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Attenuation of defined *Brucella melitensis wboA* mutants

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ABSTRACT

Rough *Brucella* mutants have been sought as vaccine candidates because they do not induce seroconversion. In this study, two defined nonreverting rough mutants were derived from virulent *Brucella melitensis* strain 16M: a *wboA* deletion mutant designated WRR51 and a *wboA purEK* dual deletion mutant designated WRRP1. Strain WRRP1 exhibited reduced survival in human monocyte-derived macrophages (hMDMs) compared with parent strain WRR51 or with Δ*purEK* strain WR201. Strain WRRP1 persisted for 1 week or less in BALB/c mice after intraperitoneal infection, while less severe attenuation was exhibited by the two single mutants in this model. *Trans* complementation of *wboA* restored the survival of WRR51 in hMDMs comparable to strain 16M and the survival of WRRP1 comparable to strain WR201.
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1. Introduction

The *Brucellae* are gram-negative coccobacilli that gain entry into phagocytes of mammalian hosts and live within these cells, causing disease in domesticated animals and marine mammals and also chronic zoonoses. Species of *Brucella* are so genetically similar that they can be seen as biovars within a single species [1], but are reliably distinguished based on animal host specificity. Of these, *Brucella melitensis*, *Brucella suis*, *Brucella abortus*, and only rarely *Brucella canis* [2,3] cause human brucellosis or undulant fever. *B. melitensis* is the major cause of human brucellosis worldwide [2]. There is no accepted vaccine to safely and effectively protect humans against brucellosis, though a variety of approaches have been pursued to this end [4]. Attenuated live vaccines have been demonstrated to be effective against bovine, caprine and ovine brucellosis [5,6]. Live veterinary vaccines were reported pathogenic in humans [3,7,8], while cattle vaccine *B. abortus* strain RB51 carries resistance to rifampin, a drug useful for human treatment [9]. The genetic basis of attenuation in these live veterinary vaccines is not defined and thus stability is uncertain. Evidence of such uncertainty was observed in derivatives of S19 that regained the ability to catabolize erythritol [10].

Rough strains of *Brucella*, deficient in the ability to synthesize or assemble intact lipopolysaccharide (LPS), tend to exhibit reduced virulence [10,11]. Exceptions to this, *B. ovis* and *B. canis*, are naturally rough and cause disease in their preferred hosts [12] but have limited virulence in humans. Multiple studies indicate a contribution of LPS O-polysaccharide (OPS) to intracellular survival and virulence of the predominant *Brucella* species that cause disease in humans [10,11,13–17]. Loss of OPS increases sensitivity of *B. abortus* to serum complement [10]. This does not hold for *B. melitensis*, though roughness increases surface binding of complement components by both species [18]. The presence of OPS on internalized *B. melitensis* inhibits apoptosis, while a lack of OPS stimulates apoptosis [19,20]. OPS also plays a role in the ability of *Brucella* to avoid or delay phagosome–lysosome fusion and thus survive within macrophages [21].

As OPS represented a valid target for attenuation to develop candidate live vaccines for humans, we deleted *wboA*, a gene encoding a glycosyl transferase essential for OPS synthesis [11], in the genome of *B. melitensis* 16M. We also combined this *wboA* deletion with a *purEK* deletion, because strain WR201 appeared a promising live vaccine candidate [22,23,24], and since this might result in a safer vaccine strain that would not cause seroconversion.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Table 1 lists strains and plasmids constructed and used in this study.

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Table 1
Bacterial strains and plasmids used in this study.

Name	Description	Source or reference
Bacterial strain		
B. melitensis 16M	Wild type strain, smooth	G.G. Schurig, VPI
B. melitensis WR201	Δ*purEK* mutant of strain16M	[22]
B. melitensis WRR51	Δ*wboA* mutant of strain16M	This study
B. melitensis WRRP1	Δ*purEK* mutant of strain WRR51	This study
Escherichia coli DH10B	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lac*X74 *endA*1 *recA*1 *deoR* Δ(*ara-leu*)7697 *araD*139 *galU galK nupG rpsL* λ⁻	Life Technologies, Inc., Rockville, MD
Plasmid		
pJM63	Clone of Tn*5*-disrupted *wboA* locus of *Brucella abortus* strain RA1	[11]
pJM83ΔClaI	*Cla*I deletion of pJM63	This study
pRFBU1	pJM83Δ*Cla*I with Cm^r cassette insert	This study
pBBR1MCS	Broad-host range vector; Cm^r	[28]
pRFBU2	*B. melitensis* 16M *wboA* amplicon inserted into pBBR1MCS	This study
pRFBUK11	Kn^r cassette inserted in pRFBU2	This study
pBBR1MCS-6Y	*Brucella* GFP expression vector	[33]
pMNWG16	GFP cassette inserted into pRFBUK11	This study
pGSG5	pBBR1MCS-based plasmid containing Ap^r cassette, *B. melitensis* *wboA* and GFP under control of P_{groES}.	This study
pUC4K	Kn^r cassette donor	Pharmacia

2.1.1. Strains

In order to introduce a *wboA* deletion into *B. melitensis* strain 16M, two internal *Cla*I sites were used to make a deletion in the *wboA* open reading frame on pJM63 (a 11.6 kbp *Eco*RI clone of a *B. abortus* strain RA1 chromosomal fragment containing the Tn*5*-disrupted *wboA* [11]) that removed the inserted Tn*5* element along with 607 bp of flanking *wboA* coding sequence. A chloramphenicol resistance cassette from pCAT19 [25] was then inserted on a *Sma*I fragment into the blunted *Cla*I deletion site of this plasmid, pJM63Δ*Cla*I, to create pRFBU1. Electroporation was used to introduce pRFBU1 into *B. melitensis* strain 16M, where pGEM-based plasmids such as pJM63 do not replicate. Plates containing 5 μg/ml chloramphenicol were used to select for replacement by homologous recombination of the intact genomic *wboA* allele with the deleted and marked allele. Thus, a chloramphenicol-resistant rough strain designated WRR51 was isolated. A second allelic replacement was then made in the *purEK* locus of strain WRR51 by introducing pURE198 [22] by electroporation. This pUC-based plasmid contains the *purEK* operon cloned from the *B. melitensis* strain 16M genome with a 248 bp region including 193 bp of the 3′ end of *purE* and 18 bp of the 5′ end of *purK* replaced with a kanamycin resistance gene. This second exchange created a chloramphenicol and kanamycin resistant rough purine auxotroph designated WRRP1.

2.1.2. Plasmids

Plasmid pRFBU2 was constructed by amplifying the *wboA* region from the *B. melitensis* strain 16M genome by polymerase chain reaction (PCR) using oligonucleotide primers previously described [11] and inserting the amplicon into pBBR1MCS, a derivative of broad host range plasmid pBBR1 [28]. Plasmid pRFBUK11 was created by inserting a kanamycin resistance cassette from pUC4K on a *BamH*I fragment into the *BamH*I site of pRFBU2 and was used in previous studies to complement the *wboA* defect in strain WRR51 [18].

2.1.3. Growth conditions

Brucella strains were grown in Brucella broth (Difco), Brucella agar (BA) or M9 minimal agar. Frozen stocks in 25% glycerol were thawed and used to inoculate. Broth cultures were shaken at 37 °C for 22–26 h to an A_{600} of 0.4–0.6. Broth media did not contain antibiotics to avoid carryover (maintenance of relevant phenotype was verified). Bacterial cells were harvested by centrifugation, washed and adjusted to the correct concentration in sterile 0.9% NaCl. Turbidity was measured by spectrophotometer to estimate viable bacterial counts. Actual colony-forming units (CFU) in inocula were determined by serial dilution and plating on BA. Plates were counted after 4 days of incubation at 37 °C unless otherwise noted.

2.2. DNA manipulation and analysis

Procedures not specified were done by standard methodology [27,29,30]. Enzymes were purchased from InVitrogen, Carlsbad, CA. Southern blots were done using positive-pressure transfer (Stratagene, LaJolla, CA) to Nytran membranes (Schleicher and Schuell, Keene, NH), UV crosslinking (Stratagene, LaJolla, CA), and chemiluminescent detection (Amersham Life Sciences).

2.3. Electroporation

Electroporation was used to introduce DNA into *E. coli* and *Brucella* strains. *Brucella* strains were grown for 20 h in YENB [26], pelleted, washed in 0.5 volume cold 10% glycerol and resuspended in 1/10 volume of the same. In cuvettes, 1 μg of plasmid DNA was added to 100 μl of the electrocompetent bacteria and then electroporated in a BioRad Gene Pulser at 2.5 kV, 25 μF and 600 Ω. One milliliter of SOC (Life Technologies, Inc., Gaithersburg, MD) was added and the mix was incubated at 37 °C with shaking for 1 h. The electroporation mix was then plated on BA containing the appropriate antibiotic selection.

2.4. DNA sequencing

The nucleotide sequence of the *B. melitensis* 16M genomic region containing the putative *wboA* gene was determined using Prism thermocycling fluorescent dideoxy chemistry and a 373A automated DNA sequencer (Applied Biosystems Inc.). Sequencing primers were made either using an oligonucleotide synthesizer (ABI model 394) or by BioServe Technologies, Laurel, MD. The sequence was verified by Veritas Labs, Bethesda, MD. Sequence was compiled using Sequencher software (GeneCodes, Madison, WI), analyzed using GCG (Genetics Computer Group, Madison, WI) and BLAST [31], submitted to GenBank on 17 December 2001 and received accession number AY065979.

2.5. Cell culture and infection

Monocytes were isolated from normal human volunteers by counterflow centrifugal elutriation [32]. Monocytes were suspended in monocyte culture medium [MCM; RPMI 1640 medium (Gibco) containing 10% heat-inactivated human AB serum (Sigma),