Recombinant Surface Proteomics as a Tool to Analyze Humoral Immune Responses in Bovines Infected by *Mycoplasma mycoides* Subsp. *mycoides* Small Colony Type*†§*

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A systematic approach to characterize the surface proteome of *Mycoplasma mycoides* subspecies *mycoides* small colony type (*M. mycoides* SC), the causative agent of contagious bovine pleuropneumonia (CBPP) in cattle, is presented. Humoral immune responses in 242 CBPP-affected cattle and controls were monitored against one-third of the surface proteins of *M. mycoides* SC in a high throughput magnetic bead-based assay. Initially, 64 surface proteins were selected from the genome sequence of *M. mycoides* SC and expressed as recombinant proteins in *Escherichia coli*. Binding of antibodies to each individual protein could then be analyzed simultaneously in minute sample volumes with the Luminex suspension array technology. The assay was optimized on Namibian CBPP-positive sera and Swedish negative controls to allow detection and 20-fold mean signal separation between CBPP-positive and -negative sera. Signals were proven to be protein-specific by inhibition experiments, and results agreed with Western blot experiments. The potential of the assay to monitor IgG, IgM, and IgA responses over time was shown in a proof-of-concept study with 116 sera from eight animals in a CBPP vaccine study. In conclusion, a toolbox with recombinant proteins and a flexible suspension array assay that allows multiplex analysis of humoral immune responses to *M. mycoides* SC has been created. *Molecular & Cellular Proteomics* 8: 2544–2554, 2009.

*Mycoplasma mycoides* subsp. *mycoides* small colony type (*M. mycoides* SC) is the causative agent of contagious bovine pleuropneumonia (CBPP), a severe respiratory disease in cattle. It is a disease requiring official declaration to the World Organization for Animal Health (OIE) and that causes vast problems in Africa with severe socioeconomic consequences (1, 2). In 2006, 15 African countries reported 186 outbreaks of CBPP to the OIE. CBPP was eradicated from Europe in the beginning of the 20th century (3) but has reemerged in every decade since (4). Eradication was largely facilitated by slaughtering infected herds, which is still considered as the most efficient means of disease control and was successfully performed in Botswana in 1995 (5). However, this campaign was directly correlated to increased malnutrition in children (6) and is also considered to be too expensive for other African countries (2, 7). The use of chemotherapy in CBPP control is a debated subject, has long been discouraged, and is even illegal in some countries (1), mainly because of the risk of creating silent carriers of the disease (8). However, new antibiotics have shown positive effects (9), but extensive vaccinations are still considered the preferred option for prevention and control of CBPP in Africa (2, 10, 11). The vaccines currently in use are based on live attenuated *M. mycoides* SC strains and have several disadvantages such as short term immunity (12), poor protection as indicated in recent trials (4, 13), and even pathogenicity (13, 14).

The two currently available tests for serological diagnosis of CBPP recommended by the OIE, the complement fixation test (15) and a competitive ELISA (16), are based on whole cell *M. mycoides* SC. For subcellular components of the organism, the genome sequence of *M. mycoides* SC strain PG1 (17) offers an emerging possibility to improve both diagnostic and therapeutic approaches with selected antigens. However, as for the 10 other *Mycoplasma* genomes sequenced, the genome sequences *per se* did not reveal any primary virulence factors common in other bacteria, such as adhesins or toxins (18). The few known molecular mechanisms of pathogenicity were recently reviewed (18) and include five lipoproteins studied in detail: LppA (19, 20), LppB (21), LppC (22) LppQ (23), and Vmm (24). Of these, LppQ has been used to develop an indirect ELISA (25), and Vmm, a variable surface protein, has recently been studied along with five novel putative variable surface proteins as
recombinant proteins expressed in *Escherichia coli* (26). That study demonstrated the feasibility of producing recombinant surface proteins from *M. mycoides* SC in *E. coli* and screening for antibodies in sera from CBPP-affected bovines by Western and dot blotting.

To explore further the immunogenicity of the *M. mycoides* SC surface proteome, a platform for multiplexed analysis of proteins using minute serum samples such as bead-based array systems (27) is desirable. One method is available from Luminex Corp. and uses spectrally distinguishable beads (28) to form an array in suspension. The array is analyzed in a flow cytometer-like instrument and can perform up to 100 simultaneous assays in a single reaction well. This platform has recently been used to determine binding specificities to antigens produced in a similar fashion (29) and to profile antibodies in serum toward six antigens of *Mycobacterium tuberculosis* (30).

The aim of this study was to develop a rapid and highly multiplex method for affinity analysis of antibody levels in serum samples from CBPP-affected bovines against recombinant *M. mycoides* SC surface proteins. To facilitate this, a large set of surface proteins were cloned, expressed in *E. coli*, and purified. Furthermore, the bead-based assay conditions had to be optimized and verified for detection of immunoglobulin levels in bovine sera. This methodology would enable monitoring and protein-specific characterization of humoral immune responses during CBPP infections. As a secondary aim, the study was expanded to include specific IgG, IgA, and IgM responses in sera from a vaccine study with time series sampling from each animal over 8 months, covering prevaccination and 4 months postinfection.

**EXPERIMENTAL PROCEDURES**

**Selection and Design of Recombinant Proteins**—The genome sequence of *M. mycoides* SC strain PG1 was retrieved from EMBL/GenBank™/DDBJ entry BX293980 and screened in three steps to select surface proteins for this investigation. Initially, the complete proteome was analyzed with SignalP (31, 32) to identify signal peptide sequences. The identified surface proteins were further analyzed using TMHMM (33) and BLASTP (34) to identify transmembrane regions. The identified surface proteins were further analyzed for similarity to proteins in related species were selected.

Recombinant proteins were designed, excluding the signal peptide only. In the case of transmembrane regions, the largest extracellular domain was selected to avoid problems in protein expression. Names of the recombinant proteins were derived from the corresponding ORF names from EMBL/GenBank/DDBJ entry BX293980.

**Cloning and Protein Expression**—All recombinant proteins were cloned from *M. mycoides* SC strain M223/90 (35) whole genomic DNA and expressed as described previously (26) except for TGA codon mutagenesis, which was adapted for higher throughput. In brief, the mutagenesis was run in two PCR steps. First a multiple mutation reaction (36) was performed with a sequence-verified plasmid containing the gene fragment of interest as template using PfX50 (Invitrogen) and AmpliBlast (Epicentre) enzymes. A secondary PCR with PfX50 and primers complementary to the previous primer handles was used to introduce a biotin, thus enabling solid phase restriction and ligation into the vector pAFF8c. After transformation, recombinant *E. coli* BL21(DE3) were cultured, expression was induced with isopropyl-1-thio-β-D-galactopyranoside, and cells were harvested and lysed followed by immobilized metal ion chromatography purification of the recombinant proteins. In the end, purified *Mycoplasma* proteins with an N-terminal hexahistidine and albumin-binding protein fusion tag (His6-ABP) were obtained.

**Sera**—A group of previously studied bovine sera was chosen for optimization and validation and as controls (26). These included CBPP cases from Namibia and Botswana as well as healthy Swedish controls. For the large screening, field samples from Tanzania and Kenya were included as well as additional positive and negative control samples (see Table I). The group of sera used in the time course study consisted of 116 serum samples from eight animals selected from a larger CBPP vaccine trial held in Kenya in 1998/99. Four animals were vaccinated with the common T1-44 and T1SR vaccine strains (two animals for each vaccine), two were intubated with a virulent local strain of *M. mycoides* SC to serve as a source of the disease, and the last two animals were untreated controls. After intubation, all animals in the vaccine trial were kept together.

**Immunoblotting**—Western blotting was performed as described previously (26) with minor modifications. In brief, 1.5 μg of the recombinant proteins was separated by SDS-PAGE on a 15% Tris/HCl gel (Bio-Rad) and electrotransferred onto a nitrocellulose membrane. Following blocking, the membranes were incubated with a 1:5000 dilution of preblocked sera, and bound antibodies were detected with secondary goat anti-bovine IgG conjugated with horseradish peroxidase (4 ng/ml; Jackson ImmunoResearch Laboratories).

**Bead Preparation**—Recombinant *M. mycoides* SC surface proteins were coupled to carboxylated magnetic beads (MagPlex-C, Luminex Corp.) according to the manufacturer’s protocol with modifications (29) as follows: 10⁶ beads per ID were transferred to separate wells in a 96-well plate (Greiner Bio-One), incubated with a magnet (Lifetech), washed, and resuspended in buffer (0.1 M NaH₂PO₄, pH 6.2). Beads were activated by addition of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (10 μl, 50 μg/ml) and N-hydroxysuccinimide (10 μl, 50 g/ml) and incubated for 20 min on a shaker (Thermomixer, Eppendorf). Beads were washed, washed, and resuspended prior to addition of individual recombinant proteins (100 μl, 100 μg/ml) to separate bead IDs. After 120 min, the beads were sedimented, washed, and resuspended to low binding microcentrifuge tubes (Starlab) for storage in a protein-containing buffer (blocking reagent for ELISA, Roche Applied Sciences) with Na₂HPO₄ (0.5%). For analysis, beads were resuspended by sonication in an ultrasonic cleaner (Branson Ultrasonic Corp.), and a bead mixture with 500 beads per ID and sample was prepared as described previously (29).

**Suspension Array Assay**—Prior to analysis, the sera were diluted 1:3000 in a protein-containing buffer (blocking reagent for ELISA, Roche Applied Sciences) and preadsorbed with His6-ABP and an *E. coli* lysate to reduce undesired signals as described previously (26). For competition experiments, preadsorption included a 20 μg/ml concentration of the respective protein(s) in question. Each serum (45 μl) in a 96-well plate was mixed, and the beads were resuspended in a 96-well plate and incubated with continuous mixing for 60 min. Beads were sedimented magnetically and washed three times in 100 μl of PBST (PBS, 0.1% Tween 20) via an automated procedure on a liquid handling system (PlateMate 2 × 2, Matrix Technologies Corp.). The magnet was then removed, and the beads were resuspended in a solution of biotinylated anti-bovine IgG (30 μl, 0.5 μg/ml; Jackson ImmunoResearch Laboratories), anti-bovine IgM (30 μl, 1.6 μg/ml; AbD Serotec), or anti-bovine IgA (30 μl, 8 μg/ml; AbD Serotec) antibodies. Anti-bovine IgM and IgA were biotinylated in-house with a 50× molar excess (EZ-Link
sulfo-NHS-LC-LC-biotin, Pierce). Following 45 min of mixing, the beads were washed and resuspended in a solution of phycoerythrin-labeled streptavidin (30 μl, 0.5 μg/ml; Pierce). After 20 min of mixing, beads were washed and resuspended in stop solution (0.2% (v/v) paraformaldehyde in PBS, 100 μl). A final incubation of 5 min was followed by sedimentation and resuspension in PBST. Samples were thereafter analyzed in a Luminex LX200 system using Luminex IS 2.3 software counting 50 events per bead ID and sample. The median fluorescence intensity (MFI) was chosen to display serum antibody-protein interactions. Data analysis and graphical presentation were performed in Microsoft Excel 2004 or R, an environment for statistical computing and graphics (37).

**RESULTS**

Selection of Target Proteins—Screening the *M. mycoides* SC strain PG1 genome with SignalP predicted 187 genes to have a signal peptide. These were ranked by ascending similarity to related *Mycoplasma* species, which in most cases was *Mycoplasma capricolum* subsp. *capricolum*. Transmembrane regions were predicted in 34 of the proteins. The number of TGA tryptophan codons as an estimate of mutations needed for expression in *E. coli* ranged from none to 27 codons per protein. From these data, 64 proteins were selected based on similarity less than 80% covering 80% of the full-length sequence and favoring few codons to mutate; however, four genes were included despite exceeding the similarity threshold. Whenever possible, recombinant proteins were designed to contain the full-length protein, excluding signal peptides only. For the 15 selected proteins that contained transmembrane regions, the largest extracellular domain was chosen for expression, resulting in amino acid coverage of 8–94%. In the full set of recombinant proteins, the mean amino acid coverage was 80% with truncations due to signal peptides and transmembrane regions. For detailed information on the recombinant proteins, see supplemental Table S1.

Cloning and Expression of Proteins—To facilitate expression of the recombinant proteins, a high throughput scheme was adopted. At first, the 64 selected gene fragments were amplified by PCR, plasmids containing the amplicons fused to a His<sub>6</sub>ABP tag were created, and sequence-verified clones were subsequently used as templates in the mutagenesis. A His<sub>6</sub>ABP tag was created, and sequence-verified clones were amplified by PCR, plasmids containing the amplicons fused to a His<sub>6</sub>ABP tag were created, and sequence-verified clones were subsequently used as templates in the mutagenesis. An antibody specific for the His<sub>6</sub>ABP tag was used to validate successful immobilization of recombinant protein on the beads, resulting in MFI signals of 800–3700 arbitrary units (AU) in all 65 bead IDs. An initial optimization and validation of the capacity of the assay for measuring specific IgG levels in sera were performed on an initial set of 30 proteins with Namibian CBPP samples and three Swedish negative controls. Evaluated parameters included buffer composition, serum incubation time, choice of detection molecule, serum and antibody dilutions, and bead amounts. The buffer composition had a minor impact on background signal levels, whereas the chosen detection molecule, phycoerythrin-labeled streptavidin, allowed a broader range of signal intensities. The effect of the incubation time of sera and beads was studied, resulting in a linear increase of signals with time (exemplified in Fig. 1). Longer incubation times were shown to increase the distance between strong, moderate, and weak signals, and an incubation time of 60 min was chosen to allow good separation of signal intensities while avoiding saturation of the signal. Finally, reproducibility was determined by triplicate analyses of 30 proteins in 10 sera to an intra-assay coefficient of variation (CV) of 5% and an interassay CV of 25%.

Performance of High Throughput Screening—To test the performance of the assay on a larger scale, analysis of 242 sera was performed on the 51 recombinant surface proteins produced at that time and the unconjugated His<sub>6</sub>ABP fusion tag as control. Signal intensities in replicates of three serum samples within the runs varied with an intra-assay CV of 5% and interserum and interassay CV of 22%; and the serum pattern was maintained with a correlation of $R^2 > 0.97$. A hierarchical cluster analysis was performed to investigate batch effects of individual runs, but no such correlations were found. Importantly, no titrations were needed to compensate for different quality of the sera (Ig content; total protein content; decay due to
transport, storage, or age, etc.), and a standard procedure and dilution were used for all samples. Serum origin-driven trends were investigated, and Swedish control sera were found to have substantially lower signal intensities than CBPP-free African field sera. The signals from the negative control bead, His$_6$ABP, were generally less than 100 AU throughout the experiment and maximum 300 AU. Binding patterns from all sera were visualized in a heat map (Fig. 2). Both serum samples and proteins were clustered hierarchically based on the Euclidean distance of log$_2$-transformed signals. Clustering of proteins showed a separation into three main groups: one group of seven proteins that obtained low

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**Fig. 2. Overview of 242 analyzed sera.** Log$_2$-transformed signals from the analysis of 242 sera (Table I) toward 51 Mycoplasma proteins and the His$_6$ABP fusion protein per se (ID 58) as a control are displayed as a heat map. Color intensity denotes signal intensity (white, low; orange, medium; red, high). A serum sample dendrogram is displayed on top, an antigen dendrogram is displayed on the left, and protein IDs are on the right. The top colored line indicates sample type as CBPP-affected (red), CBPP-free (blue), uncertain disease status (pale yellow), and serum-free blanks (green). Serum samples clustered into two groups: one lacking or having low antibody levels that bind the recombinant *M. mycoides* SC proteins and one with medium to high titers for most proteins. Proteins clustered into three major groups, presumably of poor, medium, and high immunogenicity.
TABLE I

Sera used in large screening

A selection of 242 sera from individual bovines used in the large screening is shown. CBPP status of African field samples was derived from complement fixation test, ELISA, latex agglutination test, or gross pathology results given by sample donors. Uncertain samples are from CBPP-endemic regions with conflicting or no prior test results. Positive reference samples were donated from approved diagnostic laboratories.

| Type                    | Origin                  | No.  | Comment                                      |
|-------------------------|-------------------------|------|----------------------------------------------|
| African field samples   | Kenya, Namibia, Tanzania| 206  | CBPP-positive (98), CBPP-negative (41), and uncertain (66) |
| Positive reference samples | Portugal, Spain, Tanzania  | 10   | CBPP-positive reference samples               |
| Negative control samples | Sweden, United Kingdom  | 26   | CBPP-negative; five with Mycoplasma bovis infection |
| Total                   |                         | 242  |                                              |

Signals (likely poor immunogens), one large group of 35 proteins that displayed medium signals, and a group of 10 proteins with high intensity signals. All but three of the 10 native proteins with high signals were lipoproteins with unknown function (MSC_0117, MSC_0122, MSC_0240, MSC_0397, MSC_0431, MSC_0816, and MSC_1001) whereof two were previously studied putative variable surface proteins (MSC_0117 and MSC_0816) (26). Interestingly, MSC_0122 (annotated as prolipoprotein LppC) is a truncated duplicate of the N-terminal region of LppC (MSC_0177) with 37% identity using BLAST. The remaining proteins were the C-terminal region of a hypothetical transmembrane protein (MSC_0298) where the recombinant protein covered 41% of the full-length protein and two hypothetical proteins (MSC_0576 and MSC_1052). In contrast, five of the seven native proteins corresponding to the recombinant proteins obtaining low signals were transmembrane proteins (MSC_0187, MSC_0321, MSC_0422, MSC_0707, and MSC_0910). Clustering of bovine sera showed a separation into two main clusters. One consisted of a third of the samples with low signal intensities; i.e., these sera contained limited amounts of antibodies for the M. mycoides SC surface proteins or at least not for their recombinant counterparts. It cannot be ruled out that some of these sera have lost their original antibody titers. The second cluster included two-thirds of the samples, all with high signal intensities, and obviously these bovines had evoked a humoral immune response to the majority of the M. mycoides SC proteins in this study. CBPP-positive and -negative samples (for CBPP status classification, see Table I) were present in both clusters, but the smaller cluster was predominantly CBPP-negative (15 positive and 33 negative), and the larger cluster was predominantly CBPP-positive (59 positive sera and 30 negative sera). Samples rated as uncertain were evenly distributed between both groups.

When the operating protocol was established and the assay was judged to be reproducible, 13 additional proteins had been cloned and included into the final set of 64 recombinant surface proteins. CBPP-specific humoral responses to native counterparts of the recombinant proteins and the ability to distinguish CBPP-positive and -negative sera were further scrutinized. Two of the CBPP-positive sera from Namibia and one negative Swedish serum were reanalyzed, and relative antibody responses were visualized as binding patterns where each serum showed a unique profile as shown in Fig. 3A. There was a 20-fold difference (1071–53 AU) in overall signals for the two positive sera versus the negative serum. Signals in the serum-free control, mainly derived from the intrinsic fluorescence of the beads, peaked at 25 AU. A minor cross-reactivity in the serum-free control was observed for protein 62 with an elevated background of <90 AU, indicating unspecific interaction of the secondary antibody or the streptavidin conjugate. Bead 58, displaying the fusion tag His<sub>6</sub>ABP per se, common for all recombinant surface proteins, was the control to monitor the proportion of binding to the tag. The His<sub>6</sub>ABP signal was not utilized for normalization; instead it was used to set a serum-specific internal cutoff level to determine antibody-protein-specific responses. Some protein-specific signals were observed in the negative serum, among which protein 40 showed intensities peaking at 450 AU. The corresponding signals in the positive sera were therefore judged as true positive (5600 AU) for 1MUK15A and uncertain (482 AU) for 2MUK15A. There were over 10 proteins for which a strong interaction was monitored in the two positive sera, and in both cases signals to proteins 62 and 64 were most prominent (>10,000 AU). Serum 1MUK15A had six additional positive signals at >4000 AU, whereas 2MUK15A displayed interactions with two of those six proteins and one other protein at about 3000 AU.

To further verify that signals were derived from proteinspecific antibody interactions, a competition study was performed with five proteins with high signal intensities in the CBPP-positive sera (Table II). The CBPP-positive and -negative sera were preincubated with each of the five proteins separately or with a mixture of all five. Following analysis by the bead-based assay, a clear protein-specific signal inhibition was shown. For both CBPP-positive sera, signals were reduced to less than 20% of the original signal intensity, whereas signals for all other proteins remained unaffected. In the negative serum (Swe 2), little or no reduction was observed except for a reduction to 38% of the original MFI for one of the proteins, indicating that some of the comparatively weak signals were also protein-specific. In all, these results indicate that the detection of antibody binding to the recombinant proteins was specific and thereby allowed monitoring...
of CBPP-associated IgG levels for individual *M. mycoides* SC proteins in sera.

**Comparison with Blotting Experiments**—Western blot experiments were performed with a subset of sera and proteins to enable a direct comparison of protein-specific signal patterns in sera and to further validate the bead-based assay. From the signal patterns in Fig. 3A, seven recombinant proteins corresponding to high and three corresponding to low signal intensities in the two CBPP-positive sera were selected along with the common fusion tag protein His6ABP. The binding profiles for the CBPP-positive sera were used to select seven recombinant proteins with high and three with low signal intensities for a comparison with Western blot analysis shown in B. Here, binding to selected proteins and the fusion tag as a negative control is shown for both CBPP-positive sera.

**Time Course Study of IgG, IgM, and IgA Responses**—Sera from eight bovines in a CBPP vaccine study were selected for a time course monitoring of humoral immune responses to the *Mycoplasma* proteins. Serum samples were taken at multiple time points, and animals belonged to groups T1-44- or T1SR-vaccinated, intubated, or untreated controls. In this study, different secondary antibodies allowed monitoring of IgG, IgM, and IgA responses in the bead-based assay. Reproducibility of IgM and IgA detection was verified with an interassay CV of ≤25%. Binding patterns in control sera were similar to those in Fig. 3 with an average MFI of 180 and 35 AU (CBPP-positive and -negative sera, respectively) for IgM, whereas IgA showed intensities of 164 AU (positive) and 35 AU (negative). In all, 124 sera including controls were ana-

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**Fig. 3. IgG profiles.** A shows IgG binding profiles for 64 recombinant surface proteins and a control (*), displayed as bar charts for two CBPP-positive sera (black bars, 1MUK15A; gray bars, 2MUK15A; top chart), a CBPP-negative serum (black bars) and a serum-free control (gray bars). Whiskers denote S.D. in replicate samples. The mean intensities for the CBPP-positive and -negative sera were 1071 and 53 MFI, respectively. Protein 58 (denoted *) is His6ABP, the fusion tag present in all proteins. The binding profiles for the CBPP-positive sera were used to select seven recombinant proteins with high and three with low signal intensities for a comparison with Western blot analysis shown in B. Here, binding to selected proteins and the fusion tag as a negative control is shown for both CBPP-positive sera.
lyzed for each class of antibodies, adding up to 372 samples. Reproducibility for the controls in the observed Ig classes was determined to a CV of 5%, and the median His6ABP signal was below 100 AU.

Generally, antibodies targeting Mycoplasma surface proteins were detected in all bovine sera, and changes in relative immunoglobulin levels could be monitored over the duration of the vaccine trial, although a more frequent sampling would have been desirable to allow a finer resolution of the response curves as exemplified for all 64 recombinant surface proteins in four animals (Fig. 4). A shows the humoral immune responses for all Ig classes in a T1-44-vaccinated animal following both vaccination and exposure to the infectious agent. The highest antibody levels were observed for IgG, whereas low signals for all proteins (see Fig. 2A) but still showed a specific reduction for one protein. Results for the other 59 proteins were unaffected (data not shown).—, no blocking.

| Blocking ID | Relative signal intensity per ID |
|-------------|---------------------------------|
|             | 14 | 38 | 52 | 62 | 64 | His6ABP |
| 1MUK15A     |    |    |    |    |    |         |
| —           | 100| 100| 100| 100| 100| 100     |
| 14          | 100| 100| 100| 100| 100| 100     |
| 38          | 118| 7 | 110| 100| 105| 123     |
| 52          | 111| 107| 2 | 99 | 99 | 123     |
| 62          | 117| 107| 2 | 103| 128|
| 64          | 98 | 99 | 106| 97 | 1 | 105     |
| All the above| 1  | 9  | 4  | 3  | 2  | 135     |
| 2MUK15A     |    |    |    |    |    |         |
| —           | 100| 100| 100| 100| 100| 100     |
| 14          | 100| 94 | 99 | 90 | 95 | 117     |
| 38          | 104| 22 | 105| 94 | 97 | 124     |
| 52          | 106| 102| 2 | 93 | 98 | 115     |
| 62          | 67 | 76 | 80 | 5  | 94 | 98      |
| 64          | 67 | 82 | 84 | 94 | 2  | 87      |
| All the above| 4  | 20 | 2  | 11 | 2  | 98      |
| Swe 2       |    |    |    |    |    |         |
| —           | 100| 100| 100| 100| 100| 100     |
| 14          | 100| 128| 131| 101| 120| 93      |
| 38          | 85 | 90 | 107| 103| 105| 77      |
| 52          | 98 | 96 | 76 | 108 | 161| 74      |
| 62          | 92 | 96 | 110| 74 | 108| 72      |
| 64          | 98 | 100| 105| 112| 92| 77      |
| All the above| 38 | 112| 93 | 86 | 95 | 98      |

month later. As in earlier experiments, antibodies were binding only a subset of the recombinant surface proteins, which varied among both animals and Ig classes. For all eight animals, time periods, and Ig classes, the top five recombinant proteins detected with highest peak signals are summarized in Table III. The recombinant protein R1046, which had the highest overall signal intensities, was predominant in all Ig classes for intubated animals, untreated controls, and one of the T1-44-vaccinated animals. It also showed high postvaccination IgG responses in both T1SR animals but with high signals after CBPP exposure in one animal only. The recombinant protein R1046 was designed on the prolipoprotein Q gene, and its native protein LppQ is a previously studied immunodominant protein of M. mycoides SC, supporting our results. R209 and R364 were also among the dominant proteins of the animal/Ig class/time period groups (Table III). Signals to R209 seemed to be prominent in T1-44-vaccinated animals, whereas R364 was prominent in all vaccinated animals. R209 was designed on MSC_0209, which is annotated as a conserved prolipoprotein containing Interpro motifs IPR005046 and IPR011889. Both motifs have unknown functions and are present in predicted surface proteins. Both motifs are also present in LppQ. R364 was found to be a variable surface protein in our previous study (26) and does not contain any currently known motifs.

Prior to analysis of CBPP-specific humoral responses in animals from the vaccine trial, this data set was used to determine possible correlations between signal intensity and recombinant protein size. The mean signals for the recombinant proteins in the 116 sera (IgG detection) were thus summarized (Fig. 5). No direct correlation was found between size and signal intensity, indicating that the obtained antibody responses were driven by antigen-dependent immunogenicity and not an artifact of recombinant protein sizes.

**DISCUSSION**

A fundamental part of the presented work was the successful cloning and expression of 64 surface proteins of M. mycoides SC. To ensure the greatest structural resemblance to the native protein, recombinant proteins were designed to cover the full-length protein if possible, excluding signal peptides and transmembrane regions that may affect protein expression. The proteins were also fused to an N-terminal His6ABP tag to enable IMAC purification and enhance solubility. A crucial step and a bottleneck was the mutagenesis of the 158 tryptophan codons from TGA to the universal TGG. The multiple mutation reaction method (36) was adapted to our cloning scheme, enabling high throughput substitution of up to five codons simultaneously based on sequencing of six colonies per reaction. Hence, the actual limit might be higher than five simultaneous substitutions. Protein expression and solubility did not pose a problem as all proteins were successfully expressed and purified from 100-ml cultures with a substantial yield.
The bead-based assay format was chosen for its high multiplicability in both samples and analytes as well as for its flexibility to add and remove analytes (proteins on beads). This allowed us to include new proteins in the test set whenever additional cloning and protein expression had been successful. After optimization of the assay for screening of *M. mycoides* SC surface proteins with bovine sera, it provided a setup with 64 recombinant surface proteins that enabled sep-

![FIG. 4. Time course study. IgG, IgM, and IgA responses to 64 proteins were studied with a series of samples collected over time for eight animals from a vaccine trial as exemplified in A for a T1-44-vaccinated animal. Each line represents signals obtained by one protein at the different time points. All Ig classes show specific protein responses both after vaccination (Vacc.) and following CBPP exposure. In B, protein-specific IgG responses are exemplified by data from an intubated bovine, one vaccinated with T1SR, and an untreated control animal.](image-url)
TABLE III
Dominant protein-specific humoral immune responses in each animal

For each time period, Ig class, and animal, the five recombinant proteins with highest single intensity (AU) are listed in descending order. Vacc., vaccinated.

| Animal | Post-vaccination | After CBPP exposure | Animal | After CBPP exposure |
|--------|-----------------|---------------------|--------|---------------------|
| IgA    | R1046           | R1052               | IgG    | R1046               |
| R1041  | R1052           | R1079               | R1046  | R1052               |
| R1042  | R1052           | R1079               | R1046  | R1052               |
| IgM    | R1046           | R1052               | IgG    | R1046               |
| R1041  | R1052           | R1079               | R1046  | R1052               |
| R1042  | R1052           | R1079               | R1046  | R1052               |
| IgM    | R1046           | R1052               | IgG    | R1046               |
| R1041  | R1052           | R1079               | R1046  | R1052               |
| R1042  | R1052           | R1079               | R1046  | R1052               |
| IgA    | R1046           | R1052               | IgG    | R1046               |
| R1041  | R1052           | R1079               | R1046  | R1052               |
| R1042  | R1052           | R1079               | R1046  | R1052               |
| IgM    | R1046           | R1052               | IgG    | R1046               |
| R1041  | R1052           | R1079               | R1046  | R1052               |
| R1042  | R1052           | R1079               | R1046  | R1052               |
| IgA    | R1046           | R1052               | IgG    | R1046               |
| R1041  | R1052           | R1079               | R1046  | R1052               |
| R1042  | R1052           | R1079               | R1046  | R1052               |
| IgM    | R1046           | R1052               | IgG    | R1046               |
| R1041  | R1052           | R1079               | R1046  | R1052               |
| R1042  | R1052           | R1079               | R1046  | R1052               |
| IgA    | R1046           | R1052               | IgG    | R1046               |
| R1041  | R1052           | R1079               | R1046  | R1052               |
| R1042  | R1052           | R1079               | R1046  | R1052               |

FIG. 5. Protein size. To monitor the influence of protein size on signal intensity, the recombinant proteins were divided into three groups according to molecular mass (containing 32, 19, and 14 proteins, respectively), and their mean IgG responses in all 116 time course sera were summarized as box plots. Here, the bold line represents the median signal, the boxes comprise 50% of the data set, and the whiskers extend to the furthest data point within 1.5 times the box length. Although there is a shift in the median from the first group, no obvious correlation to protein size was found.

The generation of binding patterns in 242 sera toward 52 proteins within 1 week showed the high throughput performance of the assay. All sera could be handled according to a standard dilution procedure, and the high reproducibility of the individual fingerprint-like binding patterns demonstrated the strength of the bead-based assay. By cluster analysis, sera grouped into two major clusters, a predominantly CBPP-positive cluster and a predominantly CBPP-negative cluster. Sera rated as uncertain were evenly distributed among the clusters. Because of a lack of clinical information on the sample set as well as differences in handling of sera from the sampling event until the present investigation and a shortage of accompanying documentation, it was difficult to truly assess and evaluate the discriminatory power of the assay as a diagnostic tool. It may seem as if there were many false positives and negatives according to the clustering, but false negatives may in fact have dropped in antibody titers since the diagnosis was made up to 10 years ago, and false positives could occur because this methodology is more sensitive than the original method used. It can also be mentioned that the high throughput experiment was reproduced in full with 10 times more concentrated sera; the only difference was stronger signals. Bearing in mind that cluster analysis takes the complete data set into account, reducing the number of proteins or sera in the analysis could also affect the outcome of the clustering.
and improve the separation of both CBPP positives from CBPP negatives and protein categories. A true diagnostic evaluation of the array would in essence be a reduction of the array to remove irrelevant proteins and poorly characterized sera in searching for the least number of proteins fulfilling a balance between final assay cost, highest CBPP diagnostic power, and least risk of cross-reactions to related diseases. Such an investigation was recently performed to select proteins for a diagnostic ELISA (38).

When the 10 proteins in the cluster that obtained high signal intensities were compared with the seven proteins with low signal intensities, there was no correlation with the recombinant protein size. We also did not see a correlation between signal intensity and recombinant protein size in the data set from the vaccine trial. However, five of the seven proteins in the low signal intensity group originated from transmembrane proteins, compared with only one in the high signal intensity group, which otherwise consisted of lipoproteins and two hypothetical proteins. The largest extracellular loop may not be enough to represent the native protein, and a recombinant protein consisting of all extracellular loops might be preferable in this assay. Furthermore, full-length recombinant proteins may have a higher structural resemblance to their native proteins compared with the truncated recombinant transmembrane proteins. It is also possible that the native lipoproteins have higher immunogenicity than the native transmembrane proteins.

To further benchmark the bead-based assay, it was used to monitor humoral responses over time in eight cattle from a previous CBPP vaccine study. IgG, IgM, and IgA responses to individual proteins were successfully detected throughout the study using the 64 recombinant surface proteins. With adequate clinical information available, such analyses may reveal proteins associated with protective immune responses against CBPP in contrast to unprotective responses and identify new potent markers for early and reliable diagnosis of CBPP. With assorted recombinant proteins, it is also possible to build a diagnostic system based on different technological platforms from ELISA to more advanced miniaturized tests. For each animal, information concerning onset of disease, clinical evidence, and outcome of disease are crucial, although humoral immune responses might not give the animal long term immunity for CBPP. Unfortunately, the serum set used in this study cannot be utilized for such studies as complementary and in-depth information on the animals’ health status was not available. Despite this, proteins generating highest responses among the sera were identified. The most prominent protein was R1046, which corresponds to the previously studied LppQ and demonstrates concordance to previous research efforts. Another important aspect of this study is the need for routine sampling at close intervals during a time course study. It is evident from results in Fig. 4 that a higher sampling rate following vaccination and intubation/challenge would be necessary to compensate for fluctuations in sample preparation, disclose outliers, and obtain higher precision in timing of onset and duration of responses. This would also allow comparisons among protein-specific responses and aid in judging their relative importance for immunity or disease.

In conclusion, the presented bead-based assay allows for high throughput analysis of CBPP-specific humoral immune responses toward 64 surface proteins of M. mycoides SC. Results from experiments comparing CBPP-positive and -negative sera demonstrated that the assay provides high discriminative power of protein-specific responses. The possibility for monitoring humoral responses over time was demonstrated in a proof-of-concept study, identifying immunodominant proteins in corroboration with previous research.

Acknowledgments—We gratefully thank Göran Böltske and Roger D. Ayling for providing sera.

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