Identification of *Mycoplasma mycoides* subsp. *mycoides* Small Colony Genes Coding for T-Cell Antigens\(^\text{v}\)

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Contagious bovine pleuropneumonia (CBPP), an infectious pulmonary disease caused by the *Mycoplasma mycoides* subsp. *mycoides* small colony type (*MmmSC*), is one of the most important transboundary bacterial epizootics affecting cattle in sub-Saharan Africa. CBPP represents a major constraint to cattle production due to mortality, weight loss, and reduced fertility (17) and because the countries affected are excluded from international trade (5; http://www.oie.int). Disease control through slaughtering measures, quarantine, or even strict control of animal movement is unrealistic in those parts of Africa where transhumance is widely prevalent. The most practical solution is vaccination, but the existing live, attenuated vaccines need to be improved because of their low efficacy and residual virulence (6, 13, 14). We have adopted a strategy of identifying genes of the agent coding for immunoprotective antigens of the agent coding for immunoprotective antigens. In addition to *MmmSC*-specific memory CD4\(^+\) T cells comprised of two phenotypically and functionally different subsets (19). The first subset corresponds to conventional effector memory T cell (Tem cells), which can migrate to infected tissues and produce effector cytokines such as gamma interferon (IFN-γ). Tem cells are poised to fight infection but progressively disappear when the pathogen is eliminated (8, 21, 22). The second subset shares several characteristics with central memory T cells (Tcm cells): (i) high proliferative capacities, (ii) low production of IFN-γ, and (iii) recirculation through lymph nodes (19). Tcm cells have been shown in other models to be long-lived, surviving even after complete clearance of the pathogen, and capable of rapid and strong expansion in draining lymph nodes upon reinfection in order to replenish the pool of short-lived Tem cells (15, 20, 22). Thus, both Tem and Tcm cells are important targets for vaccines that are aimed at eliciting cell-mediated immunity (CMI). Since animals that have recovered from CBPP are known to have acquired lifelong immunity (13), results from studies of T-cell responses are consistent with a role for both the Tem and Tcm subsets of CD4\(^+\) T cells in protection. This implies that *MmmSC* antigens capable of eliciting these responses have potential for incorporation into a subunit vaccine against CBPP. So far, only the lipoprotein LppA has been shown to possess some degree of T-cell immunogenicity (4), but its capacity to elicit Tcm cells has not been investigated.

Here we report the results of a study undertaken to further characterize the T-cell immunogenicity of LppA and identify other *MmmSC* genes that code for T-cell antigens. In addition to LppA, five genes (*abc*, *gapN*, *glpO*, *lppA*, and *ptsG*) previously shown to code for B-cell antigens were selected on the basis of results from previous work (11). The products of these genes were expressed as recombinant polypeptides and assessed for their capacites to elicit recall IFN-γ and proliferative responses from CD4\(^+\) Tem and Tcm cells of cattle that recovered from CBPP.

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Expression and purification of MmmSC proteins. Six MmmSC genes shown to contain B-cell epitopes were selected for this study, and their proteins were expressed in E. coli as histidine (His)-tagged recombinant polypeptides as previously described (1, 11). Apart from lppA codons, which read as stop codons in E. coli, proteins were expressed and purified from total cell extracts by Ni²⁺ affinity chromatography (Invitrogen, Carlsbad, CA). Following elution, fractions of interest were pooled and dialyzed in phosphate-buffered saline (PBS), and protein concentrations were estimated by the bicinchoninic acid method. All proteins manifested as single dominant bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels except for PtsG, which manifested as two faint bands with molecular masses of 70 and 45 kDa instead of the 28 kDa (11). Finally, ApxIVA, a cytotoxin from the porcine pathogen Actinobacillus pleuropneumoniae (16), was expressed by the same method and used as an irrelevant recombinant protein control in our system.

Peptides. Synthetic peptides spanning the 255 amino acids of the PtsG protein were purchased from Genepet (Montpellier, France). The peptides were 16 to 20 residues long and overlapped by 5 amino acids, as shown in Table 2. Purity was shown by high-performance liquid chromatography and mass spectrometry to be above 70%. The peptides were solubilized in PBS or dimethyl sulfoxide (DMSO) at concentrations of 3 to 5 mg/ml and 25 mg/ml, respectively. The peptides were used in T-cell assays as a cocktail containing 10 (pool A) and 7 (pool B) peptides at concentrations of 3 to 5 mg/ml and 25 mg/ml, respectively. The peptides were shown by high-performance liquid chromatography and mass spectrometry to be considered to be significant at a P value of <0.05.

RESULTS

Proliferation of primed CD4⁺ T cells in response to MmmSC antigens. Pilot experiments indicated that 10 μg/ml of clonal antibodies (MAbs): Mab IL-A11 (IgG2a) for CD4 and Mab BAQ92A (IgG1) for CD26L (YMRD, Pullman, WA). After being washed, cells were stained with a cocktail of fluorochrome-conjugated, isotype-specific antibodies (Tebu, Le Perray, France). Single- and three-color analyses were performed with a FACScan flow cytometer equipped with the CellQuest 3.01 software (Becton Dickinson, San Jose, CA) after the acquisition of at least 5,000 events within a typical forward- and side-scatter gate set to exclude dead cells and debris. Normal mouse serum was used to evaluate nonspecific binding and to set gates delineating positive populations.

IFN-γ assay. The secretion of IFN-γ by stimulated cells in culture supernatants was assayed by using a commercially available enzyme-linked immunosorbent assay (ELISA) (BioCore, Omaha, NE) according to the manufacturer’s instructions. Each test was performed in duplicates, and results are expressed as optical densities (OD) measured using a microplate reader at 450 nm. Statistics. A paired Student’s t test (http://www.physics.csbsju.edu/stats/) was used to analyze differences between stimulated and nonstimulated cells. A difference was considered to be significant at a P value of <0.05.

### TABLE 2. Synthetic peptides derived from the MmmSC protein PtsG

| Peptidea | Sequence |
|----------|----------|
| Soluble in PBS (pool A) | KLFNVPTRGDNGAAEAKLYT |
| p6 | AKLYTKADFASKGLNVDGS |
| p7 | NVDGSKMQADDKEQARLA |
| p10 | RLTVDAKKADDIGKLG |
| p12 | IQIVYVGGEQAIKPRM0KLL |
| p13 | MOKLLQORHEKMSHSEMK |
| p14 | HSEMKEEMKSENMGMTCES |
| p15 | MTCESNOACDKDEACSECK |
| p16 | CSEKDCMCEEPKNVEQPQ |
| p17 | EEQVPSEMKEVTKSN |
| Soluble in DMSO (pool B) | ATGALQTHITOVSGHIDD |
| p2 | GIIDYIVFPIESGAMK |
| p3 | GAMKPSAFGVGLVAVLAP |
| p4 | VVLAPIYCFAYFLFKLVN |
| p8 | QARLAKAAAIEYLGGEEN |
| p9 | GEENIVDSCASRLRTTV |
| p11 | KSLGGTGTALVKGONIQIVY |

a Numbers indicate peptide positions on the PtsG portion by increasing order from amino acids 421 to 676.
recombinant proteins was optimal for use in proliferation assays. At this concentration, LppA induced the proliferation of cells collected from lymph nodes draining the lungs of an immunized animal (Fig. 1). Proliferation to the irrelevant control protein Apx was negligible, indicating that there was little interference from *E. coli* contaminants. The level of proliferation was lower with LppA than with whole *Mmm*SC. For that reason, a longer incubation time (i.e., up to 10 days) was used in subsequent experiments. In these experiments, the proliferation of CD4⁺ T cells exposed to LppA and to the five additional *Mmm*SC recombinant proteins was investigated. At day 10, Abc (*P* = 0.025), LppA (*P* = 0.001), and PtsG (*P* = 0.003) induced a significant recall proliferation of primed CD4⁺ T cells in all animals that were tested (Fig. 2). With LppA and PtsG, the proliferation was also accompanied at day 10 by an increase in absolute cell numbers as indicated by a reduction in the time needed for the flow cytometer to acquire 1,000 CD4⁺ T cells: 100 ± 18 s for unstimulated cultures, 34 ± 15 s for LppA, and 62 ± 7 s for PtsG. In contrast, the activity of GlpO and LppB was highly variable, with some animals showing no response at all, while there was little or no response to GapN and Apx (Fig. 2). Finally, negligible responses were detected with cells collected from naive cattle (data not shown).

The yield of recombinant PtsG in *E. coli* was very low, and its unexpected molecular masses on SDS-PAGE gels cast some doubts on its purity (see Materials and Methods). For those reasons, a series of overlapping peptides that covered the expressed amino acid sequence of PtsG was synthesized (Table 2). Pool A peptides induced the recall proliferation of CD4⁺ T cells from all animals tested, whereas pool B was poorly immunogenic (Fig. 3). This was not due to residual cytotoxicity, since the addition of pool B peptides together with whole *Mmm*SC antigen triggered proliferative responses comparable to those obtained with *Mmm*SC antigen alone (data not shown).

**CD62L expression on *Mmm*SC protein-driven proliferating CD4⁺ T cells.** As shown previously (19), the surface expression of the lymph node homing receptor α-selectin (or CD62L) among *Mmm*SC-driven proliferating CD4⁺ T cells can be used to discriminate Tcm from Tem cells in animals that have re-

![FIG. 1. LppA-induced recall proliferation of lymphocytes from CBPP-immunized (a) and naïve (b) cattle. Cells were loaded with CFSE and incubated for 7 days with medium alone (no antigen [NoAg]), whole *Mmm*SC antigen (*Mmm*SC), the mitogen concanavalin A (ConA), and recombinant LppA and Apx proteins. Proliferation is associated with a decrease in CFSE fluorescence intensity (FL1) and is indicated on the plots as percentages of total cells calculated within the M1 interval (percent CFSE<sup>low</sup>).](http://cvi.asm.org/)

![FIG. 2. Recall proliferation of primed CD4⁺ T cells in response to individual *Mmm*SC recombinant proteins. Cells were loaded with CFSE and incubated for 7 and 10 days with medium alone (NoAg), whole *Mmm*SC antigen (*Mmm*SC), and recombinant proteins (Abc, Apx, GapN, GlpO, LppA, LppB, and PtsG). Results are mean percentages (± standard deviations [SD]) of CFSE<sup>low</sup> cells for four animals and two independent experiments. Asterisks indicate significant differences (*P* < 0.05) from medium-alone controls.](http://cvi.asm.org/)

![FIG. 3. Recall proliferation of primed CD4⁺ T cells in response to synthetic PtsG peptides. Cells were loaded with CFSE and incubated for 10 days with medium alone (NoAg), whole *Mmm*SC antigen (*Mmm*SC), PtsG peptides soluble in PBS (pool A), and PtsG peptides soluble in DMSO (pool B). Results are mean percentages (± SD) of CFSE<sup>low</sup> cells for three animals and two independent experiments.](http://cvi.asm.org/)
covered from CBPP. Here we found that MmmSC-primed CD4+ T cells that proliferated in vitro in the recall response to LppA comprised both CD62L-positive and CD62L-negative populations (Fig. 4). This was also observed for the Abc protein and PtsG peptides, and analysis at the group level confirmed the presence of both subsets, with a predominance of CD62L-positive cells in cultures stimulated with LppA and PtsG but not with Abc (Fig. 5).

**IFN-γ production by primed lymphocytes in response to MmmSC antigens.** Only LppA and PtsG induced sufficient secretion of IFN-γ by cells from all animals to result in a statistically significant response at the group level ([P] = 0.021 and [P] = 0.031, respectively, at day 10) (Fig. 6). IFN-γ responses to Abc were detected in one animal only and were negligible for Apx, GapN, GlpO, and LppB (Fig. 6). There was no significant response detected when cells collected from naïve cattle were used (data not shown).

The immunogenicity of PtsG was assessed further by using synthetic peptides. As observed for the recall proliferation of primed CD4+ T cells, only pool A generated sufficient secretion of IFN-γ from all animals to result in a statistically significant response at the group level (Fig. 7).

**DISCUSSION**

The identification of MmmSC genes coding for immunogenic proteins is a prerequisite for developing recombinant subunit vaccines against CBPP. Until recently, efforts have focused exclusively on identifying genes that code for B-cell antigens. Results from studies of CMI strongly suggest, however, that T-cell responses are also important in achieving long-term protection. In particular, IFN-γ production and memory CD4+ T cells appear to be associated with resistance to infection (2, 3, 18), thus providing a rationale for targeting these responses in novel vaccine strategies. Ideally, subunit CBPP vaccines should elicit not only CD4+ Tcm cells that can migrate rapidly to the site of infection and produce IFN-γ but also Tcm cells with their increased proliferative and survival capacities (19). Here we report the identification of MmmSC genes coding for proteins capable of inducing recall IFN-γ and proliferative responses from CD4+ Tem and Tcm cells prepared from cattle that have completely recovered from CBPP infection. Recall proliferation and surface expression of the lymph node homing receptor L-selectin (CD62L) were used as indicators of Tcm cell responses. Another important parameter was the capacity of MmmSC proteins to elicit these responses in all animals tested.

Among the six MmmSC genes tested, lppA, ptsG, and, to a lesser extent, abc were found to code for proteins capable of

![Fig. 4](image-url) Both CD62L-positive and CD62L-negative CD4+ T cells proliferate in response to LppA. Results of a typical three-color flow cytometric analysis are shown in density plots for one CBPP-immunized animal. Cells were loaded with CFSE and incubated for 10 days with medium alone (NoAg) or LppA. An R2 gate was set to analyze CD4+ CFSE+ cells for CD62L expression. The percentages of CD62L-positive and CD62L-negative cells within R2 are indicated on the plot in the upper and lower quadrants, respectively.

![Fig. 5](image-url) CD62L expression among CD4+ T cells proliferating (R2 gate, as in Fig. 4) in response to the Abc, LppA, and PtsG (pool A) peptides. Results are mean percentages (±SD) for four animals and two independent experiments.

![Fig. 6](image-url) Recall IFN-γ production by primed cells in response to individual MmmSC recombinant proteins. IFN-γ was measured by ELISA in 7- and 10-day-old supernatants of cultures supplemented with medium alone (NoAg), whole MmmSC antigen (MmmSC), and recombinant proteins (Abc, Apx, GapN, GlpO, LppA, LppB, and PtsG). Results are expressed as optical densities (OD) and are mean (±SD) values for four animals and two independent experiments. Asterisks indicate significant differences (P < 0.05) from medium-alone controls.
inducing a recall proliferation of primed CD4+ T cells in all animals tested. Both CD62L-positive and CD62L-negative lymphocyte subsets proliferated, indicating that both Tem and Tcm cells were present in the responding CD4 population. In addition, percentages of CD62L-positive cells were lower after stimulation with Abc than those after stimulation with LppA and PtsG. This finding is consistent with the lower overall proliferative response induced by Abc in primed CD4+ T cells. A correlation between the vigorous proliferation of MmmSC-specific memory CD4+ T cells and high levels of expression of CD62L was reported previously (18, 19). Finally, the use of synthetic peptides confirmed that the T-cell antigenicity of PtsG was not due to E. coli contaminating proteins. A further division of pool A (i.e., two pools of five peptides) substantially reduced the proliferation of primed CD4+ T cells, suggesting that PtsG contains more than one T-cell epitope (data not shown).

LppA and PtsG were the only MmmSC proteins that induced a significant production of IFN-γ by primed T cells from all the animals that were tested. Although the phenotypes of the cells that produced IFN-γ were not determined in this study, CD4+ T cells are likely to be the main source. Indeed, previous work clearly demonstrated a predominant role for CD4+ T cells in the recall IFN-γ response triggered by whole MmmSC antigens. Two-thirds of IFN-γ producers were CD4+ T cells, and in addition, they strictly control the production of IFN-γ by other cell types (18). In comparison with the responses to LppA and PtsG, the IFN-γ response to Abc was more heterogeneous and not significant at the group level, which suggests that Abc is a weaker T-cell immunogen. It should, however, be pointed out that the protein used in this study represents only 62% of the full ABC transporter protein, and the possibility that the remaining sequence may also contain T-cell epitopes cannot be excluded. This applies to all poly-peptides used in this study, except for LppA and GlpO, and warrants further studies using full-length proteins, which can be obtained only after all mycoplasmal tryptophan codons have been mutated to TGG (9). Also, the search for T-cell antigens should not be restricted to genes previously shown to code for proteins recognized by B cells but should be extended to all membrane or transmembrane proteins of the agent.

Our results confirm a previous report of LppA’s T-cell immunogenicity (4) and extend it to show that LppA is a target for both Tem and Tcm cells potentially associated with protection. Since LppA is not a variable surface protein, opportunities for MmmSC to escape from the host’s anamnestic immune response should be limited. On the other hand, the antigenic variability of PtsG was reported and shown to result from the truncation of a gene portion encompassing the portion used in this study (7). Nonetheless, PtsG was recently shown to be upregulated when MmmSC forms stress-resistant biofilms (10). It is therefore tempting to speculate that animals vaccinated against PtsG will circumvent disease persistence more efficiently.

In conclusion, LppA and PtsG consistently induced recall IFN-γ production and proliferation from immune CD4+ T cells in all animals tested. In addition, they were shown to possess epitopes recognized by both CD4+ Tem and Tcm cells. They are thus potential vaccine candidates for eliciting both T1-type effector and long-lived T-cell immunity in genetically heterogeneous cattle.

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