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A Bile Salt Hydrolase of *Brucella abortus* Contributes to the Establishment of a Successful Infection through the Oral Route in Mice▽†

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Choloylglycine hydrolase (CGH), a bile salt hydrolase, has been annotated in all the available genomes of *Brucella* species. We obtained the *Brucella* CGH in recombinant form and demonstrated in vitro its capacity to cleave glycocholate into glycine and cholate. *Brucella abortus* 2308 (wild type) and its isogenic Δ*cgh* deletion mutant exhibited similar growth rates in tryptic soy broth in the absence of bile. In contrast, the growth of the Δ*cgh* mutant was notably impaired by both 5% and 10% bile. The bile resistance of the complemented mutant was similar to that of the wild-type strain. In mice infected through the intragastric or the intraperitoneal route, splenic infection was significantly lower at 10 and 20 days postinfection in animals infected with the Δ*cgh* mutant than in those infected with the wild-type strain. For both routes, no differences in spleen CFU were found between animals infected with the wild-type strain and those infected with the complemented mutant. Mice immunized intragastrically with recombinant CGH mixed with cholera toxin (CGH+CT) developed a specific mucosal humoral (immunoglobulin G [IgG] and IgA) and cellular (interleukin-2) immune responses. Fifteen days after challenge by the same route with live *B. abortus* 2308 cells, splenic CFU counts were 10-fold lower in mice immunized with CGH+CT than in mice immunized with CT or phosphate-buffered saline. This study shows that CGH confers on *Brucella* the ability to resist the antimicrobial action of bile salts. The results also suggest that CGH may contribute to the ability of *Brucella* to infect the host through the oral route.

Bile acids are synthesized in the liver and secreted into the bile mostly as conjugated bile acids in which the steroid moiety of the acid is linked to glycine or taurine by an amide bond. The amphipathic nature of the conjugated bile acids confers on them a detergent action that helps in the dispersion and enzymatic digestion of fats. By virtue of this detergent action, bile salts also exert an antimicrobial effect, and their second physiological function is to prevent bacterial overgrowth in the small intestine (8). Conjugated bile acids are deconjugated by the intestinal microflora through the action of bile salt hydrolases (E.C. 3.5.1.24), also known as choloylglycine hydrolases (CGH) or conjugated bile acid hydrolases (14). It has been postulated that resident bacteria produce these enzymes to reduce the detergent properties of bile salts and therefore their deleterious effects on bacterial membranes, thus enhancing bacterial survival in the gut (3, 6, 8). The production of bile salt hydrolases has been described mostly for bacteria from the indigenous gut microflora, including members of the genera *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, *Clostridium*, and *Enterococcus* (5, 9, 10, 15, 17). In other bacteria, including *Francisella tularensis* and *Bordetella pertussis*, a *cgh* gene has been identified by genomic analysis, but the enzymatic activity and the biological role of the encoded protein have not been characterized.

Genes coding for a putative CGH have been described for the genomes of *Brucella abortus* 2308 (BAB1_1488), *B. abortus* 9-941 (BruAb1_1463), *Brucella melitensis* 16M (BMEI0543), and *Brucella suis* 1330 (BR1468) (1, 2, 7, 12), but no studies have been performed to characterize the encoded proteins or their role in the biology of brucellae. While *Brucella* species do not reside in the gut, oral infection is one of the main forms in which the disease is acquired, both in humans and animals (11). Animals usually smell and lick fetal and placental tissues from abortions, most of which are caused by brucellae. Among humans, *Brucella* infection is frequently acquired through consumption of raw or undercooked meat or cheese made from unpasteurized milk. After reaching the gut, the bacterium must overcome the detergent action of bile acids before leaving the gut lumen. In consequence, it can be speculated that CGH expression is important for the success of *Brucella* infection through the oral route. In spite of the importance of oral infection in brucellosis, potential virulence factors involved in this mode of infection have not been assessed. Therefore, the purposes of the present study were to confirm the enzymatic nature of the putative CGH from *B. abortus* and to analyze its

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contribution to the success of oral infection in mice. We also assessed whether a specific anti-CGH local mucosal immune response can protect mice against such infection.

MATERIALS AND METHODS

Sequence analysis of *Brucella* CGH. BLAST software was used to search for homologies between the amino acid sequence of the CGH from *B. abortus* 2308 (CAJ11444) and those from other bacterial proteins. Multiple sequence alignment with bile salt hydrolases from other bacteria was performed with CLUSTALW. The amino acid sequences of *B. abortus* CGH and its homologues were also analyzed with SignalP 3.0 to search for signal peptides.

Production of recombinant CGH. The open reading frame of *cgh* (including its signal sequence) was amplified by PCR with primers 5′GCGGCTCATATGGC TTGCACGAGCTTC3′ (NdeI) and 5′TTCATGGGATCCTTACTTTTTCAGT GA3′ (BamHI) by use of *B. abortus* genomic DNA as the template (underlining indicates restriction sites for the enzymes). The amplicon was inserted into plasmid pET17b (Novagen, Madison, WI) and the recombinant plasmid was used to transform *Escherichia coli* JM109 competent cells. Miniprep-purified plasmid DNA from a colony containing the insert was used to transform *E. coli* strain BL21(DE3) competent cells (Stratagene). Recombinant CGH was successfully expressed in inclusion bodies of *E. coli* cells after induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Inclusion bodies were solubilized in 50 mM Tris-8 M urea (pH 8.0) and refolded by dialysis against phosphate-buffered saline (PBS) containing 1 mM dithiothreitol. Recombinant CGH was purified by anion exchange chromatography and adsorbed with Sepharose-polymyxin B (Sigma) to eliminate lipopolysaccharide contamination.

Measurement of the enzymatic activity of recombinant CGH. The CGH activity of the recombinant protein was measured in vitro essentially as described by Tanaka et al. (16) with slight modifications. Briefly, 100 μg of CGH (100 μl) was incubated at 37°C with 100 μl of 20 mM sodium glycocholate (Sigma) and 1.8 ml of phosphate buffer, pH 6.0, in the presence of 10 mM dithiothreitol. At different times, samples (50 μl) were obtained and the reaction was stopped with an equal volume of 15% trichloroacetic acid. Samples were centrifuged and supernatants were separated to measure the amount of released glycine. Samples (100 μl) were mixed with 500 μl of 1% ninhydrin, 200 μl of 0.5 M citrate buffer, pH 5.5, and 1.2 ml of glycerol. The mixture was heated in boiling water for 14 min and cooled in a water bath for 3 min. The absorbance of samples at 570 nm was interpolated in a curve obtained with glycine standard solutions. In some experiments, recombinant CGH was preincubated for 15 min with a mouse anti-CGH serum or with mouse normal serum before the addition of glycocholate.

DNA manipulations. (i) Construction of the *Brucella abortus* Δ*cgh* mutant strain. For the construction of a *cgh* (BAB1_1488) unmarked chromosomal mutant from *B. abortus* 2308, two PCR fragments of approximately 500 bp were generated immediately upstream and downstream of the *cgh* gene (complete open reading frame, including the signal sequence). Oligonucleotides cghF1 (5′-CGGG ATCCGTCCAGATTATCGAAAATGTC-3′) and cghR2 (5′-TCCAGACTGCTA CGTATCGCGAGAGAGCTTTTCGTTTCCAT-3′) were used to amplify a fragment including codons 1 to 7 of *cgh*, and oligonucleotides cghF3 (5′-GCGATACG TAGCAGTCTGGAGCGCCGTCACTGAAAAAGTAA-3′) and cghR4 (5′-GGA CTAGTCCCGTGACCGTGCGAGAAAAA-3′) were used to amplify a fragment including codons 327 to 333 of *cgh*. Both fragments (containing complementary regions) were ligated by overlapping PCR using oligonucleotides cghF1 and cghR4. The resulting fragment containing the *cgh* deletion allele was cloned into pBluescript KSII-*sacB*, which encodes the levansucrase from *Bacillus subtilis*. The plasmid was introduced into *B. abortus* 2308 by electroporation. The integration of the suicide vector into the chromosome was selected by resistance to carbenicillin (50 μg/ml) and sensitivity to sucrose (10% [wt/vol]) in tryptic soy agar (TSA) plates. A selected colony was grown overnight in tryptic soy broth (TSB) without antibiotics and plated in TSA-sucrose. Sucrose-resistant colonies were replica plated in TSA-carbenicillin. Carbenicillin-sensitive colonies were selected to confirm the excision of the plasmid and the generation of the mutant strain by allelic exchange. A colony PCR with primers cghF1 and cghR4 yielded an amplicon of 1 kbp in the mutant strain and a fragment of 2 kbp in the parental wild-type strain. The mutant strain carrying an unmarked deletion of *cgh* was called the Δ*cgh* mutant.

(ii) Complementation of the Δ*cgh* mutant by knock-in. Despite several attempts, it was not possible to complement the Δ*cgh* mutant by expression of the missing gene from a plasmid. Thus, we adopted a knock-in strategy, whereby the deleted gene was replaced at the original locus. A PCR fragment containing the wild-type *cgh* gene was obtained using oligonucleotides cghF1 and cghR4. This fragment was cloned into the pGEM-T Easy (Promega) suicide vector, and the resulting plasmid was electroporated into the *B. abortus* Δ*cgh* strain. Double recombination events were selected by resistance to 3% bile salts and by sensitivity to carbenicillin (50 μg/ml) in TSA plates. Colonies were screened by PCR with oligonucleotides cghF1 and cghR4 as described above. The production of CGH in the reconstituted strain was checked by Western blotting.

Assessment of *B. abortus* resistance to bile salts in vitro. Two in vitro methods were used to test the effect of CGH deletion on the resistance of *B. abortus* to bile salts. The wild-type strain and the Δ*cgh* mutant were grown in TSB up to an optical density at 600 nm (OD_{600}) of 1.0 and were resuspended to an OD_{600} of 0.1 in TSB alone or in TSB containing 5% or 10% bovine bile. Cultures were incubated at 37°C with shaking for 18 h, and aliquots were plated on TSA for CFU counting.

In the second method, both the wild-type strain and the Δ*cgh* mutant were grown in TSB to an OD_{600} of 0.6. Aliquots (200 μl) from these cultures were uniformly spread on TSA plates. Nitrocellulose discs (5 mm) embedded with different concentrations (0%, 5%, and 10%) of bovine bile were laid on the TSA plates, which were incubated for 48 h before the diameter of the inhibition zones was measured.

Infection of mice. BALB/c mice (10 per group) were infected by intragastric delivery of wild-type *B. abortus* 2308, its isogenic Δ*cgh* mutant, or the complemented mutant (all at 1×10^8 CFU) suspended in 300 μl of 10% sodium bicarbonate by use of a plastic feeding tube introduced through the mouth. Other mice (10 per group) were infected through the intraperitoneal route with 1.4×10^8 CFU of the wild-type strain, the Δ*cgh* mutant, or the complemented mutant. Five mice from each group were sacrificed at 10 and 20 days postinfection (p.i.), and spleens were removed. Dilutions of spleen homogenates were plated in duplicate on TSA for CFU counting. Data were expressed as $\log_{10}$ CFU per spleen. Differences between groups were analyzed with the Mann-Whitney U test (Prism 4.0; GraphPad Software, Inc.).

Oral immunization of mice with CGH and oral challenge with virulent *B. abortus*. BALB/c mice (10 animals per group) were immunized intragastrically with (i) 100 μg of recombinant CGH mixed with 5 μg of cholera toxin (CT) in 500 μl of PBS, (ii) cholera toxin alone, or (iii) PBS. Animals received three consecutive weekly immunizations. Twenty days after the last immunization, mice were challenged intragastrically with 1×10^8 CFU of *B. abortus* 2308. Animals were euthanized at 21 days postchallenge, and spleen CFU counts were determined as described above. The CFU data were normalized by log transformation, and differences between groups were analyzed by analysis of variance and Dunnett's posttest (Prism 4.0; GraphPad Software, Inc.).

Fecal extracts. Fecal extracts were prepared as follows. Five fecal pellets were collected from each mouse (housed in separate cages) and were mixed with 0.5 ml of extraction buffer (30 mM disodium EDTA, pH 7.6, 100 μg/ml soybean trypsin inhibitor, and 10 mg/ml bovine serum albumin in PBS). Pellets were homogenized and centrifuged at 4°C, and supernatants were stored at −20°C until immunoglobulin A (IgA) and IgG measurements were performed.

Measurement of anti-CGH antibodies. Specific anti-CGH antibodies were measured in serum samples and fecal extracts by indirect enzyme-linked immunosorbent assay (ELISA) using polystyrene plates coated with CGH (0.5 μg per well). Sera were dispensed in the appropriate dilution, while fecal extracts were used undiluted. Plates were incubated for 1 h (IgG) or overnight (IgA). Bound antibodies were revealed by incubation with specific conjugates (horseradish peroxidase labeled) against mouse IgA or IgG followed by a chromogenic solution (*o*-phenylenediamine–H_2O_2).

Assessment of the cellular immune response to CGH. The anti-CGH cellular immune response of immunized mice was assessed in vitro using splenocytes and cells derived from mesenteric lymph nodes. Spleens were cut into small pieces, homogenized in RPMI 1640, and filtered. Splenocytes were washed with RPMI and resuspended in complete culture medium (RPMI 1640, 10% fetal calf serum, 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin). Cells from mesenteric lymph nodes were obtained by crushing the nodes against a sterile steel mesh in a petri dish filled with 3 ml of RPMI. Cells were washed and resuspended in complete culture medium.

Splenocytes or lymph node cells were placed at 4×10^6 cell/ml in polypropylene tubes and were stimulated with antigen (CGH, 0.1 μg/ml) or mitogen (concanavalin A, 5 μg/ml) or were left unstimulated. After incubation for 48 h at 37°C in a 5% CO_2 atmosphere, cells were centrifuged at $450 \times g$ at 4°C, and culture supernatants were collected and kept at −70°C until cytokine measurements. Levels of gamma interferon, interleukin-2 (IL-2), IL-5, and IL-10 were measured in culture supernatants by use of commercial sandwich ELISA kits (OptEIA; Pharmingen).

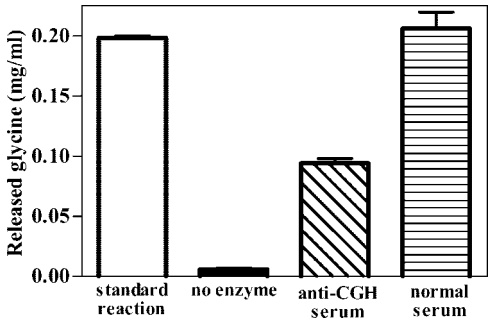

FIG. 1. In vitro cleavage of glycocholate by recombinant CGH. Released glycine was measured with the ninhydrin reaction. The same procedure was performed in the presence of mouse anti-CGH serum and normal mouse serum and in the absence of enzyme (to control for the spontaneous release of glycine).

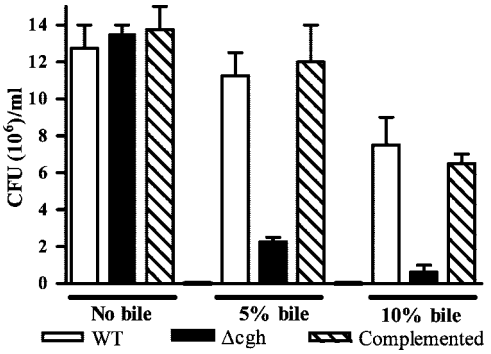

FIG. 2. Growth of *B. abortus* 2308 (WT), its isogenic Δ*cgh* mutant (Δcgh), and the complemented mutant in TSB containing different concentrations of bile. Data indicate the number of viable bacteria at 18 h of culture in each medium in triplicate experiments.

RESULTS

***B. abortus* CGH is homologous to other bile salt hydrolases.** As revealed by BLAST searches, the CGH from *B. abortus* 2308 is identical to those found in *B. abortus* 9-941 and *B. melitensis* 16M. In the CGH from *B. suis*, the Ala at position 149 is replaced by a Thr. In addition, BLAST revealed significant homologies with CGHs from *Bacillus cereus* (40% identity), *Vibrio fischeri* (37%), *Bacteroides fragilis* (36%), *Clostridium perfringens* (34%), *Lactobacillus plantarum* (30%), *Bifidobacterium bifidum* (31%), *Listeria monocytogenes* (29%), and many others.

CLUSTALW multiple sequence alignments of annotated CGHs revealed that the first 36 amino acids in the CGH sequences from *B. abortus* 9-941 and *B. melitensis* are absent in those of *B. suis and B. abortus* 2308 (see Fig. S1 in the supplemental material). SignalP analyses revealed that this sequence corresponds to a signal peptide with a probable cleavage site between Ala36 and Cys37. A review of the annotated genomes revealed that CGHs from *B. suis* and *B. abortus* 2308 also include this signal sequence, which was omitted in the annotation of these proteins (see Fig. S1 in the supplemental material). According to SignalP analyses, the CGHs from *Bordetella pertussis*, *Francisella tularensis*, *Rhodopseudomonas palustris*, and *Bacteroides thetaiotaomicron* contain signal peptides with probable cleavage sites between Ala and Cys residues homologous to those found for *Brucella* CGH (see Fig. S1 in the supplemental material).

In a previous study, the amino acid residues involved in the active site of the conjugated bile acid hydrolase from *Clostridium perfringens* were identified by X-ray crystallography and were shown to be conserved in the CGH sequences from other bacteria (13). As revealed by the multiple alignment performed in the present study (see Fig. S1 in the supplemental material), four of these six residues (Cys37, Arg53, Asn213, and Arg266) are conserved in the CGH sequences from *B. abortus*, *B. suis*, and *B. melitensis*, while a conservative change (Glu instead of Asp) is observed at position 56.

***B. abortus* CGH deconjugates bile salts.** *B. abortus* CGH was successfully expressed in inclusion bodies in *E. coli* BL21(DE3). To test the enzymatic activity of the recombinant CGH, the protein was incubated with sodium glycocholate and the released glycine was measured by the ninhydrin reaction. As shown in Fig. 1, the amount of released glycine was significantly higher in the presence of CGH than in the absence of the enzyme (virtually no glycine was released spontaneously). This enzymatic activity was reduced by a half when the enzyme was preincubated with a mouse serum against CGH but was unaltered in the presence of normal mouse serum (Fig. 1). The enzyme was active at pH values ranging from 6 to 9, but activity was somewhat higher at pH values 7 and 8 (not shown).

CGH confers on *B. abortus* resistance to bile salts. To determine whether CGH contributes to *B. abortus* resistance to bile salts, a Δ*cgh* deletion mutant was derived from *B. abortus* 2308. While the parental *B. abortus* strain grew on tryptic soy agar supplemented with 3% bovine bile, the Δ*cgh* mutant did not develop a visible growth (not shown). The complemented mutant was selected by its growth on agar containing bile, showing that the complementation restored the resistance to 3% bile observed in the wild-type strain. To obtain a quantitative measure of bile resistance, the wild-type strain of *B. abortus*, the isogenic Δ*cgh* mutant, and the complemented mutant were grown in broth containing different concentrations of bile, and growth was assessed by plating on agar and CFU counting. As shown in Fig. 2, all three strains exhibited similar growth levels in TSB in the absence of bile. The growth of the wild-type strain was virtually unaffected in the presence of 5% bile but was notably reduced in 10% bile compared to that seen when growth took place in TSB alone. The growth of the Δ*cgh* mutant was severely impaired by both 5% and 10% bile, and mean CFU counts were 5-fold and 12-fold lower, respectively, than those of the wild-type strain for the same culture conditions. Complementation restored the bile resistance of the mutant to levels similar to those observed for the wild-type strain (Fig. 2). Similar results were obtained in TSA cultures with discs embedded with bile. While the growths of the wild-type strain and the complemented mutant were unaffected under these conditions (no inhibition zones were observed), significant inhibition zones were observed for the Δ*cgh* mutant around discs embedded with 5% and 10% bile (diameters, 20 ± 5 mm and 58 ± 6 mm, respectively). These results indicate that CGH confers on *B. abortus* the capacity to resist the antimicrobial action of bile salts.

CGH deletion reduces *B. abortus* infectivity in mice. To test whether the reduced resistance of the Δ*cgh* mutant to bile

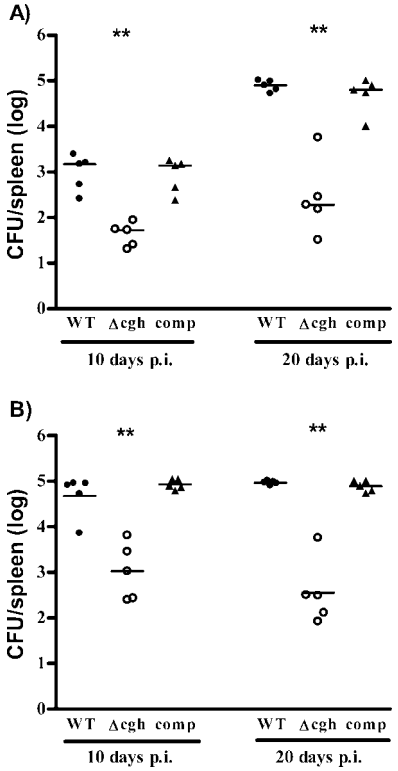

FIG. 3. Spleen colonization by *B. abortus* 2308 (WT), its isogenic Δ*cgh* mutant (Δcgh), and the complemented mutant (comp) following oral (A) or intraperitoneal (B) infection of mice.**, significantly different medians ($P = 0.0079$, Mann-Whitney U test).

could affect its infectivity through the oral route, the mutant and its parental strain were administered intragastrically to mice, and CFU counts in spleens were determined at different times p.i. As shown in Fig. 3A, at all times p.i. the splenic infection was significantly lower ($P = 0.0079$, Mann-Whitney U test) in mice infected with the Δ*cgh* mutant than in those infected with the wild-type strain. For comparison, mice were also infected intraperitoneally with both strains. As shown in Fig. 3B, splenic counts were also significantly lower ($P = 0.0079$) in mice infected with the Δ*cgh* mutant. These results suggest that CGH may be involved in *Brucella* infection through both the oral route and the intraperitoneal route.

Oral immunization with CGH protects mice from oral challenge. Since CGH is involved in the intestinal phase of *Brucella* infection through the oral route, experiments were carried out to test whether local mucosal anti-CGH immune responses could protect mice from infection. Mice were immunized intragastrically with recombinant CGH mixed with CT and were challenged by the same route with live *B. abortus* 2308. Mice receiving CT alone or PBS served as controls. At baseline, anti-CGH antibody levels were similar in the three immunization groups. As shown in Fig. 4, levels of anti-CGH IgA in serum, feces, and saliva increased after immunization in the CGH+CT group, and the same was true for serum levels of specific IgG. In contrast, anti-CGH antibodies remained at baseline levels in mice injected with CT alone or PBS (not shown). Upon in vitro stimulation with recombinant CGH, splenocytes and lymph node cells from mice in the CGH+CT group, but not those from the CT group or the PBS group, produced IL-2 levels significantly higher than those seen for unstimulated cells (Fig. 5). No specific production of gamma interferon, IL-5, or IL-10 was observed in any group of mice.

Mice were challenged 3 weeks after the last immunization. As shown in Table 1, CFU recovered from spleens 15 days after challenge were significantly lower ($P < 0.01$) in mice immunized with CGH+CT than in mice immunized with CT or PBS, yielding a level of protection of 1 log compared to the PBS group. These results, together with the attenuated oral infectivity of the CGH mutant, show that CGH is important for the success of *B. abortus* infection through the oral route.

DISCUSSION

Several bacteria that are naturally exposed to bile salts in the host express bile salt hydrolases that allow them to resist the antimicrobial action of these substances. As expected, most of these enzymes have been described and characterized for gut-dwelling bacteria, such as lactobacilli. However, the increasing number of sequenced bacterial genomes has revealed the existence of genes for such enzymes in bacteria that are not permanent gut dwellers. This is the case for *Brucella* spp., which can be acquired by ingestion but spread systemically. The bacterium must resist several noxious conditions in the gastrointestinal tract, cross the mucosal barriers, and reach the general circulation in order to disseminate. Since one of the adverse conditions faced by *Brucella* in the gut is the antimicrobial action of bile salts, the expression of a bile salt hydrolase, such as the CGH described here, would constitute an advantage for the pathogen. A *cgh* gene has been found for all the four *Brucella* species with published genomes, but no biochemical or functional characterization of the encoded enzyme has been performed.

We have found that the CGHs are virtually identical in all the four *Brucella* genomes available, although, as discussed below, some annotation differences have been detected. The enzyme has more than 50% amino acid similarity with well-described CGHs such as those from *B. fragilis*, *C. perfringens*, and *L. plantarum*. It is noteworthy that most of the amino acids previously identified as involved in the catalytic action of other CGHs (13) are conserved in the *Brucella* enzyme. Four of the six amino acids are identical, and a fifth one presents a conservative change (Glu instead of Asp at position 56). The sixth amino acid (position 119 in *Brucella* CGH) seems to be the less conserved among the different CGHs. Thus, the conservation in the *Brucella* CGH of most of the amino acids previously shown to be involved in CGH activity suggested that the protein would in fact display such enzymatic activity.

An intriguing apparent difference between the CGHs from *B. abortus* 2308 and *B. suis* and those from *B. abortus* 9-941 and *B. melitensis* was that the latter two enzymes contained a putative signal sequence while the former did not. When the annotation of the first two CGHs was checked against the corresponding genomes, it was evident that both enzymes also contain such a signal sequence, but it had been omitted during annotation in the Protein Data Bank, since an alternative starting Met (corresponding to position 35 in the CGH from *B. abortus* and *B. melitensis*) had been used. Therefore, the mature CGH would start at Cys37, which is homologous to the

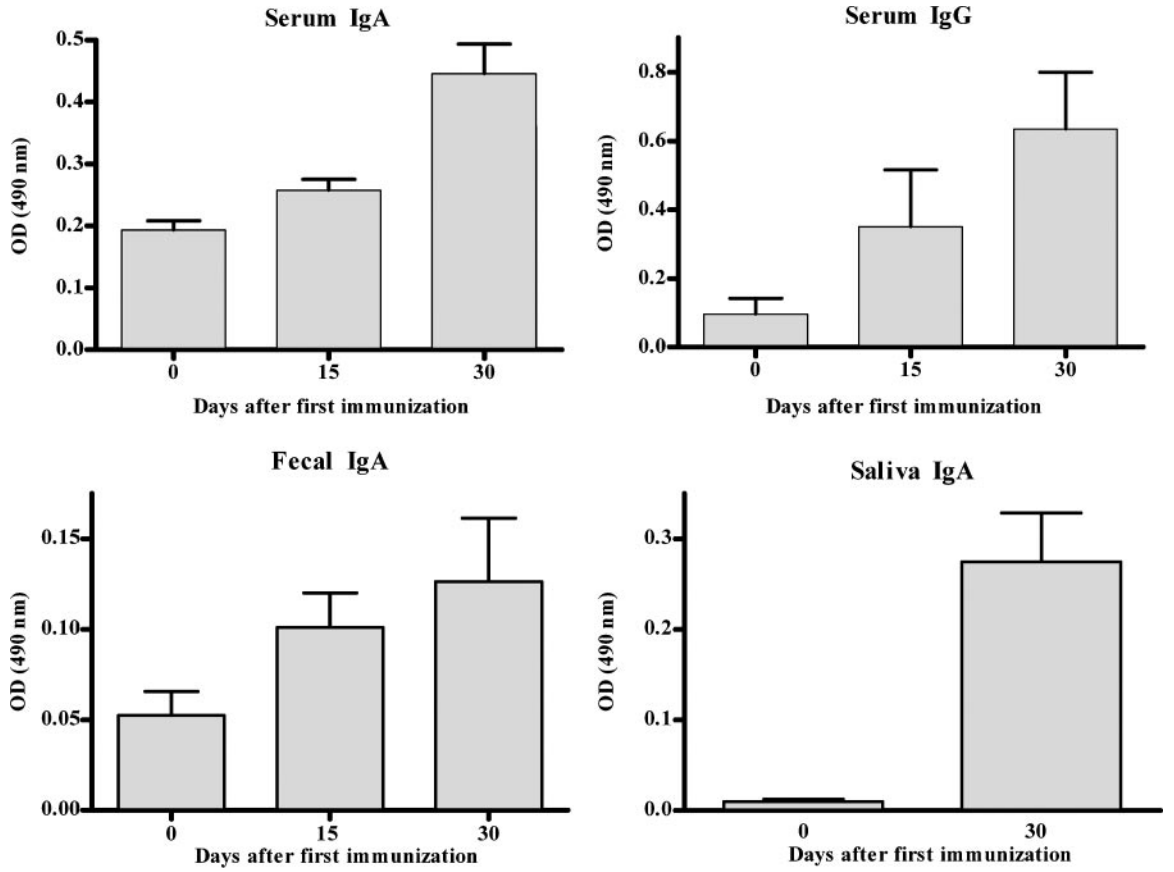

FIG. 4. Antibody responses elicited in mice by oral immunization with CGH plus cholera toxin (adjuvant). Levels of specific anti-CGH antibodies were measured by ELISA in serum, saliva, and fecal extracts.

Cys2 found for most other CGHs. In addition, CLUSTAL alignment and SignalP analyses revealed that other CGHs, such as those from *B. pertussis* and *F. tularensis*, also contain signal sequences. Notably, among CGHs containing signal peptides, the cleavage site is conserved and results in proteins with a free Cys at their N termini. The biological reasons for the existence of bacterial CGHs containing signal sequences and others lacking such sequences are currently unknown. Further studies will be necessary to assess whether such a difference determines a differential distribution of the enzyme in different species.

The enzymatic activity of the *Brucella* CGH was revealed by different experiments. The protein was obtained in recombinant form, and its capacity to cleave glycocholate into glycine and cholate was assayed in vitro. This assay revealed that the protein displays CGH activity and that this activity is partially

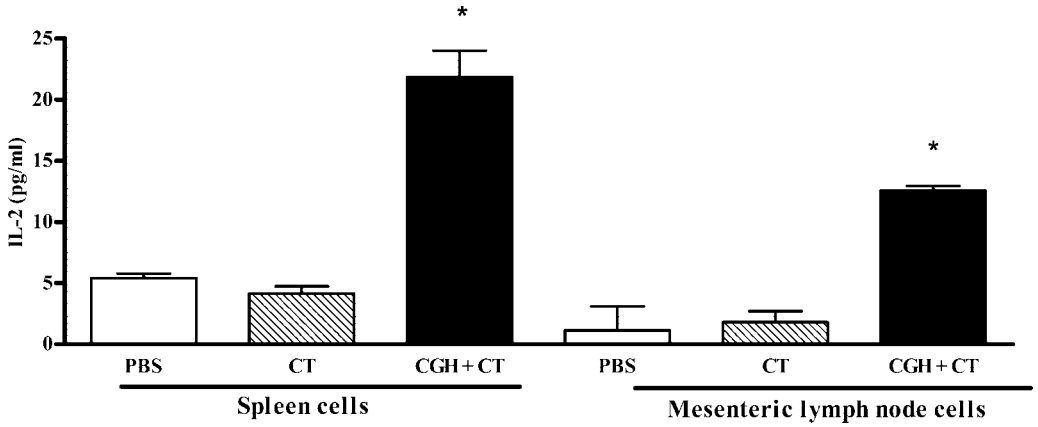

FIG. 5. Specific in vitro production of IL-2 by cells obtained from spleens and mesenteric lymph nodes of mice immunized with CGH plus cholera toxin. Cells were stimulated with recombinant CGH, and IL-2 levels were measured 48 h later in culture supernatants.

TABLE 1. Partial protection conferred by oral immunization with recombinant CGH against oral challenge with *B. abortus* 2308 in mice

Treatment group[a]	*B. abortus* organisms in spleen (log_{10})[b]	Protection level (log_{10})
PBS	4.20 ± 0.33	0
CGH+CT	3.22 ± 0.33[c]	0.98
CT	4.22 ± 0.44	0

[a] Mice (10 animals per group) were immunized with CGH+CT, CT alone, or PBS.
[b] The content of bacteria in spleens is represented as the mean log CFU ± standard deviation per group.
[c] This value was significantly different from the value for PBS-immunized mice ($P < 0.01$) as estimated by Dunnett's posttest.

inhibited by specific anti-CGH antibodies. In agreement with these results, we found that a *B. abortus* mutant lacking the *cgh* gene has reduced resistance to bile salts and that this defect is reverted in the complemented mutant. Overall, these results confirmed that the *Brucella* CGH can cleave bile salts and that this enzymatic activity enhances *Brucella* survival in a bile-containing environment. Therefore, it can be speculated that CGH expression may help *Brucella* to withstand the antimicrobial action of bile in the gut when the bacterium enters the host through the oral route. To test this hypothesis, a CGH deletion mutant was derived from *B. abortus* 2308, and mice were infected by intragastric delivery with the mutant and the parental strains. The assay revealed that CGH deletion affects *B. abortus* infectivity by the oral route, since bacterial counts from spleen were significantly lower for mice infected with the mutant. Therefore, CGH would constitute a virulence factor for *Brucella* species. In the case of *Listeria monocytogenes*, which like *Brucella* can infect through the oral route and disseminate systemically, deletion of the bile salt hydrolase reduces bacterial survival in the gut and its capacity to colonize the liver (4).

Given its action on bile salts, the participation of CGH in the infection process is assumed to take place mostly in the gut. Therefore, we decided to assess whether a specific local mucosal immune response against CGH could reduce *Brucella* infection in mice upon intragastric challenge. Mice immunized intragastrically with recombinant CGH developed a specific mucosal immune response, as revealed by the presence of anti-CGH IgA in feces and saliva and the specific production of IL-2 by cells isolated from mesenteric lymph nodes. Upon intragastric challenge with virulent *B. abortus*, the bacterial count in spleen was reduced 10-fold in immunized mice from that for nonimmunized controls. These results confirmed the involvement of CGH in the invasion capacity of *Brucella* through the gut.

While several results from this study support a role for CGH in the capacity of *Brucella* to infect through the oral route, the results of the intraperitoneal infection experiments suggest that the enzyme may also be involved in *Brucella* infectivity through systemic routes. It is possible that CGH can exert enzymatic actions other than bile salt deconjugation, and such actions could be involved in the infection process at a systemic level. CGH belongs to the family of linear amide C-N hydrolases (Pfam 02275), which also includes penicillin acylases and acid ceramidases. Members of this family are N-terminal nucleophile hydrolases, and most, including CGH, contain an N-terminal nucleophilic cysteine. In summary, members of Pfam 00275 share the ability to cleave C-N bonds and share structural motifs, including the N-terminal nucleophilic cysteine. Therefore, it can be speculated that CGH may cleave C-N bonds on substrates other than bile salts, which may eventually be directly or indirectly involved in the infectivity of *Brucella* through systemic routes. We have performed preliminary studies which indicate that the Δ*cgh* mutant has a reduced capacity of cellular invasion. At 4 h p.i. in the murine macrophage line J774, the mean intracellular CFU of the mutant were half of those of the wild-type strain. In contrast, the capacity of intracellular replication seemed to be preserved (not shown).

Overall, this study shows that the protein annotated as CGH in the genomes of different brucellae deconjugates bile salts and confers on these bacteria the ability to resist the antimicrobial action of these compounds. To our knowledge, this is the first protein described as a virulence factor of *Brucella* for oral infection. In addition, the enzyme also seems to be involved in processes relevant to infection through systemic routes. Studies are ongoing in our labs to try to elucidate the different mechanisms by which CGH confers on *Brucella* the ability to invade and survive in its mammalian hosts.

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