Production and Characterization of Monoclonal Antibodies against a Major Membrane Protein of *Mycobacterium avium* subsp. *paratuberculosis*[^7]

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The *Mycobacterium avium* subsp. *paratuberculosis* 35-kDa major membrane protein (MMP) encoded by MAP2121c is an important membrane antigen recognized in cattle with Johne’s disease. In this study, purified recombinant MMP was used to produce two stable monoclonal antibodies, termed 8G2 and 13E1, which were characterized by immunoblotting, epitope mapping, and immunofluorescence microscopy.

![Image](https://example.com/image.png)

Despite the 100-year history since the discovery of Johne’s disease and the economic consequences of its continued dissemination, there are very few reports describing specific, antigen-based detection reagents for *Mycobacterium avium* subsp. *paratuberculosis*, the causative agent of the disease. One study described the use of three monoclonal antibodies (mAbs) to characterize culture filtrate proteins of *M. avium* subsp. *paratuberculosis*, whose identities were never defined (20). Very recently, single-chain antibodies were selected by cloning heavy and light chains from sheep with Johne’s disease (7). This effort has resulted in two very promising antibodies; however, the *M. avium* subsp. *paratuberculosis* proteins with which these antibodies react remain undefined. A major program initiative, funded by the U.S. Department of Agriculture and entitled the Johne’s Disease Integrated Program, has as one of its many objectives the development of monoclonal antibody reagents. This research priority was identified because of the distinct lack of such reagents and the blockade this void creates in moving along newly developed technologies and assays. Some technologies that are especially critical for *M. avium* subsp. *paratuberculosis* research, such as immunomagnetic separation (12, 16) and biosensor detection (18), highlight the need to raise mAbs to this bacterium.

The major membrane protein (MMP), encoded by MAP2121c, is a 35-kDa surface-located mycobacterial antigen that is recognized by cells from humans and cattle infected with mycobacteria (1, 29). In addition, it has been shown to have an effect on the immune response in mice (5) as well as a delayed-type hypersensitivity reaction (6). Collectively, these findings suggest that this antigen deserves further study. However, no antibodies that specifically detect this protein have been available.

The National Animal Disease Center’s mycobacterial culture collection served as the source of all strains used in this study (Table 1). All mycobacteria were cultivated in Middlebrook 7H9 medium supplemented with OADC (Hardy Diagnostics, Santa Maria, CA). For the cultivation of *M. avium* subsp. *paratuberculosis*, mycobactin J (2 mg/liter; Allied Monitor, Fayette, MO) was added to the Middlebrook-OADC medium. Whole-cell sonicated extracts of mycobacterial species and isolates were prepared for use as antigens in immunoassays as described previously (30). Membrane-enriched and cytosol-enriched fractionated lysates were prepared as described previously (24).

Expression clones producing recombinant *M. avium* subsp. *paratuberculosis* proteins were obtained using methods previously described in detail (2). All recombinant fusion proteins contained maltose-binding protein (MBP) as the tag used in affinity purification. For cloning, the full-length gene was amplified using 2121c-F and 2121c-R primers (Table 2) whereas primers 2121c-F and 2121c-N-R were used to amplify the 5’ half and primers 2121c-C-F and 2121c-R were used to amplify the 3’ end. The central segment of the MAP2121c open reading frame was amplified using 2121c-CenF and 2121c-CenR. Details of the methods used for the induction and affinity purification of MBP-MAP2121c gene product fusion proteins have been described previously (2).

mAbs were produced using standard methods (13). Briefly, 6-week-old female BALB/c mice were immunized three times intraperitoneally with the recombinant fusion protein (100 μg per injection) suspended in 0.5 ml of phosphate-buffered saline (PBS; pH 7.3) at 14-day intervals. The antigen was emulsified.

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TABLE 1. Mycobacterial isolates used in this study

| Organism            | Isolate | Host   | Location | Reference and/or strain designation(s) |
|---------------------|---------|--------|----------|----------------------------------------|
| M. avium subsp.     | K-10    | Cow    | Feces    | ATCC 19698                              |
| paratuberculosis    | 19698   | Cow    | Feces    | ATCC 19698                              |
|                     | 6100    | Human  | Ileum    | ATCC 43015                              |
|                     | 187     | Cow    | Ileum    | ATCC 35773                              |
|                     | 523     | Cow    | Ileum    | ATCC 43015                              |
|                     | 803     | Cow    | Ileum    | ATCC 35773                              |
|                     | Linda   | Human  | Ileum    | ATCC 43015                              |
| M. avium subsp.     | 6009    | Cow    | ATCC 35716 (TMC715) |
| avium               |         |        |          |                                        |
|                     | 6003    | Chicken| ATCC 35713 (TMC702) |
|                     | 6006    | Roe deer| ATCC 35734 |
|                     |         |        |          |                                        |
| M. avium subsp.     | 6076    | Cow    | Lymph node| ATCC 19977 |
| silvaticum           |         |        |          | ATCC 19210                              |
| M. bovis BCG        |        | Cow    | Milk     | ATCC 35734                              |
| Pasteur             |         |        |          |                                        |
| M. kansasi           | 6081    | Human  | ATCC 12478  |
| M. intracellularine  | 6010    | Pig    | ATCC 35773  |
| M. phlei            | 6083    |        | ATCC 35773  |
| M. scrofulaceum     | 6077    | Human  | ATCC 35773  |

TABLE 2. Oligonucleotide primers used to amplify MAP2121c

| Primer name | Sequencea | Location within MAP2121c |
|-------------|-----------|-------------------------|
| 212G-F      | ATCCCTCTGAATGGCACTCGGTTCCAAAAGAG | 1 to 21 |
| 212G-R      | GCCGAGGGCTGAGTCTCACTTCCGCCGAG   | 904 to 924 |
| 212G-N-R    | GCGCAGGGCTGAGTCTCACTTCCGCCGAG   | 442 to 462 |
| 212G-CenF   | ATCCCTCTGAGATGCGCTGAGAAGGAGACCG | 232 to 252 |
| 212G-CenR   | GCGCAGGGCTGAGTCTCACTTCCGCCGAG   | 676 to 697 |
| 212G-C-F    | ATCCCTCTGAGATGCGCTGAGAAGGAGACCG | 463 to 483 |

a Underlined nucleotides represent restriction endonuclease cut sites. The Xbal recognition sequence is TCTAGA, and AAGCTT is the HindIII recognition site.

b The numbers represent the nucleotide positions within the gene.

detected with Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G (Invitrogen) at a dilution of 1:500 in PBS-BSA. The slide was incubated at room temperature in the dark for 1 h and then washed again three times with PBS. Excess PBS was drained away, and the slide was prepared for microscopy with a drop of Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) and a coverslip. Labeled cells were visualized by differential interference contrast (DIC) and fluorescence microscopy with a Nikon ES800 series microscope.

An M. avium subsp. paratuberculosis protein encoded by MAP2121c was previously shown to play a role in the invasion of epithelial cells (1). The protein was heterologously expressed as a full-length fusion protein in Escherichia coli to serve as an antigen. To obtain mAbs against MMP, BALB/c mice were immunized with the recombinant fusion protein. The fusion of immune splenocytes with SP2/0 myeloma cells served as an antigen. To obtain mAbs against MMP, BALB/c mice were immunized with the recombinant fusion protein. The fusion of immune splenocytes with SP2/0 myeloma cells resulted in six hybridomas initially testing positive in immunoblot screens, but only two stable cell lines secreting antibody to MMP were obtained, and these antibodies were designated 8G2 and 13E1.

Supernatants from the 8G2- and 13E1-secreting cell lines were evaluated by immunoblotting with the recombinant protein expressed in E. coli as well as whole-cell lysates of M. avium subsp. paratuberculosis. Both 8G2 and 13E1 detected a 35-kDa protein in M. avium subsp. paratuberculosis as well as the E. coli-expressed recombinant MBP-MAP2121c fusion protein (Fig. 1). However, neither antibody detected the MBP-LacZ fusion protein, suggesting that neither mAb reacts with the affinity tag (Fig. 1). As a control, a third immunoblot was probed with a mAb that binds to the MBP affinity tag. This antibody, developed in our laboratory during this study, detected both MBP-LacZ and MAP2121c gene product fusion proteins, but nothing was detected in M. avium subsp. paratuberculosis whole-cell lysates. Isotype analysis revealed that both 8G2 and 13E1 mAbs were immunoglobulin G1 heavy-chain and kappa light-chain antibodies.

Because the MAP2121c coding sequence is present in other mycobacteria, the cross-reactivities of 8G2 and 13E1 were determined. Each mAb was screened by immunoblotting with whole-cell lysate preparations from nine mycobacterial species and subspecies (Fig. 2). Both mAbs reacted to all three M. avium subsp. paratuberculosis strains, as well as a human isolate (Fig. 2A and B, lanes 5, 10, and 12). 13E1 detected a similarly sized protein in all members of the M. avium complex, which in-
FIG. 1. Immunoblot analysis of mAbs 8G2 and 13E1 shows reactivity with the E. coli-expressed recombinant protein as well as the native M. avium subsp. paratuberculosis 35-kDa protein. Shown are three identical immunoblots that were exposed to the antibodies indicated beneath each filter. Note that anti-MBP (α-MBP) is a mAb to the MBP affinity tag that does not detect any M. avium subsp. paratuberculosis proteins whereas 8G2 and 13E1 detect only the E. coli-expressed MBP-MAP2121c gene product fusion protein and the MAP2121c gene product in M. avium subsp. paratuberculosis. Lanes: 1, MBP-LacZ; 2, MBP-MAP2121c gene product fusion protein; 3, M. avium subsp. paratuberculosis whole-cell lysate. Kilodalton size markers are shown in the left margin and indicated by corresponding notches between the blots.

FIG. 2. MMP mAbs are not subspecies specific for M. avium subsp. paratuberculosis. Two identical immunoblots were probed with 13E1 (A) and 8G2 (B). In all lanes, 0.6 μg of a whole-cell lysate was loaded. Lanes: 1, protein standards; 2, M. avium subsp. silvaticum; 3, M. scrofulaceum; 4, M. abscessus; 5, M. avium subsp. paratuberculosis K-10; 6, M. avium subsp. avium (TMC702); 7, M. bovis (strain 95-1315); 8, M. phlei; 9, M. bovis BCG; 10, M. avium subsp. paratuberculosis ATCC 19698; 11, M. avium subsp. avium (TMC715); 12, M. avium subsp. paratuberculosis (isolate Linda); 13, M. intracellulare; 14, M. kansiasi. Sizes in kilodaltons of protein standards are indicated in the left margin.

includes M. avium subsp. avium, M. avium subsp. silvaticum (15), M. avium subsp. paratuberculosis, and M. intracellulare (Fig. 2A). Similar reactivity of 8G2 with M. avium complex members was observed; however, the epitope detected by this mAb may be more conserved than the one recognized by 13E1 as 8G2 also cross-reacts with M. abscessus and M. kansasi (Fig. 2B). A smaller protein than the MMP produced in M. avium complex mycobacteria was detected in those two species (Fig. 2B). Neither mAb detected any proteins in M. scrofulaceum, M. bovis, or M. phlei.

The results of specificity experiments, which showed distinct patterns of reactivity among the mycobacterial species, suggested that 8G2 and 13E1 may bind distinct epitopes within MMP. To determine if this hypothesis was true, fragments of the MAP2121c gene encoding the N-terminal, center, and C-terminal regions of the protein were cloned and expressed and the truncated proteins were purified from recombinant E. coli cells. The regions spanned by these truncated versions of MMP relative to full-length MMP are shown schematically to scale in Fig. 3A. The full-length gene is 924 bp, encoding a protein encompassing 307 amino acids. The MMP N-terminal truncation included the initial 154 amino acids, and the MMP C-terminal truncation included the final 153 amino acids. The MMP center truncation included the central 155 amino acids. These constructs were designed such that the epitopes could be quickly mapped to four specific quadrants within the protein as shown in Fig. 3A. These proteins, along with the corresponding full-length protein, were then analyzed by immunoblotting with each of the respective mAbs (Fig. 3B). These data show that, in addition to the full-length protein, 8G2 binds strongly to the N-terminal end as well as to the central region of MMP. This reactivity pattern suggests that the epitope lies within a 77-amino-acid region of overlap between the MMP N-terminal and center polypeptides (quadrant 2 in Fig. 3A). mAb 13E1 detected only the full-length protein (Fig. 3B). This experiment was repeated with polyhistidine-tagged recombinant fusion proteins, and the same results were obtained (data not shown). These data confirm that 8G2 and 13E1 recognize distinct epitopes within MMP and suggest that 13E1 may recognize either a very large linear or a conformational epitope that is disrupted in the truncated versions of MMP.

Antigen localization was performed by immunoblot analysis of cell-fractionated lysates prepared from five M. avium subsp. paratuberculosis isolates. mAb 8G2 showed that MMP was present predominantly within the membrane-enriched fraction, although some reactivity in the cytoplasm-enriched fractions was observed (Fig. 4). Results similar to that shown for 8G2 were also obtained using 13E1 (data not shown).

A major obstacle in defining more precisely the role of MMP in the pathogenesis of Johne’s disease is the lack of reagents that would facilitate the specific immunodetection of MMP. To determine if these mAbs might be useful in detecting M. avium subsp. paratuberculosis within infected macrophages, an indirect immunofluorescence assay was performed. Bovine macrophages immortalized with simian virus 40 (26) were infected with M. avium subsp. paratuberculosis and fixed in methanol 48 h postinfection. Intracellular bacilli stained equally well with Alexa Fluor 488-labeled 13E1 and 8G2 (Fig. 5). In contrast, no staining with Alexa Fluor 488-labeled 13E1 and 8G2 in neighboring uninfected cells was observed (Fig. 5), although cells labeled with 8G2 showed a very slight increase in background fluorescence relative to those labeled with 13E1.

We present the first described mAbs that detect a known M. avium subsp. paratuberculosis protein. Membrane proteins are commonly immunodominant antigens recognized following infection with any of numerous bacterial pathogens (10, 23). While the M. avium subsp. paratuberculosis genome sequence has revealed a few hundred putative cell membrane proteins, using antibodies developed in this study, we were able to confirm experimentally that MMP is a surface protein in all five isolates tested. MMP has been reported to be immunodomi-
nant in patients with leprosy (28, 29). Although a previous study using immunoblotting found that this protein also shows reactivity with samples from cattle with Johne’s disease (1), only 13 cows were clinically evaluated in that study. Therefore, more extensive studies are needed to further assess the immunodominance of this protein in the context of Johne’s disease.

Both antibodies label intramacrophage bacilli for visualization by immunofluorescence microscopy, and although the possibility has not been tested, our data suggest that these antibodies may work for immunohistochemistry with infected tissues as well.

For quickly mapping the epitopes of MMP, three truncated recombinant MMP proteins were produced in addition to the full-length protein. Although the precise epitopes for each mAb were not defined at a high resolution by using synthetic overlapping peptides (19), the production of truncated versions of MMP clearly showed that 8G2 and 13E1 recognize
distinct epitopes. The 8G2 epitope mapped to a 77-amino-acid region on the N-terminal half of MMP, whereas 13E1 detected the full-length protein but none of the truncated proteins. It is unknown why 13E1 detects only the full-length protein; however, one possibility is that the mAb detects a discontinuous epitope formed by the natural aggregation or folding of the N- and C-terminal sections that is disrupted when one section of the protein is missing. The reducing agent 2-mercaptoethanol apparently does not disrupt this conformational epitope as it is detected by immunoblotting. In addition, if conformational changes in MMP do occur by bringing each end of the protein together, this may explain why the truncated MMP observed in *M. kansasii* and *M. abscessus* may not be detected by 13E1, because one end may be missing in the MMP produced by those species. Regardless of the possible explanation, it is clear that distinct epitopes within MMP are recognized by the two mAbs.

From the immunoblot experiments with several mycobacterial species, it can be concluded that 13E1 is specific for the *M. avium* complex, comprising *M. intracellulare* and the three subspecies of *M. avium* (14, 27). However, the finding that 8G2 recognized MMP in *M. kansasii* provides evidence of determinants shared between this species and members of the *M. avium* complex. This cross-reactivity was also observed with *M. abscessus*, which was previously classified as *M. chelonae* (17). The detection of MMP in *M. abscessus* was surprising as this species is a fast-growing mycobacterium that is distinctly related to the *M. avium* complex (11). *M. kansasii* is more closely related to the *M. avium* complex than *M. bovis* and more distantly related than *M. scrofulaceum* (11), yet paradoxically, antibodies did not react with any protein in *M. bovis* or *M. scrofulaceum*. To the best of our knowledge, no antibody has been reported that detects only *M. avium* subsp. *paratuberculosis*; therefore, with the recognition of the potential value of such an antibody, the search for this elusive reagent will likely continue. This search is difficult considering the very close genetic relatedness between *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* (4); however, genomic comparisons between these two subspecies suggest that there are some proteins that may serve as good initial targets (22).

It is noteworthy that a smaller protein than the MMP produced by members of the *M. avium* complex was detected in both *M. kansasii* and *M. abscessus* (Fig. 2B). It is unclear whether the decreased size is due to proteolytic processing or if it is simply a natural truncation in these more distantly related species. Bioinformatic analysis of the MAP2121c gene product does not reveal any type of signal peptidase cleavage sequence normally associated with membrane-targeted proteins; however, it is unknown whether *M. kansasii* or *M. abscessus* may have such a cleaved signal sequence, which may account for the smaller size. An insertion sequence in the MAP2121c gene of *M. avium* subsp. *paratuberculosis* and corresponding orthologs in *M. avium* complex members seem unlikely as no similarity to any known insertion sequence elements appears upon BLAST analysis.

In conclusion, these mAb reagents will open new lines of research in Johne’s disease. They have already been shown to be useful for detection by immunofluorescence microscopy, making them useful in cell biology and pathogenesis studies. Although not subspecies specific, these mAbs nonetheless have potential clinical use as immunodiagnostic reagents for histopathology (9), sandwich enzyme-linked immunosorbent assays, and general research applications. They may be of particular importance for experimental or epidemiologic studies with samples in which enrichment with *M. avium* subsp. *paratuberculosis* among high levels of other environmental bacteria is beneficial (7). Such samples include water (31) and bulk milk tank (12, 21) samples as well as other environmental samples (8, 25).

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