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The mglA gene and its flanking regions in Brucella: The role of mglA in tolerance to hostile environments, Fe-metabolism and in vivo persistence

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Abstract

We previously demonstrated that a spontaneous smooth small-colony variant of Brucella abortus S19 is characterized by increased in vivo persistence and the differential expression of a gene predicted to encode a galactoside transport ATP binding protein (mglA). In order to further investigate the role of this gene in the context of its flanking regions, we analyzed the respective DNA sequences from the formerly described B. abortus S19 as well as from avirulent B. neotomae 5K33 and compared these with published data from other Brucella species. Deletion mutagenesis of mglA in the large-colony variant of B. abortus S19 resulted in increased tolerance of the deletion mutant to a hyperosmotic (toxic), galactose-containing medium as well as to oxidative stress (H₂O₂). Whilst the deletion mutant is characterized by reduced growth on solid Fe³⁺-containing minimal medium (small-colony morphology), in vivo studies in mice demonstrated statistical significant differences in the bacterial load of spleens in the pre-immune, but not in the late phase of the infection.

Introduction

Bacteria of the genus Brucella cause chronic infections in both humans and a variety of animal species. We previously characterized a spontaneous smooth small-colony variant of B. abortus S19 which demonstrates not only a reduced growth rate in vitro, but, most importantly, also a less effective clearance from spleens and livers of experimentally infected mice (Hort et al., 2003). Using a differential approach to analyze mRNA-derived cDNA, we identified a molecular difference in the transcription of a gene predicted to encode for a formerly described galactoside transport ATP binding protein, mglA, in the small-colony variant (Jacob et al., 2006).

In order to further characterize the genetic basis of (adoptive) tolerance of Brucella to host immune responses, we analyzed the mglA gene and its flanking regions by means of PCR-assisted DNA
sequencing in B. abortus S19 and the avirulent B. neotomae 5K33. The comparison of these data with published mglA sequences of B. melitensis 16M (DelVecchio et al., 2002), B. abortus 2308, B. abortus 9–941 (Halling et al., 2005), B. suis (Paulsen et al., 2002), B. canis (Wattam et al., 2009), B. ovis (Wattam et al., 2009), Brucella sp. 83/13 (Broad Institute of MIT and Harvard, Cambridge, USA, http://www.broad.mit.edu/annotation/genome/brucella_group/GenomeDescriptions.html#Brucella_sp_83_13_v1), and B. microti CCM 4915 (Audic et al., 2009) revealed various differences within these species.

To substantiate the assumption of a role of mglA in pathogenesis and tolerance to hostile environments, we generated a mglA deletion mutant of the formerly described (Jacob et al., 2006) large-colony variant of B. abortus S19. The behaviour of the mutant was characterized by assessing its growth rate on galactose and Fe\(^{3+}\)-containing minimal media and the tolerance to both a hyperosmotic medium and oxidative stress. Moreover, the BALBc mouse model was used to test for in vivo behaviour.

**Material and methods**

**Bacterial strains**

B. abortus 2308, B. abortus S19, B. neotomae 5K33 (Stoener and Lackman, 1957) as well as B. microti CCM 4915 (Scholz et al., 2008) were grown in trypticase soy broth (TSB) as described (Hort et al., 2003) and were used for chromosomal DNA preparation according to the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany). Minimal basic medium (MBM) is a modification of Triple Sugar Iron (TSI) available from OXOID Ltd (Basingstoke, UK, Cat. no. CM 277).

**Molecular biological techniques**

PCR was used to create overlapping PCR products of the following target genes of Brucella: nirK, nirV, nrrA, periplasmic protein precursor, mglA, and permease protein (Fig. 1). That was performed using an UNO-Thermoblock (Biometra, Göttingen, Germany). The PCR fragments were analyzed by gel electrophoresis in 1% BIO-RAD Ready-agarose TBE mini gel (BIO RAD Cat. no. 161–3010, 1 V/cm, 24 h). The cycling conditions and the complete list of primers are available on request. PCR products were sequenced with the ABI PRISM 3100 Genetic Analyzer using the PCR Cycle sequencing Big-DYE (V. 3.0) Terminator Protocol (PE Applied Biosystems, Foster City, CA, USA). The sequences were exported to and assembled, with respect to primary DNA sequence comparisons, using Sequencher (V. 4.6) DNA analysis software (Gene Code Corp., Ann Arbor, MI, USA). More detailed analysis and primer selection was done by means of MacVector Software (V. 10.6.0, Accelrys Inc., USA).
Deletion mutagenesis

For deletion mutagenesis, a PCR fragment containing mglA was cloned into pJQ200mp18 (Quandt and Hynes, 1993). The resulting plasmid p10/29 was then digested by Mv1269I/Pagl yielding p10/29 which contained Δ mglA, characterized by a deletion of 460–652 bp with respect to the intact mglA.

Then, p10/29 (Δ mglA) was mobilized into B. abortus S19A strain (the large-colony variant) from E. coli strain S17-1(-pir) (Simon et al., 1983) by conjugation on Brain Heart Agar (BHA) for 2 days at 37 °C (5% CO₂ vol.). Transconjugants were selected on BHA supplemented with nalidixic acid (5 μg/ml), polymixin-B (5 IU/ml), and gentamicin (50 μg/ml). Single colonies growing on this medium were subcultured on BHA + antibiotics with and without 5% sucrose.

Gentamicin-resistant colonies were selected, DNA extracted (High Pure PCR Template Preparation Kit, Roche) and analyzed by PCR using mglA-specific primers to confirm the deletion of the internal Mv1269I/Pagl fragment in mglA (Fig. 2).

In vitro tolerance assays

In vitro tolerance assays were performed in ‘F1-medium’ consisting of 17 g casein peptone, 3 g soybean meal peptone, 2 g yeast extract, 49 g potassium acetate, 9 g M9 minimal media salt A, and 1.7 g M9 minimal media salt B (MP Biomedicals, Illkirch, France) per litre. The medium was adjusted to pH 7.0 and subsequently autoclaved. Finally, the sugar content was adjusted to be 10 mM by means of addition of sterile filtered galactose solution. 1 × 10⁹ viable cells of each B. abortus 2308, B. abortus S19, B. neotomae 5K33, and B. abortus S19 A Δ-mglA 3.14 were used to yield an initial experimental inoculum of 1 × 10⁷ cells/ml. Experiments were performed at 37 °C under an oxygen-depleted and CO₂-enriched atmosphere in an anaerobic jar (Anaerocult C, Merck, Darmstadt, Germany). Survival of cells was determined by taking aliquots of 100 μl each and plating on trypticase soy agar (TSA) on days 1, 3, 6, 8, and 10 (Fig. 5).

In another set of experiments, H₂O₂ in concentrations of (0.6, 0.3, 0.15, 0.075% each) was added to RPMI 1640 medium to test tolerance to oxygenic stress.

Modified triple sugar iron agar (TSI, No. 227, Oxoid, Germany) was used to test for growth on this medium containing 1.8 g/l (10 mM) galactose and/or Fe³⁺ at 37 °C under both aerobic and microaerobic conditions. Fig. 4 shows Brucella which were grown aerobically only.

In vivo behaviour of bacterial strains

Female, 10–12 week-old BALBc mice were used for all experiments. They were raised in the breeding facilities of Charles River WIGA (Sulzfeld, Germany) under specified pathogen-free conditions. The experiments were performed in the L3 facilities of the Robert Koch-Institute using an IsoCage-System (Tecniplast, Hohenpeißenberg, Germany). Mice were infected with 5 × 10⁶ viable Brucella each in 0.1 ml PBS. Subsequently, on the days 7, 21, and 45, defined groups of 5, 5, and 8 animals,
respectively, were sacrificed. Spleens were removed and weighed. Numbers of viable bacteria per spleen were determined by serial dilutions plated on TSA agar. For statistical analysis, Mann–Whitney test was performed by grouping bacterial counts for both strains A (wild type, B. abortus S19) and M (Δ-mglA mutant strain, B. abortus S19 A Δ-mglA 3.14) obtained on days 7, 21, and 45 of the mouse experiment.

Immunohistology and cytology

On days 7, 21, and 45 after infection, the mice were killed and the corresponding parts of spleens were removed and immersed in O.C.T. compound (Tissue Tek, Bakura, USA), and kept at −20 °C for histologic evaluation. Cryostat sections were fixed in acetone, treated with the respective primary antibody, anti-Brucella LPS (Difco, Detroit, MA, USA; 1:100 dil.), and diluted in PBS plus 10% FCS for 45 min at room temperature in humidified air. Incubation ensued with the secondary antibody, a peroxidase-conjugated mouse anti-rabbit IgG (Dianova, Hamburg, Germany) diluted 1:100 for anti-Brucella, respectively (45 min, room temperature, humidified air). Slides were covered with 100 μl of the freshly prepared 3,3’-diaminobenzidine chromogen-substrate solution (Vector, Burlingame, CA, USA) containing 0.06% Tris buffer, 0.03% H₂O₂, at pH 7.6 for approximately 3 min. Finally, the sections were washed, counterstained with hematoxylin, and dehydrated.

Results

Differences in the mglA gene and its flanking regions in the genus Brucella

In order to further investigate formerly obtained data about mglA-related differences in B. abortus S19 variants (Jacob et al., 2006), DNA in the respective target region of chromosome II from B. abortus S19 as well as from avirulent B. neotomae 5K33 and B. microti CCM 4915 was amplified and sequenced. The data for B. abortus S19 and B. neotomae 5K33 were deposited at GenBank (EU402949 and EU410469) and compared with corresponding data from publicly available sequences from B. abortus 2308 (Chain et al., 2005), B. abortus 9–941 (Halling et al., 2005), B. abortus S19 (Crasta et al., 2008), B. melitensis 16M (DelVecchio et al., 2002), B. suis (Paulsen et al., 2002), B. ovis (Wattam et al., 2009), B. canis (Wattam et al., 2009), Brucella sp. 83/13 (http://patricbrc.vbi.vt.edu/portal/portal/patric/Taxon?cType=taxon&cld=520449), B. neotomae 5K33 (http://patricbrc.vbi.vt.edu/portal/portal/patric/Taxon?cType=taxon&cld=520456) as well as with the data obtained by sequencing of the respective genes in B. microti CCM 4915, later confirmed by Audic et al. (2009).

For further comparison, the flanking genes BMEII0981 (permease), BMEII0983 (periplasmic protein precursor), BMEII0985 (operon repressor), and BMEII0986 (transcriptional regulator, nrrA), as well as BMEII0987 (nirV), and BMEII0988 (nitrate reductase precursor, nirK) were also taken into consideration. The B. melitensis 16M genome was used as a reference.
The mglA-DNA sequence of the investigated B. abortus S19 strain was identical to the published sequence of B. abortus 9–941 while in B. abortus 2308 mglA is truncated at amino acid (AA) position 236 (Fig. 3) (Chain et al., 2005). The comparison with other Brucella biovars/species revealed that B. neotomae, B. ovis, and B. microti show the most complete sequences of the mglA gene while B. abortus, including strain B. abortus S19, B. melitensis, B. canis, and B. suis are characterized by nucleotide deletions, resulting in the loss or exchange of amino acids at various positions (Fig. 3). In the newly identified species Brucella sp. 83/13 (http://patricbrc.vbi.vt.edu/portal/portal/patric/Taxon?cType=taxon&cId=520449), mglA as well as its flanking genes (operon repressor, periplasmic protein precursor, and permease) are missing (Fig. 1, Table 1).

However, in contrast to the complete mglA gene, the avirulent B. neotomae 5K33 demonstrates a large-scale nucleotide deletion of 2.2 kb including a partial deletion of the genes BMEII0986 and BMII0988, respectively, and a complete deletion of BMEII0987 (Baek et al., 2004) resulting in the loss of putative regulator genes (nnrA, nirK, nirV) (Fig. 1, Table 1).

**Deletion mutagenesis of mglA**

To further investigate the potential role of mglA, we generated a Δ-mglA deletion mutant of the large variant of B. abortus S19 A by allelic gene replacement (Campos et al., 2002, Quandt and Hynes, 1993 and Pelicic et al., 1996). Successful deletion was confirmed by both PCR (Fig. 2) and DNA sequencing (data not shown). In Fig. 2, lanes 2, 3, and 5 demonstrate the Δ-mglA-specific PCR fragment of 1.4 kb size as a result of gene exchange, as compared to the parental strain in lanes 1, 4, and 6 (1.6 kb).

**Behaviour on Fe³⁺-containing minimal medium and tolerance to oxidative stress**

Our original investigation (Jacob et al., 2006) was initiated by the observation of a reduced growth rate of a small-colony variant of B. abortus S19. We therefore characterized the growth of B. abortus S19 A Δ-mglA 3.14 on defined galactose and/or Fe³⁺-containing minimal media. On these media, B. abortus S19 A Δ-mglA 3.14 showed a reduced growth (i.e. colony size) whenever Fe³⁺ was present in the medium (Fig. 4). In addition, B. abortus S19 A Δ-mglA 3.14 was characterized by increased tolerance to H₂O₂ (0.3% versus 0.085%, Table 2) and a higher tolerance to a galactose-containing hyperosmotic (i.e. toxic) medium (F1) (Fig. 5).

**In vivo behaviour**

The investigation of the in vivo behaviour of the Δ-mglA mutant mainly focused on differences in the persistence of the bacteria in the late phase of infection (Hort et al., 2003). Mice were infected with 5 × 10⁶ viable bacteria of B. abortus S19A and its isogenic mglA deletion mutant B. abortus S19 A Δ-mglA 3.14, and the bacterial load was determined in spleens on days 7, 21, and 45, respectively. While the bacterial load was similar on day 45 after infection, the infection with the mglA deletion
mutant resulted in a slight, but significantly higher bacterial load on day 7 after infection which represents the pre-immune phase of the infection (Fig. 6).

**Immunohistology and cytology**

In order to further investigate the differences in bacterial load on day 7 and day 21, spleens of infected mice were investigated by immunohistology using a Brucella-LPS-specific antibody (Hort et al., 2003). Histological alterations in spleens of Brucella-infected mice have been described earlier (Hort et al., 2003) and did not differ significantly between the groups investigated in this study (data not shown).

**Discussion**

The persistence of bacteria in chronic infections like brucellosis may be due to (i) inadequate host responses or (ii) a specific ability of the invader to tolerate (or evade) usually effective defence mechanisms. In addition, Brucella-induced inflammatory responses in the spleens of infected animals associated with changes in the eradication kinetics of Brucella have been described (Hort et al., 2003). In a previous paper, we demonstrated that in B. abortus S19 a gene predicted to encode for a formerly described galactoside transport ATP binding protein (mglA) is differentially expressed in small- versus large-colony variants which differ in vivo persistence (Jacob et al., 2006). In an attempt to further investigate the potential role of this gene locus, we analyzed mglA and its flanking regions in various members of the genus Brucella.

Sequencing of the mglA gene of both, the large- and the small-colony variants of strain B. abortus S19 revealed no differences on the DNA level when compared to the homologous genes of B. abortus biovar 1 strain 9–941. Compared to the attenuated vaccine strain B. abortus S19, virulent B. abortus 2308, however, demonstrates an additional deletion in the mglA gene resulting in the loss of 42 amino acids at the C-terminal end at position 236 ff (Fig. 3). In B. melitensis, a specific difference in the mglA gene is present at amino acid position 199–205 while in B. suis and B. canis, there is a deletion of amino acids at position 214/215.

Additional differences in mglA and its flanking regions became prominent when compared to B. neotomae and other members of the genus Brucella like B. ovis, B. microti, and Brucella sp. 83/13. The B. microti mglA sequence which we obtained was identical to the one from a B. microti whole-genome sequencing project (Audic et al., 2009) and, therefore, we did not submit our own results to NCBI.

Data obtained from the investigation of the flanking regions of mglA, especially in putative regulatory genes (Fig. 1, Table 1), revealed that the sequences of B. microti CCM 4915 (Audic et al., 2009) and B. ovis are similar, not only with respect to mglA, but also to the flanking regions. In this context, B. ovis is of special interest because of its narrow host range and tissue tropism (Tsolis et al., 2009), while possessing the whole mgl operon.
In summary, B. neotomae, B. ovis, and B. microti, all characterized by reduced pathogenicity, have the most complete mglA gene sequence, while B. melitensis, B. suis, B. canis, and B. abortus are characterized by specific deletions. Taken together with differences in the flanking regions, this gene locus may thus be interesting not only for diagnostic purposes (Table 1), but also with respect to virulence and pathogenesis. So far, the most relevant difference between the virulent and chronically persisting strain B. abortus 2308 and the vaccine strain B. abortus S19 has only been described for erythritol-catabolism (Crasta et al., 2008 and Sangari et al., 2000). Most interestingly, the differences in the mglA genome region enable similar conclusions as the results of a phylogenetic ‘clade’ system, based on whole-genome comparisons (Wattam et al., 2009), which describes B. ovis as an ‘ancestor’ from which other Brucella species may have diverged from.

The differences in the mglA sequences within the genus Brucella and the loss of regulatory genes as exemplified in B. neotomae 5K33 (Fig. 1 and Fig. 3, Table 1) can be interpreted as a result of ongoing genetic rearrangements in this region of chromosome II. Such mechanisms have been described as a cause for the generation of strain and species-specific differences within the genus Brucella (Chain et al., 2005, Rajashekara et al., 2005 and Tsoktouridis et al., 2003).

With respect to functional consequences of differences in this gene region, it is worth mentioning that a gene in immediate neighbourhood and functionally related to mglA, the D-galactose-binding periplasmic protein precursor (BMEII0983), has been found to be underexpressed in the attenuated vaccine strain B. melitensis Rev1 (Eschenbrenner et al., 2002) and recently in B. abortus 2308 (Lamontagne et al., 2009). Furthermore, the gene nrrA (BMEII0986), which is an immediate neighbour of the operon and potentially involved in its regulation (Fig. 1), has been demonstrated as a virulence factor (Haine et al., 2006). The absence of nrrA (BMEII0986) in the avirulent B. neotomae 5K33 (Baek et al., 2004) has now been confirmed in the context of this study (GenBank acc.no. EU410469). However, in the initial paper (Baek et al., 2004) describing the importance of nrrA, nirV, and nirK for virulence, no data were presented for mglA. Data from hybridization studies of the virulent B. melitensis and the avirulent B. neotomae demonstrate only 2 significant differences in the genome between these organisms (Baek et al., 2004). One is the region analyzed in more detail here, the other one is an additional, possibly Rhizobium-borne gene region (Rajashekara et al., 2004).

There are more hints that the region around mglA may be involved in Brucella pathogenicity as the genome region upstream of mglA has just been shown by means of DNA microarray as to be poorly expressed under laboratory conditions in B. abortus 2308 (Viadas et al., 2009) (BAB2_0941, 0942, 0943). In addition, gene norD, upstream of the operon repressor, has also been described to be involved in virulence of B. suis (Loisel-Meyer et al., 2006).

In B. suis 1330, the gene nosZ (BRA0275) was described by proteomics (Al Dahouk et al., 2009) as to be overexpressed during anaerobic survival.

In order to substantiate the assumption that mglA may be involved in the tolerance of Brucella to hostile environments, we generated an mglA deletion mutant of the large-colony type of B. abortus
S19 A (Jacob et al., 2006) and tested it in various in vitro and in vivo assays. The increased tolerance of the deletion mutant in a galactose-containing hyperosmotic medium (Fig. 5) as well as to oxidative stress (H$_2$O$_2$) (Table 2) may be taken as a hint that the region is involved in tolerance to adverse environmental conditions (Köhler et al., 2002 and Köhler et al., 2003).

Most interestingly, the reduced growth on Fe$^{3+}$-containing minimal media (Fig. 4) revealed an in vitro phenotype of the deletion mutant that has previously been described for a spontaneous small-colony variant of B. abortus S19 (Jacob et al., 2006).

Finally, in vivo experiments were performed in order to identify the effects of mglA deletion on in vivo persistence of Brucella. Whilst there was a significant difference in bacterial load of spleens in the pre-immune phase of the infection, both the vaccine strain S19 and the mglA-deletion mutant did not differ in the amount of bacteria present in the late phase of the infection, which is characterized by their residence in NO synthase-positive macrophages.

In conclusion, mglA and its flanking regions may be useful for diagnostic purposes. In addition, deletion mutagenesis of mglA resulted in a small-colony phenotype on Fe$^{3+}$-containing minimal medium.

The intracellular fate of various Brucella strains and mutants differing in the respective gene locus in the presence and absence of IFN-γ is the topic of forthcoming investigations.

**Acknowledgements**

B. neotomae 5K33 and B. microti CCM 4915 were both obtained from AFFSA (Maison-Alfort, France). Brucella sp. 83/13 was provided by VLA (Weybridge, UK).
References


**Tables and Figures**

**Figure 1.** mglA in the context of its flanking genes in chromosome II of B. melitensis 16M, B. abortus, B. neotomae, and Brucella sp. 83/13 (blue arrows indicate identical genes, red arrows indicate naturally deleted genes, slightly red arrows indicate partly deleted genes). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Figure 2. PCR-assisted mglA fragment length analysis of DNA from wild type and deletion mutants of B. abortus S19. Lanes 1, 4, 6: wild type; lanes 2, 3, 5: B. abortus S19 Δ-mglA 3.14 mutant.
Figure 3. Formatted alignment of amino acid sequences of mglA from B. neotomae 5K33, B. ovis ATCC25840 (BOV_A0241), B. microti CCM 4915, B. canis ATCC 23365 (BCAN_B0267), B. suis 1330 (BRA0266), B. melitensis 16M (BMEII0982), B. abortus 9–941 (BruAb2_0914), B. abortus 2308 (BAB2_0937), and B. abortus S19 (GenBank acc.no. EU402949).
Figure 4. Growth characteristics of B. abortus 2308, B. abortus S19A, and B. abortus S19A Δ-mglA 3.14 on galactose/Fe$^{3+}$-containing minimal medium under aerobic conditions. Equivalent numbers of B. abortus 2308, B. abortus S19, and the corresponding mglA deletion mutant (B. abortus S19 Δ-mglA 3.14) were grown on various defined media: (a) minimal basic medium (MBM), (b) minimal basic medium + 10 mmol galactose, (c) minimal basic medium + 1 mmol Fe$^{3+}$, (d) minimal basic medium + (galactose/Fe$^{3+}$), and (e) minimal basic medium + 10 mmol erythritol. Differences in erythritol metabolism have been described for both B. abortus 2308 and B. abortus S19 (Crasta et al., 2008 and Sangari et al., 2000).
Figure 5. Survival of B. abortus S19 A, B. abortus S19 A Δ-mglA 3.14, and B. neotomae 5K33 in hyperosmotic medium F1 with and without 10 mM galactose. A representative experiment out of 3 independent experiments using triplicates each is presented. Statistical analysis (Mann–Whitney U test) revealed that differences in growth kinetics between B. abortus S19 A and B. abortus S19 A Δ-mglA 3.14 were statistically significant on days 6 and 8 both in the presence of galactose (d6, p = 0.0003; d8, p = 0.0004) and without galactose in the medium (d6, p = 0.0006, d8, p < 0.0001).
Figure 6. Kinetics of bacterial eradication in spleens of BALBc mice infected i.v. with $5 \times 10^6$ B. abortus S19A resp. B. abortus S19A Δ-mglA 3.14 (mutant strain). On days 7, 21, and 45 after infection, mice were sacrificed, spleens were homogenized, and bacterial loads were determined by plating of serial dilutions on TSA. Statistical analysis (Mann–Whitney U test): (day 7, $p = 0.016$; day 21, $p = 0.032$).
**Table 1.** Differences in mglA and its flanking regions in the genus *Brucella*. AA, amino acid; p, position.

<table>
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<tr>
<th>Genes</th>
<th>Species</th>
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<tbody>
<tr>
<td>mglA</td>
<td>B. melitensis 16M (ATCC 23456)</td>
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<tr>
<td>nrrA/nirK/nirV</td>
<td>+</td>
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**Table 2.** Tolerance of *B. abortus* 2308, *B. abortus* S19, and *B. abortus* S19 ΔmglA 3.14 to H$_2$O$_2$. The results show the H$_2$O$_2$ concentrations tolerated by the respective strain in the medium.

<table>
<thead>
<tr>
<th>Tolerance to H$_2$O$_2$</th>
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<tr>
<td><em>B. abortus</em> 2308</td>
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<tr>
<td><em>B. abortus</em> S19 Gr. A</td>
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<tr>
<td><em>B. abortus</em> S19 ΔmglA</td>
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