosseray (1991) and Bosseray *et al*. (1984). To this end, groups of 32 female Swiss outbred CD-1 mice six weeks of age were subcutaneously inoculated approximately with 1×10^8 CFU in 0.1 ml of PBS (exact doses were retrospectively assessed). Lots of eight animals from each group were killed by cervical dislocation at appropriate time intervals. Spleens were aseptically removed, individually homogenized in 1 ml of PBS, seeded onto blood agar base plates, and incubations carried out at 37°C for 5–7 days in 10% CO_2 atmosphere. Animals from which at least 1 CFU was isolated were considered as infected. RT_{50} values were calculated using the PROBIT procedure of the SAS statistical package (SAS, 1989), and differences were analysed by regression line comparison using the same statistical package. For calculating the bacterial multiplication rates in spleens, groups of 25 eight-week-old female BALB/c were intraperitoneally inoculated with 0.1 ml of a suspension containing $\approx 5 \times 10^5$ CFU ml^{-1} of each bacterial strain (exact doses were retrospectively assessed). Lots of five animals of each group were killed at appropriate times and CFU numbers in spleens determined as described above. Mean values of spleen counts at each time interval were obtained after logarithmic conversion (Montaraz and Winter, 1986), and comparisons were performed between groups at each point time using two-tailed Student's *t*-test.

Cell culture and bacterial infections

Peritoneal murine macrophages were obtained from 7-week-old BALB/c mice. After cervical dislocation each mouse received an intraperitoneal injection of 15 ml of 5% sucrose in sterile PBS at 15°C, and the abdomen of each animal was massaged and the liquid extracted. Pooled fluids were centrifuged at 1000 r.p.m. for 10 min at 4°C, and the pelleted cells were washed twice with Dulbecco's minimal essential medium (Gibco) supplemented with 10% fetal calf serum, 2 mM glutamine and 10 mM HEPES without antibiotics (culture medium). Cells (5×10^5) in cell culture medium were plated over a 24-well tissue culture plate and incubated 1 h at 37°C under 5% CO_2. Non-adherent cells were removed from the wells by aspiration, and the adherent macrophages rinsed twice and reloaded with fresh culture medium. Epitheloid human HeLa cells were maintained in 24-well tissue and cultured at 37°C under 5% CO_2 for 24 h before infection.

Murine resting peritoneal macrophages (5×10^4), or subconfluent monolayers of human HeLa cells were inoculated with bacteria diluted to 1×10^7 CFU ml^{-1} in cell culture medium. Plates were centrifuged for 10 min at 1000 r.p.m. at room temperature and placed under a 5% CO_2 atmosphere at 37°C. After 4 h, wells were washed five times and further incubated with cell culture medium supplemented with 100 μg ml^{-1} gentamicin (Sigma) in 0.25% fetal calf serum to kill the remaining extracellular bacteria. The number of intracellular viable *B. abortus* was determined at different times post infection. To this end, the monolayers were washed twice with cell culture medium and PBS, and were treated for 10 min with 1 ml of 0.1% Triton X-100 (Sigma) in deionized water. Lysates were serially diluted and plated onto TSA plates for determination of CFU.

Immunofluorescence and confocal fluorescence microscopy

Murine resting macrophages or human HeLa cells grown on glass coverslips were inoculated with bacteria as described above. Coverslips were extensively washed to remove non-adherent bacteria before 3% paraformaldehyde fixation. Cells were washed once in PBS and incubated for 10 min with PBS–NH_4Cl 50 mM to quench free aldehyde groups. For quantification analyses, extracellular bacteria were detected by incubating the cells for 30 min with a serum (diluted 1:10 000 in 10% mouse serum in PBS) from a *B. abortus*-infected cow (Rojas *et al*., 1994) and by revealing the antibodies with Texas red-conjugated goat anti-cow IgG antibodies (Immunotech). Intracellular bacteria were detected by permeabilization of cells with 0.05% saponin (Sigma), incubation for 30 min with serum (at 1:5000 in 10% mouse serum in PBS) from a *B. abortus*-infected rabbit (Rojas *et al*., 1994) revealed by FITC-conjugated donkey anti-rabbit IgG (Immunotech). For confocal studies regarding the intracellular localization of internalized bacteria, two protocols were used. For double immunofluorescence analysis, cells were permeabilized with 0.05% saponin, incubated for 30 min with the cow anti-*Brucella* serum and revealed by Texas red-conjugated donkey anti-cow IgG. The presence of cathepsin D (a well-known marker for lysosomes) was detected with rabbit anti-cathepsin D antibodies (Meresse *et al*., 1995) revealed by FITC-conjugated donkey anti-rabbit IgG. For triple immunofluorescence analysis, extracellular bacteria were detected by incubating the cells for 30 min with a mouse monoclonal anti-*Brucella* antibody (diluted 1:100 in 10% horse serum in PBS) (Rojas *et al*., 1994) revealed by cyanin 5-conjugated donkey anti-mouse IgG (Immunotech). Cells were then permeabilized with 0.05% saponin and intracellular bacteria were detected by incubating cells for 30 min with the cow anti-*Brucella* serum revealed by Texas red-conjugated goat anti-cow IgG. Finally, cathepsin D was detected with rabbit anti-cathepsin D antibodies revealed by FITC-conjugated donkey anti-rabbit IgG. In all cases, samples were mounted in Mowiol and analysed under a Leica TCS 4DA confocal laser scanning microscope. Series of confocal immunofluorescence images of cells were recorded at 0.4 μm vertical steps. Projections and stereo pairs of each series were calculated.

DNA manipulations

B. abortus chromosomal DNA was purified using the CTAB (hexadecyltrimethyl-ammonium bromide)–NaCl method as described previously (Wilson, 1990). The pUT/Km DNA was isolated from *E. coli* CC118 by a rapid alkaline method (Birnboim and Doly, 1979), and other plasmid DNAs were isolated by the Wizard Minipreps DNA Purification System (Promega). Plasmid pBluescript-II KS+ and *E. coli* XL1-Blue were used routinely in cloning experiments. Restriction modification enzymes (Amersham) were used under the conditions recommended by the manufacturer, and agarose gel electrophoresis

and other recombinant DNA techniques were performed according to standard procedures (Sambrook *et al*., 1989).

For Southern hybridization, DNA was vacuum transferred from agarose gels to positively charged nylon membranes (Hybond N^+, Amersham) and probed with appropriate DNA fragments previously recovered from 0.8% agarose gels (GenecleanII kit, BIO 101) and labelled with horseradish peroxidase (ECL Direct System, Amersham). Hybridizations were carried out at 42°C for 16 h, and membranes were developed by the enhanced chemiluminescence direct nucleic acid detection system (ECL Direct System, Amersham) according to the instructions of the manufacturer. To generate clones of the Tn*5*-inserted chromosomal fragment from *B. abortus* mutants, total genomic DNA was completely or partially digested with *Eco*RI (Tn*5* lacks *Eco*RI-specific sites). The resulting fragments were ligated with dephosphorylated *Eco*RI-cut pBluescript-II KS+, transformed into *E. coli* XL1-Blue, and Amp- and Km-resistant clones were selected. Purified double-stranded plasmid DNA was sequenced on a Pharmacia ALF DNA sequencer (Pharmacia Biotech) using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham). Internal sequencing primers were synthesized to determine the DNA sequences in both strands. Also, sequencing primers complementary to one of the mini-Tn*5* ends and to the cloning vector were used. The DNA sequence was assembled and analysed by using the GENEWORK program (version 2.45n), and similarity searches were carried out at the European Bioinformatics Institute, Hinxton Hall, UK (Pearson and Lipman, 1988; http://www2.ebi.ac.uk/fasta3).

Complementation was carried out by introducing a broad host range plasmid harbouring the parental sequence into mutant 65.21 (Kovach *et al*., 1994). Briefly, a 6 kb *Eco*RI fragment obtained from plasmid pBPS2.13 (see Table 1) was ligated into *Eco*RI-digested broad host range cloning vector pBBR1MCS-4 possessing an Amp-resistant gene and a promoter sequence for the constitutive production of proteins coded for by inserted genes (Kovach *et al*., 1994). The resulting construct designated pBBR2.13 was introduced into *B. abortus* 65.21 by mating with *E. coli* SM10 (λ pir). *B. abortus* 65.21 harbouring pBBR2.13 (designated as *B. abortus* 65.21-*bvrR*) was selected by plating the mating mixture onto TSA–Amp–Nal plates which were incubated at 37°C for 3 days.

Nucleotide sequence accession number

The DNA sequence of the *bvrR* and *bvrS* genes of *B. abortus* strain 2308 Nal^r have been submitted to the GenBank and have been assigned accession numbers AF005157 and AF012539 respectively. Other amino acid and nucleotide sequences used were taken from the SWISSPROT (accession numbers *A. tumefaciens* ChvG, Q07737; *R. meliloti* ChvI, P50350; *A. tumefaciens* ChvI, Q07783; *E. coli* OmpR, P03025; *S. typhimurium* PhoP, P14146) or the GenBank (*R. meliloti* ExoS, AF027298; *S. typhimurium* PmrA, L13395; *B. melitensis* FeuP, X87324) databases.

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