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Cloning and Sequencing of *yajC* and *secD* Homologs of *Brucella abortus* and Demonstration of Immune Responses to YajC in Mice Vaccinated with *B. abortus* RB51

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To identify *Brucella* antigens that are potentially involved in stimulating a protective cell-mediated immune response, a gene library of *Brucella abortus* 2308 was screened for the expression of antigens reacting with immunoglobulin G2a antibodies from BALB/c mice vaccinated with *B. abortus* RB51. One selected positive clone (clone MCB68) contained an insert of 2.6 kb; nucleotide sequence analysis of this insert revealed two open reading frames (ORFs). The deduced amino acid sequences of the first and second ORFs had significant similarities with the YajC and SecD proteins, respectively, of several bacterial species. Both the YajC and SecD proteins were expressed in *Escherichia coli* as fusion proteins with maltose binding protein (MBP). In Western blots, sera from mice vaccinated with *B. abortus* RB51 recognized YajC but not SecD. Further Western blot analysis with purified recombinant YajC protein indicated that mice inoculated with *B. abortus* 19 or 2308 or *B. melitensis* RM1 also produced antibodies to YajC. In response to in vitro stimulation with recombinant MBP-YajC fusion protein, splenocytes from mice vaccinated with *B. abortus* RB51 were able to proliferate and produce gamma interferon but not interleukin-4. This study demonstrates, for the first time, the involvement of YajC protein in an immune response to an infectious agent.

Brucellosis, a chronic infection resulting in abortion and infertility in animals and undulant fever in humans, is caused by *Brucella* species (1). Brucellae are gram-negative, facultative intracellular bacteria which can survive in macrophages of infected animals. Six well-recognized species of the genus *Brucella* display a certain host preference, although most can infect humans (6). Humans acquire the infection by ingesting contaminated dairy products or by contact with infected abortion-related animal tissues and secretions. Although some degree of protection can be induced in animals, mainly by vaccination with live attenuated strains, no satisfactory vaccine for humans has been described (6).

Smooth strains of *Brucella* have an O-polysaccharide chain attached to the core component of lipopolysaccharide, while truly rough *Brucella* strains completely lack such a structural moiety. Infection with smooth strains usually results in the production of antibodies against the O polysaccharide (30). These antibodies can have a protective role, at least in some animal species like the mouse (3, 4). Nevertheless, there is general consensus that a cell-mediated immune (CMI) response is necessary to induce strong protective immunity in most animal species since *Brucella* is able to survive within macrophages (4, 17, 36). Adoptive immunity can be induced in naive mice by the passive transfer of either $CD4^+$ or $CD8^+$ cells from immunized mice (4). Recent experimental evidence indicates that the induction of a Th1 type of CMI response with production of gamma interferon (IFN-γ) and generation of cytotoxic $CD8^+$ T cells appears to play an important role in protection against brucellosis, with one major role for IFN-γ being the activation of macrophages (11, 17, 19, 37, 38). Therefore, *Brucella* protein antigens which can stimulate Th1 responses may have good potential to induce protective immunity if presented to the immune system in an appropriate way.

Many antigens of *Brucella* have been described and characterized (7, 14, 16, 19, 21, 23, 33, 34, 39), but our understanding regarding the specific antigens involved in the stimulation of a protective Th1 type of CMI response is minimal. Only two specific *Brucella* antigens which are able to induce a partial, protective CMI response have been described: the L7/L12 ribosomal protein (12a, 18) and certain epitopes of the Cu/Zn superoxide dismutase (31). It is therefore important to continue to search for *Brucella* antigens able to induce a specific Th1 type of response with IFN-γ production, since such proteins could be used to develop safe and effective vaccines against brucellosis in animals and humans.

Using recombinant DNA methods, we are identifying and characterizing the genes of *Brucella abortus* proteins which have the potential to stimulate a Th1 response. Our approach to isolating such proteins is screening the genomic library of *B. abortus* for expressed antigens that react with mouse immunoglobulin G2a (IgG2a) subisotype antibodies, since this subisotype is indicative of a Th1 response (29). By following this screening strategy, we isolated several positive recombinant clones. In this paper, we describe nucleotide sequence analysis of one such clone which contained the *yajC* and *secD* genes of *B. abortus*. Using purified recombinant YajC protein, we further demonstrated that *Brucella*-infected mice develop humoral and CMI responses to this protein.

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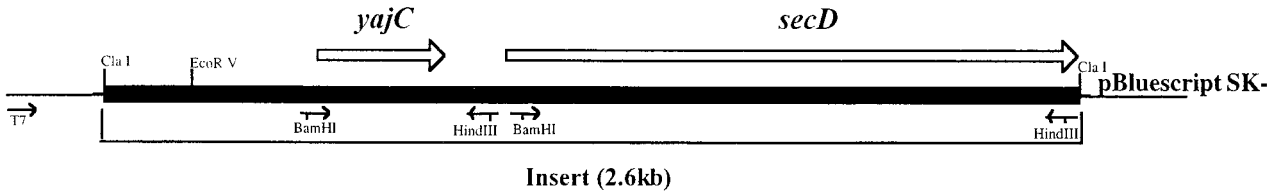
A



B

yajC	Bam HI
Forward primer	5' TCG CTC AAG GAT CCG GCA GCA TGG TTG G 3'
	Hind III
Reverse primer	5' GGG GGA TGA AGC TTT GCC GGA GTT CAC C 3'
secD	Bam HI
Forward primer	5' CTG ATA TGG GAT CCA GTC CTT GTA TGC CTC 3'
	Hind III
Reverse primer	5' TGC AGC TCA AGC TTC GAA CCG CCA C 3'

FIG. 1. (A) Schematic diagram of clone MBP68, which contains the *yajC* and *secD* genes of *B. abortus*. The locations of various primers used for PCR amplification of the genes are shown as arrows. The restriction enzyme sites engineered into the primers are indicated. (B) Nucleotide sequences of the primers used for the amplification of the *yajC* and *secD* genes.

MATERIALS AND METHODS

Bacterial strains. *B. abortus* vaccine strain RB51 and virulent strain 2308 were from our culture collection and were grown either in Trypticase soy broth or on Trypticase soy agar (TSA) plates as described elsewhere (26). *Escherichia coli* DH5α (GIBCO BRL, Bethesda, Md.) was used for the recombinant DNA manipulation of *Brucella* genomic fragments. All experiments with live *Brucella* were performed in a biosafety level 3 facility.

Antisera. Mouse antisera to *B. abortus* 2308, 19, and RB51 and to *Brucella melitensis* RM1 (a rough strain) were already available in our laboratory. These sera were collected at 8 weeks after intraperitoneal inoculation of BALB/c mice with viable bacteria equivalent to 1×10^6 CFU of strain 2308, 2×10^7 CFU of strain 19, 2×10^8 CFU of strain RB51, and 2×10^7 CFU of strain RM1 (36). For screening the plasmid library, mouse antisera to *B. abortus* RB51 were pooled and absorbed with *E. coli* DH5α/pBBR1MCS lysates to remove cross-reactive antibodies as described previously (23). Rabbit antiserum to maltose binding protein (MBP) of *E. coli* was purchased from New England Biolabs Inc., Beverly, Mass.

In conducting research using animals, we adhered to the National Research Council's *Guide for the Care and Use of Laboratory Animals* (5a).

Construction and screening of genomic library. The molecular biologic techniques performed in this study were based on the standard procedures outlined by Sambrook et al. (24). The genomic DNA of *B. abortus* 2308 was partially digested with *Cla*I, and the resulting fragments were cloned into a broad-host-range plasmid, pBBR1MCS (12). *E. coli* DH5α cells transformed with these recombinant plasmids were screened for antigen expression by a previously described colony immunoblot assay (22) with mouse antisera to strain RB51 as primary antibodies and horseradish peroxidase-conjugated goat anti-mouse IgG2a as secondary antibodies (Caltag Laboratories, San Francisco, Calif.). One such clone, MCB68, was isolated for further characterization.

Sequence analysis. The *Brucella* insert from clone MCB68 was subcloned into pBluescript SK(−) (Stratagene, La Jolla, Calif.), and unidirectional nested deletions from either ends of the insert were generated with the Erase-a-Base system (Promega Corp., Madison, Wis.). Nucleotide sequencing of the contigs was performed as previously described (25) with an automated DNA sequencer at the Walter Reed Army Institute for Research (D. Hoover). LaserGene sequence analysis software (DNASTAR, Inc., Madison, Wis.) was used for analyzing the nucleotide sequences for open reading frames (ORFs), inverted repeats, and hydropathy analyses and multiple alignment of protein sequences. Blast programs (2) were used for the homology searches in databases available at the National Biotechnology Information Center. Computer programs available on the Internet were used to predict the potential promoter sequence (the neural-network method of promoter prediction available at Lawrence Berkeley National Laboratory [http://www-hgc.lbl.gov/projects/promoter.html]) and potential transmembrane domains of YajC and SecD proteins (TMpred, prediction of transmembrane regions and orientation, available at the Swiss Institute for Bioinformatics [http://www.isrec.isb-sib.ch/software/TMPRED_form.html]).

Expression in *E. coli*. The YajC and partial SecD proteins of *B. abortus* were expressed in *E. coli* by using expression vectors $pMalP_2$ and $pMalC_2$, respectively (New England Biolabs Inc.). In these vectors, the cloned foreign gene is expressed as a fusion protein with MBP at the amino terminus so that the recombinant protein can be purified by affinity chromatography with amylose resin. The *yajC* and *secD* genes were PCR amplified from the genomic DNA of strain 2308. For each gene, a primer pair consisting of one primer upstream and one primer downstream of the gene was designed based on the nucleotide sequence of the gene (see Fig. 1). A restriction site was engineered into each primer by point mutations. PCR was performed with a 50-μl volume containing assay buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 0.1% Triton X-100), 1.5 mM $MgCl_2$, a mixture of the four deoxynucleoside triphosphates at 250 μM each, a 0.5 μM concentration of each primer, 50 ng of genomic DNA as a template, and 2.5 U of *Taq* DNA polymerase (Promega). Amplification was performed in an Omni Gene thermocycler (Hybaid Limited) at 95°C for 5 min followed by 30 cycles that each included 1 min of denaturation at 95°C, 2 min of annealing at 54°C, and 2 min of extension at 72°C. The amplified gene fragments were digested with appropriate restriction enzymes and cloned into $pMalC_2$. The recombinant plasmids were electroporated into *E. coli* DH5α, and single recombinant colonies were selected. Expression and purification of the recombinant proteins were performed according to the manufacturer's suggested procedures.

Overexpression in *B. abortus*. The YajC protein was overexpressed in *B. abortus* RB51 by cloning the gene along with its putative promoter in a broad-host-range vector, pBBR1MCS. The *yajC* gene from clone MCB68 was amplified with its downstream primer and the T_7 primer of the plasmid (see Fig. 1). The amplified fragment was digested with *Eco*RV and *Hin*dIII restriction enzymes and cloned into the same sites of pBBR1MCS. *E. coli* DH5α cells were transformed with the recombinant plasmid, and colonies containing the plasmid were selected on a TSA plate containing 30 μg of chloramphenicol per ml. From these colonies, plasmid DNA was extracted by using Mini Spinprep (Qiagen Inc., Valencia, Calif.). The plasmid was electroporated into *B. abortus* RB51 according to previously described procedures (15). *B. abortus* colonies containing the plasmid were obtained on a TSA plate containing 30 μg of chloramphenicol per ml. A single colony of transformed *B. abortus* was grown in Trypticase soy broth with chloramphenicol, and bacteria were harvested, heat killed (at 60°C for 20 min), and used for Western blot analysis. Strain RB51 harboring the pBBR1MCS plasmid containing the *yajC* gene was designated strain RB51/pBByajC.

Western blotting. Antigens of whole *Brucella* bacteria, extracts of *E. coli* expressing the MBP fusion proteins, and purified MBP-YajC protein were separated on 12.5% denaturing polyacrylamide gels by electrophoresis as previously described (13). From the gels the antigens were transferred to nitrocellulose

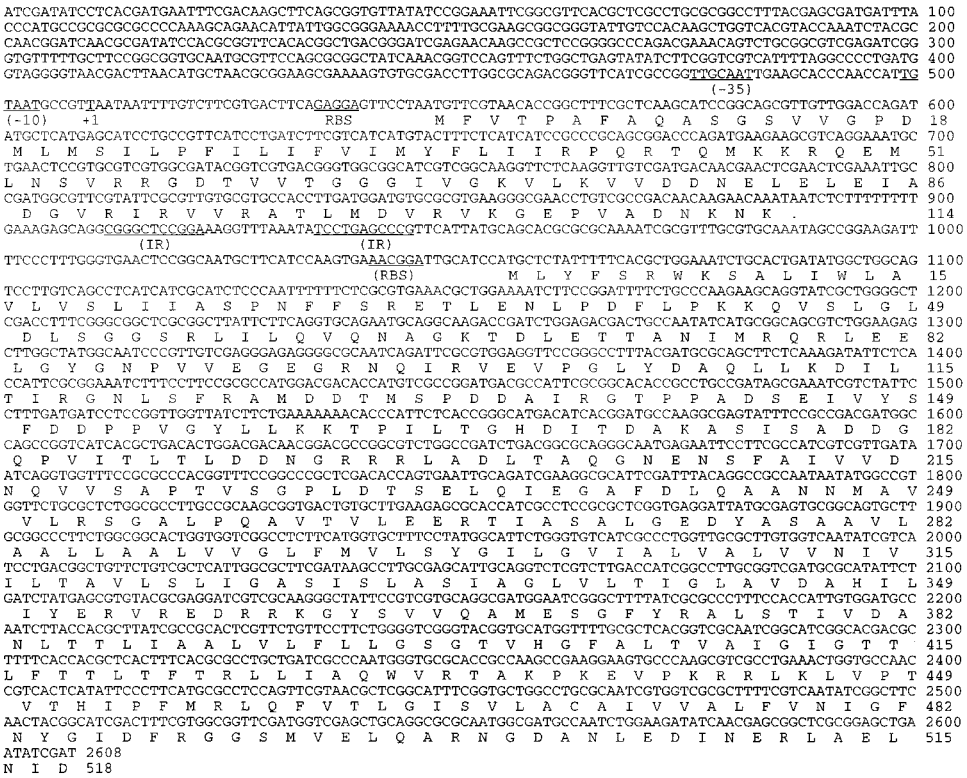


FIG. 2. Nucleotide and deduced amino acid sequences of the *yajC* and *secD* genes of *B. abortus*, present in clone MBP68. The putative promoter region (−10 and −35), transcription start site (+1), and ribosomal binding sites (RBS) are indicated. Inverted repeat sequences (IR) with the potential to form a hairpin loop are also indicated.

membranes according to a published procedure and developed (35). Briefly, the nitrocellulose membranes were blocked with 3% bovine serum albumin and reacted with various antisera and appropriate horseradish peroxidase-conjugated secondary antibodies. The membranes were developed with substrate solution containing 1-chloro-4-naphthol and hydrogen peroxide.

Lymphocyte proliferation assay. Assays were carried out as described elsewhere (28). Briefly, three 6-week-old female BALB/c mice (Charles River Laboratories, Wilmington, Mass.) were vaccinated intraperitoneally with 2×10^8 CFU of strain RB51 in 0.5 ml of saline. As a negative control, another three mice were inoculated with 0.5 ml of saline alone. Seven weeks postinoculation, the animals were killed by CO_2 asphyxiation and the spleens were obtained. Single cell suspensions were prepared from the spleens of normal and vaccinated mice. After the erythrocytes were lysed with ACK solution (150 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA [pH 7.3]), the splenocytes were cultured in 96-well plates at a concentration of 5×10^5 cells/well in the presence of 5 μg of recombinant YajC fusion protein, 5 μg of MBP (purified in a manner similar to that used for the YajC fusion protein), 10 μg of *B. abortus* RB51 crude extract, 0.5 μg of concanavalin A (ConA), or no additives (unstimulated control). RPMI 1640 medium (GIBCO BRL) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, and 50 μM 2-mercaptoethanol was used for culturing the cells. The cells were cultured for 3 or 5 days and then pulsed with 1 μCi of [^{3}H]thymidine/well for 18 h. The cells were harvested onto glass fiber filters, and the radioactivity was measured in a liquid scintillation counter and expressed as counts per minute. Assays were performed in triplicate.

The *B. abortus* RB51 crude extract used for stimulating the lymphocytes was prepared as follows. *B. abortus* RB51 grown on TSA plates was harvested in sterile distilled water, and the bacteria were killed by adding an equal volume of acetone at room temperature for 3 h with continuous stirring. The killed bacteria were washed twice in distilled water by centrifugation at 8,000 × *g* for 10 min each time. Finally, the pellet was resuspended in a solution containing 10% NaCl, 4 M urea, and 0.1% 2-mercaptoethanol. After incubation for 24 h in a 40°C water bath with shaking, the unlysed bacterial cells were removed by centrifugation at 8,000 × *g* for 10 min. The clear supernatants were collected, dialyzed against several changes of distilled water, and lyophilized. The required amount of the lyophilized extract was weighed and dissolved in RPMI 1640 medium and used for lymphocyte stimulation.

Quantitation of cytokines. After stimulation of the splenocytes with antigen or mitogen as described for the lymphocyte proliferation assay, supernatants of 3- or 5-day-old cultures were tested for the presence of IFN-γ and interleukin-4 (IL-4) by antigen-capture enzyme-linked immunosorbent assays (5). The assays were performed with plates coated with specific capture antibodies purified by protein G affinity chromatography (HiTrap Protein G column; Pharmacia Biotech, Uppsala, Sweden) from rat hybridoma culture supernatants produced in our laboratory (HB-170 [rat anti-murine IFN-γ] and HB-188 [rat anti-murine IL-4]; American Type Culture Collection, Rockville, Md.), biotinylated detection antibodies specific to IFN-γ and IL-4 (PharMingen, San Diego, Calif.), streptavidin-horseradish peroxidase conjugate (Zymed Corp.), and TMB (3,3′,5,5′-tetramethyl benzidine) one-component substrate (Dako Corp., Carpinteria, Calif.). Purified recombinant cytokines (PharMingen) were used for initial optimization of the assays and also as standards each time an assay was performed. The lower detection limits were calculated to be 100 and 10 pg for the IFN-γ and IL-4 assays, respectively. All assays were performed in triplicate, and the amounts of cytokines in the culture supernatants were calculated by using a linear-regression equation obtained from the optical density values of the standards.

Statistical analysis. The results of lymphocyte proliferation assays were analyzed by one-way analysis of variance, followed by the Student-Newman-Keuls method of all-pairwise multiple comparison. The data for IFN-γ production were analyzed by the Wilcoxon rank sum test. A P value of ≤0.05 was considered significant.

Nucleotide sequence accession number. The nucleotide sequence of the insert of clone MBP68 that contains the complete *yajC* gene and the partial *secD* gene has been submitted to GenBank. The accession no. is AF085217.

RESULTS

Clone MBP68 contains *yajC* and *secD* homologs of *B. abortus*. Clone MBP68 reacted strongly with the IgG2a subisotype antibodies of mouse antisera to strain RB51. The plasmid extracted from this clone contained an insert 2.6 kb in size. Both strands of the entire insert were sequenced. Sequence analysis revealed two ORFs (Fig. 1 and 2) separated by an intergenic sequence of 100 nucleotides. The first ORF was capable of coding for a 12.5-kDa protein consisting of 113 amino acids. The second ORF was incomplete at the 3′ end and capable of coding for a 54-kDa polypeptide with 518 amino acids. Both ORFs were preceded by a purine-rich ribosomal binding site.

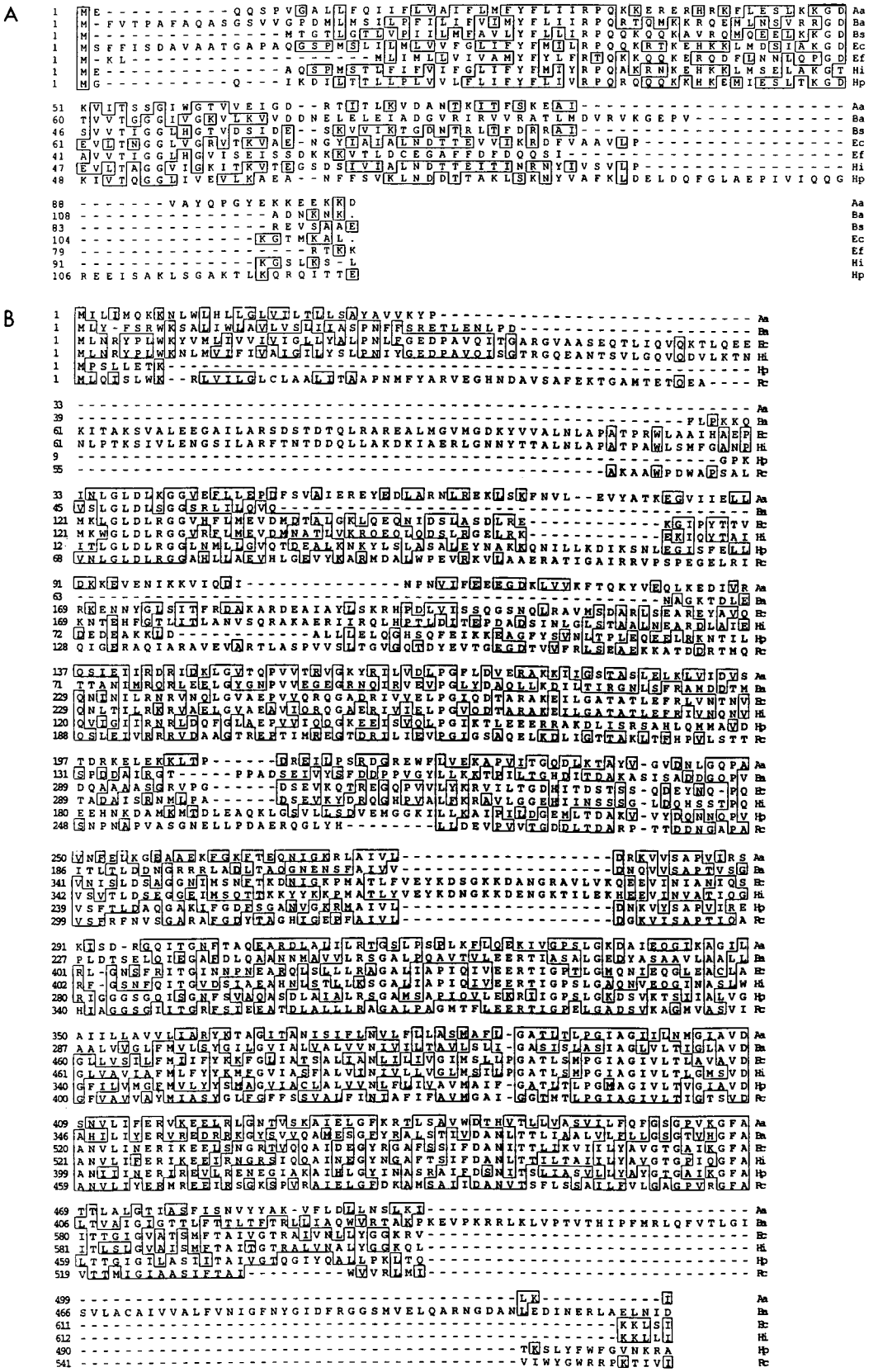


FIG. 3. Multiple alignment of deduced amino acid sequences of the YajC (A) and SecD (B) proteins. Alignment was performed with the Clustal method of the MegAlign program of LaserGene software. Aa, *Aquifex aeolicus*; Ba, *B. abortus*; Bs, *Bacillus subtilis*; Ec, *E. coli*; Ef, *Enterococcus faecalis*; Hi, *Haemophilus influenzae*; Hp, *Helicobacter pylori*; Rc, *Rhodobacter capsulatus*. Gaps are indicated by dashes. Amino acids identical for the two sequences are boxed. Based on this multiple alignment, the similarities between the YajC protein of *B. abortus* and those of the other bacteria are as follows: Aa, 26.5%; Bs, 30.3%; Ec, 27.9%; Ef, 31.7%; Hi, 22.7%; and Hp, 28.1%. The similarities between the SecD protein of *B. abortus* and those of the other bacteria are as follows: Aa, 23.4%; Ec, 24.7%; Hi, 24.9%; Hp, 26.2%; and Rc, 29.2%.

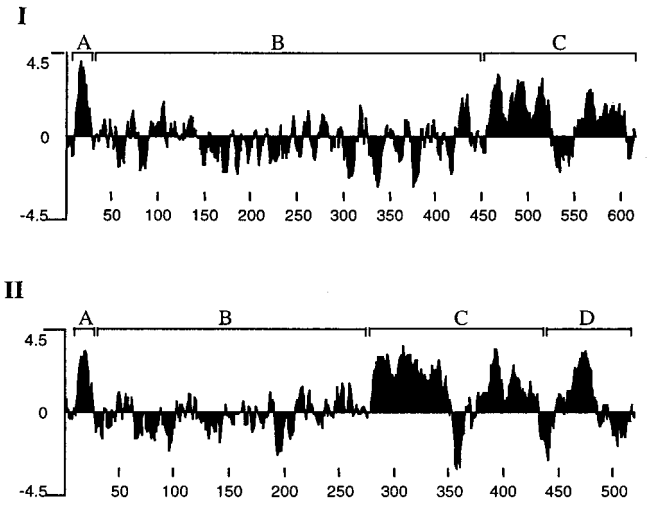


FIG. 4. Hydrophobic profiles of SecD proteins of *E. coli* (I) and *B. abortus* (II). Analysis was performed with the Kyte-Doolittle option of the Protean program of LaserGene software. The numbers at the bottom are the amino acid positions. The profiles are divided into segments for easy explanation in the text.

A putative promoter sequence was identified upstream of the first ORF, but no such obvious sequences were found in the intergenic sequence. A region containing inverted repeats that can potentially form a hairpin loop was found in the intergenic sequence downstream of the first ORF (Fig. 2). Homology searches performed with the deduced amino acid sequences of first and second ORFs revealed a significant similarity with the YajC and SecD protein homologs, respectively, of several bacterial species (Fig. 3).

Computer analysis predicted one (amino acids 19 to 38) and six (amino acids 9 to 25, 279 to 297, 301 to 317, 382 to 401, 401 to 422, and 460 to 482) potential transmembrane regions in the YajC and SecD proteins of *B. abortus*, respectively. Since the multiple alignment of the sequences of SecD proteins revealed several gaps, we compared their hydropathy profiles. As shown in Fig. 4, *B. abortus* SecD protein contained a region (segment D; segmentation was done for the purpose of easy explanation only) not contained in *E. coli* SecD protein. We included only *E. coli* SecD protein in the figure since other SecD proteins had hydropathy profiles similar to that of *E. coli* SecD. For all the SecD proteins, including that of *B. abortus*, the profiles of segments A and C were similar. However, there was variation among all SecD proteins in the length of segment B that did not contain major hydrophobic regions. Based on the topology of *E. coli* SecD protein (20), this segment is probably exposed to periplasmic space. The presence of the extra region (segment D) makes it difficult to estimate the full length of *B. abortus* SecD protein.

Expression of recombinant YajC and SecD fusion proteins. The expression of *B. abortus* YajC and that of partial SecD proteins were achieved in *E. coli* as MBP fusions. The extracts of *E. coli* cells containing the recombinant plasmids were analyzed by Western blotting with rabbit antisera to MBP (Fig. 5A). The observed molecular masses of the recombinant proteins were in accordance with the estimated molecular weights of the MBP fusions: 55 kDa for the YajC function protein and 97 kDa for the SecD fusion protein. A higher degree of proteolytic degradation was observed with the SecD fusion protein than with the YajC protein. The expressed recombinant YajC protein was purified by affinity chromatography on an amylose resin column (data not shown).

Involvement of YajC in immune responses against *Brucella* infections. To verify the involvement of YajC and SecD proteins in the immune response against *B. abortus*, Western blotting was performed with the expressed recombinant proteins and mouse sera against strain RB51. As shown in Fig. 5B, YajC, but not SecD, reacted with the immune sera. The purified recombinant YajC fusion protein also reacted with sera from mice inoculated with live *B. abortus* 19 and 2308 and *B. melitensis* RM1 (Fig. 5C). Sera from mice immunized with killed *B. abortus* RB51 and 19 did not react with the recombinant YajC protein (data not shown).

In lymphocyte proliferation assays, the purified recombinant YajC fusion protein stimulated the proliferation of splenocytes from mice vaccinated with *B. abortus* RB51 but not that of splenocytes from mice inoculated with saline only (Fig. 6). However, this proliferation was noticed in 3-day cultures but not in 5-day cultures. As a negative control, MBP alone was used to stimulate the splenocytes, and no significant proliferation was observed with either of the mouse groups. ConA significantly stimulated the proliferation of splenocytes from both vaccinated and normal mice (data not shown).

Culture supernatants of immune splenocytes stimulated with the recombinant YajC protein contained IFN-γ, but the levels were approximately 10 times lower than those induced by the RB51 crude extract or ConA in 5-day cultures and negligible in 3-day cultures (Table 1). IL-4 was not detected except in the cultures stimulated with ConA (data not shown). The observed lack of correlation between splenocyte proliferation and IFN-γ secretion after stimulation with YajC fusion protein was unexpected and probably reflects the variation in the kinetics of activation of different subpopulations of lymphocytes. Experiments with purified T-cell subpopulations need to be performed to address this possibility.

Immunodetection of YajC in *B. abortus* RB51. The presence of YajC protein in *B. abortus* RB51 was examined by Western blot analysis with mouse antisera. YajC was separated from the purified MBP-YajC fusion protein by factor Xa cleavage, and the resulting polypeptides were used as a positive antigen control for the Western blot analysis. No band of the molecular size corresponding to the YajC protein was detected in strain RB51 (Fig. 7). However, significant levels of YajC protein were detected in strain RB51/pBByajC (Fig. 7).

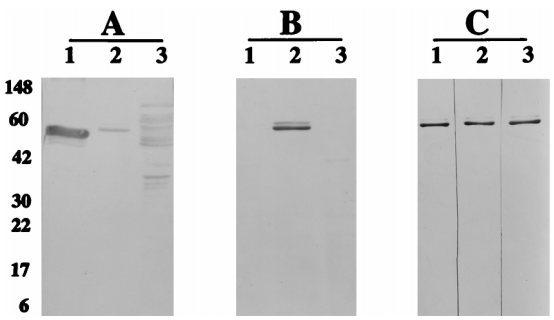


FIG. 5. Western blot reactivities of recombinant YajC and SecD proteins. (A and B) *E. coli* extracts expressing MBP fusions of LacZα peptide (lane 1), YajC (lane 2), and SecD (lane 3) were reacted with rabbit antisera to MBP (A) and antisera to *B. abortus* RB51 (B). (C) Reaction of affinity column-purified MBP-YajC fusion protein with mouse antisera to *B. abortus* 2308 (lane 1) and 19 (lane 2) and *B. melitensis* RM1 (lane 3). Horseradish-peroxidase-conjugated secondary antibodies specific to mouse IgG whole molecules and the IgG2a subisotype were used to develop the blots in panels B and C, respectively. Numbers at the left in panel A are approximate protein molecular masses, in kilodaltons. The appearance of a double band in the MBP-YajC lanes is due to some fusion protein with uncleaved signal sequence of the MBP.

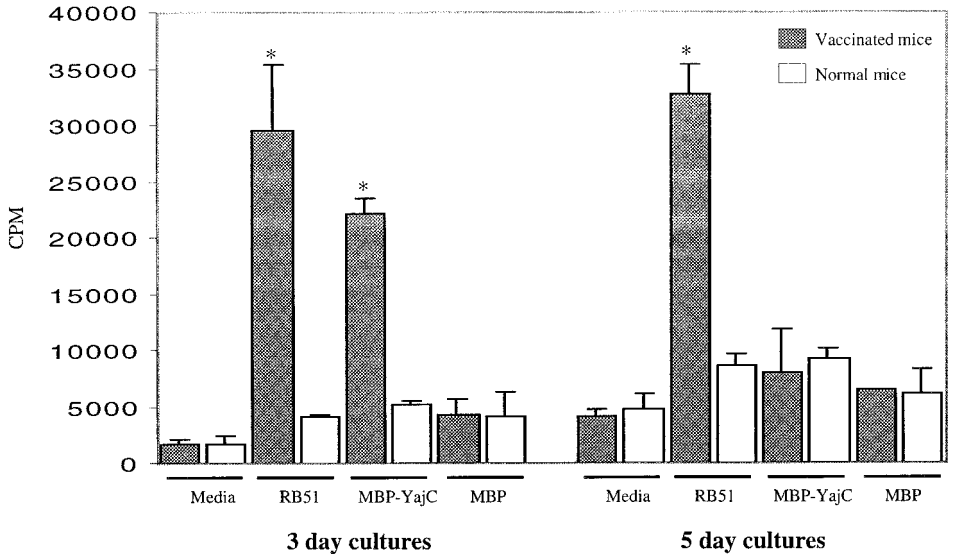


FIG. 6. In vitro proliferation of splenocytes from vaccinated and naive mice. The assays were set up in triplicate, and the cells were either left unstimulated (media alone) or stimulated with antigens. The radioactivity of incorporated [^{3}H]thymidine was measured after 3 or 5 days of culturing. Results were expressed as mean counts per minute ± standard deviation (n = 3). Groups with an asterisk are significantly different from other groups of cultures measured for the same number of days but not from each other.

DISCUSSION

In order to identify *B. abortus* protein antigens that can invoke a Th1 type of CMI response, we screened the genomic library of *B. abortus* 2308 for expressed antigens that reacted with IgG2a subisotype antibodies of mice vaccinated with protective strain RB51. Clone MBP68 was one of the several positive clones isolated from the library. The deduced amino acid sequences of the two ORFs from clone MBP68 showed homology with YajC and SecD protein homologs of several bacterial species. Even though the gene sequences of these two proteins for several bacterial species are available, studies on their structural and functional characterization have been limited to *E. coli*. The organization of these two *B. abortus* genes in an operon appears to be similar to that of *E. coli* (20). Like in *E. coli*, a potential hairpin loop structure was also present downstream of the *yajC* gene of *B. abortus*. The significance of the presence of this hairpin structure within an operon is not known. Generally, a hairpin loop structure present downstream of a gene functions as a rho-independent transcription terminator. In *E. coli*, YajC was initially identified as a hypothetical membrane protein whose gene was present in an operon along with the genes encoding SecD and SecF, members of the translocation machinery of secretory proteins (10). Genetic studies with *E. coli* suggested that YajC could also be involved in the translocation of secretory proteins (8, 9, 32). However, until now, to the best of our knowledge, the presence of YajC protein in any bacteria other than *E. coli* has not been demonstrated. The functional role of *B. abortus* YajC protein may be in the translocation of periplasmic or putative secretory proteins. Deletion mutation studies are presently ongoing in our laboratory to determine the role of YajC and SecD proteins in the extra- and intracellular survival of *B. abortus*.

Western blots developed with sera from *Brucella*-infected or RB51-vaccinated mice showed strong reaction with the recombinant MBP-YajC fusion protein (Fig. 5B and C), and the reactive antibodies included the IgG2a subisotype. Further, with the recombinant MBP-YajC fusion protein it was possible to

TABLE 1. Production of IFN-γ by splenocytes of vaccinated and naive mice after in vitro stimulation with specific antigens and mitogen

Stimulant	Concn of IFN-γ (ng/ml)[a]			
	3-day cultures		5-day cultures	
	Vaccinated mice	Naive mice	Vaccinated mice	Naive mice
Media	—[b]	—	—	—
ConA	30.55 ± 0.65*	28.31 ± 2.00*	27.96 ± 0.43*	26.05 ± 0.89*
RB51	29.63 ± 1.00*	—	29.12 ± 0.30*	—
MBP-YajC	0.42 ± 0.10**	—	2.90 ± 0.89**	—
MBP	—	—	—	—

[a] Values are means ± standard deviations for three experiments. Groups with two asterisks are significantly different from groups with one asterisk and are significantly different from each other.
[b] —, under lower detection limit.

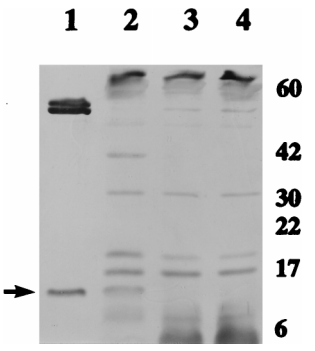


FIG. 7. Western blot detection of YajC protein in *B. abortus* RB51. The antigens present in the lanes are as follows: lane 1, MBP-YajC fusion protein partially cleaved by factor Xa; lane 2, extracts of *B. abortus* RB51/pBByajC; lane 3, extracts of strain RB51 with plasmid alone; lane 4, extracts of strain RB51. The blot was reacted with mice antisera to *B. abortus* RB51 and horseradish-peroxidase-conjugated goat antibodies specific to mouse IgG whole molecules. The arrow at the left shows the YajC band. The numbers at the right are approximate protein molecular masses, in kilodaltons.

stimulate splenocytes from *B. abortus* RB51-vaccinated mice to proliferate in vitro and produce IFN-γ. These findings clearly indicate that the YajC protein of *Brucella* is involved in stimulating a Th1 type of immune response in mice, although the levels of IFN-γ induced are low. Vaccination of mice with *B. abortus* RB51 induces protective immunity (26), and splenocytes from such mice produce substantial levels of IFN-γ when stimulated in vitro with the antigen extract of the immunizing strain, as demonstrated in this study and by others (27). This finding is consistent with the hypothesis that a protective immune response against *Brucella* needs the production of the Th1 type of cytokine IFN-γ. The levels of IFN-γ produced by the MBP-YajC-stimulated splenocytes of vaccinated mice were significantly lower than those stimulated with the strain RB51 antigen extract (Table 1). Similar observations were also reported for other recombinant *Brucella* antigens, including the L7/L12 protein, which was shown to be involved in stimulating a protective immune response (16, 18). Hence, the observed low levels of IFN-γ produced by the splenocytes stimulated in vitro do not exclude a role for YajC in protective immunity. As demonstrated before by others with a variety of other *Brucella* antigens (16, 18, 38), neither the RB51 extract nor the recombinant MBP-YajC protein stimulated the production of detectable levels of IL-4, suggesting that a Th2 type of response to these antigens is not a predominant component of the immune response. In Western blots, the lack of reactivity of mouse anti-RB51 sera with the MBP-SecD protein suggests that SecD was unable to induce antibodies in these mice. Since no further immunological studies were carried out with the MBP-SecD protein, a CMI response to this protein in the absence of a humoral response cannot be ruled out at this time; further studies are needed to confirm this.

The presence of YajC in *B. abortus* RB51 could be demonstrated only upon overexpression of the gene (Fig. 7). This indicates that, like in *E. coli*, where overexpression was also needed to demonstrate the presence of the YajC protein (8), a low level of YajC expression is seen in *B. abortus*. This low level of expression is probably responsible for the observed lack of antibody response to YajC protein in mice vaccinated with killed *B. abortus* vaccines. However, during a *Brucella* infection, YajC is produced in quantities sufficient to stimulate the immune responses demonstrated in this study. These results demonstrate, for the first time, the involvement of YajC protein in an immune response to an infectious agent. We are presently conducting investigations in our laboratory, using the purified recombinant protein, to determine the role of YajC in the induction of immune responses in vaccinated and infected animals such as cattle, pigs, and goats.

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