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# The Ton System, an ABC Transporter, and a Universally Conserved GTPase Are Involved in Iron Utilization by *Brucella melitensis* 16M

Isabelle Danese,[1]* Valerie Haine,[1] Rose-May Delrue,[1] Anne Tibor,[1] Pascal Lestrate,[1] Olivier Stevaux,[1]† Pascal Mertens,[1] Jean-Yves Paquet,[1]‡ Jacques Godfroid,[2] Xavier De Bolle,[1] and Jean-Jacques Letesson[1]

*Unité de Recherche en Biologie Moléculaire, University of Namur, Namur,[1] and Veterinary and Agrochemical Research Centre, Brussels,[2] Belgium*

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***Brucella* spp. are gram-negative intracellular facultative pathogens that are known to produce 2,3-dihydroxybenzoic acid (DHBA), a catechol siderophore that is essential for full virulence in the natural host. The mechanism of DHBA entry into *Brucella* and other gram-negative bacteria is poorly understood. Using mini-Tn*5Kmcat* mutagenesis, we created a transposon library of *Brucella melitensis* 16M and isolated 32 mutants with a defect in iron acquisition or assimilation. Three of these transposon mutants are deficient in utilization of DHBA. Analysis of these three mutants indicated that the ExbB, DstC, and DugA proteins are required for optimal assimilation of DHBA and/or citrate. ExbB is part of the Ton complex, and DstC is a permease homologue of an iron(III) ABC transporter; in gram-negative bacteria these two complexes are involved in the uptake of iron through the outer and inner membranes, respectively. DugA is a new partner in iron utilization that exhibits homology with the bacterial conserved GTPase YchF. Based on this homology, DugA could have a putative regulatory function in iron assimilation in *Brucella*. None of the three mutants was attenuated in cellular models or in the mouse model of infection, which is consistent with the previous suggestion that DHBA utilization is not required in these models.**

In various environments, iron is present in two states, and its availability is affected by pH and aeration. Fe(III) oxides present in aerobic conditions are very insoluble at neutral pH (recently reviewed in references 5 and 29). In contrast, Fe(II) is relatively soluble, and obtaining iron is a much easier task for bacteria growing anaerobically. The problems that bacteria face in acquiring sufficient iron from their surroundings are particularly acute for pathogens. Bacteria are able to deal with this iron restriction imposed by their environment, e.g., through the mechanism of ferric iron capture. They may produce iron(III)-scavenging siderophores or directly bind heme- and iron-containing compounds from the host.

In an aerobic environment, the concentration of available iron(III) is so low that energy is required for transport of the iron. An energized high-affinity iron(III) (as well as heme and vitamin $B_{12}$) transporter is conserved in many gram-negative bacteria. This system is composed of different outer membrane-specific receptors, all of which are linked to an inner membrane complex that contains at least the TonB, ExbB, and ExbD proteins (35), and of a specific periplasmic binding protein associated with an ABC transporter. It has been proposed that the binding of a siderophore to the gated outer membrane receptor enhances the interaction of this receptor with TonB (11, 48). The energy required for TonB's gatekeeper activity is provided by the proton motive force, and ExbB and ExbD proteins are essential for this function (1, 13, 39). After this, the ferrisiderophore is transported into the cytoplasm by a specific periplasmic binding protein associated with an ABC transporter. In the cytoplasm, iron is finally dissociated from the siderophore (e.g., following iron reduction). Little is known about the other steps of the pathway, and nothing is known about the recycling, storage, and modification of the siderophore; however, one pathway for secretion of the enterobactin of *Escherichia coli* has recently been shown to be mediated by the membrane exporter EntS protein (28).

*Brucella* spp. are facultative intracellular gram-negative bacteria that are responsible for worldwide zoonotic diseases. These pathogens induce abortion and sterility in domestic mammals, and some of the species, including *Brucella melitensis*, are responsible for a chronic fever in humans (25). *Brucella* can replicate in macrophages and in nonprofessional phagocytes. During its life cycle, *Brucella* probably uses different siderophores and host iron-containing compounds in the various environments that it encounters. The catecholic siderophores 2,3-dihydroxybenzoic acid (DHBA) and brucebactin, a complex siderophore structurally based on DHBA, have been reported to be produced and utilized as iron sources by *Brucella* (7, 31, 45). In addition, *Brucella abortus* also has the ability to acquire the most abundant source of iron in mammals, heme (2). The mechanism of entry of ferric DHBA through the *Brucella* envelope has not been studied, and compared to the more complex catechol-based siderophore uptake, the capture of ferric DHBA has only been marginally studied in other bacteria (32, 61). Moreover, *B. abortus* does not use enterochelin, deferoxamine (DFA), ferrichrome, or rhizobactin as an exogenous siderophore, and except for heme,

* Corresponding author. Mailing address. Unité de Recherche en Biologie Moléculaire (URBM), University of Namur, 61 Rue de Bruxelles, B-5000 Namur, Belgium. Phone: 081 72 42 83. Fax: 081 72 42 97. E-mail: Isabelle.Danese@fundp.ac.be.

† Present address: UCSF Cancer Center, San Francisco, CA 94143-0534.

‡ Present address: Entité des Eaux et Forêts, University of Gembloux, B-5030 Gembloux, Belgium.

nothing is known about utilization of host iron-containing compounds by *Brucella* spp. (2).

The role of iron metabolism in *Brucella* virulence is not well understood. *Brucella* can grow within macrophages in which iron has been depleted if gamma interferon or DFA is added (37, 38). The *ent* operon encoding DHBA and brucebactin synthesis does not appear to play an essential role in intracellular survival and in virulence in the mouse model (7, 31). However, in pregnant cattle, DHBA production is essential for complete maternal and fetal colonization (8). The *fbpA* gene, a homologue of a gene encoding an iron binding protein, may be involved in iron assimilation in the host, as it was shown to be expressed in macrophages (26).

In this report, we describe isolation and characterization of *B. melitensis* DHBA uptake transposon mutants. Our results indicate that the *exbB*, *dst*, and *dugA* genes are implicated in the use of iron sources. This report provides the first description of an iron utilization complex in *Brucella*; we describe the Ton system, an iron(III) ABC transporter, and a new partner, DugA, a probable GTPase. Since mutants with mutations in iron acquisition pathways are often described as attenuated (3, 22, 36, 57), we evaluated the survival of these mutants in bovine macrophages and in HeLa cells. The three mutants used were also tested for virulence in BALB/c mice. In these ex vivo and in vivo assays, the mutants were not attenuated compared to the wild-type strain.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *B. melitensis* 16M Nal$^r$, a spontaneous nalidixic acid-resistant mutant, is the wild-type strain that was used throughout this study; this strain was received from J.-M. Verger, Laboratoire de Pathologie Infectieuse et d'Immunologie, Institut National de Recherche Agronomique, Nouzilly, France (71). *E. coli* XL1-Blue (Stratagene) and strain S17-1 were also used in this study (63). The vectors used in this work were pUTmini-Tn*5Kmcat*, a plasmid that contains the transposase gene separated from the transposon and is unable to replicate in *Brucella* spp. (18); pBBR1MCS4, a replicative vector in *Brucella* (41); pCAT19, a pUC19 derivative containing a chloramphenicol acetyltransferase-encoding cassette (27); and plasmids pARBV, pBBR*dugA*1, and pBBR *dugA*2, which are described below.

**Media and antibiotics.** *Brucella* strains were grown on tryptic soy agar containing 0.1% yeast extract, in 2YT medium (10% yeast extract, 10 g of tryptone per liter, 5 g of NaCl per liter), and in Luria-Bertani (LB) medium (liquid or solid [13 g of agar per liter]). *E. coli* strains were manipulated as described by Sambrook et al. (25). To make LB agar iron limiting, we added the iron chelator 2,2′-dipyridyl (DIP) (0.2 μM; Sigma) or the ferric iron chelator ethylenediamine di(*o*-hydroxyphenylacetic acid) (EDDHA) (0.65 mM; Sigma). At these concentrations wild-type strain *B. melitensis* 16M was not inhibited. DIP and EDDHA are routinely used to identify and characterize iron acquisition mutants of a variety of bacteria, particularly brucellae (31, 55, 62).

The antibiotic concentrations used were as follows: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; nalidixic acid, 25 μg/ml; gentamicin, 50 μg/ml, and tetracycline, 12.5 μg/ml.

**Iron utilization assays.** Liquid growth assays to test the ability of *B. melitensis* mutants to utilize DHBA as a sole source of iron were performed as follows. Cells were grown in liquid LB medium containing the iron chelator DFA (500 μM; Sigma) alone or with DHBA (500 μM) (44). At this concentration of DFA, the growth of the wild-type strain was greatly reduced. All bacterial cultures were incubated at least in triplicate at 37°C; samples were taken at different times, and growth was determined by measuring the optical density at 590 nm.

Plate bioassay experiments were also performed with *Brucella* strains. *Brucella* cultures grown to the mid-log phase were plated on LB agar containing 1 mM EDDHA (at this concentration of EDDHA, parental strain *B. melitensis* 16M did not grow). The siderophores to be tested [10 μl of DHBA (without iron; 0.4 M) or 10 μl of iron(III) citrate (100 mM; Sigma)] were added to sterile paper disks and placed onto the solid medium. The plates were checked for the presence of a halo of growth after 4 days of incubation at 37°C.

**Transposon mutagenesis.** The transposon mini-Tn*5Kmcat*, a derivative of transposon mini-Tn*5Km*1 (19) bearing the kanamycin resistance gene and the chloramphenicol acetyltransferase reporter gene, was constructed (the resulting transposon was 2,409 bp long) and used to mutagenize *B. melitensis* 16M (18, 20, 30). Briefly, pUTmini-Tn*5Kmcat*, an ampicillin-resistant plasmid containing the transposon (without the transposase gene) was introduced into *B. melitensis* 16M Nal$^r$ by conjugation and incubated for 1 h without selection. Transconjugants resistant to kanamycin and sensitive to ampicillin were selected (18). A total of 3,040 clones were individually stored in 2YT medium containing 30% glycerol at −80°C in microtiter plates. Chromosomal DNA from 30 different mutants (including F13, F15, and F32) was prepared, digested with EcoRV or HindIII, and analyzed by Southern blot hybridization with the kanamycin or chloramphenicol resistance genes as a probe. The results demonstrated that each mutant contained a single transposon insertion. In this work, we screened 800 mutants belonging to this library of stable and independent mini-Tn*5* mutants of *B. melitensis* 16M for an iron assimilation defect.

**Molecular techniques and sequence analysis.** DNA manipulation was performed by using standard techniques (5a). Restriction enzymes were purchased from Roche and were used as described by the manufacturer.

Inverse PCRs (iPCR) were performed as described previously by Delrue et al. (20) with the transposon mutants after restriction of genomic DNA with TaqI. The oligonucleotides used were based on the *cat* gene sequence and on the *km* gene. The sequences of the oligonucleotides were 5′-GCATTCATCAGGCGG GCAAGAATGTGAAT-3′, 5′-GCCATCACGACTGTGCTGGTCAT-3′, 5′-G CCATCACGACTGTGCTGGTCAT-3′, and 5′-GATTCCGACTCGTCCAAC ATCA-3′. The PCR products were separated on agarose gels and were purified by using a High Pure PCR product kit as described by the manufacturer (Boehringer Mannheim, Roche). The sequencing reactions were performed by using an ABI DNA sequencer with a Big Dye terminator kit (Perkin-Elmer Cetus) and an ABI 377A sequencer. The oligonucleotides used for sequencing were the previously described primers used for iPCR amplification.

To obtain more genomic sequence near the site of transposition, chromosomal DNA from the F32 mutant was prepared, digested with SalI and with EcoRV (there were no SalI or EcoRV sites in the Tn*5* derivative), and ligated with pBBRMCS4 previously digested with SalI and EcoRV, respectively. Each ligation mixture was transformed into *E. coli* XL1-Blue and plated on LB medium supplemented with kanamycin. A plasmid from kanamycin-resistant colonies was prepared, and the insert was sequenced.

The iPCR with the F15 mutant was used to screen a *B. abortus* genomic library (9). One positive clone was isolated and characterized.

All the sequences obtained were analyzed by using the National Center for Biotechnology Information BLAST server, the Conserved Domain database, and GenBank (4, 46). Multiple alignment was performed by using CLUSTAL W (1.74) (66). Secondary structures were predicted by using PHD (58, 59), and PSORT (49, 50) was used to examine the signal sequence. The *B. melitensis* 16M genome was analyzed by using the server at http://serine.urbm.fundp.ac.be /~seqbruce/GENOMES/Brucella_melitensis/ (C. Lambert, E. Depiereux, J.-J. Letesson, and X. De Bolle, unpublished data).

To obtain a wild-type copy of *dugA* in the F15 mutant, the intact predicted coding sequence (CDS) of *dugA* was amplified from *B. melitensis* DNA by using the oligonucleotides 5′-CTATGACGCATAGCCACAA-3′ and 5′-TCAGGTA TTGAAGCGGAACA-3′ (sequences corresponding to start and stop codons are underlined) and cloned at the EcoRV site of pBBR1MCS4 (41). This resulted in constructs pBBR*dugA*1 and pBBR*dugA*2, in which the *dugA* CDS was cloned under control of the *E. coli lac* promoter, which allowed constitutive expression of this CDS in *Brucella* (24, 41), and in the opposite direction (no expression of *dugA*), respectively.

For Southern hybridization, the DNA probe (819 bp) specific to the chloramphenicol acetyltransferase (*cat*) gene was obtained after TaqI restriction of the pCAT19 plasmid and purification from an agarose gel with a JET sorb kit (Genomed). The probe was fluorescein labeled with a Tropix kit. Southern blotting on a positively charged nylon membrane (Tropix) was performed with a Hybaid vacuum blotter (Biozym). Membrane prehybridization, hybridization, and washing were performed under highly stringent conditions by using the Southern-Star protocol (Tropix). Hybridization was detected by autoradiography.

**Intracellular survival.** Survival of mutants was evaluated in an immortalized cell line of bovine peritoneal macrophages (65) and in HeLa cells by using previously described procedures (20). Briefly, brucellae grown in 2YT medium for 24 h were resuspended in a complete cell culture medium for inoculation. For the F32 mutant, inocula were also prepared from bacteria grown to the mid-log phase in LB broth containing 5 to 30 μg of EDDHA per ml, which induced sufficient iron starvation to reduce, but not stop, the growth of *Brucella*. Brucellae

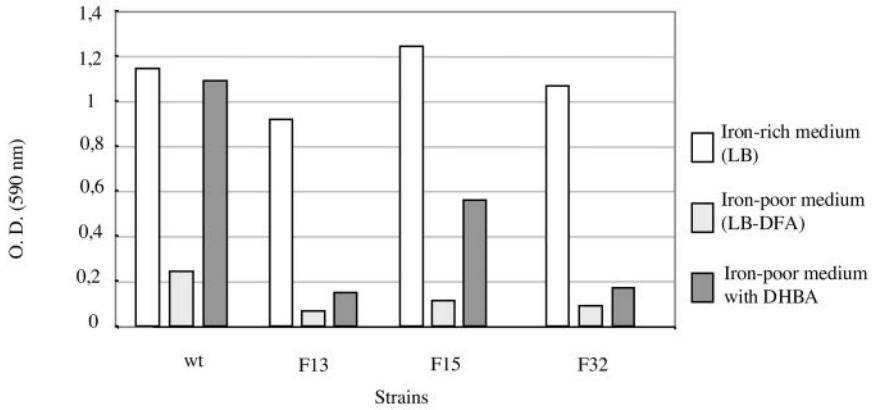



FIG. 1. Typical cell density 48 h postinoculation of *B. melitensis* wild-type and mutant strains in the absence of DFA (LB) or in the presence of DFA (500 μM) (LB-DFA) with or without DHBA (500 μM). O.D. (590 nm), optical density at 590 nm; wt, wild type.

were added to cells at a multiplicity of infection of 300:1, and culture plates were centrifuged for 10 min at 1,100 rpm at room temperature in a Jouan BR4i centrifuge. Gentamicin (50 μg/ml) was added 1 h after infection. The number of viable brucellae was estimated 3, 24, and/or 48 h postinfection (54) after the monolayers were washed with complete culture medium and lysed with 0.1% Triton X-100 in deionized water. Serial dilutions were plated on 2YT agar to determine the average number of CFU per well. The data presented below are means for at least three culture wells and are representative of two experiments.

**In vivo survival.** Groups of 7-week-old BALB/c female mice ($n = 4$) were inoculated intraperitoneally (i.p.) with $10^4$ CFU of either *B. melitensis* 16M or a mutant or intravenously (i.v.) with the F32 mutant or the wild-type strain (53). At 1 and 4 weeks postinoculation, four mice from each group were sacrificed, and spleens were collected. The spleens were homogenized in 2 ml of phosphate-buffered saline with a Stomacher 80 homogenizer. Serial dilutions of the homogenized spleens were plated on tryptic soy agar containing 0.1% yeast extract to determine bacterial counts.

**Nucleotide sequence accession number.** The *B. melitensis* sequence data reported in this paper have been deposited in the GenBank nucleotide sequence database under accession numbers AF325201 (for the *exbB*, *exbD*, and *tonB* genes), AY028973 (for the *pth* and *dugA* genes), and AF358662 (for the *tol* genes).

## RESULTS

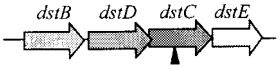
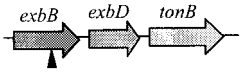
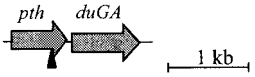
**Isolation of *B. melitensis* 16M mutants sensitive to an iron chelator.** We constructed a library of *B. melitensis* 16M mini-Tn*5Kmcat* transposon mutants. More than 800 of these mutants were individually screened for deficient growth on iron-poor medium. Thirty-two *B. melitensis* 16M mutants appeared to be deficient for growth on iron-poor DIP-containing medium. In standard media, the mutants showed no obvious growth defect. The defect was confirmed for all 32 mutants (designated F1 to F32) on medium supplemented with another iron chelator, EDDHA. These 32 mutants were screened for DHBA siderophore uptake.

**Iron source utilization deficiency of the F15, F13, and F32 mutants.** By utilizing an iron-deficient growth medium, we tested the ability of the 32 transposon mutants to use DHBA as the sole iron source. A marked increase in the growth of the wild-type strain was observed when DHBA was added to the iron-deficient medium. The growth of 29 mutants was also completely restored. In contrast, addition of DHBA did not fully restore growth of the F13 and F32 mutant strains (Fig. 1). For the F15 strain, the ability of the organism to use DHBA was also reduced, but it was reduced less than it was for the F13 and F32 mutants. In addition, the wild-type strain and the F15 mutant could grow on an iron-deficient medium when iron(III) citrate was provided in a disk assay. In contrast, growth of the F32 and F13 mutants in a plate bioassay was significantly reduced when ferric citrate or DHBA was used as the iron source compared to growth of the wild type. The diameters of growth observed in this assay were as follows: wild type, 53 to 54 mm; F15, 52 to 54 mm; F32, 21 to 22 mm; and F13, 24 to 26 mm. These results showed that *B. melitensis* 16M is able to use ferric citrate directly or indirectly as a sole iron source.

**Identification of the mutated genes in the F13, F15, and F32 mutants.** Southern blot hybridization demonstrated that each mutant contained a single transposon insertion (data not shown). iPCRs were used to clone genomic DNA flanking the transposon, and the PCR products were then sequenced. DNA and protein database searches were performed by using the flanking sequences, and the results are shown in Table 1.

A genomic DNA fragment containing the transposon from the F32 mutant (renamed the *exbB* mutant) was subcloned into pBBR1MCS4, and the DNA sequence of the regions flanking the transposon was determined by using PCR products. The sequence was determined for both strands by using two independent PCR products. Examination of this nucleotide sequence revealed three CDSs homologous to *exbB*, *exbD*, and *tonB* separated by 5 and 2 bp. Such a tight organization suggests that these three CDSs are part of an operon (60), and the transposon is inserted in the first CDS, *exbB* (Table 1). The three encoded proteins constitute the Ton system. Like its two homologues in *Pseudomonas putida* and *Bordetella pertussis* (56), *Brucella* ExbB (320 amino acids) has an N-terminal extension compared to *E. coli* ExbB. This extension contains an additional predicted transmembrane helix and an Ala-Pro-rich region with low complexity. A multiple alignment of four ExbB proteins showed that the sequence conservation occurs mainly in the three transmembrane segments common to all ExbB proteins. The 175-amino-acid *B. melitensis* ExbD protein has a hydropathy profile similar to that of *E. coli*. Like their *E. coli* homologues, the *B. melitensis* 16M ExbB and ExbD proteins also showed homology to the Tol proteins (12, 14). The TolQ and TolR sequences of *B. melitensis* 16M were obtained previously (67), and their amino acids were 44 and 41% identical

TABLE 1. *B. melitensis* genes essential for ferric DHBA utilization

| Mutant | Putative function | *B. melitensis* CDS[a] | Disrupted locus in *B. melitensis* 16M genome[b] |
|---|---|---|---|
| F13 (*dstC*) | Iron(III) ABC transporter | BMEII0603 to BMEII0606 |  |
| F32 (*exbB*) | Ton system | BMEI0365 to BMEI0367 |  |
| F15 (*pth*/*dugA*) | Peptidyl-tRNA hydrolase | BMEI0480 to BMEI0481 |  |



[a] CDS of *B. melitensis* genome on the National Center for Biotechnology Information Entrez Genome website (http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/micr.html).
[b] An arrowhead indicates the transposon localization.

to the amino acids of *B. melitensis* 16M ExbB and ExbD, respectively. As described previously for *E. coli* (10), the E/P-rich region and a K/P-rich region (at residues 82 and 119, respectively) of TonB (272 amino acids), encoded by the third CDS, are predicted to span the periplasm, and the protein is predicted to be anchored in the inner membrane via an N-terminal transmembrane segment (68). The predicted transmembrane sequence contains a conserved His residue involved in TonB activity in *E. coli*. The conserved predicted topology and sequence suggest that the Ton system of *Brucella* has a membrane arrangement similar to that of *E. coli.*

The deduced polypeptide sequence of the disrupted *B. melitensis* gene in the F13 mutant (designated the *dstC* mutant) is homologous to *fatC* (ferric anguibactin iron transport system), which encodes a permease of the anguibactin siderophore ABC transporter of *Vibrio anguillarum* (Table 1). The entire sequence of the locus designated *dstBDCE* (DHBA siderophore transport system) was obtained from the recently sequenced *B. melitensis* 16M genome (21). The polypeptide sequences deduced from the *dstBDCE* CDSs exhibit similarity with a periplasmic binding protein, with two permeases, and with the ATPase protein of an iron(III) ABC transporter. In the *B. melitensis dstBDCE* locus, all four genes are oriented in the same direction and overlap or have very short intergenic sequences (up to 59 bp long), suggesting that they are part of a single transcriptional unit encoding the proteins of a binding protein-dependent ABC transporter.

Interestingly, in the F15 mutant (designated the *pth*/*dugA* mutant), the homology of the disrupted sequence and *pth* does not suggest a direct link with iron assimilation in bacteria. The transposon insertion is located 190 bp upstream of the stop codon of the homologue of the *pth* coding sequence (753 bp), which encodes peptidyl-tRNA hydrolase, an enzyme that cleaves peptidyl-tRNA that has been abortively released from the ribosome during protein synthesis. We cloned the *B. abortus pth* homologue, as well as the gene located 41 bp downstream that we designated *dugA* and encodes a probable YchF homologue (a GTP binding protein), as described in Materials and Methods (Table 1). This genomic context is maintained in about 50% of diverse bacterial genomes (15), such as the *E. coli* (17), *Mesorhizobium loti*, and *Bradyrhizobium japonicum* genomes. DugA (DHBA utilization GTPase homologue A) possesses a domain homologue with the GTP1/OBG domain and contains the highly conserved *E. coli* YchF GTPase signature motifs G1 to G4 (Fig. 2) (16). Analysis of the *B. melitensis* 16M genome (data not shown) demonstrated that as in other bacteria, a core of 11 GTPases is universally conserved. These 11 GTPases are proposed to regulate special aspects of ribosome function and are probably RNA binding proteins (15, 16). DugA of *Brucella* belongs to the 11th conserved GTPase group, designated YchF, which has no predicted function.

**Infectivity of *B. melitensis* mutants in bovine macrophages and in HeLa cells.** We studied the ability of the *exbB*, *dstC*, and *pth*/*dugA* mutants to infect professional and nonprofessional phagocytes in order to focus attention on the importance of the iron utilization mechanism for survival in these cells. Only minor differences in intracellular growth in bovine macro-


FIG. 2. CLUSTAL W (1.74) multiple-sequence alignment of the amino acid sequences of the DugA protein of *B. melitensis* (Bru) and the YchF proteins of *E. coli* (E.coli) and *Haemophilus influenzae* (Hinfl). Identical amino acids are indicated by asterisks. The GTPase signature motifs G1 to G4 conserved in YchF of *E. coli* are enclosed in boxes (16).

TABLE 2. Differences in log CFU between the wild-type strain and *B. melitensis* 16M mutants in cellular infections

| Conditions | Time postinfection (h) | Differences in bacterial counts[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | Bovine macrophages | | | HeLa cells | | |
| | | *exbB* mutant | *dstC* mutant | *pth*/*dugA* mutant | *exbB* mutant | *dstC* mutant | *pth*/*dugA* mutant |
| Iron rich | 48 | 0.18 ± 0.16 | 0.31 ± 0.34 | −0.18 ± 0.50 | −0.32 ± 0.10 | 0.01 ± 0.13 | −0.15 ± 0.01 |
| Iron restricted | 48 | 0.17 ± 0.03 | ND[b] | ND | 0.33 ± 0.12 | ND | ND |

[a] Differences were calculated as follows: (total mean log CFU of wild type) − (total mean log CFU of mutant). The experiments were performed with bovine macrophages and HeLa cells at 48 h postinfection, and bacterial inocula were grown in iron-rich or iron-restricted conditions. The data are means ± standard deviations; the standard deviations for the wild-type strain were ±0.18 and ±0.20 for the two cellular models tested. Cellular infection experiments were performed twice in triplicate.

[b] ND, not determined.

phages and HeLa cells between the mutant and wild-type strains were observed (Table 2). Similar results were obtained after infection of cells with an *exbB* bacterial inoculum that had been grown in iron-poor medium (Table 2).

**Survival of the mutants in BALB/c mice.** We compared the numbers of bacteria in the spleens of mice infected i.p. with the wild-type strain and with the *dstC*, *pth*/*dugA*, and *exbB* mutants, as well as the numbers of bacteria in the spleens of mice infected i.v. with the wild-type strain and with the *exbB* mutant (Table 3). No significant difference in spleen colonization between the mutants and the wild-type strain was observed at 1 and 4 weeks after infection, whether the i.p. or i.v. route was used for the *exbB* mutant. This indicates that the *exbB*, *dstC*, and *pth*/*dugA* mutants were not attenuated in the BALB/c mice after i.p. infection.

**Implication of the *dugA* gene in DHBA assimilation by *B. melitensis* 16M.** In contrast to the *dstC* and *exbB* mutants, we were unable to explain the iron assimilation defect of the *pth*/*dugA* mutant on the basis of the *pth* mutation. Moreover, this gene is essential in *E. coli*, and recently, it was shown that *ychF* is cotranscribed with *pth* (17, 40). We hypothesized that transposon insertion in the 3′ end of *pth* affects expression of *dugA*. We tested this hypothesis by providing a wild-type copy of the *dugA* gene in *trans* in the *pth*/*dugA* mutant.

The *pth*/*dugA* mutant containing plasmid pBBR*dugA*1 with *dugA* under control of the *lac* promoter, which allowed expression of *dugA*, was able to grow as well as the wild-type strain in the presence of DHBA (Table 4). In contrast, the *pth*/*dugA* mutant transformed with plasmid pBBR*dugA*2, which did not allow expression of DugA (because the *dugA* CDS and the *lac* promoter are in opposite orientations), manifested no complementation of the phenotype. This result demonstrated that expression of *dugA* led to complementation of the *pth*/*dugA* mutant. We propose that transposon insertion in *pth* has a polar effect on *dugA* expression and that the *pth*/*dugA* mutant is deficient in iron assimilation because of altered expression of the *dugA* gene.

TABLE 3. Differences in bacterial counts between the wild-type and mutants in BALB/c mouse spleens

| Time postinfection (wk) | Differences in bacterial counts[a] | | | |
|---|---|---|---|---|
| | i.p. infection | | | i.v. infection with *exbB* mutant |
| | *exbB* mutant | *dstC* mutant | *pth*/*dugA* mutant | |
| 1 | 0.06 ± 0.07 | 0.16 ± 0.22 | −0.11 ± 0.13 | −0.55 ± 0.54 |
| 4 | 0.10 ± 0.11 | 0.69 ± 0.17 | 0.13 ± 0.14 | 0.10 ± 0.03 |

[a] Differences were calculated as follows: (total mean log CFU of wild type) − (total mean log CFU of mutant). Samples ($n = 4$) were examined 1 and 4 weeks after i.p. or i.v. infection.

## DISCUSSION

We identified a set of 32 *B. melitensis* transposon mutants that appear to be defective in iron acquisition or assimilation. When three of these mutants (the *exbB*, *dstC*, and *pth*/*dugA* mutants) were screened for DHBA uptake, the results allowed identification of components of a DHBA utilization pathway or a DHBA-ferric citrate utilization pathway, the Ton system and an iron(III) binding protein-dependent ABC transporter described for other bacteria and also a homologue of YchF GTPase.

Three *B. melitensis* mutants identified in this work, the *dstC*, *pth*/*dugA*, and *exbB* mutants, exhibited altered growth under in vitro iron-limiting conditions. In addition, growth of these three mutants in iron-depleted medium was not rescued by addition of DHBA (or by addition of ferric citrate for the *exbB* and *dstC* mutants). These results indicated that the gene disrupted in the *dstC*, *pth*/*dugA*, and *exbB* mutants is involved in DHBA and/or ferric citrate utilization. Iron(III) citrate has not been described previously as an iron source for *Brucella*, and our results show that utilization of this compound by *Brucella* is ExbBD-TonB complex and Dst ABC transporter dependent.

*Brucella* iron capture via DHBA is still not clearly understood. In *E. coli*, the Ton system and the ferrous uptake system

TABLE 4. Liquid growth assay in iron-poor medium (LB medium with 500 μM DFA) for utilization of the DHBA siderophore (500 μM) by *B. melitensis* wild-type strain 16M and the *exbB* mutant as controls and the *pth*/*dugA* mutant transformed with pBBR*dugA*1 and pBBR*dugA*2

| Medium | Growth of strains[a] | | | |
|---|---|---|---|---|
| | *exbB* | Wild type | *pth*/*dugA* with pBBR*dugA*1 | *pth*/*dugA* with pBBR*dugA*2 |
| Iron-rich medium (LB medium) | + | + | + | + |
| Iron-poor (LB medium with DFA) | − | + | + | − |
| Iron-poor medium with DHBA | − | + | + | − |

[a] +, growth equivalent to wild-type growth; −, growth much less than wild-type growth. Growth was examined 48 h after inoculation.

seem to be partially implicated in ferric DHBA capture, and in *Pseudomonas aeruginosa*, DHBA transport is neither iron repressible nor strongly energy dependent, unlike other siderophore uptake mechanisms (32, 33, 61). In *B. abortus*, Lopez-Goni et al. (45) did not observe any induction of outer membrane protein expression under low-iron conditions and proposed that DHBA uses a receptor-independent pathway or an unusual non-iron-repressible receptor. Our results demonstrate that the Ton protein complex and an ABC transporter (Dst) are implicated in utilization of Fe(III)-DHBA by *Brucella*. In other bacteria, this iron uptake pathway is also energy and receptor dependent. We propose that the ExbBD-TonB complex and the Dst binding protein-dependent ABC transporter are implicated in DHBA (and ferric citrate) assimilation by *B. melitensis* 16M in low-iron conditions, probably at the stage of outer membrane and inner membrane crossing, respectively. The Dst transporter could probably also be utilized by brucebactin, a more complex siderophore for which DHBA probably serves as a precursor. In *E. coli*, one iron(III) ABC transporter is also used by more than one siderophore belonging to the same structure family (hydroxamates, catecholates, or ferricitrate), and many siderophores have a specific receptor (except, for example, for DHBA and dihydroxybenzoylserine). In the *B. melitensis* genome, no sequence encoding a potential TonB-dependent receptor was found near the *dst* genes (BMEII0604 to BMEII0607). However, three CDSs encoding homologues of iron receptors were detected: BMEII0105/II0297 and BMEI0657. One part of this sequence is adjacent to CDSs encoding an iron(III) ABC transporter (BMEI0657 to BMEI0660). In addition to this ABC transporter (BMEI0657 to BMEI0660) and to the Dst transporter (BMEII0604 to BMEII0607), four other putative iron(III) ABC transporters determined by homology were detected in the *B. melitensis* genome: BMEII0535 to BMEII0537/BMEII0583 to BMEII0585/BMEII1120 to BMEII1123 and BMEII0565 to BMEII0567.

The *exbB*, *dstC*, and *pth*/*dugA* mutants of *B. melitensis* exhibited no defect in intracellular infection. Bellaire et al. (7) and González Carreró et al. (31) obtained similar results with *B. abortus entC* and *entE* mutants unable to synthesize DHBA and brucebactin, respectively. DHBA is dispensable for intracellular survival of *Brucella*. In an intracellular environment, the source of iron is not clearly identified, but a siderophore is not essential for survival, as demonstrated for *Shigella flexneri* (43, 51) and *Mycobacterium* sp. (42). However, the Ton complex, which is absent in the *exbB* mutant, is probably implicated in the acquisition of iron sources other than DHBA, like all putative endogenous-exogenous siderophores and some host iron-containing compounds. Despite this, the Ton system appears to be dispensable for intracellular growth of *Salmonella enterica* (69), as it is for *Brucella*. The strategy used by intracellular pathogens to obtain iron could be to multiply within intracellular sites where available iron is stored, and *Brucella* seems to behave in this way (43, 69). In addition, it seems that *Brucella* does not possess a second Ton complex (or protein), as described for *Vibrio cholerae* and *P. aeruginosa* (52, 73). In fact, only one copy of the *exbBD-tonB* genes was detected in the genome of *B. melitensis* 16M. However, *tol* genes are present, and in *E. coli*, TolQ/R proteins partially complement the absence of ExbB/D proteins (12, 64). Finally, brucellae could use Ton-independent iron uptake mechanisms, as suggested for heme capture by *Neisseria gonorrhoeae* and *Haemophilus ducreyi* (23, 70, 72).

Iron availability varies in the different environments encountered by a pathogen in the host, implying that the mechanisms of iron acquisition differ according to the infection route. An *S. enterica tonB* mutant is attenuated in an intragastric infection but not in an i.p. infection (69). No significant difference in spleen colonization between the *Brucella exbB*, *dstC*, and *pth*/*dugA* mutants and the wild-type strain was observed after i.p. infection. These results are also consistent with the findings of Bellaire et al. (7), who showed that biosynthesis of DHBA is not required for chronic infection in the mouse model. After i.p. inoculation, *Brucella* is probably internalized by peritoneal macrophages and rapidly transported to the spleen, where it multiplies. Our observations in cell line infection experiments suggest that the Ton complex, the Dst transporter, and the DugA protein are not required for intracellular *Brucella* replication. Following i.v. inoculation, bacteria are for a while located in the blood, in which iron is withheld by iron(III) binding proteins, and after this bacteria are also targeted to the spleen. However, with regard to the model of *Brucella* pathogenesis, blood is not a critical replication site. We concluded that the Ton complex, Dst transporter, and DugA GTPase are not required for survival or growth of *B. melitensis* in the compartments targeted in our animal models of infection. Our routes of infection bypass mucosal surfaces where the initial phase of infection takes place in a natural host. Therefore, we cannot exclude the possibility that the system altered in the mutants contributes to growth at sites, such as the mucosal epithelium, the submucosal environment, or the peripheral lymph nodes, that are not encountered by *Brucella* when the organism is administered i.p. or i.v.

Despite these results, DHBA plays a role in virulence, as demonstrated by significant attenuation of the *entC* mutant in pregnant cattle (8). A possible explanation for the requirement for DHBA in the host was the state of gestation. Indeed, erythritol, a preferred carbon source for *Brucella* produced by placental trophoblasts (but not produced in mice), stimulates the production of DHBA (6). Perhaps brucellae use sources of iron other than DHBA during infection but also need the DHBA (and brucebactin) siderophore after a change in the iron status in a different gestational stage. Consequently, *exbB*, *dstC*, and *pth*/*dugA* mutant virulence should be further evaluated in the natural host, and the effects of infection with the mutants on gestation should also be investigated.

Our data also demonstrate that a GTPase homologue is implicated in DHBA utilization. FeoB is the only G protein that has been described as a protein that is involved in iron assimilation (34, 47). This GTPase is an essential membrane protein for iron(II) uptake in *E. coli*; however, there is no similarity between DugA and FeoB. Moreover, no FeoB homologue was detected in the *B. melitensis* 16M genome sequence. DugA is the homologue of YchF, the 11th universally conserved GTPase in bacteria (16), and thus may be a regulator. Indeed, Caldon et al. (16) hypothesized that the core of 11 GTPases is necessary to regulate ribosome function or transmit signals from the ribosome to downstream effector pathways. This regulating function through interaction with RNA and/or ribosomes could explain the genomic position of *dugA* next to *pth* (encoding peptidyl-tRNA hydrolase). Finally, the

proposed function of the DugA protein is a starting point for studies of the role of its homologues.

In addition to characterization of the Ton system, Dst transporter, and DugA GTPase in *Brucella*, further genetic and phenotypic analyses of other selected mutants should provide valuable insights into the mechanisms of *Brucella* iron assimilation and into their involvement in virulence.

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