Brucellosis Vaccines: Assessment of Brucella melitensis Lipopolysaccharide Rough Mutants Defective in Core and O-Polysaccharide Synthesis and Export

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Abstract

Background: The brucellae are facultative intracellular bacteria that cause brucellosis, one of the major neglected zoonoses. In endemic areas, vaccination is the only effective way to control this disease. Brucella melitensis Rev 1 is a vaccine effective against the brucellosis of sheep and goat caused by B. melitensis, the commonest source of human infection. However, Rev 1 carries a smooth lipopolysaccharide with an O-polysaccharide that elicits antibodies interfering in serodiagnosis, a major problem in eradication campaigns. Because of this, rough Brucella mutants lacking the O-polysaccharide have been proposed as vaccines.

Methodology/Principal Findings: To examine the possibilities of rough vaccines, we screened B. melitensis for lipopolysaccharide genes and obtained mutants representing all main rough phenotypes with regard to core oligosaccharide and O-polysaccharide synthesis and export. Using the mouse model, mutants were classified into four attenuation patterns according to their multiplication and persistence in spleens at different doses. In macrophages, mutants belonging to three of these attenuation patterns reached the Brucella characteristic intracellular niche and multiplied intracellularly, suggesting that they could be suitable vaccine candidates. Virulence patterns, intracellular behavior and lipopolysaccharide defects roughly correlated with the degree of protection afforded by the mutants upon intraperitoneal vaccination of mice. However, when vaccination was applied by the subcutaneous route, only two mutants matched the protection obtained with Rev 1 albeit at doses one thousand fold higher than this reference vaccine. These mutants, which were blocked in O-polysaccharide export and accumulated internal O-polysaccharides, stimulated weak anti-smooth lipopolysaccharide antibodies.

Conclusions/Significance: The results demonstrate that no rough mutant is equal to Rev 1 in laboratory models and question the notion that rough vaccines are suitable for the control of brucellosis in endemic areas.

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Introduction

Brucellosis is a group of closely related zoonotic bacterial diseases caused by the members of the genus Brucella, a group of gram-negative bacteria that behave as facultative intracellular parasites. There are several Brucella species, and they infect a wide range of mammals in which they produce a grave and debilitating disease that requires a long and costly antibiotic therapy and that often leaves permanent sequelae [1]. Because of its high incidence in developing countries, economic consequences, and difficult eradication, the World Health Organization considers brucellosis as one of the seven neglected zoonoses, a group of diseases that contribute to the perpetuation of poverty [2].

Ruminants are highly susceptible to brucellosis. Cattle are most often infected by B. abortus whereas sheep and goats are the preferred hosts of B. melitensis, the Brucella species most virulent for
humans [3]. Although there is no human vaccine, vaccination of animals against brucellosis is one of the most cost-effective measures to improve human health in endemic areas [4] as well as an essential tool to achieve eradication [5,6]. For these purposes, vaccines B. abortus S19 and B. melitensis Rev 1 have been successfully used in some developed countries, but both induce abortions when applied during pregnancy, are virulent for humans and elicit antibodies to the smooth (S) lipopolysaccharide (LPS) of the Brucella surface that interfere in serodiagnosis. Moreover, Rev 1 is resistant to streptomycin, an antibiotic used to treat the disease. Although the serodiagnosis problem can be partially solved by using the conjunctival route, by avoiding adult vaccination, and by an individual serological follow up, the breeding conditions characteristic of small ruminants make these measures unrealistic in large areas of the world. Therefore, effective brucellosis vaccines not interfering in diagnosis would represent a major breakthrough [7,8].

Rough (R) Brucella mutants lack the LPS immunodominant N-formylperosamine O-polysaccharide (O-PS) and are attenuated. Thus, they have been the subject of great attention as alternative vaccines [7,8]. Some R vaccines or candidates are spontaneous mutants selected after repeated passage on antibiotic-containing media. This approach was used to obtain RB51, a B. abortus R mutant that carries a IS711-disrupted wboA (putative glycosyltransferase gene) as well as unknown mutations also affecting LPS. However, RB51 has yielded controversial results in cattle, is not effective in sheep and is resistant to rifampin, an antibiotic used to treat brucellosis [8]. B. melitensis RBM9, RBM11, RBM15, RBM17 and RBM19 have been obtained by a similar method and, as expected, they carry undefined LPS defects and are rifampin resistant [9]. Targeted and transposon mutagenesis have also been used. Disruption of per, wboA and wbkA (putative perosamine synthetase and glycosyltransferase genes) results in R mutants that outperform RB51 in the mouse model [10–12], showing that empirically R vaccines can be improved. However, R mutants can result from mutations affecting O-PS precursor synthesis, its polymerization and transport or from a variety of defects in the inner core oligosaccharide. Presently, it is not known which of these mutations results in the best R vaccine or how such a vaccine would compare with S19 or Rev 1. To address these questions, we investigated 14 LPS B. melitensis mutants with respect to LPS defects, intracellular multiplication and virulence and vaccine efficacy in mice. The results provide the basis for selecting the best R candidates for sheep and goat vaccination against B. melitensis, and are also relevant for an appraisal of the value of brucellosis R vaccines.

Results

Mutagenesis, mutant selection and growth characteristics

Perusal of the literature shows that 16M [reference strain of B. melitensis biovar 1 and the one sequenced] does not always show the virulence levels of other S brucellae [12–14]. Because of this, we used this strain and also B. melitensis H38 (fully virulent [15]) to obtain the nalidixic acid resistant (NalR) derivatives necessary for mutagenesis and confirmed that this selection did not affect their characteristics in mice (not shown). Then, we performed transposon mutagenesis and obtained ca. 16,500 kanamycin resistant mutants from which we selected 23 that displayed a stable R phenotype both in vitro and in mice, as judged by the crystal violet test, lack of reactivity with anti O-PS antibodies and phage typing. These mutants mapped in 16 open reading frames (ORFs) scattered into eight regions. Complementation failed to restore the S phenotype in mutants in four of these ORFs. Moreover, mutagenesis of the adjacent ORFs ruled out both possible polarity effects and the involvement in LPS synthesis of some ORFs with suggestive annotations (Table S1). We confirmed the R phenotype of the remaining mutants (Table 1) by examining the LPS by the sodium dodecylsulfate-proteinas K method and, for ORFs BMEI1326, BMEI1414, BMEI1426 and BMEI1427, by making in frame deletion mutants. Finally, since some mutants showed differences in colony size suggestive of altered growth rates, we obtained the corresponding growth curves. Mutants in BMEI1415 (wzm), BMEI1886 (pgm) and BMEI1326 (wa**) were retarded with respect to the parental strain whereas BMEI0899 (manBcore) grew faster (Figure S1).

Table 1. ORFs shown to be involved in B. melitensis LPS synthesis.

| ORF          | Strain (mutants) | Mutagenesis | Role (R phenotype) | LPS gene | Annotation          |
|--------------|------------------|-------------|--------------------|----------|---------------------|
| BMEI0997     | 16M (1)          | Transposon  | O-PS synthesis (R1) | wboB     | Mannosyltransferase |
| BMEI1098     | H38 (1)          | Transposon  | O-PS synthesis (R1) | wboA     | Mannosyltransferase |
| BMEI1126     | 16M (1)          | Transposon and in frame deletion | Core synthesis (R2) | wbk**     | Glycosyltransferase |
| BMEI1139     | H38 (2),16M (1)  | Transposon  | O-PS synthesis (R1) | wbkE     | Mannosyltransferase |
| BMEI1136     | 16M (1)          | Targeted mutagenesis | Uncertain | manB     | Phosphomannomutase  |
| BMEI1404     | 16M (1)          | Transposon  | O-PS synthesis (R1) | wbkA     | Mannosyltransferase |
| BMEI1413     | 16M (1)          | Transposon  | O-PS synthesis (R1) | gmd      | GDP-mannose dehydratase |
| BMEI1414     | 16M (1), H38 (2) | Transposon and in frame deletion | O-PS synthesis (R1) | per      | Peromannase synthetase |
| BMEI1415     | 16M (1)          | Transposon  | O-PS synthesis (R1) | wzm      | ABC transporter     |
| BMEI1426     | H38 (3)          | Transposon and in frame deletion | O-PS synthesis (R1) | wbkF     | Undecaprenyl-glycosyltransferase |
| BMEI1427     | H38 (2)          | Transposon and in frame deletion | O-PS synthesis (R1) | wbkD      | Epimerase/dehydratase |
| BMEI1886     | 16M (1)          | Transposon  | Core synthesis (R2) | pgm      | Phosphoglucomutase  |
| BMEI0899     | H38 (1)          | Transposon  | Core synthesis (R3) | manBcore | Phosphomannomutase  |

1 16M, B. melitensis 16M NalR; H38, B. melitensis H38 NalR
2 n.a., not applicable.

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The mutations block the major LPS polysaccharide synthesis pathways

In order to assign the mutations to the O-PS or the core oligosaccharide biosynthetic pathways, we first classified the mutants as R1, R2 or R3 according to the decrease in LPS molecular weight (Figure 1 and Table 1). Three R1 mutants mapped in \textit{per} (BMEI1414, Table 1). Since \textit{per} is involved in the synthesis of perosamine [16], the only \textit{Brucella} O-PS sugar [17], we took this as evidence for a complete core in R1 and, consequently, for a mutation affecting the O-PS. Likewise, the progressively defective core in R2 and R3 mutants indicated that the corresponding ORF belonged to core synthesis pathways. Consistent with these interpretations, the 2-keto-3-deoxyoctulosonic acid (Kdo) inner core marker content of solvent-extracted R-LPS of BMEI1414 and BMEI1427 (R1), BMEI1326 (R2) and BMEI10899 (R3) mutants was 3.8, 4.0, 6.8 and 9.5%, respectively.

**Mutants affected in O-PS synthesis.** Mutants in BMEI10997 and BMEI10998 and in ORFs from BMEI1393 to BMEI1427 were of the R1 type (Table 1 and Figure 2A). The two first ORFs, putatively encoding glycosyltransferases, included the previously described \textit{wbkD} (BMEI0998) [18]. According to their R1 phenotype, these two ORFs are implicated in O-PS synthesis and BMEI10997 was thus named \textit{wbkB}. Likewise, the R1 phenotype of BMEI1393 matched its annotation as a mannosyltransferase gene (mannose and perosamine are related) and its location close to \textit{wbkC} (BMEI1404), the first ORF of the O-PS \textit{wbk} region [16] (Figure 2B). Because of this, we named the gene \textit{wbkE}. The R1 phenotype of mutants in genes \textit{wbkA}, \textit{gmd}, \textit{per} and \textit{azwm}, all in the \textit{wbk} region, was also in keeping with the roles proposed in previous works (Table 1 and Figure 2A) [11,16]. Two other R1 mutants mapped close to \textit{wbk} (Figure 2B). The first one (BMEI1426), separated by several IS from \textit{wbkC} (BMEI1418), was identified before as a LPS gene in \textit{B. abortus} [19] and encodes a 335 amino acid protein with homology to polysisoprenyl-phosphate N-acetylhexosamine-1-phosphate transferases (PNPT). Known PNPTs include \textit{Pseudomonas aeruginosa} PAO1 WbpL and \textit{Escherichia coli} WecA, both involved in bactoprenol priming but belonging to two subfamilies that differ in amino sugar specificity [20]. A search for WbpL (339 amino acids) homologues in \textit{B. melitensis} identified BMEI1426 as the closest one (BLAST E-value = 4e-25). For WecA (367 amino acids), not only BMEI1426 (1e10-52) but also BMEI10899 (1e10-45) were identified. However, the only WecA match in \textit{B. abortus} genomes was the BMEI126 ortholog (BraAb1_0533 and BAB1_0539-49 in \textit{B. abortus} 9-49 and 2308, respectively). This result, which is due to a large deletion in \textit{B. abortus} chromosome II encompassing the position of the BMEI10899 ortholog [21], supports the role of BMEI1426 as the \textit{Brucella} PNPT gene. The BMEI1426 protein resembles WecA and WbpL in the Asp rich consensus motif of the catalytic site [20] but did not show the motifs thought to relate to substrate specificity in either subfamily. Since it could not be ascribed to any of these subfamilies, we named this gene \textit{wbkF}. Finally, we named the adjacent ORF (BMEI1427) \textit{wbkD}. The protein is annotated as an epimerase/dehydratase and carries a NAD or NADP binding motif, two features of the trifunctional UDP-N-\textit{D-acetylglucosamine} 4,6-dehydratase/5-epimerase/3-epimerase enzymes that take part in the synthesis of 2-acetamido-2,6-dideoxy-L-hexoses such as N-acetylglucosamine and N-acetyquinovosamine [22]. Quinovosamine (2-amino-2,6-dideoxy-D-gucose) has been reported in the LPS of \textit{S Brucella} spp. [23-27] but its location is unclear. Since N-acetyquinovosamine synthesis would require an epimerase/dehydratase using NADP as the coenzyme [22], we investigated the S-LPS hydrolytic polysaccharides of \textit{B. melitensis} and \textit{B. abortus}. In addition to the N-formylperosamine signals, a 2.06 ppm. indicated the presence of an N-acetyl group in these materials. The integrals of these signals corresponded to those from the methyl groups of perosamine at ~1.3 ppm showed that the former derived from ~1% of the material. Moreover, the 1H NMR spectrum at 25°C of the \textit{B. abortus} material (which was highly pure) showed a resonance at 4.56 ppm having \textit{H1}, \textit{H2} = 7.9 Hz. Further analysis was performed at 70°C (Figure S2) using in particular 2D 1H-1H total correlation spectroscopy (TOCSY) with spin-lock times up to 120 ms by which a complete spin-system corresponding to a quinovosamine residue was traced out. The following chemical shifts were identified: δ 4.54 (H1), 3.79 (H2), 3.66 (H3), 3.35 (H4), 3.50 (H5), and 1.30 (H6). Thus, the analysis revealed the characteristic 1H NMR chemical shifts of a \beta-linked quinovosamine, which should be N-acetylated and is presumably 3-substituted [28,29]. Indeed, this N-acetyquinovosamine could be the substrate of the putative PNPT \textit{WbkF}, since \textit{WbkF} is close to \textit{WbpL} and the latter transfers either N-acetylglucosamine or N-acetyquinovosamine [20]. Moreover, the adjacent position of \textit{wbkF} and \textit{wbkD} also suggests a functional connection.

Extension of the \textit{wbk} region up to BMEI1393 (\textit{wbkE}) (Figure 2B) suggested that BMEI1394, BMEI1395 and BMEI1396 (putative mannos-6-phosphate isomerase [\textit{manA}], mannos-1-phosphate guanylyltransferase [\textit{manC}] and phosphomannomutase [\textit{manB}]) could be involved in the synthesis of mannosose, the precursor of perosamine (Figure 2A). However, when we disrupted BMEI1393 (\textit{manA}), the mutant still expressed O-PS demonstrating that the gene was not essential for mannose synthesis.

**Mutants affected in core oligosaccharide synthesis.**

Mutant BMEI1326 had a R2 LPS like that observed before in a \textit{B. abortus} orthologous mutant [11], and encodes a predicted glycosyltransferase of family 25. This is worth mentioning because this family contains LPS glycosyltransferases, including the WaaX protein that takes part in the synthesis of some \textit{E. coli} core chemotypes [30]. BMEI1326 is isolated from other LPS genes and, in the absence of more information, we maintained its provisional...
Mutant in BME1886 was affected in a putative phosphoglucomutase (Pgm) and had a R2 LPS (Figure 2), in agreement with a previous observation in *Brucella abortus* [31] and with the presence of glucose in the *Brucella* LPS core [26]. Finally, BMEII0899 disruption generated a R3 LPS (Figure 2) like the one reported for the orthologous *B. abortus* mutants [11,19]. It is annotated as phosphomannomutase gene and, because of the severe core defect, we proposed before the name of *manBcore* [11]. However,
taking into account the lack of R phenotype in the mutant in the
manB gene of region wbk (see above) and the absence of additional
manB annotations in Brucella, manB再加上contiguous manC seem
the only genes of the pathway providing mannose for both
perosamine and core synthesis (Figure 2A).

Mutants blocked in O-PS export. To study whether the R
mutants could elicit antibodies to the O-PS, we infected mice and
tested the sera in an enzyme-linked immunosorbent assay with
Brucella native hapten, a N-formylperosamine polysaccharide that
lacks core sugars [32]. We observed reactivity in the sera of mice
infected with 10^8 or 10^10 colony forming units (CFU) of the
wzm (BMEI1415) mutant or with 10^10 CFU of the wa** (BMEI1326)
mutant (0.810 and 0.714 optical density readings, respectively;
1:25 dilution). However, the antibody levels were lower than those
induced by 10^6 CFU of the 16M Naïve parental strain (1.490
readings for the same dilution). When we tested extracts of these
mutants by gel immunodiffusion with sera from Brucella infected
cattle, we observed a component giving a reaction of identity with
the native hapten polysaccharide (Figure 3). Moreover, 1H-NMR
analysis of the extracts showed the signals of α1,2- α1,3-linked N-
formylperosamine polysaccharides [17] plus a small signal at
2.06 ppm corresponding to the N-acetyl group of an unidentified
N-acetylated aminosugar. Upon cell fractionation, the
polysaccharides were detected in the envelope and cytosol of the
wzm and wa** mutants, respectively.

Mutant designation. Based on the above analyses, we
designated the mutants according to the original strain (Bm16M
or BmH38), the phenotype (R) and the LPS gene disrupted
(Table 1 and Figure 2).

The LPS defects alter key topological, physicochemical
and biological surface properties of Brucella

It is known that outer membrane proteins (Omp) are more
exposed on R than on S brucellae [11,33], and we confirmed this for
R1, R2, and R3 mutants using monoclonal antibodies (Moabs) to
Omp1, Omp2b, Omp31, Omp25, Omp19, Omp16 and Omp10
(not shown). Less is know about the topology of R-LPS epitopes and,
therefore, we tested the mutants with the appropriate Moabs. In all
cases, absence of O-PS correlated with exposure of the outer core
(Figure 4, left panel) and the lipid A disaccharide (Moab Bala-1; not
shown). Surprisingly, the R1 and R2 but not the R3 mutants failed

![Figure 3. B. melitensis mutants in genes wa** and wzm synthesize N-formylperosamine polysaccharides. The figure shows a gel immunodiffusion analysis of the polysaccharides obtained from mutants Bm16MRwa** and Bm16MRwzm. Well 16MNaïve contained the LPS of the parental strain and shows both the slow diffusing S-LPS and the fast diffusing native hapten polysaccharide precipitin lines; (S), serum from naturally infected cattle. doi:10.1371/journal.pone.0002760.g003](image1)

![Figure 4. LPS epitopes in smooth B. abortus and B. melitensis strains and their cognate R mutants. Whole bacteria (left panel) or B. melitensis R-LPS representative of the R1, R2 and R3 types (right panel) were probed with monoclonal antibodies of the indicated specificity (Ba2308, B. abortus 2308; Bm16MNalR, B. melitensis 16M Naïve; codes for the R mutants are those used in the text). doi:10.1371/journal.pone.0002760.g004](image2)
polycation effects were clearly reduced in the S parental strains; the potential was measured in the presence of the polycation poly-L-lysine. BmH38R mutants behaved like the S. minnesota HL63 and BmH38R mutants (Figure 4 left panel). As expected, all these R mutants but wbkD [11] reacted with Baro-2. Then, we tested representative B. melitensis R-LPS by Western blot. The LPS of BmH30RwbkD (R1), Bm16MRwbkD (R1) and Bm16MwbkD* (R2) but not that of BmH30RwbkD (R3) reacted with Baro-1 (Figure 4, right panel). Conversely, Baro-2 failed to detect an inner core epitope in the R1 and R2 but not in the R3 LPS (Figure 4, right panel). These results show hitherto undescribed LPS core differences between B. abortus and B. melitensis.

All R mutants displayed an increased surface hydrophobicity, more markedly in B. melitensis than in B. abortus (Figure 5, left panel), and also in sensitivity to the polycationic lipopeptide polymyxin B (not shown). The latter effect was explained by the increase in negative Zeta potential (surface charge) of the R mutants (Figure 5 right panel). However, we did not observe a clear correlation between Zeta potential and LPS defects (~50 to ~64 mV for R1, ~53 to ~58 mV for R2, and ~66 to ~68 mV for R3), like that obtained for the S. minnesota R chemotypes (Ra, ~39 mV; Re, ~44 mV, and Re, ~55 mV). Since the LPS core is complete in Ra, lacks the distal N-acetyl-glucosamine in Re, and is reduced to two Kdo residues in R3, the LPS core does not balance the negative charge of the Kdo-lipid A section in Brucella LPS. In support of this interpretation, all Brucella R mutants behaved like the S. minnesota Re mutant when the Zeta potential was measured in the presence of the polycation poly-L-lysine (Figure 5, right panel). The Zeta potential value and the polycation effects were clearly reduced in the S parental strains S. minnesota H38 and B. melitensis H38 NaR (Figure 5, right panel) or B. melitensis 16M NaR (not shown).

Resistance to complement-mediated killing in normal serum is a protective immunity, it has to be able to multiply intracellularly.

R mutants show different patterns of attenuation

To assess attenuation, we inoculated BALB/c mice with three doses of each R mutant or with 10^6 CFU of the parental strains and vaccine Rev 1, and determined the CFU numbers in the spleens. All R mutants were less persistent than the parental strains, with differences that allowed classifying them into four broad attenuation patterns (Figure 7A). Pattern 1 comprised BmH38Rper, BmH38RwbkD and BmH38RwbkF, all of the R1 type. They multiplied at 10^6 CFU/mouse and, no matter the dose, reached spleen counts at week 2 similar to those of the parental strain. Then, the CFU decreased markedly. Pattern 2 included BmH30RwbkD, Bm16MRwbkD, Bm16MRwbkE, BmH30RwbkE, Bm16MRwbkA, Bm16MRwbkE, and Bm16MRwbkD, all of the R2 type. Although able to replicate, they never reached the parental strain level and were cleared at rates directly related to the inoculum size. Pattern 3 included Bm16MRwbkE and Bm16MRwbkD, two mutants with internal N-formylperosamine polysaccharides and reduced in vitro growth rates. Although not multiplying when inoculated at 10^6 CFU, they persisted at relatively high numbers at the end of the experiment (week 6) for the 10^5 or 10^6 CFU doses. To further examine their persistence, we inoculated mice with 10^6 CFU/mouse and examined them 6, 9 and 12 weeks after infection. The CFU/spleen at week 6 were similar to those of the first experiment, declined more than 2 logs at week 9 and the mutants were cleared by week 12. Remarkably, the results of pattern 3 mutants (10^5 or 10^6 CFU) and Rev 1 almost overlapped at weeks 2, 3, 6 (Figure 7), 9 and 12 (not shown). Finally, we separated BmH38RwbkD (R3 and R2) from the parental strain and Rev 1 and determined the CFU numbers in the spleens. All R mutants inoculated with 10^6 CFU induced transient splenomegaly similar to that of Rev 1 (Figure 7B). As reported before [36], splenomegaly caused by virulent Brucella increased throughout the experiment (Figure 7B). In contrast, the weights of the spleens of Rev 1 inoculated mice peaked at week 2 and declined afterwards. Only pattern 1 R mutants inoculated with 10^6 CFU induced transient splenomegaly similar to that of Rev 1 (Figure 7B).

Some R mutants reach the Brucella replicative niche in macrophages

Since a brucellosis vaccine must persist long enough to trigger protective immunity, it has to be able to multiply intracellularly.

Figure 5. Surface hydrophobicity (partition in hexanol/water) and charge (zeta potential in dependence of poly-L-lysine) of smooth B. abortus and B. melitensis parental strains and their cognate R mutants. For Brucella, the genes (left panel) or the strain codes (right panel) indicated are those used in the text. S. minnesota controls were: wild type, SmWT; Ra LPS mutant, SmRa; Re LPS mutant, SmRe. doi:10.1371/journal.pone.0002760.g005
Thus, we studied the multiplication of BmH38Rper, Bm16MRper and (patterns 1 and 2, both R1), Bm16MRwzm (pattern 3, R1 and internal N-formylperosamine polysaccharides), Bm16MRwzm** (pattern 3, R2 and internal N-formylperosamine polysaccharides) and BmH38RmanBcore (pattern 4, R3) in bone-marrow derived macrophages (BMDM). We found that only BmH38RmanBcore was unable to grow in BMDM, and that no mutant was as efficient as the virulent strain (Figure 8, left panel). We also examined the intracellular fate of representative mutants. After 24 hours, some bacteria of patterns 1, 2 and 3 were in calreticulin (endoplasmic reticulum marker)-positive but also in LAMP1-positive compartments and then co-localized efficiently with calreticulin, persisting intact at least 24 hours.

**R mutants can be ranked by their ability to immunize against virulent bacteria**

Using BmH38RwzbF, BmH38RwzbD (both of pattern 1), Bm16MRwzm** (pattern 3), and BmH38RmanBcore (pattern 4), we determined the conditions necessary to rank R mutants by their ability to induce protection by the most favorable (i.e. intraperitoneal) route [8]. We studied the vaccine dose (from 10^6 to 10^8 CFU), two strains of mice (BALB/c and CD-1), the vaccination-challenge interval (4 and 9 weeks), and the challenge dose (10^8 and 10^9 CFU) and strain (16M and H38). A vaccine dose below 10^6 CFU or a 10^9 CFU challenge resulted in poor or no protection, and a vaccination-challenge interval of 9 weeks did not improve the evaluation. Similarly, there was no influence of the challenge strain regardless of the 16M or H38 background of the mutants. Finally, the Brucella-susceptible BALB/c mice yielded a larger span between control groups (PBS [150 mM NaCl, 7 mM KH2PO4, 10 mM K2HPO4; pH 6.85] and Rev 1-inoculated mice) and more homogenous results than CD-1 mice, thereby allowing better statistical evaluations (see Figure S3).

Then, we performed a first experiment (Table 2) which ranked the 14 R mutants into three sets. The first one (Bm16MRwzm**, Bm16MRwzm, BmH38RwzbF, BmH38RwzbD, and Bm16MRper) generated protection not significantly different from that provided by Rev 1 (at the dose and by the route used as reference in mice). A second set (BmH38RwzbA, Bm16MRgmd, and Bm16MRgml) generated protection but less than Rev 1, and the third set (6 mutants) failed to protect. For a better discrimination, we tested the first set of mutants (plus two mutants of the third set as references) using 10 mice per group (Table 2). The results confirmed Bm16MRwzm**, Bm16MRwzm and BmH38RwzbF as the best R vaccines. However, protection by BmH38Rper and BmH38RwzbD was less than that by Rev 1.

Only attenuation pattern 3 R mutants approach Rev 1 as Brucella melitensis vaccines

Intraperitoneal inoculation is unpractical in the natural host. Thus, we tested the efficacy of the five best R mutants when administered subcutaneously. As shown in Table 3, only pattern 3 mutants compared to Rev 1 under these conditions. However, the dose used was one thousand fold higher than that of Rev 1 (10^8 and 10^9 CFU, respectively). Higher doses of these R mutants generated abscesses and other untoward effects.

**Discussion**

We present here a comprehensive study aimed to clarify the value of brucellosis R vaccines that is based on an understanding of the genetics of Brucella LPS synthesis, rather than on empirical approaches. LPS synthesis is known to occur through three main pathways that lead to the synthesis of the O-PS, core oligosaccharide and lipid A [37]. Some steps of the last two pathways overlap to generate the (Kdo)2-lipid IVa precursor on which sugars other than Kdo are then assembled to generate a complete
R-LPS (R1 in the classification used in the present work). Accordingly, deficiencies in these processes generate deeper R (R2 and R3) phenotypes than those affecting O-PS synthesis. Polymerization of the latter takes place on the bactoprenol carrier which subsequently shuttles the polymer to the periplasm, the place where it is linked to R1-type R-LPS. Therefore, mutants in O-PS synthesis generate R1 phenotypes which may be accompanied or not by accumulation of O-PS precursors and may have side effects on other envelope located functions. In addition to the above-summarized pathways, there are ancillary routes providing the core and O-PS building units. Genomic surveys show that the three main pathways are present in \textit{Brucella} \cite{38}, and the mutations studied here affect key steps of the O-PS and core pathways as well as the synthesis of precursors.

Although we screened about 16,500 transposon mutants from \textit{B. melitensis} H38 NaI\textsuperscript{R} and 16M NaI\textsuperscript{R}, and expanded the investigation to those ORFs with suggestive annotations that flanked some eventually spurious R mutants, we only found one gene (\textit{wbkD}) that, to the best of our knowledge, had not been identified as a LPS gene or as a virulence-related gene \cite{11,16,19,24,39–41}. On this basis, it seems that only the \textit{wbk} (Figure 2B) and \textit{wbo} regions encode proteins dedicated to \textit{Brucella} O-PS synthesis. Intriguingly, \textit{wbk} contains several genes that, upon disruption, do not generate R phenotypes. They include \textit{wbkB} \cite{16} and BMEI396, annotated

Figure 7. Attenuation patterns of \textit{B. melitensis} R mutants and vaccine Rev 1 in BALB/c mice in comparison with smooth \textit{B. melitensis} parental strains. Panel A, evolution of CFU/spleen; panel B, spleen weights.

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as manB. We proposed before that the putative man genes in *wbk* could be involved in O-PS synthesis [11] but the evidence that disruption of BMEI1396 fails to generate a R phenotype is against this hypothesis. It is still possible that manB core (BMEII0899) could internally complement the defect in the *wbk* manB mutant. However, since manB core mutants show a deep R phenotype, it

**Figure 8.** Multiplication and intracellular localization in BMDM of representative *B. melitensis* R mutants. Left panel, evolution of intracellular bacteria CFU numbers. Right panel, colocalization of selected R mutants (immunostained in red) with LAMP1 (immunostained in blue) or calreticulin (immunostained in green) in BMDM. doi:10.1371/journal.pone.0002760.g008

**Table 2.** Protective efficacy against *B. melitensis* of *B. melitensis* R mutants (10⁸ CFU) administered intraperitoneally.

| Vaccine       | Type of LPS | Attenuation pattern | Log₁₀ CFU in spleen (X±SD) | Units of protection * | Log₁₀ CFU in spleen (X±SD) | Units of protection * |
|---------------|-------------|---------------------|-----------------------------|-----------------------|-----------------------------|-----------------------|
| Bm16M Rwa**   | R2          | 3                   | 2.07±1.33 b, c              | 3.90                  | 1.83±1.23 b, c              | 4.37                  |
| Bm16M Rwzm    | R1          | 3                   | 2.65±1.88 b, c              | 3.32                  | 2.48±1.73 b, c              | 3.72                  |
| BmH38RvbkF    | R1          | 1                   | 2.91±0.87 b, c              | 3.06                  | 3.32±1.50 b, c              | 2.88                  |
| BmH38RvbkD    | R1          | 1                   | 3.40±1.51 b, c              | 2.57                  | 3.76±1.41 b, d              | 2.44                  |
| BmH38Rper     | R1          | 1                   | 3.70±1.72 b, c              | 2.27                  | 4.10±0.82 b, d              | 2.10                  |
| BmH38RvboA    | R1          | 2                   | 4.31±0.60 b, d              | 1.66                  |                             |                       |
| Bm16MRpgm     | R2          | 2                   | 4.62±0.56 b, d              | 1.35                  |                             |                       |
| Bm16MRgmd     | R1          | 2                   | 4.72±0.50 b, d              | 1.25                  |                             |                       |
| BmH38RvbkE    | R1          | 2                   | 4.99±0.73 d                 | 0.98                  |                             |                       |
| Bm16MRvbkA    | R1          | 2                   | 5.05±0.69 d                 | 0.92                  |                             |                       |
| Bm16MRvbkE    | R1          | 2                   | 5.06±0.43 d                 | 0.91                  |                             |                       |
| Bm16MRvboB    | R1          | 2                   | 5.38±0.36 d                 | 0.59                  |                             |                       |
| Bm16Mrper     | R1          | 2                   | 5.38±0.70 d                 | 0.59                  | 5.44±0.51 d                 | 0.76                  |
| BmH38RmanB_core | R3       | 4                   | 4.88±0.57 d                 | 1.09                  | 5.49±0.52 d                 | 0.71                  |
| Rev 1         | S           | 5                   | 2.76±0.44 b                 | 3.21                  | 2.63±0.90 b                 | 3.57                  |
| PBS           |             |                     | 5.97±0.19                   |                       | 6.20±0.08                   |                       |

*Average of log₁₀ CFU in the spleens of saline inoculated mice minus average of log₁₀ CFU in the spleens of vaccinated mice.

*P<0.005 versus PBS.

*P>0.05 versus Rev 1 vaccinated.

*P<0.005 versus Rev 1 vaccinated.

*10⁵ CFU subcutaneously.

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Table 3. Protective efficacy against *B. melitensis* of selected *B. melitensis* R mutants administered subcutaneously.

| Vaccine (dose)   | Log_{10} CFU in spleen (X ± SD) | Units of protection |
|-----------------|----------------------------------|---------------------|
| Bm16MRwaa**     | 3.19 ± 0.63 b, c                 | 3.10                |
| Bm16MRwzm       | 2.22 ± 1.48 b, c                 | 4.07                |
| BmH38Rwbf       | 4.92 ± 0.51 b, d                 | 1.37                |
| BmH38RwbfD      | 5.08 ± 0.54 b, d                 | 1.21                |
| BmH38Rper       | 4.96 ± 0.67 b, d                 | 1.33                |
| Rev 1 control   | 3.02 ± 1.01 b                    | 3.27                |
| PBS             | 6.29 ± 0.15                      | -                   |

*Average of log_{10} CFU in the spleens of saline inoculated mice minus average of log_{10} CFU in the spleens of vaccinated mice.*

1P<0.005 versus PBS.

1P>0.05 versus Rev 1 vaccinated.

1P<0.005 versus Rev 1 vaccinated.

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would remain to be explained why the converse internal complementation is not effective. Studies on the expression and activity of the proteins encoded by the putative *man* genes in *wbb* are necessary for a definitive conclusion. The *wbb* region also contains the ABC transporter genes (*wzm* and *wzt*) typical of homopolymeric O-PS, and the implication of *wzm* and *wzt* in O-PS export was established in a previous work [16]. We extended this demonstration by purifying the N-formylperosamine polysaccharide built up by Bm16MR*wzm* and showing its location in the cell envelope, in all likelihood bound to bactoprenol, a linkage that could overcome its haptenic nature and account for its immunogenicity. In contrast, the polysaccharide built up by mutant Bm16MR*rev** was found mostly in the soluble fraction. This suggests a transient bactoprenol linked state and, consistent with the R2 LPS pattern, it may be that Wa** transfers a sugar necessary for the linkage of the O-PS to the core. This step occurs in the periplasm [37], but our results are not in disagreement with this because the physical fractionation methods used do not allow to distinguish periplasmic and cytosoluble fractions in *Brucella*. Interestingly, both the *waa** and the *wzm* mutants showed delayed generation times consistent with a linkage of the O-PS precursor to bactoprenol and a reduced availability of the latter for other envelope biogenesis processes. The retarded growth of mutant *pgm*, however, can be explained by the role of *Pgm* in the metabolic steps that use glucose-nucleotides. Obviously, it can be reasoned that the *manBcore* mutant owes its accelerated growth to absence of O-PS synthesis. These differences in growth rates are relevant traits in a live vaccine candidate.

The attenuation of *R Brucella* mutants has been known for decades [5] and it evidently relates to changes in the bacterial surface that affect the interaction of bacteria with cells and soluble effectors of the immune system. Concerning the host cells, it has been observed that *R* mutants penetrate more actively and display higher adherence to nonphagocytic and phagocytic cells than *S Brucella* [42–46]. In addition, Porte et al. [39] found that, whereas virulent *B. suis* cells select lipid rafts to enter into murine macrophages, *R B. suis* *manBcore* cells do not, hence showing the critical role of the O-PS at the port of entry. This role could be that of a negative modulator of non specific adherence which would allow receptors to act, that of the ligand of an unknown cell receptor, or both [39,41]. Indeed, a definition of the nature of the surface changes should contribute to better understand this key aspect of *Brucella* virulence. Despite this, only one study conducted with the spontaneous *R* mutant *B. abortus* 45/20 has partially addressed the overall effects of the O-PS deficiency [49]. Here, we demonstrate that the surface of the *Brucella* outer membrane, once devoid of the O-PS, becomes simultaneously highly hydrophobic and negatively charged, and that the negative charge relates exclusively to inner core-lipid A groups. That this negative charge was similarly abrogated by the O-PS in *S. minnesota* HLD3 and *S Brucella* leads to the conclusion that we are dealing with general physicochemical properties not linked to the particular sugar (N-formylperosamine) in *S Brucella* O-PS. Accordingly, the overall physicochemical picture for the *R* mutant surface is that of a mosaic of negative charges scattered among hydrophobic spots. Such a surface should both allow and bring about multiple non specific interactions with eukaryotic membranes that should override any specific binding. This picture is in keeping with the observations on the invasiveness and attachment of *R Brucella* mutants and supports the hypothesis that the O-PS acts at least in part as a negative modulator. Clearly, the loss of the ability to select the port of entry and the possible unspecific interactions with the membranes of the internal compartments where *Brucella* multiplies are factors that can contribute to attenuation.

Concerning the surface topological changes, we confirmed the differences in Omp and lipid A exposure existing between *R* and *S* bacteria [11,33]. However, the differences in LPS core epitopes observed between *B. abortus* and *B. melitensis* were unexpected. This divergence adds to the absence of Omp31 and possible surface elements related to the 25 Kb deletion of *B. abortus* chromosome II [21], and both have to be considered in the light of other cell surface properties. Over 40 years ago, it was observed that normal human serum has a more powerful killing activity on *B. abortus* than on *B. melitensis* or *B. suis* and that *R Brucella* mutants are cytophatic for monocytes, a phenomenon that would leave the invaders exposed to the bactericidal action of serum [3,50–52]. These findings and hypothesis have been updated in recent works. Fernandez-Prada et al. [53] noted that *B. abortus* 2308 *wboA* mutants (R1 according to our results) are more sensitive to normal human serum than homologous *B. melitensis* 16M mutants. This species difference is supported and extended by our experiments with the R1, R2, and R3 mutants from *B. abortus* 2308 NaLR, *B. melitensis* H38 NaLR and 16M NaLR and sheep and cattle sera. Moreover, since the difference persisted in the *B. abortus* and *B. melitensis* *manBcore* mutants (which display similar core stubs), it is unlikely that core variations in *B. abortus* and *B. melitensis* could account for it. Therefore, it seems that the absences of Omp31 and possible surface elements related to the 25Kb deletion in *B. abortus* [21] account for the greater sensitivity to normal serum, as proposed by Fernandez-Prada et al. [53]. These same authors also found that *B. melitensis* 16M and its *wbdA* R mutant are resistant to normal human serum. In our conditions, however, only one of the seven *R* mutants (BmH38R*wbbD*) survived, indicating that complement sensitivity is a usual characteristic of *B. melitensis* R mutants whose contribution to attenuation cannot be dismissed.

Interestingly, not all *R* mutants were equivalent in surface properties, suggesting that they could display different degrees of cell interaction and virulence which would allow the selection of optimal vaccines. Assessment of this point required appropriate laboratory models and we used both animals and cultured cells. The animal used in the vast majority of works evaluating *Brucella* virulence is the mouse, and spleen CFU is the main criterion. However, literature perusal shows that mouse studies with *R* mutants vary in infectious dose from 10^4 to 10^8 CFU [8]. Preliminary observations and some apparently conflicting results reported in the literature led us to test the possibility that a...
combination of doses could yield better analyses. This was proved correct and we classified the mutants into four broad patterns. Pattern 1 mutants inoculated at the lower dose multiplied transiently reaching spleen counts similar to those of Rev 1 and the S virulent strains. Consistent with this, they reached compartments bearing the endoplasmic reticulum marker calreticulin and multiplied there. These R mutants were the only ones inducing a splenomegaly similar to that of Rev 1. Since splenomegaly correlates with IFN-γ and IL-12 levels in mouse brucellosis and both cytokines are decisive for mounting effective immunoresponses to Brucella [54], it seemed that pattern 1 mutants stimulated immunity better than other R mutants. This interpretation was born out by the ranking of the mutants by their immunizing ability. Despite this, pattern 1 mutants were not equivalent to Rev 1 because the latter strain persisted longer in spleens. Concerning pattern 2, a remarkable feature was a conversely related dose-persistence relationship, a trend that, although less clearly, was also perceived in pattern 1. It has been shown that Brucella behaves as a stealthy parasite that avoids detection by innate immunity at the onset of infection, thus retarding an adaptive cellular response and making possible for the parasite to reach sheltered intracellular niches [35]. This ability is not related to the induction of regulatory cytokines such as IL-10 but rather to a reduction in the pathogen-associated molecular pattern of those envelope molecules (most notably LPS) normally recognized by TLR bearing cells [35]. According to this, it can be predicted that mutants in LPS, a molecule critical in avoiding innate immunity by Brucella, could elicit innate immunity (and hence cellular immunity) in a dose-dependent fashion and be thus eliminated faster at higher than at lower doses. Indeed, our results are consistent with this prediction. Concerning pattern 3 mutants, it can be proposed that their longer generation times account for the lower CFU in spleen and, in keeping with the Brucella stealthy strategy, for a lower stimulation of innate immunity that favors persistence. These mutants did not multiply at the level of the parental strains in BMDM but they were surprisingly efficient in reaching endoplasmic reticulum-derived compartments. Although the first characteristic could relate to their long generation time, we have no clear hypothesis to propose for the second observation. We can speculate that it is somehow connected to the internal O-PS precursor they carry, since these mutants were the only ones that had detectable amounts of it. It has to be considered that a fraction of the pattern 3 bacteria was destroyed by BMDM, and that this should release the O-PS precursor within the cell. The effect of free N-formylperosamine polysaccharides within the Brucella containing vacuoles has not been studied and, in the light of our results and of the presence of these free polysaccharides in S brucellae [32], this is an aspect that deserves attention. Finally, the marked attenuation of the pattern 4 mutant was evident in all of our results and of the presence of these free polysaccharides in S. minnesota [32].

It is interesting that all pattern 1 mutants derived from B. melitensis H38 and that some homologous ones in B. melitensis 16M (like Bm16MRper) belonged to pattern 2 and were poor vaccines. These mutants behaved similarly in BMDM, suggesting that early events in host cells are not the reason for the differences in mice. It seems, therefore, that the overall genetic background of the parental strains is relevant and that selection of Brucella vaccines cannot be based only on the identification of a target gene whose dysfunction generates attenuation. In this regard, it is worth commenting that the literature reveals that strain16M stocks sometimes yield CFU/spleen lower that those obtained in our experiments with either H38 or our 16M. We have tested the virulence of 16M obtained from several laboratories and found that, in fact, some were less virulent than others, suggesting that the stability of this strain cannot be taken for granted (M.J. Grillo, and J.M. Blasco, unpublished results).

Like virulence studies, the laboratory assessment of brucellosis vaccines has been almost always based on experiments in mice, and there is a useful mouse model for the evaluation of S19 and Rev 1 vaccine stocks. This model uses a tight set of conditions chosen to reflect in mice field observations on residual virulence and immunogenicity [55]. However, there are no studies in the natural hosts with different Brucella R vaccines. Therefore, one aim of our work was to set up a model to rank R mutants for their ability to induce protection, and we defined some experimental conditions for this. Using these conditions, we found that the top candidates were Bm16MRwx**, Bm16MRwzwx, BmH38RwbkF, BmH38RwbkD, and BmH38Rper. These mutants belonged to patterns 1 and 3, were able to multiply in BMDM and to reach the niche where virulent brucellae replicate, and all but Bm16MRwx** were of the R1 phenotype. This result with Bm16MRwx** is not in contradiction with our previous observation that a complete core was required for maximal efficiency of R. B. abortus vaccines in mice [11] because Bm16MRwx** (and Bm16MRwzwx) elicited anti-O-PS antibodies, and these are known to be protective in mouse brucellosis [54]. Therefore, we cannot rule out that priming by the O-PS precursors could contribute to an antibody response to the challenge strain accounting in part for protection. In fact, these two mutants are reminiscent of a wbk complemented RB51 construct that produces internal O-PS and that has been shown to generate better protection in mice than RB51 [18]. Also in keeping with the idea that the internal O-PS set the difference with other R mutants, they were the only ones that matched Rev 1 when we assessed subcutaneously, even though at doses one thousand fold higher. Therefore, our results lead to the conclusion that no R mutant completely devoid of O-PS is equal to Rev 1 in the mouse model. On these grounds, and taking into account the range of genetic defects and properties of the R mutants tested, it seems unlikely that any R vaccine lacking the ability to induce anti S-LPS antibodies could match classical brucellosis vaccines in the natural host. Nevertheless, a definite conclusion requires studies with a definite set of R vaccine candidates in sheep and goats.

Materials and Methods

Bacterial strains and culture conditions

B. melitensis 16M (S, virulent) is the reference strain of biovar 1 [56] and B. melitensis H38 (S, virulent) is a virulent biovar 1 strain [15]. The corresponding NalR strains were selected on tryptic soy agar containing 25 μg/ml of the antibiotic. B. abortus 2308 Na1K (S, virulent) and its R mutants (Ba2308Rper [formerly Ba 9.49], Ba2308RwbkF [Ba 2.17], Ba2308RwbkD [Ba 80.16], and Ba2308Rwzb [Ba55.30]) have been described previously [11]. B. abortus bsrS::Tn5 2.13 is an avirulent S strain that carries a normal O-PS but is defective in several outer membrane proteins tightly linked to the LPS [57]. S. minnesota HL63 is a S strain and its HL100 Ra, HL105Rs Rc, and HL111Rs Re mutant is bacteria carrying a R-type LPS with intact (Ra), truncated outer core (Rc) or highly defective (deep R) core [58]. All these bacteria were kept in skim milk at −80°C, and aliquots grown on tryptic soy agar or broth when needed (repeated in vitro passage was systematically avoided).

The reference B. melitensis Rev 1 vaccine was originally obtained from the INRA station at Nouzilly (France) and conserved freeze-dried. For routine use, the stocks were rehydrated and cultured only once before preparing the appropriate bacterial suspensions (see below) [56].

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Mutagenesis, ORF identification and complementation analysis

Mini-Tn5 mutagenesis was performed by mating B. melitensis 16M NaI6 or H38 NaI6 with Escherichia coli SM10 (λpir) carrying the suicidal plasmid pUT/Km [59], and mutants selected on tryptic soy agar with nalidixic acid (25 μg/ml) and kanamycin (50 μg/ml). R mutants were identified by the crystal violet exclusion test and the lack of reactivity with anti-S-LPS IgG [11]. To test the stability of the mutants, 3×105 CFU of each mutant were first seeded on tryptic soy agar, either plain or supplemented with nalidixic acid (25 μg/ml) or kanamycin (50 μg/ml). Mutants showing no variation in CFU numbers (one-way ANOVA) on these media and a stable R phenotype were inoculated intraperitoneally into 8–10 weeks old BALB/c mice (106 CFU/mouse). Two weeks later, the spleens were seeded on tryptic soy agar and colonies restested for roughness and for the presence of Tn5 by Southern blot. The DNA flanking the mini-Tn5 insertion in the selected mutants was cloned and sequenced [45], and ORFs identified using the database and links at http://urbm59.urbm.fundap.ac.be/%7Edharbi/aPAGe/. When the regions adjacent to the mini-Tn-5 included ORFs with annotations suggestive of sugar or polysaccharide synthesis, mutants were generated in B. melitensis 16M using a disruptive strategy [60]. To this end, an internal fragment of 300 bp, localized in 5' part of the ORF was amplified by polymerase chain reaction (PCR) and cloned into the EcoRV restriction site of plasmid pSKoriTKan. Constructs were transferred into B. melitensis 16M NaI6 by conjugation with E. coli S17 (λpir), and the recombinant clones were selected on tryptic soy agar with nalidixic acid and kanamycin (see above). Chromosome insertion was assessed either by Southern blot with a probe specific for the kanamycin-resistance cassette, or by PCR using two primers annealing at the start of the disrupted ORF and a primer specific for the pSKoriTKan vector. When necessary, confirmation of the disrupted sequence was achieved by PCR. In frame deletion mutants were constructed by overlapping PCR as described previously [61].

For complementation, the PCR amplified gene was first cloned into plasmid pCR®2.1 (Invitrogen S.A., Barcelona, Spain) using T4 ligase and transformed into E. coli TOP10 F’. From this, the construct was subcloned into pBBR1MCS-4, transformed first into E. coli XLI Blue K5+ and then into E. coli SM10 λ pir for conjugation with the corresponding mutant. Alternatively, the B. melitensis ORFeme [62] was used. The appropriate clones were extracted and the ORF was subcloned into plasmid pRH001 [63]. The construct was introduced into the R mutant by mating with E. coli S17-1 and the conjugants were selected on tryptic soy agar with nalidixic acid and chloramphenicol.

Bacteriological characterization

Crystal violet exclusion and sensitivity to S (Tb, Wb, Iz) and R (R/C) brucellaphages were studied as described by Alton et al. [56]. Growth curves were obtained simultaneously for all bacteria in a Bioscreen C [http://www.bioscreen.fi] instrument. For this purpose, multi-well plates containing 250 μl/well of tryptic soy broth were prepared, and 5×106 CFU of each strain inoculated into triplicate wells. To monitor growth, the optical density at 470 nm (OD470) was measured at 10 min intervals.

LPS and polysaccharide extraction

Whole cell LPS. Bacteria (0.5 g wet weight) were extracted with 2% SDS, 60 mM Tris-HCl (pH 6.8) (10 ml), digested with DNase (30 μg), RNase (30 μg) and proteinase K (1.5 mg), the LPS precipitated with isopropanol and analyzed directly by SDS-PAGE [64].

Extraction of S- and R-LPS with organic solvents. S-LPS was obtained from the phenol phase of a water-phenol extract, and purified by nuclease and proteinase K digestion and by removal of free lipids [26,32]. LPS from R mutants was extracted with phenol-chloroform-light petroleum (2:5:3) (165 mg of freeze-dried bacteria/ml) [65].

Extraction and purification of intracellular polysaccharides. Bm16MR28R and BmR16MR28** cells were resuspended in 0.5 N trichloroacetic acid (30 g wet weight in 150 ml) and stirred at 4°C overnight. After removal of cell debris (15000× g for 15 min), the supernatant was neutralized with NaOH, mixed with 3 volumes of ethanol (24 h at −20°C), the precipitate collected (5000× g for 15 min), dialyzed and freeze-dried. This crude extract was chromatographed at room temperature on a Bio-Gel P-10 (Bio-Rad Laboratories S.A., Madrid, Spain) column (Vt 150 ml, Vo 48 ml) in 5 mM phosphate buffered saline (pH 7.2), 0.05% NaN3. Fractions (2 ml) were examined for cyclic β-glucans by high performance thin layer chromatography and for immunoreactive polysaccharides by immunodiffusion with sera from Brucella infected cattle [32], and those free from glucans were pooled, dialyzed and freeze-dried. In addition, cells were disintegrated in the presence of nucleases in a 40K French Pressure Cell Press (SLM Instruments Inc., Urbana, Ill.) operating at 140 Kg/cm2, and the soluble and cell envelope fractions separated by ultracentrifugation (60000× g, 2 h). The soluble fractions and crude trichloroacetic envelope extracts were tested for immunoreactive polysaccharides without further purification.

S-LPS hydrolytic polysaccharides and native hapten polysaccharide. The S-LPS hydrolytic polysaccharides and the native hapten polysaccharide of B. melitensis 16M were prepared as described before [32]. The hydrolytic polysaccharide of B. abortus 2.13 S-LPS, which is highly pure due to the absence of group 3 Omp tightly bound to LPS in this barS mutant [57], was obtained by a similar procedure.

LPs and polysaccharide characterization. The Kdo assay, and the SDS-PAGE and Western blot procedures have been described previously [11,32]. Moabs Baro-1 and Baro-2 recognize the outer and inner core epitopes of Brucella LPS, respectively, and Bala-1 recognizes the lipid A disaccharide [34]. Moab 31D2 (Ingenasa, S.A., Madrid, Spain) is specific for the C-epitope of the S-LPS. 1H-NMR spectra were recorded at 25 and 70°C in D2O solution using Varian Inova 600 and 800 spectrometers equipped with 5 mm PFG triple-resonance probes. Chemical shifts are reported in ppm, using external sodium 3-trimethylsilyl-2,3,3-T2-propanoate (TSP, δH 0.00). Data processing was performed using vendor-supplied software. TOCSY [66] experiments with mixing times of 10, 30, and 120 ms were used for assignment of resonances.

Surface hydrophobicity and Zeta potential

To assess cell surface hydrophobicity [67], stationary phase bacteria were inactivated in 0.5% NaNO3 at 37°C overnight, washed twice with 97 mM K2HPO4, 53 mM KH2PO4, 0.8 mM MgSO4, 21 mM urea, and adjusted to an OD470 of 1.0. Equal volumes of this suspension and n-hexadecane were mixed, stirred briefly, incubated at room temperature for 15 min, and the partition coefficient (1- [DO470 water phase]/DO470 n-hexadecane) calculated. The surface charge density was measured as the electrophoretically effective potential (Zeta potential) [68]. For this, bacteria were inactivated with 0.5% phenol, washed and resuspended in 1 mM GsCl, 10 mM HEPES 10 mM (pH 7.2) at an OD600 of 0.2. Measurements were performed at 25°C in a Zetamaster instrument using the PCS 1.27 software (Malvern
Instruments Ltd., Malvern, UK) and the settings of aqueous solutions (viscosity = 1.020 cP; dielectric constant = 80.4), either in plain buffer or in buffer supplemented with poly-L-lysine (MW 14000, Sigma-Aldrich Quimica, S.A., Madrid, Spain).

**Surface epitope mapping**

Epitope exposure was tested by dot blot [11] using the above anti-LPS Moabs and the anti-Omp Moabs Omp10 (A68/07/G11/C10), Omp16 (A68/08/C03/G03), Omp19 (A76/06/C10/A08), Omp25 (Omp25) (A70/06/B05/A07, A76/02/C12/C11, A68/04/B10/F05, A68/07/D11/B03 and A68/29/G06/C07), Omp31 (A59/10/F09/G10), Omp29 (A65/03/H02/B01), and Omp1 ([Omp89] A53/10/B02/A01) [11,33,69]. The reaction was measured (OD per mm²) using the Imagemaster system (Pharmacia Biotech, Uppsala, Sweden).

**Sensitivity to non immune serum**

Exponentially growing bacteria were adjusted to 10⁵ CFU/ml and then mixed with fresh sheep or cattle normal serum (45 μl of cells plus 90 μl of serum per well) in microtiter type plates in duplicate. After incubation for 1, 3, 6 or 18 h at 37°C h with gentle stirring, brain heart infusion broth (200 μl) was added, mixed and 100 μl aliquots plated out. The results were expressed as the % survival with respect to the CFU in the inoculum.

**Animal studies**

Female BALB/c mice of 7 weeks of age (Charles River Laboratories, Barcelona, Spain) were housed in the animal building of the CITA laboratory (registration number ES 50297012005) with water and food ad libitum. Animals were randomly allotted and acclimated for 1–2 weeks before the start of the experiments. Animal handling and experimental procedures were in accordance with European (DOCE 86/609/EEC), National (RD1201/2005), and Regional (Levy 11/2003) directives, and were supervised by the Ethical Committee of the Institution. To prepare the inocula, bacteria were suspended in PBS and adjusted spectrophotometrically to the appropriate CFU/ml (45 μl of cells plus 90 μl of serum per well) in microtiter type plates in duplicate. After incubation for 1, 3, 6 or 18 h at 37°C h with gentle stirring, brain heart infusion broth (200 μl) was added, mixed and 10 μl aliquots plated out. The results were expressed as the % survival with respect to the CFU in the inoculum.

**Virulence.** Groups of 20 BALB/c mice were inoculated intraperitoneally with ca. 10⁵, 10⁶ or 10⁷ CFU/mouse in 0.1 ml of serum per well) in microtiter type plates in duplicate. After incubation for 1, 3, 6 or 18 h at 37°C h with gentle stirring, brain heart infusion broth (200 μl) was added, mixed and 10 μl aliquots plated out. The results were expressed as the % survival with respect to the CFU in the inoculum.

**Protection.** The following conditions were determined to be optimal to rank R mutants by their ability to generate protection against virulent *B. melitensis*. Groups of 5 BALB/c mice were inoculated intraperitoneally with 10⁵, 10⁶ or 10⁷ CFU/mouse of *B. melitensis* R mutant and challenged 4 weeks later with 1×10⁸ CFU of *B. melitensis H38* injected by the same route. Two weeks later, the spleens procured for CFU counting [70]. To differentiate the challenging strain from the R mutants persisting in the spleens, samples were plated on both BAB and BAB supplemented with 25 μg/ml of kanamycin, and plates with isolated colonies flooded with a crystal violet-oxalate solution [56]. The results, expressed as the mean and SD of the log₁₀ of CFU per spleen of the challenge strain, were analyzed using the Fisher’s Protected Least Significant Differences (more than 4 groups) or Bonferroni’s test (4 or less groups). In a second experiment, the 5 top ranked mutants plus the 2 mutants inducing the lowest protection were reassessed using 10 mice per group. After ranking, the mouse model of protection was used for evaluating the efficacy of the 5 best R mutants (i.e. Bm16MRzw**, Bm16MRzw*, BmH38Rwbf, BmH38Rwbd, and BmH38Rperf) as vaccines when inoculated (10⁵ CFU/mouse) subcutaneously in mice (n = 10). In all experiments, controls were mice (n = 5 or n = 10) inoculated subcutaneously with 10⁵ CFU/mouse of Rev 1 or with 0.1 ml PBS.

**Studies in BMDM**

R mutants representative of the main attenuation patterns were studied in comparison with the parental strains. BMDM were obtained from 8 to 10 week-old female C57BL/6 black mice, infected after 6 days of *in vitro* maturation (50 CFU per cell) and, at appropriate times, coverslips were processed and immunostained [71]. Briefly, R mutants were labeled using the serum of a *B. abortus* infected sheep and a Texas Red conjugated monoclonal antibody to sheep IgG and the parental strains using the serum from a *B. abortus* infected cattle and an Alexa® 594 (Molecular Probes; Eugene, OR) conjugated anti-cattle monoclonal antibody. The lysosomal-associated membrane protein 1 (LAMP1) and the endoplasmic reticulum marker calreticulin were detected using rat anti LAMP1 MoAb plus a Cy5 conjugated anti-rat IgG monoclonal antibody and a specific rabbit anti-calreticulin serum plus an Alexa® 488 (Molecular Probes) conjugated anti- rabbit IgG monoclonal antibody. The images were obtained in a confocal microscope [71]. For mutants BmH38Rwbf, BmH38Rwbd, Bm16MRzw*, and BmH38Rperf, the images were taken at 0.5, 2, 4, 8, 12, 24, and 48 hours after infection. In addition, 24 h post-infection images were obtained for BmH38Rperf, Bm16MRzw*, and Bm16MRperf.

**Supporting Information**

**Figure S1** Growth curves of smooth parental strains and representative *B. melitensis* R mutants in tryptic soy broth at 37°C. Plots labeled as A, B and C correspond to accelerated, normal or retarded growth, respectively. Found at: doi:10.1371/journal.pone.0002760.s001 (3.20 MB TIF)

**Figure S2** 1H-NMR spectrum of *B. abortus* 213 LPS polysaccharide. Found at: doi:10.1371/journal.pone.0002760.s002 (0.09 MB TIF)

**Figure S3** BALB/c mice allow a better discrimination of vaccines than CD-1 mice. Plots represent the 50% line within box, 25 and 75 (lower and upper box limits) percentiles and minimal and maximal values (lower and upper lines) of the CFU/spleen in mice vaccinated with Rev 1 or PBS and challenged with BmH38 or Bm16M.
Author Contributions
Conceived and designed the experiments: MJG MZ JJL JPG JM BM. Performed the experiments: DJM TD VAG RMD RC PM CM. Analyzed the data: MJG ILG MI AW JJL JPG JM BM. Wrote the paper: IM.

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