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Glutamate Decarboxylase-Dependent Acid Resistance in *Brucella* spp.: Distribution and Contribution to Fitness under Extremely Acidic Conditions

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Brucella **is an expanding genus of major zoonotic pathogens, including at least 10 genetically very close species occupying a wide range of niches from soil to wildlife, livestock, and humans. Recently, we have shown that in the new species *Brucella microti*, the glutamate decarboxylase (Gad)-dependent system (GAD system) contributes to survival at a pH of 2.5 and also to infection in mice by the oral route. In order to study the functionality of the GAD system in the genus *Brucella*, 47 isolates, representative of all known species and strains of this genus, and 16 strains of the closest neighbor genus, *Ochrobactrum*, were studied using microbiological, biochemical, and genetic approaches. In agreement with the genome sequences, the GAD system of classical species was not functional, unlike that of most strains of *Brucella ceti*, *Brucella pinnipedialis*, and newly described species (*B. microti*, *Brucella inopinata* BO1, *B. inopinata*-like BO2, and *Brucella* sp. isolated from bullfrogs). In the presence of glutamate, these species were more acid resistant *in vitro* than classical terrestrial brucellae. Expression in *trans* of the *gad* locus from representative *Brucella* species in the *Escherichia coli* MG1655 mutant strain lacking the GAD system restored the acid-resistant phenotype. The highly conserved GAD system of the newly described or atypical *Brucella* species may play an important role in their adaptation to acidic external and host environments. Furthermore, the GAD phenotype was shown to be a useful diagnostic tool to distinguish these latter *Brucella* strains from *Ochrobactrum* and from classical terrestrial pathogenic *Brucella* species, which are GAD negative.**

Brucellae are the etiologic agents of brucellosis, the most widespread bacterial zoonosis, infecting livestock and humans (human incidence, 500,000/year). The disease is endemic in the Mediterranean, in Near East and Middle East countries, and in Latin America (1, 2).

These pathogens are classified as different species on the basis of specific phenotypic traits and their natural hosts (3): *Brucella melitensis* (isolated from goats and sheep), *Brucella abortus* (cattle and bison), *Brucella suis* (pigs and wild boar), and *Brucella canis* (dogs). Transmitted to humans via the mucosal, cutaneous, respiratory or, most frequently, oral route, these species may induce undulant fever (Malta fever) and a wide range of clinical manifestations, including encephalitis and endocarditis. With a few exceptions, *Brucella* species isolated from marine mammals (*Brucella pinnipedialis* and *Brucella ceti*) have not been described as human pathogens. Two other species are nonpathogenic for humans: *Brucella ovis* (sheep) and *Brucella neotomae* (desert woodrat) (3). Being known for 20 years or longer, these eight species are considered "classical". More recently, three "new" species/strains were identified: *B. microti* from common vole, red fox, and also soil (4–7) and *Brucella inopinata* BO1 and *B. inopinata*-like BO2 from patients with a breast implant infection (8, 9) and a chronic destructive pneumonia (10), respectively. Potentially novel *Brucella* spp., described as "atypical" because they do not match the phenotypical criteria defined for the classical species (like *B. melitensis*), were isolated from baboons (11), Australian rodents (12), and African bullfrogs (13). Thus, it has become increasingly clear that the genus *Brucella* comprises species which are able to occupy a vast range of niches from soil to wildlife animals, from domesticated livestock to humans.

Orally acquired bacteria, such as *Escherichia coli*, *Shigella flexneri*, and *Listeria monocytogenes*, possess the ability to withstand acidic environments, such as those found in fermented food, gastric juice, distal gut lumen, and the macrophagic phagosome. To counteract the deleterious effects of acid stress, the above-mentioned bacteria activate specific systems, among which the glutamate decarboxylase (Gad)-dependent system (GAD system) was shown to be by far the most effective (14–18). Upon exposure to an extremely low pH (mimicking that of the gastric compartment, ≤2.5), the GAD system becomes activated: a molecule of gluta-

Received 8 September 2014 **Accepted** 30 October 2014

Accepted manuscript posted online 7 November 2014

Citation Damiano MA, Bastianelli D, Al Dahouk S, Köhler S, Cloeckaert A, De Biase D, Occhialini A. 2015. Glutamate decarboxylase-dependent acid resistance in *Brucella* spp.: distribution and contribution to fitness under extremely acidic conditions. Appl Environ Microbiol 81:578–586. doi:10.1128/AEM.02928-14.

Editor: C. A. Elkins

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.02928-14.

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doi:10.1128/AEM.02928-14

mate (Glu) is taken up via the inner membrane antiporter GadC and decarboxylated into γ-aminobutyrate (GABA) by the cytosolic enzyme Gad, with concomitant intracellular consumption of a proton; GadC promotes the export of GABA in exchange for an incoming Glu molecule (19), coupled to an electrogenic antiport (20). *E. coli* possesses two cytoplasmic isoforms of glutamate decarboxylase (GadA and GadB), and the gene coding for GadC is cotranscribed with *gadB* (15).

A comparative genome analysis of *B. microti* and several classical *Brucella* species revealed the occurrence of two sequences homologous to those of *E. coli gadB* and *gadC*, which contain in all cases except for *B. microti* either stop codons or frameshift mutations, generating truncated/potentially nonfunctional proteins (21). *B. microti* grows faster and is more resistant to pH 4.5 *in vitro* than classical *Brucella* strains, such as *B. suis* 1330 (22). The *B. microti* GAD system is fully active, i.e., it confers resistance to extreme acid stress (pH 2.5), and also contributes to infection transmitted through ingestion in mice (23).

In order to elucidate whether the occurrence of an operative GAD system correlates with a better adaptation to acidic environments, a collection of 47 strains, including all currently known species of the genus *Brucella*, was thoroughly investigated for GAD and GABA export activities. Furthermore, the survival of a subgroup of 17 strains among those that were GAD positive and negative was analyzed for acid resistance (or survival) at pH 2.5 in the presence of Glu. Finally, the role of the *gadBC* locus of these strains in glutamate-dependent acid resistance (AR) was corroborated by functional complementation of an acid-sensitive *gadABC* mutant of *E. coli*.

MATERIALS AND METHODS

Bacterial strains and media. Sixty-nine bacterial strains were analyzed for the presence of GAD activity and for GABA export: 47 belong to *Brucella*, 16 to *Ochrobactrum*, and 6 to *E. coli* (see Table S1 in the supplemental material). *Brucella* and *Ochrobactrum* strains were grown in tryptic soy (TS) broth (Becton-Dickinson) and *E. coli* in Luria-Bertani (LB) broth (Difco) at 37°C, supplemented when appropriate with chloramphenicol and kanamycin at 25 and 50 μg/ml, respectively. *B. pinnipedialis* and *B. ovis* were cultured in a 5%-CO_2 atmosphere and in TS medium with 10% fetal calf serum.

Construction and complementation of *E. coli gad* triple mutant strains. A triple Δ*gadA* Δ*gadBC* mutant of the *E. coli* K-12 strain MG1655 was used for heterologous expression of the *gadBC* operon of *Brucella* species representative of the GAD-positive and GAD-negative species. This *E. coli* mutant strain was generated by using the one-step inactivation procedure of Datsenko and Wanner (24). In this specific case, two consecutive gene-specific inactivation steps using plasmid pKD13 as the template for amplification of the kanamycin cassette (Kan^r) were performed. Briefly, a *gadA* mutant was constructed by replacement of its open reading frame (ORF) with Kan^r and, after excision of this cassette, the entirety of *gadBC* was replaced with Kan^r. Thus, to accomplish the mutation in *E. coli*, a linear DNA substrate was constructed by PCR amplification of the Kan^r cassette-encoding gene using bipartite primers. Usually, these primers consist of 60 to 70 nucleotides (nt) to generate the gene replacement, with 40 to 50 nt of homology to the target region where the cassette has to be inserted, followed by 20 nt (highlighted in italics in sequences below) to prime the drug-resistant cassette in pKD13. The primers used in this study were the following: Δ*gadA*_for, 5′-TTCGAAATGGACCAGAAGCTGTTAACGGATTTCCGCTCA*TGTAGGCTGGAGCTGCTTC*-3′; Δ*gadA*_rev, 5′-TCAGGTGTGTTTAAAGCTGTTCTGCTGGGCAATACCCTGA*TTCCGGGGATCCGTCGACC*; Δ*gadB*_for, GATTTAAGGTCGGAACTACTCGATTCACGTTTTGGTGCG*TGTAGGCTGGAGCTGCTTC*-3′; Δ*gadC*_rev, 5′- TTAGTGTTTCTTGTCATTCATCACAATATAGTGTGGTGAA*ATTCCGG*GGATCCGTCGACC-3′. Homologous and heterologous complementations of the triple mutant were obtained by transformation with the vector pBBR1MCS carrying the complete *gadBC* operon of *E. coli* or *Brucella* strains, each including its native promoter, as reported (23). A 3.6-kb DNA fragment of each *Brucella* species containing the *gadB* and *gadC* genes was amplified by PCR using the long-extend and high-fidelity Platinum *Taq* polymerase (Invitrogen) and the *gadBC*-op_PstI-For (G**C**CCTGCAGCCGAGCTTATTGCGCTAATATC) and *gadBC*-op_XbaI-Rev (G**CC**TCTAGAATCGCATCTGATGAGCTTGAC) primers (Sigma). In the above primers, the sequence in bold has no homology with the target genes and is included to increase stability of the 5′ end, which contains the restriction sites (underlined) for PstI and XbaI, respectively. The resulting clones were selected on the basis of their double resistance to Kan and Cm, the latter provided by pBBR1MCS.

Glutamate-dependent acid resistance assay. Assays with *Brucella* spp. and *E. coli* wild-type and mutant strains were carried out in minimal media as previously described (15, 23), namely, modified Gerhardt's (GMM without glutamate) for *Brucella* spp. and Vogel Bonner's medium with 0.4% glucose (EG) for *E. coli*; both media were brought to pH 2.5 with HCl and used in the presence/absence of 1.5 mM Glu.

Qualitative and quantitative GAD assays and GABA measurements. The qualitative GAD assay (25) was performed as described for *E. coli* and *B. microti*. For quantitative assay of GAD activity in *E. coli* strains, the cells from a 50-ml overnight culture grown in LB broth at pH 5, containing 0.4% glucose (LBG), were harvested by centrifugation and resuspended in 1 ml of a solution containing 1 mM pyridoxal 5′-phosphate, 1 mM dithiothreitol, and protease inhibitors (cOmplete Mini, EDTA free; Roche). Bacteria were lysed by sonication and then centrifuged at 12,000 rpm for 15 min at 4°C to remove cells debris. The cleared cell lysates were immediately assayed for protein content (26) and GAD activity (27). The concentration of GABA exported by *E. coli* and by *Brucella* in acidified medium was measured using a commercial biochemical GABase assay (Sigma), as described previously (15, 23).

RESULTS

An *in silico* analysis reveals potentially functional GadB and GadC proteins in several *Brucella* species. Comparing the putative protein sequences of GadB and GadC (available from NCBI, Broad Institute, and PATRIC databases), several *Brucella* species, in addition to *B. microti*, were expected to have an active GAD system. These include *B. ceti*, *B. inopinata* BO1, *B. inopinata*-like BO2, and *B. pinnipedialis*. On the contrary, the analysis of the sequences homologous to those of the *gadB* and *gadC* genes in other classical *Brucella* species (*B. abortus*, *B. canis*, *B. melitensis*, *B. neotomae*, *B. ovis*, and *B. suis*) showed evidence of point or frameshift mutations. In *gadB*, most of the mutations fall within the nucleotide region coding for the large cofactor- and substrate-binding domain (19, 28), spanning from amino acid (aa) residue 58 to 346, referring to the numbering of *E. coli* GadB (Fig. 1 and Table 1; see also Table S1 and Fig. S1 in the supplemental material). The *gadC* sequences are much more conserved, and any change in the sequence affects the overall length of the polypeptide chain, which will result is shorter forms truncated in the N-terminal region, i.e., missing the first 42 to 117 aa in the sequence. These shorter polypeptides will likely be unable to localize in the membrane and perform an antiport (Fig. 1 and Table 1; see also Table S1 and Fig. S2), even in the presence of an active GadB.

GAD activity and GABA export in *Brucella* strains correlate with the integrity of the *gadBC* locus. To determine which *Brucella* species are GAD positive, a qualitative GAD test (Rice assay) was employed for a collection of 47 strains belonging to all 10 recognized species of the genus and also including yet-unclassified atypical strains (Table 1; see also Table S1 in the supplemental

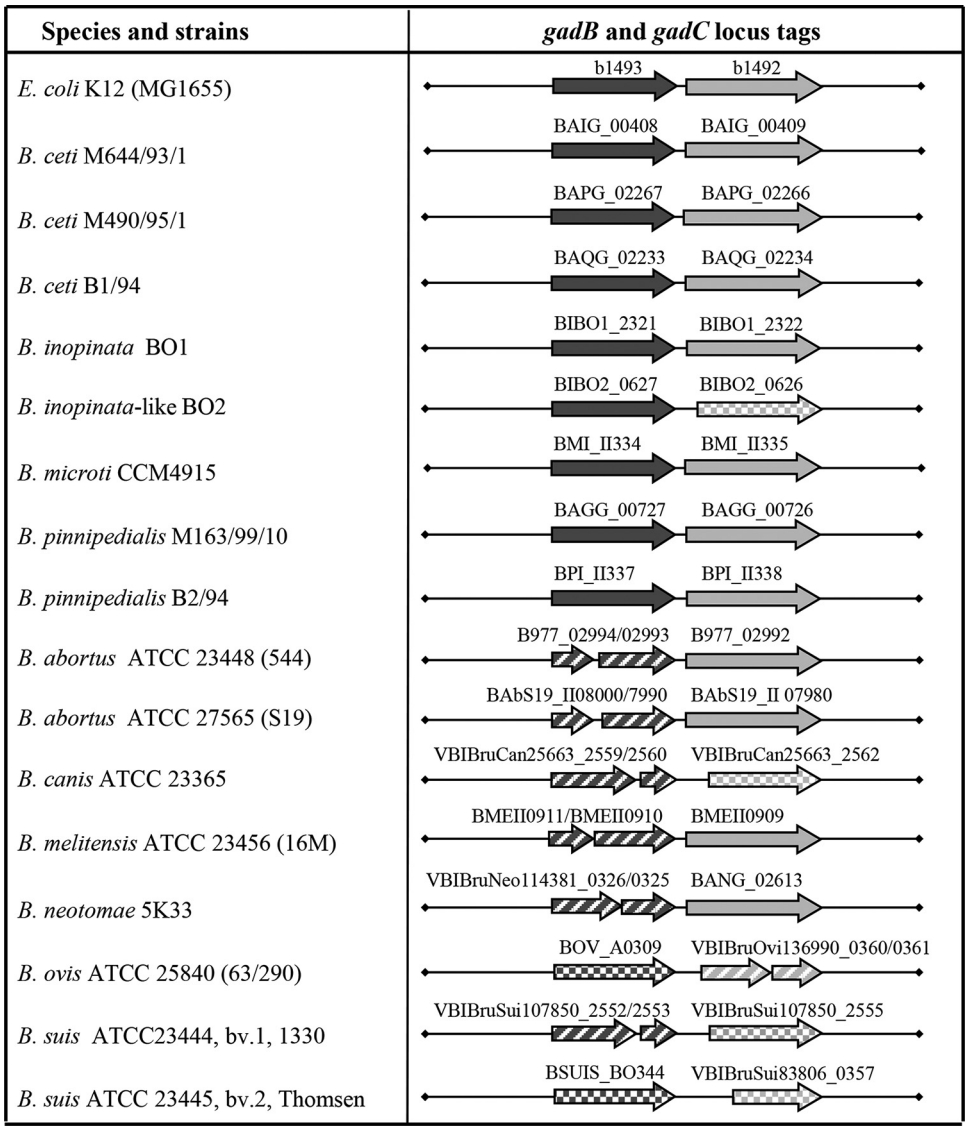

FIG 1 Schematic representation of the *gadB* (dark-gray arrow) and *gadC* (light-gray arrow) loci in the *Brucella* species and strains for which genome sequences (complete or partial) are available. The arrow length is proportional to the gene length, whereas the distance between the two genes is arbitrary, though always referring to two adjacent loci. The corresponding locus tags are shown above each arrow. Dashed interrupted arrows indicate the occurrence of frameshift mutations or stop codons. Squared arrows mark genes shorter than the canonical length for GadB (464 aa) and GadC (510 to 513 aa).

material for the full list). In this rapid test, initially described for *E. coli* (25), the proton-consuming GAD activity is indirectly demonstrated by a change in color (i.e., from yellow to green and blue) of an unbuffered assay solution (at pH 3.4) containing bromocresol green, a pH indicator, as pH increases (GAD Rice test in Table 1; see also Table S1). The test revealed that most of the strains of classical brucellae were GAD negative (yellow), even after 24 h of incubation of the bacteria in the GAD reagent. These included *B. abortus* (10/10), *B. canis* (1/1), *B. melitensis* (3/3), *B. neotomae* (1/1), *B. ovis* (1/2), and *B. suis* (4/4, one strain of each biovar, 1, 3, 4, and 5). Only the two reference strains, *B. ovis* ATTC 25840 bv. 1 (63/290) and *B. suis* ATCC 23445 bv. 2 (Thomsen), were GAD positive, with green and blue colors, respectively (Table 1; see also Table S1). In contrast, within 4 h, several strains of the newly described *Brucella* species were found to be GAD positive (blue) (Table 1; see also Table S1), i.e., *B. microti* (12/12 strains), *B. ceti* (4/4), *B. inopinata* BO1, *B. inopinata*-like BO2, and 3 out of 4 *Brucella* sp. strains isolated from bullfrogs. After a prolonged incubation period (≥8 h), the initially negatively tested strain isolated from bullfrogs (*Brucella* sp. Br1 in Table 1; see also Table S1) turned blue in the Rice test. The results of the Rice test were in agreement with the putative sequences of the GadB proteins, as far as those were available (Table 1, column 3; see also Table S1, column 5). Among the three *B. pinnipedialis* strains tested, strains M163/99/10 and M292/94/1, isolated from a hooded and a common seal, respectively, were clearly GAD positive, whereas strain B2/94, isolated from a common seal, was only weakly positive (yellow/green) despite its putatively intact GadB protein.

To evaluate the specificity of the Rice test, 16 strains of the genus *Ochrobactrum* (*O. anthropi* and *O. intermedium*), the closest phylogenetic neighbor of *Brucella*, were also analyzed. In fact, in the genome sequences of the two reference strains of these spe-

TABLE 1 Representative *Brucella* strains for which the GAD phenotype (Rice test) was assayed and exported GABA was measured[a]

Species and strain	Natural host	Protein length (aa)		GAD Rice test[b]	Exported GABA (mM)
		GadB	GadC		
B. abortus					
ATCC 23448 bv. 1 (544)	Cattle	155/286	510	−	0.00 ± 0.00
ATCC 27565 bv. 1 (S19)	Cattle	155/274	510	−	0.00 ± 0.00
B. canis					
ATCC 23365 bv.1	Dog	319/136	426	−	0.00 ± 0.00
B. ceti					
B1/94 bv.1	Porpoise	464	513	+	0.25 ± 0.00
M490/95/1	Common seal	464	513	+	0.53 ± 0.01
M644/93/1	Common dolphin	464	513	+	1.83 ± 0.37
B202R	Minke whale	ND	ND	+	0.26 ± 0.08
B. inopinata					
BO1	Human	464	509	+	0.12 ± 0.04
B. inopinata-like					
BO2	Human	464	471[c]	+	0.02 ± 0.02
B. melitensis					
ATCC 23456 bv. 1 (16 M)	Goat	167/304	510	−	0.00 ± 0.00
B. microti					
CCM4915	Common vole	464	510	+	0.20 ± 0.04
CCM4916	Common vole	ND	ND	+	0.65 ± 0.09
B. neotomae					
5K33	Desert woodrat	263/201	510	−	0.00 ± 0.00
B. ovis					
ATCC 25840 bv. 1 (63/290)	Sheep	455	291/206	+[w]	0.00 ± 0.00
B. pinnipedialis					
M163/99/10	Hooded seal	464	513	+	0.33 ± 0.06
B2/94 bv. 1	Common seal	464	510	+[w]	0.04 ± 0.04
Brucella spp.					
Br1	African bullfrog	ND	ND	+[s]	0.17 ± 0.04
Br2	African bullfrog	ND	ND	+	1.18 ± 0.47
Br3	African bullfrog	ND	ND	+	0.25 ± 0.18
Br4	African bullfrog	ND	ND	+	1.04 ± 0.25
B. suis					
ATCC 23444 bv. 1 (1330)	Swine	319/136	426	−	0.00 ± 0.00
ATCC 23445 bv. 2 (Thomsen)	Swine	454	336[d]	+	0.00 ± 0.00

[a] The natural host of each strain and the lengths of their putative GadB and GadC proteins are provided. For GadB, the numbers before and after the character "/" indicate the respective sizes of the two consecutive fragments annotated as decarboxylases in the presence of a stop codon. ND, not determined (sequence unavailable).
[b] The qualitative GAD test is based on the color change of the Rice reagent. Following the increase in pH that accompanies the decarboxylation of glutamic acid, the pH indicator bromocresol green turns from yellow (negative) to blue (positive). −, negative (yellow); +, positive (blue); +[w], weakly positive (green); +[s], slowly positive (blue color develops slowly during incubation of >8 h).
[c] Shorter form, truncated at N terminus, with probably decreased ability to export GABA.
[d] Shorter form, truncated at N terminus, probably unable to export GABA.

cies (LMG 3331 and LMG 3301), the two genes *gadB* and *gadC* of the GAD system are not present. A GAD-negative result was found for all 16 strains tested (see Table S1 in the supplemental material, notes). In contrast, the *E. coli* strains tested were all GAD positive except for CC118. These results indicated that the Rice test may be useful to distinguish environmental and atypical strains of *Brucella* from those of *Ochrobactrum*, for which they are sometimes mistaken using standard biochemical tests.

To confirm the functionality of the GAD system among *Brucella* strains, the activity of the GadC antiporter was assayed by measurement of GABA levels in the medium at pH 3.5, as described previously (23). GABA was detected in all strains with a potentially functional GAD system (i.e., intact GadB and GadC proteins), with the exception of *B. pinnipedialis* strain B2/94, for which results of the Rice test were found to be only weakly positive (Fig. 1 and Table 1), thereby justifying a very limited or null GABA export.

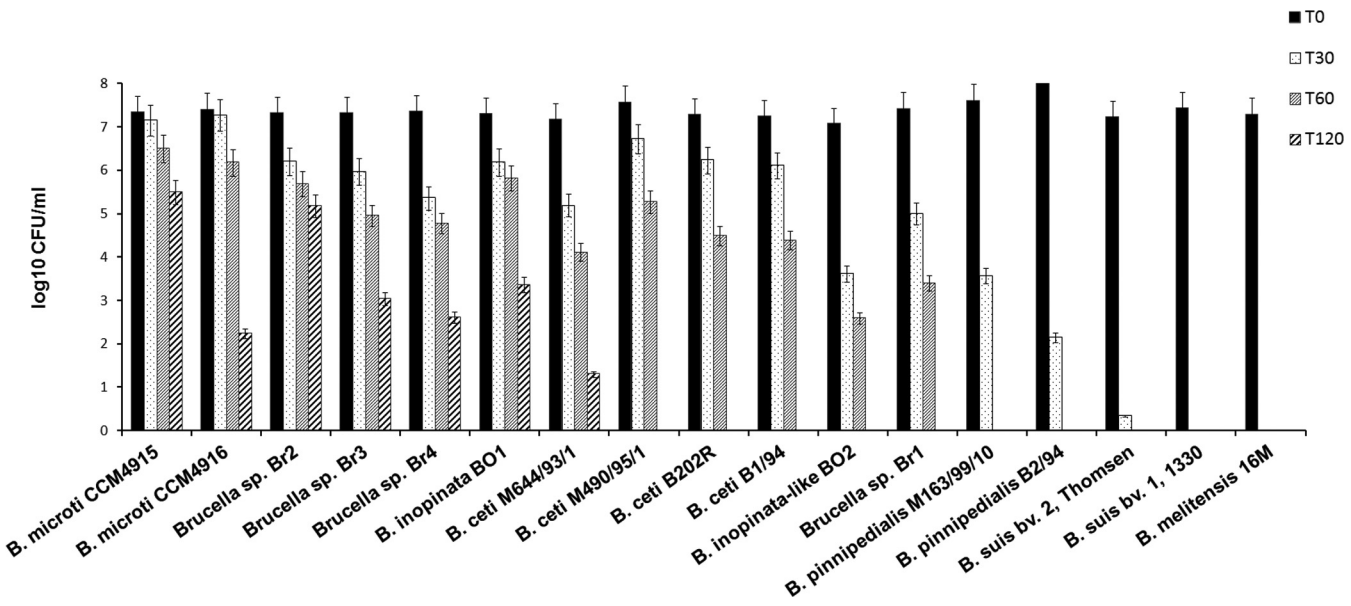

FIG 2 Survival of representative *Brucella* strains in minimal medium at pH 2.5 with 1.5 mM Glu. Decrease of viability during the acid shock (at the indicated time, T) was expressed as a $\log_{10}$ of the number of CFU (CFU/ml) at each time point (30, 60, and 120 min) versus bacteria present at time zero. *B. abortus* S19 and ATCC 23488 (544), *B. neotomae* 5K33, and *B. ovis* ATCC 25840 strains have the same acid-sensitive phenotypes as *B. suis* bv. 1, 1330 and *B. melitensis* 16M.

GAD-positive and GABA-exporting *Brucella* strains are resistant to extreme acid pH in the presence of glutamate. To identify possible glutamate-dependent AR phenotypes among *Brucella* strains, a variety of *Brucella* GAD-positive and -negative strains were subjected to acid shock in modified GMM at pH 2.5 for 30, 60, and 120 min in the presence/absence of Glu (1.5 mM). After 30 min, viable bacteria were undetectable for all classical *Brucella* species tested (*B. abortus*, *B. canis*, *B. melitensis*, *B. neotomae*, *B. ovis*, and *B. suis*), whereas a significant reduction in viability ($\geq 10^4$-fold) was observed for the *B. pinnipedialis* strains (Fig. 2).

In contrast, the GAD-positive and GABA-producing strains (*B. microti* CCM4915 and CCM4916, three strains of *Brucella* isolated from bullfrogs, *B. inopinata* BO1, *B. inopinata*-like BO2, and four strains of *B. ceti*) were acid resistant, with a reduction in viability varying from 10^2- to 10^4-fold. Some of these strains maintained viability even after 2 h of incubation at this extreme pH (Fig. 2). Incubation in modified GMM at pH 2.5 without Glu for 30 min resulted in killing of all strains, suggesting an important role of the GAD system in acid resistance. Even though we could not detect any clear correlation between a strong AR phenotype and levels of exported GABA, it was nevertheless evident that a GABA export of at least 0.12 mM/h was necessary to support AR for 1 h at extreme acid pH (Table 1, rightmost column), the only exception being *B. inopinata*-like BO2.

Heterologous expression of *gadBC* of *Brucella* species in *E. coli*. To compare the functioning of the GAD system of representative strains of *Brucella* species in a unique background genotype, their *gadBC* regions were overexpressed in an acid-sensitive triple mutant strain of *E. coli* MG1655, specifically constructed to be devoid of the GAD system (i.e., deleted in *gadA* and *gadBC*) (Table 2). In addition, four strains derived from *E. coli* MG1655 were constructed as controls: a wild-type strain and a triple mutant strain, carrying the pBBR1MCS nonrecombinant plasmid, and two triple mutant strains, carrying pBBR1MCS with the *gadBC* operon of *E. coli* MG1655 or *B. microti* CCM4915.

As expected, on the basis of our previous work with Δ*gadC* and Δ*gadA* Δ*gadB* simple and double mutant strains (23), the *E. coli* triple mutant strain (carrying [or not] the pBBR1MCS control plasmid) was GAD and GABA negative and sensitive to extreme acid stress, with no survivors after 2 h at pH 2.5 in EG in the presence of Glu. In contrast, homologous and heterologous complementations of the same strain with the *gadBC* operons of *E. coli* or *B. microti* CCM4915 resulted in GAD activity. Hence, these strains were able to export GABA in LBG and displayed AR in EG at pH 2.5 in the presence of Glu to a level identical to that of the *E. coli* wild-type strain (Table 2). The presence of the plasmid pBBR1MCS *per se* did not affect the AR phenotype of the strains investigated.

Similarly, the *E. coli* triple mutant strains carrying the *gadBC* operons of various GAD-positive and GABA-positive *Brucella* strains were able to express a functional GadB protein and export GABA (*B. ceti* B1/94, *B. ceti* M644/93/1, *B. inopinata* BO1, *B. inopinata*-like BO2, *B. microti* CCM4916, *B. pinnipedialis* M163/99/10, and *Brucella* sp. Br2). These strains were as acid resistant as the *E. coli* triple mutant strains complemented with the operon of *E. coli* or *B. microti* CCM4915 (Table 2). In contrast, the *E. coli* triple mutant strain complemented with the *gadBC* operon of *B. suis* 1330 was devoid of GAD activity, unable to export GABA, and sensitive to acid challenge (Table 2). The mutant strain complemented with the *gadBC* region of the *B. suis* bv. 2 strain (Thomsen) expressed a functional GadB protein but was unable to export GABA and was also very sensitive to pH 2.5 even in the presence of Glu. As mentioned above, this strain possesses a *gadC* gene encoding a truncated GadC, very likely inactive.

Western blot analysis with crude lysates of recombinant *E. coli* strains grown to stationary phase showed that the GadB protein was detectable with anti-GadB (*E. coli*) antibodies only if the *gadBC* operon was derived from GAD-positive *Brucella* strains (data not shown). Truncated GadB proteins were not detected by Western blotting for the *E. coli* strains complemented with *gadBC*

TABLE 2 GAD test, GAD activity, and GABA export in overnight cultures of *E. coli* strains grown in LBG, before assaying survival following exposure to extreme acid

Description of *E. coli* MG1655 wild type and *gadABC* triple mutant (Δ) and complemented strains[a]	GAD Rice test result	GAD activity (U/mg[b])	Concn (mM) of GABA[c]	% survival[d]
Wild type_p	+	1.29 ± 0.03	0.86 ± 0.35	86.6 ± 12.2
Δ_p	−	0.03 ± 0.03	0.00 ± 0.00	0.0 ± 0.0
Δ_ p*gadBC* (*E. coli* MG1655)	+	2.34 ± 0.36	3.17 ± 0.21	70.3 ± 23.7
Δ_ p*gadBC* (*B. ceti* M644/93/1)	+	0.45 ± 0.01	0.28 ± 0.15	72.5 ± 16.7
Δ_ p*gadBC* (*B. ceti* B1/94)	+	0.36 ± 0.07	0.40 ± 0.14	99.2 ± 1.2
Δ_ p*gadBC* (*B. inopinata* BO1)	+	0.54 ± 0.10	0.16 ± 0.06	60.2 ± 16.4
Δ_ p*gadBC* (*B. inopinata*-like BO2)	+	0.64 ± 0.08	0.25 ± 0.15	83.6 ± 18.4
Δ_ p*gadBC* (*B. microti* CCM4915)	+	0.55 ± 0.01	0.31 ± 0.12	79.2 ± 22.3
Δ_ p*gadBC* (*B. microti* CCM4916)	+	ND[e]	0.31 ± 0.11	95.3 ± 8.0
Δ_ p*gadBC* (*B. pinnipedialis* M163/99/10)	+	0.51 ± 0.03	0.54 ± 0.13	70.7 ± 24.8
Δ_ p*gadBC* (*B. pinnipedialis* B2/94)	+	ND	0.18 ± 0.11	62.0 ± 30.4
Δ_ p*gadBC* (*Brucella* sp. Br2)	+	0.86 ± 0.14	0.20 ± 0.09	69.0 ± 22.0
Δ_ p*gadBC* (*B. suis* bv. 1, 1330)	−	0.03 ± 0.03	0.00 ± 0.00	0.0 ± 0.0
Δ_p*gadBC* (*B. suis* bv. 2, Thomsen)	+	0.49 ± 0.05	0.10 ± 0.03	0.0 ± 0.0

[a] p, pBBR1MCS plasmid, either nonrecombinant (p only) or carrying *gadBC* (p*gadBC*) of *E. coli* MG1655 or *Brucella* strains (name of strain).
[b] Micromoles of GABA produced per minute at 37°C per mg of total protein.
[c] Concentration of GABA exported in spent medium was measured using GABase (Sigma).
[d] Bacterial survival was expressed as the percentage of viable bacteria present in EG at pH 2.5 in the presence of 1.5 mM Glu after 2 h of incubation. Mean values ± SD are from 3 to 8 independent experiments.
[e] ND, not determined.

from GAD-negative strains, probably because of instability of the synthesized polypeptide. As a matter of fact, in *B. suis* ATCC 23445 bv. 2 (Thomsen), possessing intact GadB and truncated GadC, GadB was detectable by Western blotting and activity assays (Table 2 and data not shown). This suggests that the normal regulatory machinery for expression was still intact in this otherwise acid-sensitive strain.

The results of the complementation experiments corroborated the finding that the more acid-resistant strain was not necessarily the one exporting more GABA and that an extracellular GABA concentration of 0.16 mM was sufficient to support full expression of AR.

DISCUSSION

As unicellular organisms, bacteria are more vulnerable to sudden physicochemical environmental changes. To bypass the acid stress encountered in food or in the gastrointestinal tract of animal and human hosts, orally acquired bacteria apply various mechanisms to cope with this stress, among which the hydrolysis of urea by ureases (e.g., in *Helicobacter pylori*, *Klebsiella*, and all *Brucella* species except *B. ovis*) and the decarboxylation of different amino acids are the most effective (18, 29, 30). Based on the decarboxylation of Glu by the cytosolic enzyme glutamate decarboxylase (Gad) and the Glu/GABA antiport by the inner membrane protein GadC, the GAD system is the most efficient amino acid-dependent AR system in *E. coli*, *S. flexneri*, *L. monocytogenes*, and *Lactobacillus lactis* (18). In recent work, it was demonstrated that the GAD system is also functional as a *gadBC* operon in the newly described species *B. microti* but not in strain *B. suis* 1330, contributing to extreme AR *in vitro* and to the success of orally acquired infection in mice (23).

In the present work, we studied the functionality of the GAD system among a collection of strains of the genus *Brucella*. Our *in silico* analysis revealed that all *Brucella* species for which genome sequences are available possessed a *gadBC* locus homologous to those of *B. microti* and *E. coli*. However, only a definite group of strains may possess a potentially functional GAD system on the basis of the putative Gad proteins (Fig. 3). Experimental data confirmed that all existing isolates of *B. microti* ($n = 12$), *B. inopinata* BO1, *B. inopinata*-like BO2, and also several *B. ceti*, *B. pinnipedialis*, and *Brucella* species strains isolated from bullfrogs were GAD positive and exported GABA. Representative strains of these species were acid resistant at pH 2.5 in the presence of Glu. When the *gadBC* locus of these strains was used to complement a triple mutant strain of *E. coli* MG1655 deleted of its endogenous GAD system, glutamate-dependent AR was restored in this otherwise acid-sensitive strain. Among the *B. pinnipedialis* strains investigated in this study, the B2/94 strain, isolated from a common seal, was only weakly GAD positive and unable to export GABA, despite the presence of a putatively intact *gadBC* locus; mutations in as yet unidentified transcriptional regulators might be responsible for the observed GAD-negative phenotype. According to their *gadBC* sequences, *Brucella* strain 83/13 and *Brucella* sp. NF2653, isolated from Australian rodents (12), are also expected to have a functional GAD system.

In agreement with their putative GAD sequences, the representative strains of the terrestrial classical *Brucella* species tested (*B. abortus*, *B. canis*, *B. melitensis*, *B. neotomae*, and *B. suis*) were found to have a nonfunctional GAD system, i.e., were GAD negative, unable to export GABA, and highly sensitive to extreme acidity even after short-time incubation (Fig. 3). A nonfunctional GAD system was also confirmed for the *B. ovis* ATTC 25840 (63/290) and *B. suis* bv. 2 (Thomsen) strains. According to their putative GadB proteins, these strains were GAD positive, but due to their truncated GadC proteins, they did not export GABA, which finally makes them acid sensitive, comparable to the other classical *Brucella* strains. Genome analysis of 3 recently sequenced *B. suis* bv. 2 strains suggests identical Gad phenotypes (31).

Therefore, the intriguing question is the following: why was the functional GAD system lost in these host-adapted and important human food-borne pathogens, although it might have conferred

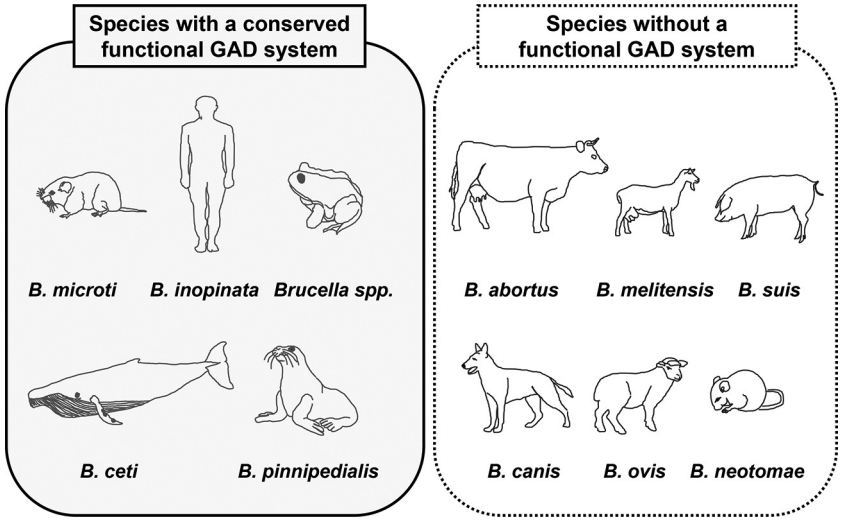

FIG 3 Distribution of *Brucella* species based on the functionality of the GAD system.

an advantage regarding survival in food and during passage through the gastrointestinal tract? The oral route is considered the most common route of infection in humans, which are only accidental hosts of *Brucella*; in livestock and domesticated animals, the pathogenic strains are preferentially transmitted by sexual or vertical routes, where the GAD system does not play a significant role. In fact, experimental infections of mice with *Brucella* spp. by the oral route succeed only with high doses of living bacteria $>10^9$ (CFU) (23, 32, 33). Thus, stomach acidity may significantly affect the survival of brucellae despite the presence of several factors allowing resistance to the acid conditions, such as urease and HdeA (19, 32–35). In addition, it is controversial that the primary invasion after ingestion of classical pathogenic *Brucella* spp. occurs through the intestine. In fact, several observations indicate that the mucosae of the face area (tonsils, lymph nodes, and conjunctival and respiratory mucosae), are the normal sites of entry of *Brucella* (36), which is consistent with the absence of a functional GAD system in most pathogenic *Brucella* species.

Our previous results with *B. microti* showed that the loss of a functional GAD system significantly reduces the number of living bacteria recovered from the target organs liver and spleen after oral infection of mice (33). Based on our present work, it seems conceivable that *Brucella* strains that have conserved this system may directly infect their hosts via the gastrointestinal tract. However, it must be stressed that the natural hosts and ecological niches of these strains are still unknown and the verification of this hypothesis is not straightforward, since appropriate animal models cannot be readily defined.

Our data suggested that the state of the GAD system may reflect the adaptive evolution of *Brucella* species for their respective environments and hosts. These agree with results of phylogenic tree analysis of the genus *Brucella*: five main groups of host-associated *Brucella* species rapidly diverged from a likely free-living ancestor close to *B. microti*, resulting in explosive radiation (37, 38). Our hypothesis is that, starting from a GAD-positive ancestor, all the strains adapted to specific hosts have lost this system, except those isolated from marine mammals, among which the *gad* locus is still conserved, though not equally expressed. Multiple alignments of the available sequences of *gadB* and *gadC* of *Brucella* strains included in this study (see Fig. S1 and S2 in the supplemental material) indicate that point mutations arose independently in each group, leading to truncated proteins. The genotypes of the two strains *B. ovis* ATTC 25840 and *B. suis* bv 2 (Thomsen) (*gadB* intact and *gadC* interrupted) indicate that in both groups of strains of *B. ovis* and *B. suis*, the loss of the GAD system is still in progress.

In several pathogenic bacteria, this evolutionary step can be accompanied by a loss or inactivation of specific genes which are not essential or even detrimental for the interaction with a host. These gene modifications, referred as "pathoadaptive mutations", may occur by point mutations, microdeletion, or insertional inactivation, as described for the lysine-cadaverine AR system of *S. flexneri* (39). However, there is no evidence indicating that this also applies to the GAD system of *Brucella*; in fact, the behaviors of the wild type and a Δ*gadBC* mutant strain of *B. microti* were identical during cellular and intraperitoneal murine infections. In addition, a mutant strain of *B. suis* 1330 expressing the functional GAD system of *B. microti* CCM4915 is neither advantaged nor affected in the cellular model of infection compared to the parental wild-type strain (23).

Because *gadBC* protects the bacterium from acid pH levels of <2.5, it may confer to marine and "new and atypical" *Brucella* species an adaptive advantage for survival in particularly acidic niches encountered in various environments, such as water, food, soil, and the gastrointestinal tract of hosts. In the case of *B. microti*, the bacterium was recovered from soil several years after its first isolation from voles (6). The other new and atypical *Brucella* species or strains may be also considered more environmental than the classical pathogenic species of the genus. Moreover, it cannot be excluded that a functional GAD system is conserved because it may play a role in the protection against other, as yet unidentified stresses. For example, it has been shown that GadC plays a role in protection against oxidative stress in *Francisella tularensis* (40) and that GadB is part of a compensatory pathway (GABA shunt) that partially overcomes metabolic defect in the interrupted TCA cycle, as in *L. monocytogenes* and *Mycobacterium tuberculosis* (41, 42).

In conclusion, our results showed that the GAD system has

been lost in classical pathogenic *Brucella* species, which are most adapted to livestock and human hosts, and it is conserved in new and in atypical strains of ancestral origin as well as in marine pathogenic *Brucella* species, which may have retained their ability to persist in acidic external and/or gastrointestinal host environments.

In addition, our data indicate that GadB activity may be useful to discriminate newly described or atypical species of *Brucella* which are GAD positive (*B. microti*, *B. inopinata* BO1, *B. inopinata*-like BO2, and strains from bullfrogs) from closely related *Ochrobactrum* species and also from classical terrestrial pathogenic species of *Brucella*, which are GAD negative. Thus, the simple, easy-to-handle, and inexpensive colorimetric Rice test may be added to other conventional and rapid biochemical tests used for the identification of new or atypical *Brucella* species. A more complete GAD database has to be established by increasing the variety and number of strains tested, including more atypical *Brucella* isolates.

ACKNOWLEDGMENTS

This work was supported by grant 1329-485 from the Federal Institute for Risk Assessment, Germany (to A.O. and S.K.), and by funding from Sapienza University of Rome and Fondazione Roma (to D.D.B.). Travels and research stays of A.O., D.D.B., M.A.D., and D.B. were supported by the 2011-2012 Galilée program of Egide (Hubert Curien program), no. 25960UE, from the French and Italian Ministry of Foreign and Europeans Affairs. M.A.D. was a recipient was awarded a postdoctoral fellowship from the foundation Infectiopôle Sud, and A.O. was awarded a Congés pour Recherches ou Conversions Thématiques (CRCT) from Montpellier University.

We thank E. Bianchini for her participation in genetic constructions of *E. coli* strains, B. Saadeh for preliminary GABA tests, C. Göllner for GAD tests, P. Bouhours for preparation of bacterial media, and I. Jacques and D. O'Callaghan for their generous gifts of several marine strains and of *B. inopinata* BO1 and *B. inopinata*-like BO2 strains, respectively.

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