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Genomic Polymorphisms for *Mycobacterium avium* subsp. *paratuberculosis* Diagnostics

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Mycobacterium avium* subsp. *paratuberculosis* is an emerging pathogen of mammals and is being actively investigated as a possible zoonotic agent. The lack of reliable diagnostic assays has hampered rational assessment of the prevalence of this organism in humans and animals. We have used a comparative genomic approach to reveal genomic differences between *M. avium* subsp. *paratuberculosis* and its close relative *M. avium* subsp. *avium*, a highly prevalent environmental organism. From computational and DNA microarray-based study of two prototype strains, *M. avium* subsp. *avium* strain 104 and *M. avium* subsp. *paratuberculosis* strain K10, we have uncovered two types of large sequence polymorphisms (LSPs): those present in the former but missing in the latter (LSP^{A}s) and those only present in the latter (LSP^{P}s). We examined the distribution of 3 LSP^{A}s and 17 LSP^{P}s across a panel of 383 *M. avium* complex isolates in order to determine their potential utility for the development of accurate diagnostic tests. Our results show that the absence of LSP^{A}8 is 100% specific for the identification of *M. avium* subsp. *paratuberculosis*. Of the 17 LSP^{P}s, 10 regions were not specific for *M. avium* subsp. *paratuberculosis* while 7 were shown to be highly specific (>98%) and, in some cases, highly sensitive as well (up to 95%). These data highlight the need to evaluate these regions across a diverse panel of clinical and environmental isolates and indicate the LSPs best suited for *M. avium* subsp. *paratuberculosis* diagnostics.

Mycobacterium avium subsp. *paratuberculosis* is a serious pathogen of domestic ruminants, in which it causes paratuberculosis (Johne's disease), a chronic and eventually fatal inflammatory bowel disease. Its rising incidence in many regions of the world has resulted in significant economic losses to the livestock industry (13). This organism also infects free-ranging wildlife species (16). The impact of *M. avium* subsp. *paratuberculosis* in nonruminant wildlife species is largely unknown; however, the potential for interspecies transmission has important implications for paratuberculosis control programs. *M. avium* subsp. *paratuberculosis* is also being actively investigated as the possible cause of a debilitating human inflammatory bowel disease, Crohn's disease (4, 5, 18).

Effective control of Johne's disease and investigation of the potential link to Crohn's disease have been hampered by the lack of effective assays to easily and accurately diagnose *M. avium* subsp. *paratuberculosis* infections. Commercially available serological assays for bovine disease are convenient but offer poor sensitivity, especially during subclinical disease. Moreover, owing to the high degree of genetic similarity between *M. avium* subsp. *paratuberculosis* and the other members of the *M. avium* complex, many of the proteins that are recognized during the early stages of infection are not specific for *M. avium* subsp. *paratuberculosis* (14). Since *M. avium* subsp. *avium* is highly prevalent in the environment but not associated with a similar clinical syndrome in mammals, the capacity to differentiate between these closely related organisms is essential for rational clinical and epidemiologic assessment.

Previous work has identified a number of *M. avium* subsp. *paratuberculosis* genetic sequences that are absent from other mycobacteria, such as the insertion sequence IS*900* (11), the F57 element (21), and the *hspX* gene (9), but the value of these sequences for diagnostics of *M. avium* subsp. *paratuberculosis* remains unclear. Although PCR testing for the multicopy insertion element IS*900* is widely used, there are concerns about its specificity and its validity as a direct proxy for *M. avium* subsp. *paratuberculosis*. IS*900*-like elements have been found in unrelated organisms (10), and other *M. avium* complex insertion elements, including IS*1311* (7) and IS*1626* (22), share considerable sequence similarity. In the cases of F57 and *hspX*, their specificity for *M. avium* subsp. *paratuberculosis* has not to date been rigorously evaluated in a large sample of clinical isolates.

More recently, data derived from genome sequencing projects have suggested a number of polymorphic genomic regions that may serve in the specific diagnosis of *M. avium* subsp. *paratuberculosis*. Our microarray-based comparisons using *M. avium* subsp. *avium* strain 104 as the reference revealed 14 large sequence polymorphisms (LSPs) that are variably present among a small collection of *M. avium* isolates. Three of

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these regions appeared to be present in *M. avium* subsp. *avium* (LSP^As) and absent from *M. avium* subsp. *paratuberculosis* isolates (23). Conversely, computational comparisons of the genome sequences of *M. avium* subsp. *paratuberculosis* strain K10 (GenBank accession no. NC_002944) and *M. avium* subsp. *avium* strain 104 (http://www.tigr.org) have identified DNA sequences that are present in the former but missing or divergent in the latter (2, 3, 19). We have to date identified 17 regions varying in length from 2.9 to 66 kb that are unique to *M. avium* subsp. *paratuberculosis* strain K10; we call these LSP^Ps. Such sequences can be expected to lend themselves to nucleic acid-based diagnostic tests and, if they encode immunogenic proteins, to immunological assays as well. One of these, a 19-kb sequence that we call LSP^P12, has been previously documented (8, 23). Another, a 98-kb segment of the genome that encompasses elements we call LSP^P14 and LSP^P15, also includes within it a 38-kb element that was recently described as an *M. avium* subsp. *paratuberculosis*-specific putative pathogenicity island (24).

The objective of our study was to assess the value of these LSPs for the specific diagnosis of *M. avium* subsp. *paratuberculosis*. To be diagnostically specific for *M. avium* subsp. *paratuberculosis*, an LSP^P must be found only in *M. avium* subsp. *paratuberculosis* isolates and should be absent from non-*M. avium* subsp. *paratuberculosis* isolates. Conversely, for an LSP^A to serve in the molecular diagnosis of *M. avium* subsp. *paratuberculosis*, it should be consistently present in non-*M. avium* subsp. *paratuberculosis* isolates but missing from a broad collection of *M. avium* subsp. *paratuberculosis* isolates. We therefore tested the distribution of the 17 LSP^Ps and 3 LSP^As across a panel of 383 *M. avium* complex isolates. Our results indicate that many LSP regions, although distinct between prototype genome sequences, are heterogeneously distributed across geographically diverse isolates and so lack the specificity required for diagnostics. However, a subset of LSPs do appear highly specific for *M. avium* subsp. *paratuberculosis* and should prove useful in the development of effective diagnostics for Johne's disease and evaluation of the Crohn's disease hypothesis.

MATERIALS AND METHODS

Bacterial isolates. A panel of 107 *M. avium* subsp. *paratuberculosis*, 260 *M. avium* subsp. *avium*, 4 *M. avium* subsp. *silvaticum*, and 12 *M. intracellulare* isolates was assembled (Table 1). Isolates were first identified as *M. avium* complex species by the laboratories providing them using the commercial nucleic acid probe AccuProbe (Gen-Probe Inc., San Diego, CA). Initial dependence on Mycobactin J and the presence of the insertion sequence IS*900* were determined by these laboratories and used to identify *M. avium* strains to the subspecies level as *M. avium* subsp. *paratuberculosis*. Designation as *M. avium* subsp. *avium* was supported by phenotypic characteristics (including lack of Mycobactin J dependency) and the presence of IS*1245*. Additional extensive testing, including sequencing of the 16S-23S internal transcribed spacer (ITS) region, was completed for 160 of these isolates in the context of another study (17). *M. avium* subsp. *silvaticum* strains exhibited dependence on Mycobactin J on primary isolation and were IS*900* negative. *M. intracellulare* strains were identified using the *M. intracellulare* AccuProbe, and species designation was confirmed by 16S rRNA gene sequencing.

Genomic DNA was extracted from each isolate using previously published methods (27). The amount of DNA in each sample was quantified using a spectrophotometer, and each sample was normalized to a concentration of 1 ng/μl prior to PCR testing.

Discovery of LSP^P and testing across *M. avium* complex isolates. As part of our ongoing work to characterize *M. avium* complex organisms, we compared the sequenced genomes of *M. avium* subsp. *avium* strain 104 (http://www.tigr.org) and *M. avium* subsp. *paratuberculosis* strain K10 (GenBank accession no. NC_002944). Artemis software (http://www.sanger.ac.uk/Software/Artemis/) was used for visualization of the *M. avium* subsp. *paratuberculosis* strain K10 genome data and annotation of *M. avium* subsp. *avium* strain 104. Genome sequence comparison, via visual inspection in Artemis and with BLASTN, permitted identification of regions present in *M. avium* subsp. *paratuberculosis* strain K10 but lacking a homologous region in *M. avium* subsp. *avium* strain 104 (LSP^P). Additional homology analyses (using BLASTN) were used to confirm that LSP^P regions were truly missing and not simply displaced (e.g., due to genomic rearrangement). BLAST2 was used to precisely define the boundaries of these novel LSP^Ps. For the 17 regions and their locations in the *M. avium* subsp. *paratuberculosis* strain K10 genome, see Table 3.

To screen for the specificity of LSP^Ps, we initially tested for their presence by PCR targeting a short internal sequence across a panel of 96 *M. avium* subsp. *avium* isolates. LSP^Ps for which a screening PCR was negative for these *M. avium* subsp. *avium* strains were then tested across the extended panel of 383 *M. avium* complex isolates. Since LSP^P14, which includes but is not restricted to a known 38-kb island (24), and LSP^P12, a 19-kb sequence (8, 23), were previously postulated to be of potential diagnostic utility, we tested each of these LSPs with two and three different sets of primers targeting distinct loci, respectively. Primer sequences for each of the LSP^Ps tested are listed in Table 2.

Testing of LSP^A across *M. avium* complex isolates. Our previous DNA microarray-based analysis revealed numerous large sequences variable among *M. avium* isolates. LSP^A8, LSP^A13, and LSP^A14 appeared potentially specific for *M. avium* subsp. *paratuberculosis* (23). When aligned against the genome of *M. avium* subsp. *paratuberculosis* strain K10, these *M. avium* subsp. *avium* strain 104 sequences were confirmed to be absent from the *M. avium* subsp. *paratuberculosis* K10 genome. Testing for these regions employed PCR with primers designed toward the flanking regions of the sequence, such that an amplicon would be generated only if the region was indeed missing. The PCR test was considered positive (region missing) if amplification generated a product of the expected molecular weight. Furthermore, for each set of primers, a random selection of 25 PCR products, originating from 25 different strains, was sequenced using ABI

TABLE 1. Sources of *M. avium* complex isolates studied

Name (no. of isolates) and source	No. of isolates
M. avium subsp. *avium* (260)	
Bird	33
Human	73
Pig	96
Reference	32
Cow	5
Cigarette	5
Deer	3
Dog	2
Cat	2
Fish	1
Sheep	1
Environmental	3
Unknown	4
M. avium subsp. *paratuberculosis* (107)	
Cow	65
Sheep	14
Goat	2
Deer	12
Human	8
Pig	1
Rabbit	1
Unknown	4
M. avium subsp. *silvaticum* (4)	
Wood pigeon	4
M. intracellulare (12)	
Human	12
Total	383

TABLE 2. Primers used for testing of LSPs

Sequence	PCR target	Forward primer	Reverse primer	Product size (bp)
LSP^{P}1	MAP0095	GCGATGTTCAAGAAGCCAGT	GGAGGTAATAGTCGCGTTCG	442
LSP^{P}2	MAP0284	CCAGTTACCTTCGACGAGGA	CTCTGTTGGATTCCCGTTTG	600
LSP^{P}3	MAP0388	GCAATCATCCCGACAACTG	TTCAGGTCGAGGTGTGACAA	166
LSP^{P}4	MAP0865	TCCTCTCCTTCGTCACCAAC	ATCTCAGACAGTGGCAGGT	597
LSP^{P}5	MAP0963	GCCACGAAGGACATCAATTC	GGTCGGGGTTGCTGATCTAT	592
LSP^{P}6	MAP1233	TCGTAACGTTAGCACCGAGA	AGCTTGAAGCATTCGACCAT	661
LSP^{P}7	MAP1345	GCACGATTCCGGTTCTTCTA	GCGGCGTTGAAATAGGTAGT	579
LSP^{P}8	MAP1633	AAAAGGGGAACGGTACAACC	ATTCACGCTGATCCTCGAAC	653
LSP^{P}9	MAP1718	TCCAATACAACCCGAAGACC	TTCCACGCTTTGGATAGACC	625
LSP^{P}10	MAP2027	CGACACCTCGGTGTTCATC	CCGAATAATCGCCAGGTTC	373
LSP^{P}11	MAP2154	GTCGGACCAGGGTTGAGAT	GGATAAGGCCGCATACAAAC	375
LSP^{P}12	MAP2181c	GCTTCGTCGGAATGTACGAG	AGCATCAGCCTGACTTGGTT	595
	MAP2182c	TCTGAACCCGGCTACACAC	CATGCCGGTGTGAGTACAA	430
	MAP2188c	GCCCGTAGAAGTGGACGAT	GATGACGGTCGCCAATCC	719
LSP^{P}13	MAP2765	ACTGATCCTGCCATGCCTAC	GCGTAACTCAGCGAACAACA	451
LSP^{P}14	MAP3726	GGGCTATCAGACGATGTTCC	TACCAGCAGTATCGCCGAAT	211
	MAP3750	CACTGAAGCTCGGCATGTAA	CGACGACGAGTCCTATCCAG	674
LSP^{P}15	MAP3774	AGCAGTGGACGAGGCAAC	GAGGGCGTAGAACTCTGTG	621
LSP^{P}16	MAP3815	GTAATGGCGCATTCTCTGGT	CCCTTGATCTCGACACTGGT	611
LSP^{P}17	MAP4269	GGAAACCAAGGAAGGGAAAG	GCTAGACGGGTGATCGTGTT	592
LSP^{A}8	Flanking sequence	TCTGCAGAGCGGTGACATC	ATACCGCCAACGACATCTTC	474
LSP^{A}8	Internal sequence	ACCGCCAGATGTTTCTCATC	GACTCGGTGCTGCTGGTC	166
LSP^{A}13	Flanking sequence[a]	ACGAATGACGCCGTAGTAGC	GTCACGAGCGTCCATCCTAT	444
LSP^{A}13	Internal sequence	AGCTCAGAGGGCACTTTCCTT	CCAGGTCGATCAGGTCCTC	236
LSP^{A}14	Flanking sequence set A	ATATCGAGGCGTTCATCCTG	CACTCAACGAAGATCCAGCA	427
LSP^{A}14	Internal sequence	ATCACAGGTAGGAGCCTTGG	CACTTCAATGCCGAAATCAA	299
LSP^{A}14	Flanking sequence set B	GGCCTGGTATTCAACCTCA	CCGTTGACGTTGTGTTCAAG	718

[a] The sequence of the reverse flanking primer used to amplify across LSP^{A}13 contains one SNP in comparison to the sequence of *M. avium* subsp. *paratuberculosis* strain K10.

dye terminator chemistry. The resulting sequences were aligned with the sequenced genomes of *M. avium* subsp. *paratuberculosis* strain K10 and of *M. avium* subsp. *avium* strain 104 to determine if the junction of the deleted regions was identical across the different isolates.

For isolates in which amplification did not occur using the flanking primers, additional PCR testing was performed (using sets of primers targeting sequences within the LSP^A) to determine whether the intervening sequence was present. An example is LSP^{A}8 in Fig. 1, with corresponding positions on the genomes of *M. avium* subsp. *avium* strain 104, *M. avium* subsp. *paratuberculosis* strain K10, and *M. tuberculosis* strain H37Rv. The primer sequences used for all reactions are given in Table 2.

PCRs. All PCRs were performed in 50-μl volumes, using 5 μl of template DNA (5 ng) and 1 U *Taq* polymerase (MBI Fermentas), with 5 μl of 10× PCR buffer (MBI Fermentas), 2.5 mM $MgCl_2$, 5 μl acetamide 50% (wt/vol), 0.2 mM deoxynucleoside triphosphates, and each primer at 0.5 μM. PCRs were conducted in 96-well plates. Amplification consisted of an initial denaturation step of 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30s, annealing at 58°C for 45s, and extension at 72°C for 2 min. PCR products were separated by electrophoresis in precast 1% (wt/vol) agarose gels containing ethidium bromide (ReadyAgarose gels; Bio-Rad Laboratories, Hercules, CA). Samples that had bands of the expected molecular weight were considered positive, whereas those with bands of different size or where no bands were seen were considered negative. When results were ambiguous, PCRs were repeated using individual closed tubes.

M. avium subsp. *paratuberculosis* strain K10 and *M. avium* subsp. *avium* strain 104 were used as positive and negative controls for each set of primers used to test for the LSPs.

RESULTS

LSP^{P}s: regions present in *M. avium* subsp. *paratuberculosis* K10 and absent from *M. avium* subsp. *avium* 104. LSP^{P}s represent four types of polymorphisms with respect to the sequenced genome of *M. avium* subsp. *avium* 104. For certain regions, the *M. avium* subsp. *paratuberculosis* K10 genome simply contains an additional element when aligned against the corresponding positions on the genomes of *M. avium* subsp. *avium* strain 104 and *M. tuberculosis* strain H37Rv; the element is flanked by regions of high homology and synteny among the three genomes (schematically depicted by the example of LSP^{P}4 in Fig. 2A). For other regions, the *M. avium* subsp. *paratuberculosis* K10 genome does not contain additional DNA, but rather, there is a region of extremely low sequence similarity (<20%) with the best hit against the *M. avium* subsp. *avium* 104 genome, flanked by regions of high homology (>97%) between the two sequences (depicted as the example of LSP^{P}2 in Fig. 2B). Some regions are characterized by both additional DNA and sequence with low similarity (examples of LSP^{P}14 and LSP^{P}15 in Fig. 3A). Finally, one region is the result of additional DNA in the *M. avium* subsp. *paratuberculosis* K10 genome and loss of DNA from the *M. avium* subsp. *avium* genome and thus is characterized by the replacement of a DNA segment with another (example of LSP^{P}12, Fig. 3B).

For the 17 LSP^{P}s, a positive PCR using primers internal to these sequences indicated the presence of at least part of the region tested. From the screening panel of 96 *M. avium* subsp. *avium* isolates, 10 of these sequences were variably present in *M. avium* subsp. *avium* isolates while 7 could not be amplified from the screening panel (LSP^{P}2, LSP^{P}4, LSP^{P}11, LSP^{P}12, LSP^{P}14, LSP^{P}15, and LSP^{P}16; Table 3). These regions were thus investigated using an extended panel of 383 *M. avium* complex isolates; results are summarized in Table 4, with more detailed descriptions below.

LSP^{P}2, LSP^{P}4, and LSP^{P}15 could not be amplified from any

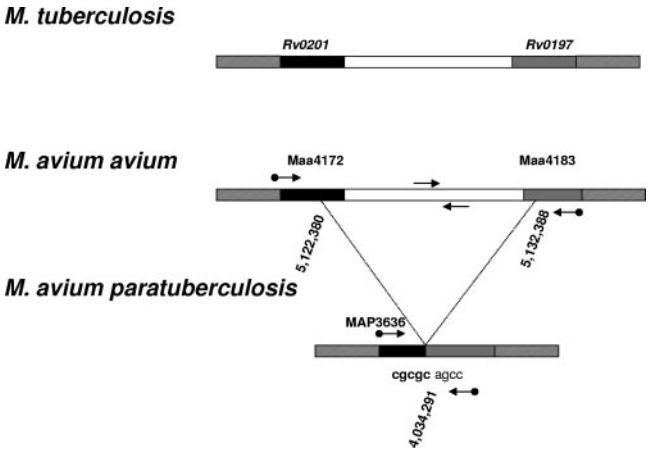

FIG. 1. Schematic representation of LSP^{A}8 in *M. avium* subsp. *avium* 104, *M. avium* subsp. *paratuberculosis* K10, and the homologous region in *M. tuberculosis* H37Rv. Coordinates on the genome are given as base pairs starting from the first nucleotide of the start codon of *dnaA* in *M. avium* subsp. *avium* 104 and *M. avium* subsp. *paratuberculosis* K10, respectively. Striped boxes represent homologous sequences; the 10-kb sequence (LSP^A 8) that is present in *M. avium* subsp. *avium* 104 but missing in *M. avium* subsp. *paratuberculosis* strain K10 is depicted by the white box. Black and gray boxes represent genes that are truncated in *M. avium* subsp. *paratuberculosis*. Thick arrows represent primers flanking the LSP (flanking primers); a PCR product is obtained if the region is missing (as in *M. avium* subsp. *paratuberculosis* K10). Thin arrows represent primers targeting a sequence that is within LSP^{A}8 (internal primers).

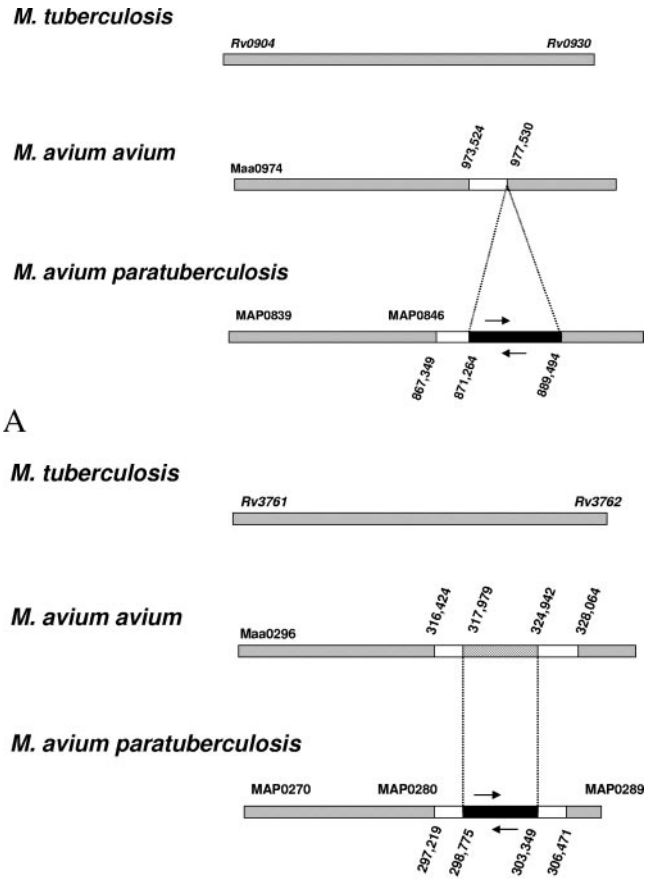

FIG. 2. Schematic representation of two LSP^P elements, as illustrated by the alignment of *M. avium* 104 (middle), *M. avium* subsp. *paratuberculosis* K10 (bottom), and the homologous region in *M. tuberculosis* H37Rv (top). Coordinates on the genome are given as base pairs starting from the first nucleotide of the start codon of *dnaA* in *M. avium* subsp. *avium* 104 and *M. avium* subsp. *paratuberculosis* K10, respectively. Gray striped boxes represent a sequence that is shared and syntenic among the three genomes. White boxes represent a sequence that is shared between *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis*. Black boxes represent a sequence that is found only in *M. avium* subsp. *paratuberculosis*. Thin arrows represent primers targeting a sequence within the LSP^P (internal primers). (A) An additional 18-kb sequence (LSP^{P}4) is found in *M. avium* subsp. *paratuberculosis* strain K10 compared to the *M. avium* subsp. *avium* 104 genome. (B) The *M. avium* subsp. *paratuberculosis* strain K10 and *M. avium* subsp. *avium* 104 genomes contain discrete regions nearly equivalent in size but extremely low in sequence similarity (LSP^{P}2).

of the 276 non-*M. avium* subsp. *paratuberculosis* isolates, demonstrating 100% specificity for this subspecies.

LSP^{P}11 and LSP^{P}16 also exhibited high specificity but unexpectedly amplified from a group of six non-*M. avium* subsp. *paratuberculosis* strains: one isolate gave amplification products for both LSP^{P}11 and LSP^{P}16; four isolates gave amplification products for LSP^{P}11 only, and one gave a product for LSP^{P}16 only. The five LSP^{P}11 and two LSP^{P}6 amplicons obtained from these isolates were identical to *M. avium* subsp. *paratuberculosis* strain K10 sequences. To further characterize these six isolates, all clinical human isolates collected in Montreal, we performed additional analysis, including 16S rRNA sequencing (to confirm species designation) and 16S-23S ITS sequencing to identify their sequevar. By 16S analysis, one isolate (13373) had a novel 16S rRNA sequence, similar to *M. chimaera* (previously known as MAC-A) (26) and a unique ITS sequence, closest to the MAC-C sequevar. Four isolates had a 16S sequence consistent with *M. avium* and had ITS sequences identical to the common Mav-B sequevar. Finally, we determined that one isolate (9033) was potentially mixed, based on the presence of double peaks in the DNA sequence chromatograms, with features of both *M. avium* subsp. *avium* and *M. intracellulare* (Min-A sequevar).

The presence of LSP^{P}12, a 19-kb sequence that is found in lieu of a 197-kb sequence in *M. avium* subsp. *avium* 104, was evaluated with three distinct sets of internal primers targeting different genes. Across the entire panel of *M. avium* complex isolates, amplification of two target genes (MAP2182c and MAP2187c) was restricted to *M. avium* subsp. *paratuberculosis* strains, resulting in a calculated specificity of 100%. However, primers targeting MAP2181c (predicted to encode a transcriptional regulator of the TetR family) unexpectedly produced an amplicon from two non-*M. avium* subsp. *paratuberculosis* isolates, clinical specimens 13373 and 9033 noted above. Sequencing of the MAP2181c amplicons revealed numerous sequence level polymorphisms shared across these isolates and only 89% sequence identity to *M. avium* subsp. *paratuberculosis* strain K10.

LSP^{P}14 is a heterogeneous region (Fig. 3A). Some portions are extra to *M. avium* subsp. *paratuberculosis* K10, including a previously described 38-kb island (24), while other segments exhibit moderate sequence similarity to *M. avium* subsp. *avium* 104. LSP^{P}14 was tested using two different sets of primers

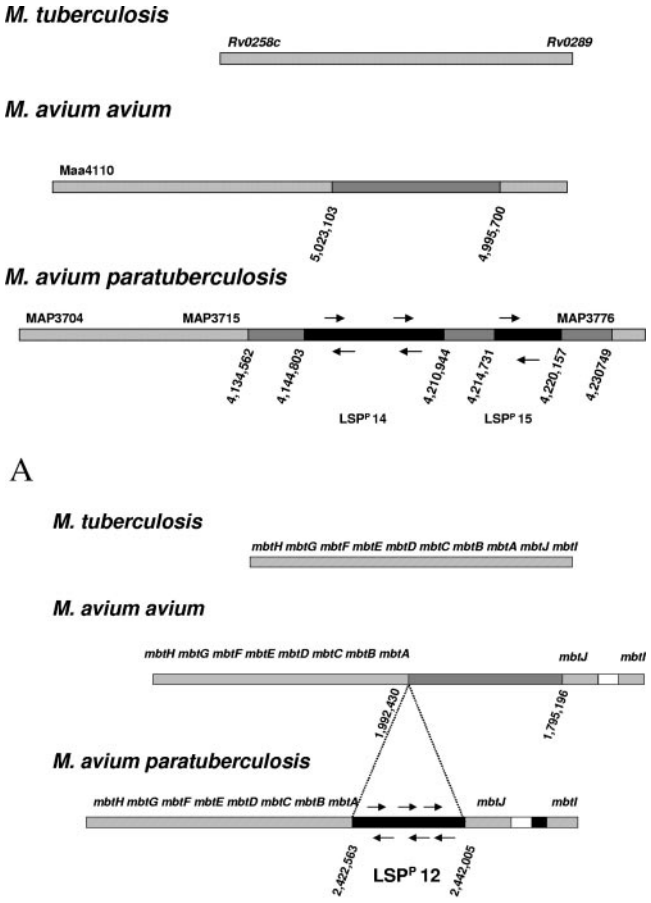

FIG. 3. Schematic representation of LSP^{P}14 and LSP^{P}15 (A) and of LSP^{P}12 (B), illustrated by the alignment of *M. avium* subsp. *avium* 104 (middle), *M. avium* subsp. *paratuberculosis* K10 (bottom), and the homologous region in *M. tuberculosis* H37Rv (top). Coordinates on the genome are given as base pairs starting from the first nucleotide of the start codon of *dnaA* in *M. avium* subsp. *avium* 104 and *M. avium* subsp. *paratuberculosis* K10, respectively. Striped boxes represent a sequence that is shared and syntenic among the three genomes. White boxes represent a sequence that is shared between *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis*. Black boxes represent a sequence that is found only in *M. avium* subsp. *paratuberculosis*. Gray boxes represent a sequence of low similarity between *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis*. Thin arrows represent primers targeting a sequence within the LSP^P (internal primers).

targeting MAP3726 and MAP3750 within the additional segment of DNA. Seven non-*M. avium* subsp. *paratuberculosis* isolates tested positive for at least one of the two genes. One *M. avium* subsp. *avium* isolate was positive for both MAP3726 and MAP3750, while six other isolates were positive for one of the two genes tested. In the case of MAP3726, a sequence with 97% similarity to that of *M. avium* subsp. *paratuberculosis* strain K10 was amplified from three *M. avium* subsp. *avium* strains. One of these isolates was from a pig in The Netherlands and had an ITS sequence identifying it as a Mav-B sequevar, while the other two were isolated from birds and had Mav-A sequevars. For MAP3750, sequences with less than 93% similarity to that of *M. avium* subsp. *paratuberculosis* strain K10 were amplified from five *M. avium* subsp. *avium* isolates, including the pig isolate described above. The presence of polymorphic sequence within *M. avium* subsp. *avium* isolates, and the fact that only a subset of *M. avium* subsp. *paratuberculosis* isolates were positive for these two genes, prompted us to look for polymorphisms within *M. avium* subsp. *paratuberculosis* isolates. Amplicons obtained from eight different isolates for each of these two genes also revealed several single-nucleotide polymorphisms (SNPs) compared to the genome of *M. avium* subsp. *paratuberculosis* K10. Specifically, of the eight isolates tested, one (obtained from a deer) had a SNP noted within MAP3726 (at position 400 starting from the first base pair of the gene) and an additional two SNPs within MAP3750 (positions 331 and 396). Three other isolates (two from cows, one from a sheep) had a shared SNP within MAP3750 (at position 384). These findings suggest that LSP^{P}14 is polymorphic within *M. avium* subsp. *paratuberculosis*.

Of the seven LSP^{P}s tested extensively, only LSP^{P}12 and LSP^{P}15 could be amplified from the majority of *M. avium* subsp. *paratuberculosis* isolates, suggesting either that the other LSP^{P}s were not universally present in this subspecies or that sequence level polymorphisms prevented their amplification across some isolates. Despite our efforts to identify a pattern, it was not possible to correlate the distribution of these variable LSP^P regions with respect to the host source of the isolate tested.

LSP^{A}s: regions present in *M. avium* subsp. *avium* 104 and absent from *M. avium* subsp. *paratuberculosis* K10. A positive PCR with primers flanking the LSP^{A}s indicated the absence of the region. PCR with flanking primers demonstrated the absence of LSP^{A}8 in 94 *M. avium* subsp. *paratuberculosis* isolates. For the 289 isolates (including 13 of *M. avium* subsp. *paratuberculosis*) which failed to amplify using the flanking primers, we performed internal PCR testing using two different sets of primers targeting sequences within LSP^{A}8 and found the sequence was present in all 276 non-*M. avium* subsp. *paratuberculosis* isolates. The 13 *M. avium* subsp. *paratuberculosis* isolates did not generate PCR products with either set of primers internal to this region. Therefore, it appears that this sequence is consistently missing from *M. avium* subsp. *paratuberculosis* isolates and is present in every other *M. avium* complex strain tested. Sequencing of 25 of the amplicons generated from *M. avium* subsp. *paratuberculosis* strains using the flanking primers revealed the same polymorphism in all *M. avium* subsp. *paratuberculosis* strains, with the junction point truncating two genes (called Maa4183 [the ortholog of MAP3636] and Maa4172 [not annotated in *M. avium* subsp. *paratuberculosis*]), homologs of *Rv0197* and *Rv0201* in *M. tuberculosis*, respectively. These results strongly suggest that absence of LSP^{A}8 is the result of a genomic deletion from the ancestral genome.

LSP^{A}13, a 19.5-kb sequence, was confirmed as missing in 167 isolates by PCR using primers flanking this sequence. Of these, 102 were *M. avium* subsp. *paratuberculosis* isolates, but also 64 of *M. avium* subsp. *avium* and 1 of *M. avium* subsp. *silvaticum* were shown to lack this region. Sequencing of the amplicons generated using the flanking primers in 25 *M. avium* subsp. *paratuberculosis* and 25 *M. avium* subsp. *avium* isolates demonstrated the exact same sequence, which is also identical to that of the *M. avium* subsp. *paratuberculosis* K10 type strain. LSP^{A}13 is located at the tRNA-*argV* gene and is flanked by

TABLE 3. Locations of LSP^P s and their distribution across a screening panel of 96 non-*M. avium* subsp. *paratuberculosis* isolates[a]

Sequence	Start	End	Open reading frames	Presence in non-*M. avium* subsp. *paratuberculosis* isolates	Characteristic of LSP^P
LSP^{P}1	101,281	117,322	MAP0094-0107	21	
LSP^{P}2	298,775	303,349	MAP0282-0284	0	Segment with low homology
LSP^{P}3	409,982	413,017	MAP0387-0389	4	
LSP^{P}4	874,118	889,494	MAP0851-0866	0	Additional segment
LSP^{P}5	990,416	1,003,949	MAP0957-0967	51	
LSP^{P}6	1,292,643	1,300,357	MAP1231-1237	56	
LSP^{P}7	1,441,715	1,449,170	MAP1344-1349	19	
LSP^{P}8	1,785,287	1,792,800	MAP1631-1637	40	
LSP^{P}9	1,877,241	1,894,258	MAP1718-1732	44	
LSP^{P}10	2,233,452	2,236,388	MAP2027-2029	10	
LSP^{P}11	2,378,964	2,391,994	MAP2148-2158	0	Additional segment
LSP^{P}12	2,422,564	2,442,004	MAP2179-2196	0	Replacement of a segment
LSP^{P}13	3,081,818	3,099,481	MAP2751-2768	8	
LSP^{P}14	4,144,803	4,209,911	MAP3725-3764	0	Additional and low-homology segments
LSP^{P}15	4,214,731	4,220,156	MAP3771-3776	0	Additional and low-homology segments
LSP^{P}16	4,261,192	4,267,858	MAP3814-3818	0	Additional segment
LSP^{P}17	4,734,953	4,738,741	MAP4266-4270	5	

[a] Start and end refer to base pairs from the first nucleotide of the start codon of *dnaA* on the genome of *M. avium* subsp. *paratuberculosis* strain K10. Gene names are those given to *M. avium* subsp. *paratuberculosis* strain K10 (GenBank accession number NC_002944).

direct repeats, features characteristic of mobile genetic elements. Whatever its origin, the absence of this region was not specific to *M. avium* subsp. *paratuberculosis*.

LSP^{A}14, a 23-kb region, was initially judged to be missing in 101 isolates, all of which were *M. avium* subsp. *paratuberculosis*, by PCR using flanking primers (set A). Upon sequencing, the amplicons obtained from 25 of these *M. avium* subsp. *paratuberculosis* isolates were identical to that of the prototype, *M. avium* subsp. *paratuberculosis* K10. When testing for the presence of LSP^{A}14, an intervening sequence was shown to be present in 215 isolates (all non-*M. avium* subsp. *paratuberculosis*). However, 67 isolates did not give products with either the flanking or the internal primers, leading us to hypothesize that the sequence may be missing (as in *M. avium* subsp. *paratuberculosis*) but associated with polymorphisms in the flanking regions. To test the latter possibility, we designed a new set of primers (set B) to amplify across LSP^{A}14, such that the resulting PCR products would encompass the sequences of the original flanking primers (set A). Using flanking primer set B, we determined that the region was indeed missing in these isolates. Upon sequencing the resulting PCR products, we noted that the LSP^{A}14 junction point in these isolates was identical to that in *M. avium* subsp. *paratuberculosis*. However, the sequence of the flanking region in these isolates was only 97% identical to that of *M. avium* subsp. *paratuberculosis*. Further, two of the SNPs noted to be common to these non-*M. avium* subsp. *paratuberculosis* isolates occurred along the recognition site for one of the original flanking primers (set A). These results indicate that a positive PCR using flanking primer set A appears to be specific for *M. avium* subsp. *paratuberculosis*; however, absence of this region is not specific to *M. avium* subsp. *paratuberculosis*.

DISCUSSION

Members of the *M. avium* complex form a closely related group of bacteria, with *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis* sharing the same species designation. Despite their taxonomic relationship, *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis* exhibit markedly different capacities to cause disease across a number of hosts. In particular, *M. avium* subsp. *paratuberculosis* is emerging as a serious threat to many mammalian species, but control programs to detect and eradicate this pathogen have been hampered by the lack of sensitive, specific, and practical assays. The availability of sequence information for two prototype strains of the *M. avium* complex offers the opportunity to uncover *M. avium* subsp. *paratuberculosis*-specific sequences that may be employed for nucleic acid-based tests and development of immunological assays.

Using a comparative genomic approach, we have identified two types of markers: large sequences present in *M. avium* subsp. *paratuberculosis* K10 but not in *M. avium* subsp. *avium* 104 (LSP^{P}s) and large sequences present in *M. avium* subsp. *avium* strain 104 but not in *M. avium* subsp. *paratuberculosis* (LSP^{A}s). We evaluated the distribution of these markers across a large panel of *M. avium* complex isolates and determined that, of the latter, PCR-based testing for the loss of LSP^{A}8 was 100% specific for the identification of *M. avium* subsp. *paratuberculosis*. Akin to the RD1 region of the *M. tuberculosis* complex, which permits accurate differentiation between virulent *M. bovis* and BCG strains (25), our results for LSP^{A}8 have immediate applicability in the diagnostics of *M. avium* subsp. *paratuberculosis*. First, the presence of this region (by PCR testing of a sequence internal to LSP^{A}8) can be used to determine that an isolate is highly unlikely to be *M. avium* subsp. *paratuberculosis*. Second, the absence of LSP^{A}8 (by PCR testing using primers flanking these sequences) can be used to diagnose *M. avium* subsp. *paratuberculosis* with 100% specificity.

Of the 17 LSP^{P}s, 10 had poor specificity for *M. avium* subsp. *paratuberculosis*, based on the amplification of genomic targets from these regions in non-*M. avium* subsp. *paratuberculosis* isolates, while 7 of the LSP^{P}s showed a high degree of speci-

TABLE 4. Results of PCR testing of selected LSPs across an extended panel of *M. avium* complex isolates[a]

Name of region	Open reading frame tested	PCR result	No. of isolates		Sensitivity (%)	Specificity (%)
			M. avium subsp. *paratuberculosis*	Non-*M. avium* subsp. *paratuberculosis*		
LSP^{P}2	MAP0284	+	44	0		
		−	63	276		
		Total	107	276	41.1	100.0
LSP^{P}4	MAP0865	+	73	0		
		−	34	276		
		Total	107	276	68.2	100.0
LSP^{P}11	MAP2154	+	62	5		
		−	45	271		
		Total	107	276	57.9	98.2
LSP^{P}12	MAP2181c	+	102	2		
		−	5	274		
		Total	107	276	95.3	99.3
	MAP2182c	+	99	0		
		−	8	276		
		Total	107	276	92.5	100.0
	MAP2187	+	74	0		
		−	33	276		
		Total	107	276	69.2	100.0
LSP^{P}14	MAP3726	+	78	3		
		−	29	273		
		Total	107	276	72.9	98.9
	MAP3750	+	83	5		
		−	24	271		
		Total	107	276	77.6	98.2
LSP^{P}15	MAP3774	+	97	0		
		−	10	276		
		Total	107	276	90.7	100.0
LSP^{P}16	MAP3815	+	48	2		
		−	59	274		
		Total	107	276	44.9	99.3

[a] Testing for large sequences of *M. avium* subsp. *paratuberculosis* strain K10 absent from *M. avium* subsp. *avium* strain 104 (LSP^{P}s). A positive PCR test signifies that the gene tested for is present. Sensitivity was calculated as follows: number of *M. avium* subsp. *paratuberculosis* isolates with positive PCR test/number of *M. avium* subsp. *paratuberculosis* isolates tested. Specificity was calculated as follows: number of non-*M. avium* subsp. *paratuberculosis* isolates with negative PCR test/number of non-*M. avium* subsp. *paratuberculosis* isolates tested.

ficity for *M. avium* subsp. *paratuberculosis*. In recent microarray-based genomic comparisons, Paustian et al. also identified genomic regions divergent between *M. avium* subsp. *paratuberculosis* and other members of the *M. avium* complex (20). The seven regions they describe as potentially unique to *M. avium* subsp. *paratuberculosis* represent a subset of our LSP^{P}s. However, our confirmatory testing of these regions across an extended panel revealed that sequences identical or nearly identical to LSP^{P}11, LSP^{P}14, and LSP^{P}16 (which encompass the regions called MAP_RD3, MAP_RD6, and MAP_RD7 by Paustian et al.) can be occasionally found in other closely related bacteria, which were isolated from clinical specimens from various hosts.

LSP^{P}14, a 66-kb sequence rich in genes encoding proteins of the PE/PPE family, includes a previously described 38-kb element (24). It has recently been suggested that one gene from this region, MAP3732, encodes an immunogenic protein of potential utility for the diagnosis of *M. avium* subsp. *paratuberculosis* infection (19). By PCR testing of two different gene loci within LSP^{P}14, we were able to detect sequences with homology to *M. avium* subsp. *paratuberculosis* K10 in some *M. avium* subsp. *avium* isolates, with one isolate being positive for two of the genes tested. Additionally, only 67 of the 107 (63%) *M. avium* subsp. *paratuberculosis* isolates tested positive for both targets; 30 isolates amplified with only parts of this sequence, and 10 isolates did not amplify with any part of this sequence. These findings, combined with variable sequence data obtained from *M. avium* subsp. *paratuberculosis* isolates, confirm that there is sequence divergence in this region, which may undermine its potential utility in the accurate diagnosis of *M. avium* subsp. *paratuberculosis* infection.

LSP^{P}2, LSP^{P}4, and LSP^{P}15 were 100% specific for *M. avium* subsp. *paratuberculosis*. The presence of LSP^{P}12, a previously described 19-kb island (8, 23), was also highly predictive of the identification of *M. avium* subsp. *paratuberculosis*. Two of the targeted genes for this region, MAP2187c and MAP2182c, were only detected in *M. avium* subsp. *paratuberculosis*, while an element similar but not identical to MAP2181c, a putative

transcriptional regulator of the TetR family, can be present in a minority of non-*M. avium* subsp. *paratuberculosis* isolates. MAP2182c encodes a protein (HspX) possibly involved in mediating cell attachment and has been shown in a previous PCR-based study to be specific for *M. avium* subsp. *paratuberculosis* (9). In that study, where PCR tests were done in two different laboratories, *hspX* was detected in 10 out of 12 (83.3%) *M. avium* subsp. *paratuberculosis* isolates, which is comparable to the sensitivity of our assay (92.5%). Of note, three *M. avium* subsp. *paratuberculosis* isolates failed to amplify with all three primer pairs used in testing for LSP^{P}12 yet had an LSP^{A} profile suggestive of *M. avium* subsp. *paratuberculosis* (absence of LSP^{A}8); this suggests that a small proportion of *M. avium* subsp. *paratuberculosis* isolates may be truly devoid of this sequence or that there is sequence level polymorphism in certain isolates.

Of the sequences noted to be highly specific for *M. avium* subsp. *paratuberculosis*, LSP^{P}12 and LSP^{P}15 consistently gave positive results across *M. avium* subsp. *paratuberculosis* isolates, while amplification of most other LSP^{P}s was variable. The low observed sensitivity of PCR-based testing for these LSP^{P}s may reflect technical limitations of a PCR-based approach. However, in this work we deliberately opted for PCR parameters (such as low annealing temperatures) that maximize sensitivity and thereby achieved successful and consistent amplification of numerous other targets across a wide range of isolates. Moreover, in preliminary microarray-based studies of *M. avium* subsp. *paratuberculosis* strains, certain isolates that gave negative amplification results for the LSP^{P} regions studied in this investigation also failed to hybridize to probes designed for these same regions (unpublished observations). Together, these observations argue that certain elements are truly missing from certain *M. avium* subsp. *paratuberculosis* strains. In fact, many of the LSP^{P}s we describe have characteristics of mobile genetic elements and include genes annotated as encoding transposases and phage-like integrases. LSP^{P}1 is most likely a prophage. LSP^{P}13 is composed almost entirely of IS elements. The extra sequence represented by LSP^{P}4 is, as is common with mobile elements, inserted at a tRNA gene and flanked by long direct repeats. Testing for such sequences may be specific, but consistent with their mobile nature, they may be variably present in *M. avium* subsp. *paratuberculosis*.

LSP^{P}12 was the most widely conserved *M. avium* subsp. *paratuberculosis*-specific region. Besides the previously described *hspX* gene, this sequence also includes a cluster of six homologs of the mammalian cell entry gene (*mce*) family (MAP2189 to MAP2194), as well as genes predicted to encode potentially useful metabolic functions, more specifically, genes involved in lipid metabolism. The former have previously been identified as important for invasion, survival within macrophages, and possibly virulence (6), although some homologs of *mce* genes present in the opportunistic pathogen *M. avium* subsp. *avium* were noted to be missing from *M. avium* subsp. *paratuberculosis* (23). Our analysis indicates that there are nine *mce* gene clusters in the *M. avium* subsp. *avium* strain 104 genome, including four that are homologous to those identified in *M. tuberculosis*. The genes present in LSP^{P}12 represent an *mce* cluster unique to *M. avium* subsp. *paratuberculosis*. Although the functional importance of this requires further study, work done by others has shown that there are immunodominant epitopes within *mce* genes (12), suggesting that these could potentially be exploited as a source of antigenic proteins for the diagnosis of *M. avium* subsp. *paratuberculosis*. LSP^{P}12 is also of interest because it truncates *mbtA*, which mediates the first step in mycobactin biosynthesis (Fig. 3B). This truncation is potentially sufficient to prevent siderophore biosynthesis and may account for the dependence on Mycobactin J for the in vitro growth of *M. avium* subsp. *paratuberculosis*.

LSP^{P}15, for which amplification was observed in over 90% of *M. avium* subsp. *paratuberculosis* isolates, includes genes likely required for the transport of iron. Since iron plays a key role in the balance between pathogen survival and host immunity, and since *M. avium* subsp. *paratuberculosis*, unlike other mycobacteria, lacks cell-wall associated mycobactin and siderophores, the proteins encoded by these genes may serve in iron acquisition in the intracellular environment. These findings suggest that LSP^{P}12 and LSP^{P}15 are of great potential not only for diagnostics but possibly also for studies of the pathogenesis of *M. avium* subsp. *paratuberculosis*.

Identification of the isolates used in our study to the species level was based partly on the presence of IS*900*, a multicopy insertion element widely used in the molecular diagnosis of *M. avium* subsp. *paratuberculosis*. Therefore, the sensitivity and specificity of our LSPs have not been directly compared to those of this element. However, insertion elements of different bacterial species share sequence similarities, a feature which can confound diagnostic testing relying on PCR amplification of small fragments of these elements. This issue has previously been raised in the molecular diagnostics of *M. avium* subsp. *paratuberculosis* relying on IS*900* (7, 10) and also for the use of IS*6110* as a marker of *M. tuberculosis* (15). Furthermore, as has been shown to be the case with some members of the *M. tuberculosis* complex which do not contain IS*6110* (1), it is possible some strains of *M. avium* subsp. *paratuberculosis* are either low copy number or IS*900* negative, providing additional argument for the need to develop molecular tests that rely on genomic characteristics other than mobile elements.

In summary, we have evaluated the distribution of numerous LSPs across a large panel of *M. avium* complex isolates. Our findings emphasize that in silico identification of a species-specific sequence in a prototype genome does not guarantee its utility as a diagnostic marker. Although most polymorphisms lack the sensitivity and specificity necessary for reliable diagnosis of *M. avium* subsp. *paratuberculosis* infection, the proteins they encode may contribute to the virulence of *M. avium* subsp. *paratuberculosis* and still prove valuable as markers of infection or disease. In contrast, seven of the LSP^{P}s and LSP^{A}8 revealed themselves to be exquisitely specific for *M. avium* subsp. *paratuberculosis*. Further work geared toward the characterization of these and other polymorphisms should facilitate the development of effective diagnostics.

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