Distinct Differences in Repertoires of Low-Molecular-Mass Secreted Antigens of *Mycobacterium avium* Complex and *Mycobacterium tuberculosis*

INGRID OLSEN,[1,2]* LIV J. REITAN,[1] AND HARALD G. WIKER[2,3]

National Veterinary Institute,[1] Institute of Immunology (IGRI), The National Hospital,[2] and Department of Environmental Medicine, National Institute of Public Health,[3] Oslo, Norway

Antigens in a 4-week-old culture filtrate (CF) of *Mycobacterium avium* subsp. *avium* were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and identified by Western blotting. The culture had minimal lysis of bacilli, giving a CF preparation consisting mainly of secreted proteins. Comparison with a similar CF of *Mycobacterium tuberculosis* with almost no contamination with intracellular proteins showed the presence of cross-reactive antigens homologous to the four components of the antigen 85 complex, as well as MPT32. These were major constituents of the *M. avium* subsp. *avium* CF. In addition, there were several low-molecular-mass bands (<15 kDa) in both species that did not cross-react with polyclonal and polyvalent rabbit antibodies in Western blotting. Furthermore, these bands were not detected in corresponding sonicate preparations, indicating high localization indexes, which is typical of soluble secreted proteins. A 14-kDa protein was selected for purification and more detailed characterization. The N-terminal amino acid sequence was determined, and a matching gene was found within the genomic sequence of *M. avium* subsp. *avium* which was highly homologous to Rv0455c of *M. tuberculosis*. The gene encoded a signal peptide typical of secreted mycobacterial proteins. A rabbit antiserum was raised against the purified protein, and the antigen was demonstrated by Western blotting in CFs of *M. avium* subsp. *avium*, *Mycobacterium avium* subsp. *paratuberculosis*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum* but was not detected in *M. tuberculosis*. This is a new example of a highly homologous gene being differentially expressed by different mycobacterial species.

The *Mycobacterium avium* complex consists of the subspecies *Mycobacterium avium* subsp. *avium*, *Mycobacterium avium* subsp. *paratuberculosis*, and *Mycobacterium avium* subsp. *silvaticum* (29). *M. avium* subsp. *avium* causes disseminated disease in birds and also granulomatous lesions in various animals. The bacterium can also cause severe infections in immunocompromised humans, and the human immunodeficiency virus epidemic has led to an increasing interest in this bacterium that contributes considerably to mortality in human immunodeficiency virus-infected patients (4). *M. avium* subsp. *paratuberculosis* is a pathogenic bacterium which causes a chronic granulomatous infection of the intestines of ruminants. Only 10 to 15% of infected animals develop overt paratuberculosis, or Johne's disease, but subclinically infected animals result in decreased production, with financial consequences for farmers. *M. avium* subsp. *paratuberculosis* has also been suggested as the etiologic agent of Crohn's disease in humans, which has similarities to paratuberculosis in ruminants (5, 18). Yet another subspecies of the *M. avium* complex, *M. avium* subsp. *silvaticum*, can cause enteritis in ruminants, as well as disseminated infections in other hosts.

Protection against mycobacterial infections is dependent on cellular immune responses, and early diagnosis of tuberculosis in animals is currently based on the skin test measuring delayed-type hypersensitivity reactions or in vitro tests measuring gamma interferon production (37). Tests measuring humoral immune responses have little value, especially in subclinically infected animals. A number of studies have thus focused on identifying the antigens responsible for the cellular immune

responses seen in the early stages of the infections. Proteins secreted by the bacteria during growth may be important in this respect, and several secreted antigens from the *Mycobacterium tuberculosis* complex have been identified and characterized (10, 20, 25–27, 31, 34). These antigens have also received a lot of attention in attempts to improve diagnostic tests and in the design of new vaccines against tuberculosis (2, 11, 13). In comparison, relatively little is known about secreted proteins in the *M. avium* complex. Such proteins have been demonstrated by using monoclonal antibodies (19) or 35S methionine labeling of proteins in short-term cultures (30). Only a few secreted proteins have been further characterized, including components of the antigen 85 complex, which are major antigens of *Mycobacterium bovis* BCG (7, 8, 20, 32). Ohara et al. have described the presence of 85B and 85D (MPT51) (22) in *M. avium* subsp. *avium* culture fluid, and a component of the 85 complex from *M. avium* subsp. *paratuberculosis* has also been purified and characterized (28). Plum et al. have described a macrophage-induced protein of *M. avium* subsp. *avium* grown in human macrophages (24). A ferric reductase (12), as well as a putative serine protease (3), from *M. avium* subsp. *paratuberculosis* has also been studied. Some of these proteins have been shown to induce humoral immune responses in animals with paratuberculosis, but the applicability of the proteins in diagnostic assays has not been evaluated.

The aim of this study was to identify secreted proteins in the *M. avium* complex using polyclonal antisera raised against culture filtrate (CF) proteins and antisera against individual secreted proteins from *M. tuberculosis* and *M. bovis*. Secreted proteins that are present in the *M. avium* complex and not in *M. bovis* and *M. tuberculosis* could help distinguish between paratuberculosis and tuberculosis in cattle and also between *M.*

* Corresponding author. Mailing address: National Veterinary Institute, P.O. Box 8156 Dep., 0033 Oslo, Norway. Phone: 47 22 96 46 34. Fax: 47 22 46 88 90. E-mail: Ingrid.Olsen@vetinst.no.

avium complex infections and tuberculosis in humans by using assays measuring cellular or humoral immune responses.

MATERIALS AND METHODS

Bacterial culture and antigen preparation. *M. tuberculosis* and *M. bovis* CF proteins have been described previously (20, 33). *M. tuberculosis* CF was a gift from S. Nagai, Osaka, Japan. The other strains were obtained from the National Veterinary Institute, Oslo, Norway. All strains were kept on Middlebrook's 7H10 medium (with mycobactin [2 µg/ml] for the *M. avium* subsp. *paratuberculosis* strain). The reference strains *M. avium* subsp. *paratuberculosis* strain 2E and *M. avium* subsp. *avium* strain D4 were cultivated on the surface of synthetic Reid's medium at 37°C for 1 month. *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum*, *Mycobacterium fortuitum*, *Mycobacterium kansasii*, and *Mycobacterium phlei* were cultured on the same medium for 1 to 3 weeks until sufficient growth was obtained. The bacteria were sequentially removed by paper filtration, and the CF was subsequently passed through a sandwich filtration system (pore sizes, 1.0, 0.5, and 0.22 µm). Concentration of CF was done by precipitation with 80% ammonium sulfate (550 g/liter). The bacteria were washed three times in phosphate-buffered saline (PBS), and 2 g of bacteria in 10 ml of PBS was kept on ice and sonicated 20 times for 1 min each time. The samples were centrifuged, followed by filtration (0.22-µm-pore-size filter) of the supernatant. The protein concentration was determined by the bicinchoninic acid assay (Pierce, Rockford, Ill.).

Antisera. The antisera against cellular antigens of *M. avium* subsp. *paratuberculosis* strain 2E and *M. avium* subsp. *avium* strain D4 were obtained from Dako (Glostrup, Denmark). Antisera against CF proteins from *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* were produced in rabbits by three subcutaneous administrations of concentrated CF proteins (0.5 mg) in Freund's incomplete adjuvant, with 2 weeks between injections. The rabbits were bled 2 weeks after the last injection, and the sera were used in Western blotting at a dilution of 1:1,000. Antisera against purified *M. tuberculosis* and *M. bovis* CF proteins have been described previously (20, 33). Antiserum against a purified 14-kDa protein was made by immunizing two rabbits with 40 µg of purified protein in Freund's incomplete adjuvant three times, with 2 weeks between injections. The rabbits were bled 2 weeks after the last injection, and the sera were used in Western blotting at a dilution of 1:200.

SDS-PAGE with immunoblotting. The antigens were separated under reducing and nonreducing conditions by horizontal SDS-PAGE in precast gradient Excel gel 8 to 18% (Pharmacia, Uppsala, Sweden), using a Multiphor II unit model 2117 (Pharmacia). After separation, the proteins were transferred to a nitrocellulose membrane (pore size, 0.2 µm) (Schleicher & Schuell, Dassel, Germany) by diffusion blotting (23), and the proteins remaining in the gel were stained with Coomassie brilliant blue (CBB). The membranes were blocked with PBS containing 2% bovine serum albumin and 1% gelatin and incubated with antisera over night. Bound antibodies were recognized by horseradish peroxidase-labeled anti-rabbit immunoglobulins. As a substrate, 3,3′ diaminobenzidine was added to visualize the bound antibodies.

Purification of a 14-kDa protein. The purification procedures for the 14-kDa protein were developed on the basis of combinations of ion-exchange chromatography and gel filtration using a Gradi Frac system equipped with a P-50 pump, a UV monitor, and a conductivity monitor (Pharmacia). Fractions were analyzed by SDS-PAGE with diffusion blotting (23), and the nitrocellulose membrane was incubated with polyvalent anti-*M. avium* subsp. *avium* antiserum against CF proteins. Proteins remaining in the gel after blotting were stained with CBB. The fractions containing proteins with the estimated molecular masses were collected.

In a typical run, the initial protein concentrate (100 mg of *M. avium* subsp. *paratuberculosis* CF proteins) was applied to a column of Q Sepharose Fast Flow (Pharmacia) with a bed volume of 15 ml and eluted at 7.5 ml/min using a gradient of 0 to 500 mM NaCl with 30 mM Tris-HCl, pH 8.7, and 3% Methyl Cellosolve (Sigma Chemical Co., St. Louis, Mo.). The fractions containing the protein were diluted 1:10 in 30 mM Tris-HCl, pH 8.7, and 3% Methyl Cellosolve and separated on a 6-ml Resource Q column (Pharmacia) followed by elution at 3 ml/min using a gradient of 0 to 400 mM NaCl. The fractions containing the protein were concentrated to a sample of 5 ml on the Resource Q column by first diluting the sample 1:5 with 30 mM Tris-HCl, pH 8.7, and 3% Methyl Cellosolve followed by elution in the same buffer supplemented with 200 mM NaCl. The concentrated sample containing the 14-kDa protein was gel filtered on a Superdex 75 column (26/60) in PBS at a flow rate of 2 ml/min and then applied to the Resource Q column and eluted at 3 ml/min using a gradient of 0 to 300 mM ammonium sulfate in Tris-HCl, pH 7.5. The 14-kDa protein was present in two fractions.

N-terminal amino acid sequencing. Automatic Edman degradation was performed by Knut Sletten at the Biotechnology Centre, University of Oslo, Oslo, Norway, using a 477A protein sequencer with an on-line 120-A phenylthiohydantoin amino acid analyzer (Applied Biosystems, Foster City, Calif.).

Databases. The Institute for Genomic Research and Sanger databases http://www.tigr.org/tdb/mdb/mdb.htlm and http://www.sanger.ac.uc/Datasearch/, respectively) were searched using the N-terminal amino acid sequence.
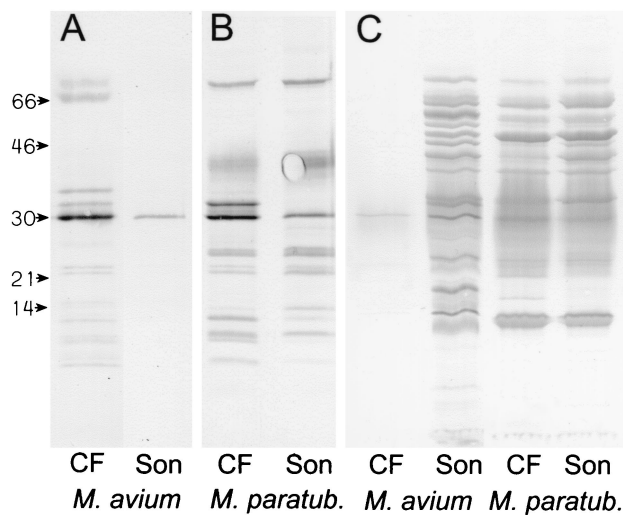

FIG. 1. *M. avium* subsp. *paratuberculosis* (*M. paratub.*) and *M. avium* subsp. *avium* (*M. avium*) CF and sonicate proteins were separated in SDS-PAGE and blotted onto nitrocellulose membranes. The amounts of total protein applied were 1 µg for lanes A and B and 5 µg for lanes C. The molecular masses (in kilodaltons) are indicated. The two lanes A were incubated with antiserum against *M. avium* subsp. *avium* CF, and the two lanes B were incubated with antiserum against *M. avium* subsp. *paratuberculosis* CF. The four lanes C were incubated with antiserum against *M. avium* subsp. *avium* sonicate (Dako).

RESULTS

Characterization of *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis* CFs and antisera. Concentrated CF protein samples were tested in SDS-PAGE and Western blotting using the antisera raised against the CF proteins. The antiserum against *M. avium* subsp. *avium* CF proteins reacted mainly with CF proteins and only to a limited extent with sonicate antigens, while antiserum against *M. avium* subsp. *paratuberculosis* CF reacted with several bands in both the CF and the sonicate preparations (Fig. 1). The Dako antiserum against *M. avium* subsp. *avium* was raised against a sonicate preparation of bacilli and hence consisted mainly of antibodies directed against cellular proteins. In Western blotting, this antiserum gave multiple bands, with the sonicate preparations from both subspecies showing the large extent of cross-reactions. The antiserum also reacted with many bands in *M. avium* subsp. *paratuberculosis* CF, while only one distinct band at 30 kDa was seen in *M. avium* subsp. *avium* CF (Fig. 1). This showed that the *M. avium* subsp. *avium* CF was not contaminated with any substantial amounts of intracellular antigens while the *M. avium* subsp. *paratuberculosis* CF contained a large number of cellular proteins, probably due to extensive lysis of the bacteria in the culture.

Comparison of *M. avium* subsp. *avium* CF and *M. tuberculosis* CF. The various bands in *M. avium* subsp. *avium* CF were tested with rabbit antisera made against purified proteins from *M. tuberculosis* or *M. bovis* BCG (Fig. 2). The mobility in SDS-PAGE for the four bands reacting with sera against the 85 complex corresponded to molecular masses of 36, 33, 30, and 26 kDa. The most prominent band of the 85 complex seemed to be 85B, with a mobility corresponding to 30 kDa, while the band reacting weakly with anti-85D had a mobility corresponding to 26 kDa. The two bands that reacted strongly with the MPT32 antiserum had molecular masses of about 65 and 74 kDa, which are considerably higher than the molecular masses of 43 and 45 kDa of this antigen in *M. tuberculosis*. No bands were detected in *M. avium* subsp. *avium* CF with sera against
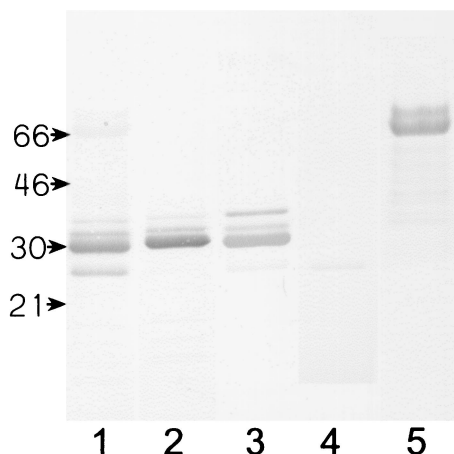
FIG. 2. *M. avium* subsp. *avium* CF proteins (5 μg of total protein) were separated in SDS-PAGE, blotted onto nitrocellulose membranes, and incubated with various antisera. Lanes: 1, *M. tuberculosis* CF antiserum; 2, anti-85B antiserum; 3, anti-85C antiserum; 4, anti-85D antiserum; 5, anti-MPT32 antiserum. The molecular masses (in kilodaltons) are indicated.

MPB83, MPB70, MPT63, MPT46, and ESAT6, but a weak band with a molecular mass of 26 kDa was detected with rabbit MPT64 that was probably due to a cross-reaction with antigen 85D, since an MPT64 homologue was not found in the *M. avium* subsp. *avium* genomic sequence.

M. avium subsp. *avium* CF and *M. tuberculosis* CF were also tested in SDS-PAGE with Western blotting, using the polyclonal antisera against CF from the two species in order to identify possible *M. avium* complex-specific proteins (Fig. 3). The *M. avium* subsp. *avium* CF antiserum reacted with several bands with molecular masses of less than 15 kDa in *M. avium* subsp. *avium* that were not present in *M. tuberculosis* CF, in addition to the already identified antigen 85 complex and MPT32. The *M. tuberculosis* CF antiserum reacted with the antigen 85 complex and MPT32 in *M. avium* subsp. *avium* and
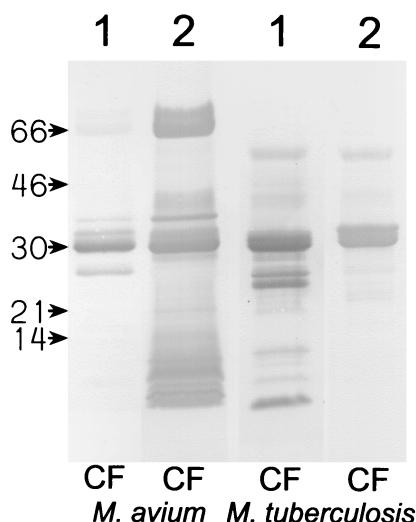

FIG. 3. *M. avium* subsp. *avium* (*M. avium*) CF proteins and *M. tuberculosis* CF proteins (5 μg of total protein) were separated in SDS-PAGE and blotted onto nitrocellulose membranes. The molecular masses (in kilodaltons) are indicated. Lanes 1 were incubated with antiserum against *M. tuberculosis* CF proteins, and lanes 2 were incubated with antiserum against *M. avium* subsp. *avium* CF proteins.

gave faint staining of two or three bands among the low-molecular-mass proteins. In contrast, this antiserum reacted distinctly with several low-molecular-mass proteins in *M. tuberculosis* that did not have apparent counterparts in *M. avium* subsp. *avium*. These results indicate that there are major differences in the low-molecular-mass areas of the two species.

Purification and N-terminal sequence of a CF protein. A 14-kDa protein was purified from *M. avium* subsp. *paratuberculosis* CF as described above. The purified protein was run in SDS-PAGE and stained with CBB and also blotted onto nitrocellulose and incubated with *M. avium* subsp. *paratuberculosis* antiserum. Only a 14-kDa band was detected using either method. The N-terminal amino acid sequence was obtained by Edman degradation, and homologous proteins with unknown functions were found in *M. avium* subsp. *avium* and *M. tuberculosis* when the Institute for Genomic Research and Sanger databases were searched (Fig. 4). The calculated molecular mass of the mature protein in *M. avium* subsp. *avium* was 14.1 kDa, fitting with its observed mobility in SDS-PAGE. The gene encoded a signal peptide typical of secreted proteins, with a cleavage site consisting of the amino acids AVA just upstream of the mature protein. The predicted cleavage site was confirmed by the N-terminal sequence of the mature protein, and the first 20 N-terminal amino acids were identical to those found in the *M. avium* subsp. *avium* genome. The overall homology between the proteins from *M. avium* subsp. *avium* and *M. tuberculosis* was 76% by the Lipman and Pearson alignment (16).

Specificity of the 14-kDa protein. CFs and sonicated antigens of several mycobacterial strains were tested in SDS-PAGE with Western blotting using a monospecific antiserum raised in rabbits against the purified protein in order to identify cross-reacting proteins in the various species (Fig. 5). The monospecific antiserum reacted with a 14-kDa band in *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *avium*, *M. intracellulare*, and *M. scrofulaceum* but not with the other strains that were tested, including *M. tuberculosis*. The bands were distinct in the CFs from these four species and barely visible in the sonicate when the same amount of total protein was applied to the gel, indicating that the 14-kDa protein is actively secreted by the bacteria during growth. This method has been shown to be reliable in *M. tuberculosis* for identification of secreted proteins (35).

DISCUSSION

There are several possible approaches to an attempt to identify secreted proteins. Today, when the whole genome of *M. tuberculosis* has been sequenced, secreted proteins can be recognized by searching the genome for proteins with a typical signal sequence (9). The secretory pathway is probably the main mechanism for protein export in mycobacteria, and most of the actively secreted proteins will have a typical signal sequence (21). Construction of libraries with *M. tuberculosis* genomic DNA fused with leaderless genes for phosphatase A (15) or beta-lactamase (6) have been successfully used to identify secreted proteins and have allowed a more detailed characterization of the mycobacterial signal peptide (15, 36). A reliable approach is to search for proteins found in abundance in CFs and in minor quantities in cellular extracts. The classification of such proteins as cellular or secreted can be based on calculation of a localization index (LI) as described earlier (33). The low-molecular-mass proteins identified in *M. avium* subsp. *avium* CF in the present study clearly had high LIs. They were found in abundance in CF, while they were barely detectable in the corresponding sonicate preparation. With
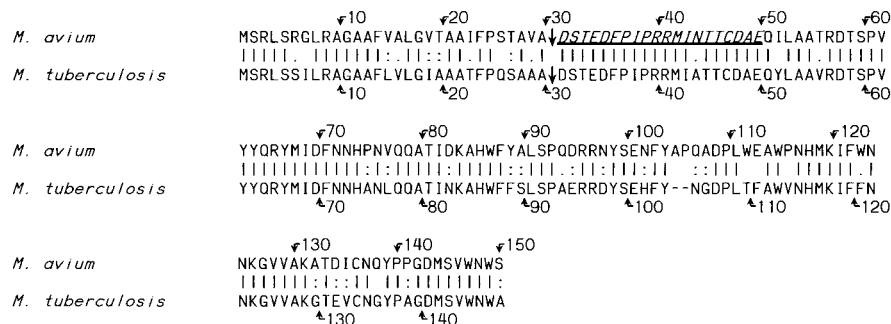
FIG. 4. Complete amino acid sequence of the 14-kDa protein identified in *M. avium* subsp. *avium* and the homologous protein of *M. tuberculosis*. The N-terminal amino acid sequence obtained by Edman degradation is italicized and underlined, and straight arrows indicate the cleavage sites for the signal peptide. The positions of amino acids are indicated with bent arrows. |, identical amino acid; :, conservative exchange of amino acid; ., less conservative exchange of amino acid.

proper antibody reagents, secreted proteins can be identified in old lysed cultures by the LI method, but it is helpful to use cultures with a minimal extent of cellular lysis, especially when trying to identify novel proteins. This can sometimes be achieved by using short-term cultures (1), but it can also be obtained by cultivating the bacteria as a surface pellicle (33).

The present study has focused on identifying secreted antigens in the *M. avium* complex using polyclonal antisera raised against crude CF proteins from this species and antisera against known proteins from *M. tuberculosis* and *M. bovis*. The extensive cellular lysis seen in the culture of *M. avium* subsp. *paratuberculosis*, with release of cellular proteins to the CF, made the identification of novel secreted proteins in this subspecies difficult. This extensive lysis was in marked contrast to what was seen in *M. avium* subsp. *avium* CF, which appeared to consist mainly of secreted proteins. The quality of the *M. avium* subsp. *avium* CF in this study is comparable to that of the *M. tuberculosis* CFs produced by S. Nagai (20, 33), which also had

negligible amounts of intracellular proteins. Both the CF made here and Nagai's CFs were produced by traditional surface pellicle cultures grown on synthetic media for several weeks. This shows that it is not necessary to use short-term cultures to obtain CFs specially adapted for studying secreted proteins. The advantage of using longer cultivation times is a higher yield.

It is not known whether a low degree of lysis can be obtained by refining the cultivation technique or whether it is a feature of particular strains. If there is variation in lysis between strains and species of bacteria, it will be of consequence for the amounts of cellular proteins present in CFs and therefore also in purified protein derivatives made from the various cultures. Therefore, caution should be taken in interpretation of immune responses elicited against purified protein derivative as responses towards secreted proteins.

The diagnosis of mycobacterial disease, especially in subclinically infected animals, is still a problem and is often based on
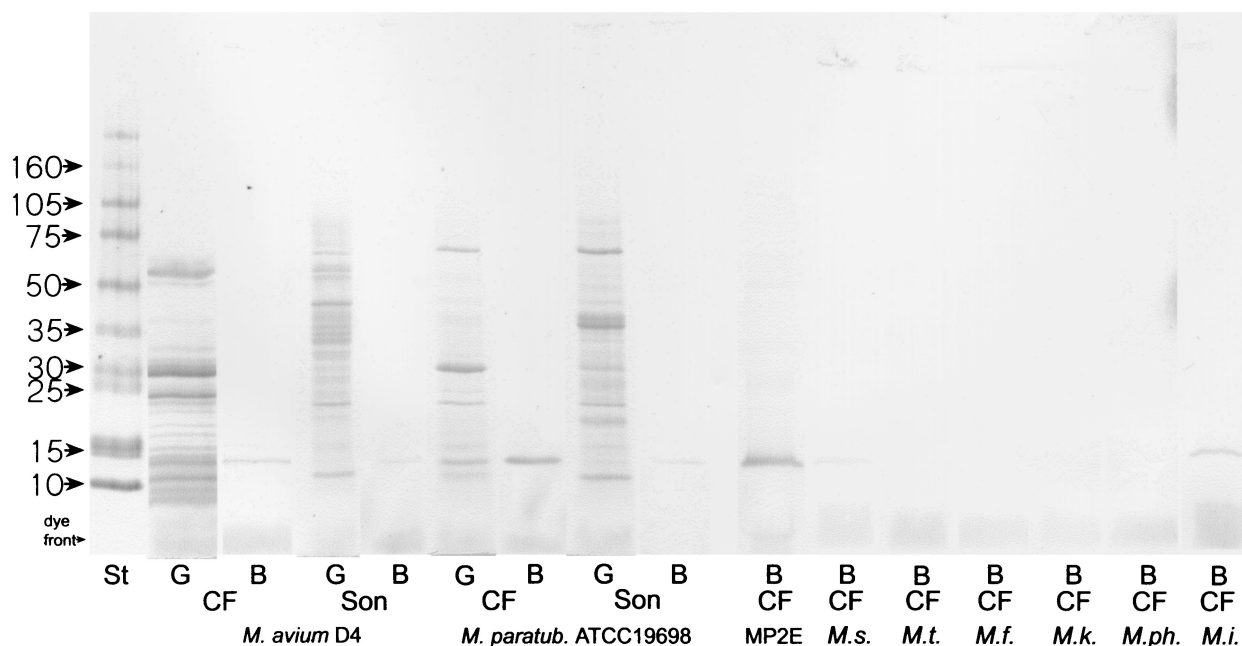

FIG. 5. SDS-PAGE with CBB staining and Western blot of sonicate (Son) and CF antigens of various mycobacterial species. The molecular masses (in kilodaltons) of the standard (St) are indicated. G, proteins in gel stained with CBB; B, lanes blotted onto nitrocellulose and incubated with antiserum against the 14-kDa protein isolated from *M. avium* subsp. *paratuberculosis*; MP2E, *M. avium* subsp. *paratuberculosis* strain 2E; *M.s.*, *M. scrofulaceum*; *M.t.*, *M. tuberculosis*; *M.f.*, *M. fortuitum*; *M.k.*, *M. kansasii*; *M.ph*, *M. phlei*; *M.i*, *M. intracellulare*.

measuring cellular immune responses. The specificity of such tests is dependent on the antigens used to stimulate the cells, and specific proteins from *M. tuberculosis* have been shown to be able to differentiate between tuberculosis and *M. avium* complex infections (14, 17). We have shown that several secreted low-molecular-mass proteins present in the *M. avium* complex are without overt counterparts in *M. tuberculosis*. It is likely that some of these are also good candidates for use in diagnostic assays based on cellular and humoral immune responses to distinguish between infections with these bacteria. We have identified and purified one 14-kDa protein that was detected by Western blotting only in the *M. avium-M. intracellulare* complex including *M. scrofulaceum*. It is a secreted protein found in much larger amounts in the CF than in the sonicate and with a signal sequence typical of secreted mycobacterial proteins, thus fulfilling the basic criteria to establish it as a secreted protein (35). A homologous gene (Rv0455c) with an unknown function is present in the *M. tuberculosis* H37Rv genome, but the monospecific and polyclonal antiserum raised against the purified protein did not react with any proteins in CF from *M. tuberculosis*. Given the strong homology between the proteins, a likely explanation for this finding is that the protein is expressed in significantly larger amounts in the *M. avium* complex. However, it might also be due to structural differences between the two proteins. A Jameson-Wolf antigenic index showed that one of the most likely areas for B-cell epitopes is between amino acids 90 and 100, and the differences in amino acid sequence between *M. tuberculosis* and *M. avium* subsp. *avium* in this area were quite extensive.

The four components of the antigen 85 complex and an MPT32 homologue were the quantitatively dominant secreted proteins in *M. avium* subsp. *avium*. Ohara et al. have previously claimed that only 85B and 85D are expressed in large amounts in *M. avium* subsp. *avium* based on the fact that they managed to obtain N-terminal sequences of only these two proteins when eluting spots from a two-dimensional nitrocellulose blot (22). We found that antisera raised against 85A, 85B, 85C, and 85D reacted with at least four bands in *M. avium* subsp. *avium* CF, showing that all four of these proteins probably are expressed in this species. The genes encoding the four proteins are present in *M. avium* subsp. *avium* (22), which is consistent with our findings. We cannot exclude the possibility that our results are due to the presence of other proteins that cross-react with the antigen 85 complex, but no genes encoding other homologous proteins were found when the *M. avium* subsp. *avium* genome was searched.

The present work has shown that the low-molecular-mass areas of the *M. avium* complex and *M. tuberculosis* contain different repertoires of secreted proteins. These findings are probably due to differences in protein expression, as well as the existence of species-specific proteins. It is likely that some of these proteins can be useful in the attempt to improve current immunological assays to help differentiate among infections with these species. We have purified and characterized one of these proteins with a molecular mass of 14 kDa that will be further evaluated for its potential role as an antigenic target in mycobacterial infections.

ACKNOWLEDGMENTS

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