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Genetic Analysis of *Mycobacterium avium* Complex Strains Used for Producing Purified Protein Derivatives

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For over a century, purified protein derivatives (PPD) have been used to detect mycobacterial infections in humans and livestock. Among these, reagents to detect infections by *Mycobacterium avium* complex organisms have been produced, but the utility of these reagents has not been clearly established due in part to limited biologic and immunologic standardization. Because there is little information about the strains used to produce these reagents (avian PPD, intracellulare PPD, scrofulaceum PPD, and Johnin), we have performed genetic characterizations of strains used to produce these products. Sequence analysis of 16S rRNA and the *hsp65* gene provided results concordant with species designations provided for *M. avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum* organisms. For *M. avium* strains, comparative genomic hybridization was performed on a whole-genome DNA microarray, revealing one novel 7.9-kilobase genomic deletion in certain Johnin-producing strains, in addition to genomic variability inherent to the particular *M. avium* subspecies. Our findings indicate that considerable genomic differences exist between organisms used for reagents and the infecting organism being studied. These results serve as a baseline for potency studies of different preparations and should aid in comparative studies of newly discovered antigens for the diagnosis of infection and disease by *M. avium* complex organisms.

The successful control of mycobacterial diseases is contingent on early diagnosis of infection and/or the successful application of treatment to contagious cases. In the case of tuberculosis, both strategies are applied, with immunologic testing being used to inform about infection and microbiologic diagnosis guiding the detection of active disease. The cornerstone of immunologic diagnosis of mycobacterial infection has been skin testing to detect cell-mediated immune responses to constituents of the bacillus. Several types of products were used for skin testing during the past century, with Koch's "old tuberculin" being replaced first by heat-concentrated synthetic medium tuberculins and later by purified protein derivatives (PPDs) (13, 30). Interpretable skin test results may be ensured, at least in part, by careful standardization of the active reagent and the application of potency assays for quality control. For tuberculin PPD, a WHO International Standard termed PPD-M is widely used throughout the world (10).

Infection and disease due to organisms of the *Mycobacterium avium* complex (MAC) are recognized to be health concerns for humans and livestock; however, testing for MAC infection has been less standardized than tuberculin testing. Traditionally, MAC responsiveness was studied to guide the interpretation of tuberculin skin testing by assaying to distinguish sensitization to environmental mycobacteria from infection with members of the *Mycobacterium tuberculosis* complex. This could be accomplished by comparative testing using PPD-Battey, a reagent derived from the Battey bacillus now known as *Mycobacterium intracellulare*, in parallel with tuberculin skin testing (23). The same approach was later used in comparative skin testing using *M. avium* PPD RS10 (Statens Serum Institut, Copenhagen, Denmark) (43–46) and, more recently, in the first generation of in vitro assays (QuantiFERON-TB; Cellestis Limited, Victoria, Australia), wherein one determines the ratio of gamma interferon responses to tuberculin PPD versus those to avian PPD (20).

There has also been a more direct interest in determining the prevalence of infection with members of the MAC in humans, not only in the context of AIDS but also in understanding the epidemiology of pulmonary MAC disease and cervical adenitis in children (18, 44, 46). To address this interest, investigators have applied different PPD reagents in epidemiologic surveys, although none of these have been rigorously standardized or extensively tested. In livestock, *M. avium* reagents have also been used for discriminating between tuberculous and nontuberculous exposure in cattle (as in the commercial gamma interferon assay Bovigam; Prionics AG, Switzerland) (28) but their greater utility may be in direct detection of disease due to *M. avium* organisms themselves. In the case of *M. avium* subsp. *paratuberculosis*, the etiological agent of a chronic inflammatory bowel disease in ruminants called Johne's disease, early infection is characterized by a cell-mediated immune response, so PPD-based skin tests (or interferon-based assays) promise the greatest likelihood of detecting subclinically infected hosts (3, 35). Unlike bovine or human tuberculosis, however, such tests are not currently in wide use, having been abandoned as a result of reported problems with the sensitivity and specificity of skin-based testing (4, 5, 11, 16).

A critical concern in the use of these tests has been the availability of standardized PPDs to be used towards diagnostic

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TABLE 1. Characteristics of strains used to produce *M. avium* PPD reagents[a]

Reagent	Reference(s)	Strain name	Provider	16S rRNA designation	*hsp65* designation	ML-SSR genotype[b]	LSP-based identification	Microarray-based genomic profile
Avian PPD	11, 20, 25, 36, 46	10	SSI	*M. avium*	Code 4	NA	*M. avium* subsp. *avium* bird type	Similar to prototype strain (*M. avium* subsp. *avium* strain R13)[c]
Intracellulare PPD	9	23	SSI	*M. intracellulare*	NA	NA	NA	ND
Scrofulaceum PPD	9	95	SSI	*M. scrofulaceum*	NA	NA	NA	ND
Johnin	15, 16	18	M. Hines	*M. avium*	Code 4	NA	*M. avium* subsp. *avium* bird type	ND
Johnin (NL)	12	C	CIDC-Lelystad	*M. avium*	Code 5	ggt5 tgc5	*M. avium* subsp. *paratuberculosis* cattle type	Deletion of LSP^{jn}
		3+5	VLA-Weybridge	*M. avium*	Code 5	ggt5 tgc5	*M. avium* subsp. *paratuberculosis* cattle type	Deletion of LSP^{jn}
Johnin (NVI)	29	316	NVI	*M. avium*	Code 5	ggt5 tgc5	*M. avium* subsp. *paratuberculosis* cattle type	Similar to prototype strain (*M. avium* subsp. *paratuberculosis* strain K10)
		2E	NVI	*M. avium*	Code 5	ggt5 tgc5	*M. avium* subsp. *paratuberculosis* cattle type	Similar to prototype strain (*M. avium* subsp. *paratuberculosis* strain K10)
Johnin (USDA)	26, 27, 37	19698	ATCC	*M. avium*	Code 5	ggt5 tgc5	*M. avium* subsp. *paratuberculosis* cattle type	Similar to prototype strain (*M. avium* subsp. *paratuberculosis* strain K10)
Johnin (CFIA)	5, 21, 47	II	CFIA	*M. avium*	Code 5	ggt4 tgc5	*M. avium* subsp. *paratuberculosis* cattle type	Similar to prototype strain (*M. avium* subsp. *paratuberculosis* strain K10)
		III	CFIA	*M. avium*	Code 5	ggt5 tgc5	*M. avium* subsp. *paratuberculosis* cattle type	Deletion of LSP^{jn}
		IV	CFIA	*M. avium*	Code 5	ggt5 tgc5	*M. avium* subsp. *paratuberculosis* cattle type	Deletion of LSP^{jn}
		C286	CFIA	*M. avium*	Code 5	ggt5 tgc5	*M. avium* subsp. *paratuberculosis* cattle type	Deletion of LSP^{jn}
		C300	CFIA	*M. avium*	Code 5	ggt5 tgc5	*M. avium* subsp. *paratuberculosis* cattle type	Deletion of LSP^{jn}
		III.V	CFIA	*M. avium*	Code 5	ggt5 tgc5	*M. avium* subsp. *paratuberculosis* cattle type	Deletion of LSP^{jn}

[a] SSI, Statens Serum Institut, Copenhagen, Denmark; CIDC-Lelystad, Central Institute for Animal Disease Control, Lelystad, The Netherlands; VLA-Weybridge, Veterinary Laboratory Agency, Weybridge, United Kingdom; NVI, National Veterinary Institute, Oslo, Norway; CFIA, Canadian Food Inspection Agency, Ontario, Canada; NA, not applicable; ND, not done.

[b] ML-SSR, multilocus short sequence repeats. The genotypes given are based on numbers of repeats for locus 8 (ggt repeat) and locus 9 (tgc repeat).

[c] See reference 33 for details.

TABLE 2. Primers used to genotype *M. avium* strains

Target sequence[a]	Primer 1 (forward)	Primer 2 (reverse)	Primer 3 (reverse)
LSP^{in}	ACACACCGACCAGAGAGGAG	CGATCCAGAGGCTGAAGATG	ACAACTTCGCCAAGGTCATC
SSR locus 1	GTGTTCGGCAAAGTCGTTGT	TCAGACTGTGCGGTATGGAA	
SSR locus 2	GTGACCAGTGTTTCCGTGTG	TGCACTTGCACGACTCTAGG	
SSR locus 8	AGATGTCGACCATCCTGACC	AAGTAGGCGTAACCCCGTTC	
SSR locus 9	GACAAGTTCGGGTTGACCAC	AGTTCCTCGACCCAGTCGT	
16S rRNA	AGAGTTTGATCCTGGCTCAG	GTATTACCGCGGCTGCTG	
Hsp65 (3′ end)	CGGTTCGACAAGGGTTACAT	ACGGACTCAGAAGTCCATGC	

[a] Testing for the presence or absence of LSP^{in} was performed using three primers in a multiplex PCR reaction, such that a 233-bp product is expected if the sequence is present (primers 1 and 3) and a 396-bp product is expected if the sequence is missing (primers 1 and 2). SSR, short sequence repeat. Locus 1 and locus 2 are mononucleotide (G) repeats; locus 8 and locus 9 are variable-number trinucleotide repeats.

tests for these organisms. A number of different MAC antigen preparations have been used, including the use of avian PPD for Johne's surveillance by some groups (11, 12, 36) and the use of different Johnin preparations by others. For example, in Canada, Johnin has been produced by six strains that have been continuously passaged in the laboratory for more than 70 years (II, III, IV, C286, C300, and III.V) (6, 47). Little information is available about the history of strains used elsewhere, although one of the strains used in The Netherlands to produce Johnin is thought to have originated in Canada (strain C), while another was provided by the Veterinary Laboratory Agency in the United Kingdom (strain 3+5). Because of this confusing history, we have employed genetic and genomic modalities to genotype the organisms used to produce the *M. avium* complex PPD reagents as a first step towards an improved understanding of these reagents.

MATERIALS AND METHODS

Bacterial isolates and their genetic identification. We collected killed bacterial cells from 15 strains in total (Table 1); 6 were used for the production of Johnin in Canada (Canadian Food Inspection Agency, Nepean, Ontario), 2 were used in The Netherlands (CIDC-Lelystad, The Netherlands), 2 were used in Norway (National Veterinary Institute, Norway), 2 were used in the United States (strain 18, a gift from M. E. Hines III, Georgia, and strain ATCC19698, purchased from the American Tissue Culture Collection), and 3 were used at the Statens Serum Institut (SSI, Copenhagen, Denmark) for the production of PPDs from *M. avium*, *M. intracellulare*, and *Mycobacterium scrofulaceum*. DNA extraction was performed according to a standard protocol (42). Each strain was characterized by PCR and sequencing of 16S rRNA and the 3′ end of *hsp65*, a typing scheme that we recently proposed to classify members of the *M. avium* complex (40). Additionally, the characterization into subspecies and distinct types was determined using three-primer PCR assays testing for the presence or absence of large sequence polymorphisms LSP^{A} 8 (specifically missing from *M. avium* subsp. *paratuberculosis*), LSP^{A} 20 (missing from *M. avium* subsp. *paratuberculosis* of the sheep type but present in the cattle type) and LSP^{A} 17 (missing from *M. avium* subsp. *avium* of the bird type), according to a previously described diagnostic algorithm (33).

Microarray-based genomic comparisons. The test isolates were compared to *M. avium* 104 (the reference sequenced strain, a clinical isolate from a human AIDS patient in the United States) in cohybridization experiments on a whole-genome DNA microarray representative of 98% of the open reading frames (ORFs) for the genome of *M. avium* subsp. *paratuberculosis* strain K10 and 93% of the predicted ORFs for the genome of *M. avium* strain 104 (Table 1 shows a list of the strains tested by microarray). Fluorescence labeling of the DNA samples, hybridizations, and scanning were performed according to previously described methods (33, 34). Sequences that were identified by microarray analysis as potentially divergent and not representative of previously described polymorphisms were flagged for further study using a PCR approach. In this study, we opted to analyze only sequences that were greater than three ORFs, as these are more likely to represent truly divergent sequences rather than experimental artifacts. We first verified that these sequences were missing from test isolates with primers designed towards a sequence internal to these regions. In a second step, we performed PCR using primers designed towards the flanking regions, such that an amplicon would be obtained only if the region was missing. Primers were designed using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer sequences are given in Table 2. The PCR products were sequenced in a core sequencing facility (McGill University and Génome Québec Innovation Centre) on a 3730XL DNA analyzer system using ABI dye terminator chemistry. The resulting sequences were aligned to completed genome sequences (*M. avium* subsp. *paratuberculosis* K10 [AE016598]) and *M. avium* 104 (www.tigr.org) to identify the exact site at which the sequence polymorphism occurs.

Molecular subtyping of isolates. Molecular subtyping of the *M. avium* subsp. *paratuberculosis* strains tested was done by multilocus short sequence repeat analysis, a tool that is expected to generate a greater degree of genetic diversity than genotyping methods based on insertion elements (IS*900* restriction fragment length polymorphism and multiplex PCR of IS*900* integration loci). We selected four loci suggested in a previous study to be the most discriminatory: locus 1, locus 2, locus 8, and locus 9 (1). The first two loci consist of mononucleotide (G) repeats, while the last two are variable-number trinucleotide (GGT and TGC) repeats. We performed PCR amplification and sequencing of these loci using previously described primers (1). The resulting chromatograms were edited manually for accuracy and added to the alignment component of MEGA3 (14). For each strain tested, the number of nucleotide repeats was analyzed and an allele number was assigned to reflect the number of copies per locus.

PCRs. PCRs were performed in 50-μl volumes, using 5 μl (equivalent to about 20 ng) of DNA, 1 U *Taq* polymerase (MBI Fermentas), 5 μl of 10× PCR buffer (MBI Fermentas), 2.5 mM $MgCl_2$, 5 μl acetamide 50% (wt/vol), 0.2 mM deoxynucleoside triphosphates (dNTPs), and 0.5 μM of each primer. PCR amplification consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, with annealing at 55°C (for amplification across large sequence polymorphisms) or 60°C (for amplification across short sequence repeats) for 45 s, elongation at 72°C for 2 min, and a final elongation step at 72°C for 10 min. PCR products were separated by electrophoresis in 1.5% (wt/vol) agarose gels containing ethidium bromide.

RESULTS

Genetic characterization of strains. From 16S rRNA sequencing, all but two of the strains studied were identified as *M. avium*: strains 23 and 95 were identified as *M. intracellulare* and *M. scrofulaceum*, respectively, consistent with their previous designations. Since both of these species exhibit a high level of sequence difference from *M. avium* proper, these two isolates were not submitted to further comparative genomic studies. Strain 10, used to produce avian PPD, and strain 18, previously used to produce both an antigenic reagent and a vaccine for Johne's disease, were both identified as *M. avium* subsp. *avium* (bird type) based on *hsp65* sequencing (code 4) and PCR for LSPs (LSP^{A} 17 missing). The strains used in Canada, Norway, and The Netherlands and one strain recently used in the United States for the production of Johnin were all determined to be *M. avium* subsp. *paratuberculosis* of the cattle type (*hsp65* code 5 and LSP^{A} 20 present) (Table 1).

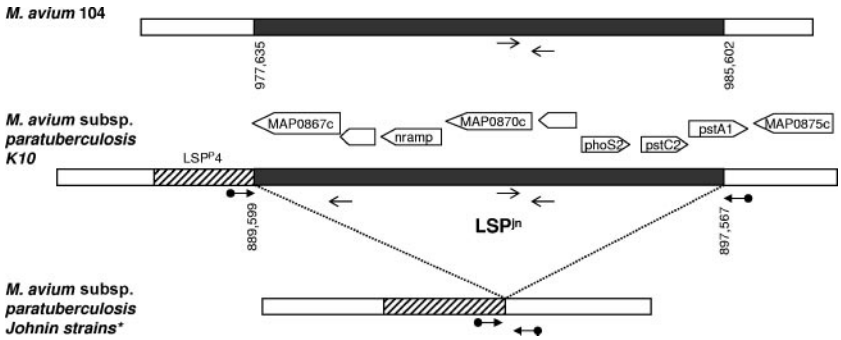

FIG. 1. Schematic representation of large sequence polymorphism Johnin. Coordinates on the genome are given as base pairs starting from the first nucleotide of the start codon of *dnaA* in *M. avium* 104 and *M. avium* subsp. *paratuberculosis* K10, respectively. White boxes represent homologous sequences across *M. avium* 104, *M. avium* subsp. *paratuberculosis* K10, and *M. avium* subsp. *paratuberculosis* strains used to produce Johnin. The striped box represents a large sequence (LSP^P4) that is present in *M. avium* subsp. *paratuberculosis* but missing in *M. avium* 104. LSP^{jn} is depicted by the gray box and is missing from *M. avium* subsp. *paratuberculosis* strains III, IV, C286, C300, III.V, 3+5, and C. Thick arrows represent primers flanking LSP^{jn} (bridging primers); a PCR product is obtained if the region is missing. Thin arrows represent primers targeting a sequence within $LSP^{jn;}$ a PCR product is obtained if the sequence is present.

Genomic characterization of *M. avium* strains. Using *M. avium* strain 104 as the referent, microarray-based study revealed that the strains tested had genomic profiles consistent with their subspecies and type designations (33, 34) (Table 1). Additionally, we identified one novel large sequence that was deleted in the following seven strains: III, IV, C286, C300, III.V, 3+5, and C. This sequence, which we called large sequence polymorphism Johnin (LSP^{jn}), is a 7,968-bp sequence spanning MAP0867c to MAP0874 on the genome of *M. avium* subsp. *paratuberculosis* strain K10 (AE016598). This sequence is adjacent to a previously described large sequence, LSP^P4, that is specific to *M. avium* subsp. *paratuberculosis* (missing from all nonparatuberculosis strains of *M. avium*). The sequence LSP^{jn} is conserved and syntenic in *M. avium* 104 and the *M. tuberculosis* complex and encompasses an operon predicted to carry a membrane-associated phosphate transport complex called the Pst system and a gene predicted to be involved in manganese transport (*mntH* or bacterial NRAMP). Sequence-based analysis revealed that the polymorphism occurs at the same locus in all seven strains in which LSP^{jn} is missing (corresponding to positions 889599 to 897567 in the *M. avium* subsp. *paratuberculosis* K10 genome) and truncates MAP0874 (Fig. 1). Testing for this sequence using a multiplex PCR approach in a panel of 20 *M. avium* subsp. *paratuberculosis* isolates (cattle- and sheep-type strains) characterized in a recent study (32) showed this region to be present and intact in a diverse collection of field isolates.

Molecular subtyping of strains. From analysis of two of the short sequence repeat loci (locus 8 and locus 9), we identified two genotypes among the isolates tested. All but one of the *M. avium* subsp. *paratuberculosis* strains tested clustered into a predominant genotype (ggt5, tgc5), while strain II (a strain in which LSP^{jn} was intact) was determined to be of a different genotype (ggt4 tgc5).

The other two loci (mononucleotide repeats) could not be analyzed due to inconsistent and illegible sequences immediately following the mononucleotide stretch. This is consistent with previous reports of higher error rates in PCR amplification and sequencing of long mononucleotide repeats due to polymerase "slippage," presumably caused by slipped-strand mispairing (2).

DISCUSSION

In this study, we formally analyzed the genetic and genomic characteristics of strains used to produce *M. avium* complex PPD reagents. Although these reagents have frequently been used to distinguish *M. tuberculosis* complex infection from exposure to nontuberculous mycobacteria, they are also used to detect MAC infection both in humans and in livestock (8, 23, 46), with recent reports suggesting that their utility has previously been underappreciated (12, 26, 27, 35, 36). Furthermore, while the postgenomic identification of specific antigens offers an opportunity for improved diagnostic assays, the utility of novel antigens will likely benefit from direct comparison against cocktails of antigens, as has been done in the case of ESAT-6 and tuberculin testing (41).

One of the strains most commonly used to produce the *M. avium* sensitin PPD or avian PPD, the SSI strain 10, was determined by genomic analysis to be *M. avium* subsp. *avium* of the bird type. There is extensive evidence that *M. avium* subsp. *avium* bird-type strains are the cause of tuberculosis in birds and are associated with severe disease in many other animals including livestock. However, these strains have rarely been encountered in human disease, although the SSI strain 10 was isolated from a Danish child in 1944 (19). This suggests either that human exposure is quite rare or that humans are inherently more resistant to these strains (7, 22, 39). Given that SSI strains 23 and 95 are indeed environmental organisms occasionally associated with human disease (*M. intracellulare* and *M.* subsp. *scrofulaceum*, respectively), it is surprising that reagents based on these two strains are rarely used, while avian PPD based on a strain seldom encountered in humans is used more widely. Nevertheless, studies conducted in different geographical regions using avian PPD produced from SSI strain 10 have indicated that certain individuals do mount a skin reaction to this sensitin (43–46). In a recent skin survey, skin test positivity to this reagent was not more frequent in those at risk

of disseminated MAC disease (such as human immunodeficiency virus-infected persons) or pulmonary MAC disease but rather was associated with other, less obvious risk factors, such as black race and exposure to soil, the epidemiological relevance of which is not entirely clear (25).

The strain that was extensively used in earlier studies of skin testing for Johne's disease in livestock, strain 18, is now well documented to be *M. avium* subsp. *avium* of the bird type. Results from these skin test studies were disappointing (15, 16), but the use of avian PPD made from *M. avium* subsp. *avium* bird-type strains (such as SSI strain 10) to test for paratuberculosis continues with, perhaps predictably, variable results (11, 12, 36). Because of the complexity of PPD products, in which production methods and other factors are critical, it is difficult to relate the biological effect of a PPD reagent to the strain used to produce it. However, there are large regions of genomic differences between *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis*, with some large sequences present in the former but absent in the latter and others uniquely present in *M. avium* subsp. *paratuberculosis* (31, 33). So, while such reagents undoubtedly help to discriminate between tuberculous and nontuberculous infection in cattle (as is done through the commercial gamma interferon assay Bovigam [Prionics A.G. Switzerland]), genomic considerations predict that reagents based on *M. avium* subsp. *avium* may suffer poor specificity in detecting infection with *M. avium* subsp. *paratuberculosis*.

Among the *M. avium* subsp. *paratuberculosis* strains used to produce Johnin PPD by several different laboratories, we identified a large sequence, LSP^{jn}, missing from seven of the Johnin production strains. Since the precise coordinates of this deletion are shared among these seven strains and the locus is otherwise intact in field isolates, this most probably represents a genomic deletion. In a historical context of collaborations and strain sharing between different veterinary laboratories producing diagnostic reagents during the 20th century, we conclude that the LSP^{jn}-deleted strains share a common origin, with the deletion likely having occurred during prolonged in vitro passage. The sequence encodes proteins putatively involved in the transport of phosphate. The homologous genes in *M. tuberculosis* encode proteins that are present at the mycobacterial surface as well as in the culture filtrate, have been associated with virulence in an experimental infection model, and are potentially immunostimulatory antigens of *M. tuberculosis* (17, 24, 38). The occurrence of this polymorphism in *M. avium* subsp. *paratuberculosis* may have occurred as a result of continuous passaging in synthetic medium rich in inorganic phosphate. Of more practical interest is the observational report that Johnin reagents produced from these strains may suffer from poor sensitivity (5), in contrast with studies using *M. avium* subsp. *paratuberculosis* strains genomically intact for this locus (26, 27).

In conclusion, in this study we show that the strains used to produce sensitins used for diagnosis of *M. avium* infections, both in humans and in livestock, are not always based on strains more commonly associated with disease in these hosts. We also show that some *M. avium* subsp. *paratuberculosis* strains used to produce Johnin PPD have suffered a genomic deletion, the biological impact of which needs further study. Genetically and genomically well-characterized strains should be utilized to produce standardized *M. avium* PPD reagents, and strict care should be taken in maintaining these production strains to prevent the creation of genetically divergent strains.

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