

## Interplay between Two RND Systems Mediating Antimicrobial Resistance in *Brucella suis*<sup>∇</sup>

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The RND-type efflux pumps are responsible for the multidrug resistance phenotype observed in many clinically relevant species. Also, RND pumps have been implicated in physiological processes, with roles in the virulence mechanisms of several pathogenic bacteria. We have previously shown that the BepC outer membrane factor of *Brucella suis* is involved in the efflux of diverse drugs, probably as part of a tripartite complex with an inner membrane translocase. In the present work, we characterize two membrane fusion protein-RND translocases of *B. suis* encoded by the *bepDE* and *bepFG* loci. MIC assays showed that the *B. suis*  $\Delta$ *bepE* mutant was more sensitive to deoxycholate (DOC), ethidium bromide, and crystal violet. Furthermore, multicopy *bepDE* increased resistance to DOC and crystal violet and also to other drugs, including ampicillin, norfloxacin, ciprofloxacin, tetracycline, and doxycycline. In contrast to the  $\Delta$ *bepE* mutant, the resistance profile of *B. suis* remained unaltered when the other RND gene (*bepG*) was deleted. However, the  $\Delta$ *bepE*  $\Delta$ *bepG* double mutant showed a more severe phenotype than the  $\Delta$ *bepE* mutant, indicating that BepFG also contributes to drug resistance. An open reading frame (*bepR*) coding for a putative regulatory protein of the TetR family was found upstream of the *bepDE* locus. BepR strongly repressed the activity of the *bepDE* promoter, but DOC released the repression mediated by BepR. A clear induction of the *bepFG* promoter activity was observed only in the BepDE-defective mutant, indicating a regulatory interplay between the two RND efflux pumps. Although only the BepFG-defective mutant showed a moderate attenuation in model cells, the activities of both *bepDE* and *bepFG* promoters were induced in the intracellular environment of HeLa cells. Our results show that *B. suis* harbors two functional RND efflux pumps that may contribute to virulence.

*Brucella* is a facultative intracellular pathogen taxonomically classified within the *Alphaproteobacteria*, along with other intracellular pathogens, such as *Rickettsia*, *Bartonella*, and several plant symbionts and pathogens (7). *Brucella* spp. are the etiological agents of brucellosis, a major zoonotic disease distributed worldwide and transmitted from domestic, farm, and wild animals to humans. *Brucella* enters the host through the nasal, oral, and pharyngeal cavities and from there is transported to the proximal lymph nodes. Early during infection, host innate immunity mechanisms contribute to reduce the initial number of infecting brucellae (26). Once in contact with the organism, *Brucella* is able to invade professional and non-professional phagocytes (10). Within the cells, *Brucella* is found in a membrane-associated vacuole called a *Brucella*-containing vacuole. The *Brucella*-containing vacuole is able to subvert the normal phagocytic pathway to form a vacuole with endoplasmic reticulum markers suitable for *Brucella* sp. replication (13, 50). This strategy helps the bacteria to escape from the bactericidal mechanisms used by the host (7).

Host barriers range from antimicrobial products of the innate immune system to toxic compounds, such as bile salts, in the gastrointestinal tract. These barriers constitute effective defense mechanisms that a pathogen must overcome to survive, colonize, and replicate. In several bacterial species, low outer membrane permeability to hydrophobic (and toxic) compounds accompanies active efflux of the noxious agent (37), allowing pathogens to eliminate toxic compounds by pumping them from the cytoplasm back to the external environment. Efflux pumps have been classified into five families according to amino acid sequence homology and their mechanisms of action. Three-component pumps of gram-negative bacteria traverse both inner and outer membranes and form a continuous channel through which the substrate is transported without a periplasmic intermediate. These systems operate with an inner membrane transporter (IM), a protein from the membrane fusion protein (MFP) family that is mostly periplasmically anchored to the inner membrane, and an outer membrane factor (OMF), which is recruited by the IM-MFP complex to form the channel (1, 4, 63). Within the IM components, those belonging to the resistance-nodulation-cell division (RND) superfamily have been shown to exhibit unusually broad substrate spectra, resulting in a multiple drug resistance phenotype (42, 55). The uncontrolled expression of RND-MFP-OMF tripartite efflux pumps has been associated with the multiple drug resistance phenotype in many clinically

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or description	Reference or source
<i>E. coli</i> DH5 $\alpha$	$\lambda^-$ $\phi$ 80 <i>dlacZ</i> $\Delta$ M15 $\Delta$ ( <i>lacZYA-argF</i> ) <i>U169 recA1 endA1 hsdR17</i> ( $r_K^- m_K^-$ ) <i>supE44 thi-1 gyrA relA1</i>	Invitrogen
<i>B. suis</i> 1330	Biotype 1; ATCC 23444T; wild-type strain	ATCC
$\Delta$ <i>bepE</i>	Deletion of <i>bepE</i> gene in 1330	This study
$\Delta$ <i>bepG</i>	Deletion of <i>bepG</i> gene in 1330	This study
$\Delta$ <i>bepE</i> $\Delta$ <i>bepG</i>	Deletion of both <i>bepE</i> and <i>bepG</i> in 1330	This study
$\Delta$ <i>bepE</i> $\Delta$ <i>bepC</i>	Deletion of both <i>bepE</i> and <i>bepC</i> in 1330	This study
Plasmids		
pGEM-T Easy	Cloning vector; Amp <sup>r</sup>	Promega Inc.
pBBAD22k	Broad-host-range vector	61
pSDM3005	pBGS19 derivative containing <i>sacB</i> gene from <i>B. subtilis</i>	66
pKGFP	Broad-host-range vector containing a promoterless <i>gfpmut3</i>	28
pK18mobsacB	Allelic-exchange vector	59
pFC26	pGEM-T Easy with a 2,553-bp fragment containing the <i>bepC</i> gene	This study
pFC70	pGEM-T Easy with a 4.6-kb fragment containing the <i>bepD</i> and <i>bepE</i> genes	This study
pFC138	pBBAD22k with a 4.6-kb fragment containing the <i>bepD</i> and <i>bepE</i> genes	This study
pSD <b><math>\Delta</math>bepE</b>	pSDM3005 with a 488-bp fragment containing 5' and 3' regions of <i>bepE</i> gene	This study
pSD <b><math>\Delta</math>bepG</b>	pSDM3005 with a 548-bp fragment containing 5' and 3' regions of <i>bepG</i> gene	This study
pFC165	pGEM-T Easy containing a deletion of the <i>bepC</i> gene with 367 bp upstream and 778 bp downstream of the <i>bepC</i> gene	This study
pFC166	pK18mobsacB containing a SphI-PstI fragment of pFC165	This study
pAV45	Broad-host-range vector containing <i>gfpmut3</i> under the control of <i>tacp</i> ; Cm <sup>r</sup>	A. Vergunst
pbepD-GFP	pKGFP with a 194-bp fragment containing the intergenic region between <i>bepD</i> and <i>bepR</i> genes ( <i>bepDE</i> promoter activity)	This study
pbepR-GFP	pKGFP with a 194-bp fragment containing the intergenic region between <i>bepD</i> and <i>bepR</i> genes ( <i>bepR</i> promoter activity)	This study
pbepRD-GFP	pKGFP with a 824-bp fragment containing the <i>bepDE</i> promoter and <i>bepR</i> gene	This study
pbepF-GFP	pKGFP with a 462-bp fragment containing the <i>bepFG</i> promoter	This study

relevant strains (48, 69). In addition, recent works have reported a role of the RND-MFP-OMF in bacterial pathogenesis (49).

Compared with other gram-negative bacteria and with the closely related *Ochrobactrum*, *Brucella* shows an outer membrane with higher permeability to hydrophobic compounds (38, 65). This characteristic has been associated with the properties of the lipopolysaccharide, which allows exposure of hydrophobic patches (41). Conversely, this feature was associated with an increase in resistance to polycations and EDTA (40). The elevated permeability to hydrophobic molecules of the *Brucella* outer membrane makes efflux pumps particularly relevant to survival within the host. In a previous study, we showed that the unique OMF identified in *Brucella suis*, named BepC (for *Brucella* efflux protein C) is involved in drug efflux. A mutational approach has shown that a BepC-deficient mutant was more sensitive to hydrophobic compounds, including antimicrobials, several dyes, and intercalators. Furthermore, survival of the *bepC* mutant was affected in the mouse model, probably due to its inability to expel toxic compounds during the course of infection (54). Since BepC must interact with an inner membrane translocase (for example, an RND-MFP complex), a question arises as to which partners of BepC are involved in the efflux of toxic compounds.

Analysis of the of *B. suis* genome sequence revealed the presence of at least six putative RND-MFP translocases that may interact with BepC. In this study, we show that two RND-MFP translocases of *B. suis* are involved in the efflux of several

toxic compounds that partially account for the defects observed in the *bepC* mutant. In addition, we present evidence of notable regulatory interplay between the two translocases.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** All *Brucella* strains used in this study were derived from *B. suis* 1330 (ATCC 23444T) and are listed in Table 1. The *Brucella* strains were grown in tryptic soy broth (TSB) (Bacto) medium or in modified minimal medium E (MME) (29) in combination with the appropriate antibiotics (chloramphenicol, 6  $\mu$ g ml<sup>-1</sup>; kanamycin, 25  $\mu$ g ml<sup>-1</sup>). *Escherichia coli* strain DH5 $\alpha$  was used as the recipient strain for all cloning experiments and was routinely grown in Luria-Bertani (Bacto) medium with the appropriate antibiotics (ampicillin, 50  $\mu$ g ml<sup>-1</sup>; chloramphenicol, 50  $\mu$ g ml<sup>-1</sup>; and kanamycin, 50  $\mu$ g ml<sup>-1</sup>). All bacterial strains were grown at 37°C and 200 rpm when needed. DNA manipulation was performed according to standard techniques (58). All experiments with viable brucellae were performed in a biosafety level 3 containment facility. Since the plasmid pFC138 with cloned *bepDE* conferred a reduction in sensitivity to some antibiotics, including tetracycline, the stocks and cultures of *Brucella* harboring cloned *bepDE* were destroyed immediately after these observations were made.

**Gene cloning and generation of unmarked deletion mutants.** The *bepD-bepE* locus, corresponding to BR0291-BR0292 according to The Institute for Genomic Research annotation (<http://www.tigr.org>) (GenBank accession no. AAN29240.1 and AAN29241.1), was amplified from a *B. suis* 1330 (ATCC 23444T) genomic DNA sample using bepDfw (CGATTTCCGTCAGTGTGGA) and bepErv (AAATACCGCCGCCGTA). MFP-RND loci were amplified using Pfx DNA Polymerase (Invitrogen Life Technologies) for accurate amplification and cloned into the pGEM-T Easy vector (Promega Inc.) according to the manufacturer's procedure to obtain pFC70 containing *bepDE*. The fidelity of the amplification reaction was confirmed by sequencing both inserts. Thereafter, a 4,667-bp fragment was excised from the pFC70 plasmid with SpeI and SphI and subcloned into the pBBAD22K plasmid (pFC138). Isogenic mutants in *bepE* or *bepG* were

generated by unmarked deletions using a similar deletion strategy. A PCR fragment of approximately 250 bp was obtained in the 5' region of *bepE* using the *bepE*5'fw (CGGGATCCGCCAATATCCCGAGCTGAC) and *bepE*5'linkrv (TCCAAGACTGCTACGATTCGCAGTGCAGGTCACGGTCA) primers for the *bepE* deletion and *bepG*5'fw (CGGGATCCTGTCCGCCACGAGTTTCTGA) and *bepG*5'linkrv (TCCAAGACTGCTACGATTCGCAGGTCACGGTCA) primers for the *bepG* deletion. A second amplicon of approximately 250 bp was generated in the 3' regions of both *bepE* and *bepG* using *bepE*3'rv (GGACTAGTCCGCATCAGGCTGTAGAA) and *bepE*3'linkfw (CGGATACGTCGTAGTCTGGAGGATCAATTCACCGACA) or *bepG*3'rv (GGACTAGTACGTTCTGGCCGTCGGTGA) and *bepG*3'linkfw (CGGATACGTCGTAGTCTGGAGGATCAATTCACCGACA) for each gene. Both fragments including the 5' and the 3' region of each gene contained complementary regions (underlined in the *bepE*5'linkrv, *bepG*5'linkrv, *bepE*3'linkfw, and *bepG*3'linkfw sequences) and were ligated by overlapping PCR and amplified using the external oligonucleotides for each sequence. The resulting fragment was cut with BamHI (sites underlined in *bepE*5'fw and *bepG*5'fw) and SpeI (*bepE*3'rv and *bepG*3'rv) and cloned into pSDM3005 (66), which carries the suicide *sacB* gene encoding the levansucrase from *Bacillus subtilis*. The plasmid was introduced into *B. suis* 1330 by electroporation. The integration of the suicide vector into the chromosome was selected for resistance to kanamycin (25 µg/ml) and sucrose sensitivity (10%) in tryptic soy agar (TSA) plates. A single colony was grown overnight in TSB without antibiotics and plated on TSA-sucrose. The sucrose-resistant and kanamycin-sensitive colonies were selected. The excision of the plasmid and the generation of the mutant strain by allelic exchange were confirmed by colony PCR and Southern blotting.

A *bepC* mutant was generated by allelic exchange of an unmarked deletion. The pFC26 plasmid harboring a 2,512-bp DNA region from *B. suis* 1330 containing *bepC* and surrounding regions was used as a template. Briefly, the oligonucleotides GCGGGATCCTGATCGGCTCTGCAAACAA and GCGGGATCCATCTCAGAACAAACGAATCCA were used in an inverse PCR outward from the start and stop codons of the *bepC* gene (boldface in the oligonucleotide sequences). The amplicon was cut with BamHI (the sites are underlined in the oligonucleotide sequences) and religated to obtain the pFC165 plasmid. The fidelity of the reaction was confirmed by sequencing. A 1,215-bp SphI and PstI fragment was cut from pFC165 and ligated into the pK18mob*sacB* vector (59), which carries the *sacB* counterselecter marker, and introduced into *B. suis* 1330 by electroporation. The integration of the pFC166 plasmid into the chromosome was selected for resistance to kanamycin (25 µg/ml) and sucrose sensitivity in TSA plates. A single colony was grown overnight in TSB without antibiotics and plated on TSA-sucrose. Sucrose-resistant (and kanamycin-sensitive) colonies were selected. The *bepC* unmarked deletion mutant was confirmed by colony PCR. To generate a *bepE* *bepC* double mutant, the pSDΔ*bepE* plasmid (Table 1) was introduced into the *bepC* unmarked deletion mutant, and the *bepE* deletion allelic exchange was generated as described above.

**Construction of GFP reporter plasmids.** The promoter regions of the *bepDE* and *bepFG* genes were amplified using *Pfu* DNA polymerase (Promega Inc.). A region containing exactly the intergenic region between *bepD* and divergently transcribed *bepR* was amplified with the oligonucleotides pr\_ *bepDf* (CGGGATCCCGCATGGTGGGAATTCGC) and pr\_ *bepDr* (CGGGATCCCGGCTTTTGTTCGCGCAT). The same region, but including the complete open reading frame coding for *BepR*, was amplified with the oligonucleotides pr\_ *bepDf* and pr\_ *bepRDr* (CGGGATCCCGGTGGGAATTCGCCCTTACT). Both amplicons were digested with BamHI (the recognition sequence [underlined] was included in the oligonucleotides) and ligated into the pKGFP (Kan<sup>r</sup>) vector (28) upstream of the promoterless green fluorescent protein (GFP) gene to generate the p*bepD*-GFP, p*bepR*-GFP, and p*bepRD*-GFP plasmids. The 462 bp upstream of *bepF* was amplified using the oligonucleotides pr\_ *bepFf* (CGGGATCCCGCAAACAGCTTGCAATTCGA) and pr\_ *bepFr* (CGGGATCCCGATGAAAATG CCGGAACCA). The amplicon was cloned into the BamHI site (the recognition site is underlined) of the pKGFP vector to generate p*bepF*-GFP. In all cases, the sequence and the correct orientation of each amplicon were confirmed by sequencing.

**Antimicrobial sensitivity assays.** The MICs for *B. suis* 1330 and for the corresponding isogenic mutants were evaluated following the recommendations established by the CLSI (Clinical Laboratory Standards Institute) standard M7-A7. The MICs were determined by the agar dilution test method using TSA (Bacto) containing graded concentrations of the drug to be tested. An inoculum of 10<sup>4</sup> CFU per spot was applied in triplicate in each plate. All plates were incubated for 48 h at 37°C. The MIC was considered to be the concentration of the first plate in which no visible growth was observed.

**Cell infection assay.** J774 or HeLa cells were seeded in 24-well plates and inoculated (at multiplicities of infection of 20:1 and 50:1, respectively) with the

wild-type *B. suis* 1330 or the respective isogenic mutants. A standard gentamicin protection assay was performed as previously described (45).

**Promoter activity assay.** In vitro assay of GFP reporter activity was performed as follows. *B. suis* harboring the indicated plasmid containing each GFP transcriptional fusion was grown for 16 h in TSB, and a 1:100 dilution of this culture was made in 2 ml of TSB. For MME, cells were grown for 48 h in TSB, and a 1:100 dilution was made in 2 ml fresh MME. In all cases, the cells were harvested at early exponential phase for fluorescence activity determination. The cultures were washed twice with phosphate-buffered saline and concentrated five times in phosphate-buffered saline. Fluorescence was determined using a Mithras LB940 multiplate reader (Berthold Technologies). The results are expressed as relative fluorescence units divided by the optical density at 600 nm (RFU/OD<sub>600</sub>). Analysis of expression in the presence of deoxycholate (DOC) was performed in MME. All determinations were made at least three times in triplicate. For in vivo observation, HeLa cells were infected with *B. suis* harboring the indicated construction as indicated above. After either 5 or 48 h, the cells were fixed with 3% paraformaldehyde for 30 min. The cells were observed with a fluorescence microscope (Leica DM IRB). Statistical significance was analyzed by one-way analysis of variance. *P* values of <0.05 were considered statistically significant.

**Bioinformatics analysis.** Computer-assisted analysis of nucleotide sequences was performed with VectorNTI (Invitrogen Life Technologies), and the Basic Local Alignment Search Tool (BLAST) algorithm (3) was used to compare the sequences with the nucleotide and amino acid sequences currently deposited in GenBank. A domain search was performed using the KEGG database (<http://www.kegg.com>). Protein sequence alignment was performed using ClustalX (30) and T\_coffee (44).

## RESULTS

**Sequence analysis.** The *B. suis* 1330 genome sequence annotation suggests the presence of six putative RND-MFP translocases. BLAST analysis (3) against the Swiss-Prot protein database (20) with the six putative proteins from the RND family showed that AAN29241.1 (*BepE*) and AAN33534.1 (*BepG*) had the strongest homology to AcrB (36) from *E. coli* (47 and 45% identity, respectively) and MexB (51) from *Pseudomonas aeruginosa* (45 and 43%, respectively). Both AcrB and MexB, together with their cognate MFP components, have been shown to confer a multidrug-resistant phenotype. *BepE* and *BepG* also showed considerable identity to the aromatic hydrocarbon TtgB transporter (56) from *Pseudomonas putida* (43 and 37%, respectively) and the broad-spectrum CmeB RND efflux pump (34) from *Campylobacter jejuni* (51 and 38%). Sequence analysis using the DAS server (14) revealed that *BepE* and *BepG* possess the classical pattern of an RND transporter (15) with 12 hydrophobic transmembrane segments (TMS) and two large loops (~300 amino acids) between TMS 1 and 2 and TMS 7 and 8. Multiple alignments of *B. suis* *BepE* and *BepG* with the MexB and AcrB RND proteins also showed the characteristic pattern of the RND family described by Putman et al. (55), with high levels of amino acid conservation between the *Bep* proteins and AcrB and MexB (Fig. 1). An open reading frame encoding an MFP protein was identified upstream of and in the same orientation as both the *bepE* and *bepG* genes (Fig. 2). Accordingly, the products of these genes were named *BepD* and *BepF*. Both *BepD* and *BepF* contain most of the traits conserved among the MFP proteins (2, 6, 17): an amino-terminal region with a hydrophilic amino acid stretch, followed by a nonconserved hydrophobic region at the amino-terminal end. However, *BepD*, but not *BepF*, showed a predicted lipoprotein signal peptidase cleavage site motif in its amino-terminal region. Similar to what was observed for the RND partners, the first homologues of *BepD* and *BepF* were the MFP proteins AcrA (36) from *E. coli* (34

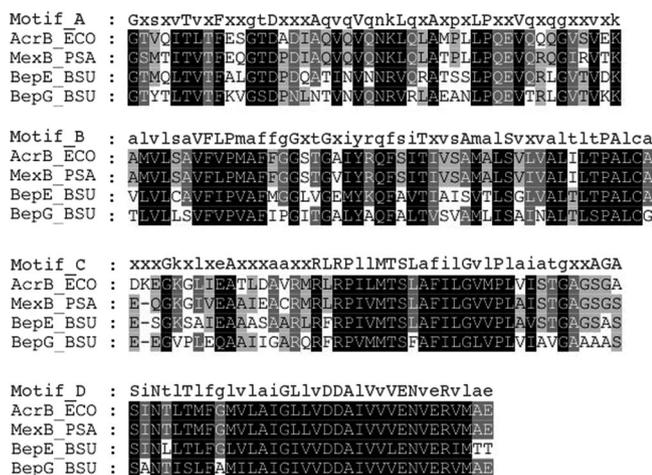


FIG. 1. Multiple-sequence alignment of the BepE and BepG proteins with AcrB from *E. coli* and MexB from *P. aeruginosa*. The regions displayed were selected according to the motifs described by Putman et al. (55) for the RND superfamily. x indicates any amino acid; capital letters denote amino acids that occur in 70% of the examined sequences; lowercase letters represent amino acids that occur in 40% of the sequences used to generate the motifs. The sequences were aligned using ClustalX and T-coffee software and manually edited with GeneDoc (43).

and 35%, respectively), AcrE from *E. coli* (34 and 32%), TtgA (56) from *P. putida* (36 and 32%), and CmeA (34) from *C. jejuni* (27 and 28%).

A divergent open reading frame (named *bepR*) with similarity to those of many known bacterial transcriptional repressors from the TetR family was located upstream of the *bepD* gene (Fig. 2). BepR has the characteristic DNA binding helix-turn-helix motif (23) near its amino-terminal region and showed significant similarity to AcrR from *E. coli* (35) (33%), TtgR from *P. putida* (18) (33%), MtrR (46) from *Neisseria gonorrhoeae* (27%), and QacR (22) from *Staphylococcus aureus* (26%), which are involved in the regulation of inner membrane efflux translocases. Genome analysis of the adjacent regions of

*bepFG* showed no evidence of a putative regulator from the TetR family or any other type.

The genes of RND-MFP translocases are mostly organized in operons. Based on the genetic arrangements of the *bepDE* and *bepFG* loci and sequence similarities with other efflux pumps of the RND family, it is very likely that both *bepDE* and *bepFG* are transcribed as operons (Fig. 2).

**BepDE and BepFG are implicated in the resistance to structurally unrelated compounds.** Our sequence analysis suggests that both the putative translocases BepDE and BepFG of *B. suis* might be implicated in antimicrobial resistance. To gain insight into the possible functions of these translocases, deletion mutants in the RND genes (*bepE* and *bepG*) were generated as described in Materials and Methods. To investigate the effects of the *bepE* and *bepG* deletions on the sensitivity profile of *B. suis* to different dyes, detergents, and antimicrobials, the MICs were assayed by the agar dilution test. Within the antimicrobial group, we tested different classes of antibiotics, including those used to treat human brucellosis. The  $\Delta$ *bepE* mutant showed increased sensitivity to DOC (MIC, 250  $\mu$ g/ml), ethidium bromide (MIC, 3.125  $\mu$ g/ml), and crystal violet (MIC, 3.125  $\mu$ g/ml) in comparison with the wild-type strain of *B. suis* (MICs, 1,000, 25, and 6.25  $\mu$ g/ml, respectively) (Table 2). We did not observe differences between the MICs of the rest of the evaluated drugs for the wild type and the  $\Delta$ *bepE* mutant (Table 2). The  $\Delta$ *bepG* mutant showed no differences in the resistance profile compared with that of the wild-type strain (data not shown). However, the mutant in both RND transporters ( $\Delta$ *bepE*  $\Delta$ *bepG*) showed a marked increase in sensitivity to nalidixic acid (MIC < 0.48  $\mu$ g/ml) and sodium dodecyl sulfate (SDS) (MIC, 62.5  $\mu$ g/ml) compared with *B. suis* 1330 (MICs, 15.625 and 1,000  $\mu$ g/ml, respectively), two compounds for which no differences were observed between the single mutants and the wild type. Interestingly, the  $\Delta$ *bepE*  $\Delta$ *bepG* double mutant showed higher sensitivity to DOC (MIC, 62.5  $\mu$ g/ml) than the single  $\Delta$ *bepE* isogenic mutant (MIC, 250  $\mu$ g/ml) (Table 2).

The resistance of the *B. suis*  $\Delta$ *bepE* mutant to ethidium bromide was restored with pFC138 containing the *bepDE* lo-

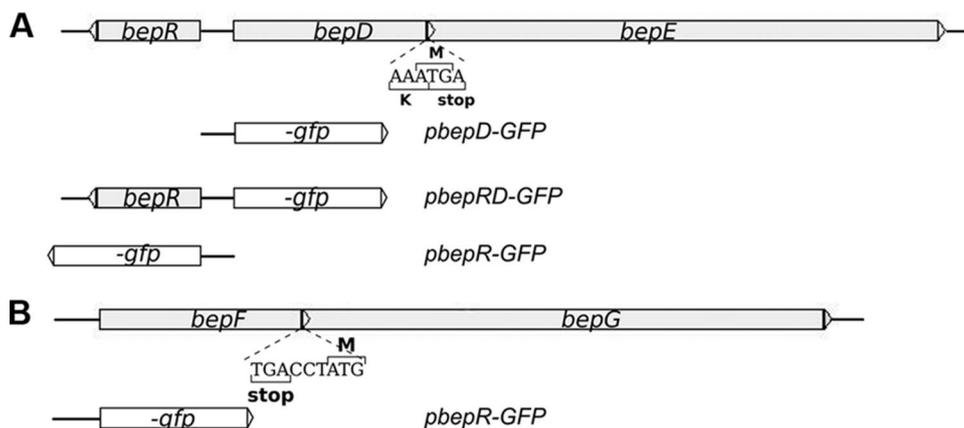


FIG. 2. Schematic representation of the *bepDE* (A) and *bepFG* (B) genomic sequences from *B. suis*. The open arrowheads represent coding sequences and indicate the sense of transcription. The overlapping region between *bepD* and *bepE* and the proximity of *bepF* and *bepG* are shown. The *bepF* and *bepG* genes are separated by 2 bases. In both cases, the organization suggests the presence of an operon. Transcriptional fusions to the GFP gene used to analyze in vitro and in vivo *bepDE* and *bepFG* expression are shown.

TABLE 2. MIC determinations of antimicrobials, dyes, and detergents for *B. suis* 1330 and derivative strains

Drug	MIC <sup>a</sup> (μg/ml) for <i>B. suis</i>			
	1330	<i>ΔbepE</i>	<i>ΔbepE</i> (pFC138)	<i>ΔbepE</i> <i>ΔbepG</i>
<b>Antimicrobials</b>				
Ampicillin	0.78	0.78	<b>6.25</b>	0.78
Chloramphenicol	>25	>25	>25	>25
Ciprofloxacin	0.313	0.313	<b>0.626</b>	ND
Doxycycline	0.325	0.325	<b>0.650</b>	ND
Erythromycin	0.078	0.078	0.078	ND
Nalidixic acid	15.625	15.625	15.625	<b>0.48</b>
Norfloxacin	0,625	0,625	<b>1.25</b>	ND
Novobiocin	12.5	12.5	<b>50</b>	12.5
Thiamphenicol	3.125	3.125	<b>50</b>	3.125
Tetracycline	<0.19	<0.19	<b>1.56</b>	<0.19
Trimethoprim	50	50	50	50
Polymyxin B	17.5	17.5	<b>140</b>	17.5
Rifampin	0.15	0.15	0.15	ND
Streptomycin	0.52	0.52	0.52	ND
<b>Dyes</b>				
Acriflavine	12.5	12.5	<b>50</b>	12.5
Crystal violet	6.25	<b>3.125</b>	<b>100</b>	3.125
Ethidium bromide	25	<b>3.125</b>	12.5	3.125
<b>Detergents</b>				
DOC	1,000	<b>250</b>	<b>4,000</b>	<b>62.5</b>
SDS	250	250	<b>1,000</b>	<b>62.5</b>

<sup>a</sup> The MICs of the different antimicrobials were determined by the agar dilution test in triplicate in TSA following CLSI standards. Three different independent experiments gave similar results. The numbers in boldface indicate higher or lower MICs than those for the wild-type *B. suis* 1330. ND, not determined.

cus. Moreover, resistance to DOC and crystal violet increased severalfold in the presence of multicopy *bepDE* compared with the wild-type strain. In addition, the pFC138 plasmid conferred increased resistance to other compounds to which the *B. suis* *ΔbepE* (and the *ΔbepG*) single mutant showed no phenotype. Among these compounds, we found an increase in the resistance of *B. suis* to ampicillin, norfloxacin, ciprofloxacin, novobiocin, polymyxin B, tetracycline, doxycycline, thiamphenicol, acriflavine, and SDS. Taken together, these observations indicate that the RND-MFP BepDE translocase is involved in resistance to several unrelated toxic compounds. In addition, the effect of the *bepG* deletion in the *bepE* mutant background on the sensitivity of *B. suis* to nalidixic acid, SDS, and DOC suggests that the BepFG translocase also contributes to resistance to some drugs.

**Resistance conferred by BepDE is dependent on BepC.** RND transporters require an OMF to expel the substrate. As was noted previously, the downstream regions of *bepDE* and *bepFG* do not contain a gene that might encode an OMF. In fact, our previous report indicated that BepC, which is encoded by an open reading frame separated from those of inner membrane translocases, is the unique OMF present in *B. suis* (54). In order to investigate whether the resistance conferred by BepDE requires BepC to exert its function, we made use of the increased resistance to DOC conferred by *bepDE* cloned into pFC138 and tested whether inactivation of the *bepC* gene prevents this effect. We generated a *ΔbepE ΔbepC* double mutant, which was transformed with the pFC138 plasmid. As expected, the sensitivity to DOC of the *B. suis* *ΔbepE ΔbepC* double

mutant (MIC, 31.25 μg/ml) was significantly higher than that of the *B. suis* *ΔbepE* single mutant (MIC, 250 μg/ml). The pFC138 plasmid did not increase the resistance to DOC of the *B. suis* *ΔbepE ΔbepC* double mutant (MIC, 31.25 μg/ml), strongly suggesting that BepC is necessary for BepDE to confer resistance. The increased sensitivity to DOC of the *ΔbepE ΔbepC* double mutant may account for the sensitivity phenotype of the mutant defective in both BepDE and BepFG RND translocases (*ΔbepE ΔbepG*) (Table 2), suggesting that both BepDE and BepFG interact with BepC to form tripartite pumps.

**Survival of RND mutants in macrophages J774 and HeLa cells.** To determine the roles of BepDE and BepFG translocases in the survival of *B. suis* in model cells, the abilities of *bepE* and *bepG* isogenic mutants to enter and replicate in J774 macrophages and HeLa cells were analyzed. The wild-type strain showed the typical biphasic curve of viable brucellae recovered after infection of cultured cells. A slightly lower number of CFU of the BepE-deficient strain than of the wild type were recovered from HeLa cells after 5 h of infection, but the CFU level was able to recover after 24 h, displaying a level similar to that of the wild-type strain (Fig. 3A). Similarly, no significant differences in CFU numbers between the wild type and the *ΔbepE* mutant were observed in J774 macrophages (Fig. 3B). The *B. suis* *ΔbepG* mutant showed a lower number of recovered brucellae than the wild-type strain recovered from HeLa cells after 5, 24, and 48 h of infection (Fig. 3A). This

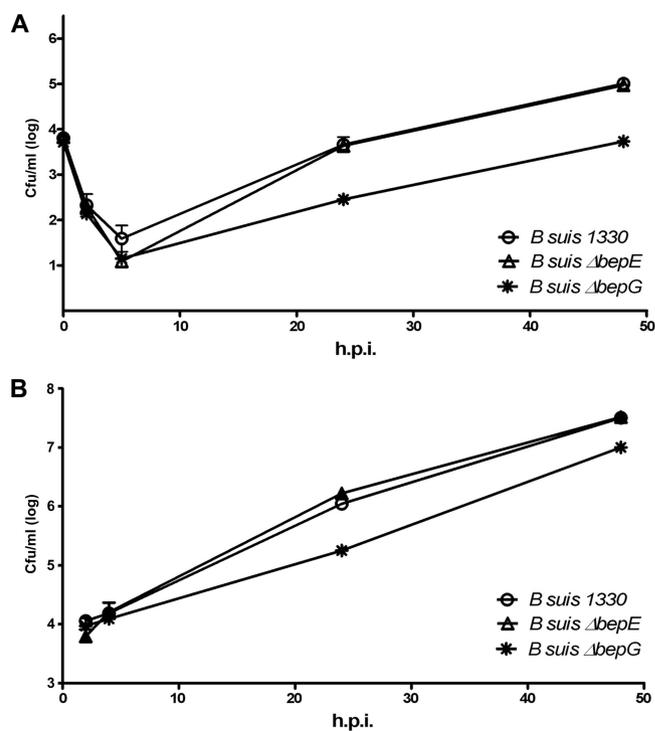


FIG. 3. Intracellular replication of *B. suis* *ΔbepE* and *ΔbepG* mutants in HeLa cells (A) and murine J774 macrophages (B). The numbers of viable intracellular bacteria were determined 2, 5, 24, and 48 h postinfection (p.i.). The experiment was repeated three times, and the data presented are the results of a representative experiment done in triplicate. The results are expressed as geometric means and standard deviations (error bars) of plate counts.

suggests that the  $\Delta bepG$  mutant is more sensitive to the initial intracellular killing than the wild type. The intracellular survival of the *B. suis*  $\Delta bepE \Delta bepG$  double mutant did not differ from that of the  $\Delta bepG$  isogenic mutant (data not shown), indicating that in this case there was not the same additive effect that we found for the sensitivity phenotype to certain drugs (Table 2). As in HeLa cells, a lower number of CFU of the *B. suis*  $\Delta bepG$  mutant than of the wild-type strain were recovered from J774 macrophages (Fig. 3B), which reinforces the idea that BepFG is required for optimal intracellular growth.

***bepDE* and *bepFG* expression in vitro and in vivo.** To further assess the biological significance of BepDE and BepFG, in vitro and in vivo expression analyses were carried out. Sequence analysis of the *bepDE* upstream region showed the presence of a divergent gene named *bepR*, encoding a putative repressor from the TetR family that may be involved in the regulation of *bepDE*. Our analysis using the promoter prediction software BPROM (SoftBerry Inc.) indicated the presence of two overlapping and divergent putative promoters within the 172-bp intergenic region between *bepR* and *bepD* (Fig. 2). To investigate the expression (in vitro and in vivo) of both *bepDE* and *bepR*, we constructed three different transcriptional fusions to the GFP reporter gene in the pKGFP vector containing the promoterless *gfpmut3* reporter gene (28). The GFP gene was placed under the control of the putative promoters within the intergenic region between the *bepR* and *bepD* genes in both 5'-3' (pbepD-GFP) and 3'-5' (pbepR-GFP) orientations (Fig. 2A) to analyze the expression of *bepDE* and *bepR*, respectively. In order to prevent a stoichiometric imbalance between the promoter activity of *bepDE* expressed in a multicopy plasmid and a single chromosomal copy of the putative repressor gene (*bepR*), we also generated a construction with the *bepDE* transcriptional fusion, together with a complete copy of the *bepR* gene (pbepRD-GFP) (Fig. 2A).

To validate the reporter expression analysis, quantification of the fluorescence levels of *B. suis* harboring the pKGFP promoterless vector (28) and the pAV45 plasmid containing the GFP gene under the control of the *tac* promoter (Annette Vergunst, personal communication) was performed over a time course experiment. The normalized RFU count of *B. suis* containing pAV45 was fivefold higher ( $5 \times 10^4$  RFU/OD<sub>600</sub>) than that of bacteria containing pKGFP ( $1 \times 10^4$  RFU/OD<sub>600</sub>) after 10 h of incubation, indicating that pKGFP is suitable to analyze promoter activities in *B. suis*.

The expression of the *bepDE* promoter (in pbepD-GFP) was sixfold higher than that of the negative control in both rich and minimal media (Fig. 4A). However, with the pbepRD-GFP plasmid, which also carries the *bepR* gene, we saw only fluorescence levels similar to those of the negative control in both media tested (Fig. 4A). These results strongly suggest that BepR acts as a repressor, preventing *bepDE* expression. As judged by the effect of cloned *bepR* on *bepDE* expression, a considerable level of *bepR* expression was predicted. In fact, significant expression of the *bepR* promoter was observed in both media (Fig. 4A).

To analyze the relevance of BepDE in vivo, we sought to determine whether *bepDE* is expressed in HeLa cells by using the pbepRD-GFP reporter plasmid. HeLa cells infected with *B. suis* harboring pbepRD-GFP were fixed after 5 and 48 h postinfection and examined by fluorescence micros-

copy. HeLa cells were also infected with brucellae containing the empty pKGFP vector as a negative control. *B. suis* harboring pbepRD-GFP showed consistent reporter expression, as judged by the fluorescent bacteria observed after 5 h (data not shown) and 48 h (Fig. 4B) of infection. Reporter expression was not observed in HeLa cells infected with *B. suis* harboring pKGFP (data not shown). This leads to the conclusion that the intracellular environment induces *bepDE*. The same strategy was used to analyze the expression of *bepR* in HeLa cells. *B. suis* harboring pbepR-GFP also showed a clear level of fluorescent bacteria (Fig. 4C), indicating that the repressor gene is also expressed in vivo within HeLa cells.

To evaluate the in vitro expression of *bepFG*, the GFP reporter gene was placed under the control of the 462-bp upstream region of *bepF* to generate the pbepF-GFP plasmid. Expression from the *bepFG* putative promoter over the level of the pKGFP-containing bacteria was not detected in rich or minimal medium, indicating that *bepFG* is not expressed in vitro under these conditions (Fig. 4D). However, *bepFG* promoter expression was clearly induced intracellularly in HeLa cells after 5 h (data not shown) or 48 h (Fig. 4E) of infection.

We were unable to identify any putative regulator in the genome sequences upstream or downstream of *bepFG*. We showed that a single deletion of the *bepG* gene had no effect on the sensitivity profile of *B. suis*. Nevertheless, the  $\Delta bepE \Delta bepG$  double mutant showed a further reduction in resistance to some drugs (Table 2). One possibility is that disruption of *bepE* induces *bepFG* expression, contributing to resistance to some drugs in the absence of BepDE. In this scenario, an additional mutation in *bepG* might result in a more severe phenotype. To test this hypothesis, we transformed the *B. suis*  $\Delta bepE$  isogenic mutant with the reporter fusion cloned into pbepF-GFP to evaluate the expression of *bepFG* in a *bepDE*-defective background. Interestingly, the promoter activity of *bepFG* significantly increased in the  $\Delta bepE$  mutant background (Fig. 4D). This observation indicates that the absence of the BepDE translocase induces *bepFG* expression.

**Expression of *bepDE* is induced in the presence of the DOC bile salt component.** We showed that the activity of the *bepDE* promoter in the presence of stoichiometric amounts of *bepR* is not significant in either rich or minimal medium. Likewise, expression of the *bepFG* promoter in these media was also below the level of the negative control. Detergent activities of conjugated and unconjugated bile salts encountered in the intestine are a primary defense mechanism against pathogenic bacteria, and DOC (an unconjugated bile salt) is one of the major components of bile (8). Since DOC seems to be a substrate of both BepDE and BepFG and might be a physiological substrate of these pumps encountered by *Brucella* in the intestine, we aimed to evaluate whether this compound could induce the expression of the RND efflux pumps. Surprisingly, after 12 h of incubation in the presence of 40  $\mu$ M DOC, reporter expression from the *bepDE* promoter cloned into pbepRD-GFP showed a ninefold increase compared with the expression in the absence of DOC (Fig. 5). In addition, a significant increase in the activity of the *bepR* promoter was observed (Fig. 5). Conversely, the *bepFG* promoter was not induced by DOC (Fig. 5). As expected, expression of *bepFG* was also induced by the *bepE* deletion in the presence of DOC

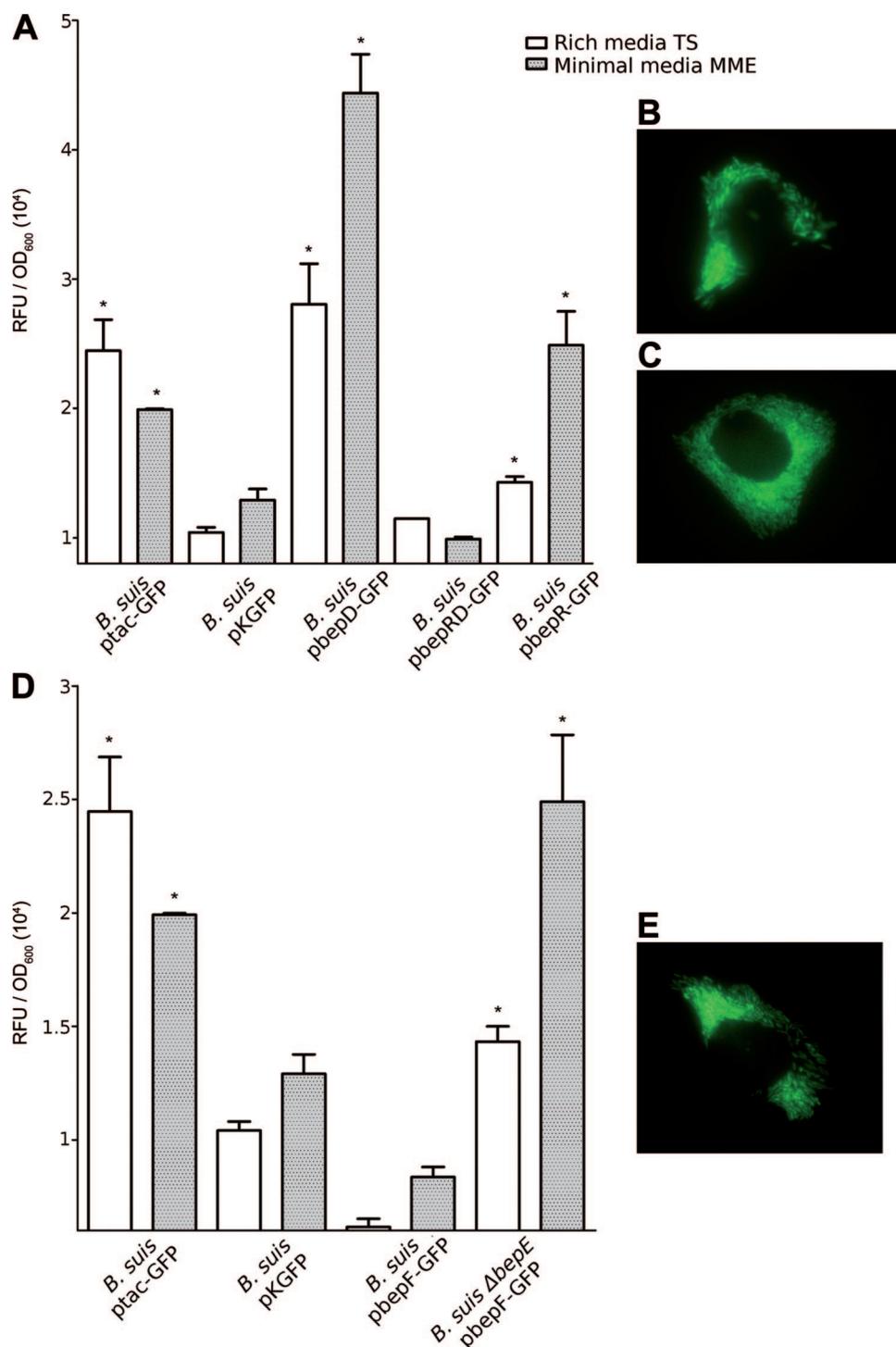


FIG. 4. In vitro and in vivo expression of *bepDE* and *bepR* (A, B, and C) and *bepFG* (D and E). Bacteria containing the GFP promoter fusion were incubated in rich (TS) or minimal (MME) medium for 12 h, and the fluorescence was determined in a Mithras LB 940 (Berthold Technologies). The results are expressed as RFU relative to the OD<sub>600</sub> plus the standard deviation of each bacterial culture. All samples were compared with the negative control, pKGFP (\*,  $P < 0.05$ ). (B, C, and E) Active expression of intracellular bacteria after 48 h of infection with *B. suis* pbepRD-GFP (B), *B. suis* pbepR-GFP (C), and *B. suis* pbepF-GFP (E).

(not shown), confirming that BepFG may compensate for the absence of BepDE (see above). The presence of 44  $\mu$ M SDS did not activate the *bepDE* promoter (Fig. 5), implying that the activation mediated by DOC is substrate specific and is not due merely to the detergent activity of DOC.

## DISCUSSION

**BepDE and BepFG: two RND-MFP pumps.** Detailed analysis of the BepE and BepG peptide sequences suggests that both proteins are complete and functional RND transporters.

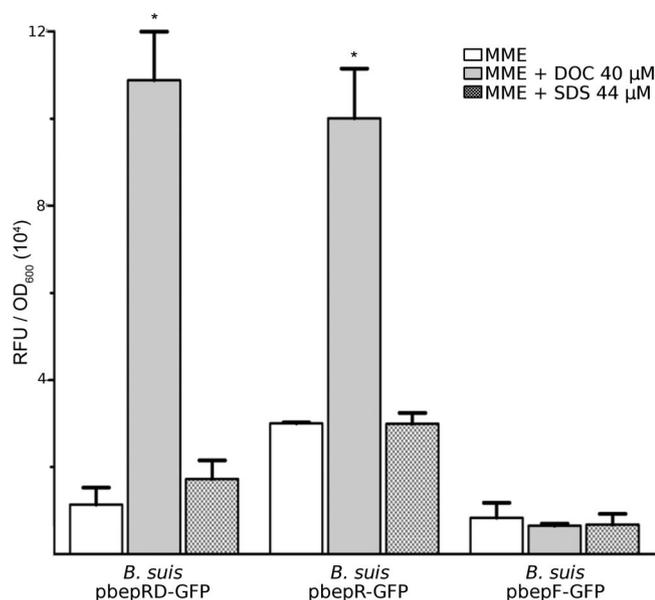


FIG. 5. Induction of *bepDE* expression by DOC. Samples were incubated for 12 h in the presence or absence of 40  $\mu$ M DOC or 44  $\mu$ M SDS. The results are expressed as RFU relative to the OD<sub>600</sub> plus the standard deviation of each bacterial culture. \*,  $P < 0.05$  compared with the same samples incubated in the absence of substrate.

Comparison of these proteins with the AcrB (36) and MexB (31) proteins showed high conservation of key residues involved in protein structure (19, 39, 70) and proton relay transfer (24) that sustain a possible role of BepE and BepG in multidrug efflux. A similar conclusion was drawn for the BepD and BepF MFP components. The deletion approach confirmed that the BepDE and probably the BepFG translocases are involved in resistance to several compounds. Deletion of *bepE* makes *B. suis* more sensitive to DOC, crystal violet, and ethidium bromide. In contrast, deletion of *bepG* did not confer any particular sensitivity phenotype. However, the  $\Delta$ *bepE*  $\Delta$ *bepG* double mutant showed a more severe sensitivity phenotype to DOC than the single  $\Delta$ *bepE* mutant. Furthermore, the  $\Delta$ *bepE*  $\Delta$ *bepG* double mutant was clearly more sensitive to SDS and nalidixic acid than the wild-type *B. suis*, two drugs for which no differences were observed between the single mutants and the wild-type strain. Additional evidence of multidrug efflux mediated by BepDE was the effect of multicopy *bepDE* in the  $\Delta$ *bepE* mutant, conferring an increase in the resistance of *B. suis* to ampicillin, norfloxacin, ciprofloxacin, novobiocin, polymyxin B, tetracycline, doxycycline, thiamphenicol, acriflavine, and SDS compared with the *B. suis* wild-type strain. Taken together, these results show that BepDE is able to confer resistance to several toxic compounds, such as dyes, detergents, and antibiotics. Moreover, the sensitivity phenotypes of the double mutant are consistent with the idea that efflux of some drugs in the *bepE* single mutant of *B. suis* might be partially or totally compensated for by BepFG. Therefore, BepFG may contribute to the resistance to some drugs, like DOC, SDS, and nalidixic acid, in the absence of BepDE. The interplay between the two translocases was further supported by the in vitro expression analysis (see below).

BepC is the unique OMF harbored in the *B. suis* genome.

This is a situation similar to that seen in *E. coli*, where RND-MFP translocases rely on the presence of TolC to exert their functions (33, 67). On the other hand, four out of the six RND-MFP translocases described in *P. aeruginosa* are encoded by an MFP-RND-OMF operon (52). We have previously shown that BepC is responsible for resistance to several drugs, dyes, and detergents (54). Our genetic analysis showed that BepDE requires BepC to confer DOC resistance. Since the *bepC* mutant showed an augmented sensitivity to DOC compared with that of the single *bepE* mutant, another translocase may be a partner of BepC contributing to DOC efflux. The further sensitivity to DOC of the  $\Delta$ *bepE*  $\Delta$ *bepG* double mutant supports the idea of BepFG as a second partner of BepC to exclude DOC and probably other compounds.

**Regulation: in vitro and in vivo expression of *bepDE* and *bepFG*.** To give more insight into the roles of the RND-MFP translocases, we investigated the regulation of these pumps by using transcriptional fusions of GFP to the *bepDE* and *bepFG* putative promoters. At first glance, the context of *bepDE* showed the presence of a gene encoding a putative repressor from the TetR family named BepR, while the regions around *bepFG* did not suggest the presence of any putative regulator. No *bepFG* expression was observed in vitro in either rich or minimal medium. We found that *bepDE* promoter activity was completely repressed by BepR in both culture media. However, the expression of both promoter fusions showed early induction in the intracellular environment of HeLa cells, suggesting that both the BepDE and BepFG pumps are functional in vivo. Surprisingly, only the  $\Delta$ *bepG* mutant showed a moderate but reproducible attenuation in HeLa cells. The observed interplay between these systems would explain these data in part, i.e., that both BepDE and BepFG can transport (or efflux) a set of substrates but the transport of other, more specific physiological substrates inside the cell can be performed only by BepFG. Hence, although there are some interchangeable functions between BepDE and BepFG, the transport of a specific substrate by BepFG would explain the moderate attenuation of the  $\Delta$ *bepG* mutant in model cells. Our data are consistent with the idea that both BepDE and BepFG might protect brucellae from some toxic intracellular compound. Alternatively, the possibility exists that these RND systems are involved in the transport of other metabolites within the host cell. It has been proposed that RND pumps participate in the transport of autoinducers, such as acyl-homoserine lactones (11, 47). In fact, *Brucella* produces a C<sub>12</sub> acyl-homoserine lactone that may need a transport mechanism to exert its function as a signaling molecule (62).

The mechanism by which the expression of *bepDE* and *bepFG* is induced within the host cell is unknown. In some cases, the expression of RND efflux pumps has been shown to be responsive to general stress conditions or induced by a specific substrate recognized and transported by the pump (23). During cell infection, early phagosome acidification (9, 27, 53) and the oxidative burst (21) generated by the macrophage are necessary for virulence gene activation in *Brucella* spp. Our preliminary data indicate that the expression from the *bepDE* or *bepFG* promoter is not induced at low pH, and only a 10% increase in the expression of *bepDE* was observed in the presence of hydrogen peroxide. Further studies are required to

better define the effects of these or other stress conditions on *bepDE* and *bepFG* expression.

***bepDE* induction by DOC.** Although a considerable level of *bepDE* promoter activity was observed with the reporter plasmid that lacks a copy of *bepR*, expression of *bepDE* was strongly repressed in the presence of equivalent amounts of *bepR*, indicating that BepR is a local repressor of *bepDE* expression. Interestingly, 40  $\mu$ M DOC released the repression mediated by BepR. The simplest interpretation, based on evidence accumulated for other TetR regulators (23), is that DOC binds directly to BepR, promoting BepR release from the *bepDE* promoter. In other species, DOC and other components of bile salts were shown to be efficient inducers of RND systems (32, 57). SDS, which is also a detergent and a substrate of BepDE, was not able to activate *bepDE* expression, indicating that induction by DOC is substrate specific.

One puzzling observation is the increased expression of *bepR* in the presence of DOC. The concomitant increase of *bepR* expression, along with *bepDE*, was somehow unexpected. However, a similar observation was described in the regulatory circuit of other RND systems. Induction conditions increased the transcription of the *acrR* repressor gene, along with the *acrAB* efflux genes (35). It was proposed that AcrR functions as a secondary modulator to fine-tune the level of *acrAB* transcription to prevent an excess of *acrAB* expression and, as a consequence, deleterious effects on cell viability. In the TetR/TetA system, the increased expression of the *tetR* repressor gene in the presence of the inducer ensures strict control of *tetA* transcription at all times (25). It has also been reported that the expression of *bpeR* of *Burkholderia pseudomallei* was induced in late exponential phase, probably due to the accumulation of a metabolite that is a substrate of the BpeAB-OprB efflux system (12).

It is becoming clear that DOC and other bile salts may represent physiological substrates of BepDE and BepFG. Brucellosis can be acquired by ingestion of contaminated products. Therefore, during the initial stages of infection, *Brucella* is exposed to antimicrobials, including bile salts and fatty acids. The RND pumps may contribute to resistance to DOC and other bile components, along with the bile salt hydrolase that has recently been described in *Brucella abortus* (16). Although our results strongly suggest a role of BepDE and BepFG in protecting bacteria from some intracellular antimicrobial compound, a function of the RND pumps in survival during the extracellular stage of *Brucella* infection cannot be ruled out.

***bepFG* expression is induced in the absence of BepDE.** It was interesting to find that disruption of *bepE* induces *bepFG* expression. A possible explanation for this activation is that *bepDE* and *bepFG* are under the control of a common DOC-independent level of regulation, resulting in a compensatory mechanism in the absence of BepDE. We hypothesize that an unknown global regulator may mediate this compensatory mechanism. The affinity of this hypothetical regulator for the *bepDE* promoter would be significantly higher than for the *bepFG* promoter. Thus, upon activation of the global regulator, an initial increase of *bepDE* expression is induced. In the absence of BepDE, the inducer is accumulated in the cytoplasm, favoring the ability of the regulator to bind and activate the *bepFG* promoter.

**Resistance to antibiotics mediated by RND systems in *B. suis*.** The resistance profile of *B. suis* in the presence of multicopy *bepDE* indicates that BepDE is able to exclude antibiotics, including tetracycline, doxycycline, and fluoroquinolones.

Antibiotic resistance associated with mutations in repressors from the TetR family has been previously described in *E. coli* (68) and *Klebsiella pneumoniae* (60). This implies that a “resistance” phenotype to tetracyclines, fluoroquinolones, or other antibiotics due to the selection of a strain with *bepR* mutations can be predicted. Therefore, special attention should be paid to a possible effect of *bepDE* overexpression on resistance to antibiotics, such as tetracyclines and fluoroquinolones, two broad types of antibiotics used in clinical treatment for brucellosis. In fact, resistance to fluoroquinolone in two *Brucella* strains has been associated with an energy-dependent efflux mechanism (64). The agents recommended by the World Health Organization guidelines for more than a decade have included rifampin (rifampicin) or streptomycin plus doxycycline for the management of human brucellosis (5). Although tetracyclines have been proven to be efficient in *Brucella* treatment during randomized trials, our analyses suggest that a tetracycline resistance mechanism in *Brucella* is possible.

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