Phage Library Screening for the Rapid Identification and In Vivo Testing of Candidate Genes for a DNA Vaccine against *Mycoplasma mycoides* subsp. *mycoides* Small Colony Biotype

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A new strategy for rapidly selecting and testing genetic vaccines has been developed, in which a whole genome library is cloned into a bacteriophage λ ZAP Express vector which contains both prokaryotic (P_{lac}) and eukaryotic (P_{CMV}) promoters upstream of the insertion site. The phage library is plated on *Escherichia coli* cells, immunoblotted, and probed with hyperimmune and/or convalescent-phase antiserum to rapidly identify vaccine candidates. These are then plaque purified and grown as liquid lysates, and whole bacteriophage particles are then used directly to immunize the host, following which P_{CMV} -driven expression of the candidate vaccine gene occurs. In the example given here, a semirandom genome library of the bovine pathogen *Mycoplasma mycoides* subsp. *mycoides* small colony (SC) biotype was cloned into λ ZAP Express, and two strongly immunodominant clones, λ -A8 and λ -B1, were identified and subsequently tested for vaccine potential against *M. mycoides* subsp. *mycoides* SC biotype-induced mycoplasmaemia. Sequencing and immunoblotting indicated that clone λ -A8 expressed an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible *M. mycoides* subsp. *mycoides* SC biotype protein with a 28-kDa apparent molecular mass, identified as a previously uncharacterized putative lipoprotein (MSC_0397). Clone λ -B1 contained several full-length genes from the *M. mycoides* subsp. *mycoides* SC biotype pyruvate dehydrogenase region, and two IPTG-independent polypeptides, of 29 kDa and 57 kDa, were identified on immunoblots. Following vaccination, significant anti-*M. mycoides* subsp. *mycoides* SC biotype responses were observed in mice vaccinated with clones λ -A8 and λ -B1. A significant stimulation index was observed following incubation of splenocytes from mice vaccinated with clone λ -A8 with whole live *M. mycoides* subsp. *mycoides* SC biotype cells, indicating cellular proliferation. After challenge, mice vaccinated with clone λ -A8 also exhibited a reduced level of mycoplasmaemia compared to controls, suggesting that the MSC_0397 lipoprotein has a protective effect in the mouse model when delivered as a bacteriophage DNA vaccine. Bacteriophage-mediated immunoscreening using an appropriate vector system offers a rapid and simple technique for the identification and immediate testing of putative candidate vaccines from a variety of pathogens.

Whole bacteriophage (phage) λ particles have recently been described as highly efficient DNA vaccine delivery vehicles (6, 7, 17, 20), inducing significant antibody responses in both laboratory mice (7) and larger animals such as rabbits (20) and sheep (33). Bacteriophages are viruses of bacteria and are metabolically inert, requiring the host bacterium for growth and propagation. Using standard “naked” DNA vaccination, purified genetic material in the form of a plasmid encoding protective antigens is given to the host and used to stimulate an immune response and give protection against pathogens (37, 42). With bacteriophage genetic immunization, expression cassettes containing a vaccine gene under the control of a suitable eukaryotic promoter (e.g., the cytomegalovirus [CMV] promoter) are simply cloned into a standard λ DNA cloning vector. The DNA is subsequently packaged in vitro, and the vaccine phage is then easily propagated on an *Escherichia coli*

host. When whole recombinant bacteriophage particles containing DNA vaccines are injected into a host (phage vaccination), the protein coat of the bacteriophage protects the DNA from degradation. Being particulate antigens, bacteriophage are taken up by antigen-presenting cells (APCs) (3), cleared from the circulation, and targeted to the spleen and liver Kupffer cells (9, 12, 14). Thus, phages represent an extremely simple and effective technology for targeting DNA vaccines to APCs. Bacteriophage vaccines are cheap, stable (13), and easy to produce, permit the cloning of relatively large inserts (up to 20 kb for lambda vectors), do not require the presence of antibiotic resistance genes, and are unable to replicate in eukaryotic cells. Vaccination with phage particles also induces a highly immunogenic signal (against phage coat proteins), which provides an easily assayable marker to confirm the vaccination status of animals. Previous research has indicated that high phage antibody titers do not interfere with the immune response against the expressed DNA vaccine antigen, and if anything, are more likely to efficiently target the phage to APCs (via phage-antibody complexes [20]).

As a research tool, phage λ vectors are ideal for constructing genomic expression libraries. In a vector such as λ ZAP Express (Stratagene), the DNA insert is under the transcriptional

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control of both an inducible prokaryotic promoter (P_{lac} , inducible with isopropyl- β -D-1-thiogalactopyranoside [IPTG]) and the strong constitutive cytomegalovirus promoter (P_{CMV}) to drive eukaryotic transcription. Once a suitable library has been constructed, whole-library genetic immunization can be carried out without any further manipulation (17). Alternatively, the same library can be rapidly screened by plating phage on *E. coli* in the presence of IPTG and immunoblotting with convalescent-phase or hyperimmune sera to identify immunodominant clones. These potential vaccine candidates can then be isolated, the insert DNA rapidly sequenced, and whole phage particles produced and immediately screened in vivo for protective efficacy, either singly or as a mixture, without the need for further manipulation. If required, the recombinant proteins can also be expressed and purified directly from *E. coli* by excising the integral pBK-CMV phagemid vector by using a helper phage. We have already performed preliminary immunogenicity testing in mice of a whole bacteriophage λ semirandom genomic library of *Mycoplasma mycoides* subsp. *mycoides* small colony (SC) biotype (5), the causal agent of contagious bovine pleuropneumonia (CBPP). This is an economically important disease of cattle currently affecting much of Africa and, until recently, southern Europe for which current live attenuated vaccines offer poor efficacy (for a recent review, see reference 27). The aforementioned *M. mycoides* subsp. *mycoides* SC biotype phage vaccine study has now been extended by picking two clones from the library expressing immunodominant proteins which have been tested as whole phage λ vaccines for their immunogenicity and protective efficacy against *M. mycoides* subsp. *mycoides* SC biotype-induced mycoplasmaemia, using a mouse infection technique (36).

MATERIALS AND METHODS

Mycoplasma strains and growth conditions. *M. mycoides* subsp. *mycoides* SC biotype challenge strain N6 (19) was grown in HEPES-Gourlay's broth or agar (1%) medium (38) at 37°C in an atmosphere containing 5% CO₂. For the challenge experiment, the mycoplasma culture was grown to mid-logarithmic phase, concentrated 10-fold by centrifugation, and resuspended in fresh medium immediately before challenge to give a titer of 5×10^{10} organisms/ml.

Antisera. Rabbit hyperimmune serum (R55) was raised against *M. mycoides* subsp. *mycoides* SC biotype strain N6 and has already been described (19). The immunoglobulin G (IgG) fraction was purified using a protein A column (Roche Diagnostics Ltd.) following the manufacturer's recommended protocol and stored at 10 mg/ml. CBBP-specific convalescent-phase antiserum was obtained from naturally infected cattle from field outbreaks in Tanzania and was provided by Benedict Lema, Animal Diseases Research Institute, Dar-es-Salaam, Tanzania.

Preparation of bacteriophage λ *M. mycoides* subsp. *mycoides* SC biotype genomic library. DNA was prepared from *M. mycoides* subsp. *mycoides* SC biotype vaccine strain T₁₄₄ by previously described methods (19) and partially digested with Tsp509I (New England Biolabs), which has the recognition sequence AATT. The DNA was size fractionated in an agarose gel, and fragments in the range of 6 to 8 kb were excised, purified, and then cloned into the unique EcoRI site of the expression vector λ ZAP Express (Stratagene), in which inserts are under the control of both a prokaryotic (*lacZ*) and a eukaryotic (CMV) promoter. The resulting library was amplified and plated on *E. coli* strain XL1-Blue (Stratagene) in the presence of IPTG to induce prokaryotic expression. The plaques were then blotted onto nitrocellulose membranes and incubated with purified rabbit anti-*M. mycoides* subsp. *mycoides* SC biotype IgG (R55), and standard immunoscreening protocols were used to identify *M. mycoides* subsp. *mycoides* SC biotype-expressing clones (approximately one-third of clones gave a signal, with various degrees of intensity). The most strongly positive clones were picked, replated on *E. coli*, and screened with CBPP-specific convalescent-phase sera from three different cattle (preadsorbed with *E. coli* cells lysed with a λ ZAP Express vector without any insert to reduce the nonspecific background). The λ clones which gave a strong (immunodominant) signal with all three bovine

convalescent-phase sera were selected for further study. Phagemids were excised from these clones directly into *E. coli* strain XL0LR (Stratagene), using the recommended protocol (λ ZAP Express instruction manual, Stratagene). Insert protein expression was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with R55 IgG, while the insert DNA was sequenced using the phagemid T7 and T3 sequencing primers adjacent to the insert multicloning site on a PE Biosystems 377 DNA sequencer. Open reading frames for both *E. coli* and mycoplasma codon usage were determined using the ORF-finder web-based sequence analysis tool at the NCBI website (www.ncbi.nlm.nih.gov/gorf/gorf.html), while sequences were aligned to the published *M. mycoides* subsp. *mycoides* SC biotype strain PG1 sequence (40) by using BLAST searches.

Immunoblots. *E. coli* strain XL0LR cells containing phagemids of interest were grown in 10 ml LB-kanamycin broth to an optical density at 600 nm of 0.4 to 1.0. IPTG (0.2 mM) was then added, and incubation was continued for a further 3 to 4 h. Cells were pelleted and resuspended in a small volume (100 to 400 μ l, depending on the cell density) of SDS-PAGE sample buffer (0.1 M Tris-HCl, pH 6.8, 40% [vol/vol] glycerol, 4% [wt/vol] SDS, 0.25% bromophenol blue, 2% β -mercaptoethanol). The samples were boiled for 5 min, separated by SDS-PAGE, and immunoblotted using standard procedures as previously described (20). Blots were probed using rabbit (R55) or mouse primary antiserum and a relevant secondary antiserum (DAKO A/S, Glostrup, Denmark).

Growth and purification of bacteriophage λ for vaccine preparation. The negative control phage λ -gt11, which does not contain any *M. mycoides* subsp. *mycoides* SC biotype DNA, was produced on *E. coli* strain LE392 (26), while λ ZAP Express *M. mycoides* subsp. *mycoides* SC biotype clones were grown on strain XL1-Blue (Stratagene). Large-scale liquid lysates of phage λ were prepared, purified using previously published protocols (essentially polyethylene glycol precipitation followed by ultracentrifugation) (20), and stored at 4°C until use.

Mouse challenge experiment. The mice used were female BALB/c mice of 10 weeks of age, with eight mice used per group. Three mouse groups were tested. Group 1 mice were immunized with λ ZAP Express clone A8, group 2 mice were immunized with clone B1, and group 3 mice were negative control mice and were injected with λ gt-11. All mice were injected intramuscularly on weeks 0, 4, and 11 with 50 μ l SM saline buffer containing 5×10^9 bacteriophage particles. Mice were bled from the tail prevaccination at week 0, postvaccination at weeks 4, 8, and 13 (immediately prior to challenge at week 13), and postchallenge at 43, 68, and 92 h.

At week 13, all three groups of BALB/c mice were challenged by intraperitoneal injection of 0.5 ml (2.5×10^{10} organisms) of *M. mycoides* subsp. *mycoides* SC biotype strain N6. Previous research had indicated that this strain produced a particularly high degree of mycoplasmaemia in mice (18). All mice were held in negative-pressure isolators (one group per isolator) to satisfy current disease security requirements. A drop of blood from tail-tip bleeds was placed in 3 ml of liquid growth medium before being diluted 10^{-1} and 10^{-2} in the same medium and incubated at 37°C for 7 days. Growth in liquid medium (observed as a color change from red to yellow of 1-in-10 dilutions) was confirmed with an *M. mycoides* subsp. *mycoides* SC biotype-specific latex agglutination test (21) and by plating on solid medium and observing mycoplasma colonies. Finally, all mice were humanely killed and bled at 92 h postchallenge, and spleens were obtained for stimulation assays.

ELISA analysis of sera. Antibody titers against bacteriophage λ coat proteins and whole *M. mycoides* subsp. *mycoides* SC biotype antigens (strain T₁₄₄) were measured by an indirect enzyme-linked immunosorbent assay (ELISA) following previously published procedures (20). Microtiter plates (96-well; Greiner Ltd., Brunel Way, Stonehouse, Gloucestershire, United Kingdom) were coated overnight at 4°C with 0.05 M sodium carbonate buffer at pH 9.2 and either whole sonicated *M. mycoides* subsp. *mycoides* SC biotype (N6) at a concentration of 5 μ g/ml or 10^9 λ gt-11 bacteriophage (50 ng) per well.

Lymphocyte stimulation assay (LSA). Spleens were harvested from each mouse at 96 h postchallenge and combined for each of the three groups (eight spleens per group). Splenocytes were recovered from the spleens via injection with 2 ml mouse wash medium (Hanks balanced salt solution, 2% fetal bovine serum, 2% penicillin-streptomycin, 0.25% nystatin, and 0.2% gentamicin [Lloyds Chemist PLC, United Kingdom]) and rubbing between sterile microscope slides. This cell suspension was then filtered through lens tissue (Whatman International Ltd., England) before being centrifuged for 5 min at 1,500 rpm at 16°C. The pellet was resuspended in lysis buffer (9 parts 0.16 M ammonium chloride [Sigma] and 1 part 0.17 M Tris-HCl [Sigma] at pH 7.65). After 10 min at 4°C, lysis was stopped by the addition of mouse wash medium. Following centrifugation at 1,500 rpm for 5 min at 16°C, the pellet was resuspended in complete RPMI (RPMI 1640, 10% fetal bovine serum, 2% glutamine, 1% penicillin-

FIG. 1. Immunoblots of *E. coli* whole-cell extracts containing excised *M. mycoides* subsp. *mycoides* SC biotype genomic phagemids grown in either the presence (a) or absence (b) of IPTG, using rabbit hyperimmune *M. mycoides* subsp. *mycoides* SC biotype antiserum.

streptomycin, 1% gentamicin, 0.5% 2-mercaptoethanol, 2.5% sodium bicarbonate, and 1.2% 1 M HEPES [Sigma]). This cell suspension was centrifuged again (as described above), and the pellet was resuspended in 1 ml complete RPMI. A 1:10 dilution of cell suspension in 0.1% nigrosin (Sigma) in phosphate-buffered saline was prepared for a viability count using a modified Neubauer counting chamber. The cell concentration was adjusted to 1×10^6 cells/ml in complete RPMI. Using sterile 96-well tissue culture plates, 100 μ l (10^5) of viable splenocytes was seeded into 100- μ l aliquots of sterile diluted antigens containing 10^6 live *M. mycoides* subsp. *mycoides* SC biotype cells or 5 or 2.5 μ g/ml λ gt-11 particles. All assays were performed in triplicate, with average values and standard deviations being calculated. Samples were taken daily and cultured for mycoplasma growth (none could be detected; *M. mycoides* subsp. *mycoides* SC biotype strain N6 is streptomycin sensitive [data not shown]). As a positive control, cells were cultured with concanavalin A (Sigma), which was present at a final concentration of 2.5 μ g/ml. Plates were incubated in a humid 5% CO₂ environment at 37°C for 96 h and then pulsed for 18 h with 18.25 kBq (1 μ Ci) [³H]thymidine (Amersham Biosciences, United Kingdom) per well. The cells were harvested with a Packard Harvester onto glass fiber filters (Packard, The Netherlands), and activity was counted in a direct beta counter (Packard). Results were expressed as average counts per minute (cpm) $\pm$ standard deviations, from which stimulation indices (SI) were calculated using the following equation: SI = average cpm of test/average cpm of control medium without antigenic stimulus.

Statistical analysis. Comparisons of group ELISA titers were analyzed using Student's two-tailed *t* test. The mean values of triplicate ELISA titers for serum samples were compared between groups (*n* = 8 samples per group). For lymphocyte stimulation assays, pooled mouse spleens from each group were assayed in triplicate, and values between groups were compared using a two-tailed *t* test. For the challenge experiment, Fisher's exact probability test was used to compare the numbers of mycoplasmaemic mice in different groups.

RESULTS

Isolation and expression profiles of λ -A8 and λ -B1 clones. Following immunoscreening of the *M. mycoides* subsp. *mycoides* SC biotype λ ZAP Express library (containing ca. 10^6 primary clones), the most strongly positive clones were grown up, recombinant phagemids were excised, and the *M. mycoides* subsp. *mycoides* SC biotype genomic inserts were sequenced. All inserts were given a specific letter and number for identification (e.g., A8). When this insert was contained within a bacteriophage λ vector, it was known as λ -A8, and when it was contained within the excised phagemid vector, it was known as p-A8. The expression profiles of the *M. mycoides* subsp. *mycoides* SC biotype proteins encoded by these clones were ex-

amined by immunoblotting of whole *E. coli* extracts containing the individual phagemids grown in the presence (Fig. 1a) or absence (Fig. 1b) of IPTG, using rabbit hyperimmune *M. mycoides* subsp. *mycoides* SC biotype-specific antiserum. Not all phagemid clones were positive following immunoblotting compared to plaque lifts (e.g., clones B2, B6, and C9) due to extremely poor growth of cells harboring these cloned inserts as plasmids.

Only clone p-A8 appeared to express an IPTG-inducible *M. mycoides* subsp. *mycoides* SC biotype protein (28 kDa), indicating expression originating from the P_{lac} promoter of the phagemid vector. For most of the clones, similar expression profiles were observed in the presence and absence of IPTG, suggesting that most *M. mycoides* subsp. *mycoides* SC biotype protein expression was being internally primed. Since *M. mycoides* subsp. *mycoides* SC biotype DNA is highly AT rich (75%) (40), this was not unexpected, with aberrant transcription initiation often occurring with mycoplasma DNA when maintained within an *E. coli* host (25). However, it makes specific identification of the expressed *M. mycoides* subsp. *mycoides* SC biotype proteins more difficult since it is not obvious where the transcriptional start site lies within the insert. The identification of expressed proteins is further hampered by the fact that in mycoplasmas the typical UGA stop codon encodes a tryptophan. In most expression systems, mycoplasma proteins containing UGA are expressed as truncated proteins unless suppressor strains are used.

Based on the published *M. mycoides* subsp. *mycoides* SC biotype strain PG1 DNA sequence (40) and our own sequence analysis, it appears likely that the 28-kDa protein (P28) observed in clone A8 (from *M. mycoides* subsp. *mycoides* SC biotype strain T₁₄₄) represents the PG1 *lpp* MSC_0397 gene product (714 bp encoding a prolipoprotein of 237 amino acids [25.6 kDa]). In a comparison of both DNA sequences, only a single change was observed (a T-A substitution at position 439, resulting in a change of leucine to isoleucine from strain PG1 to T₁₄₄). This protein has the same predicted size using both mycoplasma and *E. coli* codon usage, since the *lpp* gene contains no UGA codons and the start of the open reading

FIG. 2. Arrangement of ORFs found in the four overlapping λ ZAP Express clones covering the pyruvate dehydrogenase (PDH) region of the genome. The large arrows represent the proteins expressed in mycoplasmas. In total, the four clones cover a region putatively expressing the C termini of MSC_0265 (*pdhA*, encoding 259 of 371 amino acids of the EI α lipoyamide dehydrogenase subunit of pyruvate dehydrogenase), MSC_0266 (*pdhB*, encoding the EI β lipoyamide dehydrogenase subunit of pyruvate dehydrogenase), MSC_0267 (*pdhC*, encoding the EII dihydrolipoyamide *S*-acetyltransferase subunit of pyruvate dehydrogenase), MSC_0268 (*pdhD*, encoding the EIII dihydrolipoyamide dehydrogenase subunit of pyruvate dehydrogenase), MSC_0269 (*pta*, encoding a phosphate acetyltransferase), and MSC_0270 (*ackA*, encoding acetate kinase) and the N terminus of the MSC_0271 *orfA* product, a prolipoprotein with high (57.4% identity) homology to the putative *Mycoplasma capricolum* lipoprotein OrfA (data not shown).

frame (ORF) is located immediately downstream of the P_{lac} promoter.

Clones p-A1, p-A3, p-B1, and p-B21 all exhibited identical expression profiles, with *M. mycoides* subsp. *mycoides* SC biotype proteins seen at 29 kDa (P29) and 57 kDa (P57), although with clone p-A3 P57 was expressed at a greatly reduced level compared to P29 (Fig. 1). When the inserts were sequenced, each clone was found to contain a differently sized insert (clone B21, 6.8 kb; clone A1, 5.8 kb; clone B1, 6.7 kb; clone A3, 5.6 kb). However, there was a region of approximately 3.7 kb which was common to all four clones (Fig. 2) and which presumably contains the coding regions for P29 and P57. Using the previously annotated PG1 sequence as a guide, only two mycoplasmal ORFs are wholly contained within the 3.7-kb overlapping region: they are MSC_0268 (*pdhD*, encoding the EIII dihydrolipoyamide dehydrogenase subunit of pyruvate dehydrogenase [64.3 kDa]) and MSC_0269 (*pta*, encoding a phosphate acetyltransferase [35.7 kDa]). It is possible that P57 and P29 represent these two full-length gene products, respectively, with any size discrepancy being due to limitations of SDS-PAGE, since no UGA codons are present internally in the correct locations to account for these sizes (data not shown).

Antibody responses following vaccination of mice with λ -A8 and λ -B1. Three groups of mice were vaccinated intramuscularly at weeks 0, 4, and 11 with the following whole phage λ particles: clones λ -A8 (group 1) and λ -B1 (group 2) and a negative control, λ -gt11 (group 3). Following experimental challenge at week 13 with *M. mycoides* subsp. *mycoides* SC biotype strain N6, antibody responses (at 92 h postchallenge) against whole *M. mycoides* subsp. *mycoides* SC biotype proteins in the three vaccinated mouse groups were measured by ELISA (Fig. 3). Compared to those for the mouse group vaccinated with the control phage λ -gt11, higher antibody titers against whole mycoplasma proteins were observed for the groups vaccinated with λ -A8 ($P = 0.04$) and λ -B1 ($P = 0.08$), although the intensities of the responses were relatively low overall. All mice showed high-level antibody responses against phage coat proteins when these were used as the ELISA antigen (data not shown).

In order to demonstrate the specificity of these immune responses, whole-cell extracts of *E. coli* containing recombinant phagemid p-A8 or p-B1 were separated by SDS-PAGE

and immunoblotted using sera from vaccinated and challenged mice. Cells were grown in the presence of IPTG to induce *M. mycoides* subsp. *mycoides* SC biotype recombinant protein expression where necessary. The *E. coli* p-A8 extract was probed with sera from group 1 mice 4 and 8 (vaccinated with λ -A8) and with a negative control serum (group 3 mouse 4 [vaccinated with λ -gt11]). The results are shown in Fig. 4a. It is clear that a specific protein band, at approximately 28 kDa, was detected with the sera from two mice immunized with λ -A8 but was not recognized with the serum from a mouse given the λ -gt11 negative control phage. This 28-kDa protein is almost certainly the same IPTG-inducible P28 recognized by rabbit

FIG. 3. Individual antibody responses against whole sonicated *M. mycoides* subsp. *mycoides* SC biotype antigen in vaccinated mouse groups ($n = 8$ mice per group). The standard deviations of individual mouse values are shown. Overall group responses were compared by a t test. Group 1 mice were vaccinated with λ -A8 ($P = 0.04$ compared to group 3), group 2 mice were vaccinated with λ -B1 ($P = 0.08$ compared to controls), and group 3 (negative control) mice were vaccinated with λ -gt11. OD, optical density.

FIG. 4. Immunoblots of *E. coli* whole-cell extracts containing phagemid p-A8 (a) or p-B1 (b). Immunoblots were probed with two separate sera from mice immunized with λ -A8 (a) or λ -B1 (b). Specific *M. mycoides* subsp. *mycoides* SC biotype proteins are shown. A negative control lane (using serum from a mouse immunized with λ -gt11) is also shown.

anti-*M. mycoides* subsp. *mycoides* SC biotype serum when used to probe the *E. coli* p-A8 extract (Fig. 1), indicating that both prokaryotic and eukaryotic expression of this protein occurs from the construct (i.e., the expression of P28 must also have occurred in the mouse vaccinated with λ -A8 in order to lead to specific P28 antibodies being produced). Similarly, in Fig. 4b, the results of probing an immunoblot of *E. coli* p-B1 antigen extract with mouse sera are shown. In this case, specific protein bands at 29 kDa and 57 kDa were seen by using sera from two group 2 mice (λ -B1) and were absent with the negative control serum (group 3 mouse 4), indicating both prokaryotic and eukaryotic expression of P29 and P57 (even though expression may be internally primed rather than via the P_{lac} or CMV promoter of the vector since the expression of these proteins in *E. coli* does not appear to be IPTG inducible [Fig. 1a and b]).

LSA. The cellular proliferation of pooled mouse splenocytes from each mouse group (at 92 h postchallenge) was measured

by the incorporation of [3 H]thymidine (cpm) in response to stimulation from whole *M. mycoides* subsp. *mycoides* SC biotype or whole bacteriophage λ -gt11 particles. From these values, the SI compared to a medium-only value of 1.0 was calculated for each mouse group. The responses against a whole *M. mycoides* subsp. *mycoides* SC biotype antigenic stimulus are shown in Fig. 5a. In common with the antibody response data (Fig. 3), greater proliferation ($P = 0.053$) was seen in mice vaccinated with λ -A8 (SI = 2.5 ± 0.1) than in the control group given λ -gt11 (SI = 1.6 ± 0.4). In contrast, no stimulation was observed for mice vaccinated with λ -B1 (SI = 0.5 ± 0.7).

In contrast to the low SI values seen with whole *M. mycoides* subsp. *mycoides* SC biotype, very high SI values were observed for group 1 mice (λ -A8) with whole phage λ particles as the antigenic stimulus (Fig. 5b). SI values of approximately 300 were observed, which were comparable to those seen with the positive control stimulus, concanavalin A, indicating that whole phage λ particles are an extremely effective proliferative agent. Bacteriophages in general are known to be highly immunogenic (6), and our data reinforce such findings, although the effect of contaminating bacterial lipopolysaccharide should not be discounted. High SI values against whole phage particles were also observed for mouse groups 2 and 3 (given phage λ -B1 and λ -gt11, respectively), although overall the SI values for splenocytes from group 1 animals were much larger, irrespective of the stimulus (whole phage particles or concanavalin A). During the dissection of group 1 mice following challenge with live *M. mycoides* subsp. *mycoides* SC biotype, it was noted that the spleens were generally very large compared to those of the λ -gt11-vaccinated group (approximately twice the size overall; 2×10^9 splenocytes total for group 1 compared to 1.1×10^9 for group 3), in agreement with the LSA results, which suggested that splenocytes from group 1 animals were generally in a more activated state than those from group 2 and 3 animals. This could indicate more effective priming against *M. mycoides* subsp. *mycoides* SC biotype by phage λ -A8 than that against the other immunogens.

FIG. 5. LSAs using pooled mouse splenocytes ($n = 8$) from mouse groups 1, 2, and 3. Stimulation indexes compared to a medium-only value of 1 are shown. Standard deviation values are shown for each sample (performed in triplicate). The antigen used was whole *M. mycoides* subsp. *mycoides* SC biotype (a), whole bacteriophage particles, or concanavalin A (b). Borderline significance values were observed between group 1 (λ -A8) and group 3 (λ -gt11) mice in panel (a) ($P = 0.053$ by a t test).

TABLE 1. Number of mice in each vaccinated group exhibiting mycoplasmaemia after intraperitoneal challenge

Mouse group	Vaccine	No. of mice in a group of 8 showing mycoplasmaemia on the stated day after challenge		
		Day 2	Day 3	Day 4
1	λ -A8	8	5	1
2	λ -B1	7	7	5
3	λ -gt11	7	6	4

Challenge experiment. Table 1 shows the results of the challenge experiment (week 13). All mice were negative for *M. mycoides* subsp. *mycoides* SC biotype immediately prior to challenge. Group 1 mice, immunized with the λ -A8 vaccine, showed a clear trend toward protection, with much reduced mycoplasmaemia over the course of the experimental challenge (8/8 mice were positive on day 2, with a reduction to 1/8 by day 4) compared to mouse groups 2 and 3 (7/8, with a reduction to 5/8 and 4/8, respectively, by day 4). Due to the small number of mice in these groups, the difference between groups was not statistically significant ($P = 0.12$ [comparing groups 1 and 3] and $P = 0.28$ [comparing groups 2 and 3]), even though the data suggested that vaccination with λ -A8 reduced the duration of observed mycoplasmaemia.

DISCUSSION

Whole bacteriophage particles have previously been described as a new and advantageous delivery method for DNA vaccines (6). The methodology has been used here for the rapid isolation and screening of candidate vaccines against *Mycoplasma mycoides* subsp. *mycoides* SC biotype, the causal agent of CBPP. A semirandom whole genomic library of *M. mycoides* subsp. *mycoides* SC biotype was cloned into the expression vector λ ZAP Express, in which the insert is under the transcriptional control of both a prokaryotic (P_{lac}) and a eukaryotic (P_{CMV}) promoter. The CMV promoter allows the immunogenicity and/or protective efficacy of this library to be directly tested in vivo by simply vaccinating animals with the whole phage library without any further manipulation, and we have already demonstrated this whole phage library immunization procedure using a *Mycoplasma capricolum* subsp. *capripneumoniae* λ ZAP Express library (17). Obviously, a whole cDNA expression library can also be tested if required. This system also allows rapid immunoscreening of the library when plated on *E. coli*, using P_{lac} -driven expression to identify putative vaccine candidates. Initially, rabbit *M. mycoides* subsp. *mycoides* SC biotype-specific hyperimmune antiserum was used to identify clones capable of expressing *M. mycoides* subsp. *mycoides* SC biotype proteins, followed by bovine convalescent-phase antiserum to specifically identify those clones expressing CBPP-immunodominant antigens (assuming that immunodominance correlates with protection [the positive signal observed with bovine convalescent-phase antiserum indicates that these antigens must be expressed by *M. mycoides* subsp. *mycoides* SC biotype during a natural infection]). Following subsequent phagemid excision from the λ ZAP Express vector, inserts were sequenced and protein expression profiles

were determined by immunoblotting of phagemid-expressed proteins in *E. coli* extracts. In this instance, two clones were selected for further study, namely, λ -A8 (containing a 7.1-kb insert) and λ -B1 (containing a 6.7-kb insert). These constructs were then grown and tested for their protective efficacy as purified whole phage lysates in mice against an *M. mycoides* subsp. *mycoides* SC biotype mycoplasmaemia, with both cell-mediated and humoral immune responses being studied and compared to those observed in mice given a nonexpressing λ -gt11 control bacteriophage.

Phagemid p-A8 appeared to express a major protein band recognized by *M. mycoides* subsp. *mycoides* SC biotype antiserum on an immunoblot at approximately 28 kDa (P28), with a slightly smaller band (26 kDa) of much lower intensity also present (Fig. 1a). Since these proteins are IPTG inducible, their expression must be controlled from the P_{lac} promoter of the vector. From an analysis of the DNA sequence of the A8 insert (data not shown) and the published *M. mycoides* subsp. *mycoides* SC biotype genomic sequence (40), it appears that the entire open reading frame of *lpp* MSC_0397 (encoding a previously uncharacterized prolipoprotein with an unprocessed molecular mass of 25.6 kDa) is present, with the ATG start codon about 50 bp into the cloned fragment. This gene does not encode any tryptophan residues (encoded by UGA codons in *M. mycoides* subsp. *mycoides* SC biotype), meaning that the protein should be translated in its entirety in nonmycoplasma hosts. In addition to this full-length protein, a translational fusion comprising 34 amino acids of the vector β -galactosidase α protein fragment, 16 amino acids from noncoding *M. mycoides* subsp. *mycoides* SC biotype DNA, and the entire *lpp*-encoded protein should also be generated, which would create a larger fusion protein of about 31 kDa. The most likely explanation is that P28 seen on the immunoblot represents the latter fusion protein, with translation initiating at the β -galactosidase ATG, while the smaller and less intense band (P26) represents the pure *M. mycoides* subsp. *mycoides* SC biotype *lpp*-encoded protein sequence. Alternatively, it is also possible that posttranslational modification may also occur (e.g., cleavage of a signal peptide), with P28 representing full-length Lpp and P26 representing a processed version.

Lpp MSC_0397 is an extremely basic protein, with an expected pI of 9.58. A preliminary analysis (data not shown) of the predicted structure of this protein revealed two transmembrane helices, one at the N terminus (amino acids 4 to 24) and one at the C terminus (amino acids 208 to 230). However, the N-terminal helix has a significant level of homology with the N-terminal signal sequences of the variable surface protein Vmm (30) and LppC (31) of *M. mycoides* subsp. *mycoides* SC biotype. A sequence comparison with the published genome sequence of *M. mycoides* subsp. *mycoides* SC biotype also reveals N-terminal homology with several other putative lipoproteins of *M. mycoides* subsp. *mycoides* SC biotype (40). The Lpp protein has a predicted VVA-C signal peptidase II cleavage site at amino acids 21 to 24. This strongly suggests that mature Lpp is a surface-exposed lipoprotein which is modified with lipid at the cysteine at residue 24 after cleavage of the 23-amino-acid signal peptide. We are currently attempting to determine the exact function of the *lpp* gene product.

Four separate overlapping clones were also isolated which contained a common region of 3.7 kb of *M. mycoides* subsp.

mycoides SC biotype DNA (Fig. 2) encoding the pyruvate dehydrogenase (Pdh) complex (*pdhB*, *pdhC*, and *pdhD*). Two full-length ORFs were present in this common 3.7-kb region, namely, *pdhD*, encoding the EIII dihydrolipoamide dehydrogenase subunit of Pdh (64.3 kDa), and *pta*, encoding a phosphate acetyltransferase (35.7 kDa). On an immunoblot, these clones expressed two *M. mycoides* subsp. *mycoides* SC biotype proteins (P57 and P29) which may represent the full-length polypeptides (with size discrepancies due to limitations in the SDS-PAGE system). Alternatively, they may represent N- or C-terminally truncated versions of these proteins, although it is not clear exactly where transcription/translation is occurring since neither of these proteins is IPTG inducible and therefore must be internally primed. One possibility is that P57 represents a dimer of P29. An analysis of Fig. 1 reveals a faint high-molecular-weight "ladder" above the P57 protein which could support this theory. However, the expression of P57 is reduced in clone p-A3 compared to that in the other three constructs. Uniquely, this clone lacks *pdhD* upstream DNA (likely to contain a promoter region), which would agree with the hypothesis that P57 is derived from the *pdhD* gene product.

The Pdh complex, particularly the lipoyl-binding domain, is known to be highly immunogenic in other bacterial species (e.g., *Neisseria meningitidis* [1], *Mycoplasma capricolum* [43], and *Mycoplasma hyopneumoniae* [24]). In *M. mycoides* subsp. *mycoides* SC biotype, lipoyl-binding domains are found at the N termini of both the EII (*pdhC*) and EIII (*pdhD*) subunits (using a conserved domain database search [22] through the NCBI website). The pfam reference for the biotin-lipoyl binding domain is pfam00364.11. In *Mycoplasma pneumoniae*, recent research has identified two fibronectin binding proteins, one with an observed molecular mass of 30 kDa, which was subsequently determined to be the EI β (*pdhB*) subunit of the Pdh complex (8). Several studies have suggested that the Pdh complex may be membrane bound in *M. pneumoniae* and form part of an attachment organelle (16, 32). A membrane-bound location for the *M. mycoides* subsp. *mycoides* SC biotype Pdh complex would agree with its immunogenic nature, since 4 of 10 λ clones identified using CBPP convalescent-phase antiserum carried genes encoding proteins from this region.

Clones λ -A8 (containing *lpp*) and λ -B1 (containing full-length *pdhC*, *pdhD*, *pta*, and *ackA* ORFs) were used to vaccinate mice, and humoral and cellular responses were assessed. Since whole *M. mycoides* subsp. *mycoides* SC biotype was used as the ELISA/LSA antigen rather than the purified recombinant proteins encoded by the λ vaccines (e.g., Lpp, PdhD, and Pta), it is likely that the low signal intensities observed for both procedures (Fig. 3 and 5a) may be due to the limiting amount of specific antigen, particularly since it is not known how prevalent these proteins are among the general *M. mycoides* subsp. *mycoides* SC biotype antigen mix (assuming that 500 different polypeptides at equal proportions and a binding capacity of 50 ng per well on an ELISA plate would mean only 0.1 ng of antigen per well). In support of this, strong specific antibody responses were observed against recombinant proteins (P28 [Lpp], P29, or P57) following immunoblotting. A higher level of cellular proliferation was also observed in mice vaccinated with λ -A8 than in mice given λ -gt11 control phage when whole *M. mycoides* subsp. *mycoides* SC biotype was used as the antigen. When the cellular responses of the λ -A8 group were

measured against whole phage particles, a very high stimulation index of up to 350 was noted, in contrast to the medium-only control (Fig. 5b), indicating excellent cellular priming by phage particles (which are known to be highly immunogenic) (15, 41, 44). Interestingly, following challenge of this mouse group with whole live *M. mycoides* subsp. *mycoides* SC biotype strain N6 cells, a reduced level of mycoplasmaemia was observed compared to those for the other two groups, indicating a trend toward protection for this group (due to the small number of mice per group, statistical significance was not achieved). In addition, spleens from this group were approximately twice the size of controls, indicating a more activated state suggestive of more efficient priming by λ -A8.

Current mycoplasma vaccines are generally either live or inactivated preparations of whole organisms (10, 23, 29, 34). There has been little success with subunit vaccines or recombinant proteins (35), and this is especially true for CBPP, for which the only Office International des Epizootes-recommended vaccines are freeze-dried live formulations (strain T₁44 or its streptomycin-resistant derivative T₁SR). Research designed to produce "next-generation" vaccines (e.g., immunostimulating complex [ISCOM], capsular polysaccharide conjugate, or a whole-cell inactivated vaccine) has proceeded, although with little apparent success (2, 11, 28, 39). It is possible that a genetic immunization approach may offer the potential for success in the field of mycoplasmal vaccines, since a successful plasmid-based whole-library immunization against *Mycoplasma pulmonis* was reported previously (4). The approach utilized here, in which rapid whole-library immunoscreening is followed by immediate in vivo testing of isolated clones, may offer great potential in the future. This is especially so for a disease like CBPP, which predominantly affects the developing world, where the high stability and ease and cheapness of production of bacteriophage vaccines would be a great benefit. While results obtained in a mouse model may not necessarily represent the situation in the target species, they do demonstrate the validity of the strategy, which may also be applied to both human and veterinary pathogens for which potential vaccine candidates have not yet been identified.

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