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The TolC Homologue of *Brucella suis* Is Involved in Resistance to Antimicrobial Compounds and Virulence▽

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***Brucella* spp., like other pathogens, must cope with the environment of diverse host niches during the infection process. In doing this, pathogens evolved different type of transport systems to help them survive and disseminate within the host. Members of the TolC family have been shown to be involved in the export of chemically diverse molecules ranging from large protein toxins to small toxic compounds. The role of proteins from the TolC family in *Brucella* and other α-2-proteobacteria has been explored little. The gene encoding the unique member of the TolC family from *Brucella suis* (BepC) was cloned and expressed in an *Escherichia coli* mutant disrupted in the gene encoding TolC, which has the peculiarity of being involved in diverse transport functions. BepC fully complemented the resistance to drugs such as chloramphenicol and acriflavine but was incapable of restoring hemolysin secretion in the *tolC* mutant of *E. coli*. An insertional mutation in the *bepC* gene strongly affected the resistance phenotype of *B. suis* to bile salts and toxic chemicals such as ethidium bromide and rhodamine and significantly decreased the resistance to antibiotics such as erythromycin, ampicillin, tetracycline, and norfloxacin. Moreover, the *B. suis bepC* mutant was attenuated in the mouse model of infection. Taken together, these results suggest that BepC-dependent efflux processes of toxic compounds contribute to *B. suis* survival inside the host.**

Brucella spp. are responsible for a zoonosis that causes a serious economical impact worldwide and a human disease that is difficult to treat (8, 16, 22). *Brucella* enters the host via the nasal, oral, and pharyngeal cavities, and after penetrating the mucosal epithelium, the organism is transported to the lymph nodes. During early infection, innate immunity mechanisms from the host contribute to reduce the initial number of infecting *Brucella* cells (38). *Brucella* has the ability to invade and survive within macrophages and nonphagocytic cells. After entering the host cell, *Brucella* is found in a membrane-associated vacuole (phagosome). *Brucella* subverts the intracellular endocytic pathway, bypassing the classical lysosomal pathway; the bacteria multiplicate and survive in a compartment associated with rough endoplasmic reticulum (for recent reviews, see references 33 and 69). By using this strategy, the bacterium escapes some bactericidal mechanisms (10).

The outer membrane of *Brucella* is considerably hydrophobic compared to those of other α-2-proteobacteria and therefore more permeable to lipophilic compounds (54). Conversely, the hydrophobic character of the outer membrane makes *Brucella* relatively resistant to polycationic peptides. In other species, low permeability to hydrophobic compounds goes together with efflux systems to increase protection against toxic molecules (47). Therefore, this unusual characteristic of the outer membrane raises interesting questions about the physiology of *Brucella* spp. Some outer membrane proteins (76) and the O side chain component of the lipopolysaccharide (4, 32, 52) have been shown to influence intracellular survival, probably altering the outer membrane properties.

In addition to the characteristics of outer membrane permeability, transport processes are crucial for protecting pathogens against toxic compounds. In fact, several gram-negative bacteria expel a broad range of antimicrobial compounds through the expression of different efflux systems (57, 83). Most of these systems are tripartite efflux pumps, in which an outer membrane protein channel from the TolC family works in association with inner membrane complexes to extrude different types of toxic compounds. The inner membrane complexes are formed by an inner membrane protein belonging to the RND (*r*esistance *n*odulation *d*ivision) or MFS (*m*ajor *f*acilitator *s*uperfamily) family and a protein from the MFP (*m*embrane *f*usion *p*rotein) family that expands the periplasmic space (27, 60, 83). The paradigm of members from the outer membrane channel family is the multifunctional TolC of *Escherichia coli*. High-resolution crystal structure revealed that the TolC homotrimer has a 140-Å-long cylinder which is made up of a 100-Å-long α-helical barrel (the tunnel domain) extending the

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periplasmic space and a 40-Å-long β-barrel channel in the outer membrane (7). TolC together with an ABC-MFP complex is responsible for α-hemolysin (HlyA) translocation across both inner and outer membranes in a mechanism bypassing the periplasmic space (49, 79). From biochemical studies, it was shown that protein export was achieved by the recruitment of a trimeric TolC by the inner membrane translocase after it binds to its substrate (75). TolC works also in association with RND-MFP or MFS-MFP complexes to pump antimicrobial drugs outside the cell (40). It was proposed that substrate binding induces an open state by untwisting the tunnel α-helices of TolC; this conformation change allows the direct passage of proteins and drugs from the cytosol out of the cell (7, 41).

So far, only one efflux system belonging to the MATE (*m*ultidrug *a*nd *t*oxic compound *e*xtrusion) family has been characterized in *Brucella* spp. This system was shown to be efficient in the elimination of drugs such as norfloxacin, ciprofloxacin, gentamicin, and acriflavine (18). In α-2-proteobacteria, the role of tripartite transport systems, particularly of TolC homologues, has been explored little. In this work, we investigated the role of the unique member of the TolC family identified in the *Brucella suis* genome. Heterologous and mutational approaches showed that the TolC homologue from *B. suis* is involved in the efflux of toxic and relatively hydrophobic compounds, influencing the survival of *B. suis* inside the host.

MATERIALS AND METHODS

Growth conditions, bacterial strains, and reagents. The *Brucella* strains used in this study (*B. suis* 1330 [ATCC 23444] and derived mutants) were all grown in tryptic soy (TS; Bacto) medium in combination with the appropriate antibiotics (spectinomycin, 50 μg ml^{-1}; chloramphenicol, 6 μg ml^{-1}). *E. coli* strain DH5α was used as the recipient strain for cloning and was routinely grown in Luria-Bertani (LB) medium. The appropriate antibiotics (ampicillin, 50 μg ml^{-1}; spectinomycin, 200 μg ml^{-1}; chloramphenicol, 50 μg ml^{-1}; tetracycline, 5 μg ml^{-1}; and kanamycin, 50 μg ml^{-1}) were added when needed. *E. coli* strain C600 and the *tolC* mutant (C600 *tolC*::Tn*5*) used in heterologous functional complementation assays were kindly provided by Philippe Delepelaire. The standard growth temperature for all bacterial strains was 37°C. Mueller-Hinton broth was obtained from Britania. The following drugs were used in sensitivity tests, with the compound class in parentheses: acriflavine (intercalator), ethidium bromide (intercalator), sodium deoxycholate (detergent, bile salt), erythromycin (macrolide), berberine hemisulfate (plant alkaloid), nalidixic acid (quinolone precursor), norfloxacin (fluoroquinolone), amikacin disulfate (aminoglucoside), cetyltrimethylammonium bromide (detergent), and crystal violet (dye, intercalator) were obtained from Sigma; carbenicillin (β-lactam) from Fisher Biotech; ampicillin (β-lactam) from Bago; sodium dodecyl sulfate (SDS) (detergent) from Promega; bile salts from Britania; chloramphenicol and rifampin from Parafarm; streptomycin (aminoglucoside) from Lab Richet; rhodamine 6G (dye) from Allied Chemical; and tetracycline from Amersham.

Cloning and disruption of *bepC* from *B. suis*. A 1,932-bp DNA region containing the *tolC* homologue gene (*bepC*), corresponding to BR0945 of the annotated *B. suis* genome (http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?database=gbr), was amplified from *B. suis* 1330 genomic DNA by PCR, using the specific primer pair 5′-GAACGGGATGACGGGAA and 5′-GGCGTACCGTTTTCAATGCA, and cloned into pGEM-T Easy vector (Promega). The fidelity of the amplification reaction was confirmed by sequencing. The amplicon was subcloned as a SalI-SphI fragment in pBBAD18T vector (tetracycline resistance) (72) under the *araC*-inducible promoter. This construction (pFC25) was used for heterologous functional complementation assays. To generate a knockout mutant in the *bepC* gene, the 2-kb SmaI fragment containing the spectinomycin resistance cassette (Ω) (63) was ligated into the unique StuI site of *bepC* (712 bp downstream from the ATG start codon) cloned in pGEM-T Easy. The recombinant plasmid was electroporated into *B. suis* M1330 cells, and since the p-GEM-T-*bepC*::Ω plasmid cannot replicate in *Brucella*, the *bepC* insertional mutant (Br1) was obtained by selecting clones that were spectinomycin resistant and ampicillin sensitive. The Ω insertion was confirmed by Southern blot analysis. Briefly, chromosomal DNA was completely digested with EcoRI and ClaI, electrophoresed in 0.6% agarose, and capillary transferred to positively charged nylon membranes (Hybond-N). Membranes were hybridized with a 1.9-kb complete gene fragment labeled with radioactive phosphor using Prime-a-Gene (Promega) and washed with 2× SSC-0.1% SDS solution (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature and once more with 0.1× SSC-0.1% SDS at 65°C. The washed blots were exposed on storage phosphor screen autoradiography and screened in a Storm 820 optical scanner (Amersham Pharmacia Biotech). For genetic complementation studies with *Brucella*, the SalI-SphI fragment containing the *bepC* gene (see above) was cloned into the broad-host-range pBBR1MCS vector, which confers resistance to chloramphenicol (42). The resulting plasmid (pFC115) was electroporated into the *bepC* Br1 mutant strain, and transformants were selected on TS agar supplemented with chloramphenicol and spectinomicin.

α-Hemolysin secretion and colicin E1 uptake. Colicin E1 sensitivity was determined by spotting twofold serial dilutions of the colicin stock solution (Sigma) on bacterial lawns. Killing zones were recorded after 8 h of incubation at 37°C. α-Hemolysin (HlyA) secretion was analyzed using sheep blood agar plates (5% defibrinated blood); hemolysis zones around the colonies were observed after 10 h of incubation at 37°C. The presence of the 107-kDa HlyA polypeptide in the supernatant of assayed strains was also examined. Culture supernatant proteins were concentrated by precipitation with 10% trichloroacetic acid as described previously (70). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) with 12% acrylamide and visualized by staining with Coomassie brilliant blue R-250.

Microdilution assay for drug susceptibility. The MICs of drugs for *E. coli* strains were determined by a broth microdilution assay performed in Mueller-Hinton broth (Britania) supplemented with 0.2% L-arabinose. MIC was defined as the lowest concentration of a drug that completely inhibited growth. All tests were done by triplicate in accordance with the procedures established by the CLSI (formerly NCCLS). Briefly, the MIC was determined in microtiter plates with 96 flat-bottom wells in a final volume of 0.2 ml. Except for the growth controls in the absence of the drug, 100 μl of a twofold dilution of the drug was added to the wells. Next, except for the sterility (uninoculated) controls, 100 μl of a bacterial suspension (10^5 CFU/ml of *E. coli* cells grown in Mueller-Hinton broth) was added to the wells. The microtiter plates were shaken at 200 rpm during incubation, and bacterial growth was examined by measuring the optical density at 600 nm (OD_{600}) with a microplate reader after 16 h of incubation at 37°C. The growth index was calculated by dividing the OD of the culture in the presence of drug by the OD in the absence of drug.

Disk diffusion tests. Disk diffusion tests for *E. coli* were performed as outlined in the CLSI standard M2-A9, using Mueller-Hinton agar supplemented with 0.2% L-arabinose. Bacterial lawns of *B. suis* were grown on TS agar. Sterile paper disks (Whatman filters) 6 mm in diameter were placed on bacterial lawns, and 5 μl of each drug solution was pipetted onto separate disks on bacterial lawns. The plates were incubated at 37°C for 16 h for *E. coli* or 24 h for *B. suis*, and the diameters (in millimeters) of the inhibition zones were measured. Experiments were repeated at least twice, and all tests were performed in triplicate.

Infection in BALB/c mice. Eight-week-old female BALB/c mice (5 mice per group) were inoculated intraperitoneally with 0.2 ml of a phosphate-buffered saline (PBS) suspension containing 5×10^5 CFU of wild-type *B. suis* 1330, the *bepC* mutant Br1, or Br1 complemented with *bepC* cloned in pFC115. At 2, 3, 5, and 7 weeks after infection, groups of five mice were sacrificed for spleen collection. The spleens were homogenized in 5 ml of PBS, and serial dilutions of the homogenates were plated on TS agar with the corresponding antibiotics to determine bacterial counts.

Cell infection assays. Murine J774 macrophages seeded in 24-well plates (10^5 cells per well) were inoculated with 2×10^6 CFU (multiplicity of infection, 20:1) of wild-type *B. suis* 1330, the *bepC* mutant Br1, or Br1 complemented with *bepC* cloned in pFC115 in 0.5 ml of minimal essential medium (GIBCO, Paisley, Scotland) supplemented with 5% fetal calf serum and 2 mM glutamine (cell culture medium) without antibiotics. A similar procedure was followed for infecting HeLa cells, except that the inoculum size was 10^7 CFU (multiplicity of infection, 100:1). In order to ensure close contact between cells and bacteria, multiwell plates were centrifuged for 10 min at 141 × *g* at room temperature and placed in a 5% CO_2 atmosphere at 37°C. After 1 h, the wells were washed three times with sterile PBS (pH 7.4) and further incubated with cell culture medium containing 50 mg of gentamicin per ml and 50 mg of streptomycin per ml to eliminate the remaining extracellular brucellae. At different times, the number of intracellular viable *B. suis* bacteria was determined as follows: cells were washed three times with PBS and treated for 10 min with 0.5 ml of 0.1% Triton X-100

in deionized sterile water, and lysates were serially diluted in PBS and plated on TS agar with the appropriate antibiotic to determine the number of CFU.

Phylogenetic analysis. Molecular evolutionary relationships between 20 protein members of the TolC family were examined by the neighbor-joining method of tree construction. Alignment of the proteins was performed with ClustalX (1.81). Phylogenetic trees, bootstrap, and jackknife analysis to determine the statistical stability of each node were done using Paup 4.0b10. Trees were displayed by TreeView 1.6.6.

RESULTS

Phylogenetic analysis of BepC. Proteins from the TolC family have been shown to be involved together with an inner membrane translocase in the export of different and diverse type of substrates, such as proteins, hydrophobic compounds, and cations. Available *Brucella* sp. genomes contain a unique protein from the TolC family, displaying 99% sequence identity with one another (not shown). The closest relatives to the TolC homologue of *Brucella* spp. detected by a BLAST search against the Swiss-Prot data bank were the multifunctional TolC from *E. coli* (25% identity), AprF from *Pseudomonas aeruginosa* involved in protease secretion (25% identity) (25), and TolC from *Vibrio cholerae* involved in the efflux of antimicrobial agents and cytotoxin secretion (24% identity) (14). The identity of the *Brucella* TolC homologue with TolC from *E. coli* was relatively low compared with other members of this family. In fact, TolC homologues from *V. cholerae*, *Erwinia chrysanthemi*, and *Salmonella enterica* serovar Typhimurium share more than 70% similarity with TolC of *E. coli* (9, 14, 71). Accordingly, these proteins were named "TolC." The TolC homologue component from *B. suis* was named BepC for *B*rucella efflux *p*rotein (see below).

To investigate evolutionary relationships from which we can infer the possible substrates of BepC, a phylogenetic analysis of representative and characterized members of the TolC family was performed. We also included NodT and a TolC homologue from the close relatives *Rhizobium leguminosarum* and *Bradyrhizobium japonicum*, respectively, whose substrates have not been identified. In fact, although a role in Nod factor secretion by NodT was suggested, this has not been experimentally proved (67). BepC did not clearly cluster with any of the characterized TolC homologues (Fig. 1) but clustered in another group together with NodT and the TolC homologue from *B. japonicum*. As expected, members from the TolC family that participate in protein export clustered together and the same observation was made with proteins involved in drug efflux or cation export (Fig. 1). TolC from *E. coli* has the peculiarity to interact with many different inner membrane complexes to export either proteins or hydrophobic compounds (40) and to participate in the uptake of colicins (44). Interestingly, TolC formed a subcluster with HasF from *Serratia marcescens*, involved in both lipase secretion and drug efflux, and with the *Salmonella enterica* serovar Typhimurium $TolC_{St}$, which was capable of pumping hydrophobic compounds and secreting a heterologous protein (Fig. 1). Therefore, phylogenetic analysis did not allow the prediction of a possible substrate; BepC could be involved in one or several of the functions so far described for this family of proteins. To investigate potential roles for BepC, functional complementation studies with *E. coli* were performed.

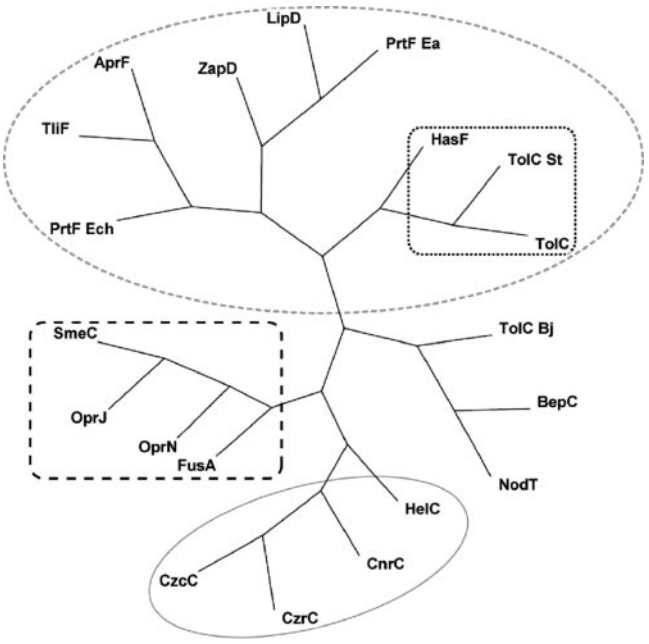

FIG. 1. Phylogenetic tree of the TolC family. Twenty protein members from the TolC family for which function is known, or strongly implicated by the location of their genes, are sorted by TreeView based on sequence alignment using ClustalX, and the tree is arranged using Paup 4.0. Sequence similarity correlates with substrate specificity; indeed, proteins can be grouped into three subfamilies corresponding to their roles. -----, group 1, protein secretion; ·····, subgroup of multifunctional proteins; ——, group 2, cation efflux; – – –, group 3, drug efflux. The substrate(s) and organism of the TolC homologues from group 1 are as follows: TliF, lipase, *Pseudomonas fluorescens* (1); AprF, alkaline protease, *Pseudomonas aeruginosa* (25); PrtF, protease, *Erwinia chrysanthemi* (45); LipD, lipase, *Serratia marcescens* (3); PrtF, protease, *Erwinia amylovora* (84); ZapD, metalloprotease, *Proteus mirabilis* (80); TolC, α-hemolysin, multiple drugs, multifunctional, *Escherichia coli* (40, 79); HasF, multifunctional, *Serratia marcescens* (2, 15, 43); and $TolC_{St}$, multifunctional, *Salmonella enterica* serovar Typhimurium (11), (12). The substrate(s) and organism of the TolC homologues from group 2 are as follows: CzrC, cadmium and zinc, *Pseudomonas aeruginosa* (36); CzcC, divalent cations, *Ralstonia metallidurans* (55); CnrC, cobalt and nickel efflux, *Cupriavidus metallidurans* (34); and HelC, heme, *Legionella pneumophila* (19). The substrate(s) and organism of the TolC homologues from group 3 are as follows: OprN, multiple drugs, *Pseudomonas aeruginosa* (51); OprJ, multiple drugs, *Pseudomonas aeruginosa* (62); SmeC, multiple drugs, *Xanthomonas maltophilia* (*Stenotrophomonas maltophilia*) (46); and FusA, fusaric acid, *Burkholderia cepacia* (78). BepC corresponds to the TolC homologue from *B. suis* M1330. This protein does not clearly belong to any of those groups and forms a new group with NodT from *Rhizobium leguminosarum* (unknown substrate) (73) and TolC from *Bradyrhizobium japonicum* (unknown substrate) (37).

***B. suis* BepC restores drug efflux in *tolC*-deficient *E. coli*.** Since TolC of *E. coli* has the distinctive feature of participating in many transport processes, the potential role of BepC was initially analyzed by heterologous complementation of a *tolC* mutant. The *bepC* gene of *B. suis* (BR0945) (http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?database=gbr) was amplified by PCR and cloned into pBBad-18T, under the control of the inducible *araC* promoter. The resulting plasmid (pFC25) was electroporated into the hypersensitive *tolC*::Tn*5* mutant of *E. coli* (40), and drug sensitivity was assayed by using an agar diffusion plate assay. The *bepC* gene cloned in pFC25 restored

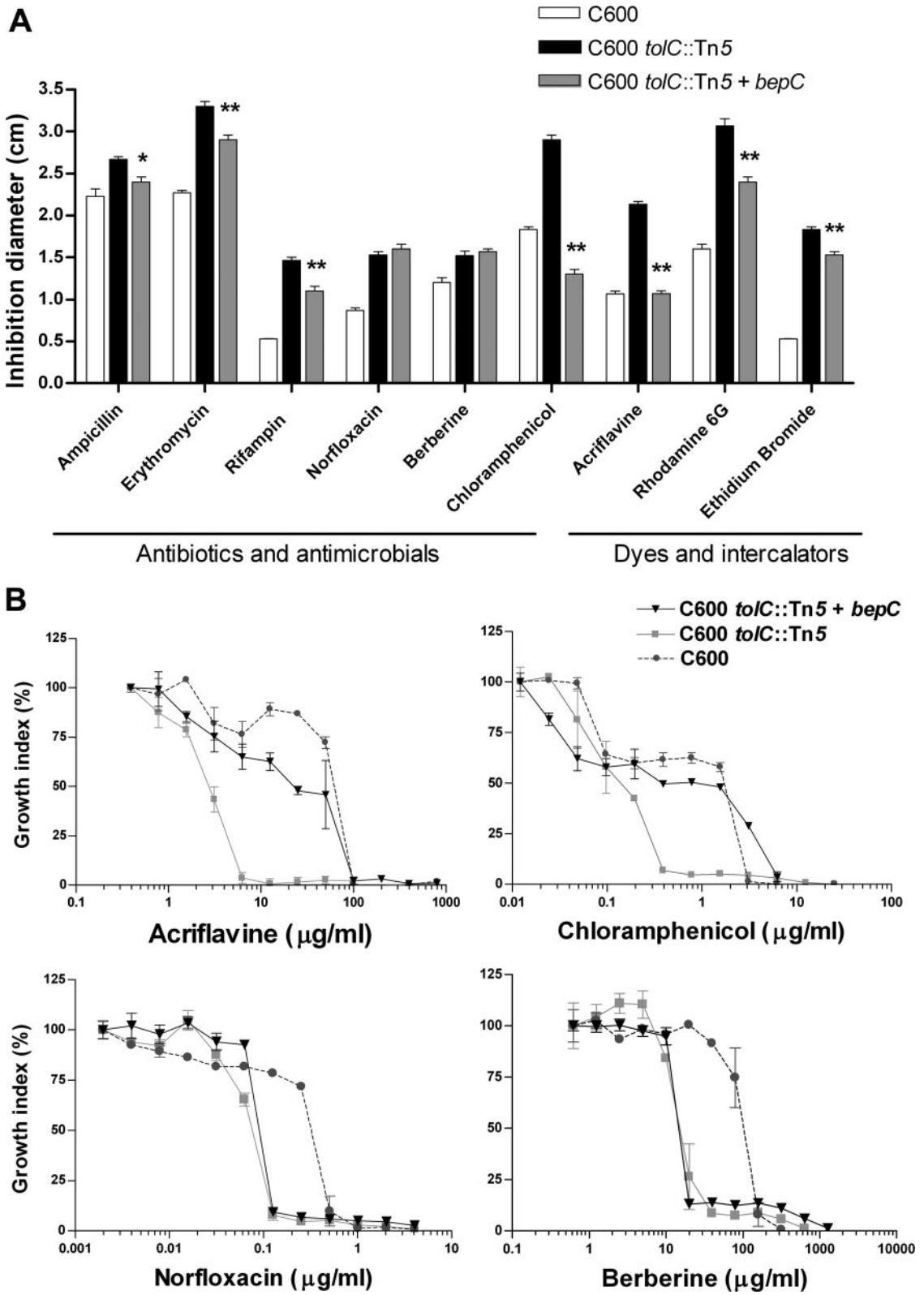

FIG. 2. Heterologous complementation of the resistance phenotype of the *E. coli tolC* mutant by *bepC* from *B. suis*. (A) The sensitivities of the *E. coli* wild type, the *tolC* mutant, and the *tolC*-plus-*bepC* strain were evaluated by the disk diffusion test in triplicate. The inoculated plates were incubated at 37°C for 16 h, and inhibition zones were compared. While partial complementation of the sensitive phenotype was observed for several drugs, sensitivity to acriflavine and chloramphenicol was fully complemented. A representative experiment is shown. For each drug, the values corresponding to the *tolC* mutant harboring the *bepC* gene marked with asterisks were significantly different from that of the *tolC* mutant (**, $P < 0.001$; *, $P < 0.05$). The statistical analysis was done by one-way analysis of variance and Bonferroni's multiple-comparison test. (B) MICs were

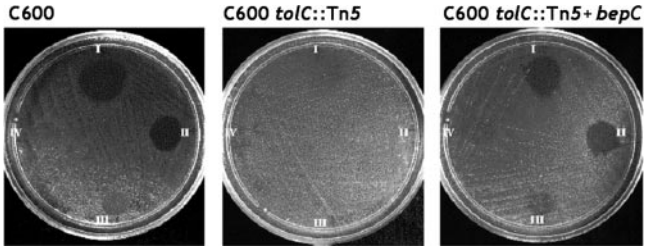

FIG. 3. Heterologous complementation of colicin E1 susceptibility. Colicin E1 sensitivity in wild-type *E. coli* (C600) and the lack of sensitivity in the *tolC* mutant (C600 *tolC*::Tn*5*) are shown. Cloned *bepC* is capable of restoring sensitivity to colicin E1. The concentration of colicin E1 decreases clockwise.

the wild-type levels of resistance to drugs such as chloramphenicol and acriflavine (Fig. 2A). The presence of the *bepC* gene also resulted in a considerable reduction of susceptibility to other hydrophobic compounds such as ampicillin, erythromycin, rifampin, rhodamine 6G, and ethidium bromide (Fig. 2A). No effect of *bepC* expression on the hypersensitivity phenotypes of the *tolC* mutant to other compounds such as norfloxacin and berberine (Fig. 2A) as well as to sodium deoxycholate, nalidixic acid, crystal violet, SDS, streptomycin, gentamicin, and carbenicillin (data not shown) was observed. These results were confirmed using drug microdilution assays in a multiwell plate. We studied the effect of *bepC* expression on the growth of the *tolC* mutant in the presence of twofold serial dilutions of both chloramphenicol and acriflavine, using berberine and norfloxacin, two drugs that were equally toxic in the presence and absence of *bepC*, as controls (Fig. 2A). Expression of *bepC* resulted in an increase of the MIC of chloramphenicol to 3.12 μg/ml, a value higher than that for the wild type (1.56 μg/ml) (Fig. 2B). Likewise, *bepC* was also able to complement the susceptibility phenotype to acriflavine, resulting in an MIC very similar to that of the wild type (50 μg/ml) (Fig. 2B). As expected, *bepC* expression in the *tolC* mutant had no effect on the MICs of berberine and norfloxacin (Fig. 2B). These results indicate that BepC reverts the *tolC*-deficient *E. coli* hypersusceptibility to several diverse hydrophobic compounds.

BepC fully complemented colicin E1 uptake in the *tolC* mutant of *E. coli*. Colicins are citotoxic peptides against susceptible relatives produced by bacteria of the *Enterobacteriaceae* family (31). TolC is involved in the uptake of colicins E1 and 10 by a specific interaction with the colicin amino-terminal translocator domain (56, 82). Consequently, mutagenesis of *tolC* results in a colicin-tolerant phenotype. In order to assess whether BepC complements TolC absence, different colicin E1 quantities were spotted on agar plates seeded with the *E. coli* wild type (C600), the *tolC* mutant, or the *tolC* mutant harboring the *bepC* gene on pFC25. As shown in Fig. 3 wild-type *E. coli* as well as the *tolC* mutant carrying *bepC* showed similar levels of colicin E1 susceptibility. This was an interesting result because HasF, a TolC homologue from *Serratia marcescens* that shares higher identity with TolC from *E. coli* (74%) than with BepC, was unable to complement colicin E1 sensitivity in the *tolC* mutant (15). This result suggests that although TolC and BepC share a low global degree of amino acid sequence similarity, some local sequence similarity or structural features within the putative colicin E1 receptor (and probably translocator) domain are conserved in both proteins. To assess a possible biological implication of such similarity, we examined the sensitivity of *B. suis* to colicin E1. Wild-type brucellae spread on a TS agar plate were not susceptible to colicin E1 over a concentration range similar to that used in experiments with *E. coli* (data not shown). This observation indicates that the receptor and uptake activities conferred by BepC are not sufficient to mediate colicin susceptibility and that other bacterial components required for colicin sensitivity (31) are probably not conserved in *B. suis*. In addition, the possibility that BepC of *B. suis* may be recognized as a specific receptor by other cytotoxic peptides cannot be rule out.

Hemolysin secretion in the *E. coli tolC* mutant was not restored by BepC. Most gram-negative bacteria, including members of the *Rhizobiaceae* family, export proteins to the extracellular medium through tripartite (ABC-MFP-TolC homologue) type I secretion systems. In *R. leguminosarum*, a close relative of *Brucella* spp., the inner membrane PrsD-PrsE translocase is responsible for the secretion of several proteins involved in biofilm formation and nodulation (28, 29, 70). However, the TolC homologue that works in association with PrsD-PrsE has not been identified. In *E. coli*, TolC together with the inner membrane translocase HlyB-HlyD is responsible for alpha-hemolysin (HlyA) secretion (79). Since BepC is the unique member of the TolC family that came out from the *B. suis* genome analysis, we explored the possibility that BepC was involved in protein secretion by testing whether BepC can restore hemolysin secretion in the *tolC* mutant expressing the *hlyCABD* genes (48, 79). The locus encoding HlyC, HlyA, HlyB, and HlyD cloned in pSF4000 was transferred by electroporation into the *tolC* mutant of *E. coli*. Hemolysin secretion was analyzed by a blood plate assay and by SDS-PAGE analysis of proteins secreted in the presence or the absence of *bepC* cloned in the pFC25 plasmid, which is compatible with pSF4000. A low level of hemolysis around the colony of the *tolC* mutant harboring both pSF4000 and pFC25 was observed, while no halo was detected around the *E. coli tolC* mutant containing only pSF4000 (data no shown). However, analysis of the trichloroacetic acid-precipitated extracellular proteins by SDS-PAGE clearly showed that secretion of HlyA was not restored by pFC25 in the *tolC* mutant (data not shown). The faint halo of hemolysis observed around the colonies might be due to a deleterious effect on cell membrane integrity by the expression of several genes encoding membrane proteins. Therefore, BepC was not capable of replacing TolC for HlyA secretion. This result suggests that TolC domains responsible

determined for acriflavine and chloramphenicol as well as for two negative controls (norfloxacin and berberine). Microdilution assays were performed in Mueller-Hinton broth by broth microdilution tests in the presence of 0.2% L-arabinose. The growth index was calculated by dividing the OD_{600} of the culture in the presence of drug by the OD_{600} of the culture in the absence of drug. MIC was defined as the lowest concentration that completely inhibited growth. The experiments were performed at least twice in triplicate, and the values are means ± standard deviations.

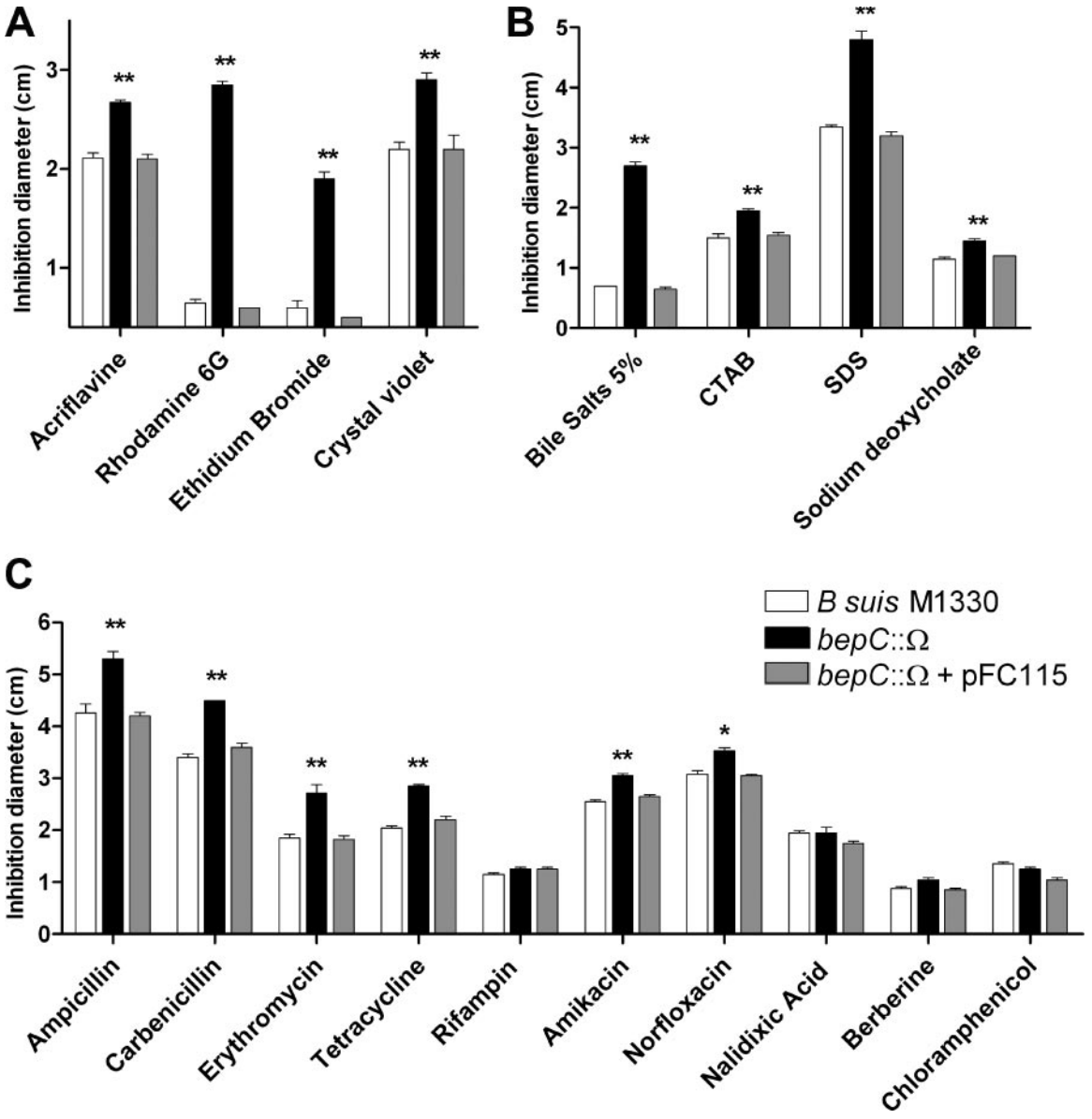

FIG. 4. Susceptibility phenotypes of the *bepC* mutant of *B. suis*. The drug sensitivity of the *bepC*::Ω insertional mutant was evaluated in agar plate diffusion tests. The mutation in the *bepC* gene significantly affected the resistance phenotype of *B. suis* to different compounds such as intercalators and dyes (A), detergents and bile salts (B), and antibiotics and antimicrobials (C). The experiments were repeated at least twice in triplicate. The data shown are expressed as mean values ± standard errors; asterisks above the values corresponding to the *bepC* mutant indicate that they were significantly different from the values for the wild-type strain 1330 (*, $P < 0.01$; **, $P < 0.001$). The statistical analysis was done by one-way analysis of variance and Bonferroni's multiple-comparison test. CTAB, cetyltrimethylammonium bromide.

for the interaction with the HlyB-HlyD translocase are not conserved in BepC or, alternatively, that some other intrinsic structural features of BepC do not allow for the secretion of such large and hydrophilic molecules.

Sensitivity of the *bepC*::Ω mutant to different hydrophobic compounds. The heterologous complementation studies suggest that BepC is more likely to have a role in the transport of small and relatively hydrophobic compounds. A mutational approach was used to further investigate the role of BepC of *B. suis* in the efflux of toxic compounds. The protein secretion phenotype was not investigated since we and others have observed that the amount of protein exported by *Brucella* spp. to the extracellular medium during in vitro cultivation is very small (24; D. J. Comerci and R. A. Ugalde, personal communication). The spectinomycin cassette (Ω) was inserted in the StuI site of *bepC*, and a knockout mutant was generated by double recombination of the *bepC*::Ω allele into the *B. suis* genome. Mutation of *bepC* did not affect the growth rate of *B. suis* in tryptic soy broth-rich medium (data not shown). The sensitivity phenotype of the *bepC*::Ω mutant to several chemicals was analyzed in tryptic soy broth by the disk diffusion plate assay. A marked growth inhibition phenotype of the *bepC*::Ω mutant by the rhodamine 6G dye and the ethidium bromide intercalator was observed (Fig. 4A). Crystal violet

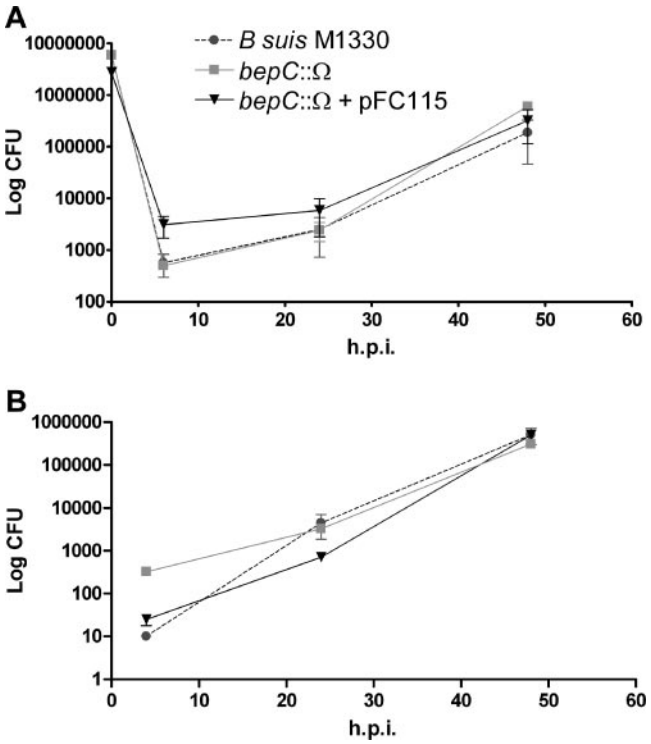

FIG. 5. Intracellular replication of *B. suis* M1330, the *bepC*::Ω mutant, and the complemented strain (*bepC*::Ω + pFC115) in murine J774 macrophages or HeLa cells. The number of intracellular viable bacteria was determined at 6, 24, and 48 h postinfection in J774 macrophages (A) or at 4, 24, and 48 h postinfection in HeLa cells (B). No significant differences in the uptake and intracellular growth in both cell types between the *bepC* mutant and the parent strain were seen. The data presented are the results of a representative experiment and are means ± standard deviations (error bars) of plate counts.

(dye) and acriflavine (intercalator) also induced halos of growth inhibition greater than those seen for the wild-type strain (Fig. 4A). The sensitive phenotypes to all of these drugs were complemented by pFC115 carrying the *bepC* gene (Fig. 4A). Interestingly, the *bepC*::Ω mutant showed a hypersensitive phenotype towards a mixture of bile salts (Fig. 4B). A significant increase in sensitivity of the *bepC* mutant was also observed in the presence of the pure steroid sodium deoxycholate and detergents such as SDS and cetyltrimethylammonium bromide (Fig. 4B). In addition, the *bepC*::Ω mutant was significantly more sensitive than the wild-type strain to β-lactam antibiotics such as carbenicillin and ampicillin, a macrolide (erythromycin), tetracycline, an aminoglycoside (amikacin), and a quinolone (norfloxacin) (Fig. 4C). The *bepC* gene cloned in pFC115 restored the resistance level to bile salts, the detergents, and all of the antibiotics (Fig. 4B and C). Mutation in *bepC* did not alter the susceptibility phenotypes of *B. suis* to berberine (plant antimicrobial), rifampin, chloramphenicol, and nalidixic acid (quinolone precursor) (Fig. 4C). These results confirm that the BepC outer membrane protein of *B. suis* plays a role in the efflux of toxic compounds and suggest that BepC is more efficient in extruding relatively hydrophobic or amphipathic molecules.

Survival of the *bepC*::Ω mutant in cultured cells. To analyze whether mutation in *bepC* affects intracellular survival, murine J774 macrophages or HeLa cells were infected with wild-type *B. suis* 1330, the *bepC*::Ω mutant, or the *bepC*::Ω mutant harboring the pFC115 complementing plasmid. Figure 5A shows similar biphasic curves of viable brucellae recovered from macrophages infected with the wild type, the *bepC* mutant, and the complemented mutant over a 48-h experiment. Similarly, no significant differences in the numbers of brucellae recovered from HeLa cells infected with wild-type *B. suis* 1330, the *bepC* mutant, or the complemented strain were observed (Fig. 5B). This indicates that the BepC protein is not crucial for in vitro intracellular survival.

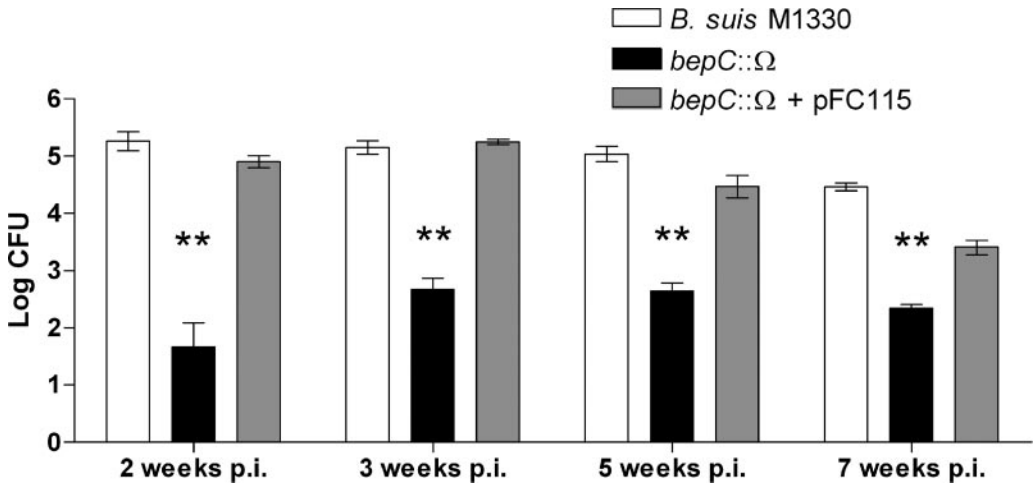

FIG. 6. Virulence of *B. suis* M1330, the *bepC* mutant, and the complemented strain in BALB/c mice. Mice were infected by intraperitoneal injection with 10^5 brucellae. At 2, 3, 5, and 7 weeks after infection, groups of five mice were sacrificed for spleen collection and bacterial counts were determined. Values are means (log number of CFU per spleen) ± standard deviations (error bars) ($n = 5$). The mean numbers of bacteria in the spleens of *bepC*-infected mice were always significantly lower (**, $P < 0.001$) than the mean numbers of bacteria in the spleens of wild-type strain 1330- or complemented strain-infected mice. These experiments also showed a clear 2- to 3-log reduction of CFU by the *bepC* mutant.

Virulence of *bepC*::Ω in the mouse model. In the mouse model of infection, *Brucella* is able to establish a chronic infection characterized by a large accumulation of brucellae in the spleen. To study the role of the BepC outer membrane protein in *B. suis* survival in vivo, groups of five mice were infected intraperitoneally with 5×10^5 CFU of the wild-type strain, the *bepC*::Ω mutant, or the complemented strain. The number of CFU recovered from spleens was determined at 2, 3, 5, and 7 weeks postinfection (p.i.). Recovery of the *bepC*::Ω mutant from the spleen was reduced 3 logs relative to that of the wild type at 2 weeks postinoculation and was reduced 2 to 2.5 logs at 3, 5, and 7 weeks postinoculation. The *bepC* gene cloned in pFC115 fully complemented the virulence phenotype at 2, 3, and 5 weeks p.i. and partially complemented the virulence phenotype at 7 weeks p.i., probably due to some plasmid instability (Fig. 6). These observations show that the BepC outer membrane channel might be required for in vivo *B. suis* survival.

DISCUSSION

In this work, we have analyzed the possible roles of the unique member of the TolC family (BepC) encoded by the *B. suis* genome. Members of the TolC family are recruited by different types of inner membrane translocases to allow a direct passage of diverse substrates from the cytoplasm to the external medium. Phylogenetic analysis of BepC did not clearly show a possible substrate for BepC. However, functional complementation studies with the hypersensitive and protein secretion-defective *tolC* mutant from *E. coli* support a role for BepC in the efflux of small and hydrophobic molecules. Mutation in the *bepC* gene of *B. suis* strongly increased the sensitivity of *B. suis* to bile salts, dyes such as rhodamine 6G, and ethidium bromide and significantly affected its resistance to antimicrobials such as erythromycin, tetracycline, and norfloxacin. These results indicate that BepC is capable of participating in the efflux of small and relatively hydrophobic compounds.

One obvious question is the following: which translocases are the partners of BepC? Or, more precisely, which are the putative inner membrane transporters encoded by the *B. suis* genome that work in association with BepC to pump toxic compounds? Our genomic analysis and the annotation of the *B. suis* genome (58) indicate that there are possible ABC-MFP and several putative RND-MFP "translocases" encoded by the *B. suis* genome. In theory, all of these candidates could work with BepC. In fact, our preliminary data suggest that at least one ABC-MFP complex and one of the RND-MFP complexes are involved in the extrusion of toxic molecules (F. A. Martín et al., unpublished data). Therefore, these inner membrane translocases probably form transenvelope tripartite complexes with BepC, allowing for the efflux of toxic compounds.

Detoxifying mechanisms in bacteria are of increasing interest because augmented resistance of several human pathogens to diverse antibiotics often involves the overexpression of efflux systems, including tripartite pumps (50, 61, 81). However, it is clear that this bacterial strategy emerged after the extensive use of antibiotics. Therefore, another question commonly raised about efflux systems concerns the in vivo physiological substrates. BepC-dependent efflux processes might contribute to resistance to compounds naturally present in the mammalian host. We have found that while the wild-type strain of *B. suis* was considerably resistant to 5% of a mixture of bile salts, the growth of the *bepC* knockout mutant was strongly inhibited by 5% of the bile salts. This observation suggests that BepC together with an inner membrane translocase may efficiently extrude bile salts produced by the host. Bile salts are very abundant in the mammalian intestine, which is not a preferred host niche for *Brucella* replication. Nevertheless, the efflux of bile salts may be important for *Brucella* survival during the intestinal transit of the bacterium in orally acquired infections. Another possibility is that BepC participates in the efflux of other steroid-like molecules encountered within the host during infection (26). In any case, these abilities may be particularly relevant for survival inside the host since the outer membrane of *Brucella* spp. was found to be particularly permeable to hydrophobic molecules (54). A similar feature was also reported for the outer membrane of *Vibrio cholerae*, an enteropathogen that circumvents the bactericidal activity of bile salts in the intestine through efflux pumps (21).

Another alternative role has been proposed for tripartite systems; these systems might be responsible of exporting hydrophobic quorum sensing signals, such as long-chain *N*-acylhomoserine lactones and quinolones (39, 59). Indeed, analysis by high-performance liquid chromatography and mass spectrometry of a dichloromethane extract of a spent culture supernatant from *B. melitensis* identified an *N*-dodecanoylhomoserine lactone (74). The transport of this putative signal to the extracellular milieu by *Brucella* spp. may be BepC dependent.

BepC did not complement the protein secretion phenotype of the *tolC* mutant, suggesting that BepC may not be involved in protein secretion. A protein secretion phenotype of the *bepC* mutant was not analyzed because it is well known that cultured *Brucella* does not secret detectable amounts of protein (24; Comerci and Ugalde, personal communication). Besides, our own analysis and description of predicted proteins by the published *Brucella* genomes (20, 23, 24, 35, 58) support the idea that there are no ABC-MFP candidates that could recruit BepC for protein secretion.

Our observations indicate that the BepC outer membrane protein significantly contributes to the intrinsic resistance/susceptibility phenotype of *B. suis* to several antibiotics. Human brucellosis is normally treated with doxycycline (a tetracycline), streptomycin, rifampin, fluoroquinolones, or combinations of these antibiotics. Emerged resistance to these or other antimicrobials in *Brucella* spp. is not a serious cause for concern. However, the presence of BepC-dependent efflux pumps in *B. suis* that are able to extrude antibiotics make this subject deserving of special attention. In fact, some strains nonsusceptible to rifampin have been isolated in Turkey and South Arabia (13, 53). In addition, a lack of effective bactericidal activity of fluoroquinolones against *Brucella* spp. has often been reported (5, 6, 30, 64, 68). In this regard, it should be noted that TolC-dependent efflux processes (Fig. 1) are responsible for the multiresistant phenotype of the *Salmonella enterica* serovar Typhimurium DT104 strain (12). In addition, it has been shown that ciprofloxacin-resistant *Salmonella enterica* serovar Typhimurium strains are difficult to select in the absence of AcrB and TolC (66).

The observation that BepC is able to replace TolC for the

sensitivity phenotype of *E. coli* towards colicin is intriguing. The biological significance of this is not known since *B. suis* was not found to be affected by colicin E1. To enter cells, colicin parasitizes multiprotein systems used by sensitive cells for important biological functions (17, 44). The fact that BepC was functional for this role might be fortuitous or may imply other unknown receptor and/or uptake functions.

Significantly fewer brucellae were recovered at 2, 3, 5, and 7 weeks p.i. from spleens of mice infected with the *bepC* mutant than from spleens of mice infected with the wild-type strain 1330. This phenotype was fully complemented by the cloned *bepC* mutant, indicating a direct association between mutation in the *bepC* gene and attenuation. However, no difference in the abilities to invade and replicate intracellularly in murine macrophages and HeLa cells between the *bepC* mutant and the parental strain was observed. This discrepancy between the in vitro and in vivo virulence phenotypes could be explained by different hypotheses. First, different surface molecules were proposed to control the initial number of infecting bacteria (38). In fact, a knockout mutant in the gene encoding the outer membrane lipoprotein Omp10 of *Brucella abortus* showed a phenotype similar to that of the *bepC* mutant, i.e., it was significantly attenuated in vivo but not in vitro. Similarly, a mutant in another outer membrane lipoprotein (Omp19) of *B. abortus* showed an in vitro phenotype indistinguishable from that of the parental strain by using bovine macrophages but was significantly attenuated in vivo. It was suggested that the attenuation associated with the lack of these outer membrane proteins is due to an increased sensitivity to serum complement (76). Another class of mutants that was frequently attenuated in vivo but not in vitro is related to lipopolysaccharide-altered phenotypes (32, 77). The lipopolysaccharide mutants may be more sensitive to mechanisms of the immune response in the early stage of infection. In all of these cases, there might be a contribution in vivo of lysis mediated by complement, a lectin pathway, or other host factors that influence extracellular survival (38). These processes may have an impact on *Brucella* dissemination (65) and on the number of bacteria contacting host target cells, especially during the initial phase of infection. The BepC outer membrane protein may have a similar immunomodulatory effect.

A relatively larger increase in the CFU of mice infected with the *bepC* mutant from 2 to 5 weeks p.i. than in the CFU of mice infected with the wild-type or complemented strain was observed. The comparatively larger error in the first time point may explain to some extent this difference. In addition, this difference may be due to a shift of the intracellular growth curve of the *bepC* mutant relative to the curve of the wild-type strain. During *Brucella* infection in mice, an initial increase of spleen CFU is usually observed, which is followed by a slow but steady decrease beginning around weeks 2 to 4 p.i. (76). In every case, the splenic load seems to reach a maximum and to decline later. In our case, the number of spleen CFU of wild-type *B. suis* seemed to have reached a plateau between weeks 2 and 3 p.i. In contrast, the splenic load of the *bepC* mutant showed a slight increase between these time points. This difference may be due to a lower initial inoculum of splenic cells in mice infected with the *bepC* mutant than in mice infected with the wild-type strain (because of the immunomodulatory effect described above). The maximum capacity of spleen cells to sustain *Brucella* replication may have been reached at 2 weeks for the wild-type infection but not for the infection with the *bepC* mutant.

Another plausible hypothesis for a role for BepC in extracellular survival would be more directly related with its efflux role. Toxic compounds encountered in the host during infection or other endogenously generated compounds might be removed by BepC-dependent efflux mechanisms. This, in turn, could have a direct influence on survival and dissemination within the host (65). In addition, the evidence presented in this work does not completely rule out a role for BepC in intracellular survival. BepC may participate in the efflux of antimicrobial compounds produced by macrophages induced only in vivo upon stimulation by cytokines generated locally by other cells in response to infection.

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