# Polymorphisms in *Brucella* Carbonic Anhydrase II Mediate $CO_2$ Dependence and Fitness *in vivo*

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Some *Brucella* isolates are known to require an increased concentration of $CO_2$ for growth, especially in the case of primary cultures obtained directly from infected animals. Moreover, the different *Brucella* species and biovars show a characteristic pattern of $CO_2$ requirement, and this trait has been included among the routine typing tests used for species and biovar differentiation. By comparing the differences in gene content among different $CO_2$-dependent and $CO_2$-independent *Brucella* strains, we have confirmed that carbonic anhydrase (CA) II is the enzyme responsible for this phenotype in all the *Brucella* strains tested. *Brucella* species contain two CAs of the β family, CA I and CA II; genetic polymorphisms exist for both of them in different isolates, but only those putatively affecting the activity of CA II correlate with the $CO_2$ requirement of the corresponding isolate. Analysis of these polymorphisms does not allow the determination of CA I functionality, while the polymorphisms in CA II consist of small deletions that cause a frameshift that changes the C-terminus of the protein, probably affecting its dimerization status, essential for the activity. $CO_2$-independent mutants arise easily *in vitro*, although with a low frequency ranging from $10^{-6}$ to $10^{-10}$ depending on the strain. These mutants carry compensatory mutations that produce a full-length CA II. At the same time, no change was observed in the sequence coding for CA I. A competitive index assay designed to evaluate the fitness of a $CO_2$-dependent strain compared to its corresponding $CO_2$-independent strain revealed that while there is no significant difference when the bacteria are grown in culture plates, growth *in vivo* in a mouse model of infection provides a significant advantage to the $CO_2$-dependent strain. This could explain why some *Brucella* isolates are $CO_2$ dependent in primary isolation. The polymorphism described here also allows the *in silico* determination of the $CO_2$ requirement status of any *Brucella* strain.

Keywords: *Brucella*, carbonic anhydrase, $CO_2$ requirement, fitness, protein structure

## INTRODUCTION

*Brucella* species are facultative intracellular Gram-negative coccobacilli that cause brucellosis, the most prevalent zoonosis with more than 500,000 human cases reported worldwide every year (Pappas et al., 2006). *Brucella* isolates are routinely identified and classified by biochemical and phenotypical characteristics like urease activity, $CO_2$ dependence, $H_2S$ production, erythritol and

dye sensitivity, lysis by *Brucella*-specific bacteriophages, agglutination with monospecific sera, or even host preference (Alton et al., 1988). The first observations pertaining to *Brucella* and $CO_2$ were made by Nowak in 1908 (Duncan, 1928), who noticed that *Brucella abortus* was more easily isolated from the host tissues when the concentration of oxygen in the atmosphere was reduced, but it was Wilson (1931) who established the requirement of $CO_2$ for growth in these isolates. This requirement is not universal within *Brucellaceae*, and the different species and biovars show a characteristic pattern of $CO_2$ dependence. Within the classical species, *B. abortus* biovars 1, 2, 3, and 4 and some isolates from biovar 9, as well as *Brucella ovis*, require an increased concentration of $CO_2$ for growth, especially in the case of primary cultures obtained directly from infected animals. Within the more recently described species, most strains of *Brucella pinnipedialis* require supplementary $CO_2$ for growth, and most of *Brucella ceti* do not (Foster et al., 1996). The $CO_2$ dependence may be lost by subculturing *in vitro*, with an estimated frequency of $3 \times 10^{-10}$ per cell division (Marr and Wilson, 1950), and this is what happened with well-known laboratory *B. abortus* biovar 1 strains like 2308 or S19, which grow in ambient air.

Facultative intracellular bacteria face two environmental conditions with very dissimilar concentrations of carbon dioxide ($CO_2$). Inside mammalian cells, $CO_2$ concentration may be as high as 5%, while atmospheric concentration is currently estimated at 0.04%. $CO_2$ and bicarbonate ($HCO_3^-$) are essential growth factors for bacteria, and they can be interconverted spontaneously at significant rates (Gladstone et al., 1935; Smith et al., 1999). The reversible hydration of $CO_2$ into $HCO_3^-$ can also be catalyzed by carbonic anhydrase (CA), a ubiquitous metalloenzyme fundamental to many biological functions including photosynthesis, respiration, and $CO_2$ and ion transport. The CA superfamily (CAs, EC 4.2.1.1) has been found in all the three domains of life (Eubacteria, Archaea, and Eukarya), and it currently includes seven known families (α-, β-, γ-, δ-, ζ-, η-, and θ-CAs) of distinct evolutionary origin (Supuran, 2018). The conversion of $CO_2$ into $HCO_3^-$ is accelerated in the presence of CA and has the effect of ensuring correct $CO_2$ concentration for carboxylating enzymes involved in central, amino acid, and nucleotide metabolism (Merlin et al., 2003).

Carbonic anhydrase has been shown to be required to support growth under ambient air in a number of microorganisms like *Ralstonia eutropha* (Kusian et al., 2002), *Escherichia coli* (Hashimoto and Kato, 2003; Merlin et al., 2003), *Corynebacterium glutamicum* (Mitsuhashi et al., 2004), and *Saccharomyces cerevisiae* (Aguilera et al., 2005). Growth of CA mutants of these organisms was only possible under an atmosphere with high levels of $CO_2$, a phenomenon that is explained by the availability of bicarbonate, which is a substrate for various carboxylation reactions of physiological importance. These reactions are catalyzed by several housekeeping enzymes, like 5′-phosphoribosyl-5-amino-4-imidazole carboxylase (EC 4.1.1.21), phosphoenolpyruvate carboxylase (EC 4.1.1.31), carbamoyl phosphate synthetase (EC 6.3.4.16), pyruvate carboxylase (EC 6.4.1.1), and acetyl-CoA carboxylase (EC 6.4.1.2). They catalyze key steps of pathways for the biosynthesis

of not only physiologically essential but also industrially useful metabolites, such as amino acids, nucleotides, and fatty acids (Mitsuhashi et al., 2004). A role for CA in the intracellular pH regulation has also been demonstrated in some bacteria (Marcus et al., 2005).

*Brucella* species contain two different β-CAs, first identified in *Brucella suis* 1330 and thus named $_{Bs1330}$CAI and $_{Bs1330}$CAII. Both CAs contain the amino acid residues involved in the binding of the Zn ion (typical of the β family of CAs), as well as those involved in the catalytic site. Their activity has been verified *in vitro*, and it is slightly higher in $_{Bs1330}$CAII than in $_{Bs1330}$CAI (Joseph et al., 2010, 2011). Pérez-Etayo et al. (2018) compared CA I and CA II activity (activity defined empirically as that allowing growth in a normal atmosphere, the same definition used throughout this study) in several strains of *B. suis*, *B. abortus*, and *B. ovis* and determined that CA II is not functional in $CO_2$-dependent *B. abortus* and *B. ovis*, thus establishing a correlation between CA activity and $CO_2$ dependence. They also observed that CA I is active in *B. suis* 1330 or 513, but not in *B. abortus* 2308W, 292, and 544. Moreover, although an active CA I alone is enough to support $CO_2$-independent growth of *B. suis* in rich media, it is not able to do it in minimal media or to support $CO_2$-independent growth of *B. abortus* at all. A similar result was also obtained by Varesio et al. (2019) that identified BcaA$_{BOV}$ (CA II) as the enzyme responsible for the growth of *B. ovis* in a standard, unsupplemented atmosphere (0.04% $CO_2$), in this case, by whole-genome sequencing (WGS) of $CO_2$-independent mutants. Interestingly, they also reported that a $CO_2$ downshift *B. ovis* initiates a gene expression program that resembles the stringent response and results in transcriptional activation of its type IV secretion system. This shift is absent in *B. ovis* strains carrying a functional copy of CA.

The classical biotyping mentioned above, despite its limitations and the emergence of new molecular approaches to identify and classify *Brucella* at different taxonomic levels, is still extensively used by reference laboratories, often side by side with the molecular methods (Garin-Bastuji et al., 2014). However, although there is a known link between phenotype and its genetic cause in some traits like urease activity or erythritol sensitivity (Sangari et al., 1994, 2007, 2010), there is still a gap between the information provided by the molecular methods and the phenotype of *Brucella* isolates. With the availability of more genome sequences, it should be possible to reduce this gap by comparing the phenotypic characteristics of *Brucella* strains with their genome content. Comparative genomics of whole-genome sequences is especially interesting in bacterial pathogenesis studies (Hu et al., 2011). Pathogenomics can be considered as a particular case of comparative genomics, and it has been extensively used for the identification of putative virulence factors in bacteria, by comparing virulent and avirulent isolates (Pallen and Wren, 2007), although in principle it could be applied to the elucidation of any phenotypic trait. The genus *Brucella* is a very homogeneous one, with over 90% identity on the basis of DNA–DNA hybridization assays within the classical species, and this results in relatively minor genetic variation between species that sometimes result in striking differences. As an example, only 253 single-nucleotide polymorphisms

(SNPs) separate *Brucella canis* from its nearest *B. suis* neighbor (Foster et al., 2009), but their host specificity differs widely, while *B. canis* is almost entirely restricted to the Canidae family, *B. suis* has a wide host range that includes pigs, dogs, rodents, hares, horses, reindeer, musk oxen, wild carnivores, and humans. Similarly, there are only 39 SNPs consistently different between the vaccine strain *B. abortus* S19 and strains *B. abortus* 9-941 and 2308, two well-known virulent isolates (Crasta et al., 2008). In the last years, a large number of *Brucella* genomes representing all species and biovars have been sequenced, and all this wealth of information is already resulting in new molecular epidemiology and typing methods (O'Callaghan and Whatmore, 2011). We have tested the potential of pathogenomics to unveil phenotypic traits in *Brucella* by defining the pangenome/pseudogenes of a set of *Brucella* strains and comparing it with the $CO_2$ dependence of those strains. This process has allowed us to identify CA II as the enzyme responsible for growth of the bacteria at atmospheric $CO_2$ concentrations and extend the analysis to new species of *Brucella*. All the sequenced genomes of *Brucella* contain two β-CA genes, but only those that carry a defective β-CA II require supplemental $CO_2$. Reversion of this phenotype happens *in vitro* at a low frequency and is accompanied by a compensatory mutation that results in a full-length β-CA II product. We have also tested the hypothesis that the presence of a truncated β-CA II would have a competitive advantage *in vivo*, as a way to explain why a mutation with such a low frequency could get fixed in some *Brucella* species and biovars. A competitive assay shows that one of such mutants is significantly enriched in a mouse model of infection when compared with its corresponding full-length β-CA II strain. This could explain why $CO_2$-dependent strains are selected *in vivo*. The polymorphisms affecting β-CA II encoding genes allow the prediction of the $CO_2$ dependence status of any given strain, thus having the potential to replace the classical assay to characterize *Brucella* isolates.

## MATERIALS AND METHODS

### Bacterial Strains and Growth Conditions

The bacterial strains and plasmids used in this work are listed in **Table 1**. *Brucella* strains were grown at 37°C for 48–96 h in a 5% $CO_2$ atmosphere in *Brucella* broth (BB) or agar (BA) medium (Pronadisa, Spain). Media were supplemented with 10% fetal bovine serum (FBS) to grow *B. ovis*. All experiments with live *Brucella* were performed in a Biosafety Level 3 (BSL3) facility at the Department of Molecular Biology of the University of Cantabria, and animal infections with *Brucella* were conducted at the University of Cantabria animal facilities, also under BSL3 conditions.

### Bioinformatic Methods

Genomic and protein sequences of the different *Brucella* species were obtained from GenBank and the Broad Institute[1]. To allow easy comparison between the genes and pseudogenes in the different *Brucella* species, we constructed the panproteome of

[1]https://www.broadinstitute.org/projects/brucella

**TABLE 1 |** Strains and plasmids used in this study.

| Strains | Main characteristics | References |
|---|---|---|
| ***Brucella*** | | |
| *Brucella abortus* 544 | Biotype 1, $CO_2$ dependent | Alton et al. (1988) |
| *B. abortus* 86/8/59 | Biotype 2, $CO_2$ dependent | Alton et al. (1988) |
| *B. abortus* Tulya | Biotype 3, $CO_2$ dependent | Alton et al. (1988) |
| *B. abortus* 292 | Biotype 4, $CO_2$ dependent | Alton et al. (1988) |
| *B. abortus* A-579 | Biotype 3, $CO_2$ dependent | Alton et al. (1988) |
| *B. abortus* 2308W | Biotype 1, $CO_2$ independent | Suárez-Esquivel et al. (2016) |
| *Brucella ovis* 63/290 | $CO_2$ dependent | Alton et al. (1988) |
| *Brucella pinnipedialis* B2/94 | $CO_2$ dependent | Foster et al. (1996) |
| *B. abortus* 2308 $\Delta_{Ba2308w}CAI$ mutant | $CO_2$ independent | This study |
| *B. abortus* 292mut1 | $CO_2$ independent | This study |
| ***Plasmids*** | | |
| pDS132 | Suicide mobilizable plasmid | Philippe et al. (2004) |
| pFJS267 | pDS132:$\Delta_{Ba2308w}$CA | This study |

a selected set of 10 strains with the most complete genome annotation at that time (**Supplementary Table S1**). To construct this set, we started with all the coding sequences (CDSs) annotated in the *B. suis* 1330 genome. Next, we found the most probable functional counterparts for the *n* pseudogenes annotated in *B. suis* 1330. The pseudogene list was taken directly from the original annotation of the *B. suis* 1330 genome. Finally, we added those CDSs in indels from the other genomes not present in *B. suis* 1330. We assigned a new gene name to every CDS in our set following the Bru1_xxxx and Bru2_xxxx nomenclature, depending on the location of the gene in the *B. suis* genome. CDSs from indels were also renamed with a nomenclature, BRU1_iXXXX, the "i" indicating their origin from indels absent in *B. suis* 1330. The file pan_pep provided in the **Supplementary Material** is a multifasta protein file containing the sequence of all 3,496 CDSs present at least once in any of the used genomes and constitutes the first version of the *Brucella* panproteome. The genes and pseudogenes annotated in these genomes were tabulated and assigned to one of the different gene families present in those genomes. In this way, we constructed a spreadsheet with the pseudogenes in each genome using a uniform nomenclature. The analysis of the CA sequences at both the DNA and protein levels was extended to a group of 35 *Brucella* genomes (**Supplementary Table S2**).

A structural theoretical model of *Brucella* $_{Ba2308}$CAII was generated by molecular threading using the protein homology and recognition engine Phyre2 (Kelley et al., 2015), taking the atomic coordinates of the best hit as template. The pdb model generated was visualized using the PyMOL Molecular Graphics System, version 1.3 (Schrödinger, LLC, Portland, OR, United States).

Primers used in this study (**Table 2**) were designed with Primer 3[2] and synthesized by Sigma-Aldrich.

[2]http://bioinfo.ut.ee/primer3-0.4.0/

**TABLE 2** | Oligonucleotides used in this work.

| | |
|---|---|
| BS192_0456.F | CATGCTGGAACCAAAGTTGA |
| BS192_0456.R | CGTTTCCGCAAGCTGTAAAT |
| BS191_1911.F | GGGTTCCACAGGGTTCATTT |
| BS191_1911.R | GACGGCAAATAATTGCATGA |
| U_BAB2_0449. *Sac*I.F | GAGCTCTGAGCACAACCTGCAAAATC |
| U_BAB2_0449.R | GGCGAATGATCGTTCTTCAT |
| D_BAB2_0449.F | ATGAAGAACGATCATTCGCCGGATCCTGCCAAC CGGCAGAATAAT |
| D_BAB2_0449. *Xba*I.R | TCTAGAGGGGGAGGCTTCCTAGTTT |

## Isolation of $CO_2$-Independent Mutants in $CO_2$-Dependent *Brucella* Strains

Different $CO_2$-dependent *Brucella* strains from our collection were streaked onto BA plates and grown in a 5% $CO_2$ atmosphere. Individual colonies were then re-streaked in duplicate plates and incubated at 5% $CO_2$ and ambient atmosphere to check for the correct $CO_2$ dependence phenotype. They were grown as a lawn in fresh BA plates, and the growth was resuspended in phosphate-buffered saline (PBS). The suspension was serially diluted, and each dilution seeded in duplicate in BA plates. One dilution series was incubated at 5% $CO_2$ to enumerate the number of bacteria in the inoculum, while the second was incubated at ambient atmosphere to select for $CO_2$-independent colonies. The mutation rate was expressed as number of mutants per number of initial bacteria. Individual mutants were selected, and genomic DNA was obtained by using the InstaGene matrix as described by the supplier (Bio-Rad Laboratories, United Kingdom). *CA I* and *CA II* complete sequences from the different strains were amplified by PCR with oligonucleotides BS192_0456.F/R and BS191_1911.F/R, respectively, and sequenced to determine if there was any change compared to the corresponding parental sequence.

## Infection and Intracellular Viability Assay of *Brucella abortus* in J774 Cells

J774.A1 macrophage-like cells [American Type Culture Collection (ATCC), TIB-67] were cultured in RPMI medium with 2 mM L-glutamine and 10% FBS at 37°C in 5% $CO_2$ and 100% humidity. Confluent monolayers were trypsinized, and $2 \times 10^5$ cells per well were incubated for 24 h before infection in 24-well tissue culture plates. Macrophages were infected with *Brucella* strains in triplicate wells at an MOI of 50. After infection for 30 min, the wells were washed five times with sterile PBS and further incubated for 30 min in RPMI with 2 mM L-glutamine, 10% FCS, and 50 μg gentamicin per milliliter to kill extracellular bacteria. That was taken as time 0 post infection, and the medium was changed to contain 10 μg gentamicin per milliliter. The number of intracellular viable *B. abortus* was determined at different time points by washing three times with PBS and lysing infected cells with 0.1% Triton X-100 in $H_2O$ and plating a series of 1:10 dilutions on BA plates for colony-forming unit (CFU) determination.

## Competitive Infection Assays

The following protocol was approved by the Cantabria University Institutional Laboratory Animal Care and Use Bioethics Committee and was carried out in accordance with the Declaration of Helsinki and the European Communities Council Directive (86/609/EEC). Comparison of fitness between $CO_2$-dependent and isogenic $CO_2$-independent strains was done through a competitive infection assay in order to minimize animal-to-animal variation. BALB/c mice (CIFRA, Spain) were injected with 1:1 mixtures of *B. abortus* 292 ($CO_2$ dependent, wild type) and *B. abortus* 292mut1 (a spontaneous $_{Ba292}CAII$ $CO_2$-independent mutant). Two hundred microliters of a suspension containing approximately $10^8$ bacteria was administered intraperitoneally to a group ($n = 6$) of 6- to 8-week-old female BALB/c mice. Mice were sacrified 8 weeks after infection, and the liver and spleen were removed aseptically and homogenized with 5 ml of BB containing 20% glycerol. Samples were serially diluted and plated in quadruplicate on BA plates. Half of the plates were incubated with 5% $CO_2$, and the other half at ambient atmosphere. Additionally, colonies grown at 5% $CO_2$ were replica-plated and incubated at both $CO_2$ concentrations, to measure the ratio of $CO_2$-dependent and $CO_2$-independent colonies in two independent ways. For *in vitro* competitive index (CI) assays, BA plates were seeded, forming a lawn with the infection mix, and incubated at 37°C with 5% $CO_2$ for 8 weeks, with repeated subculture in fresh BA plates every 4–5 days in the same conditions. The ratio of $CO_2$-dependent and $CO_2$-independent colonies was determined with the same protocol as the *in vivo* CI. The CI was calculated as the ratio of mutant to wild-type bacteria recovered at the end of the experiment divided by the ratio of mutant to wild-type bacteria in the inoculum, and the differences between groups were analyzed by Student's two-tailed *t* test with significance set at $P < 0.05$.

## RESULTS

## Identification of the Gene Responsible for the $CO_2$ Dependence in *Brucella abortus*

The first evidence of the involvement of CA in the $CO_2$ dependence phenotype came from the analysis of pseudogenes in the 10 fully annotated *Brucella* genomes (**Supplementary Table S1**). After tabulation of the pseudogenes, their presence along with the different species was compared with the target phenotype; in this particular case, we interrogated the spreadsheet n_pseudos.xls (**Supplementary Material**) to find out which genes are pseudogenes only in those strains in our list that are $CO_2$ dependent, *B. abortus* 9-941, and *B. ovis*. Three genes met this criterion, namely, Bru1_1050, which encodes for a multidrug resistance efflux pump; Bru1_1827, which encodes for CA II; and Bru2_1236, encoding for an adenosylmethionine-8-amino-7-oxononanoate aminotransferase.

Given the requirement of CA for growth of other microorganisms at ambient $CO_2$ concentrations and to check if Bru1_1827 could be responsible for the $CO_2$ dependence
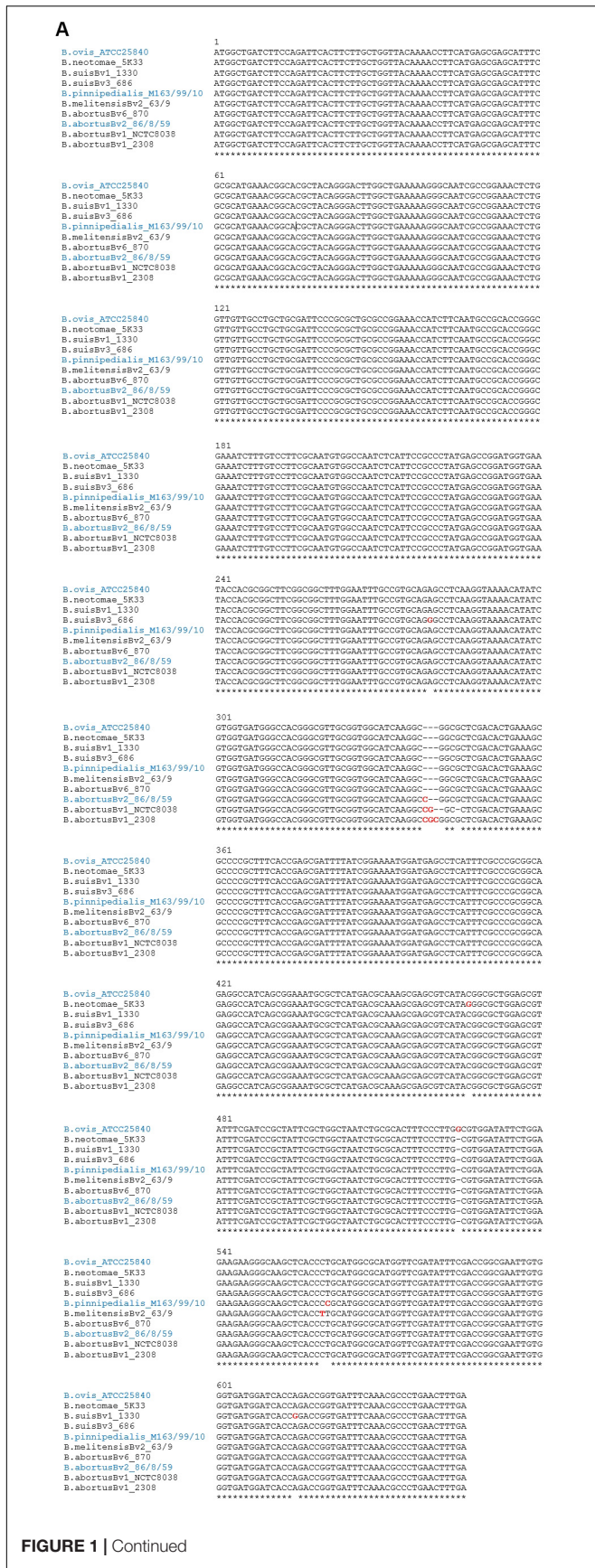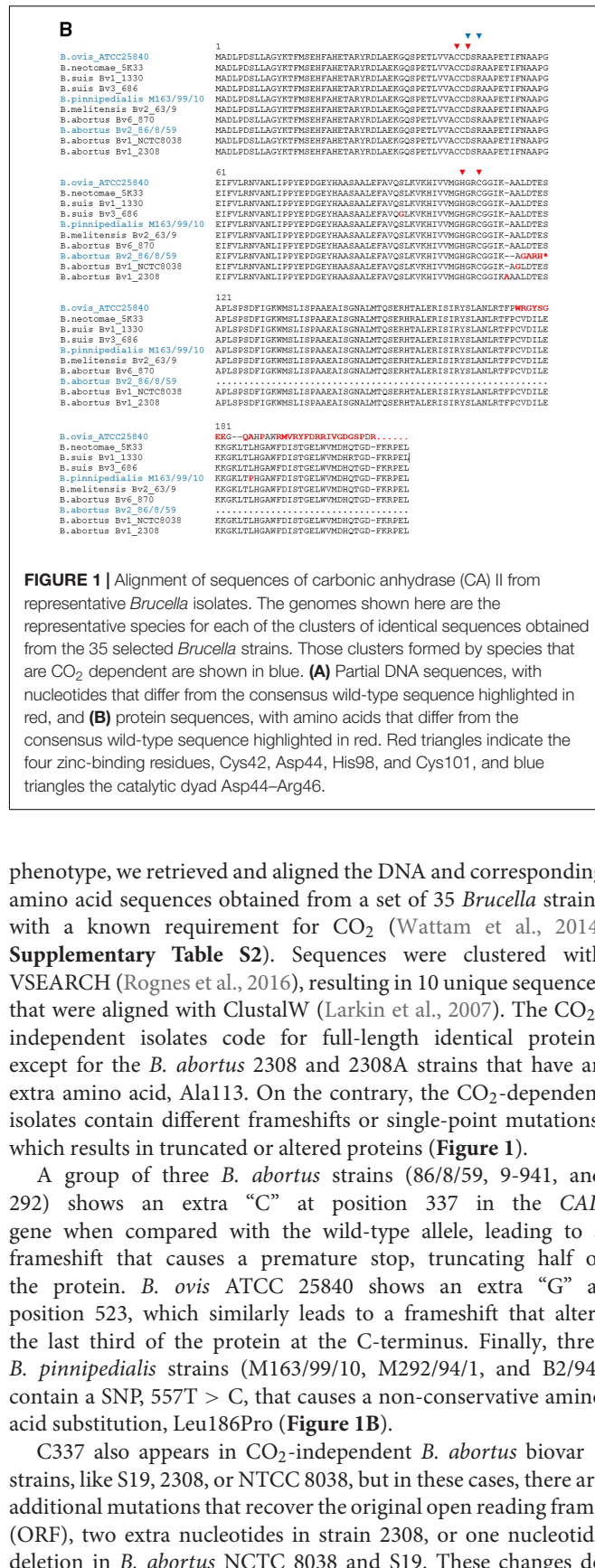
**A**

**FIGURE 1 |** Continued

**B**

**FIGURE 1 |** Alignment of sequences of carbonic anhydrase (CA) II from representative *Brucella* isolates. The genomes shown here are the representative species for each of the clusters of identical sequences obtained from the 35 selected *Brucella* strains. Those clusters formed by species that are $CO_2$ dependent are shown in blue. **(A)** Partial DNA sequences, with nucleotides that differ from the consensus wild-type sequence highlighted in red, and **(B)** protein sequences, with amino acids that differ from the consensus wild-type sequence highlighted in red. Red triangles indicate the four zinc-binding residues, Cys42, Asp44, His98, and Cys101, and blue triangles the catalytic dyad Asp44–Arg46.

phenotype, we retrieved and aligned the DNA and corresponding amino acid sequences obtained from a set of 35 *Brucella* strains with a known requirement for $CO_2$ (Wattam et al., 2014; **Supplementary Table S2**). Sequences were clustered with VSEARCH (Rognes et al., 2016), resulting in 10 unique sequences that were aligned with ClustalW (Larkin et al., 2007). The $CO_2$-independent isolates code for full-length identical proteins except for the *B. abortus* 2308 and 2308A strains that have an extra amino acid, Ala113. On the contrary, the $CO_2$-dependent isolates contain different frameshifts or single-point mutations, which results in truncated or altered proteins (**Figure 1**).

A group of three *B. abortus* strains (86/8/59, 9-941, and 292) shows an extra "C" at position 337 in the *CAII* gene when compared with the wild-type allele, leading to a frameshift that causes a premature stop, truncating half of the protein. *B. ovis* ATCC 25840 shows an extra "G" at position 523, which similarly leads to a frameshift that alters the last third of the protein at the C-terminus. Finally, three *B. pinnipedialis* strains (M163/99/10, M292/94/1, and B2/94) contain a SNP, 557T > C, that causes a non-conservative amino acid substitution, Leu186Pro (**Figure 1B**).

C337 also appears in $CO_2$-independent *B. abortus* biovar 1 strains, like S19, 2308, or NTCC 8038, but in these cases, there are additional mutations that recover the original open reading frame (ORF), two extra nucleotides in strain 2308, or one nucleotide deletion in *B. abortus* NCTC 8038 and S19. These changes do

not affect the conserved amino acid residues typical of β-CAs involved in the catalytic cycle, that is, the four zinc-binding residues, Cys44, Asp46, Hys105, and Cys108, and the catalytic dyad Asp46 and Arg48 (**Figure 1B**). Some of the strains analyzed here (*B. suis* 1330 and *B. abortus* strains 2308W, 292, and 544) were also analyzed by Pérez-Etayo et al. (2018), and our results are in complete agreement.

There is one discrepancy involving *B. abortus* Tulya, a biovar 3 strain that according to the literature (Alton et al., 1988) should be $CO_2$ dependent, but according to our analysis, it codes for a full-length CA II, thus being grouped with the $CO_2$-independent isolates. To solve this apparent puzzle, we plated a sample of *B. abortus* Tulya from our laboratory stock and determined its $CO_2$ dependence. Contrary to the original reference strain phenotype and in agreement with our *in silico* analysis, this isolate was indeed $CO_2$ independent. The complete $_{BaTulya}CAII$ was amplified by PCR from our strain and sequenced, confirming the published sequence. This strain originated from the collection kept in the Centro de Investigación y Tecnología Agroalimentaria of Aragón (CITA), Zaragoza, Spain, where it is also labeled as being $CO_2$ independent, suggesting that this is not the result of a contamination or selection of a $CO_2$-independent mutant in our hands.

As *Brucella* species code for two different CAs (Joseph et al., 2010), we repeated the analysis for the CA I CDSs. Although several isolates contain a polymorphism consisting of a 24-nt deletion between two 11-nt direct repeats or different SNPs (**Supplementary Figure S1**), there was no obvious correlation between the presence of these polymorphisms and $CO_2$ dependence. Pérez-Etayo et al. (2018) demonstrated that CA I from *B. abortus* strains 2308W, 292, and 544 is inactive, while that from *B. suis* strains 1330 and 513 is active, although it can only mediate $CO_2$ independence in complex media and in a rather prototrophic host. Comparison of $_{Bsuis513}CAI$ and $_{Babortus2308W}CAI$ reveals a difference of only one amino acid, the valine at position 74 being replaced by a glycine.

## *Brucella* $CO_2$-Independent Spontaneous Mutants Present a Modified CA II Sequence

Comparison of some of the CA II sequences of *B. abortus* biovar 1 $CO_2$-independent strains like 2308, S19, or NCTC 8038 with those of the other *Brucella* $CO_2$-dependent and independent isolates suggests that reversion of the $CO_2$ requirement is coincidental with the introduction of compensatory mutations able to reverse the initial frameshift described above. $CO_2$-independent mutants have been previously reported to appear at a low frequency ($3 \times 10^{-10}$) in cultures of $CO_2$-dependent strains by subculturing *in vitro* in the absence of supplementary $CO_2$ (Marr and Wilson, 1950). We measured the frequency of the reversion in six $CO_2$-dependent strains from our laboratory collection, by growing duplicate cultures with or without $CO_2$. We first checked the phenotype of all the strains by streaking them in a BA that was incubated without added $CO_2$. All the strains but *B. abortus* Tulya, as reported above, failed to grow in these conditions, in agreement with the published phenotype.

**TABLE 3 |** Observed frequency of appearance of $CO_2$-independent mutants in different *Brucella* strains.

| Strain | Frequency of mutants |
|---|---|
| *Brucella abortus* 292 | $2.72 \times 10^{-8}$ |
| *B. abortus* 544 | $5.95 \times 10^{-8}$ |
| *B. abortus* 86/8/59 | $1.14 \times 10^{-9}$ |
| *B. abortus* A-579 | $2.29 \times 10^{-10}$ |
| *Brucella ovis* 63/290 | $3.38 \times 10^{-6}$ |
| *Brucella pinnipedialis* B2/94 | $5.5 \times 10^{-6}$ |

We then plated o/n cultures from the $CO_2$-dependent strains to obtain colonies grown at ambient atmosphere and calculated the frequency of revertants for those strains (**Table 3**). The *B. abortus* strains had a similar frequency to the one described by Mar and Wilson (1950), $10^{-8}$ to $10^{-10}$, but *B. ovis* and *B. pinnipedialis* had a higher frequency of reversion, $10^{-6}$. In an exploratory effort to identify a possible cause for these differences in mutation rates, we analyzed the presence and identity between strains of the most obvious proteins that could be involved in this phenotype, like DNA polymerases, MutT, MutS, and MutD. Blastp analysis showed that, in all the cases, the protein not only was present in all strains but also had a 100% identity, so we could not find any difference that could explain our results. Maybe the analysis of the frequency of reversion in more $CO_2$-dependent strains will reveal if this is a species, biovar, or even isolate phenotype. We selected a few revertants from each strain and amplified by PCR the CA I and CA II coding regions. The amplicons were then sequenced to determine if any compensatory mutation had appeared in those loci. In all cases, we found compensatory mutations in the same region, around nucleotides 333–343. All mutations in this hot spot resulted in full-length CA II proteins (**Figure 2**), or in the case of *B. pinnipedialis*, a C-to-T change that reverts the Leu-to-Pro substitution. In this case, we also found the insertion of a nucleotide triplet (CGC or CCG) at the hot spot, which results in the addition of an extra amino acid, either Ala113 or Arg113, that is, the same position where the extra codon in $_{Ba2308}CAII$ is located. Although some of the compensatory mutations appear several times, the most common situation was to find different mutations for the same sequence.

As expected, reversion of the $CO_2$ dependence phenotype did not produce any change in the CDSs of CA I, reinforcing the hypothesis that CA II plays the main role in $CO_2$ independence.

## Structural Modeling of $_{Babortus2308}CAI$ and $_{Babortus2308}CAII$

A single amino acid substitution, Val74 in $_{Bsuis513}CAI$ to Gly74 in $_{Babortus2308W}CAI$, putatively renders the protein inactive, while the mutations in CA II in $CO_2$-dependent *Brucella* isolates do not affect the region where the active center is located, at the N-terminal part of the protein (**Figure 1B**). Moreover, a non-conservative Leu186Pro substitution, far from the active center, is enough to induce $CO_2$ dependence in the *B. pinnipedialis* strains analyzed. To better understand the effect of the observed mutations, a structural theoretical model of $_{Ba2308}CAI$ and $_{Ba2308}CAII$ was built with Phyre2. The modeled structures closely
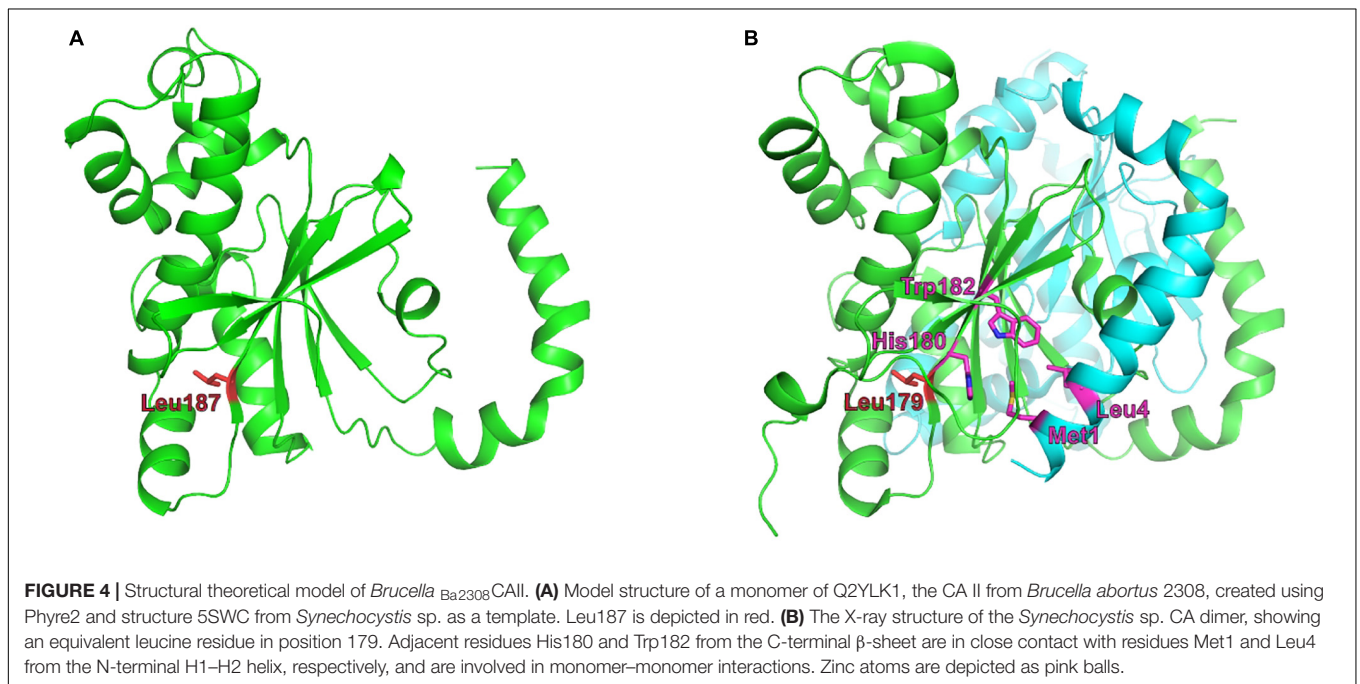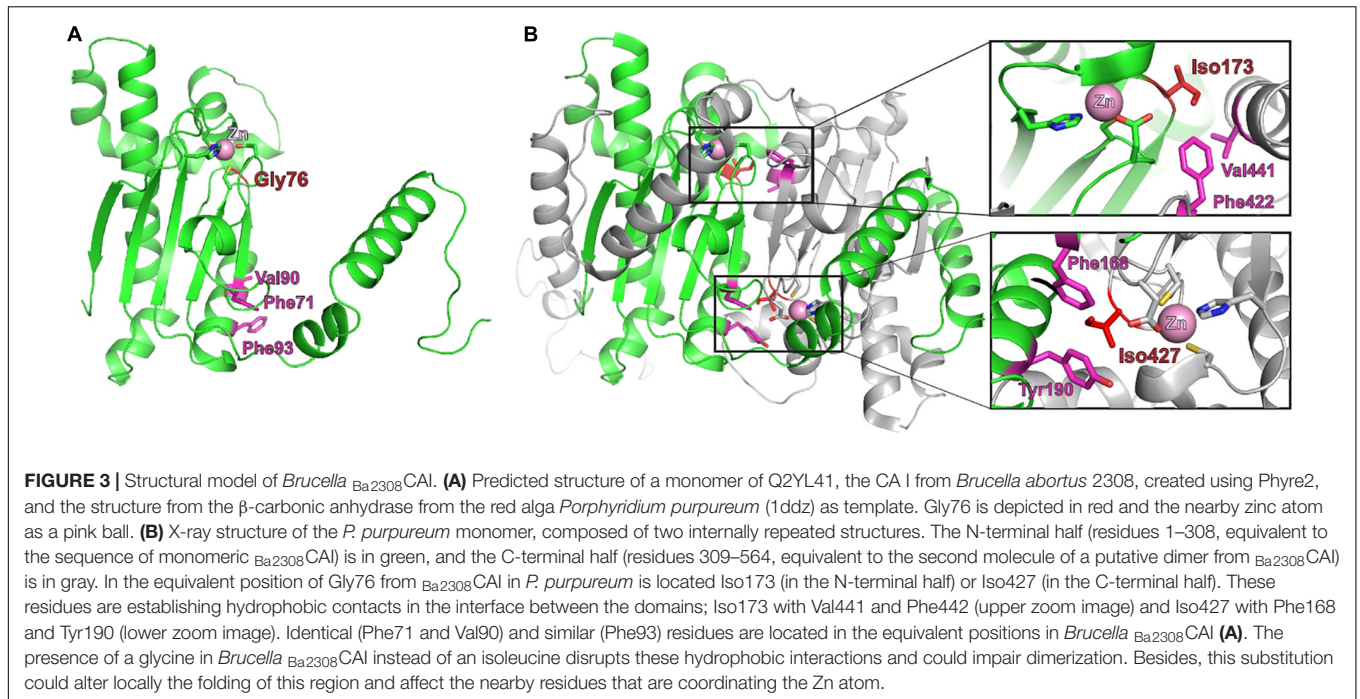
**FIGURE 2 |** Nucleotide changes in CA II from selected $CO_2$-independent mutants of different *Brucella* strains. Partial sequence of the regions where the original $CO_2$-dependent strains had the mutations that caused the defective phenotype (shown in red), and the changes observed after selection and sequencing of different spontaneous $CO_2$-independent mutants (shown in blue).

resembled those of other β-CAs that have been crystalized, displaying matches with a 100% confidence.

The closest structural homolog to $_{Ba2308}$CAI is 1DDZ, a β-CA from the red alga *Porphyridium purpureum* (Mitsuhashi et al., 2000), with a 45% identity. Each 1DDZ monomer contains two internally repeated structures, each one homologous to $_{Ba2308}$CAI. Overlapping of the modeled structures shows how the mutated residue Gly76 lies in close proximity to the coordinated zinc atom and also to the dimer interface (**Figure 3**). In the

equivalent position of Val76 in $_{Bsuis513}$CAI, 1DDZ contains Ile173 or Ile427, both among the most hydrophobic of amino acids. These residues are establishing hydrophobic contacts in the interface between the domains: Iso173 with Val441 and Phe442 (upper zoom image) and Iso427 with Phe168 and Tyr190 (lower zoom image). Identical (Phe71 and Val90) and similar (Phe93) residues are located in the equivalent positions in *Brucella* $_{Ba2308}$CAI. The presence of a glycine in *Brucella* $_{Ba2308}$CAI instead of an isoleucine disrupts these hydrophobic interactions

**FIGURE 3 |** Structural model of *Brucella* $_{Ba2308}$CAI. **(A)** Predicted structure of a monomer of Q2YL41, the CA I from *Brucella abortus* 2308, created using Phyre2, and the structure from the β-carbonic anhydrase from the red alga *Porphyridium purpureum* (1ddz) as template. Gly76 is depicted in red and the nearby zinc atom as a pink ball. **(B)** X-ray structure of the *P. purpureum* monomer, composed of two internally repeated structures. The N-terminal half (residues 1–308, equivalent to the sequence of monomeric $_{Ba2308}$CAI) is in green, and the C-terminal half (residues 309–564, equivalent to the second molecule of a putative dimer from $_{Ba2308}$CAI) is in gray. In the equivalent position of Gly76 from $_{Ba2308}$CAI in *P. purpureum* is located Iso173 (in the N-terminal half) or Iso427 (in the C-terminal half). These residues are establishing hydrophobic contacts in the interface between the domains; Iso173 with Val441 and Phe442 (upper zoom image) and Iso427 with Phe168 and Tyr190 (lower zoom image). Identical (Phe71 and Val90) and similar (Phe93) residues are located in the equivalent positions in *Brucella* $_{Ba2308}$CAI **(A)**. The presence of a glycine in *Brucella* $_{Ba2308}$CAI instead of an isoleucine disrupts these hydrophobic interactions and could impair dimerization. Besides, this substitution could alter locally the folding of this region and affect the nearby residues that are coordinating the Zn atom.



**FIGURE 4 |** Structural theoretical model of *Brucella* $_{Ba2308}$CAII. **(A)** Model structure of a monomer of Q2YLK1, the CA II from *Brucella abortus* 2308, created using Phyre2 and structure 5SWC from *Synechocystis* sp. as a template. Leu187 is depicted in red. **(B)** The X-ray structure of the *Synechocystis* sp. CA dimer, showing an equivalent leucine residue in position 179. Adjacent residues His180 and Trp182 from the C-terminal β-sheet are in close contact with residues Met1 and Leu4 from the N-terminal H1–H2 helix, respectively, and are involved in monomer–monomer interactions. Zinc atoms are depicted as pink balls.

and could impair dimerization. Besides, this substitution could locally alter the folding of this region and affect the nearby residues that are coordinating the Zn atom. In both cases, the structure and consequently the activity of the protein would be affected. Indeed, a Val-to-Gly substitution, located in the dimerization surface, was shown to interfere with dimerization of citrate synthase from *Thermoplasma acidophilum* (Kocabiyik and Erduran, 2000), not only reducing its catalytic activity (about 10-fold) but also decreasing its thermal and chemical stability.

The model structure obtained for $_{Ba2308}$CAII is shown in **Figure 4**, along with the dimer structure of the best hit obtained, 5SWC, showing a 29% of identity and 100% confidence. 5SWC is the β-CA CcaA from *Synechocystis* sp. PCC 6803. As $_{Ba2308}$CAII contains an extra codon, the residue highlighted in red, Leu187, is the equivalent residue to the Leu186Pro change that is present in the $CO_2$-dependent *B. pinnipedialis* strains.

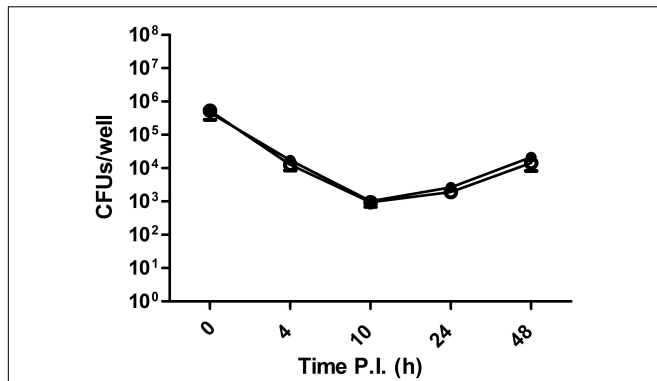In this structure, the protein crystalizes as a dimer, with the N-terminal arm composed of two α-helical segments (H1

**FIGURE 5 |** Infection and intracellular viability assay of *Brucella abortus* in J774 mouse macrophages *in vitro*. J774 macrophages were infected with either *B. abortus* 292 or the $CO_2$-independent spontaneous mutant *B. abortus* 292mut1, at a multiplicity of infection (MOI) of 50. Samples from triplicate wells were obtained at 0, 4, 10, 24, and 48 h post infection and enumerated by dilution and plating. ●, *B. abortus* 292; ○, *B. abortus* 292mut1.
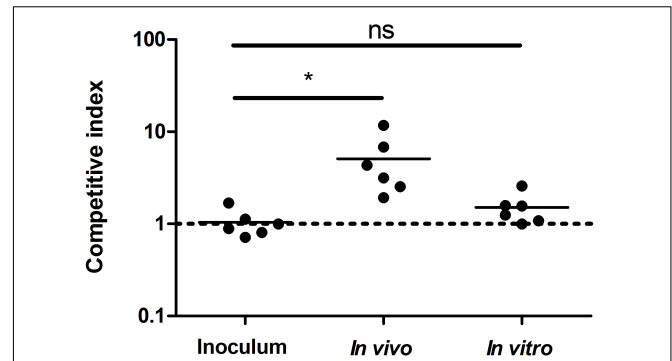


**FIGURE 6 |** Competitive index assay of a mixture of *Brucella abortus* 292 and its corresponding $CO_2$-independent spontaneous mutant *B. abortus* 292mut1. The competitive index was calculated by dividing the output ratio of mutant to wild-type bacteria by the input ratio of mutant to wild-type bacteria, in the two groups tested, regarding the original inoculum. Thus, for strains with the same fitness, the result should be 1. The differences between groups were analyzed by Student's two-tailed *t* test with significance set at *$P < 0.05$. ns, non-significant.

and H2) that extend away from the rest of the molecule and make significant contacts with the last β-sheet with an adjacent monomer (in the case of $_{Ba2308}$CAII His188 with Met1 and Trp191 with Leu4). This interaction between monomers has been determined as crucial for the establishment of the dimer (Cronk et al., 2001). In the case of *B. abortus* strains 86/8/59, 9-941, and 292, the premature stop would cause the complete loss of the C-terminal end of the protein, including the last β-sheet, involved in the formation of the dimer. *B. ovis* ATCC 25840 shows also a completely altered C-terminus, and although the new amino acid sequence would remain folded as a β-sheet, it shows a completely different amino acid composition that would prevent the establishment of the right molecular interactions between the adjacent monomers. Regarding the last mutation observed in $CO_2$-dependent strains, the SNP present in *B. pinnipedialis* strains M163/99/10, M292/94/1, and B2/94 causes a non-conservative amino acid substitution, Leu186Pro. The model predicts that this change will occur at the last β-sheet, in the area of interaction with the N-terminus of the adjacent monomer. Proline is an amino acid that confers an exceptional conformational rigidity and as such is a known disruptor of both α-helices and β-sheets. This being the case, this substitution is predicted to disrupt the dimerization of *Brucella* CA II.

## Competitive Infection Assays

Strain 2308 is not only a $CO_2$-independent *Brucella* isolate but also one of the most widely used virulent challenge strains, while S19, also a $CO_2$-independent *Brucella* isolate, is an attenuated vaccine strain. *In vitro* cell assays using J774 macrophages did not detect any difference in virulence between a $CO_2$-dependent *B. abortus* 292 strain and its corresponding $CO_2$-independent revertant (**Figure 5**). Additionally, we could not find any report in the literature that suggests that the $CO_2$ dependence phenotype is related to virulence; however, there is one puzzling fact; despite the expected low frequency of a frameshift mutation, somehow, this mutation is fixed in several species and biovars of

*Brucella*. It is then reasonable to think of it as having a biological advantage in specific situations. CI assays have been used to reveal subtle differences in fitness between two strains, and intra-animal experiments help to minimize inherent inter animal biological variation and also improve the identification of mutations or isolates with reduced or improved competitive fitness within the host (Falkow, 2004). As this could be the case with $_{Ba2308W}$CAII, we performed a CI experiment using *B. abortus* 292 and one of its $CO_2$-independent mutants, 292mut1. As a control, we grew the same initial mixture in BA plates that were incubated at 37°C with 5% $CO_2$, to know if any change in CI could be attributed to just the $CO_2$ concentration, or there was some other factor that could be attributed to growth within an animal. Results are shown in **Figure 6**. During the course of the experiments in mice, there was a significant enrichment of the strain carrying the truncated form of $_{Ba2308W}$CAII *B. abortus* 292, when compared with the $CO_2$-independent revertant able to produce a complete active form of $_{Ba2308W}$CAII. There was not a significant change in the ratio of both strains in liver or spleen, so the colony counts were combined in each mouse to show the ratio in that mice. At the same time, there was no significant enrichment/change in the ratio in cultures grown on plates. This suggests that inactivation of $_{Ba292}$CAII has some fitness advantage *in vivo* and could eventually result in the displacement of its corresponding $CO_2$-independent counterpart. This hypothesis could explain why, despite the low frequency of mutation, $CO_2$-dependent strains appear on primary isolation. As there are some other species and biotypes of *Brucella* that are $CO_2$ dependent on primary isolation, we could infer that the fitness advantage is also present in those species and biotypes.

## DISCUSSION

Diagnosis of brucellosis is usually achieved by serological detection in both animals and humans. This could be enough

to warrant the initiation of response measures, like the start of antibiotic therapy in humans or immobilization or sacrifice of animals. However, isolation, identification, and subtyping of brucellae not only are definitive proof of infection but also allow epidemiological surveillance. Depending on the laboratory, this process is carried out by a combination of classical and modern molecular methods. The classical typing methods consist in the phenotypic characterization of the isolates, using biochemical and immunological tests ($CO_2$ requirement; $H_2S$ production; urease activity; agglutination with monospecific A, R, and M sera; growth on media with thionin or basic fuchsin; or sensitivity to erythritol), and susceptibility to lytic *Brucella* phages (Alton et al., 1988). These methods require culture of the bacteria, are usually time-consuming and laborious, and do not offer good discriminatory power. Moreover, in the last few years, the field has experienced a revolution with the advent of new molecular methods, resulting in the description of new species and a better understanding of the population structure of the genus *Brucella*. Thus, the classical methods are being replaced or complemented by modern molecular methods. These methods range from PCR detection systems targeting different loci (like *ery*, *bcsp31*, or IS711), which allow species and even biovar differentiation (Mayer-Scholl et al., 2010; López-Goñi et al., 2011), to the multilocus sequence analysis (MLSA) that has been successfully used to describe the phylogenetic relationships of isolates and the global population structure of the genus *Brucella* (Whatmore et al., 2016). More recently, with the advent of WGS and especially with the drop in sequencing prices, WGS has been proposed to be the new routine typing method, particularly in groups with a high degree of similarity at the biochemical or serological levels (Chattaway et al., 2017), like *Brucellaceae*. But these methods are still far from being routine in most brucellosis laboratories, particularly in developing countries, and the classical methods are still routinely used in reference laboratories. Although genomic information offers the potential to unveil most of the phenotypic traits in bacteria, there are still important attributes that are not evident in the genome sequence. Thus, there is a gap between the classical typing schemes and the molecular methods, and some features still cannot be attributed to any specific genetic trait. In the case of *Brucella*, host specificity is particularly interesting, as it is yet impossible to predict from the genome sequence. It is reasonable to think that as molecular typing improves, we should advance in closing the gaps between classical and molecular typing, and we would be able to predict the full virulence and host specificity of a given isolate by analyzing the genome content. We have started to address this gap by looking at the genomic differences between *Brucella* isolates regarding one of the classical tests for typing, namely, $CO_2$ requirement.

*Brucella abortus* biovars 1, 2, 3, and 4 and some isolates from biovar 9, as well as *B. ovis*, require an increased concentration of $CO_2$ for growth, as do most strains of *B. pinnipedialis*, but only some of *B. ceti*. We selected 10 *Brucella* strains, which have been sequenced and annotated and whose $CO_2$ dependence status was known, to construct a *Brucella* pangenome based on the *B. suis* 1330 genome annotation. This resulted in a collection of 3,496 CDSs. We next compared the distribution

of pseudogenes (as annotated in the databases) and absent genes with $CO_2$ dependence, resulting in only three candidate genes: Bru1_1050, which encodes for a multidrug resistance efflux pump; Bru1_1827, which encodes for CA II; and Bru2_1236, which encodes for an adenosylmethionine-8-amino-7-oxononanoate aminotransferase. The most obvious candidate was CA II, as it has been shown to be required to grow under ambient air in a number of microorganisms. To confirm our initial result, we extracted and aligned the DNA and amino acid sequences of CA II from an extended set of sequenced strains with a known $CO_2$ phenotype. Those strains that are able to grow in atmospheric concentrations of $CO_2$ carry a full-length copy of the protein, while those that are not contain truncated or mutated versions of the proteins. *Brucella* species also carry a second CA, CA I, but the polymorphisms found at both the DNA and protein levels do not allow us to infer $CO_2$ dependence. This result is in agreement with that reported by Pérez-Etayo et al. (2018) and Varesio et al. (2019) and further extends the range of strains tested.

A direct application of this result would be the determination of the $CO_2$ dependence status of any given strain by determining the sequence at the CA II locus. This is actually the case in *B. abortus* Tulya, where our analysis predicted that our stock should be $CO_2$ independent, as it was the original stock from CITA. Laboratory determination of the phenotype confirmed the *in silico* result. This approach could be used to determine or at least narrow down candidate genes for different phenotypes, obviously with monogenic traits being the easier to determine.

We have found three different mutations that caused dependence of added $CO_2$, two independent insertions (C337 and G523) that either cause a premature stop or change completely the C-terminus of the protein, and an SNP that changes a leucine for a proline in the last β-sheet. All bacterial β-CAs crystallized so far are active as dimers or tetramers and inactive as monomers, and all of them have the N-terminal α-helix arm that extends away from the rest of the molecule and makes significant contact with the last β-sheet of an adjacent monomer (Supuran, 2016). In all the cases observed in this work, the mutations do not affect the active site, but all of them potentially change the sequence and structure of the protein at the C-terminus, so the most obvious hypothesis is that it is the modified structure of the proteins that causes the loss of activity. Inactive *Brucella* CA II proteins either lack the last β-sheet completely or have a very different sequence composition that disrupts this last β-sheet. The substitution of a leucine by a proline in the β-sheet is a particular example of this latter case, as proline is known to be a very disruptive amino acid for both α-helix and β-sheet structures. As these contacts seem to be important for dimerization, we can hypothesize that all the mutations found in CA II will have a strong impact in the dimerization or multimerization of CA II that will remain as a monomer, losing its activity (which we have defined as that allowing growth in a normal atmosphere). But there is a caveat in this reasoning. We, as well as others (Pérez-Etayo et al., 2018), have been unable to obtain a full-length mutant of CA II, despite being able to obtain a CA I (both data not shown). Moreover, a transposon sequencing analysis shows that CA II is essential, at

least for *B. abortus* 2308 (Sternon et al., 2018). This experiment was apparently carried out without added $CO_2$, so the result is not unexpected. It would be interesting to know if, performed in the presence of 5–10% $CO_2$, they would have observed insertions only in the C-terminus of the protein, where the mutations in the natural $CO_2$-dependent isolates accumulate. This means that the C-terminal part of the protein still carries out at least some of its functions as a monomer. We have not found any information regarding the activity of β-CAs as monomers, but in the α-CA from *Thermovibrio ammonificans*, the destabilization of the tetramer by reduction of the cysteines results in the dissociation of the tetrameric molecule into monomers with lower activity and reduced thermostability. It seems reasonable to think that this is the case also for *Brucella* CA II.

Carbonic anhydrase II catalyzes the fixation of $CO_2$ with high efficiency when forming dimers, but the low efficiency of the carboxylation reaction when acting as a monomer would require the presence of higher amounts of $CO_2$.

A similar situation could be taking place in the case of CA I. Modeling of the structure of $_{Babortus2308}$CAI allows us to hypothesize the role of the only residue of difference with $_{Bsuis513}$CAI that has to be responsible for the absence of activity in the first one. Its localization close to the Zn atom and to the dimer interface probably results in the destabilization of the dimer, lowering, or abolishing its activity. However, it would be necessary to purify and characterized biochemically the monomers of both $_{Babortus2308}$CAI and $_{Babortus2308}$CAII to confirm our model.

These mutations can only be selected in high-$CO_2$ environments, like those present inside animals, where high CA II activity would be dispensable, as this atmosphere generates enough bicarbonate in solution as to fulfill the metabolic requirement of the bacteria (Nishimori et al., 2009). We have determined the frequency of appearance of $CO_2$-independent isolates, and although there is a huge variation between strains, it ranges from $10^{-6}$ to $10^{-10}$, as previously described. Despite their low frequency, somehow these mutations got selected in several species and biovars of *Brucella*, suggesting that they provide some biological advantage. To test this hypothesis, we performed a competitive assay both *in vitro* and *in vivo*. This assay resulted in a significant enrichment of the strain carrying an inactive CA in animals, but not in cultured plates. Pérez-Etayo et al. (2018) assayed the bacterial loads of *B. ovis* PA and *B. ovis* PA Tn7$_{Ba2308W}$CAII in the spleens of BALB/c mice at 3 and 8 weeks post infection and found that there was no significant difference between a $CO_2$-dependent strain and its corresponding $CO_2$-independent strain at the level of multiplication in the mouse model. This apparent contradiction with our own results could be due to the different species used or to the different experiment used to test this hypothesis. When researchers try to determine subtle differences in fitness between two given strains, a competitive assay has a higher discrimination power (Eekels et al., 2012; Shames et al., 2017), as any effect is amplified over time. Although the ultimate reason behind this

competitive advantage is currently unknown, it would explain why some strains and biovars of *Brucella* are dependent of $CO_2$ in primary isolation, despite the low frequency of mutation. It is also noteworthy that this phenotype is only observed in certain species and biovars, suggesting that the competitive advantage of the CA II mutants only applies to a subset of host/pathogen pairs. As CA II is essential, the mutant strains still would have to produce the protein, and thus, the metabolic gain should be negligible for them. Another possibility would be that the dimer form of the enzyme is too active in a high-$CO_2$ environment and causes a deleterious acidification in the bacteria. By evolving this sophisticated system that reversibly alters the dimerization state of the protein, *Brucella* is able to adjust to the different requirements encountered during its biological cycle.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Cantabria University Institutional Laboratory Animal Care and Use Bioethics Committee.

## AUTHOR CONTRIBUTIONS

FS conceived and coordinated the study, conducted the bacteriology work, and wrote the manuscript. JG analyzed the data and wrote the manuscript. YO, CG-R, AS, and BA-R conducted the bacteriology work. All authors interpreted the data, corrected the manuscript, and approved the content for publication.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02751/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.