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Candidate Antigens for Q Fever Serodiagnosis Revealed by Immunoscreening of a *Coxiella burnetii* Protein Microarray[▽]

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Q fever is a widespread zoonosis caused by *Coxiella burnetii*. Diagnosis of Q fever is usually based on serological testing of patient serum. The diagnostic antigen of test kits is formalin-fixed phase I and phase II organisms of the Nine Mile reference strain. Deficiencies of this antigen include (i) potential for cross-reactivity with other pathogens; (ii) an inability to distinguish between *C. burnetii* strains; and (iii) a need to propagate and purify *C. burnetii*, a difficult and potentially hazardous process. Consequently, there is a need for sensitive and specific serodiagnostic tests utilizing defined antigens, such as recombinant *C. burnetii* protein(s). Here we describe the use of a *C. burnetii* protein microarray to comprehensively identify immunodominant antigens recognized by antibody in the context of human *C. burnetii* infection or vaccination. Transcriptionally active PCR products corresponding to 1,988 *C. burnetii* open reading frames (ORFs) were generated. Full-length proteins were successfully synthesized from 75% of the ORFs by using an *Escherichia coli*-based in vitro transcription and translation system (IVTT). Nitrocellulose microarrays were spotted with crude IVTT lysates and probed with sera from acute Q fever patients and individuals vaccinated with Q-Vax. Immune sera strongly reacted with approximately 50 *C. burnetii* proteins, including previously identified immunogens, an ankyrin repeat-domain containing protein, and multiple hypothetical proteins. Recombinant protein corresponding to selected array-reactive antigens was generated, and the immunoreactivity was confirmed by enzyme-linked immunosorbent assay. This sensitive and high-throughput method for identifying immunoreactive *C. burnetii* proteins will aid in the development of Q fever serodiagnostic tests based on recombinant antigen.

Coxiella burnetii is a gram-negative obligate intracellular bacterium and the etiological agent of the zoonosis Q ("query") fever. Human populations most at risk for infection are those routinely exposed to infected animals and their products. The organism has a diverse animal reservoir that includes domestic livestock such as dairy cattle, goats, and sheep. Chronically infected dairy cattle shed *C. burnetii* in milk and other secretions, and the products of livestock parturition can deposit tremendous numbers of the organisms into the environment. The insidious nature of *C. burnetii* is further exacerbated by the organism's aerosol route of infection, low infectious dose, and pronounced extracellular stability. Q fever most commonly manifests as a self-limiting but debilitating influenza-like illness that includes signs and/or symptoms of prolonged high fever, headache, and malaise. Chronic infection can occur, normally in predisposed individuals, that typically presents as a life-threatening endocarditis (reviewed in reference 17).

Two advancements that would aid in control of Q fever are (i) a specific and sensitive serodiagnostic test based on recombinant antigen and (ii) an efficacious and safe vaccine that does not require prevaccination skin testing. Human Q fever is currently diagnosed by clinical presentation and supporting serological responses against fixed, whole-cell phase I and phase II forms of the *C. burnetii* Nine Mile reference strain. Platforms for serological testing include immunofluorescence, complement fixation, enzyme-linked immunosorbent assay (ELISA) and microagglutination (21). Unfortunately, the complex nature of the whole-cell antigen results in lack of uniformity and specificity in test results. There is currently not a Food and Drug Administration-approved Q fever vaccine for use in the United States, although a killed-cellular vaccine (Q-Vax) is licensed in Australia (15). Q-Vax, along with other investigational vaccines based on formalin-inactivated organisms, are highly efficacious in prevention of Q fever by inducing both robust humoral and cell-mediated immune responses to *C. burnetii* antigens (33). However, these vaccines can cause severe local and occasionally systemic reactions in individuals previously sensitized to *C. burnetii.* Thus, skin testing for preexisting immunity against *C. burnetii* is required prior to vaccination (16). Since both current commercial serological tests and vaccines rely on intact organisms, a biosafety level 3 laboratory is required for antigen production.

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With the ready availability of pathogen genome sequences, reverse approaches to vaccine and diagnostic antigen discovery are feasible (18). Pathogen antigen screens using *E. coli*-expressed recombinant protein have used bioinformatic tools to prioritize proteins for screening. Reductive strategies are used to remove housekeeping proteins and other proteins present in nonpathogens, while retaining proteins predicted to interact with the host immune system, such as known or suspected virulence-associated proteins that are surface exposed or secreted (11). For example, by screening 156 bioinformatically selected *Chlamydia trachomatis* recombinant proteins with sera from urogenitally infected patients, novel antigens were revealed, some of which are secreted during the course of infection (24). Unbiased genome-scale antigen discovery using *E. coli*-expressed antigen has also been performed. A near-total proteome screen using purified recombinant protein was conducted with *Treponema pallidum*, wherein ca. 85% of the organism's predicted proteins were tested for serological reactivity in a 96-well plate platform (4). That study identified novel antigens that are differentially recognized by sera from patients in primary, secondary, and latent disease stages of syphilis.

Although informative, genome-scale antigen discovery using purified recombinant protein is labor intensive and *E. coli* expression of heterologous proteins can be problematic due to toxicity and insolubility. To circumvent these problems and to provide a more high-throughput platform, protein microarrays utilizing recombinant protein expressed by in vitro transcription and translation (IVTT) have been developed (2, 7, 8). In this procedure, whole-genome DNA libraries consisting of either individual plasmid-cloned genes or gene-specific transcriptionally active PCR (TAP) products are used as templates in IVTT reactions (7, 20). Nanoliter amounts of crude IVTT lysates containing synthesized protein are then spotted onto nitrocellulose-coated glass slides in a microarray format and screened for antibody reactivity using a fluorescence scanner (7). Davies et al. (7, 8) were first to describe an unbiased "immunoproteome" serological screen using IVTT synthesized protein. Using high-throughput methods of PCR amplification and in vivo recombinational cloning into a T7 promoter expression plasmid, these researchers identified 14 proteins within the vaccinia proteome that strongly react with vaccinia virus immun.globulin. This technology was later used to identify 48 and 103 immunodominant antigens of *Francisella tularensis* and *Borrelia burgdorferi*, respectively, from >80% of these pathogen's predicted proteomes (2, 10).

A more thorough understanding of the humoral response to *C. burnetii* infection is necessary for development of a new generation of Q fever diagnostics and vaccines based on recombinant antigen. To this end, we developed a *C. burnetii* protein microarray to comprehensively identify immunodominant antigens recognized in the context of *C. burnetii* infection or vaccination. A subset of these immunodominant antigens was expressed as recombinant proteins, purified, and examined for reactivity in a standardized ELISA format to correlate the predictive ability of the array-identified proteins to serve as defined antigen diagnostic reagents.

MATERIALS AND METHODS

Organism cultivation and chromosomal DNA isolation. The *C. burnetii* Nine Mile isolate (RSA493) in phase I was used in the present study. Organisms were propagated in African green monkey kidney (Vero) fibroblasts (CCL-81; American Type Culture Collection) grown in RPMI medium (Invitrogen, Carlsbad, CA) supplemented with 2% fetal bovine serum. Organisms were purified by Renografin density gradient centrifugation as previously described (5, 23). Total genomic DNA was isolated directly from purified *C. burnetii* using an UltraClean microbial DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA). An additional heating step (85°C for 30 min) was added before physical disruption of the bacterial cells. All DNA was resuspended in distilled H_2O and frozen at −20°C.

Generation of TAP products. TAP products were generated by using a modification of the Roche rapid translation system (RTS) *E. coli* linear template generation set, the His_6-Tag system (Roche Applied Science, Indianapolis, IN). This system generates TAP products compatible with Roche's RTS. The 5′ and 3′ gene-specific primers for first-round PCR were optimally designed by Sigma-Genosys (St. Louis, MO) to specifically amplify each *C. burnetii* ORF. Up to five codons were removed from the 5′ and/or the 3′ end of some ORF sequences to allow specific amplification of similar genes (e.g., paralog gene families). The 5′ end of each 5′ primer also contains the sequence CACCATGGGCGGC, which encodes tandem glycine codons (GGC), an ATG start codon, and the sequence CACC to allow potential directional TOPO cloning (Invitrogen) for downstream applications. The GGC codons were added to extend the length of common sequence on 5′ primers to allow annealing of primers for second-round PCR. The 5′ end of each 3′ primer contains the sequence TCCAGCAATAGTTGG GTTAAG, which encodes the epitope tag LNPTIAG. This peptide sequence constitutes an immunodominant epitope of the *C. trachomatis* major outer membrane protein and reacts with the monoclonal antibody L2I-10 (1). PCR products of *C. burnetii* ORFs were generated by using 12 pmol of ORF-specific primers, 20 ng of *C. burnetii* genomic DNA, and *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Reactions were conducted in 96-well plates, and the resulting PCR products were analyzed on a 0.8% agarose gel for the correct size. Aliquots of both positive and negative PCRs were transferred by using a multichannel pipetter to new 96-well plates for second-round PCR. Complementary second-round primers were used that incorporate unique overlap regions onto amplicon ends to allow subsequent annealing of DNA fragments encoding T7 regulatory elements and a C-terminal His_6 tag. Aliquots of second PCRs were transferred by using a multichannel pipetter to new 96-well plates for third-round PCR. Regulatory element and His_6 tag-encoding DNA fragments were added to wells and incorporated in the final TAP product by a third round of PCR using complementary primers. Second- and third-round PCR were conducted using Accuprime *Pfx* DNA polymerase (Invitrogen) and 2 μl of the previous round PCR as a template. Final PCR products were purified by using a Roche High-Pure 96 UF cleanup kit to remove nucleotides and primer dimers. TAP products were analyzed on a 0.8% agarose gel for the correct size, and aliquots of all final PCR products (both positive and negative for TAP product) were used in subsequent IVTT reactions. A schematic of the TAP product synthesis is shown in Fig. 1.

Generation of plasmid expression library. A revised version of a previously published high-throughput recombination cloning method (7) was used to generate plasmids expressing a subset of *C. burnetii* antigens. In this revised method, recombination was accomplished in vitro using the In-Fusion recombinase enzyme from Clontech. Custom PCR primers comprising 20 bp of gene-specific sequence with 33 bp of "adapter" sequences were used in PCRs with *C. burnetii* genomic DNA as a template. The adapter sequences, which become incorporated into the termini flanking the amplified gene, were homologous to the cloning site of the linearized T7 expression vector pXT7 (7). A total of 40 ng of PCR-generated linear vector was mixed with 10 to 50 ng of PCR-generated ORF fragment (molar ratio, 1:1; vector, 1-kb ORF fragment) and 0.1 U of In-Fusion enzyme (In-Fusion CF liquid PCR cloning kit; Clontech, Mountain View, CA) in a total volume of 10 μl. The mixture was incubated at 25°C for 30 min. For transformation, 2 μl of this mixture was added to 10 μl of competent *E. coli* DH5α cells. The mixture was incubated on ice for 45 min, heat shocked at 42°C for 1 min, and then chilled on ice for 1 min. Transformed cells were mixed with 250 μl of super optimal catabolizer medium (2% tryptone, 0.55% yeast extract, 10 mM NaCl, 10 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose), incubated at 37°C for 1 h, and then diluted into 3 ml of LB medium supplemented with 50 μg of kanamycin per ml. Cultures were incubated overnight with shaking. Plasmids were isolated and purified from cultures without colony selection. We have found that this method using In-Fusion can reduce the amount of linear vector and the amount of ORF PCR product needed for transformation and greatly reduce the background level of empty vector in the plasmid preparation. It also enables shorter "adapter" sequences to be used, which reduces the cost of the primers.

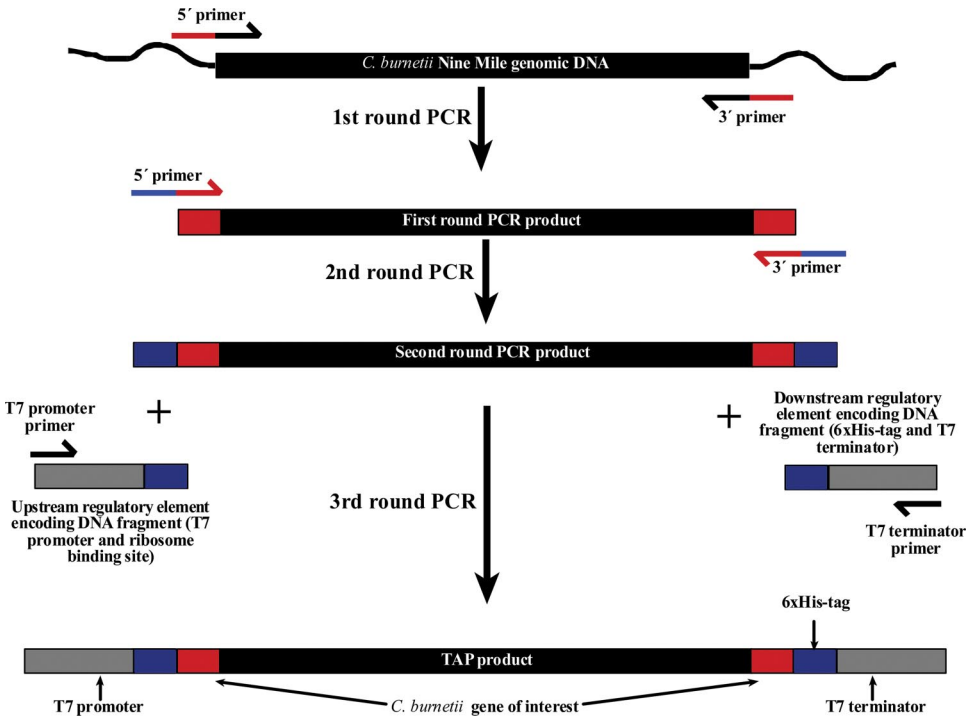

FIG. 1. Schematic of TAP product synthesis. *C. burnetii* Nine Mile ORFs were amplified in a first round of PCR using genomic DNA as a template and PCR primers consisting of gene-specific sequence and common 5′ overlap regions. TAP products were generated by an two additional rounds of PCR. Second-round primers were used that incorporate unique overlap regions onto amplicon ends to allow subsequent annealing of DNA fragments encoding T7 regulatory elements and a C-terminal His_6 tag that were incorporated into the final TAP by a third round of PCR using complementary primers.

IVTT expression of *C. burnetii* proteins. To produce IVTT protein from expression plasmids or TAP fragments, 10 μl of DNA template was added to individual wells of a 96-well Roche RTS 100 *E. coli* HY kit. IVTT reactions were incubated for 8 h at 30°C with shaking (600 rpm) in a Roche RTS Proteomaster. Samples were analyzed for the presence of C-terminal His_6-tagged protein by dot immunoblotting. Briefly, 3 μl of each IVTT reaction was spotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA), which was blocked for 1 h in PBST (10 mM sodium phosphate, 150 mM sodium chloride [pH 7.4], and 0.1% Tween 20) containing 5% nonfat dry milk. One microgram of Penta-His mouse monoclonal antibody (Qiagen, Valencia, CA) was then added, and the membrane was incubated for 1 h with rocking. The membrane was washed three times in PBST and then probed for 1 h with a peroxidase-conjugated anti-mouse immunoglobulin G (IgG) secondary antibody diluted 1:5,000 in PBST. Bound antibodies were detected by chemiluminescence using Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and Hyperfilm ECL (Amersham, Piscataway, NJ).

Human sera. Serum samples from acute and chronic Q fever patients, Q-Vax vaccinees, and naive individuals are part of an archived Australian serum bank and were obtained from B. Marmion. Additional naive serum samples were obtained from donors at the University of California–Irvine. Seroreactivity (IgG) of serum samples against *C. burnetii* was determined by using an indirect fluorescent antibody (IFA) kit (Focus Diagnostic, Cypress, CA) according to the manufacturer's instructions. Fifty-five acute Q fever patient sera collected between 38 and 172 days after the onset of clinical symptom were confirmed as having phase II IgG IFA titers ranging from 1:160 to 1:5,120. Five chronic Q fever endocarditis patient sera had both phase I and phase II IFA IgG titers ranging from 160 to 1,280 and from 640 to 5,120, respectively. Thirty-two serum samples from naive individuals were confirmed as IFA negative. Human serum samples were used according to Institutional Review Board protocol 2003-0419 (J. E. Samuel).

Microarray detection of immunoreactive *C. burnetii* antigens. Portions (10 μl) of 0.125% Tween 20 were mixed with 15 μl of each IVTT reaction (both His tag-positive and -negative reactions) to give a final concentration of 0.05% Tween 20. Then, 15-μl portions of IVTT reaction-Tween mixtures were then transferred to 384-well plates that were centrifuged at 1,600 × *g* to pellet any precipitated material. The supernatant was printed without further purification onto nitrocellulose-coated FAST glass slides (Schleicher & Schuell, Keene, NH) by using an OmniGrid 100 microarray printer (Genomic Solutions, Ann Arbor, MI). Protein microarray chip printing was conducted by Douglas Molina at Antigen Discovery, Inc., Irvine, CA. Prior to use with arrays, human sera (1:200 dilution) were incubated for 30 min with constant mixing in protein array blocking buffer (Whatman, Florham Park, NJ) that was supplemented with a lysate of *E. coli* at a final protein concentration 5 mg/ml (7). The arrays were rehydrated in blocking buffer for 30 min, incubated with the pretreated sera for 12 h at 4°C with constant agitation, washed in 10 mM Tris (pH 8.0)–150 mM NaCl containing 0.05% Tween 20 buffer, and then incubated with biotin-conjugated goat anti-human IgG (Fc-γ fragment-specific) serum (Jackson Immunoresearch, West Grove, PA) that was diluted 1:200 in blocking buffer. After the array slides were washed in 10 mM Tris (pH 8.0)–150 mM NaCl, bound antibodies were detected with streptavidin conjugated with the dye PBXL-3 (Martek, Columbia, MD). The washed and air-dried slides were scanned with a Perkin-Elmer ScanArray Express HT apparatus at a wavelength of 670 nm and with an output of RGB format TIFF files that were quantitated by using ProScanArray Express software (Perkin-Elmer, Waltham, MA) with correction for spot-specific background.

Cloning and expression of recombinant proteins. ORFs corresponding to *C. burnetii* immunoreactive proteins were amplified by PCR and cloned into the pBAD/TOPO ThioFusion expression vector (Invitrogen). Recombinant proteins were expressed as His_6-tagged fusion proteins in *E. coli* Top10 and purified by nickel affinity chromatography (Invitrogen).

Production of *C. burnetii* soluble antigen. A bead-beaten solubilized fraction of *C. burnetii* termed CBUSF was prepared as a positive control antigen. Briefly, formaldehyde-inactivated *C. burnetii* bacteria were frozen in liquid nitrogen for 1 min, thawed at 37°C for 1 min, and then disrupted by using FastProtein Blue Matrix in a FastPrep instrument (Q-Biogene, Irvine, CA) according to the manufacturer's instructions. This cycle was repeated three times. The final pellet was resuspended at 2% (wt/vol) in *N*-lauroylsarcosine (Sigma, St. Louis, MO) in distilled H_2O and mixed for 1 h. The supernatant was collected by centrifugation at 13,000 × *g* for 5 min and then dialyzed against distilled H_2O. The final product was quantified by using a Micro-BCA protein assay kit (Pierce).

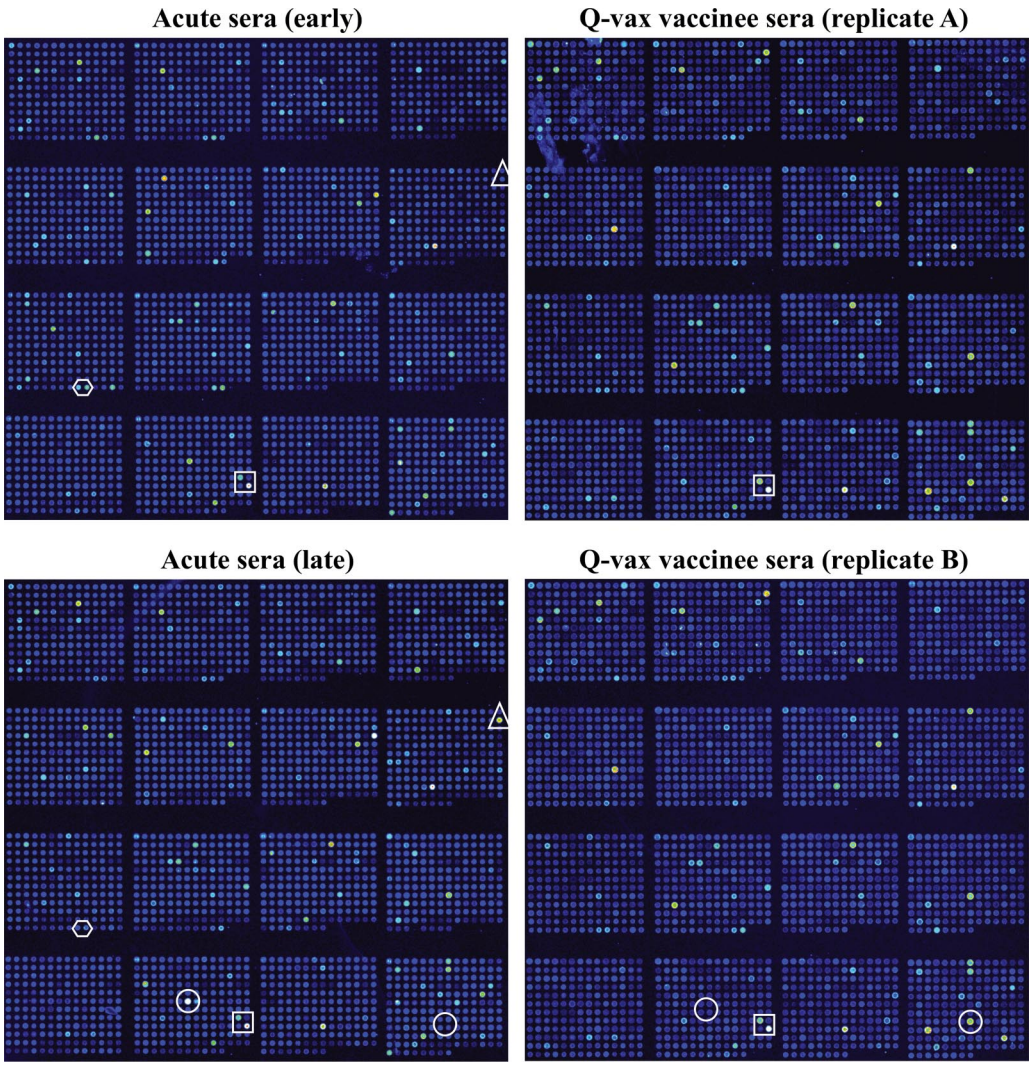

FIG. 2. Protein microarray analysis of the human humoral response to *C. burnetii* infection or vaccination. Microarrays were probed with paired "early" and "late" acute Q fever patient sera that was obtained at 20 and 96 days, respectively, after the onset of clinical Q fever (left panels) or in duplicate with serum from a human Q-Vax vaccinee (right panels). A laser confocal scanner was used to visualize reactive proteins with bound PBXL-3-conjugated streptavidin. Proteins within boxes are recognized by both Q fever patient and vaccinee sera. Proteins within circles are differentially recognized by Q fever patient and vaccinee sera. Proteins within hexagons and triangles are differentially recognized by early and late Q fever patient sera, respectively.

ELISA. Microplates (96 well; Fisher Scientific, Pittsburgh, PA) were coated overnight at 4°C with 100 μl of a 2-μg/ml antigen solution. Plates were then blocked with 200 μl of 0.5% nonfat milk for 2 h at 37°C. Then, 50 μl of a 1:50 dilution of human serum was added to each well, and the plates were incubated for 1 h at 37°C. Plates were washed three times with PBST, and then 100 μl of a 1:5,000 dilution of biotin-labeled goat anti-human IgG gamma chain-specific antibody (Sigma) was added to each well. Plates were incubated for 1 h at 37°C, wells were washed three times with PBST, and then 100 μl of ABC solution from a Vectastain Elite PK-6100 kit (Vector Laboratories, Inc., Burlingame, CA) was added to each well. After a 1-h incubation at room temperature, the plates were washed with PBST, and the peroxidase activity was detected with *o*-phenylenediamine dihydrochloride (Sigma). Substrate reactions were developed for 15 min and then stopped with 100 μl of 1 M H_2SO_4. Reactions were measured at 490 nm by using a Spectra Max M2 (Molecular Devices, Sunnyvale, CA), and data were analyzed by GraphPad Prism (San Diego, CA). A positive cutoff for a given antigen was defined as the mean absorbance of IFA-negative samples plus two standard deviations. The percent sensitivity for a given antigen was calculated as the number of ELISA-positive sera from the IFA-positive pool divided by the number of IFA-positive sera. The percent specificity for a given antigen was calculated as the number of ELISA-negative sera from the IFA-negative pool divided by the number of IFA-negative sera.

Bioinformatics. Potentially secreted *C. burnetii* antigens were identified by using a list of predicted *C. burnetii* secreted proteins compiled by the PathoSystems Resource Integration Center (PATRIC) (http://patric.vbi.vt.edu/special_projects/?amode=view&spId=8). The subcellular location of immunogenic proteins was predicted by using PSORTb.

RESULTS AND DISCUSSION

Synthesis and characterization of TAP products. A total of 1,988 ORFs representing 97.2% of *C. burnetii*'s 2,046 coding sequences were successfully amplified into TAP products (Fig. 1). Protein was synthesized from TAP products by IVTT and dot blots performed to ascertain the number of reactions that produced full-length recombinant protein. Based on a positive reaction between the Penta-His monoclonal antibody and the

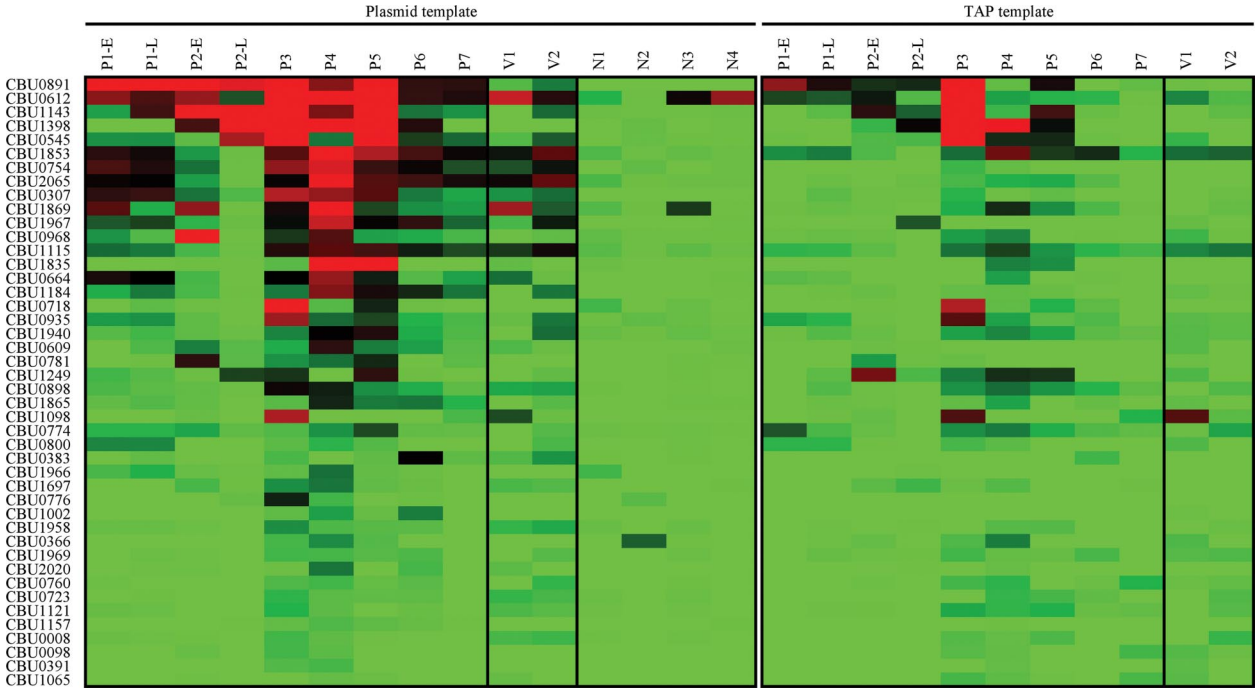

FIG. 3. Fluorescence intensity plots of plasmid- and TAP fragment-based protein microarrays probed with human sera. Plasmid- and TAP fragment-based protein microarrays were probed individually with sera from two Q-Vax vaccinees (V1 and V2) and seven acute Q fever patients (P1 to P7) that included paired early (E) and late (L) serum from patients 1 and 2. Arrays were also probed with sera from 16 *C. burnetii*-naive individuals (N). The average fluorescence intensity values were determined for each protein, with the intensity plot showing the most reactive proteins in red. Proteins are ordered from top (most reactive) to bottom (least reactive) according to the fluorescence intensity values of plasmid-based arrays. Four representative intensity plots of plasmid-based arrays probed with naive serum samples are shown, while only the intensity plots of TAP-fragment-based arrays probed with patient sera are shown.

C-terminal His_6 tag, full-length recombinant protein was produced from 1,492 TAP products representing 72.9% of the predicted *C. burnetii* proteome (data not shown). Based on signal intensities, there was obvious variation in the amount of protein produced by IVTT from individual TAP products. There were deficiencies in the production of the previously described *C. burnetii* antigens Hsp60 (CBU1718) (32), which was not amplified, and Com1 (CBU1910) (12), which was amplified into a TAP product but poorly expressed by IVTT.

Immunoproteome characterization by protein microarray. Protein microarrays were generated by spotting His_6 tag-positive and -negative IVTT reactions onto nitrocellulose-coated glass slides. Antigen-specific antibody responses to *C. burnetii* infection or vaccination were then assessed by individually probing microarrays with sera derived from five Q-Vax vaccinees, five Q fever patients (both early and late serum samples), and six naive individuals.

Representative fluorescence signals of probed microarrays are depicted in Fig. 2. Arrays probed with naive serum showed few immunoreactive proteins (data not shown). Conversely, arrays probed with early (20 days after the onset of clinical symptoms) and late (96 days after the onset of clinical symptoms) acute Q fever serum showed multiple immunoreactive proteins with stronger signal intensities for individual proteins generally associated with arrays probed with late immune sera. A subset of proteins was selectively recognized by early or late sera. Temporal development of antigen-specific antibody responses has been previously described in a guinea pig model of Q fever (6). Early antigens would likely be optimal as Q fever serodiagnostic antigens, while late antigens, with their more prominent role in development of protective immunity, may prove more efficacious in subunit vaccines. Although there was obvious overlap between proteins recognized by Q fever convalescent and Q-Vax vaccinee sera, each serum type also recognized a unique subset of proteins. Proteins selectively recognized by patient sera may represent secreted proteins that are present at low levels in killed whole-cell vaccine preparations (discussed in more detail below). Arrays probed in duplicate showed nearly identical fluorescence patterns, demonstrating the reproducibility of the microarray procedure. Some immunoreactive proteins were His tag negative, indicating IVTT can incompletely synthesize proteins to result in C-terminal truncations (data not shown).

Comparison of TAP product and plasmid-based arrays. Results in our laboratory suggested that protein is more efficiently synthesized by IVTT from expression plasmids than from TAP fragments. Therefore, to directly compare the two methods, we cloned ORFs encoding the 44 most immunoreactive proteins identified above from the TAP product proteome array into a T7 expression plasmid. Proteins were then generated by IVTT from both plasmid and TAP templates and printed as single replicates onto smaller arrays. Arrays were then probed with 9 sera from acute Q fever patients (including two pairs of early and late sera), two Q-Vax vaccinee sera, and 16 naive sera. As depicted in Fig. 3 and detailed in Table 1, patient and vaccinee sera generally recognized the same proteins on plasmid- and

TABLE 1. Forty-four immunoreactive *C. burnetii* proteins identified by protein microarray[a]

ORF no.	Protein function	ORF name	Signal peptide[b]	Predicted subcellular location[c]	Avg array signal intensity[d]					
					Plasmid			TAP		
					P	V	N	P	V	N
CBU0891	Hypothetical exported membrane-associated protein		+	Unknown	35,248	3,618	124	12,752	111	–
CBU0612	Outer membrane protein OmpH	*ompH*	+	Outer membrane	24,825	20,955	2,881	9,277	3,540	762
CBU1143	Preprotein translocase, YajC subunit	*yajC*	+	Noncytoplasmic	23,756	3,222	665	9,927	521	10
CBU1398	2-Oxoglutarate dehydrogenase	*sucB*		Cytoplasmic	21,849	–[e]	224	11,471	–	31
CBU0545	LemA protein	*lemA*		Unknown	15,922	4,348	248	7,689	1,393	–
CBU1853	GtrA family protein			Cytoplasmic membrane	15,594	14,574	563	6,644	6,529	443
CBU0754	Efflux transporter, RND family, MFP subunit		+	Cytoplasmic membrane	13,898	8,861	297	374	–	–
CBU2065	Hypothetical exported protein			Cytoplasmic membrane	13,577	15,646	440	1,276	–	35
CBU0307	OmpA-like transmembrane domain protein		+	Outer membrane	12,930	5,479	60	643	219	–
CBU1869	Hypothetical exported protein		+	Noncytoplasmic	12,281	15,781	778	2,309	342	247
CBU1967	Drug resistance transporter, Bcr/CflA family		+	Cytoplasmic membrane	10,013	6,071	46	812	322	–
CBU0968	Phospholipase D		+	Cytoplasmic membrane	9,850	634	265	1,213	1,360	18
CBU1115	Hypothetical protein			Unknown	9,085	10,329	498	3,435	5,584	227
CBU1835	Protoporphyrinogen oxidase			Cytoplasmic	8,558	427	28	1,148	–	–
CBU0664	Transposase, ISAs1 family			Cytoplasmic	8,422	3,334	712	740	–	73
CBU1184	Acyltransferase family protein			Unknown	7,432	2,943	49	296	495	–
CBU0718	Hypothetical membrane-associated protein			Unknown	6,335	–	199	3,461	–	–
CBU0935	RNA-binding protein		+	Unknown	5,997	3,258	304	3,619	1,182	311
CBU1940	ATP synthase F0, C subunit			Cytoplasmic membrane	4,539	3,128	401	1,931	1,364	76
CBU0609	Mevalonate kinase			Unknown	4,234	1,206	73	54	793	–
CBU0781	Ankyrin repeat protein	*ankG*		Secreted	4,186	–	420	561	152	52
CBU1249	DNA-binding protein			Unknown	4,017	467	324	5,260	1,002	95
CBU0898	Thyroglobulin type 1 repeat domain protein			Unknown	3,914	3,834	135	2,427	900	58
CBU1865	Hypothetical membrane-associated protein			Cytoplasmic membrane	3,225	758	246	688	444	85
CBU1098	Hypothetical cytosolic protein			Cytoplasmic	3,142	4,049	71	2,468	9,747	19
CBU0774	Stress-responsive transcriptional regulator PspC	*pspC*		Unknown	2,983	835	335	2,984	2,145	237
CBU0800	Hypothetical protein			Unknown	1,817	1,064	31	1,051	945	27
CBU0383	DNA-3-methyladenine glycosidase I	*tag*		Unknown	1,790	3,220	58	285	–	152
CBU1966	Glutamyl-tRNA reductase	*hemA*		Cytoplasmic	1,624	–	172	23	–	–
CBU1697	Endonuclease III	*nth*		Unknown	1,620	1,961	22	669	–	72
CBU0776	ABC transporter, ATP-binding protein			Cytoplasmic membrane	1,448	–	94	2	–	9
CBU1002	Biotin operon repressor/biotin synthetase	*birA*		Unknown	1,254	–	8	3	163	–
CBU1958	Hypothetical ATPase			Unknown	1,248	3,259	119	394	225	46
CBU0366	Phosphate regulon sensor protein PhoR	*phoR*		Cytoplasmic membrane	1,010	–	456	944	1,009	400
CBU1969	DnaK suppressor protein	*dksA*		Cytoplasmic	1,007	765	81	793	1,732	26
CBU2020	Glutamate/gamma-aminobutyrate antiporter			Cytoplasmic membrane	1,004	601	–	78	–	57
CBU0760	Sensor protein GacS			Unknown	749	1,423	37	1,036	640	23
CBU0723	Hypothetical protein			Unknown	704	2,520	126	683	920	172
CBU1121	Hypothetical protein			Unknown	672	1,724	79	1,295	778	90
CBU1157	Hypothetical exported lipoprotein			Unknown	522	–	26	21	–	23
CBU0008	Hypothetical protein			Unknown	513	2,408	109	494	1,377	89
CBU0098	Nicotinate-nucleotide pyrophosphorylase	*nadC*		Cytoplasmic	468	–	11	504	1,010	5
CBU0391	Riboflavin biosynthesis protein RibF	*ribF*		Cytoplasmic	453	–	6	96	588	22
CBU1065	2′-5′ RNA ligase			Cytoplasmic	158	323	45	643	726	2

[a] Immunoreactive proteins ordered from top (most reactive) to bottom (least reactive) based on average fluorescence intensity values of plasmid-based arrays probed Q fever patient sera.
[b] Predicted signal peptide as determined by SignalP.
[c] Predicted subcellular location as determined by Psort.
[d] P, Q fever patient sera; V, Q-Vax vaccinee sera; N, naive sera.
[e] –, signal absent with all sera.

TAP fragment-based arrays. However, the fluorescence intensity of individual proteins was generally greater on plasmid-based arrays, indicating more efficient IVTT protein production from plasmid templates.

Features of immunoreactive proteins. Thirteen of the forty-four immunogenic proteins are annotated as hypothetical proteins (22) (Table 1). Based on predicted signal peptides, 9 immunoreactive proteins among the top 44 (20.5%) were predicted to be secreted into the periplasm or beyond. Eight of these proteins were among the top fifteen (53.3%), a finding which contrasts markedly with the percentage of predicted secreted proteins within the entire proteome (10.2%). A similar bias in signal sequence-containing proteins was observed in a proteome microarray screen of *F. tularensis* antigens (10, 25). In addition to a type II secretory pathway, *C. burnetii* encodes a Dot/Icm type IV secretion system that translocates effector molecules into the host cell cytosol (19). Immunoreactive CBU0781 (AnkG), an ankyrin repeat domain-containing pro-

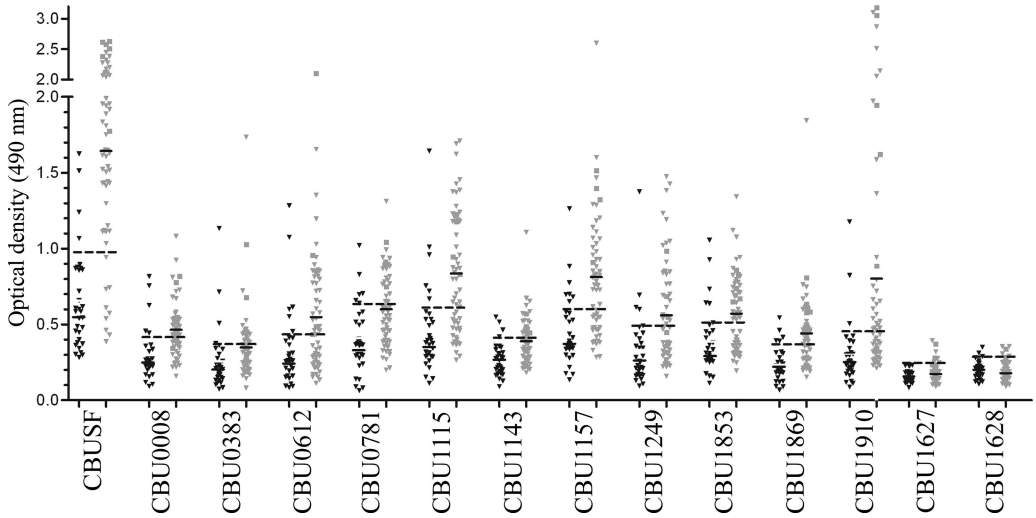

FIG. 4. ELISA validation of immunogenic proteins identified by microarray. Recombinant proteins corresponding to 10 antigens identified by protein microarray were generated and tested by ELISA for IgG reactivity of human sera. A sampling of proteins that ranged from strongly (e.g., CBU1143) to weakly (e.g., CBU0008) reactive were tested. Recombinant Com1 (CBU1910), a characterized *C. burnetii* protein antigen, was included as a positive control. Recombinant IcmE and IcmK, which tested negative by protein microarray, were included as controls. ELISA was conducted on 55 and 5 serum samples from human acute (gray triangles) and chronic Q fever patients (boxes), respectively, that tested positive for *C. burnetii* antibodies by indirect immunofluorescence assay (see Materials and Methods). ELISA was also conducted on 32 IFA-negative sera (black triangles). Black lines denote the mean reactivity of positive and negative sera. Dashed black lines denote the positive cutoff, which was calculated as the mean ELISA reactivity of IFA-negative sera samples plus two times the standard deviation. CBUSF, *C. burnetii* soluble fraction.

tein, has been recently shown to be secreted in a Dot/Icm-dependent manner (19). Secreted immunogens that disassociate with *C. burnetii* are likely present in very low amounts in the Q-Vax vaccine. Consistent with this idea, AnkG's patient to vaccinee average fluorescence array signal ratio was 489 to 1. This contrasts with a ratio of 2.1 to 1 for the most reactive protein CBU0891, a hypothetical membrane associated protein (Table 1).

A few *C. burnetii* antigens have been identified by immunoscreening a phage expression library (32) and proteins separated by two-dimensional gel electrophoresis (6). Among the 16 proteins showing the strongest average fluorescence signals by microarray, CBU0891, CBU1398 (2-oxglutarate dehydrogenase, SucB), and CBU0664 (ISAs1 family transposase) were previously identified as immunogens by Zhang et al. (32), indicating reproducibility between microarray and conventional antigen screening procedures. Antibody recognition of SucB indicates cytosolic housekeeping enzymes are exposed to the host's immune system. Indeed, in this report and elsewhere (6, 32), additional *C. burnetii* housekeeping enzymes, such as isoleucyl-tRNA synthetase and isocitrate dehydrogenase, have been identified in antigen screens. Moreover, metabolic enzymes of other bacterial pathogens are known to trigger B-cell responses (26, 30).

ELISA validation of protein microarray data. To validate protein microarray results using another serological method, 10 reactive proteins showing a range of average fluorescence intensities were purified as recombinant His_6-tagged protein and tested by ELISA. The 10 proteins chosen for testing were: CBU0008 (hypothetical protein), CBU0381 (DNA-3-methyladenine glycosidase), CBU0612 (outer membrane protein OmpH), CBU0781 (AnkG), CBU1115 (hypothetical protein), CBU1143 (preprotein translocase YajC), CBU1157 (hypothetical exported lipoprotein) CBU1249 (putative DNA-binding protein), CBU1853 (GtrA family protein), and CBU1869 (hypothetical exported protein). CBU0891 was not tested because of difficulty in obtaining recombinant protein. Recombinant His_6-tagged Com1 (CBU1910), a characterized *C. burnetii* protein antigen (12), and *C. burnetii* cell extracts (CBUSF), were included as positive controls. As negative controls, recombinant IcmE and IcmK, two array-negative proteins that are part of the organism's type IV secretion system (29), were included.

ELISA was conducted using 55 and 5 serum samples from human acute and chronic Q fever patients, respectively, that tested positive for *C. burnetii* antibodies by immunofluorescence assay (see Materials and Methods). ELISA was also conducted using 32 IFA-negative human sera, including the 16 naive sera used in microarray screens. Seven of ten recombinant proteins were positive by ELISA when tested with sera from acute Q fever patients. When tested with sera from endocarditis patients, all recombinant proteins were positive (Fig. 4 and Table 2). Consistent with microarray results, the negative control proteins IcmE and IcmK were negative by ELISA. We did not observe a clear correlation between IFA titer and responses by ELISA to specific recombinant proteins. However, convalescent human sera with high IFA titers consistently demonstrated positive reactivity against all recombinant proteins, whereas several convalescent-phase sera with low IFA titers did not react strongly with any recombinant protein (data not shown). Sensitivity and specificity for the 10 recombinant proteins ranged from 31.6 to 61.6% to 78.1 to 90.0%, respectively. CBUSF gave the highest sensitivity at 85%. While none of the individual recombinant proteins provided complete coverage for IFA-positive samples, six out ten proteins showed greater sensitivities than the positive control CBU1910 (Com1). Combining several recombinant proteins

TABLE 2. ELISA reactivity of human sera to recombinant *C. burnetii* proteins

ORF	Protein function	%		Ratio[c] (positive/negative)
		Specificity[a]	Sensitivity[b]	
CBUSF[d]		87.5	85	2.9
CBU0008	Hypothetical protein	84.0	60.0	1.8
CBU0383	DNA-3-methyladenine glycosylase	87.5	31.6	1.7
CBU0612	Outer membrane protein OmpH	81.2	51.6	2.2
CBU0781	Ankyrin repeat protein	81.3	40.0	1.8
CBU1115	Hypothetical protein	81.2	56.6	2.3
CBU1143	Protein translocase subunit YajC	90.6	33.3	1.6
CBU1157	Hypothetical exported lipoprotein	78.1	61.6	2.2
CBU1249	DNA-binding protein	87.5	45.0	2.1
CBU1853	GtrA family protein	84.3	56.6	1.8
CBU1869	Hypothetical exported protein	90.0	55.0	2
CBU1910	Outer membrane protein Com1	90.0	50.0	3.1
CBU1627	IcmE	96.9	11.6	1.1
CBU1628	IcmK	90.6	8.3	0.9

[a] The percent specificity was calculated as the number of ELISA-negative sera from the IFA-negative pool divided by the number of IFA-negative sera.
[b] The percent sensitivity was calculated as the number of ELISA-positive sera from the IFA-positive pool divided by the number of IFA-positive sera.
[c] The positive/negative ratio was calculated as the mean OD_{490} of proteins probed with IFA-positive sera.
[d] That is, the *C. burnetii* soluble fraction.

significantly increased sensitivity but also moderately reduced specificity (data not shown). While a final recombinant protein mixture equal to or superior to CBUSF was not defined, these studies did support the concept that a limited multiplex assay can be developed to replace whole-cell reagents in a new Q fever diagnostic.

While the primary goal of the present study was to identify *C. burnetii* proteins with serodiagnostic potential, some of the identified immunogens may be candidates for a Q fever subunit vaccine. Both cell-mediated and humoral immune responses are important for protection against Q fever (33), and accumulating evidence suggests that an efficacious vaccine based on recombinant antigen is feasible (27, 31, 34). Indeed, several purified proteins including CBU0781 (AnkG), CBU1157 (lipoprotein), and CBU1143 (YajC) induce strong gamma interferon recall responses in purified $CD4^+$ T cells of vaccinated or infected mice (J. E. Samuel, unpublished data). Interestingly, YajC, an inner membrane protein involved in Sec-dependent secretion (9), is both a B-cell and a T-cell antigen of *Brucella abortus* (28). Testing of microarray-identified antigens for T-cell antigenicity does not require purified recombinant protein as IVTT-produced antigen, either coupled to latex beads by an affinity tag or contained within in crude IVTT lysates, both effectively stimulate T cells in proliferation assays (13, 14).

In summary, we have used a high-throughout proteome microarray screening method to identify *C. burnetii* proteins recognized by the human humoral immune response to *C. burnetii* infection and vaccination. This screening method allows profiling of antibody responses of large cohorts of infected and/or vaccinated animals and humans to rapidly identify consensus immunodominant antigens on a whole-proteome basis. Identification of immunodominant antigens of *C. burnetii* will aid in the development of safe and effective recombinant protein-based vaccines and reliable serodiagnostic tests that do not require biosafety level 3 facilities for antigen production. We are currently developing a second-generation *C. burnetii* IVTT-based microarray that includes proteins unique to two chronic Q fever endocarditis isolates (G and K) (3). Comparisons of the immunoproteome of acute and chronic disease isolates may identify unique serodiagnostic antigens that allow differentiation of acute and chronic Q fever.

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