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# Deletion of *purE* Attenuates *Brucella melitensis* 16M for Growth in Human Monocyte-Derived Macrophages

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**We constructed a defined purine-auxotrophic mutant of *Brucella melitensis* 16M by chromosomal gene replacement. We electroporated *B. melitensis* 16M with suicide plasmids containing a kanamycin resistance cassette that replaced 226 bp at the carboxyl end of *purE*, the intergenic region, and 18 bases of the *purK* open reading frame. Recombinant *B. melitensis* Δ*purE201* required exogenous purines for growth on minimal media. Purine auxotrophy was complemented by electroporation of *B. melitensis* Δ*purE201* with a plasmid, pSD5, carrying only the wild-type *purE* gene. In in vitro assays of virulence, *B. melitensis* Δ*purE201* failed to grow in human monocyte-derived macrophages, while the growth of wild-type 16M and the complemented strain, Δ*purE201*(pSD5), increased by nearly two logs. These results suggest that *B. melitensis* Δ*purE201* will be attenuated in animals and humans and thus may be useful as a live attenuated vaccine.**

Bacterial vaccines are based on either live or inactivated whole-cell or subunit preparations (4). Live vaccines for intracellular organisms are generally more efficacious and consist of attenuated variants of particular pathogens which have lost the ability to cause clinical disease but are still able to establish self-limiting infection and hence induce an immune response in the host (4). Brucellae are facultative intracellular parasites which can survive and replicate within phagocytic cells. The majority of human brucellosis cases are caused by *Brucella melitensis*, *B. suis*, and *B. abortus* and most commonly involve the reticuloendothelial system (26). As we define the genes required for the intracellular growth and survival of brucellae, we can create attenuated variants harboring defined genetic lesions. The rational genetic attenuation of pathogens will lead to a new generation of live bacterial vaccines which are safer and do not revert to full virulence. Using information from the development of live attenuated *Salmonella* vaccines (2, 8, 12, 15, 18, 25), we designed experiments to produce a live, attenuated, genetically defined mutant of *Brucella melitensis* 16M for use in a vaccine.

Attenuated live *Brucella* vaccines are currently available for use in animals, *B. abortus* 19 and *B. melitensis* Rev1 (20). However, these strains are not genetically defined. Furthermore, they retain their virulence for humans (19, 27). Ideally, a live-vaccine strain conferring solid immunity without host restrictions would be a significant improvement over available vaccines.

The *purEK* operon of *B. melitensis* 16M has been isolated and sequenced. Defined deletions in the cloned *purE* and *purK* genes were made. Additionally, a defined *B. melitensis purK* mutant required an elevated $CO_2$ concentration but not supplemental purines for growth on minimal media (13). Here we report the characterization of a defined mutation in the *purE* gene in *B. melitensis* 16M. This mutant required purines for growth and had a decreased ability to replicate in human monocyte-derived macrophages (MDM).

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## MATERIALS AND METHODS

**Construction and characterization of *B. melitensis purE* mutants.** Plasmids pURE198 and pURE201 contain the mutated *purEK* operon of *B. melitensis* 16M in which the 3′ half of the *purE* gene, the intergenic region, and 18 bases of the *purK* gene were replaced by a kanamycin resistance cassette, in either forward (pURE198) or reverse (pURE201) orientation, in a *Bgl*II deletion site of pURE197 (13). Wild-type *B. melitensis* 16M was grown in brucella broth for 24 h to approximately $10^8$ cells per ml. Cells were prepared for electroporation by pelleting and washing, first with 1/5 volume and then with 1/10 volume of ice-cold 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, with subsequent resuspension in 1/25 volume of ice-cold 10% glycerol. One microgram of pURE198 or pURE201 plasmid DNA was added to 100 μl of cell suspension and electroporated at 2.5 kV, 25 μF, and 600 Ω. One milliliter of SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) (Gibco BRL Life Technologies, Inc., Gaithersburg, Md.) was added, and cells were grown with shaking at 37°C for 1 h and plated on brucella agar containing 50 μg of kanamycin per ml.

Transformants were examined by replica plating on brucella agar containing 50 μg each of kanamycin and ampicillin per ml and on M9 minimal media containing kanamycin. Chromosomal DNA minipreps of colonies were prepared to examine the mutated region (7). Chromosomal DNA (2 μg per lane) was double digested with *Hin*dIII and *Eco*RI, separated on a 1.2% agarose gel in TAE buffer at 20 V for 18 h (1), and passively transferred to a Nytran membrane (Schleicher & Schuell, Keene, N.H.). The *Eco*RI fragment containing the kanamycin cassette from plasmid pUC4K and the cloned wild-type *purE* on a *Hin*dIII-*Eco*RI fragment were prepared as probes for Southern hybridization by purification from a low-melting-point agarose gel. Probes were nonisotopically labeled (Genius; Boeringer Mannheim Corp., Indianapolis, Ind.), and hybridizations were conducted at 68°C.

**Complementation of the *purE* mutation.** To verify that purine auxotrophy and attenuation were due to gene replacement and not to a secondary mutation, we complemented the *B. melitensis* Δ*purE* mutant with pSD5. We constructed pSD5 by PCR amplification of the wild-type *B. melitensis purE* gene, including 140 bp upstream and 94 bp downstream of the coding sequence (GenBank accession number U10241). The 722-bp amplification product and plasmid pCm2.6, containing a chloramphenicol resistance gene (24), were double digested with *Sal*I and *Xba*I and ligated to yield pSD5, which was electroporated into *Escherichia coli* DH10B (Gibco BRL Life Technologies, Inc.). Transformants were recovered on Luria-Bertani agar containing 25 μg of chloramphenicol per ml. Plasmids from these transformants were isolated (Magic; Promega Corp., Madison, Wis.) and analyzed by agarose gel electrophoresis of restriction endonuclease digests. We electroporated *B. melitensis* Δ*purE198* and Δ*purE201* with pSD5. Transformants were selected on brucella agar containing 5 μg of chloramphenicol per ml and 50 μg of kanamycin per ml. Recombinants were examined on minimal agar (1) in the presence and absence of purines (plate concentrations as

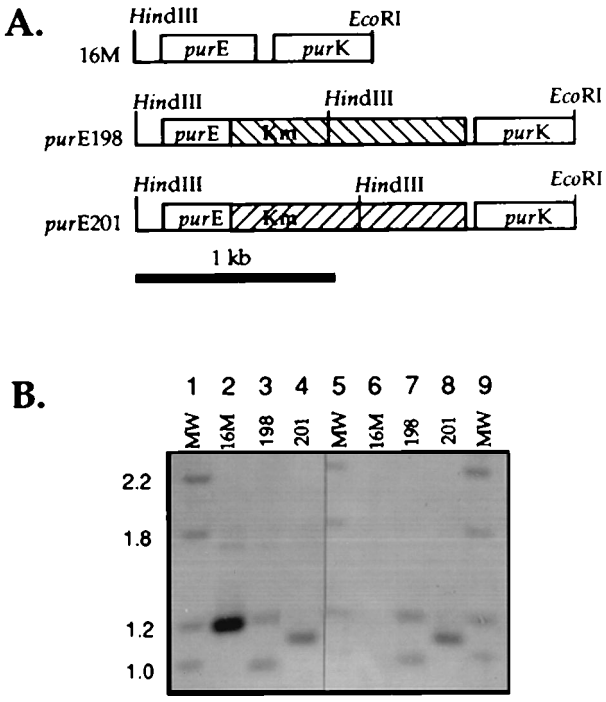


FIG. 1. Southern hybridization of *B. melitensis* chromosomal DNA. (A) *Brucella* mutants with the kanamycin resistance cassette inserted into *purE* in either orientation were recovered. (B) A PCR-generated *purE* probe hybridized to a 1.2-kb *Hin*dIII-*Eco*RI fragment in wild-type strain 16M (lane 2). An asymmetrical *Hin*dIII site in the kanamycin resistance cassette generated two reactive fragments in the *purE* replacement mutants Δ*purE198*, of 1.0 and 1.2 kb (lane 3), and Δ*purE201*, of 1.1 kb each (lane 4). The kanamycin cassette probe did not hybridize to wild-type 16M (lane 6) but did hybridize to the *purE* probe-reactive fragments in Δ*purE198* (lane 7) and Δ*purE201* (lane 8). Lanes 1, 5, and 9 contained digoxigenin-labeled DNA molecular size standards II and VI (MW; in kilobases).

follows: adenine, 5 mM; guanine, 0.3 mM; hypoxanthine, 0.7 mM; xanthine, 0.6 mM) and $CO_2$.

**Human MDM infection model.** Human mononuclear cells were prepared from leukopacks from normal donors by centrifugation over lymphocyte separation medium (Organon Teknika, Durham, N.C.). Monocytes were further purified by counterflow centrifugal elutriation (29). This procedure resulted in monocyte preparations of >95% viability by trypan blue exclusion and <10% lymphocyte contamination. Approximately $10^5$ monocytes per well were cultured for 7 days as adherent monolayers in 10% heat-inactivated human serum (Sigma) in antibiotic-free RPMI 1640 medium containing 10 ng of recombinant human macrophage colony-stimulating factor (kindly supplied by Jay Stoudemire, Genetics Institute, Boston, Mass.) per ml. Every 3 days, one-third of the spent medium from MDM was replaced with fresh complete medium. *Brucella* strains were grown for 24 h in brucella broth in the presence of antibiotics when appropriate. Brucellae were washed in saline, resuspended in complete cell culture medium at $5 \times 10^8$ CFU/ml, and added to MDM at a multiplicity of infection of 10:1. After 60 min, the monolayers were washed with complete cell culture medium three times, and complete cell culture medium containing 0 to 50 μg of gentamicin per ml was added. At various time points, monolayers were washed with complete culture medium and lysed with 0.1% Triton X-100. Serial dilutions were plated on brucella agar. The data presented are means of duplicate plate counts from each of two culture wells and representative of three to five experiments.

## RESULTS

**Characterization of *B. melitensis purE* mutants.** A defined kanamycin-resistant *purE* deletion mutant of *B. melitensis* 16M was constructed by chromosomal gene replacement (Fig. 1A). These constructions were confirmed by DNA hybridization. DNAs from parental and kanamycin-resistant, ampicillin-sensitive recombinants were isolated. Southern hybridization of *Hin*dIII-*Eco*RI-digested chromosomal DNA with a *purE*$^+$ probe produced a 1.2-kb band for *B. melitensis* 16M (Fig. 1B, lane 2). The *purE*$^+$ probe hybridized to 1.0- and 1.2-kb fragments from *B. melitensis* Δ*purE198* (Fig. 1B, lane 3) and two 1.1-kb fragments from *B. melitensis* Δ*purE201* (lane 4), consistent with these mutants containing the kanamycin cassette in opposite orientations. A probe for the kanamycin resistance gene did not hybridize to *B. melitensis* 16M DNA (Fig. 1B, lane 6) but did hybridize to the same-sized fragments recognized by the *purE*$^+$ probe from *B. melitensis* Δ*purE198* (lane 7) and *B. melitensis* Δ*purE201* (lane 8). The Δ*purE* replacement mutants did not grow on minimal media in the absence of purines, regardless of $CO_2$ concentration. In contrast, the Δ*purK* mutant grew on minimal media only when incubated in 5% $CO_2$ (Table 1). Minimal media containing adenine, guanine, or both purines supported the growth of Δ*purE*, Δ*purK*, and parental strains. Additionally, minimal media supplemented with hypoxanthine or xanthine supported the growth of strain Δ*purE201*. Within 7 days, strains Δ*purE201* and 16M grew equally well on adenine- plus guanine- or hypoxanthine-supplemented minimal media. In contrast, growth on adenine-, guanine-, or xanthine-supplemented media was observed only after extending incubation for 3 to 4 weeks.

TABLE 1. Complementation analysis of purine auxotrophs of *B. melitensis*[a]

| Strain | Growth in the absence of purines in[b]: | |
|---|---|---|
| | Air | $CO_2$ |
| 16M | + | + |
| Δ*purK* | − | + |
| Δ*purE198* | − | − |
| Δ*purE201* | − | − |
| Δ*purE198*(pSD5) | + | + |
| Δ*purE201*(pSD5) | + | + |

[a] Strains were grown for 7 to 10 days on minimal media. Minimal media supplemented with adenine and guanine supported the growth of all strains.
[b] +, growth; −, no growth.

**Complementation of the *purE* mutation.** The insertion of a kanamycin cassette into the *purE* deletion site disrupted the *purEK* operon by removing half of *purE*, the intergenic region, and the first 18 bases of the *purK* open reading frame and could block the transcription or translation of *purK*. Therefore, the purine mutants of *B. melitensis* would be either PurE$^-$K$^+$ or PurE$^-$K$^-$. To determine which of these possibilities had occurred, we transformed *B. melitensis* Δ*purE198* and Δ*purE201* with the intact *purE*$^+$ gene cloned in pSD5. Transformants containing pSD5 grew on minimal media regardless of $CO_2$ concentration, indicating the presence of a functional *purK* gene (Table 1). The orientation of the kanamycin cassette had no effect on growth on minimal media.

**Attenuation of *Brucella* infection of human MDM.** Monolayers of adherent human MDM were exposed to *B. melitensis* 16M, Δ*purE198*, Δ*purE201*, and the *purE* complemented strain, Δ*purE201*(pSD5). Bacterial survival was determined at various time points. At 1 h postinfection without gentamicin, the number of intracellular *B. melitensis* 16M colonies recovered was $\log_{10}$ 4.9 (Fig. 2A). Within 48 h, recoverable *B. melitensis* 16M colonies reached $\log_{10}$ 6.1, indicating that intracellular replication had occurred. The Δ*purE201* strain was internalized at slightly lower levels ($\log_{10}$ 4.6) than was the parent strain. We observed only a slight increase in the number of recoverable purine mutants at 48 h, from both infected MDM ($\log_{10}$ 5.1) and extracellular tissue culture media ($\log_{10}$ 3.2 at 24 h and $\log_{10}$ 3.8 at 48 h), during which time the number of extracellular 16M colonies increased from $\log_{10}$ 4.6 to $\log_{10}$

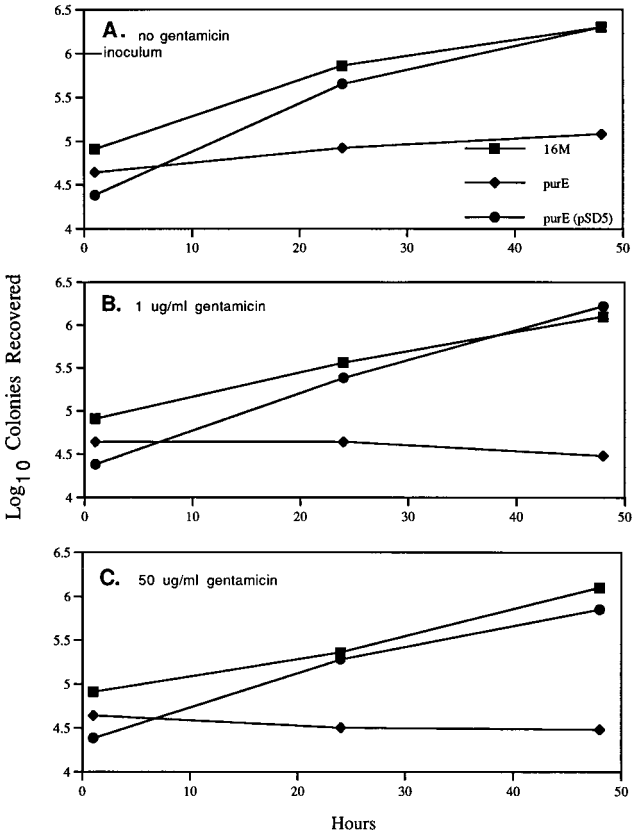


FIG. 2. Growth of *B. melitensis* in MDM. *B. melitensis* 16M, Δ*purE201*, Δ*purE201*(pSD5) were incubated with MDM at a multiplicity of infection of 10:1. After 1 h, nonadherent brucellae were removed by three washes with complete culture medium. Incubation was continued in complete medium containing 0 (A), 1 (B), or 50 μg of gentamicin per ml (C). At 24 and 48 h, MDM were washed, lysed with 0.1% Triton X-100, diluted in saline, and plated on agar for bacterial counts. The data presented are means of duplicate plate counts from each of two culture wells and representative of three to five experiments.

6.3. Similar results were obtained with Δ*purE198* (data not shown).

To verify that the *purE* defect was responsible for attenuating *B. melitensis* 16M, we introduced the cloned *purE*$^+$ gene into the Δ*purE201* strain and performed the MDM virulence assay. Even though Δ*purE201*(pSD5) was internalized at a level that was approximately one-half log lower ($\log_{10}$ 4.4) than that of 16M, equivalent intracellular levels of these strains were attained at 48 h ($\log_{10}$ 6.3). Furthermore, the extracellular growth of Δ*purE201*(pSD5) was identical to that observed for 16M. Therefore, the wild-type *purE* gene complemented the Δ*purE* defect and restored the ability of the *B. melitensis* purine auxotroph to survive and replicate within cultured monocytes.

Since brucellae can grow extracellularly and intracellularly, we examined the effect of gentamicin by the MDM virulence assay. All three *Brucella* strains were killed in cell culture medium containing 1 or 50 μg of gentamicin per ml (data not shown). As shown in Fig. 2B, the addition of 1 μg of gentamicin per ml to the virulence assay did not diminish the recovery of internalized 16M ($\log_{10}$ 6.3) or Δ*purE201*(pSD5) ($\log_{10}$ 6.1), while the recovery of the Δ*purE201* strain in this medium was approximately one-half log lower than that in gentamicin-free medium. The difference between the parent or *purE*-complemented strains and the deletion mutant was approximately 1.5 logs. The assay results in the presence of 50 μg of gentamicin per ml (Fig. 2C) were equivalent to those seen with 1 μg of gentamicin per ml. These data indicate that the diminished number of *purE* colonies at 48 h reflected reduced intracellular growth rather than reduced extracellular growth.

## DISCUSSION

There is a relationship between auxotrophy and intracellular survival or the growth of several facultative intracellular pathogens (5, 12, 16, 17, 28). These studies provide a basis for developing a genetically defined, live, attenuated vaccine strain of *B. melitensis*. Auxotrophic mutations in essential metabolic pathways generate attenuated *Salmonella* mutants that require aromatic compounds (*aroA*) and/or adenine (*purA*) for growth. These mutants, when administered parenterally, infect mice. The *aroA* strain stimulates a protective immune response. However, neither the *purA* mutant nor *purA aroA* double mutant is efficacious as a parenteral or oral vaccine (8, 12, 15). In contrast, a Tn*10*-induced *purE* deletion mutant is less attenuated than an *aroA* or *purA* strain but is considered too virulent for use as a live attenuated vaccine in humans (18, 25). Like the *purE* mutant, a *purD*::Tn*10* mutant has reduced survival in murine macrophages and reduced virulence in mice. Mice surviving vaccination with the *purD*::Tn*10* strain are protected from challenge with virulent *Salmonella* organisms, but this strain is considered too virulent for use as a live attenuated vaccine in humans (2). Similarly, mutations late in nucleotide biosynthesis overattenuate *Yersinia pestis*, and mutations in early steps reduce virulence by only 1 to 2 $\log_{10}$ (5, 28). In contrast, a *Listeria monocytogenes* adenine auxotroph is 1.5 $\log_{10}$ less infectious intravenously for mice than is the wild type (14), whereas *Salmonella* and *Yersinia purA* mutants are 4 to 5 $\log_{10}$ less virulent (5, 28). The reduced effectiveness of the *Listeria* adenine auxotroph reflects the capacity of *Listeria* organisms to escape from the phagolysosome into the cytosol, where purines are more available (14). These studies indicate that purine auxotrophy attenuates intracellular pathogens. Both the position of the mutation in the biosynthetic pathway and the intracellular compartmentalization of the organism affect the level of attenuation.

Since plasmids of pUC18 origin (pURE198 and pURE201) are unable to replicate in brucellae, pUC18 constructs are suicide plasmids in this species. These plasmids can be used to insert modified host DNA back into wild-type cells as single or double recombination events. The modified *purE* contains a kanamycin resistance gene, allowing selection of kanamycin-resistant *B. melitensis* 16M transformants. Single crossovers are distinguished from double crossovers by screening for vector-conferred ampicillin resistance. Fifty percent of kanamycin-resistant, purine auxotrophic transformants were ampicillin sensitive. In these transformants, the plasmid vector is lost and the altered *purE* gene is substituted for the wild-type gene in a double crossover event, generating a genetically defined mutant of *B. melitensis*. The gene deletion-replacement technique used in construction of this mutant eliminates the possibility of reversion to purine prototrophy. We confirmed the insertion of the altered *purE* gene by restriction digestion analysis and Southern blotting. Since the kanamycin cassette contains an asymmetric *Hin*dIII site, two patterns of hybridization to Δ*purE* DNA are possible, depending on the orientation of the kanamycin cassette in the chromosome. Δ*purE198* yields bands of 1.0 and 1.2 kb. This banding pattern occurs because the cassette is in the 5′-to-3′ orientation. The generation of two 1.1-kb bands from the Δ*purE201* strain occurs because the cassette is inserted in the opposite orientation. Growth on

purine-supplemented media is unaffected by the orientation of the resistance gene.

Complementation with the cloned *purE* gene shows that *B. melitensis* Δ*purE201* is phenotypically $PurE^{-}K^{+}$. This was an unexpected result since plasmid pURE201 does not complement the *purK*::Tn*10* mutation in *E. coli* RR1 (13). This suggests that the transcription or translation of this mutated operon in brucellae is different than in *E. coli.*

The addition of adenine plus guanine or hypoxanthine to minimal agar supported the wild-type growth of Δ*purE201*, whereas growth on media supplemented with either adenine, guanine, or xanthine was observed only after extended incubation. These results indicate that purine auxotrophy caused by a mutation in the de novo pathway leading to inosine biosynthesis is effectively overcome by the addition of both end products (adenine and guanine) or the IMP precursor (hypoxanthine). In contrast, supplementation with one of the purines synthesized after IMP (adenine, guanine, or xanthine) results in poor growth. These results suggest that the salvage pathway for purine synthesis in brucellae is less efficient than that observed in *E. coli*, which may explain the decreased survival of the *Brucella purE* mutant in our virulence model.

Since brucellae are taken up and replicate within phagocytic cells of the immune system (11, 21, 23), growth in macrophages has been used as a model system for measuring the attenuation of virulent strains of brucellae. Early studies with normal guinea pig macrophages demonstrated that rough *B. abortus* grow more slowly than does the smooth parent (9). Mouse peritoneal macrophages (30) and human polymorphonuclear leukocytes (31) kill opsonized *B. abortus*. In contrast, opsonized *B. melitensis* is phagocytized but not killed by human polymorphonuclear leukocytes (31). Campbell and Baldwin (6) established a bovine MDM system and demonstrated intracellular survival of nonopsonized *B. abortus* in cells from susceptible cattle.

In this report, we have demonstrated that wild-type *B. melitensis* is internalized, survives, and replicates within human MDM. Using this model, we have shown that a defined *purE* mutation of *B. melitensis* has minimal effect on internalization but effectively blocks replication in human MDM. The slight increase in recovered purine mutants in the absence of antibiotic may be due to replicating extracellular bacteria in this model. The inclusion of gentamicin in culture medium eliminated this potentially confounding variable. Interestingly, the identical growth in cultures treated with 1 or 50 μg of gentamicin per ml suggests that even high concentrations of gentamicin have very little effect on the growth of intracellular wild-type or auxotrophic brucellae in MDM.

Brucellae may survive within phagocytes by blocking phagosome-lysosome fusion. Frenchick et al. (10) showed that soluble extracts of *B. abortus* inhibit phagosome-lysosome fusion in murine macrophages. In addition, *B. abortus* expresses nucleotide-like compounds that inhibit primary granule release and myeloperoxidase-$H_2O_2$-halide bacterial killing by bovine polymorphonuclear leukocytes (3). Lysosomal enzyme secretion of macrophages is inhibited by adenosine and purine nucleosides convertible to adenosine (22). These observations raise the fascinating possibility that brucellae inhibit phagosome-lysosome fusion by releasing purines. The reduction of *B. melitensis* Δ*purE* growth in human MDM that we have observed may then be due to the inability of these mutants to produce excess purines, with the consequent failure to block phagosome-lysosome fusion. Alternatively, the intracellular environment occupied by brucellae may be purine deficient; intracellular bacteriostasis of the Δ*purE* mutant would then occur as a result of purine starvation.

The data presented here describe a *purE* deletion mutant of *B. melitensis* attenuated for replication within human MDM. Normal internalization in the absence of intracellular replication within human MDM suggests that this *B. melitensis* Δ*purE* strain causes limited infection when introduced into a host. We are investigating the levels of attenuation, immunologic response, and protective capability of this strain in mice and goats.

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