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(54) Title: A MODIFIED *BRUCELLA* VACCINE STRAIN FOR THE TREATMENT OF BRUCELLOSIS

(57) Abstract: The present application provides a modified *Brucella* strain, its use as a medicament, and its use as a medicament for the treatment and/or prevention of brucellosis. The *Brucella* strain has been modified through an inactivation of the *wzm* gene. Further, the present application provides a pharmaceutical composition which comprises the modified *Brucella* strain, its use as a medicament, and its use as a medicament for the treatment and/or prevention of brucellosis. The present application also provides a kit which comprises the modified *Brucella* strain and a pharmaceutically acceptable carrier or diluent and its use for the treatment and/or prevention of brucellosis.

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A modified *Brucella* vaccine strain for the treatment of brucellosis

Technical field

The present invention can be included in the field of new therapeutics for the treatment and/or prevention of brucellosis. Specifically, the present application relates to a new vaccine strain of the genus *Brucella*. The strain can be used as a medicament, specifically for the treatment and/or prevention of brucellosis.

Background art

Brucellosis is a zoonotic disease. In animals, *Brucella* infection causes abortions, infertility, decreased production and limitations to the trading of animals and animal products. In addition, the bacteria are transmitted from infected animals to humans, thereby inflicting a debilitating and often disabling disease, against which there is no vaccine and whose treatment requires high doses of antibiotics for prolonged periods with frequent relapses.

Therefore, brucellosis is a significant public health problem. It has been shown that the prevalence of human brucellosis is directly related to the prevalence of animal brucellosis. Thus, and in the absence of vaccines for use in humans, prevention of the disease requires the control of the infection in animals. In most socio-economic contexts, the only feasible way to control brucellosis is through programs based on the vaccination of farm animals, either through mass vaccination programs or through programs for the vaccination, diagnosis and slaughter of infected animals.

The reference vaccines against animal brucellosis are the smooth (S) strains *Brucella abortus* S19 for cattle and *Brucella melitensis* Rev1 for sheep and goats (OIE Terrestrial Manual, 2016 - chapters 2.4.3. and 2.7.2). Both are live attenuated vaccines, adjuvant-free, with a low cost of production and acquisition, and highly effective against infection by field strains in ruminants (main source of infection for humans). However, a technical drawback is that they generate an immune response after vaccination indistinguishable from that induced after virulent infection by field strains, generating a problem for differentiating between infected and vaccinated animals (DIVA). To solve this problem, numerous scientific efforts have been made. One strategy has consisted in the development of rough (R) strains of *Brucella*, which, due to the absence of the O-Polysaccharide (O-PS) of lipopolysaccharide (LPS) - a known virulence factor of *Brucella* and the main antigen used in tests for serological diagnosis of infection- has led to attenuated strains usable as live vaccines, which do not significantly interfere in the serological diagnostic tests. In this context, in the 90's, the spontaneous mutant with an R phenotype known as *B. abortus* RB51 was developed by subculturing (Schurig et al., 1991. Veterinary Microbiology, 28: 171-188). Strain RB51 has been used in some countries against bovine brucellosis, with controversial results. Both RB51 and a collection of R mutants derived from

B. melitensis genetically well characterized in the different LPS synthesis pathways (Godfroid et al., 2000. Res Microbiol, 151: 655–668; González et al., 2008. PLoS One, 3(7): e2760), reduce interference problems in the serological diagnosis of virulent infection, due to the absence of O-PS antigen. However, it has been shown that R vaccines are not ideal, because the protection they confer against virulent infections is well below that of the reference vaccines *B. abortus* S19 and *B. melitensis* Rev1 (González et al., 2008. PLoS One, 3(7): e2760; Barrio et al., 2009. Vaccine, 27: 1741-1749).

On the other hand, bacterial tagging with the xenogenic protein Green Fluorescent Protein (GFP) has been proposed to solve the DIVA problem (Chacón-Díaz et al., 2011. Vaccine. 29(3): 577-82).

Moreover, current vaccines also have other issues such as to induce abortions and to be present in the milk of adult animals previously vaccinated (OIE Terrestrial Manual, 2016 - chapters 2.4.3. and 2.7.2), to generate human infections and, in the case of *B. melitensis* Rev1, to be resistant to streptomycin (antibiotic of choice). Therefore, there is currently a need for an effective brucellosis vaccine and/or therapeutic which does not have all of the aforementioned drawbacks.

It is an objective of the present invention to provide a superior *Brucella* strain for the treatment and/or prevention of brucellosis.

Figures

Figure 1: Diagram showing the strategy for the deletion method by in-frame double recombination used to obtain the *Brucella*Δ*wzm* and sibling (non- mutated) strains.

Figure 2: Genetic assessment of *Brucella*Δ*wzm* mutants and strains complemented with plasmids pSRK-*wzm* or pBBR-*wzm*. The presence (319 bp) or absence (no amplification) of the complete *wzm* gene was assessed by PCR with F9 and R5 (Table 1). MW: Molecular Weight marker; 1: Rev1 sibling; 2: Rev1Δ*wzm*; 3: Rev1Δ*wzm*-pSRK-*wzm*; Pc1: Plasmid for complementation pSRK-*wzm*; Pm: Plasmid for mutation pJQKm-Δ*wzm*; C-: PCR negative Control; 4: 16M sibling; 5: 16MΔ*wzm*; 6: 16MΔ*wzm*-pBBR-*wzm*; Pc2: pBBR-*wzm*; 7: 2308 sibling; 8: 2308Δ*wzm*; 9: 2308Δ*wzm*-pBBR-*wzm*; 10: S19 sibling; 11: S19Δ*wzm*; 12: S19Δ*wzm*-pBBR-*wzm*.

Figure 3: *Brucella*Δ*wzm* mutants (upper panels) showed a rough phenotype by crystal violet-oxalate staining, in contrast to the sibling strains (lower panels).

Figure 4: *Brucella*Δ*wzm* mutants have R-LPS with an intact core and synthesize free O-PS that accumulates inside the bacteria. Complementation of these *B. melitensis* and *B. abortus* Δ*wzm* strains with plasmids pSRK-*wzm* or pBBR-*wzm* restores the S-LPS phenotype. Representative images of (A)

LPS silver staining of Rev1Δ*wzm*, 16MΔ*wzm*, 2308Δ*wzm* and S19Δ*wzm*. (B) Western Blot with sera recognizing O-PS epitopes M in Rev1Δ*wzm*, C in 16MΔ*wzm*, or A in both 2308Δ*wzm* and S19Δ*wzm*; 1: Rev1 sibling; 2: Rev1Δ*wzm*; 3: Rev1Δ*wzm*-pSRK-*wzm*; 4: 16M sibling; 5: 16MΔ*wzm*; 6: 2308 sibling; 7: 2308Δ*wzm*; 8: 2308Δ*wzm*-pBBR-*wzm*; 9: S19 sibling; 10: S19Δ*wzm*; 11: S19Δ*wzm*-pBBR-*wzm*. (C) Immunofluorescence and epifluorescence microscopy of 16MΔ*wzm*::*gfp*-pBBR-*wzm*, 16MΔ*wzm*::*gfp*, and 16M sibling, using a primary MoAb anti-C O-PS epitope and a secondary antibody labelled with Texas Red.

Figure 5: Rev1Δ*wzm* is more susceptible to streptomycin than Rev1. Exponentially growing bacteria were adjusted to $\approx 2\times10^3$ CFU/mL in sterile PBS and 100 µL by triplicate were plated in BAB and BAB supplemented with 2.5 µg/mL of streptomycin (BAB-$Str_{2.5}$). Rev1 sibling strain (Rev1) was used as control. After 5 days of incubation at 37ºC, the number of CFU/mL was calculated. Data points represent the mean ± standard deviation (n=3). The results are representative of three independent experiments. Statistical comparisons of means were performed by ANOVA and PLSD tests.

Figure 6: 16MΔ*wzm* is more susceptible to desiccation than 16M, but not Rev1Δ*wzm* with respect to Rev1. Suspensions containing $\approx 10^9$ CFU/mL of 16MΔ*wzm*, 16M, Rev1Δ*wzm* or Rev1 were allowed to dry in 12-well polystyrene plates and then maintained at room temperature under dark conditions. The number of viable cells was quantified after rehydration of the dried pellet in PBS, and the percentage of surviving bacteria was determined six days later. Data points represent the mean ± standard deviation (n=3). Statistical comparisons of means were performed by ANOVA and PLSD tests.

Figure 7: Rev1Δ*wzm* is more susceptible to Polymyxin B than 16MΔ*wzm*, and Rev1 and 16M parental and sibling strains, as a model of susceptibility to the cationic peptides of the innate immune system. Cultures with $2\text{-}3\times10^3$ CFU/mL in PBS were incubated (1 h, 37ºC) with different concentrations of Polymyxin B prepared in PSA and the number of viable cells was then quantified by plating in BAB and incubating the plates (5 days, 37ºC). Data points represent the mean ± standard deviation (n=3) of CFU/mL at each Polymyxin B concentration. Statistical comparisons of means were performed by ANOVA and PLSD tests: $^*p < 0.0001$.

Figure 8: Rev1Δ*wzm* and 16MΔ*wzm* are more susceptible than Rev1 and 16M parental strains to conventional sheep and cattle sera, mainly due to the effect of the serum complement. Bacterial cultures containing $\approx 10^4$ CFU/mL in PBS were mixed with normal or decomplemented (heat inactivated 56ºC, 1 h) sera from sheep (A) or cows (B). After incubation (18 h, 37ºC), each suspension was plated onto BAB and plates were incubated (5 days, 37ºC) to determine the number of CFU/mL and the percentage of bacterial survival. A *B. melitensis* mutant with minimal core (C+) was used as a control of susceptibility to normal serum. Results are expressed as the mean ± standard deviation

(n=3) of survival percentage. Statistical comparisons of means were performed by ANOVA and PLSD tests.

Figure 9: Rev1Δ*wzm* is more attenuated in BALB/c mice than other *Brucella*Δ*wzm* mutants and induced a peak of transient splenomegaly, usually associated with an effective immunogenic potency. Panels represent the bacterial burden in spleens and spleen weights in BALB/c mice intraperitoneally inoculated with (A-B) Rev1Δ*wzm* and Rev1Δ*wzm*::*gfp* vs. Rev1 parental strain; (C-D) 16MΔ*wzm* and 16MΔ*wzm*::*gfp* vs. 16M parental strain; (E-F) 2308Δ*wzm* vs. 2308 parental strain; and (G-H) S19Δ*wzm* vs. S19 strain. The Δ*wzm* mutants were injected at doses of 10^8 CFU/mouse, and the S-LPS strains, at 10^6 CFU/mouse. Results are expressed as the mean ± standard deviation (n=5) of the log CFU/spleen or weight grams/spleen at each selected time point. Similar results were obtained with the corresponding *Brucella*::Tn*7*-*gfp* tagged strains, indicating that *gfp* tagging does not affect the biological properties of *Brucella*.

Figure 10: Rev1Δ*wzm* and 16MΔ*wzm* (right panel) do not induce placental macroscopic lesions, in contrast to Rev1 or 16M parental or sibling strains (left panel). Arrows indicate macroscopic lesions in individual placentas, in contrast to healthy placentas in Rev1Δ*wzm* or 16MΔ*wzm*.

Figure 11: Serological response against *Brucella* LPS in sheep vaccinated with Rev1Δ*wzm*::*gfp* or 16MΔ*wzm*::*gfp*. Lambs 3-4 months-old were vaccinated subcutaneously with $1\text{-}2\times10^{10}$ CFU of Rev1Δ*wzm*::*gfp* (n=14) or 16MΔ*wzm*::*gfp* (n=8). Groups of lambs non-vaccinated (n=13) or vaccinated with $1\text{-}2\times10^9$ CFU of Rev1::*gfp* (n=12) were used as controls. Innocuousness was assessed during the first month after vaccination by clinical inspection (rectal body temperature and palpation of the inoculation site) and testicles palpation. Serum samples were taken periodically for serological analysis by (A) standard Rose Bengal (sRBT); (B) Complement Fixation (CFT); (C) Gel Diffusion Tests against R-LPS antigen (GDT-R/LPS) and (D) anti-GFP ELISA tests (only for lambs vaccinated with 16MΔ*wzm*::*gfp*).

Figure 12: Bacterial killing experiment. Heat-treated (referred to as Serum-treated in the Figure) and untreated immune sera from lambs vaccinated with Rev1Δ*wzm* was incubated for 18 h at 37ºC, and 10% CO_2 with *B. melitensis* H38 and *B. abortus* 2308 virulent infections. The results were expressed as the standardized percentage of bacteria counts with respect to initial count in the inocula. The immune sera from lambs treated with Rev1Δ*wzm* were capable of killing either *B. melitensis* H38, *B. abortus* 2308 or *B. ovis* PA.

Summary of the invention

The present application provides a modified *Brucella* strain, its use as a medicament, and its use as a medicament for the treatment and/or prevention of brucellosis. The *Brucella* strain has been modified through an inactivation of the *wzm* gene. Further, the present application provides a pharmaceutical composition which comprises the modified *Brucella* strain, its use as a medicament, and its use as a medicament for the treatment and/or prevention of brucellosis. The present application also provides a kit which comprises the modified *Brucella* strain and a pharmaceutically acceptable carrier or diluent and its use for the treatment and/or prevention of brucellosis.

Detailed description of the invention

Definitions

The terms "*treatment*" and "*therapy*", as used in the present application, refer to a set of hygienic, pharmacological, surgical and/or physical means used with the intent to cure and/or alleviate a disease and/or symptoms with the goal of remediating the health problem. The terms "*treatment*" and "*therapy*" include preventive and curative methods, since both are directed to the maintenance and/or reestablishment of the health of an individual or animal. Regardless of the origin of the symptoms, disease and disability, the administration of a suitable medicament to alleviate and/or cure a health problem should be interpreted as a form of treatment or therapy within the context of this application.

The term "*prevention*", as used in the present application, refers to a set of hygienic, pharmacological, surgical and/or physical means used to prevent the onset and/or development of a disease and/or symptoms. The term "*prevention*" encompasses prophylactic methods, since these are used to maintain the health of an animal or individual.

The term "*therapeutically effective amount*" refers to an amount of matter which has a therapeutic effect and which is able to treat and/or prevent brucellosis.

The term "*brucellosis*" refers to an infectious disease caused by bacteria from the genus *Brucella*. Brucellosis may occur in individuals or animals.

The terms "*individual*", "*patient*" or "*subject*" are used interchangeably in the present application and are not meant to be limiting in any way. The "*individual*", "*patient*" or "*subject*" can be of any age, sex and physical condition. The term "*animal*", as used in the present application, refers to any multicellular eukaryotic heterotroph which is not a human.

The term "*vaccine*", as used in the present application, refers to both "*therapeutic vaccines*", which are intended to treat an existing disease and/or infection by strengthening the body's natural immune

response, and "*prophylactic vaccines*", which are intended to prevent a disease and/or infection from developing in a healthy individual or animal.

The term "*modified*" refers to any matter which has been altered from its original form. In the present application, the term *"modified*" refers to any alteration which relies on human intervention.

As used herein, "*pharmaceutically acceptable carrier*" or "*pharmaceutically acceptable diluent*" means any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed and, without limiting the scope of the present invention, include: additional buffering agents; preservatives; co-solvents; antioxidants, including ascorbic acid and methionine; chelating agents such as EDTA; metal complexes (e.g., Zn-protein complexes); biodegradable polymers, such as polyesters; salt-forming counterions, such as sodium, polyhydric sugar alcohols; amino acids, such as alanine, glycine, glutamine, asparagine, histidine, arginine, lysine, ornithine, leucine, 2-phenylalanine, glutamic acid, and threonine; organic sugars or sugar alcohols, such as lactitol, stachyose, mannose, sorbose, xylose, ribose, ribitol, myoinisitose, myoinisitol, galactose, galactitol, glycerol, cyclitols (e.g., inositol), polyethylene glycol; sulfur containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, [alpha]-monothioglycerol, and sodium thio sulfate; low molecular weight proteins, such as human serum albumin, bovine serum albumin, gelatin, or other immunoglobulins; and hydrophilic polymers, such as polyvinylpyrrolidone. In a preferred embodiment, the pharmaceutically acceptable carrier or diluent is Phosphate Buffered Saline (PBS). Preferably, the pH of the PBS is 6.85

The term "*pharmaceutically acceptable adjuvant*" refers to any and all substances which enhance the body's immune response to an antigen. Non-limiting examples of pharmaceutically acceptable adjuvants are: Alum, Freund's Incomplete Adjuvant, MF59, synthetic analogs of dsRNA such as poly(I:C), bacterial LPS, bacterial flagellin, imidazolquinolines, oligodeoxynucleotides containing specific CpG motifs, fragments of bacterial cell walls such as muramyl dipeptide and Quil-A®.

Modified *B. melitensis* Rev1 strain

In a first aspect, the present application provides a modified *Brucella melitensis* Rev1 strain, wherein the *wzm* gene has been inactivated.

Brucella melitensis Rev1 is a strain which was spontaneously attenuated and obtained from the virulent strain *B. melitensis* 6056, through successive spontaneous mutations associated with

streptomycin (Str) dependence and the subsequent reversal of that dependency (Herzberg and Elberg 1953. Journal of Bacteriology 66: 585-599; Herzberg and Elberg 1953. Journal of Bacteriology 66: 600-605). The Rev1 strain has been used worldwide since the 50's as the only effective vaccine to prevent brucellosis in small ruminants, and is internationally considered the standard vaccine to control ovine and caprine brucellosis (OIE Terrestrial Manual, 2016 - chapters 2.4.3. and 2.7.2). The original seed lots of Rev1are available at the Brucellosis Reference Laboratory of the OIE of AFSSA (94706 Maisons-Alfort, France) or at the European Pharmacopoeia (BP 907, 67029 Strasbourg Cedex 1, France). Further, the Rev1strain is commercially available and can be bought from various vendors. For example, the strain can be bought from CZV Veterinaria in Spain under the name "*CZV Rev1*".

The *wzm* gene encodes for part of the two-component ABC transporter system required to export the O-PS to the periplasm where the O-PS is then assembled onto a core moiety to generate a S-LPS. The *wzm* gene may have the following sequence (SEQ ID NO: 1):

ATGATATCGTATATGGCTAATGTCTGGAAGGTACGCCACTTCTGGTGGCACCTTTCAATGT
CTGATTTACGTGGGCGCTTCAGGCGGTCCTCCTTGGGAATATTATGGGCAGTTATACAGC
CACTAGCGCTCACGCTGCTACTGTCTTTCGTGTTTTCTAAATTGTTGAATCAAAGTATATC
TGCATATGCCCCCTATATTCTATCTGGGATTATTATCTGGGAATACATATCATTTACAGTG
GTTGGTGGCTCAACAGCGCTTGTGCAAGCCGATGCATATATAAAGCAAACCAGAAATCCT
CTTGCAATTTACACGCTTAGGAACACTGTTTCTGGCTTGGTCGTATTATCCGTAGCAAGTA
TCTCCCTATTCGGGTGGGTACTTATCATGTTTCCTGAAAACTTCTCGCTTTCATGGTTAGC
AATACCAACTTTGCTACCCATCCTTGCTTTGATAGTTTGGCCGCTTGCCACAATCGTCGGC
TACATCGGCGCAAGATTTCGAGATCTGCCGAATGCTCTGGCGCTCGTGTTACAGGCAGCT
TGGTTTGTTTCGCCGGTCTATTTTAAAGAATCGATGTTCAGGCAGGGTGGATTGAATGCAT
TCGTTGATTATAACCCTATTTACCACGTGATGCAGATTCTAAGAGCCCCTGTCCTTTATGG
GGAATGGCCTACGGCTACCAATTACATTTGGTGCTTAGGTGTGAGCCTCCTCCTAACCTGC
GTGGCAGTAGCTGTGGGGATGCGTGCGGAGAAGAGAGCCATTTTTTACCTATGA

The *wzm* gene may be inactivated through any form of genetic modification known in the art. The inactivation may involve the partial or complete deletion of the gene from the host genome. The inactivation may involve a single none-sense mutation which renders the expressed protein non-functional. The inactivation may involve the mutation and/or deletion of the promoter, ribosome binding site or other transcriptional regulators which are involved in the transcription of the *wzm* gene. The inactivation may involve the insertion of a sequence which causes a frame shift and/or makes the resultant nascent protein non-functional. Any of the aforementioned deletions, insertions or mutations can be performed using allelic exchange (Hmelo et al., 2015. Nature Protocols, 10(11): 1820-41) or by

using the CRISPR/Cas9 system (Wang et al., 2016. ACS Synthetic Biology, 5(7): 721-32). In a preferred embodiment, the *wzt* gene is not inactivated.

In a preferred embodiment, the inactivation of the *wzm* gene is due to a partial deletion of the gene. Preferably, the partial deletion involves the deletion of at least 50, 60 or 70 % of SEQ ID NO: 1. More preferably, the partial deletion involves the deletion of at least 80 % of SEQ ID NO: 1. In a preferred embodiment, the inactivation of the *wzm* gene is not achieved by inserting a transposon into the coding sequence of the gene.

In the Examples of the present invention, nucleotides 80-721 of SEQ ID NO: 1 have been deleted in *B. melitensis* Rev1 and 16M using the allelic exchange plasmid pJQKmΔ*wzm,* which generates the correspondent Δ*wzm* mutants (Figure 1). Therefore, in a preferred embodiment, the inactivation of the *wzm* gene is achieved through the deletion of nucleotides 80-721 of SEQ ID NO: 1.

In a preferred embodiment, the modified *B. melitensis* Rev1 strain has been further modified to inactivate *znuA*, *norD*, *bip*, *tcpB*, *cgs*, *ricA*, *bvrR*, *bvrS*, one or more of the genes encoding the virB type IV secretion system selected from the group consisting of the *B. melitensis* 16M ORFs: BMEII0025, BMEII0026, BMEII0027, BMEII0028, BMEII0029, BMEII0030, BMEII0031, BMEII0032, BMEII0033, BMEII0034, and BMEII0035, and/or one or more of the genes involved in the formation, modification and/or assembly of LPS and/or metabolic pathways, including but not limited to *ppdK*, *wbdR*, *gmd*, *manA*, *manB*, *manC*, *per*, *pgm*, *wbkA*, *wbkB*, *wbkC*, *wbkD*, *wbkF*, *wadC*, and *wzt* (chromosomic regions *wbk*, *wbo* and *wad*).

In a preferred embodiment, the modified *B. melitensis* Rev1 strain has been further modified so that the autologous N-formyltransferase activity has been suppressed and a heterologous gene encoding a N-acyltransferase other than a N-formyltransferase enzyme is functionally expressed. For example, the *wbkC* may be inactivated and a heterologous *wbdR* may be introduced and expressed in the strain (see WO 2017/108515 A1).

In a preferred embodiment, the modified *B. melitensis* Rev1 strain has been further modified to express a fluorescent protein, preferably GFP. The expression of the fluorescent protein in the modified strain could be used to further distinguish Rev1 inoculated individuals or animals from infected individuals or animals (Chacón-Díaz et al., 2011. Vaccine. 29(3): 577-82; EP 2 508 201 A1). Briefly, this approach can be described as follows: when an individual or animal is vaccinated with the further modified strain, antibodies will be raised against the modified strain as well as the fluorescent protein. The antibodies raised against the fluorescent protein can be used in a serological test to test whether the individual or animal has been infected with a naturally occurring Brucella strain or with

the further modified strain of the present invention. Thus, a kit which comprises the modified *B. melitensis* Rev1 strain which has been further modified to express a fluorescent protein may further comprise antibodies which bind to the fluorescent protein, and/or the fluorescent protein. Preferably, the fluorescent protein is GFP.

In a preferred embodiment, the strain has been lyophilized. Lyophilization can be used to increase the stability and shelf-life of the strain.

In a second aspect, the present invention provides a pharmaceutical composition which comprises the modified strain in accordance with any of the previously disclosed embodiments and a pharmaceutically acceptable carrier or diluent and/or a pharmaceutically acceptable adjuvant.

In a preferred embodiment, the pharmaceutical composition comprises the modified *B. melitensis* Rev1 strain which has been further modified to express a fluorescent protein.

A pharmaceutical composition as described herein may also contain other substances. These substances include, but are not limited to, cryoprotectants, lyoprotectants, surfactants, bulking agents, anti-oxidants, and stabilizing agents. In some embodiments, the pharmaceutical composition may be lyophilized.

The term "*cryoprotectant*" as used herein, includes agents which provide stability to the strain against freezing-induced stresses, by being preferentially excluded from the strain's surface. Cryoprotectants may also offer protection during primary and secondary drying and long-term product storage. Non-limiting examples of cryoprotectants include sugars, such as sucrose, glucose, trehalose, mannitol, mannose, and lactose; polymers, such as dextran, hydroxyethyl starch and polyethylene glycol; surfactants, such as polysorbates (e.g., PS-20 or PS-80); and amino acids, such as glycine, arginine, leucine, and serine. A cryoprotectant exhibiting low toxicity in biological systems is generally used.

In one embodiment, a lyoprotectant is added to a pharmaceutical composition described herein. The term "*lyoprotectant*" as used herein, includes agents that provide stability to the strain during the freeze-drying or dehydration process (primary and secondary freeze- drying cycles), by providing an amorphous glassy matrix and by binding with the strain's surface through hydrogen bonding, replacing the water molecules that are removed during the drying process. This helps to minimize product degradation during the lyophilization cycle, and improve the long-term product stability. Non-limiting examples of lyoprotectants include sugars, such as sucrose or trehalose; an amino acid, such as monosodium glutamate, non-crystalline glycine or histidine; a methylamine, such as betaine; a lyotropic salt, such as magnesium sulfate; a polyol, such as trihydric or higher sugar alcohols, e.g.,

glycerin, erythritol, glycerol, arabitol, xylitol, sorbitol, and mannitol; propylene glycol; polyethylene glycol; pluronics; and combinations thereof. The amount of lyoprotectant added to a pharmaceutical composition is generally an amount that does not lead to an unacceptable amount of degradation of the strain when the pharmaceutical composition is lyophilized.

In some embodiments, a bulking agent is included in the pharmaceutical composition. The term "*bulking agent*" as used herein, includes agents that provide the structure of the freeze- dried product without interacting directly with the pharmaceutical product. In addition to providing a pharmaceutically elegant cake, bulking agents may also impart useful qualities in regard to modifying the collapse temperature, providing freeze-thaw protection, and enhancing the strain stability over long-term storage. Non-limiting examples of bulking agents include mannitol, glycine, lactose, and sucrose. Bulking agents may be crystalline (such as glycine, mannitol, or sodium chloride) or amorphous (such as dextran, hydroxyethyl starch) and are generally used in formulations in an amount from 0.5% to 10%.

Other pharmaceutically acceptable carriers, excipients, or stabilizers, such as those described in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980) may also be included in a pharmaceutical composition described herein, provided that they do not adversely affect the desired characteristics of the pharmaceutical composition. As used herein, "*pharmaceutically acceptable carrier*" means any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed and include: additional buffering agents; preservatives; co-solvents; antioxidants, including ascorbic acid and methionine; chelating agents such as EDTA; metal complexes (e.g., Zn-protein complexes); biodegradable polymers, such as polyesters; salt-forming counterions, such as sodium, polyhydric sugar alcohols; amino acids, such as alanine, glycine, glutamine, asparagine, histidine, arginine, lysine, ornithine, leucine, 2-phenylalanine, glutamic acid, and threonine; organic sugars or sugar alcohols, such as lactitol, stachyose, mannose, sorbose, xylose, ribose, ribitol, myoinisitose, myoinisitol, galactose, galactitol, glycerol, cyclitols (e.g., inositol), polyethylene glycol; sulfur containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, [alpha]-monothioglycerol, and sodium thio sulfate; low molecular weight proteins, such as human serum albumin, bovine serum albumin, gelatin, or other immunoglobulins; and hydrophilic polymers, such as polyvinylpyrrolidone.

In a preferred embodiment, the pharmaceutical composition further comprises an adjuvant. Preferably, the adjuvant is selected from the list consisting of Alum Hydroxide, Freund's Incomplete Adjuvant,

MF59®, synthetic analogs of dsRNA such as poly(I:C), bacterial LPS, bacterial flagellin, imidazolquinolines, oligodeoxynucleotides containing specific CpG motifs, fragments of bacterial cell walls such as muramyl dipeptide and Quil-A®.

The pharmaceutical composition may be prepared for oral, sublingual, buccal, intravenous, intramuscular, subcutaneous, intraperitoneal, conjunctival, rectal, transdermal, topical and/or inhalation-mediated administration. In a preferred embodiment, the pharmaceutical composition may be a solution which is suitable for intravenous, intramuscular, conjunctival, transdermal, intraperitoneal and/or subcutaneous administration. In another embodiment, the pharmaceutical composition may be a solution which is suitable for sublingual, buccal and/or inhalation-mediated administration routes. In an alternative embodiment, the pharmaceutical composition may be an aerosol which is suitable for inhalation-mediated administration. In a preferred embodiment, the pharmaceutical composition may be prepared for subcutaneous and/or intraperitoneal administration.

The pharmaceutical composition may further comprise common excipients and carriers which are known in the state of the art. For solid pharmaceutical compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For solution for injection, the pharmaceutical composition may further comprise cryoprotectants, lyoprotectants, surfactants, bulking agents, anti-oxidants, stabilizing agents and pharmaceutically acceptable carriers. For aerosol administration, the pharmaceutical compositions are generally supplied in finely divided form along with a surfactant and propellant. The surfactant must, of course, be nontoxic, and is generally soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery. For suppositories, traditional binders and carriers may include, for example, polyalkalene glycols or triglycerides.

In a preferred embodiment, the pharmaceutical composition is a vaccine capable of inducing an immune response. The design of pharmaceutical compositions for vaccines is well established, and is described, for example, in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Co., Easton, PA, and in Plotkin and Orenstein's book entitled Vaccines, 4th Ed., Saunders, Philadelphia, PA (2004).

Medical uses of the modified Rev1 strain

In a third aspect, the strain or pharmaceutical composition of the present invention can be used as a medicament. In a fourth aspect, the modified strain or pharmaceutical composition of the present invention can be used to treat and/or prevent brucellosis.

In a preferred embodiment, the infectious agent causing brucellosis is selected from the group consisting of *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis, B. neotomae, B. microti, B. ceti and B. pinnipedialis*. Preferably, the infectious agent causing brucellosis is selected from the group consisting of *B. abortus, B. melitensis* and *B. ovis*.

In a preferred embodiment, the strain or pharmaceutical composition is used to treat and/or prevent brucellosis in humans, cattle, goats, sheep, pigs, and/or dogs. Preferably, the strain or pharmaceutical composition is used to treat and/or prevent brucellosis in goats and/or sheep.

In a preferred embodiment, the individual or animal is inoculated with at least 10^4 CFU (colony forming units) of the strain. Preferably, the individual or animal is inoculated with at least 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11} or 10^{12} CFU of the strain. More preferably, the individual or animal is inoculated with at least 10^9 CFU of the strain. In an alternative embodiment, the individual or animal is inoculated with 10^4 to 10^{12} CFU of the strain.

In a preferred embodiment, the strain or pharmaceutical composition is administered subcutaneously, intradermally, intravenously, intraperitoneally, by mucosae and/or conjunctively. Preferably, the strain or pharmaceutical composition is administered subcutaneously. In an alternative embodiment, the pharmaceutical composition is administered via a conjunctival administration.

In a preferred embodiment, the strain or pharmaceutical composition is used to prevent brucellosis. In this embodiment, the strain or pharmaceutical is administered as a prophylactic vaccine. Preferably, the strain or pharmaceutical composition is administered to an individual or animal who/which is at risk of becoming infected with bacteria of the genus *Brucella*.

In one embodiment, the modified strain or pharmaceutical composition is used to treat brucellosis. In a preferred embodiment, the treatment of brucellosis also involves the administration of a drug. Embodiments where the drug is administered at the same time or at different times as the modified strain or pharmaceutical composition are also envisioned. The drug may be selected from the group consisting of corticosteroids, penicillins, cephalosporins, macrolides, chloramphenicol, tetracyclines, aminoglycosides, trimethoprim, rifampin, quinolones and sulfamethoxazole.

Kit comprising the modified Rev1 strain

In a fifth aspect, the present invention provides a kit comprising (i) a modified *B. melitensis* Rev1 strain, wherein the *wzm* gene has been inactivated and (ii) a pharmaceutically acceptable carrier or diluent.

The modified strain may be in accordance with any of the aforementioned embodiments outlined in this application. Further, the pharmaceutically acceptable carrier or diluent may be any of the aforementioned pharmaceutically acceptable carriers or diluents described in this application. In a preferred embodiment, the kit comprises instructions on how to combine the strain with the pharmaceutically acceptable carrier or diluent.

In a preferred embodiment, the modified strain is a lyophilisate. The lyophilisate may be contained in a separate container from the pharmaceutically acceptable carrier or diluent. Further, the kit may comprise instructions on how to combine the lyophilized strain with the pharmaceutically acceptable carrier or diluent.

In a preferred embodiment, the kit may further comprise an adjuvant. Preferably the adjuvant is selected from a list consisting of Alum, Freund's Incomplete Adjuvant, MF59, synthetic analogs of dsRNA such as poly(I:C), bacterial LPS, bacterial flagellin, imidazolquinolines, oligodeoxynucleotides containing specific CpG motifs, fragments of bacterial cell walls such as muramyl dipeptide and Quil-A®.

In a preferred embodiment, the instructions included with the kit may also outline the administration of the strain to an individual or animal. The outline of the administration may include the dosage to be used, the frequency of administration and/or the administration route to be used.

In a sixth aspect, the present invention provides the use of any of the described kits for the treatment and/or prevention of brucellosis. The use of the kit may be in line with any of the medical uses and methods of administration outlined in this application.

Modified *B. melitensis* 16M strain

In a seventh aspect, the present application provides a modified *B. melitensis* 16M strain, wherein the *wzm* gene has been inactivated.

"*B. melitensis* 16M" is well characterized and freely available at the American Type Culture Collection (ATCC 23456).

The *wzm* gene may be inactivated through any form of genetic modification known in the art. The inactivation may involve the partial or complete deletion of the gene from the host genome. The inactivation may involve a single none-sense mutation which renders the expressed protein non-functional. The inactivation may involve the mutation and/or deletion of the promoter, ribosome binding site or other transcriptional regulators which are involved in the transcription of the *wzm* gene. The inactivation may involve the insertion of a sequence which causes a frame shift and/or makes the resultant nascent protein non-functional. Any of the aforementioned deletions, insertions or mutations can be performed using allelic exchange (Hmelo et al., 2015. Nature Protocols, 10(11): 1820-41) or by using the CRISPR/Cas9 system (Wang et al., 2016. ACS Synthetic Biology, 5(7): 721-32). In a preferred embodiment, the *wzt* gene is not inactivated.

In a preferred embodiment, the inactivation of the *wzm* gene is due to a partial deletion of the gene. Preferably, the partial deletion involves the deletion of at least 50, 60 or 70 % of SEQ ID NO: 1. More preferably, the partial deletion involves the deletion of at least 80 % of SEQ ID NO: 1. In a preferred embodiment, the inactivation of the *wzm* gene is not achieved by inserting a transposon into the coding sequence of the gene.

In a preferred embodiment, the inactivation of the *wzm* gene is achieved through the deletion of nucleotides 80-721 of SEQ ID NO: 1.

In a preferred embodiment, the modified *B. melitensis* 16M strain has been further modified to inactivate *znuA*, *norD*, *bip*, *tcpB*, *cgs*, *ricA*, *bvrR*, *bvrS*, one or more of the genes encoding the virB type IV secretion system selected from the group consisting of the *B. melitensis* 16M ORFs: BMEII0025, BMEII0026, BMEII0027, BMEII0028, BMEII0029, BMEII0030, BMEII0031, BMEII0032, BMEII0033, BMEII0034, and BMEII0035, and/or one or more of the genes involved in the formation, modification and/or assembly of LPS and/or metabolic pathways, including but not limited to *ppdK*, *wbdR*, *gmd*, *manA*, *manB*, *manC*, *per*, *pgm*, *wbkA*, *wbkB*, *wbkC*, *wbkD*, *wbkF*, *wadC*, and *wzt* (chromosomic regions *wbk*, *wbo* and *wad*).

In a preferred embodiment, the modified *B. melitensis* 16M strain has been further modified so that the autologous N-formyltransferase activity has been suppressed and a heterologous gene encoding a N-acyltransferase other than a N-formyltransferase enzyme is functionally expressed. For example, the *wbkC* may be inactivated and a heterologous *wbdR* may be introduced and expressed in the strain (see WO 2017/108515 A1).

In a preferred embodiment, the modified *B. melitensis* 16M strain has been further modified to express a fluorescent protein, preferably GFP. The expression of the fluorescent protein in the modified strain

could be used to further distinguish 16M inoculated individuals or animals from infected individuals or animals (Chacón-Díaz et al., 2011. Vaccine. 29(3): 577-82; EP 2 508 201 A1). Thus, a kit which comprises the modified *B. melitensis* 16M strain which has been further modified to express a fluorescent protein may further comprise antibodies which bind to the fluorescent protein, and/or the fluorescent protein. Preferably, the fluorescent protein is GFP.

In a preferred embodiment, the strain has been lyophilized. Lyophilization can be used to increase the stability and shelf-life of the strain.

In an eighth aspect, the present invention provides a pharmaceutical composition which comprises the modified strain in accordance with any of the previously disclosed embodiments and a pharmaceutically acceptable carrier or diluent and/or a pharmaceutically acceptable adjuvant.

In a preferred embodiment, the pharmaceutical composition comprises the modified *B. melitensis* 16M strain which has been further modified to express a fluorescent protein.

A pharmaceutical composition as described herein may also contain other substances. These substances include, but are not limited to, cryoprotectants, lyoprotectants, surfactants, bulking agents, anti-oxidants, and stabilizing agents. In some embodiments, the pharmaceutical composition may be lyophilized.

In one embodiment, a lyoprotectant is added to a pharmaceutical composition described herein. In some embodiments, a bulking agent is included in the pharmaceutical composition. Other pharmaceutically acceptable carriers, excipients, or stabilizers, such as those described in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980) may also be included in a pharmaceutical composition described herein, provided that they do not adversely affect the desired characteristics of the pharmaceutical composition.

In a preferred embodiment, the pharmaceutical composition further comprises an adjuvant. Preferably, the adjuvant is selected from the list consisting of Alum, Freund's Incomplete Adjuvant, MF59®, synthetic analogs of dsRNA such as poly(I:C), bacterial LPSs, bacterial flagellin, imidazolquinolines, oligodeoxynucleotides containing specific CpG motifs, fragments of bacterial cell walls such as muramyl dipeptide and Quil-A®.

The pharmaceutical composition may be prepared for oral, sublingual, buccal, intravenous, intramuscular, subcutaneous, intraperitoneal, conjunctival, rectal, transdermal, topical and/or inhalation-mediated administration. In a preferred embodiment, the pharmaceutical composition may

be a solution which is suitable for intravenous, intramuscular, conjunctival, transdermal, intraperitoneal and/or subcutaneous administration. In another embodiment, the pharmaceutical composition may be a solution which is suitable for sublingual, buccal and/or inhalation-mediated administration routes. In an alternative embodiment, the pharmaceutical composition may be an aerosol which is suitable for inhalation-mediated administration. In a preferred embodiment, the pharmaceutical composition may be prepared for subcutaneous and/or intraperitoneal administration.

The pharmaceutical composition may further comprise common excipients and carriers which are known in the state of the art. For solid pharmaceutical compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For solution for injection, the pharmaceutical composition may further comprise cryoprotectants, lyoprotectants, surfactants, bulking agents, anti-oxidants, stabilizing agents and pharmaceutically acceptable carriers. For aerosol administration, the pharmaceutical compositions are generally supplied in finely divided form along with a surfactant and propellant. The surfactant must, of course, be nontoxic, and is generally soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery. For suppositories, traditional binders and carriers may include, for example, polyalkalene glycols or triglycerides.

In a preferred embodiment, the pharmaceutical composition is a vaccine capable of inducing an immune response. The design of pharmaceutical compositions for vaccines is well established, and is described, for example, in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Co., Easton, PA, and in Plotkin and Orenstein's book entitled Vaccines, 4th Ed., Saunders, Philadelphia, PA (2004).

Medical uses of the modified 16M strain

In a ninth aspect, the strain or pharmaceutical composition of the present invention can be used as a medicament. In a tenth aspect, the modified strain or pharmaceutical composition of the present invention can be used to treat and/or prevent brucellosis.

In a preferred embodiment, the infectious agent causing brucellosis is selected from the group consisting of *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, *B. neotomae*, *B. microti*, *B. ceti and B. pinnipedialis*. Preferably, the infectious agent causing brucellosis is selected from the group consisting of *B. abortus*, *B. melitensis* and *B. ovis*.

In a preferred embodiment, the strain or pharmaceutical composition is used to treat and/or prevent brucellosis in humans, cattle, goats, sheep, pigs, and/or dogs. Preferably, the strain or pharmaceutical composition is used to treat and/or prevent brucellosis in goats and/or sheep.

In a preferred embodiment, the individual or animal is inoculated with at least 10^4 CFU (colony forming units) of the strain. Preferably, the individual or animal is inoculated with at least 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} or 10^{11} CFU of the strain. More preferably, the individual or animal is inoculated with at least 10^9 CFU of the strain. In an alternative embodiment, the individual or animal is inoculated with 10^4 to 10^{12} CFU of the strain.

In a preferred embodiment, the strain or pharmaceutical composition is administered subcutaneously, intradermally, intravenously, intraperitoneally, by mucosae and/or conjunctively. Preferably, the strain or pharmaceutical composition is administered subcutaneously. In an alternative embodiment, the pharmaceutical composition is administered via a conjunctival administration.

In a preferred embodiment, the strain or pharmaceutical composition is used to prevent brucellosis. In this embodiment, the strain or pharmaceutical is administered as a prophylactic vaccine. Preferably, the strain or pharmaceutical composition is administered to an individual or animal who/which is at risk of becoming infected with bacteria of the genus *Brucella*.

In a preferred embodiment, the strain or pharmaceutical composition is used to treat brucellosis. In this embodiment, the strain or pharmaceutical is administered as a therapeutic vaccine. Preferably, the strain or pharmaceutical composition is administered to an individual or animal who/which suffers an infection from bacteria of the genus *Brucella*.

Kit comprising the modified 16M strain

In an eleventh aspect, the present invention provides a kit comprising (i) a modified *B. melitensis* 16M strain wherein the *wzm* gene has been inactivated; and (ii) a pharmaceutically acceptable carrier or diluent.

The modified strain may be in accordance with any of the aforementioned embodiments outlined in this application. Further, the pharmaceutically acceptable carrier or diluent may be any of the aforementioned pharmaceutically acceptable carriers or diluents described in this application. In a preferred embodiment, the kit comprises instructions on how to combine the strain with the pharmaceutically acceptable carrier or diluent.

In a preferred embodiment, the modified strain is a lyophilisate. The lyophilisate may be contained in a separate container from the pharmaceutically acceptable carrier or diluent. Further, the kit may comprise instructions on how to combine the lyophilized strain with the pharmaceutically acceptable carrier or diluent.

In a preferred embodiment, the kit may further comprise an adjuvant. Preferably the adjuvant is selected from a list consisting of Alum, Freund's Incomplete Adjuvant, MF59, synthetic analogs of dsRNA such as poly(I:C), bacterial LPSs, bacterial flagellin, imidazolquinolines, oligodeoxynucleotides containing specific CpG motifs, fragments of bacterial cell walls such as muramyl dipeptide and Quil-A®.

In a preferred embodiment, the instructions included with the kit may also outline the administration of the strain to an individual or animal. The outline of the administration may include the dosage to be used, the frequency of administration and/or the administration route to be used.

In a twelfth aspect, the present invention provides the use of any of the described kits for the treatment and/or prevention of brucellosis. The use of the kit may be in line with any of the medical uses and methods of administration outlined in this application.

PCR-Multiplex diagnostic kit

In a thirteenth aspect, the present invention provides a kit for the identification of *Brucella* strains which comprise a partial or complete deletion of *wzm*. In a preferred embodiment, the kit comprises a forward and reverse primer which anneal to the regions flanking the *wzm* in the genome of a species of the genus *Brucella*. Preferably, the kit comprises SEQ ID NO: 2 as the forward primer and, SEQ ID NO: 3 and/or SEQ ID NO: 4 as the reverse primer(s). The primer sets may amplify an amplicon of about 1,573 bp (F1 and R4; AMP NO: 1) or about 724 bp (F1 and R5; AMP NO: 2) in Rev1 or 16M wild type strain; or a band of 931 bp (F1 and R4; AMP NO: 3) in the Δ*wzm* mutants (Table 1). Since the identity between BMEI1415 and the corresponding orthologs in other *Brucella* species is at least 99.6%, this kit could be used to differentiate the wild type *Brucella* strains and *Brucella*Δ*wzm* mutants.

Examples

Example 1: Culturing of *E. coli* and *Brucella* strains

All strains were preserved in cryovials at -20°C, harvesting bacterial cultures in skimmed milk supplemented with 3% sterile lactose (Applichem Panreac) or with 20-40% glycerol (v/v).

To prepare suspensions with known concentration of *Brucella*, a preculture from a cryovial was prepared by growing bacteria onto a Blood Agar Base number 2 (Oxoid) plate, either plain (BAB) or

supplemented with 5% Fetal Bovine Serum (Gibco) (BAB-S) for *B. ovis*. After incubation (3-5 days, at 37°C, and in atmosphere supplemented with 10% CO_2 for *B. ovis*) of this preculture, 2-3 colonies from BAB or BAB-S plates were transferred onto fresh BAB or BAB-S plates and incubated for 2 days as before. The grown colonies were harvested in sterile PBS pH 6.85 to obtain a concentrated suspension of bacteria, which was diluted in the same diluent up to obtain a suspension with around 0.170 units of absorbance at Optical Density of 600 nm adjusted by spectrophotometry. In our conditions, this suspension contains around 10^9 CFU/mL. The exact number of bacteria was always determined retrospectively by serial dilutions in PBS, plating (100 µL by triplicate) and incubation of plates for 3-5 days at 37°C. The number of CFU/mL was calculated.

For the liquid culture of *Brucella*, 4-5 colonies of a BAB or BAB-S plate were inoculated into a trypticase soy broth (TSB, Pronadisa) and were incubated overnight at 37°C and 150 rpm.

E. coli was cultured in Luria Bertani (LB, Pronadisa) broth or on LB agar plates. *E. coli* cultures were incubated at 37°C.

Appropriate concentrations of antibiotics were included in the agar plates and cultures for selection. All antibiotics were purchased from Sigma.

Example 2: Sequencing of the *wzm* gene

The genome of Rev1 was extracted using the PureLink™ Microbiome DNA Purification Kit (Thermo Fisher Scientific). The *wzm* gene was amplified using primers F1 gcaaattgaaatggcagatg and R4 atgaaacgtggcgttagtcc (Table 1) and the PCR product was purified using a QIAquick PCR purification kit (Qiagen) and sequenced by Sanger method.

The resultant sequence is AMP NO: 1 (Table 1) including the SEQ ID NO: 1 described in the present application. This sequence in Rev1 is identical to that of the *wzm* gene in the *B. melitensis* 16M virulent strain (Genbank: CP007763.1) as well as 99.6% identity to that of *B. abortus* 2308 (Genbank: NC_007618) and vaccine S19 (Genbank: CP000887.1).

According to sequencing, the same allelic exchange plasmid can be used to partially delete *wzm* in both *B. melitensis* (16M and Rev1 strains) and *B. abortus* (2308 and S19 strains) species.

Table 1. Amplicon Numbers (AMP NO) used in this work and the correspondent DNA fragment size obtained by sequencing or by PCR with the indicated primer pairs with DNA from wild-type *wzm* or Δ*wzm* genes.

AMP NO:	DNA amplicon size	PCR primer pairs*	Primers nucleotide sequence (5'-3')
1	1,573 bp (in wt)	F1/ R4	F1: gcaaattgaaatggcagatg (SEQ ID NO: 2) R4: atgaaacgtggcgttagtcc (SEQ ID NO: 3)
2	724 bp	F1/ R5	F1: gcaaattgaaatggcagatg (SEQ ID NO: 2) R5: gcgtgtaaattgcaagagga (SEQ ID NO: 4)
3	931 bp (in Δ*wzm*)	F1/ R4	F1: gcaaattgaaatggcagatg (SEQ ID NO: 2) R4: atgaaacgtggcgttagtcc (SEQ ID NO: 3)
4	484 bp	F1/ R2	F1: gcaaattgaaatggcagatg (SEQ ID NO: 2) R2: agcgcccacgtaaatcag (SEQ ID NO: 5)
5	465 bp	F3/ R4	F3: ctgatttacgtgggcgcttaacctgcgtggcagtagc (SEQ ID NO: 6) R4: atgaaacgtggcgttagtcc (SEQ ID NO: 3)
6	319 bp	F9/ R5	F9: atgatatcgtatatggctaatg (SEQ ID NO: 7) R5: gcgtgtaaattgcaagagga (SEQ ID NO: 4)
7	816 bp	rrnBP1-F/ Gfp_F-R2	rrnBP1-F: gttgcgcggtcagaaaattatttta (SEQ ID NO: 8) Gfp_F-R2: ttatttgtatagttcatccatgcca (SEQ ID NO: 9)

*F: *forward*; R: *reverse*.

Example 3: Cloning of allelic exchange plasmids

All the *Brucella*Δ*wzm* strains were constructed via a double recombination event using the allelic exchange plasmid pJQKm-Δ*wzm* (Figure 1). The primers required to construct the *wzm* truncated form (Δ*wzm*) were designed using Primer3 on the basis of available sequence information of the *B. melitensis* 16M strain in the Kyoto Encyclopaedia of Genes and Genomes (KEGG) and the National Center for Biotechnology Information (NCBI).

The genome of *Brucella* strains was extracted and purified using a PureLink™ Microbiome DNA Purification Kit (Thermo Fisher Scientific). Alternatively, the DNA was extracted by re-suspending the bacteria in ultrapure water and boiling the suspension (100 °C, 20 minutes) and then centrifuging (4,000 rpm, 10 minutes). DNA was recovered by collecting the supernatant.

To clone the allelic exchange plasmid for the partial deletion of *wzm*, a 484 bp fragment (AMP NO: 4, Table 1) was amplified using PCR with the primers F1 gcaaattgaaatggcagatg and R2 agcgcccacgtaaatcag (AMP NO: 3, Table 1). A 465 bp fragment (AMP NO: 5, Table 1) was amplified using PCR and primers F3 ctgatttacgtgggcgcttaacctgcgtggcagtagc and R4 atgaaacgtggcgttagtcc (Table 1). The two PCR products were combined by using overlap extension PCR and the full-length product

was amplified using F1 gcaaattgaaatggcagatg and R4 atgaaacgtggcgttagtcc to produce the Δ*wzm* cassette (Figure 1). The Δ*wzm* fragment had a deletion of 82% of the *wzm* wild-type gene (Δ80-721 from the total 783 bp of the *wzm* wild type). The Δ*wzm* cassette was cloned into a pCR2.1 plasmid using the manufacturer's instructions (TOPO® TA Cloning®, Thermo Fisher Scientific).

The pJQKm suicide vector has been described previously (Scupham and Triplett, 1997. Gene, 202,53-59). The pCR2.1Δ*wzm* and pJQKm were digested using BamHI and XbaI and the Δ*wzm* insert and pJQKm vector were gel extracted and purified. The Δ*wzm* insert and pJQKm vector were ligated using a T4 DNA ligase resulting in the allelic exchange plasmid pJQKmΔ*wzm*.

To insert *gfp* into the genome of the *B. melitensis* strains, the mini-Tn*7* system was used by adaptation of the method described previously (Choi et al, 2005. Nature Methods, 2(6):443-8). The constitutive *gfp* expression was controlled by the *rrnB* P1 promoter contained in the fragment *rrnB* P1-*gfp*. The *rrnB* P1-*gfp* construct was extracted from the plasmid pUC18T-mini-Tn*7*-*gfp*-Gm^r (GenBank: DQ493877.2). Thus, the *rrnB* P1-*gfp* fragment was amplified with the primers rrnBP1-F gttgcgcggtcagaaaattatttta and Gfp_F-R2 ttatttgtatagttcatccatgcca (AMP NO: 7) and cloned into a pCR2.1 plasmid using the manufacturer's instructions (TOPO® TA Cloning®, Thermo Fisher Scientific), and then subcloned into pUC18R6KT-mini-Tn*7*-Km^r resistant to 50 µg/mL kanamycin (Km^r) (Llobet et al, 2009. Antimicrobial Agents and Chemotherapy, 53(1): 298–302) using EcoRI. This resulted in the plasmid pUC18R6KT-mini-Tn*7*-*gfp* (Table 1).

Example 4: Conjugation of *E. coli* with *Brucella* strains and PCR assessment of mutants

The pUC18R6KT-mini-Tn*7*-*gfp* or pJQKmΔ*wzm* were transformed into *E. coli* S17 (λpir) and the plasmids were transferred into the receiving *Brucella* strains via conjugation. In the case of the partial deletion of *wzm* using the pJQKmΔ*wzm* allelic exchange plasmid, 1 mL of overnight liquid culture of *B. melitensis* was cultured with 0.5 mL of overnight liquid cultures of *E. coli* S17 (λpir)-pJQKmΔ*wzm*.

For *Brucella*Δ*wzm* strains, the selection of transconjugants after the first recombination (integration of the suicide vector in the chromosome) was selected by growing the bacteria in BAB supplemented with 50 µg/mL kanamycin (Km^r) and susceptibility to 5% sucrose (Sac^s). The second recombination (excision of the mutator plasmid and leading to construction of the mutant strain by allelic exchange) was selected by sucrose resistance and kanamycin sensitivity (Figure 1). Finally, the resulting colonies were screened by two PCR, i.e. one PCR with primers F1 gcaaattgaaatggcagatg and R4 atgaaacgtggcgttagtcc which amplified a fragment of 931 bp in the Δ*wzm* mutants and a fragment of 1,573 bp in the parental strain (Table 1) and other PCR with F9 atgatatcgtatatggctaatg and R5 gcgtgtaaattgcaagagga amplifying a 319 bp PCR product in the wild type and sibling strains (Figure 2). For PCR, genomic DNA was extracted by re-suspending the bacteria in ultrapure water and boiling the

suspension at 100°C for 20 minutes and then centrifuging the resultant liquid at 4,000 rpm for 10 minutes and collecting the supernatant.

In all cases, three clones of each mutant and one sibling strain (i.e. submitted to the same subcultures than the correspondent mutant; Figure 1) as control were used in most of experiments, to assess the absence of uncontrolled changes during the genetic manipulations.

A Rev1 strain containing the partial deletion (82%) of the *wzm* gene was identified and called Rev1Δ*wzm*. This strain was also deposited at a depository in accordance with the Budapest Treaty.

*Brucella*Δ*wzm* mutants were complemented by conjugation with the donor *E. coli* S17pBBR-*wzm* or *E. coli* S17pSRK-*wzm*. The complemented strains were selected on BAB plates supplemented with 20 µg/mL of chloramphenicol (BAB-Cm_{20}) or 50 µg/mL of kanamycin (BAB-Km_{50}) allowing the selection of conjugants carrying the non-integrative plasmid pBBR-*wzm* or pSRK*wzm*, respectively. Transconjugants were checked by PCR (Figure 2) with F9 atgatatcgtatatggctaatg and R5 gcgtgtaaattgcaagagga primers (Table 1), amplifying DNA fragments of 319 bp (AMP NO: 6) exclusively in strains carrying the wild type *wzm* gene (parental and sibling strains) or the pBBR-*wzm* or pSRK-*wzm* complementation plasmids (Figure 2).

Example 5: Phenotypic characterization of the *Brucella*Δ*wzm* mutants

The selected clones of Δ*wzm* mutants were analyzed by the classical markers for *Brucella* typing following the standard protocols (Alton *et al.*, 1988. Techniques for the Brucellosis. Laboratory Paris: INRA) of crystal violet-oxalate exclusion, catalase, oxidase, urease and acriflavine tests (all from Sigma Aldrich), sensitivity to Tb, Wb, Iz and R/C phages, agglutination with anti-A and anti-M monospecific sera, both CO_2- and serum- dependence, susceptibility to dyes (i.e. thionine blue 10, 20 and 40 µg/mL, fuchsine 10 and 20 µg/mL, and safranin 100 µg/mL; Sigma) and to the antibiotics penicillin 5 mg/mL (P_5) and streptomycin 2.5 µg/mL ($Str_{2.5}$), as shown in Table 2.

Moreover, Rev1Δ*wzm*, 16MΔ*wzm*, 2308Δ*wzm* and S19Δ*wzm* strains showed a R-LPS phenotype, regarding positive staining with crystal violet-oxalate technique (Figure 3).

Also, the LPS structure of all Δ*wzm* mutants and complemented strains was studied by SDS-PAGE and silver staining modified for LPS. As shown in Figure 4A, both Rev1Δ*wzm*, 16MΔ*wzm*, 2308Δ*wzm* and S19Δ*wzm* mutants showed a R-LPS with an intact core identical to that of the parental and/or sibling strains. However, when analysed antigenically by Western Blot using anti-M, anti-C or anti-A O-PS epitopes, all Δ*wzm* mutants showed O-PS in lower amounts or antigenically different epitopes (Cloeckaert et al., J Gen Microbiol. 1992 Jun;138(6):1211-9) to those present in the O-PS of the

S-LPS (Figure 4B). Moreover, 16MΔ*wzm*::*gfp*-pBBR-*wzm* complementation was assessed by epifluorescence microscopy, by using a primary MoAb (Monoclonal Antibody) anti-C O-PS epitope and a secondary antibody labelled with Texas Red. 16MΔ*wzm*::*gfp* and 16M sibling strains were used as controls (Figure 4C).

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Besides PCR assessment (see Example 4, Figure 2), the phenotypic characterization showed that the complemented strains restored the S-LPS phenotype (Figures 3, 4B and 4C).

Table 2. Phenotypic characterization of Rev1Δ*wzm*, 16MΔ*wzm*, 2308Δ*wzm* and S19Δ*wzm*

STRAIN	Phage lysis				CO_2 dependent	Catalase/ Oxidase/ Urease	Agglutination with			Growth in dyes in BAB-S in presence/absence of CO_2					
	Tb	Wb	Iz	R/C			Acriflavine and crystal violet staining	Sera anti-		Thionine			Basic fuchsine		Safranin
								A	M	10	20	40	10	20	100
16M	-	-	-2	-	-	+/+/+	-/-	-	+	+/+	+/-	-/-	+/+	+/+	+/+
16MΔ*wzm*	-	-	-	-3	-	+/+/+	+/+	-	-	+/+	+/-	-/-	+/+	+/+	+/-
Rev1	-	-	0	-	-	+/+/+	-/-	-	+	+/+	+/-	-/-	+/+	+/-	+/-
Rev1Δ*wzm*	-	-	-	-4	-	+/+/+	+/+	-	-	+/+	-/-	-/-	+/+	+/+	-/-
2308	-3	-4	-4	-	-	+/+/+	-/-	+	-	-/-	-/-	-/-	+/+	+/+	+/+
2308Δ*wzm*	ND	ND	ND	ND	-	+/+/+	+/+	-	-	-/-	-/-	-/-	-/-	+/+	+/+
S19	-3	-3	-2	-	-	+/+/+	-/-	+	-	-/-	-/-	-/-	+/+	+/+	+/+
S19Δ*wzm*	-	-	-	-3	-	+/+/+	+/+	-	-	-/-	-/-	-/-	+/+	+/+	+/+

ND: Not Determined

Example 6: Deletion of *wzm* in Rev1Δ*wzm* and 16MΔ*wzm* is stable after subcultures *in vitro* and *in vivo* in mice.

*Brucella*Δ*wzm* mutants were subcultured for 20 consecutive passages in BAB plates by transferring colonies onto fresh plates every 3-4 days, after the plates were incubated at 37ºC. Besides analysis of these cultures, the grown bacteria were kept for 2 months at 4ºC to assess their stability after storage. Moreover, representative number of CFU isolated from mice spleens in the experiments described in Examples 12 and 13 were selected for stability assessment.

Each selected culture was analysed for the presence of the deletion by PCR with primers F1 gcaaattgaaatggcagatg and R4 atgaaacgtggcgttagtcc, allowing DNA amplification in both wt and Δ*wzm* bacteria (Table 1), and by phenotypic analysis, i.e. colony size after incubation at 37ºC and crystal violet-oxalate staining. Finally, inocula containing ≈ 2×10^3 CFU/mL were adjusted by spectrophotometry and plated in triplicate (3 × 100 µL) in five plates, in order to analyse the colony size after 5 days of incubation at 37ºC and colony phase by crystal violet staining in around 3,000 CFU.

All the colonies analysed showed the expected genotype and phenotype, indicating that the genetic modification is stable.

Example 7: The growth of 16MΔ*wzm* but not Rev1Δ*wzm* is inhibited by the presence of 10% CO_2 in the atmosphere of incubation. This defect is restored by growing in agar supplemented with bovine foetal sera.

Bacterial growth of Δ*wzm* mutants were studied in BAB plates incubated in normal atmosphere or supplemented with 10% CO_2. For this, 100 µL of bacterial suspensions containing ≈5×10^2 CFU/mL were plated in triplicate, and the number of CFU/100 µL determined after incubation (3-5 days, 37ºC). Moreover, 16MΔ*wzm* was analysed to determine the frequency of inhibition (i.e. the number of CFU/mL isolated after CO_2 incubation with respect to the number of CFU/mL isolated after incubation in normal atmosphere) by seeding all the bacterial dilutions prepared in both BAB and BAB-S. Each counting was repeated three times. Results are presented as mean ± standard deviation (n=9) of individual counts. Statistical comparisons of means were performed by a one-way ANOVA and PLSD tests.

As shown in Table 3, 16MΔ*wzm* but not Rev1Δ*wzm* was unable to growth in BAB under CO_2 incubation conditions.

Table 3. Growth in BAB plates incubated in normal atmosphere or supplemented with 10% CO_2. Number of CFU/100 µL of bacterial suspensions containing ≈5x10^2 CFU/mL. Mean and standard deviation of three experiments by triplicate plating of 100 µL in BAB.

	No. CFU/100 µL (mean ± SD)	
Strain	Normal atmosphere	10% CO_2
Rev1	70.1 ± 5.4	62.9 ± 8.3
Rev1-sibling	53.8 ± 3.0	61.8 ± 4.5
Rev1Δ*wzm*	42.3 ± 2.3	28.6 ± 4.2
Rev1Δ*wzm*::*gfp*	37.7 ± 2.4	21.5 ± 1.3
16M	57.9 ± 2.8	67.8 ± 2.5
16M-sibling	50.5 ± 1.8	73.1 ± 3.1
16MΔ*wzm*	31.7 ± 3.5	0 [a]
16MΔ*wzm*::*gfp*	21.4 ± 1.5	0 [a]

[a] PLSD tests: p<0.0001 vs. normal atmosphere and vs. 16M and Rev1 sibling strains

The frequency of inhibition of 16MΔ*wzm* after incubation of BAB plates in CO_2-atmosphere was of 1-0.39×10^{-2} CFU/mL. This phenotype did not occur when 16MΔ*wzm* was cultured in BAB-S plates (Table 4).

Table 4. Inhibition frequency of 16MΔ*wzm* and 16MΔ*wzm*::*gfp* in BAB and BAB-S plates incubated in atmosphere normal or supplemented with a 10% CO_2.

	CFU/mL			**CFU/mL**	
Strain	**Normal atmosphere**	**10% CO_2 (Inhibition frequency)**	**Strain**	**Normal atmosphere**	**10% CO_2 (Inhibition frequency)**
16M			**16MΔ*wzm***		
BAB	4.3×10^8	4.2×10^8 (1×10^0)	BAB	4.3×10^8	4.4×10^6 (1×10^{-2})
BAB-S	4.9×10^8	4.2×10^8 (1×10^0)	BAB-S	3.4×10^8	4.5×10^8 (1×10^0)
16M sibling			**16MΔ*wzm*::*gfp***		
BAB	5.4×10^8	6.2×10^8 (1×10^0)	BAB	4.1×10^8	1.6×10^6 (0.39×10^{-2})
BAB-S	6.6×10^8	6.2×10^8 (0.9×10^0)	BAB-S	3.9×10^8	4.5×10^8 (1×10^0)

Example 8: Rev1Δ*wzm* is more susceptible to streptomycin than Rev1

In contrast to other *Brucella* species such as *B. melitensis* 16M, and *B. abortus* 2308 and S19, Rev1 presents a relative resistance to 2.5 μg/mL of streptomycin ($Str_{2.5}$) when incubated in normal atmosphere (in 10% CO_2 all the *B. melitensis* strains show similar resistance). This relative resistance to $Str_{2.5}$ *in vitro* is directly related to the inefficiency of streptomycin-based treatments (an antibiotic of choice in humans) against Rev1 infections in both humans and in animal models (Grilló et al. 2006. J Antimic Chemother. 58 (3):622–626).

To assess this property in Rev1Δ*wzm*, bacterial suspensions containing ≈2×10^3 CFU/mL were prepared in PBS and cultured by plating 100 μL in triplicate in BAB and BAB supplemented with $Str_{2.5}$ (BAB-$Str_{2.5}$). The Rev1 sibling strain was used as control. Plates were incubated at 37°C, for 5 days, in normal atmosphere and the mean ± standard deviation (n=3) number of CFU/mL was determined. The experiment was repeated three times. Statistical comparisons of means were performed by ANOVA and PLSD tests.

As result, Rev1Δ*wzm* was more ($p<0.001$) susceptible to $Str_{2.5}$ than Rev1 sibling (Figure 5).

Example 9: 16MΔ*wzm* is more susceptible to desiccation than 16M, and Rev1Δ*wzm* is as susceptible as Rev1.

Desiccation resistance Rev1Δ*wzm*, 16MΔ*wzm*, Rev1 and 16M was tested by aliquoting 200 μL/well of a ≈10^9 CFU/mL suspension in TSB in 12-well polystyrene plates. The suspensions were allowed to dry at room temperature in the dark for 6 days. Then, the pellet was rehydrated in PBS, serially diluted, and plated on BAB plates in order to determine the number and percentage of surviving bacteria.

As can be seen in Figure 6, the partial deletion of the *wzm* gene in 16MΔ*wzm* further decreased ($p<0.001$) the 16M sibling strain's ability to survive in dry environments. However, Rev1Δ*wzm* is as susceptible as Rev1 sibling. These findings provide evidence that the 16MΔ*wzm* would be less likely to persist in the environment than the 16M virulent strain.

Example 10: Rev1Δ*wzm* is more susceptible than 16MΔ*wzm* to the bactericidal cationic peptides of the innate immune system. Both Δ*wzm* mutants are more susceptible than parental or sibling strains.

Polymyxin B was used as model of bacterial susceptibility to cationic peptides of the innate immune system. For this, exponentially growing Rev1Δ*wzm* or 16MΔ*wzm* were adjusted to 2-3×10^3 CFU/mL in PBS and mixed with different concentrations from 3 to 0.188 mg/mL Polymyxin B in Phosphate Saline Acid buffer (PSA; 0.133M NaCl, 0.1M; NaH_2PO_4, pH 4.6) in 24-well microtiter plates in

duplicate. Suspensions (100 µL, in triplicate) were plated in BAB and the number of viable CFU was recorded after incubation for 1 h at 37° C. Both parental and sibling strains were used as controls. Data points represent the mean ± standard deviation (n=3) of CFU/mL.

Figure 7 shows that Rev1Δ*wzm* strain is more susceptible than 16MΔ*wzm* to the detrimental effects of Polymyxin B. Moreover, both Δ*wzm* mutants were far more susceptible than Rev1 and 16M parental and sibling strains. In fact, Rev1Δ*wzm* failed to grow after incubation in the presence of the lowest concentration tested (0.188 mg/mL) while 16MΔ*wzm* was not totally inhibited up to a Polymyxin B concentration of 0.750 mg/mL, under the same experimental conditions (Figure 7). In contrast, Rev1 parental and sibling strains were inhibited at 3 mg/mL, while the virulent 16M resisted even at this high concentration. These results are in concordance with the different *in vivo* persistence of Rev1Δ*wzm* and 16MΔ*wzm* observed in mice (Figure 9).

Example 11: Rev1Δ*wzm* and 16MΔ*wzm* are more susceptible than Rev1 and 16M parental strains to conventional sheep and cattle sera.

Bacteria in exponential phase were adjusted to a concentration of $\approx 10^4$ CFU/mL in PBS and dispensed in microtiter plates (45 µL/well) by mixing with either normal or decomplemented (1 hour, 56ºC) ovine or bovine sera (90 µL/well). After incubation (18 h, 37ºC), 65 µL of TSB was dispensed into each well, the bacterial suspension mixed, 50 µL/well was plated onto BAB, and plates were incubated (5 days, 37ºC) to determine the number of CFU/mL and the percentage of bacterial survival. A *B. melitensis* mutant with minimal core was used as positive control (C+) of high susceptibility to normal serum. Results are expressed as the mean ± standard deviation (n=3) of survival percentage. Statistical comparisons of means were performed by ANOVA and PLSD tests.

As can be seen in Figure 8, the Rev1Δ*wzm* and 16MΔ*wzm* strains are more susceptible than the Rev1 or 16M parental strains to the bactericidal effect of both sheep and cow normal serum.

Example 12: Rev1Δ*wzm* is more attenuated than 16MΔ*wzm* in BALB/c mice

Female BALB/c mice of 7 weeks of age (Charles River Laboratories, Barcelona, Spain) were housed in the animal building of the Instituto de Agrobiotecnología (registration number ES/31-2016-000002-CR-SU-US) 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 (RD 1201/2005) and Regional (Ley 11/2003) directives, and were supervised by the Ethical Committee of the Institution.

Mice were inoculated intraperitoneally with $\approx 10^8$ CFU/mouse of Rev1Δ*wzm*, Rev1Δ*wzm*::*gfp*, 16MΔ*wzm* or 16MΔ*wzm*::*gfp* (R-LPS strains) and 10^6 CFU/mouse of Rev1 or 16M sibling strains (S-

LPS strains). Additional groups of mice inoculated with ≈10^8 CFU/mouse of S19Δ*wzm* or 2308Δ*wzm* *B. abortus* mutants and with 10^6 CFU/mouse of S19 or 2308 sibling strains were used to compare the effect of this mutation in different *Brucellae* backgrounds. At selected intervals, groups of 5 mice were necropsied to determine the number of viable bacteria present in the spleens as well as the spleens weights, as previously reported (Grilló et al., 2012. Veterinary Research, 43 (1): 29). Viable bacteria were identified on BAB plates. The rough identity of the spleen isolates was confirmed by the crystal violet-oxalate staining method as well as by PCR. Results were expressed as the mean ± standard deviation (n=5) of individual log CFU/spleen or grams/spleen. Statistical comparison of means was performed by a one-way ANOVA followed by the Fisher Protected Least Significant Differences (PLSD) tests.

As can be seen in Figures 9A and 9C, the partial deletion of the *wzm* gene led to a decrease in the number of *B. melitensis* present in the mice spleens in comparison to the correspondent Rev1 or 16M sibling strains. However, Rev1Δ*wzm* was much more attenuated than 16MΔ*wzm*, since complete clearance of infections from spleens occurred before week 4 or week 12 for Rev1Δ*wzm* or 16MΔ*wzm*, respectively. In contrast to *B. melitensis*, both *B. abortus* 2308Δ*wzm* and S19Δ*wzm* mutants persisted in the spleens similarly, i.e. somewhat more than 8 weeks or less than 9 weeks, respectively (Figures 9E and 9G). These findings indicated that both *B. abortus* mutants were more attenuated than the 16MΔ*wzm* mutant but less than Rev1Δ*wzm*.

Unexpectedly, the higher attenuation of Rev1Δ*wzm* was accompanied by the induction of a transient splenomegaly that peaked at week 2 post-infection (Figure 9B). This finding is generally associated with the triggering of an effective immune-response (Conde-Álvarez et al. 2012. PLoS Pathog. 8(5): e1002675). This splenic reaction was not observed in 16MΔ*wzm* (Figure 9D) neither in *B. abortus* 2308Δ*wzm* and S19Δ*wzm* mutants (Figure 9F and 9H).

The Rev1Δ*wzm*::*gfp* and 16MΔ*wzm*::*gfp* strains showed similar virulence and splenomegaly than Rev1Δ*wzm* and 16MΔ*wzm*, respectively (Figures 9A-9D), indicating that the insertion of the mini-Tn*7*-*gfp* in the genome did not affect the biological properties of Rev1Δ*wzm* and 16MΔ*wzm*.

Example 13: Rev1Δ*wzm* does not infect placentas or foetuses of pregnant mice

CD1 female mice (n=7) at 4.5 days of pregnancy were intraperitoneally infected with ≈7×10^6 CFU/mouse of Rev1Δ*wzm* or 16MΔ*wzm* or with ≈7×10^5 CFU/mouse of Rev1 or 16M. All mice were sacrificed at term pregnancy to assess macroscopic lesions at necropsy as well as bacteriology of spleen, placenta and foetus samples. The number of viable bacteria (log CFU/organ) in each tissue was determined by plating on BAB. Moreover, the number of pregnant females, the infected placentas, and the dams carrying infected foetuses were recorded.

As can be seen in Table 5, all mice showed well-established infections in spleens at similar levels (≈4-5 logs) between groups. However, the splenomegaly generated by Rev1Δ*wzm* and 16MΔ*wzm* in pregnant dams were moderate or low, respectively, in contrast to Rev1 and 16M parental strains (Table 5). Surprisingly, Rev1 (and to less extend 16M) induced higher splenomegaly in pregnant (Table 5) than in non-pregnant mice at week 2 (Figure 9). However, Rev1Δ*wzm* and 16MΔ*wzm* mutants induced similar splenomegaly in pregnant and non-pregnant mice, showing higher spleen weights at week 2 in Rev1Δ*wzm* than in 16MΔ*wzm* (0.30 vs 0.16 grams/spleen, Table 5). Full term pregnancies were observed in most of the mice vaccinated with Rev1Δ*wzm* or 16MΔ*wzm*, as well as in those vaccinated with Rev1, but only few dams infected with 16M parental achieved full-term pregnancy. Moreover, while Rev1Δ*wzm* and 16MΔ*wzm* were practically unable to colonize the placentas and foetuses at the dose administered, infection with one logarithm less of Rev1 allowed colonization of these tissues at very high levels (6-8 logs of infection) in all dams (Table 5).

These levels of infection were accompanied by macroscopic lesions in Rev1 and 16M infected placentas but not in those from dams inoculated with Rev1Δ*wzm* or 16MΔ*wzm* mutants (Figure 10). All these results indicated that Rev1Δ*wzm* or 16MΔ*wzm* mutants were safer in pregnant mice than both virulent and vaccine reference strains.

Table 5. Spleen, placental and foetal infections, and term pregnancies in CD1 mice infected with Rev1Δ*wzm*, 16MΔ*wzm*, Rev1 or 16M, at day 4.5 of pregnancy and slaughtered 15 days later.

Strain	Inoculation	Spleen		Pregnancy	Placenta		Fetuses	
B. melitensis	Dose /route	log CFU/spleen	Spleen weight (grams)	No. of pregnant/ total dams	No. of dams with infected placentas/ pregnant	log CFU/ gram of placenta*	No. of dams with infected fetuses/ pregnant	log CFU/ gram of fetus*
Rev1 (vaccine)	$6.0x10^5$ / IP	5.70 ± 0.80	0.89 ± 0.29	10/14	10/10	8.5 ± 0.8	10/10	6.71 ± 0.49
Rev1Δ*wzm*	$6.3x10^6$ / IP	4.70 ± 0.80	0.30[a] ± 0.16	12/14	0/12	1.52 ± 0[a]	0/5	1.52 ± 0[a]
16M (virulent)	$6.9x10^5$ / IP	4.10 ± 0.50	0.83 ± 0.43	4/14	4/4	6.0 ± 1.42	2/2	6.16 ± 1.21
16MΔ*wzm*	$6.5x10^6$ / IP	3.60 ± 0.70	0.16[a] ± 0.08	5/7	0/5	1.52 ± 0[a]	0/5	1.52 ± 0[a]

IP: intraperitoneal; a: $p < 0.001$ vs. 16M (virulent) or Rev1 (vaccine) infected mice; *Limit of Detection = 1.52 logs (i.e. no CFU isolated)

Example 14: Rev1Δ*wzm* and 16MΔ*wzm* confer solid protection against S and R virulent infections in mice, equivalent or better than that conferred by Rev1.

Vaccine efficacy of the *Brucella*Δ*wzm* mutants was evaluated in 8-10 week-old female BALB/c mice (n=5) by intraperitoneal or subcutaneous vaccination with $\approx 10^8$ CFU/mouse of the corresponding mutant. Mice (n=5) vaccinated subcutaneously with 2×10^5 CFU/mouse of Rev1 or S19 were used as reference vaccinated controls against either *B. melitensis* H38 and *B. ovis* PA or *B. abortus* infections, respectively. Three groups of mice (n=5) inoculated with 0.1 mL of sterile PBS were used as non-vaccinated controls in the corresponding experiment. Four weeks after vaccination, all mice were challenged intraperitoneally with $\approx 1\times10^4$ CFU/mouse of *B. melitensis* H38::Gm^r, 2×10^5 CFU/mouse of *B. ovis* BoPA::Gm^r or 5×10^4 CFU/mouse of *B. abortus* 2308::Gm^r, challenge strains resistant to 15 µg/mL of gentamycin (Gm_{15}). Finally, the number of virulent bacteria in the spleens were determined at 2 (H38::Gm^r and 2308::Gm^r) and 3 (BoPA::Gm^r) weeks after the challenge by plating each spleen onto BAB-S-Gm_{15}.

As can be seen in Table 6A, both Rev1Δ*wzm* and 16MΔ*wzm*::*gfp* mutants showed a degree of protection against a *B. melitensis* virulent infection similar to that showed by the Rev1 reference vaccine strain, not only by intraperitoneal but also by subcutaneous vaccination. Moreover, both *B. melitensis* Δ*wzm* mutants conferred a superior protection against *B. ovis* infection ($p<0.001$) to that conferred by the Rev1 strain. In contrast, surprisingly, the Δ*wzm* mutation in both *B. abortus* 2308Δ*wzm* and S19Δ*wzm* strains did not confer adequate protection against *B. abortus* virulent infection in mice (Table 6B).

Table 6A. Efficacy of vaccination against virulent infection by *B. melitensis* H38 (S-LPS virulent strain) or *B. ovis* PA (R-LPS virulent strain).

	Vaccination Dose/ route	Challenge H38::Gm^r log H38/spleen (mean±SD)	Uninfected[a]/totals	UP[b]	Challenge *B. ovis* PA::Gm^r log BoPA/spleen (mean±SD)	Uninfected/totals [a]	UP[b]
Rev1Δ*wzm*	10^8 / IP	1.44 ± 1.28 [c]	3/5	4.32	0.7 ± 0.00 [a]	5/5	5.37
	10^8 / SC	1.93 ± 1.43 [c]	2/5	3.83	ND		
16MΔ*wzm*::*gfp*	10^8 / IP	1.75 ± 1.52 [c]	3/5	4.01	0.62 ± 0.05 [a]	5/5	5.45
	10^8 / SC	2.05 ± 1.00 [c]	1/5	3.71	1.91 ± 1.76 [a]	2/5	4.16
Rev1	2×10^5 / SC	1.39 ± 1.16 [c]	3/5	4.37	2.58 ± 1.88 [a]	2/5	3.49
PBS Control	-	5.76 ± 0.58	0/5	-	6.07 ± 0.24	0/5	

IP: intraperitoneal; SC: subcutaneous; [a] < 5CFU/spleen; [b] Units of Protection = log CFU/spleen in unvaccinated control – in tested group; [c] $p<0.001$ vs. PBS control by PLSD test.
ND: Not Determined

Table 6B. Efficacy of vaccination with 2308Δ*wzm* or S19Δ*wzm* against virulent infection by *B. abortus*2308::Gm^R (S-LPS virulent strain)

Vaccine strain	Vaccination Dose/ route	Challenge 2308::Gm^r	
		log 2308/spleen (mean±SD)	Uninfected/totals*
2308Δ*wzm*	10^8/IP	2.97 ± 1.41 [b]	1/5
	10^8/SC	4.13 ± 1.08	0/5
S19Δ*wzm*	10^8/IP	4.18 ± 1.91	0/5
S19	10^5/SC	1.57 ± 1.96 [a]	4/5
PBS Control	-	5.87 ± 0.26	0/5

IP: intraperitoneal; SC: subcutaneous; *< 5CFU/spleen; **Units of Protection = log CFU/spleen in unvaccinated control – in tested group; [a]: p<0.001; [b]: p<0.05 vs. PBS control by PLSD test

Example 15: Serological response of lambs inoculated with Rev1Δ*wzm*::*gfp* or 16MΔ*wzm*::*gfp* vs. Rev1::*gfp*

Rasa Aragonesa male and female breed lambs born in the experimental flock of the Centro de Investigación y Tecnología Agroalimentaria (CITA) del Gobierno de Aragón (Zaragoza, Spain) were used in these experiments, at 3-4 months of age. These animals were housed in the authorized facilities of the CITA (registration number ES/50-2970-12005), handled and manipulated according to the FELASA (www.felasa.eu) and ARRIVE (Kilkenny et al., 2010. PLoS Biology, 8: e1000412) recommendations.

Lambs were vaccinated by subcutaneous inoculation of a suspension containing 1-2×10^{10} CFU of Rev1Δ*wzm*::*gfp* (n=14) or 16MΔ*wzm*::*gfp* (n=8). Groups of lambs non-vaccinated (n=13) or vaccinated with 1-2×10^9 CFU of Rev1::*gfp* (n=12) were used as controls. Thereafter, innocuousness was assessed by clinical inspection (rectal body temperature and palpation of the inoculation site) for one month after vaccination, and by periodical examination of epididymis and testicles all throughout the experiment. Moreover, blood samples were taken just before vaccination and, thereafter, weekly or every two weeks, by jugular vein puncture by using Venojet® (Terumo) vacuum tubes. After draining at room temperature for 24 h, blood samples were centrifuged at 3,500 rpm for 10 minutes and the resultant serum was conserved at -20°C until its analysis.

The anti-LPS response was measured using a standard Rose Bengal Test (sRBT) and Complement Fixation Test (CFT), recommended by the WHO/OIE. Additionally, Gel Diffusion Tests (GDT) with R-LPS antigen was carried out in order to assess by seroconversion that vaccination was effective. Details on these serological tests can be found in the "*Manual of diagnostic tests and vaccines for*

terrestrial animals" of the World Organization for Animal Health (OIE, 2016). An ELISA for anti-GFP antibodies in the serum was also performed on samples obtained from the serum of the lambs inoculated with 16MΔ*wzm*::*gfp*.

Figures 11A and 11B show that, in contrast to Rev1::*gfp*, vaccination with Rev1Δ*wzm*::*gfp* or 16MΔ*wzm::gfp* induced (if any) minimal serological interference in S-LPS *Brucella* tests. In fact, the serological response induced by Rev1Δ*wzm* vaccination did not produce any interference in the sRBT (Figure 11A) and only three lambs elicited anti-S/LPS antibodies detectable by CFT. In contrast, three animals vaccinated with 16MΔ*wzm::gfp* were positive in sRBT (Figure 11A) and one of them was also positive in CFT. In any case, these four CFT positive lambs vaccinated with the Δ*wzm* mutants showed very low anti-S/LPS titres, which persisted for less than 6 weeks post-vaccination (Figure 11B). The 100% animals positive in GDT-R/LPS (Figure 11C) demonstrated that all lambs were correctly vaccinated with the Rev1Δ*wzm*::*gfp* or 16MΔ*wzm*::*gfp* mutant, indicating that the absence of S/LPS-reactions would be due to the nature of O-PS accumulated in Δ*wzm* mutants.

Figure 11D shows that lambs which were inoculated with 16MΔ*wzm*::*gfp* also produced antibodies that bind to GFP. These antibodies could be used in a serological test to discriminate between vaccinated and infected lambs. Further, this sort of test could also be used to discriminate between lambs inoculated with Rev1Δ*wzm*::*gfp* and infected lambs.

Example 16: Efficacy of Rev1Δ*wzm*::*gfp* vaccination against a *B. ovis* PA experimental infection in rams.

Rasa Aragonesa male and female breed lambs born in the experimental flock of the Centro de Investigación y Tecnología Agroalimentaria (CITA) del Gobierno de Aragón (Zaragoza, Spain) were used in these experiments, at 3-4 months of age. These animals were housed in the authorized facilities of the CITA (registration number ES/50-2970-12005), handled and manipulated according to the FELASA (www.felasa.eu) and ARRIVE (Kilkenny et al., 2010. PLoS Biology, 8: e1000412) recommendations.

Lambs (n=14) were vaccinated by subcutaneous injection of $1\text{-}2\times10^{10}$ CFU of Rev1Δ*wzm*::*gfp*. One group (n=13) kept unvaccinated were used as control. Thereafter, innocuousness was assessed by clinical inspection (rectal body temperature and palpation of the inoculation site) for one month after vaccination, and by periodical examination of epididymis and testicles all throughout the experiment. At 8 months after vaccination, all lambs were experimentally challenged with 2×10^{9} CFU de *B. ovis* PA by conjunctival and preputial routes (30 µL/each route) and slaughtered 2 months later for bacteriological purposes. Samples of spleens, epididymis, seminal vesicles and cranial, prescapular, crural, iliac and scrotal lymph nodes were taken, homogenized in sterile PBS and cultured by duplicate

in CITA medium (De Miguel et al. 2011. Journal of Clinical Microbiology. 49(4): 1458–1463). The number and percentage of infected animals and samples were determined as previously described (Grilló et al. 2009. Vaccine, 27: 187-191) and the mean infection index was calculated as the sum of the infection levels assigned to each sample divided by all the samples processed from each group. The infection level was assigned as follows: 1 (1-5 CFU), 2 (6-25 CFU); 3 (26-125 CFU); 4 (126-300 CFU); 5 (>300 CFU). The statistical comparisons of percentages and means were performed by the Chi-square and Kruskal-Wallis tests, respectively.

As shown in Table 7, vaccination of lambs at 3-4 months of age with Rev1Δ*wzm*::*gfp* induces significant protection against a challenge infection by *B. ovis* PA at 11-12 months of age, regarding not only the number of animals found infected but also the number of samples detected as infected by *B. ovis* PA. Moreover, the level of infection observed in both vaccine groups were lower than that observed in the unvaccinated control group.

Table 7. Efficacy against *B. ovis* PA infection in rams vaccinated with Rev1Δ*wzm*::*gfp* at 3-4 months old.

Vaccination group[1]	**No. (%) Infected/ total animals**	**No. (%) Infected/ total samples**	**Mean Infection Index** [2]
Rev1Δ*wzm*::*gfp*	5/14 (35.7%) [a]	15/ 112 (13.4%) [b]	0.29
Unvaccinated	12/13 (92.3%)	47/ 104 (45.2%)	1.00

[1] Male lambs of 3-4 months old were vaccinated subcutaneously with 2×10^{10} CFU of Rev1Δ*wzm*::*gfp*, challenged 8 months post-vaccination, and analysed by bacteriology 2 months after challenge; [2] Mean Infection Index = the sum of the infection levels assigned to each sample divided by all the samples processed from each group; Statistical comparisons: [a] $p=0.002$; [b] $p<0.0001$.

Example 17: Immune sera from lambs vaccinated with Rev1Δ*wzm* are effective against *B. melitensis* H38 and *B. abortus* 2308 S-LPS virulent strains.

Exponentially grown *B. melitensis* H38, *B. abortus* 2308 and *B. ovis* PA were adjusted to 10^4 CFU/mL in PBS and mixed, in triplicate in microtiter plates (45 µL/well), with 90 µL/well of normal or heat-treated (1 h, 56ºC) sera extracted from lambs showing anti- R/LPS antibodies in GDT- R/LPS (one of them was positive in CFT as well) at 2 weeks after inoculation with Rev1Δ*wzm*. After 18 h of incubation at 37ºC, and 10% CO_2 for *B. ovis*, 65 µL of TSB were dispensed into each well, the bacterial suspension was mixed, and 50 µL were plated on BAB in triplicate. The results were expressed as the standardized percentage of bacteria counts with respect to initial count in the inocula. As it can be seen in the Figure 12, the immune sera from lambs treated with Rev1Δ*wzm* were capable of killing either *B. melitensis* H38, *B. abortus* 2308 or *B. ovis* PA.

Claims

1. A modified *Brucella melitensis* Rev1 strain, wherein the *wzm* gene has been inactivated, for use in the prevention of brucellosis.

2. The strain for use according to claim 1, wherein the *wzm* gene has been partially deleted.

3. The strain for use according to claim 2, wherein at least 50 % of SEQ ID NO: 1 has been deleted.

4. The strain for use according to any one of the preceding claims, wherein the strain has been further modified to express a fluorescent protein.

5. The strain for use according to any one of the preceding claims, wherein the strain has been lyophilized.

6. The strain for use according to any of claims 1 to 5, wherein the infectious agent causing brucellosis is selected from the group consisting of *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Brucella canis*, *Brucella ovis*, *Brucella neotomae*, *Brucella microti*, *Brucella ceti* and *Brucella pinnipedialis*.

7. The strain for use according to any of claims 1 to 6, wherein the strain is used to prevent brucellosis in humans, cattle, goats, sheep, pigs, and/or dogs.

8. A kit comprising:
(i) a modified *Brucella melitensis* Rev1 strain, wherein the *wzm* gene has been inactivated, and
(ii) a pharmaceutically acceptable carrier or diluent; for use in the prevention of brucellosis

9. The kit for use according to claim 8, wherein the *wzm* gene has been partially deleted.

10. The kit for use according to claim 9, wherein at least 50 % of SEQ ID NO: 1 has been deleted.

FIGURES

Figure 1

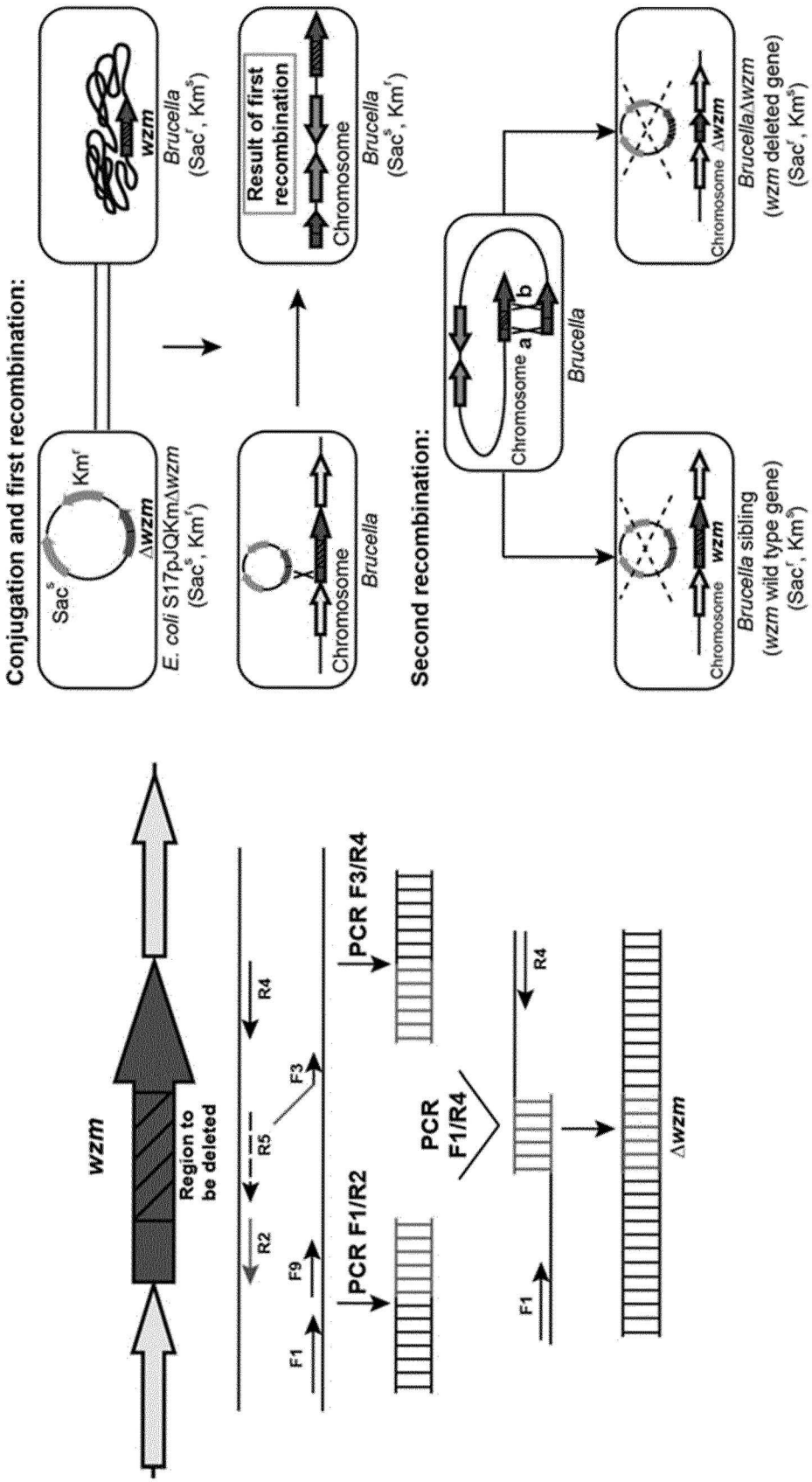


Figure 2

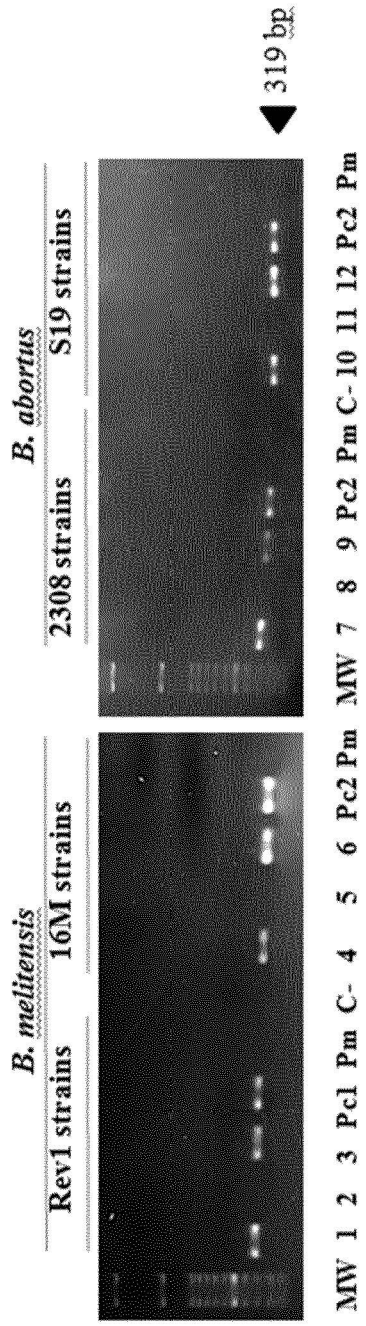

319 bp
B. abortus
2308 strains
S19 strains
B. melitensis
Rev1 strains
16M strains
MW 1 2 3 Pc1 Pm C- 4 5 6 Pc2 Pm MW 7 8 9 Pc2 Pm C- 10 11 12 Pc2 Pm

Figure 3

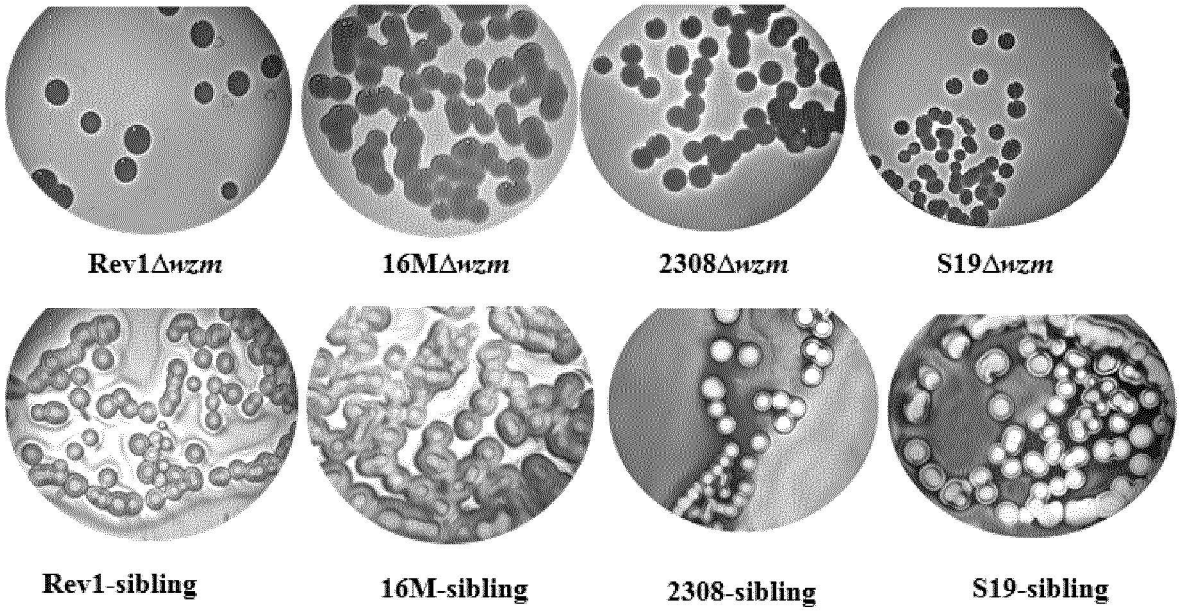
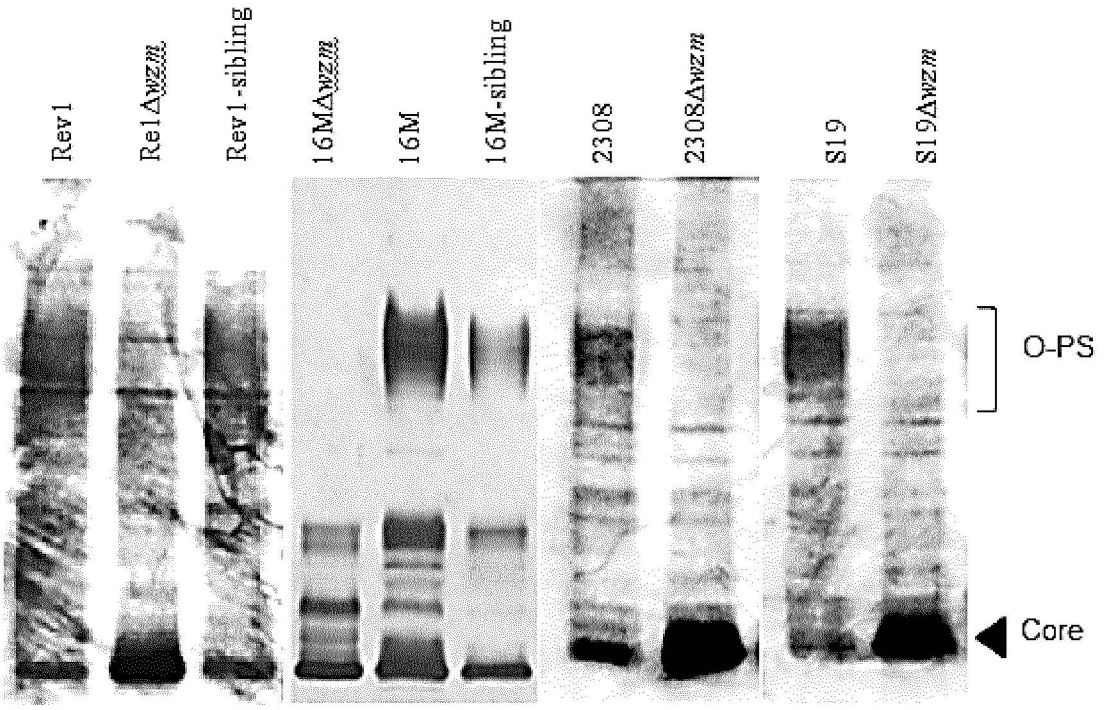

Rev1Δ*wzm*
16MΔ*wzm*
2308Δ*wzm*
S19Δ*wzm*
Rev1-sibling
16M-sibling
2308-sibling
S19-sibling

Figure 4

A)


Rev1
Re1Δwzm
Rev1-sibling
16MΔwzm
16M
16M-sibling
2308
2308Δwzm
S19
S19Δwzm
O-PS
Core

B)

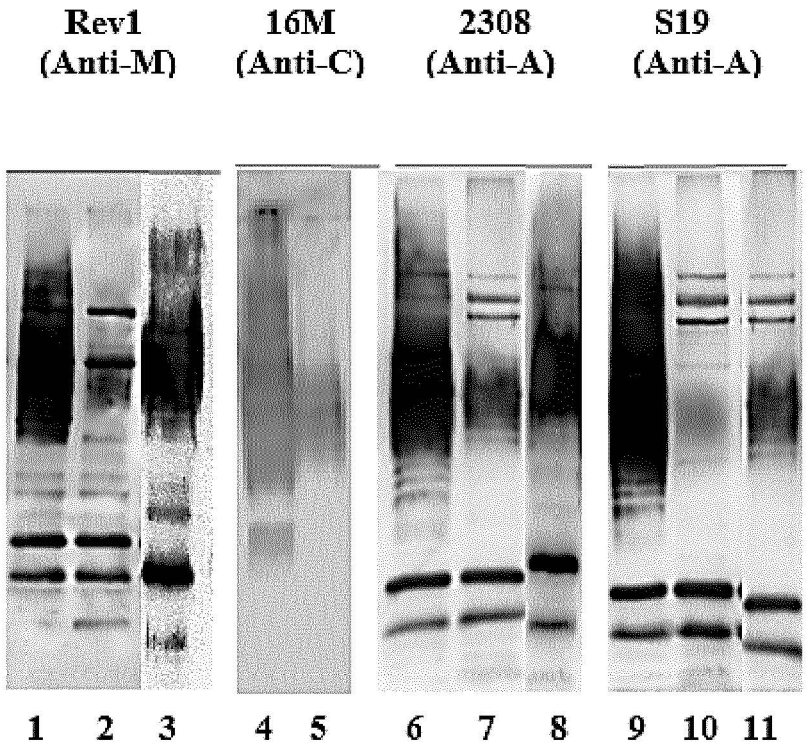

Rev1
(Anti-M)
16M
(Anti-C)
2308
(Anti-A)
S19
(Anti-A)
1 2 3 4 5 6 7 8 9 10 11

Figure 4 (Cont.)

C)

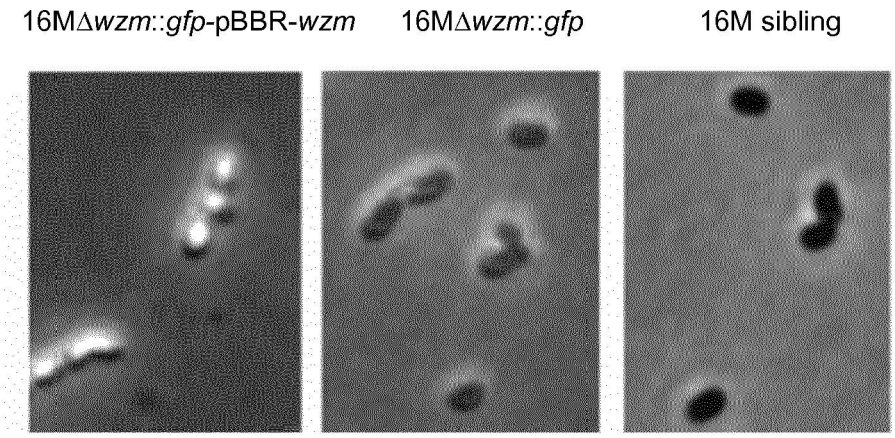


Figure 5

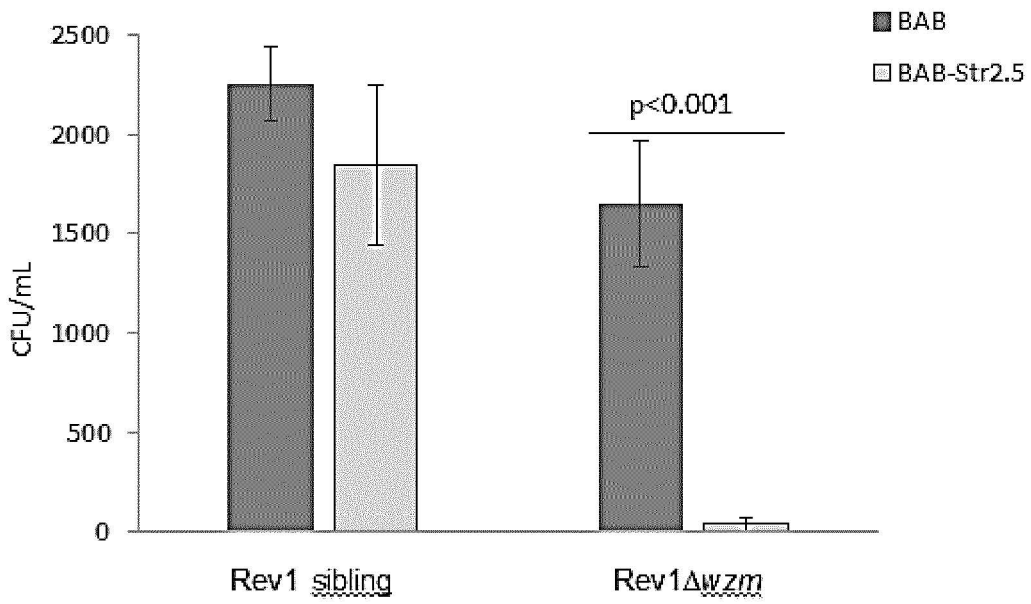

BAB
BAB-Str2.5
p<0.001
2500
2000
1500
1000
500
0
CFU/mL
Rev1 sibling
Rev1Δwzm

Figure 6

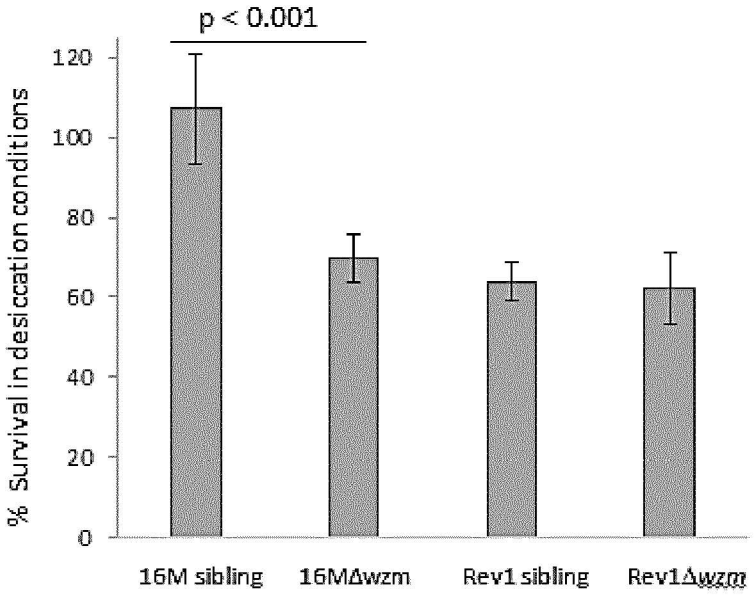

p < 0.001
% Survival in desiccation conditions
120
100
80
60
40
20
0
16M sibling
16MΔwzm
Rev1 sibling
Rev1Δwzm

Figure 7

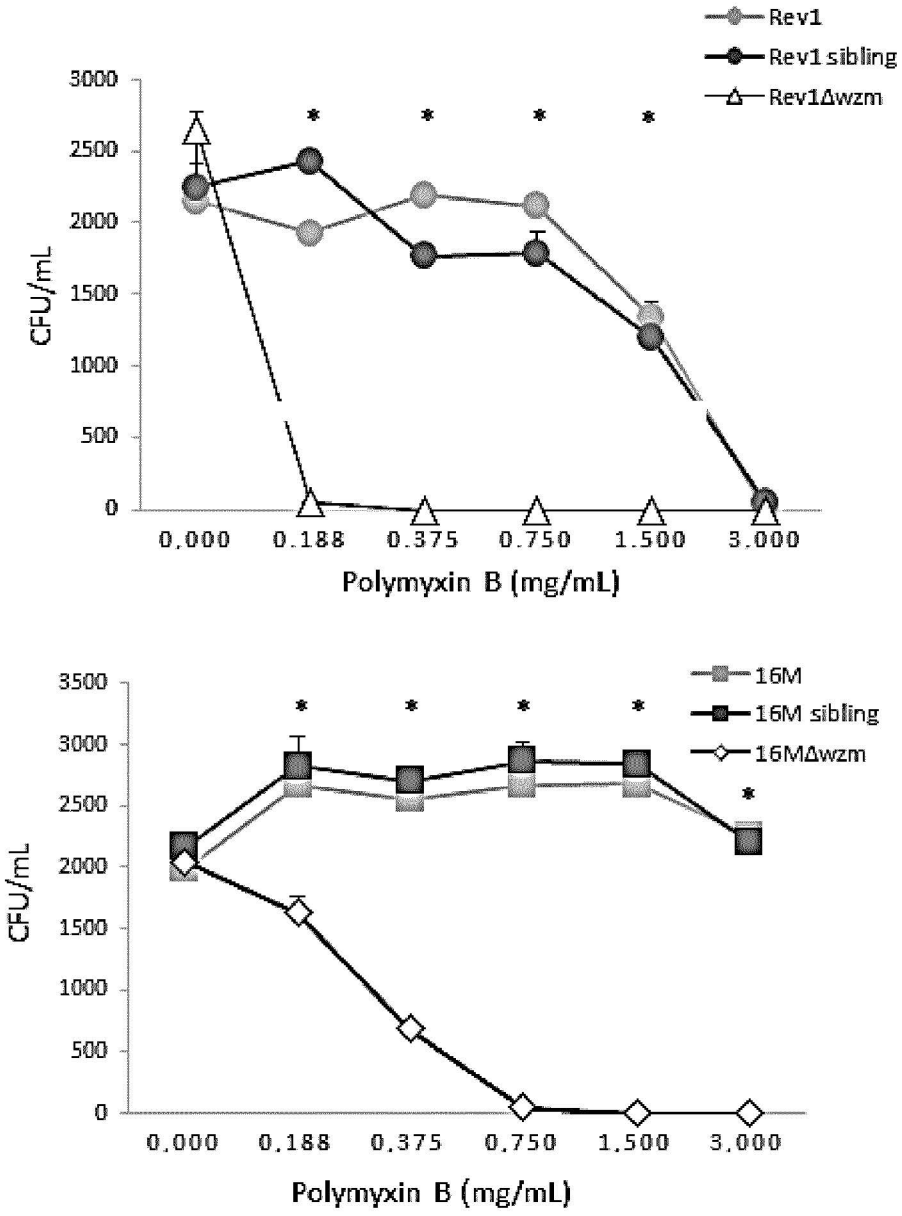

Rev1
Rev1 sibling
Rev1Δwzm
CFU/mL
3000
2500
2000
1500
1000
500
0
0.000
0.188
0.375
0.750
1.500
3.000
Polymyxin B (mg/mL)
16M
16M sibling
16MΔwzm
CFU/mL
3500
3000
2500
2000
1500
1000
500
0
0.000
0.188
0.375
0.750
1.500
3.000
Polymyxin B (mg/mL)

Figure 8

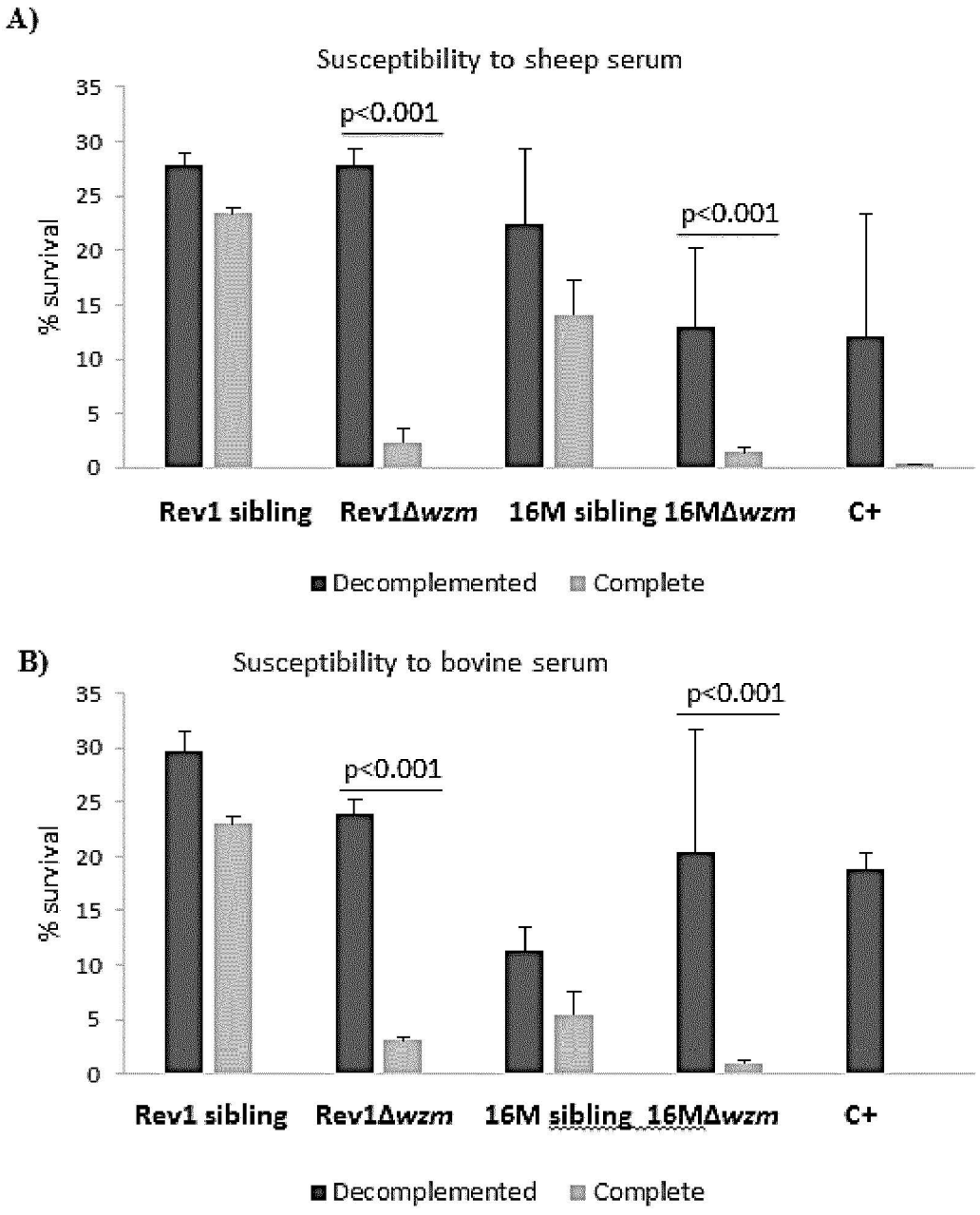

A)
Susceptibility to sheep serum
% survival
35
30
25
20
15
10
5
0
p<0.001
p<0.001
Rev1 sibling
Rev1Δwzm
16M sibling
16MΔwzm
C+
Decomplemented
Complete
B)
Susceptibility to bovine serum
% survival
35
30
25
20
15
10
5
0
p<0.001
p<0.001
Rev1 sibling
Rev1Δwzm
16M sibling
16MΔwzm
C+
Decomplemented
Complete

Figure 9

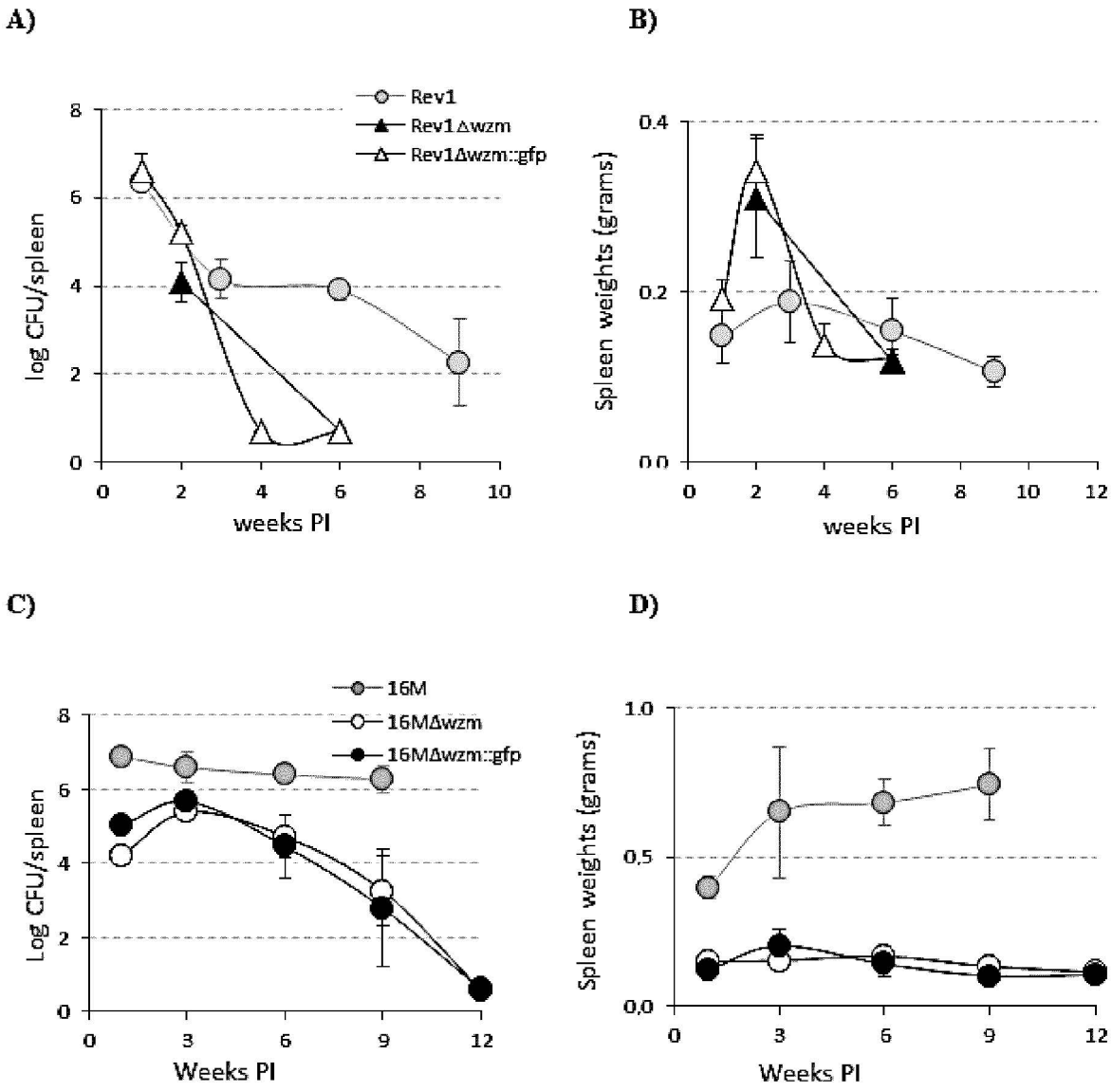

A)
Rev1
Rev1Δwzm
Rev1Δwzm::gfp
log CFU/spleen
weeks PI
B)
Spleen weights (grams)
weeks PI
C)
16M
16MΔwzm
16MΔwzm::gfp
Log CFU/spleen
Weeks PI
D)
Spleen weights (grams)
Weeks PI

Figure 9 (Cont.)

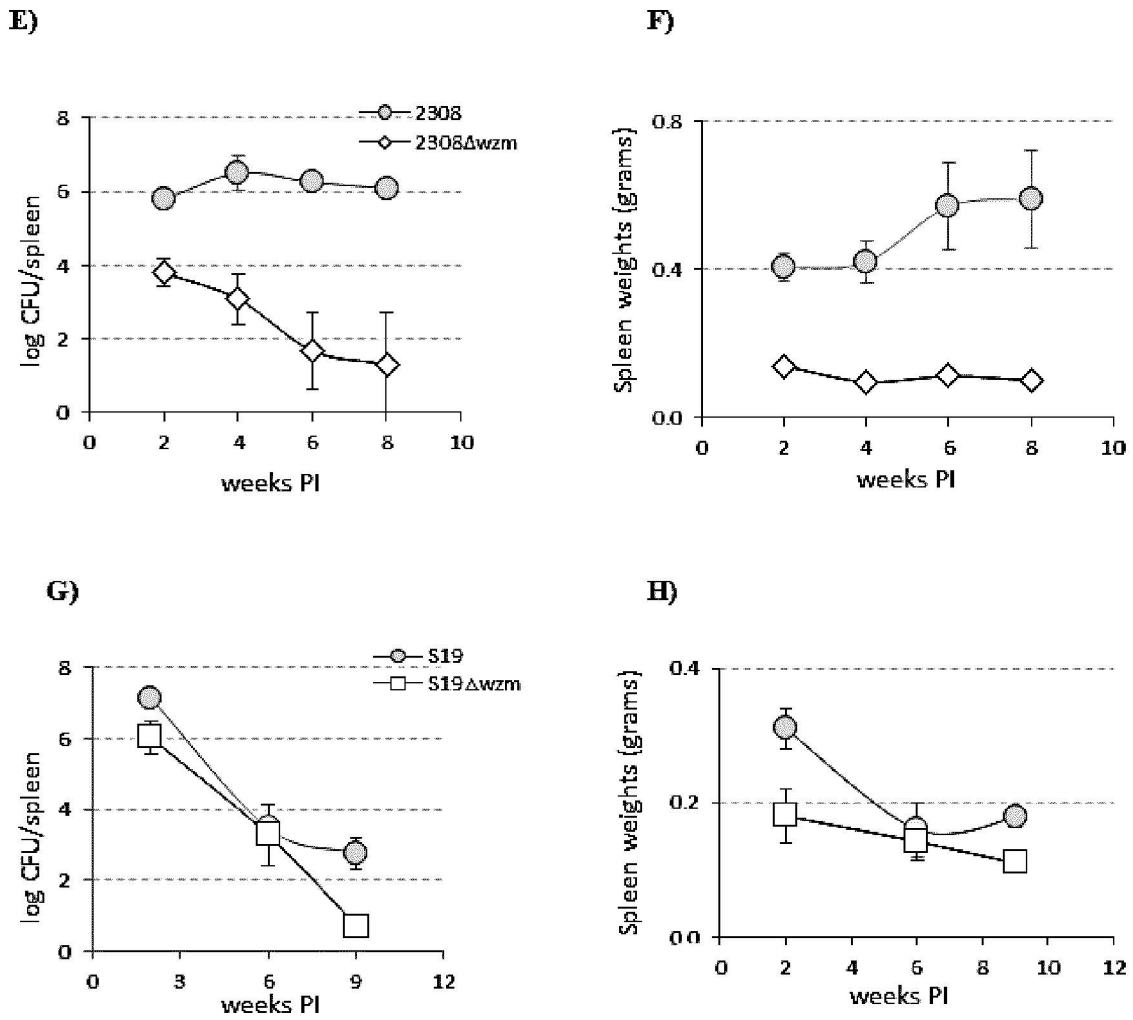
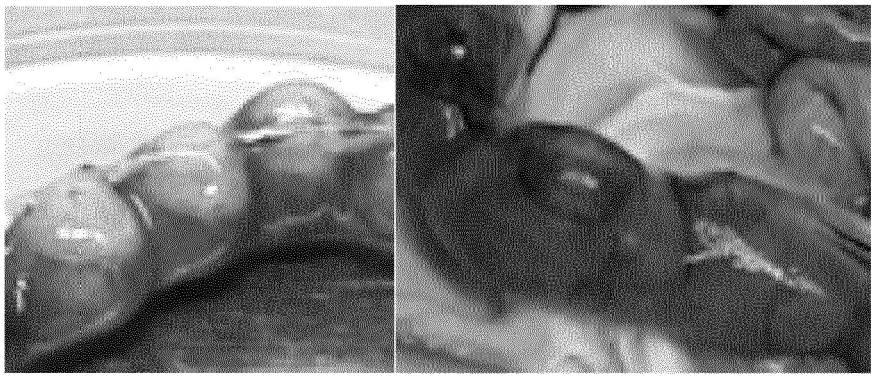
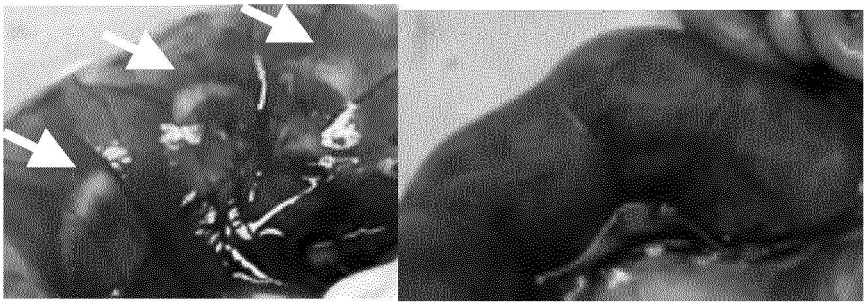


Figure 10



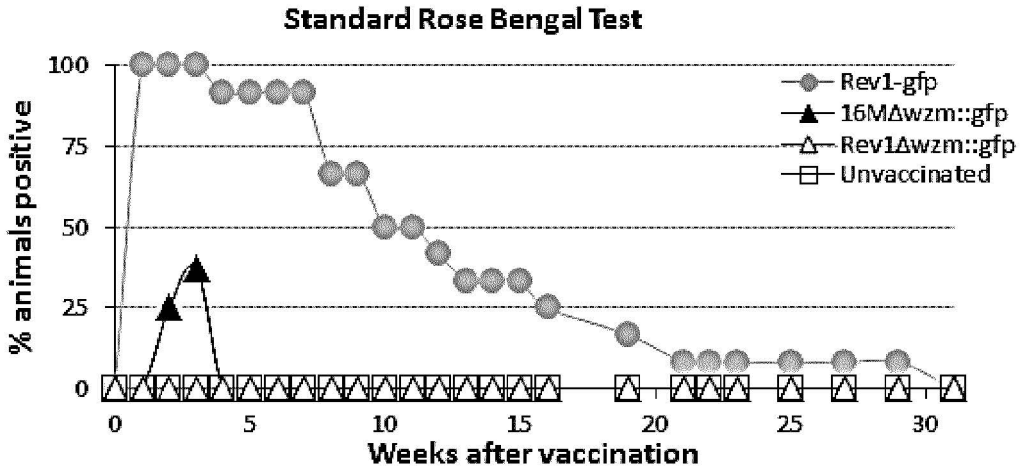
Rev1 Rev1Δ*wzm*



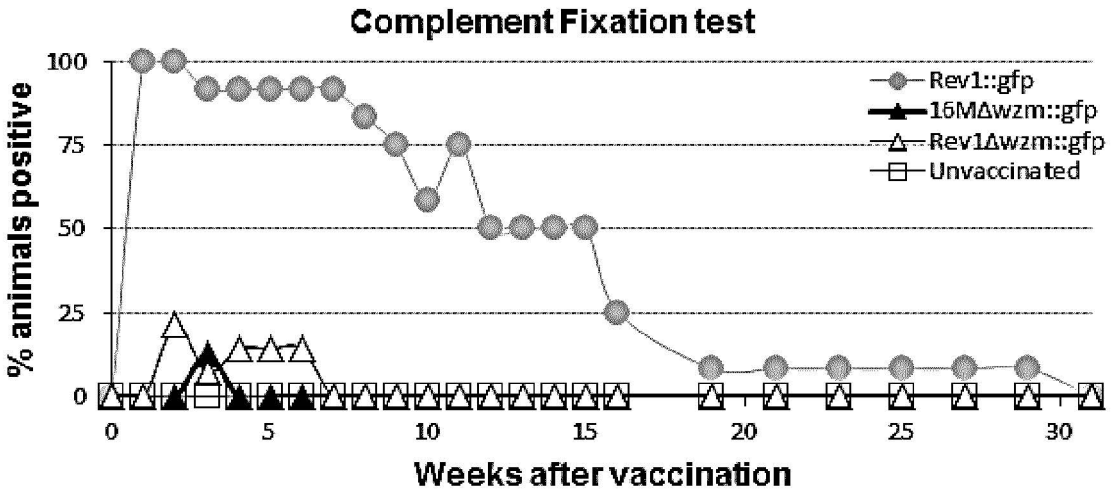
16M 16MΔ*wzm*

Figure 11

A)


Standard Rose Bengal Test
% animals positive
100
75
50
25
0
0
5
10
15
20
25
30
Weeks after vaccination
Rev1-gfp
16MΔwzm::gfp
Rev1Δwzm::gfp
Unvaccinated

B)


Complement Fixation test
% animals positive
100
75
50
25
0
0
5
10
15
20
25
30
Weeks after vaccination
Rev1::gfp
16MΔwzm::gfp
Rev1Δwzm::gfp
Unvaccinated

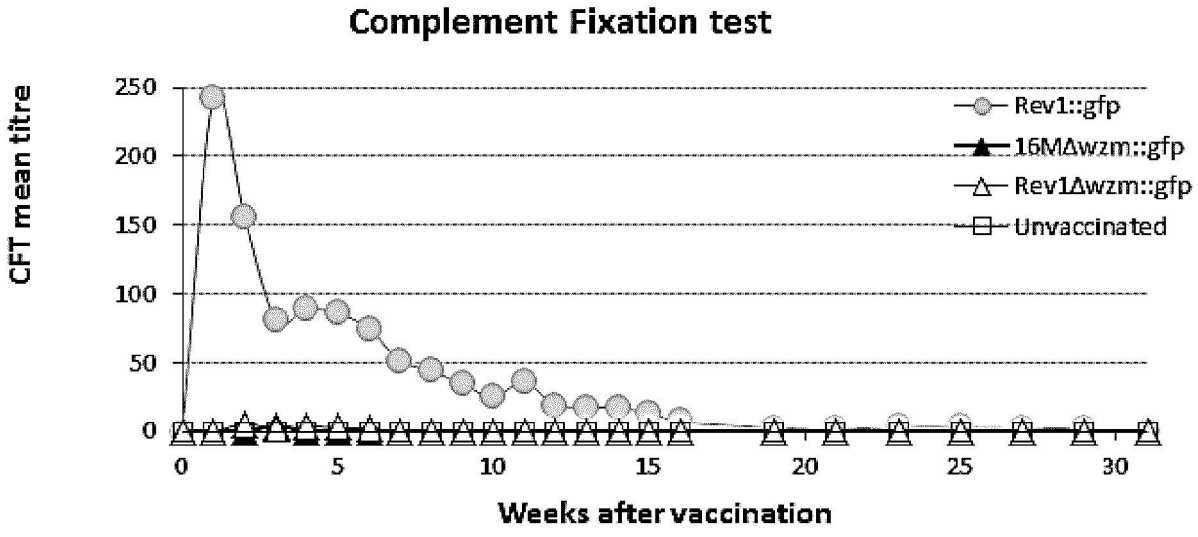
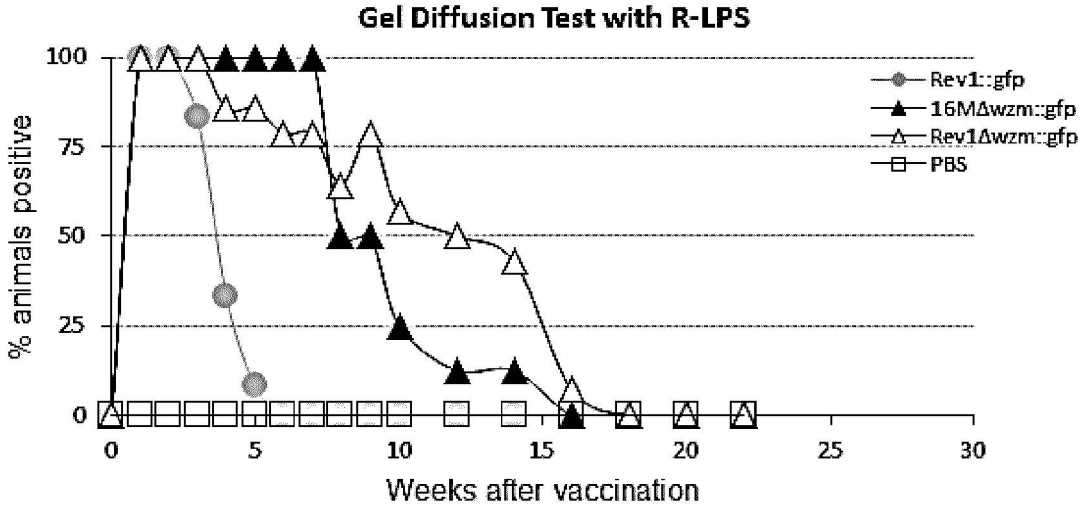

Complement Fixation test
CFT mean titre
250
200
150
100
50
0
0
5
10
15
20
25
30
Weeks after vaccination
Rev1::gfp
16MΔwzm::gfp
Rev1Δwzm::gfp
Unvaccinated

Figure 11 (Cont.)

C)



D)

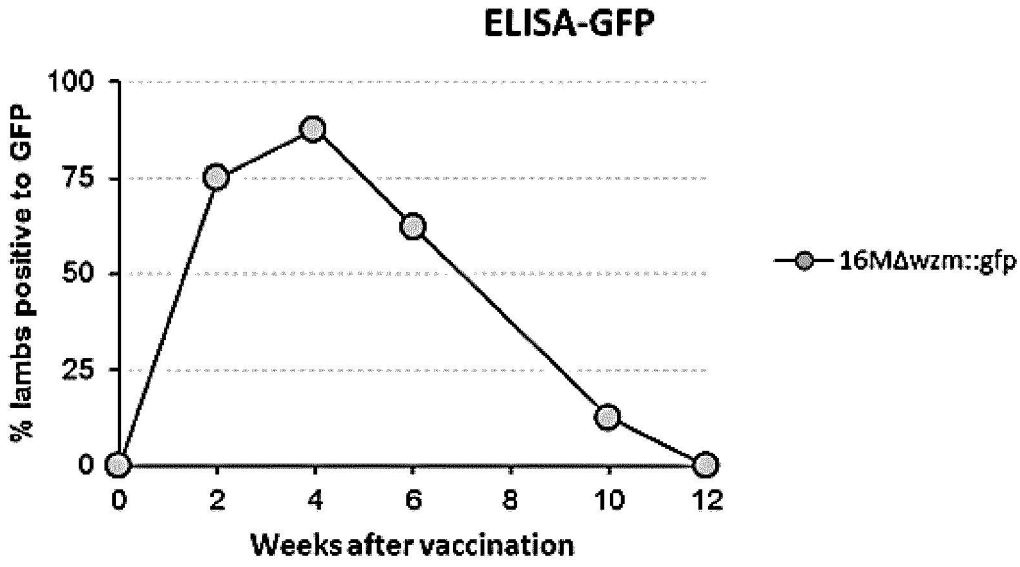
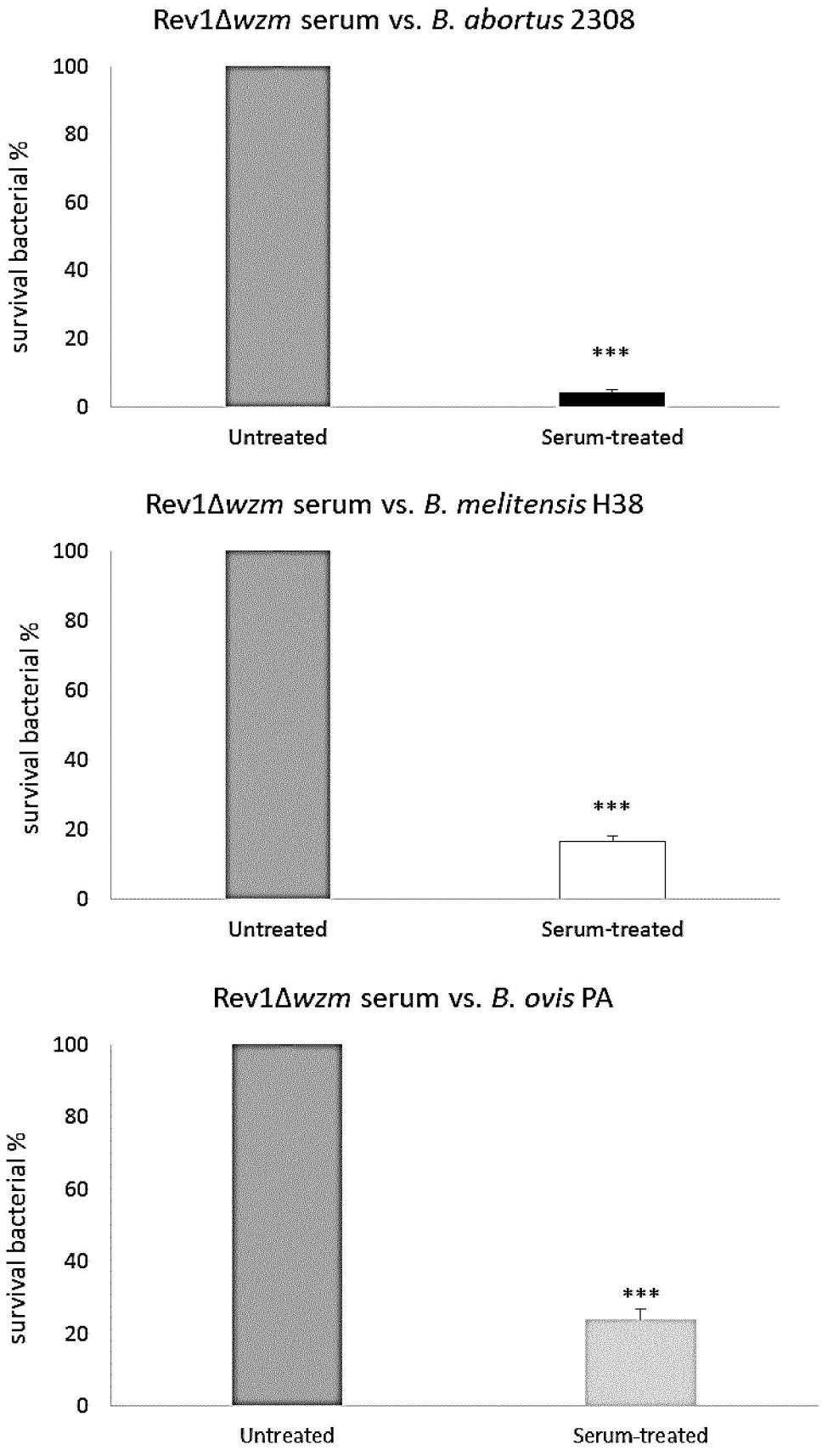


Figure 12


Rev1Δwzm serum vs. B. abortus 2308
survival bacterial %
100
80
60
40
20
0
***
Untreated
Serum-treated
Rev1Δwzm serum vs. B. melitensis H38
survival bacterial %
100
80
60
40
20
0
***
Untreated
Serum-treated
Rev1Δwzm serum vs. B. ovis PA
survival bacterial %
100
80
60
40
20
0
***
Untreated
Serum-treated

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/082539

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/02
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GONZÁLEZ DAVID ET AL: "Brucellosis vaccines: assessment of Brucella melitensis lipopolysaccharide rough mutants defective in core and O-polysaccharide synthesis and export", PLOS ONE, PUBLIC LIBRARY OF SCIENCE, vol. 3, no. 7, 23 July 2008 (2008-07-23), pages E2760.1-E2760.15, XP002573331, ISSN: 1932-6203 cited in the application table 1 page 7, right-hand column, paragraph 3 page 11, right-hand column, paragraph 2 ----- -/--	1-10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
4 April 2019	17/04/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Rojo Romeo, Elena

4

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	XIURAN WANG ET AL: "Effects of partial deletion of the wzm and wzt genes on lipopolysaccharide synthesis and virulence of Brucella abortus S19", MOLECULAR MEDICINE REPORTS, vol. 9, no. 6, 2 April 2014 (2014-04-02), pages 2521-2527, XP055466718, GR ISSN: 1791-2997, DOI: 10.3892/mmr.2014.2104 Discussion table 1	1-10

Y	XIU-RAN WANG ET AL: "Immunogenic response induced by wzm and wzt gene deletion mutants from Brucella abortus S19", MOLECULAR MEDICINE REPORTS, vol. 9, no. 2, 18 November 2013 (2013-11-18), pages 653-658, XP055466720, GR ISSN: 1791-2997, DOI: 10.3892/mmr.2013.1810 Discussion table 1	1-10

Y	CHACON-DIAZ C ET AL: "The use of green fluorescent protein as a marker for Brucella vaccines", VACCINE, ELSEVIER, AMSTERDAM, NL, vol. 29, no. 3, 10 January 2011 (2011-01-10), pages 577-582, XP027575663, ISSN: 0264-410X [retrieved on 2010-11-04] cited in the application abstract	4-10

Y	IGNACIO MORIYÓN ET AL: "Rough vaccines in animal brucellosis: Structural and genetic basis and present status", VETERINARY RESEARCH., vol. 35, no. 1, 1 January 2004 (2004-01-01), pages 1-38, XP055347505, NL ISSN: 0928-4249, DOI: 10.1051/vetres:2003037 abstract	1-10

	-/--	

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ADONE R ET AL: "Evaluation of Brucella melitensis B115 as rough-phenotype vaccine against B. melitensis and B. ovis infections", VACCINE, ELSEVIER, AMSTERDAM, NL, vol. 26, no. 38, 8 September 2008 (2008-09-08), pages 4913-4917, XP024340969, ISSN: 0264-410X, DOI: 10.1016/J.VACCINE.2008.07.030 [retrieved on 2008-08-15] abstract -----	1-10
A	P. M. MUNOZ ET AL: "Efficacy of Several Serological Tests and Antigens for Diagnosis of Bovine Brucellosis in the Presence of False-Positive Serological Results Due to Yersinia enterocolitica O:9", CLINICAL AND VACCINE IMMUNOLOGY, vol. 12, no. 1, 1 January 2005 (2005-01-01), pages 141-151, XP055466844, US ISSN: 1556-6811, DOI: 10.1128/CDLI.12.1.141-151.2005 the whole document -----	1-10