Rapid Expression of *Mycobacterium avium* subsp. *paratuberculosis* Recombinant Proteins for Antigen Discovery

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Received 1 April 2006/Returned for modification 8 May 2006/Accepted 19 October 2006

*Mycobacterium avium* subsp. *paratuberculosis* is the causative agent of Johne's disease, a chronic granulomatous enteritis of ruminants and other species. Detection of infection in animals is hampered by the lack of sensitive and specific diagnostic assays. We describe here an approach that utilizes translationally active PCR fragments for the rapid in vitro transcription and translation of recombinant proteins for antigen discovery in *M. avium* subsp. *paratuberculosis*. The investigations showed that the MAP1272c protein selectively reacts with sera from Johne's disease-positive cattle and represents an antigen of potential utility in *M. avium* subsp. *paratuberculosis* immunodiagnostics.

Johne's disease is a chronic gastrointestinal inflammatory disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (reviewed in reference 5). The disease occurs in wild and domestic ruminants, including dairy cattle, and has considerable impact on the global agricultural economy. The slow growth of the organism in laboratory culture and its extensive genetic relatedness with *Mycobacterium avium* subsp. *avium* (4, 7) have hindered diagnosis of Johne's disease using methods such as bacterial isolation, genomic assays, and serology. Recent investigations showed that the current enzyme-linked immunosorbent assay (ELISA)-based immunoassays have poor sensitivity, detecting fewer than one-third of all infected cattle (6). Furthermore, the use of crude *M. avium* subsp. *paratuberculosis* protein mixtures as antigens compromises assay specificity due to conservation of proteins across *Mycobacterium avium* complex organisms. Hence, identification of suitable *M. avium* subsp. *paratuberculosis* antigens that could enable early, sensitive, and specific detection of *M. avium* subsp. *paratuberculosis* infection is critically needed to facilitate adequate disease control measures.

In order to capitalize on the availability of the complete genome sequence of *M. avium* subsp. *paratuberculosis* (10) for novel antigen discovery, we describe here the application of an in vitro transcription and translation system that enables expression of *M. avium* subsp. *paratuberculosis* recombinant proteins directly from transcriptionally active PCR (TAP) fragments (11). This approach obviates the need for cloning of individual genes and expression of proteins in a heterologous system. It is also amenable for adaptation to a high-throughput format and enables the expression of hundreds of genes in days versus the months needed for cloning-based expression. Hence, this method is labor-, time-, and cost-effective and is ideal for large-scale antigen discovery.

In order to evaluate the utility of this approach for rapid expression and screening of potential antigens for use in *M. avium* subsp. *paratuberculosis* immunodiagnostics, we chose two candidate *M. avium* subsp. *paratuberculosis* open reading frames (ORFs), MAP1272c and MAP2121c, that have not previously been characterized either functionally or immunologically. Preliminary computational and comparative genomic analyses suggested that the MAP1272c gene product is an ~33.3-kDa protein that belongs to the NlpC/P60 superfamily. Orthologs of this protein are believed to function as putative secreted invasins and virulence factors in other mycobacteria, based on their homology with the p60 invasion protein of *Listeria monocytogenes* (9). The predicted MAP2121c gene product is an ~33.5-kDa protein with homology to the 35-kDa major membrane protein 1 (MMP-1) of *M. avium* and *Mycobacterium leprae*. MMP-1 has been shown to be a surface protein that plays a role in virulence by mediating invasion of epithelial cells (3). Previous studies have also suggested that MMP-1 may be an important antigen and a target of the immune response to *M. leprae* (14). Computational analysis of antigenic profiles can be performed with several programs, including DNAStar, DS Gene, PEOPLE, CEP, BEPITOPE, and PREDITOP (1, 8, 12, 13). In the current investigation, the MAP1272c and MAP2121c proteins were analyzed by Protean (DNAStar, Madison, WI), using the Jameson-Wolf method. The computational analyses of MAP1272c and MAP2121c protein sequences showed the presence of a large number of antigenic regions (Fig. 1A), and hence these two ORFs represented attractive candidates for an initial screen of antigens in *M. avium* subsp. *paratuberculosis*.

TAP fragments were generated by two sequential PCR steps for MAP1272c (GenBank accession no. NP_960206) and MAP2121c (GenBank accession no. NP_961055). In the first step, MAP1272c and -2121c were PCR amplified with custom oligonucleotides, with the 5′ primer comprising a universal overlapping sequence and 18 nucleotides from the specific gene, beginning after the start codon, as described below. The 3′-end primer contained a C-terminal His tag overlapping sequence and 18 nucleotides of the specific gene be-
fore the stop codon. The 5′ primer for MAP1272c had the sequence 5′-AGAAGGAGATAATACGTAGTGCAGTCC AGCGTGGG-3′ (a total of 36 nucleotides, with the start codon shown in bold). The 3′ primer for MAP1272c had the sequence 5′-TTAATGTATGATGATGATCGGGGGT GAGTCCGGGCC-3′ (39 nucleotides, with the stop codon shown in bold). The sequences for the 5′ and 3′ primers for MAP2121c were 5′-AGAAGGAGATAATACGTAGTGCAGTCC AGCGTGGG-3′ (a total of 36 nucleotides, with the start codon shown in bold). The 3′ primer for MAP2121c was 5′-TTAATGTATGATGATGATCGGGGGT GAGTCCGGGCC-3′ (39 nucleotides, with the stop codon shown in bold). The 5′ primer for MAP1272c had the sequence 5′-AGAAGGAGATAATACGTAGTGCAGTCC AGCGTGGG-3′ (a total of 36 nucleotides, with the start codon shown in bold). The 3′ primer for MAP1272c had the sequence 5′-TTAATGTATGATGATGATCGGGGGT GAGTCCGGGCC-3′ (39 nucleotides, with the stop codon shown in bold). The sequences for the 5′ and 3′ primers for MAP2121c were 5′-AGAAGGAGATAATACGTAGTGCAGTCC AGCGTGGG-3′ (a total of 36 nucleotides, with the start codon shown in bold). The 3′ primer for MAP2121c was 5′-TTAATGTATGATGATGATCGGGGGT GAGTCCGGGCC-3′ (39 nucleotides, with the stop codon shown in bold). The 5′ primer for MAP1272c had the sequence 5′-AGAAGGAGATAATACGTAGTGCAGTCC AGCGTGGG-3′ (a total of 36 nucleotides, with the start codon shown in bold). The 3′ primer for MAP1272c had the sequence 5′-TTAATGTATGATGATGATCGGGGGT GAGTCCGGGCC-3′ (39 nucleotides, with the stop codon shown in bold). The sequences for the 5′ and 3′ primers for MAP2121c were 5′-AGAAGGAGATAATACGTAGTGCAGTCC AGCGTGGG-3′ (a total of 36 nucleotides, with the start codon shown in bold). The 3′ primer for MAP2121c was 5′-TTAATGTATGATGATGATCGGGGGT GAGTCCGGGCC-3′ (39 nucleotides, with the stop codon shown in bold). The 5′ primer for MAP1272c had the sequence 5′-AGAAGGAGATAATACGTAGTGCAGTCC AGCGTGGG-3′ (a total of 36 nucleotides, with the start codon shown in bold). The 3′ primer for MAP1272c had the sequence 5′-TTAATGTATGATGATGATCGGGGGT GAGTCCGGGCC-3′ (39 nucleotides, with the stop codon shown in bold). The sequences for the 5′ and 3′ primers for MAP2121c were 5′-AGAAGGAGATAATACGTAGTGCAGTCC AGCGTGGG-3′ (a total of 36 nucleotides, with the start codon shown in bold). The 3′ primer for MAP2121c was 5′-TTAATGTATGATGATGATCGGGGGT GAGTCCGGGCC-3′ (39 nucleotids, with the stop codon shown in bold).

The results show that both MAP1272c and MAP2121c were successfully amplified by two-step PCR. As expected, the am-
kDa and 30 kDa that correspond well with the two protein bands observed on the immunoblots. Since both protein products react with anti-His antibodies as well as serum samples, the smaller band may well represent the product of the slightly smaller ORF. Regardless, the data show that the MAP2121c gene product is immunoreactive and represents a potential candidate for use in the development of the next generation of immunoassays for Johne’s disease. In contrast to the MAP2121c protein, the results show that although the recombinant MAP2121c-His fusion protein had the expected mass of ~34.5 kDa (Fig. 1B), it did not react with the pooled positive or negative sera (Fig. 1C).

To further examine the MAP2121c and MAP2121c proteins as diagnostic antigens, both proteins were tested by ELISA. The TAP expression system is essentially a screening tool and allows expression of proteins in relatively small quantities. In order to test these antigens by ELISA, the MAP2121c and MAP2121c proteins were expressed in E. coli, using the pMAL-c2 vector (New England Biolabs, Ipswich, MA) as previously described (2). Seven positive and two negative individual serum samples obtained from cattle that were confirmed to be positive or negative for Johne’s disease by bacterial isolation were tested by ELISA. ELISA plates were coated overnight with 100 ng of each of the recombinant proteins. The plates were blocked for 2 h with 2% bovine serum albumin–phosphate-buffered saline–Tween 20 (2% BSA-PBST), and 50 μL of each serum sample diluted 1:500 in BSA-PBST was added to individual wells in duplicate. After 2 h of incubation at room temperature, plates were washed nine times with distilled water. Next, horseradish peroxidase-labeled anti-goat immunoglobulin G (IgG; Vector Laboratories, Burlingame, CA) diluted 1:5,000 in 2% BSA-PBST was added and incubated for 2 h at room temperature. After a washing step, 200 μL of 3,3’5,5’-tetramethylbenzidine (TMB) solution (Sigma, St. Louis, MO) was added, and absorbance was measured at 655 nm. The two known Johne’s disease-negative serum samples were found to be negative by both MAP2127c and MAP2121c antigen-based ELISAs (Table 1). Of the seven known positive sera, MAP2127c and MAP2121c antigen ELISAs were able to detect MAP antibodies in seven and five serum samples, respectively (Table 1). Furthermore, the mean positive-to-negative ratio of the optical density values obtained for MAP2127c antigen ELISA was higher than that obtained for MAP2121c antigen ELISA. Thus, more sensitive detection of M. avium subsp. paratuberculosis antibodies by the MAP2127c antigen than by the MAP2121c antigen in ELISA correlates well with the immunoblotting results, where MAP2121c antigen could not detect M. avium subsp. paratuberculosis antibodies in pooled positive serum samples. Several reasons could account for this relatively poor immune recognition by the MAP2121c protein. First, the most plausible explanation is that the MAP2121c protein is either not expressed during infection or is poorly antigenic. Second, since the incubation period of Johne’s disease can extend to several years, it is also possible that the bacterium may express proteins such as the MAP2121c protein only during, for instance, early subclinical stages of infection rather than during clinical disease. This hypothesis should be tested by further screening of the MAP2121c protein, using a larger number of serum samples, preferably from animals during various phases of disease. Third, it is possible that the MAP2121c protein is posttranslationally modified in M. avium subsp. paratuberculosis in a manner that is different from the protein produced through the in vitro approach so as to alter its antigenicity. Furthermore, the improper folding of the MAP2121c protein may also contribute to a weak or diminished antibody response. At the present time, it is unclear which, if any, of these hypotheses for the lack of seroreactivity of the MAP2121c protein are true, and further investigations will be needed to address this issue.

**Conclusion.** Taken together, the results of our investigation provide compelling evidence that in vitro expression of proteins directly from linear TAP fragments is an efficient time- and cost-effective technology for M. avium subsp. paratuberculosis recombinant protein production and screening. The protocols can be adapted to run in a high-throughput format to rapidly screen for potential antigens and are currently being used in our laboratory to express more M. avium subsp. paratuberculosis proteins. However, it must be noted that TAP protein expression is a screening tool that enables initial screening of proteins on a large scale for antigen identification and expresses proteins in relatively small quantities. Once identified, the selected antigens have to be cloned and expressed in larger quantities, using a suitable system, for further characterization. Future studies for M. avium subsp. paratuberculosis antigen discovery will entail large-scale screening of proteins by the TAP system, evaluation of their cross-reactivities with other mycobacteria, and determination of their ability to detect M. avium subsp. paratuberculosis antibodies in serum samples from cattle shedding low, medium, and high levels of organisms. Based on the results of our current study, the identification of the MAP2127c protein as a potential M. avium subsp. paratuberculosis antigen for use as an immunodiagnostic reagent may have important implications in the development of improved Johne’s disease immunological assays.

This research was supported, in part, by competitive research grants from the U.S. Department of Agriculture-CSREES-NRI-CAP JDIP program.

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