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Mycobacterium avium in the Postgenomic Era

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INTRODUCTION

Past reviews on the species *Mycobacterium avium* have typically focused on two distinct aspects. The first examines organisms classically called *M. avium* and their role in human disease, such as disseminated disease in AIDS and pulmonary disease (87, 124). This focus has also included other genetically distinct species, such as *M. intracellulare* and related species that are grouped together as the *M. avium* complex (MAC). The other focus has been on the Johne's bacillus, previously known as *M. paratuberculosis*, in the context of veterinary medicine (36, 46, 112). For a number of reasons, spanning from tradition to tools, these two organisms are still usually studied as separate entities, although by genetic criteria they have been

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TABLE 1. Nomenclature applied to MAC organisms

Accepted name	Basonyms or synonyms
M. avium subsp. *avium* and/or *M. avium* subsp. *hominissuis*	Avian tubercle bacillus, *M. tuberculosis avium*, *M. avium*
M. avium subsp. *silvaticum*	Wood pigeon bacillus, *M. avium* subsp. *columbae*, *M. silvaticum*
M. avium subsp. *paratuberculosis*	Johne's bacillus, *M. enteritidis*, *M. johnei*, *M. paratuberculosis*
M. intracellulare	Battey bacillus, *Nocardia intracellularis*, *M. battey*
Collective designation	Battey-avian mycobacteria, *M. avium-intracellulare*, *M. avium-intracellulare-scrofulaceum*, *M. avium* complex

classified as subsets of the same species for over a decade (267). As a result, clinical and epidemiologic studies of human exposure, infection, and disease have largely ignored the now-renamed *M. avium* subsp. *paratuberculosis*. In parallel, research on *M. avium* subsp. *paratuberculosis* often overlooks the existence of other closely related *M. avium* organisms and the potential impact of these other *M. avium* organisms on diagnostic and epidemiologic findings.

The advent of genome sequencing projects and comparative genomic tools has provided a renewed opportunity to firmly classify mycobacteria. In the case of the *M. tuberculosis* complex (MTBC), comparative genomics has provided genomic signatures that define members of the complex (14, 25, 102, 183), and these genomic signatures now serve in diagnostic laboratories to assign identity to clinical isolates (198). Phenotypically ambiguous organisms can now be classified confidently based on their genomic signatures (185), leading to the recognition that certain organisms previously grouped together due to insufficiently discriminatory methods (184, 245) in fact consist of genetically distinct host-associated variants or ecotypes (adapted to a specific habitat), such as the vole bacillus, seal bacillus, dassie bacillus, oryx bacillus, and *M. caprae* in goats (2, 55, 182). With the recent availability of complete genome sequences for the two principal *M. avium* subspecies (152) (The Institute for Genomic Research [TIGR] [http://www.tigr.org/]) and results from comparative genomic studies (236, 240, 304), it is now possible to reconsider *M. avium* in a similar manner. The existence of natural variants of *M. avium* is expected to initially pose new challenges in taxonomy and diagnostics. However, once the nomenclature is resolved, a postgenomic phylogenetic framework should serve towards improved diagnostics and strain tracking and, additionally, provide a context for studies of disease pathogenesis. The aim of this review is to address current misconceptions and confusion in *M. avium* taxonomy, to place emphasis on the importance of recognizing the diversity within *M. avium* strains, and to highlight the opportunities to study *M. avium* by exploiting the existence of phenotypically variant members of the same species. In addition, we examine how genomic data provide opportunities and challenges for the derivation of novel diagnostic tools, noting in particular the distinction between elements specific by in silico analysis of genome sequence data and those specific by validated laboratory assays. Finally, in the face of accumulating reviews and rebuttals about the potential role of *M. avium* subsp. *paratuberculosis* in human Crohn's disease (CD), we consider it especially valuable to reassess the definition of this organism, the methods used for its detection, and the applicability of these methods for epidemiologic investigation of this association.

TAXONOMY AND CLINICAL SIGNIFICANCE

Mycobacteria are defined by their acid-fast properties, cell walls containing mycolic acids, and high (~61 to 71%) genomic C+G contents (149). There are now over 130 established and validated species and subspecies of mycobacteria (J. P. Euzéby, *List of Prokaryotic Names with Standing in Nomenclature* [http://www.bacterio.cict.fr]), with the most commonly isolated species in clinical laboratories consisting of members of the MTBC and members of the MAC. Originally described in two separate veterinary settings, MAC organisms have long been recognized as professional pathogens of birds and ruminants. Based on their source of isolation and pathology in animal models, two distinct organisms, namely, the avian tubercle bacillus, the agent of tuberculosis (TB) in birds, and Johne's bacillus, agent of Johne's disease in ruminants, were recognized. With the recognition that the avian tubercle bacillus could occasionally be isolated from human diseases, MAC organisms were also considered opportunistic pathogens of humans. In order to determine the potential sources of human exposure, environmental surveys were undertaken, revealing viable or culturable MAC organisms in a number of sources, including water (reviewed in reference 208). The latter observation led to the concept or belief that MAC organisms are fundamentally environmental mycobacteria. While it appears that some MAC organisms reside primarily in the environment, other subsets are veterinary pathogens with a limited capacity to survive in the environment (143, 300). Therefore, to best appreciate the natural variability among MAC organisms, it is safest to consider the MAC as a microcosm of the mycobacterial genus including both environmental mycobacteria and host-associated pathogens with their own distinct genomic identities.

The definition of MAC varies with the context in which it is discussed (Table 1). Clinicians and health care workers consider MAC to include *M. avium*, *M. intracellulare*, and miscellaneous related species. In veterinary medicine, MAC may be recognized the same way but, notably, is distinct from "*M. paratuberculosis*." The taxonomist may consider the MAC to contain only the subspecies of *M. avium*, as the designation implies, including *M. avium* subsp. *paratuberculosis*, and recognize that *M. intracellulare* is a related but clearly distinct species from *M. avium*. The scientist may or may not adopt any of the definitions described above, depending on the research question being addressed. Confusion sets in when new advances redefine the classic nomenclature. With this being said, we believe that sufficient data now exist to provide clarity in *M. avium* taxonomy and that a revised taxonomic approach will

benefit research into the epidemiology and pathogenesis of diseases due to *M. avium*.

Classical Definition of MAC Species

A milestone in the characterization and definition of *M. avium* occurred in 1990 with a publication by Thorel et al. which defined three principal subsets of *M. avium*, as revealed by prior molecular analyses, such as DNA-DNA hybridization (122, 231, 307), on the basis of growth characteristics and biochemical tests (numerical taxonomy analysis) (267). These three subsets consist of *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *silvaticum*.

***M. avium* subsp. *avium*.** Before the establishment of the *M. avium* subsp. *avium* designation, this organism was simply referred to as *M. avium* and was recognized to be the cause of avian TB and occasional infections in other animals. The type strain, ATCC 25291, was isolated from a diseased hen. The designation includes the standard *M. avium* subspecies causing disease in birds but also includes agents of disseminated disease in patients with AIDS, cervical lymphadenitis in children, and chronic lung disease in several settings in adolescents with cystic fibrosis and in older adults. Classically, the designation *M. avium* subsp. *avium* has not distinguished avian from human or environmental isolates, and hence, sensitization to *M. avium* is used as a proxy of exposure to environmental mycobacteria, even though avian purified protein derivative (PPD) was derived from a bird isolate (237). As discussed in greater detail in this review, the failure to distinguish between the environmental and host-associated ecotypes of *M. avium* is especially problematic for interpreting and comparing data from past studies.

***M. avium* subsp. *paratuberculosis*.** *M. avium* subsp. *paratuberculosis* refers to the etiologic agent of Johne's disease or paratuberculosis, a chronic granulomatous enteric disease of ruminant livestock and wildlife (112). Difficulties surrounding paratuberculosis control lie primarily in aspects of diagnosis; assays are most accurate when the disease is well established, but detection of subclinical infection is hampered by poor sensitivity (251, 294, 295) and specificity (168). *M. avium* subsp. *paratuberculosis* is one of the slowest growing mycobacterial species, such that primary isolation from specimens can take several months (173, 298). The distinguishing phenotype of *M. avium* subsp. *paratuberculosis* has classically been an in vitro growth dependency on mycobactin, an iron-chelating agent first obtained from *M. phlei* (90, 173) which was subsequently replaced by mycobactin J, currently used today, obtained from a strain of *M. avium* (41, 174). Notably, the type strain of the species, ATCC 19698, isolated from the feces of a cow with paratuberculosis (172), has lost its mycobactin dependency. From phenotypic analysis, the *M. avium* subsp. *paratuberculosis* group has been subdivided into two main types, bovine and ovine, that vary in hosts, diseases caused, and growth phenotypes (260, 297, 298).

***M. avium* subsp. *silvaticum*.** *M. avium* subsp. *silvaticum* applies to the previously named wood pigeon bacillus, an acid-fast organism causing TB-like lesions in these wood pigeons that were not initially successfully cultured in vitro (44, 167). Cultures were obtained for the first time when medium for "*M. paratuberculosis*" was used for cultivation and were observed for 5 months (249). Subsequently, the organisms were recognized by their mycobactin dependency upon primary isolation, gradually losing this phenotype upon subculture (165). Conflicting experimental data in attempting to classify the organisms led to the performance of DNA-DNA homology studies, ultimately revealing that they belonged to the same species as *M. avium* and *M. avium* subsp. *paratuberculosis* (231, 307). Support for the distinctiveness of *M. avium* subsp. *silvaticum*, however, was advanced by distinct patterns obtained with genetic tools such as pulsed-field gel electrophoresis (150), although this type of method is typically used for epidemiological purposes, not to delineate species. Finally, a thorough phenotypic evaluation of the *M. avium* species revealed that only *M. avium* subsp. *silvaticum* was distinct from classical *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis* based on an inability to grow on egg media and the stimulation of growth at pH 5.5 (267). The type strain, ATCC 48898, represents strain 6409, isolated from the liver and spleen of a wood pigeon and characterized in the numerical taxonomy study (267).

***M. intracellulare*.** Unlike the *M. avium* subsets, for which the type strains were isolated from nonhuman hosts, the type strain of *M. intracellulare* (ATCC 13950) was isolated from a human, specifically a child who died from disseminated disease (63). This organism was initially named *Nocardia intracellularis*, until Runyon made the link between an atypical mycobacterium called the "battey bacillus" and "*N. intracellularis*" based on similarities with *M. avium* and subsequently established the *M. intracellulare* species (223). Since then, *M. intracellulare* organisms have been isolated from a variety of animal hosts and environmental sources (225, 266, 269). In general, *M. intracellulare* has been subject to less study than *M. avium*, as the latter is more prevalent in clinical and environmental samples, has a wider apparent host range, and contributes almost exclusively to disseminated MAC disease in human immunodeficiency virus patients (276, 305). However, when identification to the species level is performed, *M. intracellulare* is an important contributor to MAC-associated pulmonary infections in immunocompetent or non-human immunodeficiency virus patients (108, 166, 207, 269, 290). *M. intracellulare* also appears to have a distinct environmental niche, as it has been found to be more prevalent in biofilms and at significantly higher CFU numbers than *M. avium* (88). The clinical designation MAC or MAI, used to group *M. avium* and *M. intracellulare*, largely reflects the conventional inability of the diagnostic laboratory to distinguish these organisms and the use of the same therapeutic regimens. Sequence-based analysis reveals *M. intracellulare* as a distinct out-group for resolving subsets of *M. avium* (277) (for example, see Fig. 1). The implications of blurring the species barrier in clinical, epidemiological, immunological, or bacteriologic studies are unknown but clearly important.

New Designations and Related Species

Many strains or groups of strains have been described that share similarity with the MAC, which often results in frustration, confusion, and at times, misleading data and results (137, 147, 250, 292, 293). However, as more sophisticated molecular tools become available, important (or less important) subsets can be identified with greater confidence. Examples of newer and/or less-well-recognized members of *M. avium* or species associated with MAC include the following.

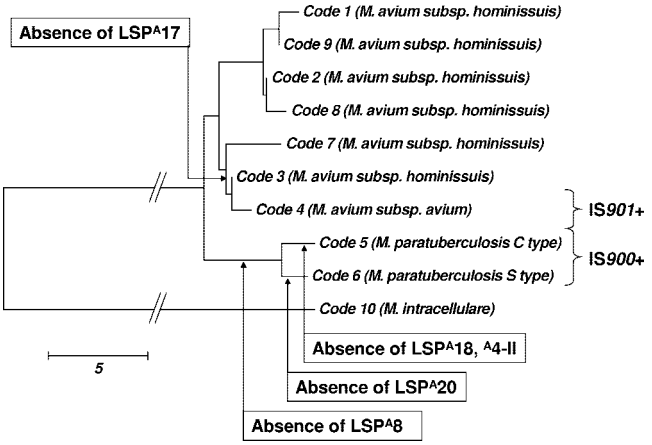

FIG. 1. *hsp65* gene phylogeny based on nucleotide differences (277), superimposed with genetic variation based on LSPs (239). IS*901*$^+$ strains cluster in one lineage, and all lack LSP^{A}17. IS*900*$^+$ strains cluster in a lineage identified by codes 5 and 6, and all lack LSP^{A}8. *M. intracellulare* serves as the outgroup for the *M. avium* subspecies. *M. avium* subsp. *silvaticum* presents with an identical genetic profile to that of code 4. Bar, 5 nucleotides.

***M. avium* subsp. *hominissuis*.** *M. avium* subsp. *hominissuis* was proposed to distinguish organisms found in humans and pigs from those isolated from birds. The hypothesis that there might be host-specific differences within *M. avium* was suggested when laboratories using genotypic methods noted that *M. avium* isolates from humans rarely shared the genetic profiles of organisms found in birds (22, 107, 139, 219). To study this further, Mijs et al. undertook a first comprehensive study that encompassed phenotypic assessment as well as several genetic tools (IS*1245* restriction fragment length polymorphism (RFLP) analysis, commercial assays, and sequencing) previously used for MAC against a large set of isolates from different hosts and geographical origins (176). This study confirmed that classical avian strains are distinct from human, other mammalian, and environmental MAC isolates. The distinguishing features of *M. avium* subsp. *hominissuis* are (i) a multiple copy number of IS*1245*, (ii) a variable 16S-23S internal transcribed spacer (ITS) sequence (the sequence in avian strains is invariant), and (iii) the ability to grow at a wider temperature range (24 to 45°C) (176). The *M. avium* strain chosen for genome sequencing, strain 104, is of the *M. avium* subsp. *hominissuis* subtype (277). Another important distinguishing feature of *M. avium* subsp. *hominissuis* from *M. avium* subsp. *avium* is that it does not possess the IS*901* insertion sequence (IS) (10, 178, 277), which is occasionally used as a marker of the *M. avium* species (243, 254). No type strain has been designated to represent *M. avium* subsp. *hominissuis*, and consequently, this designation has yet to be formally validated. However, reference strains do exist that represent this subset (Table 2).

***M. lepraemurium*.** *M. lepraemurium* refers to the agent of rodent leprosy, which was later suspected of causing skin disease in cats and dogs. To date, this organism is generally considered unculturable and can be identified reliably only by sequencing methods (119, 120). The association between *M. lepraemurium* and MAC stems from a likeness in serological groupings (103) and genetic relatedness by DNA-DNA hybridization methods (3, 123). The organism is characterized by only two single-nucleotide polymorphisms (SNPs) in the 16S rRNA gene from that of *M. avium* but bears a highly divergent *hsp65* sequence (170). While this species is genetically closely related to MAC organisms, it is not typically considered part of the MAC.

Undefined species and novel designations. Over the years, the MAC has had other taxonyms, including *M. avium-intracellulare* (MAI), *M. avium-intracellulare-scrofulaceum* (MAIS), and MAIX, where "X" represents the ambiguous MAC species that could not be assigned as *M. avium* or *M. intracellulare*. These isolates typically have similar characteristics to MAC organisms but lack features that typify the species. For example, isolates positive in the MAC AccuProbe (GenProbe) test but not in the species-specific *M. avium* or *M. intracellulare* AccuProbe test would fit within this rubric (12, 93, 147, 247, 285). *M. scrofulaceum* was once grouped with *M. avium* and *M. intracellulare* on the basis of phenotypic similarity and its ability to be serotyped, but it has long been accepted as a separate entity from MAC. The recently described species *M. palustre* (270) and *M. saskatchewanense* (278) may be confused with MAC due to their positive reactions with the MAC AccuProbe test, but they are otherwise genetically distant from MAC. Other recently described species that are genetically and phenotypically related to MAC, such as *M. chimaera* (274) and *M. colombiense* (186), highlight the fact that outliers or MAC-like organisms continue to be isolated and characterized, defying simple classification. While it is tempting to consider the latter to be the same as other MAC organisms, such a simplification risks overlooking potentially informative differences between organisms that may not yet be apparent. Therefore, when faced with such an organism, the clinical or reference laboratory may best report a MAC-like organism.

MAC Terminology Used for This Review

For the remainder of this review, we use the more encompassing term MAC to include the species and subspecies that preceded but focus mostly on the species *M. avium*. When discussing subsets of *M. avium*, we use the following terminology. *M. avium* subsp. *avium* refers to the avian subtype, includ-

TABLE 2. Commonly used reference *M. avium* strains[a]

Strain no.	True subspecies designation
ATCC 25291^T, TMC724^T, or DSM 44156^T	*M. avium* subsp. *avium*
"ATCC 12227" or strain 18	*M. avium* subsp. *avium*
ATCC 15769 or DSM 44157	*M. avium* subsp. *avium*
ATCC 19421, DSM 44158, or NCTC 8559	*M. avium* subsp. *avium*
ATCC 35713 or PPD-avian	*M. avium* subsp. *avium*
ATCC 35718	*M. avium* subsp. *avium*
104	*M. avium* subsp. *hominissuis*
ATCC 49601	*M. avium* subsp. *hominissuis*
ATCC 700897	*M. avium* subsp. *hominissuis*
ATCC 700898 or MAC 101	*M. avium* subsp. *hominissuis*
K-10 or ATCC BAA-968	*M. avium* subsp. *paratuberculosis*, C type
ATCC 19698^T	*M. avium* subsp. *paratuberculosis*, C type

[a] Identities of the strains are based on collective information and results from *hsp65* gene sequencing (277), IS*901* positivity, IS*1245* RFLP profiles, and/or the source of isolation (i.e., diseased bird or AIDS patient).

ing the type strain ATCC 25291 or TMC724. *M. avium* subsp. *hominissuis* refers to human, porcine, and environmental isolates, including the strain used for the genome sequence, which we refer to as *M. avium* 104. *M. avium* subsp. *paratuberculosis* refers to bovine and ovine subtypes of Johne's bacillus, including the strain used for the genome sequence, known as *M. avium* subsp. *paratuberculosis* K-10. Designations for commonly studied organisms, including type strains, are presented in Table 2.

LABORATORY ASPECTS OF THE MAC

Serotyping and Other Traditional Methods

Prior to the era of molecular diagnostics, identification of mycobacteria to the species level was based on morphology and a set of in vitro biochemical tests (135). These tests were not useful in subidentifying members of the MAC, since MAC organisms are generally nonreactive or produce variable results with most tests used to differentiate between species. Morphologically, the MAC presents with a wide range of colony variability, from smooth to rough and from nonpigmented to cream-colored to bright yellow, and can appear like many other mycobacterial species. High-performance liquid chromatography (HPLC) of mycolic acids became popular as a diagnostic method for species identification of mycobacteria and can distinguish MAC organisms from other mycobacteria (100). HPLC patterns of *M. avium* and *M. intracellulare* are very similar, although differentiation may be achieved by using precise interpretive criteria (32). *M. avium* subsp. *paratuberculosis* cannot be differentiated from the other subspecies of *M. avium* by this method (65). Serotyping, one of the earliest typing tools for the MAC (232), was based on differences in the sugar residue compositions of surface glycopeptidolipids (GPLs) and became the preferred method of MAC identification in the premolecular era. More than 30 serovars have been described (reviewed in reference 38). Based on a variety of tests, including DNA probing, serotype numbers could generally be assigned to the following MAC species: serotypes 1 to 6, 8 to 11, and 21 are classical *M. avium*; serotypes 7 and 12 to 20 are *M. intracellulare*; and serotypes 26, 27, and 41 to 43 are *M. scrofulaceum* (225, 293). Interlaboratory reproducibility in serotype numbers, however, was poor, and issues with autoagglutination, failure to react with any serum, or agglutination with two or more antisera were common (276, 293). Also, many serotypes could not be assigned confidently to a MAC species due to a poor consensus or to nonreaction with species-specific antisera, resulting in the ambiguous designation "MAC" for these strains. It was concerning that serotyping in tandem with RFLP methods used for epidemiological purposes was noted to generate different serotypes for isolates with identical RFLP profiles (79, 110). Conversely, the same serotype can be represented across the two subgroups of *M. avium* subsp. *avium* and *M. avium* subsp. *hominissuis*. Multilocus enzyme electrophoresis could differentiate strains of the MAC into many electrophoretic types on the basis that the enzymes chosen had multiple alleles (291, 306). This technique likely reflected variability at the geographic or ecotype level but did not appear to provide the level of resolution desirable for epidemiological tracking of isolates.

Genetic Methods To Detect IS Elements

When well characterized and used in the proper context, the species-specific IS elements described below can serve as a useful classification tool to distinguish subsets of the MAC (10, 49, 84). However, two problems have consistently hampered their utility for this purpose. First, a number of IS elements have been uncovered in strains considered to be MAC organisms, but without adequate strain characterization, it is difficult to judge which organisms harbor such elements. Second, IS elements are by nature mobile elements, so there is a risk that similar elements are found in unrelated bacteria because of mobility to or from MAC organisms. Therefore, while studies may report on the specificity of these elements across MAC organisms, this degree of specificity is not assured in diagnostic laboratories classifying unknown clinical isolates unless the organisms have first been shown to be MAC organisms by other methods. For instance, a newly discovered element may be found only in *M. intracellulare* among a panel of MAC strains and therefore appear to be a promising target for PCR-based detection directly from broth culture. However, until it can be ascertained that this element, or something genetically similar, is not found among the over 130 mycobacteria that may present to a reference laboratory, a positive PCR for this element should not on its own be considered sufficient evidence to state that *M. intracellulare* has been detected.

IS*900*. IS*900* was the first IS characterized within the *Mycobacterium* genus (51, 104). It was identified from a pMB22 clone derived from a genomic library from a human *M. avium* subsp. *paratuberculosis* isolate from a CD patient and was found to be specific to *M. avium* subsp. *paratuberculosis*. Just as IS*6110* has been used successfully for genotyping *M. tuberculosis* strains (280), RFLP analysis of the IS*900* element has been used a molecular tool to type *M. avium* subsp. *paratuberculosis* isolates. Based on IS*900* RFLP patterns, *M. avium* subsp. *paratuberculosis* has been divided into two main groups, namely, those isolates represented by a cattle-associated profile (C) and those represented by a sheep-associated profile (S) (11, 52, 57, 202, 297). A third RFLP genotype, called intermediate (I), was also identified from sheep (11, 67). The IS*900* element is by far the most widely used target for the molecular detection of *M. avium* subsp. *paratuberculosis* and has been used in the form of direct PCR (161, 235, 284), in situ PCR (228), sequence/hybridization capture PCR (109, 159, 161, 177), nested PCR (28, 187, 224), and real-time PCR (89, 214), with the references listed representing only a small portion of what is available in the literature. However, other similar elements found across other mycobacteria, including *M. terrae*, *M. xenopi*, *M. scrofulaceum* and related strains, *M. chelonae*, and strain 2333 (related to *M. cookii*), have been shown to cross-react with IS*900* primers used for detection of *M. avium* subsp. *paratuberculosis* (56, 85, 258). In these cases, the elements were not 100% identical with IS*900*, with different regions of the elements showing variable sequence identity. Sequencing of the amplified product for IS*900* is therefore necessary to confirm that the amplicon is truly IS*900*. A few studies have reported SNPs in the IS*900* element (19, 187), posing a problem for sequence-based verification of IS*900*-PCR results for molecular detection of *M. avium* subsp. *paratuberculosis*. Because sequencing directly from a single-round PCR product (i.e., not

from cloned PCR products or nested PCR products) revealed only two specific SNPs, dividing ovine and bovine forms of *M. avium* subsp. *paratuberculosis*, the relevance of these other reported polymorphisms is presently unclear (238).

IS*901*. Kunze et al. discovered the IS*901* element by performing a Southern blot with the pMB22 probe containing the IS*900* element across various MAC isolates under low-stringency conditions (142). This element shows ~60% sequence identity to IS*900*. Screening across a larger panel of isolates revealed that most isolates from birds and some animals contained the element, whereas isolates obtained from AIDS patients or the environment did not. Furthermore, it was found that most bird isolates had similar IS*901* patterns. These isolates were also shown to be strikingly more virulent than AIDS patient isolates in BALB/c mice (142, 204). Evidence that a more pathogenic subset of *M. avium* exists has been advanced numerous times since, leading some to simply divide *M. avium* isolates into those that are IS*901*$^+$ and those that are IS*901*$^-$ (68). In general, isolates from diseased birds and animals with macroscopic lesions are IS*901*$^+$, while those from humans, swine, or other animals without lesions are IS*901*$^-$ (22, 49, 189, 203). The virulence of IS*901*$^+$ strains has also been confirmed experimentally (79, 203).

Simultaneous to the publication of the IS*901* element, Moss et al., who were also screening for IS*900* under low-stringency conditions, observed cross-hybridization with a strain of *M. avium* subsp. *silvaticum* and designated the related element IS*902* (181). They determined that the element was present in all *M. avium* subsp. *silvaticum* isolates they tested, although no other MAC strains were included in the study set. Sequence alignment of the IS*901* (X59272) and IS*902* (X58030) sequences indicates 99% sequence identity, and upon closer inspection, their differences consist of several sequence gaps and four pairs of GC switches, suggestive of editing errors. IS*901* and IS*902* are most likely the same element, in which case the existence of an IS*902* element specific for *M. avium* subsp. *silvaticum* would not be a valid distinction. Consequently, claims that *M. avium* subsp. *silvaticum* has been detected in samples based on the presence of IS*902* should be interpreted with caution, with a more likely scenario being the detection of a strain containing IS*901* or related elements.

IS*1311*. IS*1311* was first reported as a GenBank entry in 1994 (U16276) and was subsequently used for RFLP analyses (73, 220). The element is present in all members of the *M. avium* subspecies, including *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, and *M. avium* subsp. *paratuberculosis* (49), and is not present in *M. intracellulare* (73, 296). The element itself has 85% sequence identity to IS*1245* (described below) and therefore results in cross-hybridization with the conventional IS*1245* probe (130). With the wide range of *M. avium* hosts for this element, it is possible that IS*1311* represents an "older" IS element which may have been present prior to subspecies divergence. A longer evolutionary time span is consistent with the presence of mutations in some of the IS*1311* elements among distinct subsets within the MAC. This was first observed by Whittington et al., who noted one polymorphism specific to the *M. avium* subsp. *paratuberculosis* cow or "C" type (a C-to-T change at bp 223 of the U16276 sequence) and other polymorphisms common to both the "C" and "S" types of *M. avium* subsp. *paratuberculosis* compared to other *M. avium* organisms (296). RFLP analysis of IS*1311* also revealed distinct pattern types corresponding to cattle and sheep strains of *M. avium* subsp. *paratuberculosis* (49). A simple PCR and restriction enzyme analysis (PCR-REA) using the restriction enzyme HinfI was then developed as a rapid diagnostic tool to distinguish bovine *M. avium* subsp. *paratuberculosis* isolates from the ovine type (158). Distinct growth characteristics of *M. avium* subsp. *paratuberculosis* isolates from bison in Montana prompted investigation using IS*1311* PCR-REA and revealed a third IS*1311* genotype, "B" (299), and *M. avium* subsp. *paratuberculosis* strains obtained from armadillos in Wisconsin were reported to have yet another IS*1311* PCR-REA allele (54). In agreement with IS*900* RFLP analysis (reviewed in reference 297), cattle and goats have predominantly the C type and sheep have predominantly the S type, while the B type has been found not only in American bison but also in goats and sheep in India (241, 296). Other animals, when tested, generally have the C type (296).

IS*1245*. First described in 1995 (107), IS*1245* was presented as having a more restricted range than IS*1311*, being limited to the subspecies of *M. avium*, i.e., *M. avium* subsp. *avium* (that would include *M. avium* subsp. *hominissuis*), *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *silvaticum*. By PCR analysis, this element was not found in *M. intracellulare* or 17 other mycobacterium species. This element, however, has high DNA sequence identity with IS*1311*, with both belonging to the IS*30* family, and it was shown that cross-hybridization of IS*1245* probes with IS*1311* is widespread; for instance, *M. avium* subsp. *paratuberculosis* does not contain the IS*1245* element (130). Nonetheless, from this first publication on IS*1245*, Guerrero et al. observed that human and swine strains contained an elevated number of copies (more than eight; "multicopy"), whereas bird strains, including *M. avium* subsp. *silvaticum*, presented a three-band pattern (107). The observation that human and swine strains (now called *M. avium* subsp. *hominissuis*) differ from avian strains has since been confirmed numerous times (139, 178, 190, 219, 263), also with the added dimension that environmental strains have similar characteristics to those of the *M. avium* subset from humans and swine (78, 164). Standardization of IS*1245* RFLP analysis was proposed in 1998 as a tool for MAC molecular epidemiology (283). To eliminate cross-hybridization with IS*1311*, the method was modified, leading to the recognition that the three-band bird type IS*1245* RFLP profile in fact consisted of a single IS*1245* copy and two copies of IS*1311* (130). It remains to be seen if epidemiological value would be added by using an IS*1245*-specific protocol instead of the standard protocol.

As for any widely tested insertion element, the "presence" of the IS*1245* element in species outside its typical host has been documented, although this was not confirmed by sequencing and may have been related elements, such as IS*1311* (12, 134). Some *M. avium* isolates have been documented as being IS*1245* negative, but only a few such reports have presented further documentation of strain identity by a sequence-based method (12). In some reports, IS*1245*-negative isolates have been described that contain an *hsp65* sequence identical to that of *M. avium* but that differ from *M. avium* in other taxonomic targets, such as the 16S rRNA gene and the ITS sequence (147, 277).

Other insertion elements described for the MAC. Many other IS elements have been described or detected in various members of the MAC. In most cases, their distribution is either unknown or only partially known. We attempt to put some emphasis on their most likely distribution or lack thereof. When stating that some elements are similar to others, we refer to similarity on the order of 80 to 85% identity at the nucleotide level.

(i) IS elements of rare distribution. The IS*1110* element was identified from a single strain, designated *M. avium* LR541 (116). It has some similarity to IS*900* and IS*901* and was found in only a small proportion of *M. avium* strains. However, which subset of *M. avium* contains this element is not clear, and screening was done by Southern hybridization, where the signal could have resulted from other related elements. IS*1110*-like elements have been reported for many species of mycobacteria, but these either have not been sequence confirmed (117) or have been confirmed but do not correspond to IS*1110* per se (194, 253). The only information available for the IS*1141* element is a GenBank entry dated 1995 (L10239). It was found in a strain identified as *M. intracellulare* strain Va14. Since then, no new data have been presented on this element or on the strain of *M. intracellulare* in which it was found. Unfortunately, no IS element has been identified to date that is present in all strains of *M. intracellulare* or even in any single well-known strain representative of the species. IS*1626* was discovered in the same manner as IS*901* (and IS*902*), by Southern blotting of 66 MAC isolates with an IS*900* probe (210), and has some similarity to IS*900* and to IS*1613* (below). Strong hybridization occurred for only one strain, subsequently characterized as *M. avium* by a variety of molecular tests. It is unclear, however, if this strain or any of the others screened were *M. avium* subsp. *avium* or *M. avium* subsp. *hominissuis*. This element appears to be uncommon in MAC organisms in general. IS*1613* is another element for which very little information is available: a GenBank submission exists (AJ011837), and one publication mentions that it was isolated from an AIDS patient (28), indicating a probable *M. avium* subsp. *hominissuis* strain. It is similar to both IS*1626* and IS*900*. None of these elements is present in the genome sequence of strain 104 or K-10.

(ii) IS elements of partially known distribution. The element IS*1612*, identified in a strain of *M. avium* subsp. *silvaticum* and in *M. avium* subsp. *avium* TMC724 (30), corresponds to IS*2534* (80), similarly found in strain TMC724. Proper IS nomenclature (244) was eventually assigned to this element, now referred to as IS*Mav1* (ISFinder [http://www-is.biotoul.fr/]). IS*Mav1* is present in at least one *M. avium* subsp. *hominissuis* strain, the *M. avium* subsp. *avium* type strain, and also the *M. avium* 104 genome sequence. The distribution of this element across a panel of MAC isolates is undetermined. The element IS*666* was identified in *M. avium* isolates from humans (36%), pigs (5%), cattle (12%), and the environment (78%) but not from avian strains (227). Therefore, IS*666* is likely present only in some subsets of *M. avium* subsp. *hominissuis*, as it was present in 21% of *M. avium* strains tested. The IS*1601* element was identified during a study of the genetic mechanisms behind the variable morphology of *M. avium* and was implicated in the smooth-to-rough switch in some strains (81). The IS*1348* element was uncovered upon further sequencing of the *ser2* GPL gene cluster (81). Both IS*1601* and IS*1348* are present in the *M. avium* 104 genome but not in *M. avium* subsp. *paratuberculosis* K-10. On this basis, these elements appear to be present in at least a subset of *M. avium* subsp. *hominissuis* strains and not in *M. avium* subsp. *paratuberculosis* strains. IS*Mav2* is a potentially *M. avium* subsp. *paratuberculosis*-specific element, as it was detected in all *M. avium* subsp. *paratuberculosis* strains but not in strains of *M. avium* subsp. *avium* (243, 254). Unfortunately, IS*901*-negative strains were not evaluated, and therefore the distribution of IS*Mav2* in *M. avium* subsp. *hominissuis* isolates is unknown. The IS*999* element was found in isolates presumed to be *M. avium* subsp. *hominissuis* since they were from human clinical samples and was absent from one strain known to be *M. avium* subsp. *avium* (144). While its distribution in *M. avium* subsp. *paratuberculosis* was not evaluated, it is not present in the K-10 genome sequence. The element IS*Mpa1*, with 80% sequence identity with IS*1601*, was found in all *M. avium* subsp. *paratuberculosis* strains tested, 2 of 13 MAC organisms tested, and no other mycobacterial species (191). The true distribution of this element within the MAC is unknown since only a small panel of isolates was evaluated.

(iii) IS elements newly discovered via genome sequencing projects. The *M. avium* subsp. *paratuberculosis* K-10 genome sequence contains three insertion elements that were previously described, namely, the *M. avium* subsp. *paratuberculosis*-specific elements IS*900* and IS*Mav2* and the pan-*M. avium* element IS*1311*. Sixteen additional insertion elements were identified and named IS_MAP01 through -16 (152). Of note, IS_MAP12 corresponds to the previously described IS*Mpa1* (191). The most abundant IS family represented in the K-10 genome is the IS*110* family, which includes IS*900*, IS*Mpa1*, and IS_MAP14 to -16 (152) but also the IS*1110*, IS*901*, IS*1613*, and IS*1626* elements described for other MAC strains. A few K-10 IS elements correspond to some found in the *M. avium* 104 genome, while others have low or no similarity to other bacteria, including mycobacteria, and may potentially serve in the specific diagnosis of *M. avium* subsp. *paratuberculosis*. With the 14 IS elements described for the MAC in the pregenomic era, 15 novel IS elements identified in the genome sequence of *M. avium* subsp. *paratuberculosis* K-10, and more to be found through the genome sequence of *M. avium* 104, it is clear that MAC organisms contain a very large number of IS elements, many of which are related to each other. As new genome sequences become available at an increasing pace, so will the number of related insertion elements. For example, IS*1110*, which was first identified because of its similarity with IS*900* and IS*901*, is now known to have much higher similarity to one of the new insertion elements in *M. avium* subsp. *paratuberculosis* K-10 (IS_MAP15) and to also share high similarity with an element in the recently sequenced *Mycobacterium* sp. strain MCS (GenBank accession no. CP0003841; *M. monacense* by 16S rRNA gene sequencing). This example illustrates an important limitation of targeting IS for diagnostics, as cross-hybridization with closely related elements has been documented by both PCR and Southern hybridization.

Non-IS-Based PCR Differentiation of MAC

Apart from insertion elements, other genes have been used as diagnostics or to differentiate MAC organisms and can be a

more attractive option due to concerns of nonspecificity associated with IS elements. A single-copy sequence named F57 was identified as specific for *M. avium* subsp. *paratuberculosis* (206) and later used in a duplex PCR that differentiated the MTBC, *M. avium*, and *M. avium* subsp. *paratuberculosis* (47, 101). More recently, a real-time PCR assay based on the F57 element was developed for the detection of *M. avium* subsp. *paratuberculosis* in milk, feces, and tissue (23, 259). Another *M. avium* subsp. *paratuberculosis*-specific genetic target showed similarity to the *dnaJ* family of heat shock protein genes and was designated *hspX* (83). The specificity of *hspX* for *M. avium* subsp. *paratuberculosis* was subsequently confirmed across a large panel of MAC strains with various genetic and host characteristics (84). To distinguish bovine and ovine *M. avium* subsp. *paratuberculosis* strains, a three-primer PCR assay was developed that yields PCR products of different sizes in a single reaction tube (50, 66).

In an attempt to find an *M. intracellulare*- and *M. avium*-specific target for use in clinical laboratory diagnostics, Southern hybridization of genomic fragments cloned from the avian type strain revealed two fragments specific for the MAC that were not found in other mycobacterial species (265). Fragment DT1 was specific for all isolates of *M. intracellulare* and *M. avium* strains of serotypes 2 and 3, while DT6 was specific for all *M. avium* isolates and *M. avium* subsp. *paratuberculosis* (265). Although it was not considered at the time, most isolates of *M. avium* tested were from human clinical isolates (276), therefore most likely consisting of *M. avium* subsp. *hominissuis* isolates, which could explain why few *M. avium* strains were positive for DT1. While DT1 appears to be a marker of *M. intracellulare* (71, 248) and may possibly be a marker of *M. avium* subsp. *avium*, the presence of DT1 was also found in several other mycobacterial species closely related to the MAC (72) and was lacking in some *M. intracellulare* isolates (97). DT1 reveals no similarity to *M. avium* 104 (the highest match is 61%) and no similarity to *M. avium* subsp. *paratuberculosis* K-10 or other sequences available in GenBank to date. Conversely, DT6 is found in both sequenced genomes and the avian type strain and therefore may serve as a marker for subspecies of *M. avium*.

Sequence-Based Classification

The ribosomal operon. The 132 established mycobacterial species at last count are known to present almost as many different 16S rRNA gene sequences. Additionally, many other mycobacterial 16S rRNA genotypes are thought to exist for which the organisms are not yet recognized as species (199, 272). Yet the *M. avium* subspecies (*M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *hominissuis*) share an identical 16S rRNA gene sequence and hence cannot be differentiated by 16S rRNA gene sequencing. The unvalidated species "*M. brunense*" (ATCC 23434) also shares 100% identity with the *M. avium* 16S rRNA gene sequence (279), although it is unclear to which subspecies it corresponds. The closest relatives to *M. avium* by 16S rRNA gene sequence vary by 6 bp (*M. colombiense*), 9 bp (*M. intracellulare*), and 10 bp (*M. chimaera*) and are considered part of the MAC in a clinical setting. Restricting analysis to the type strains of validated species, the next closest species are *M. bohemicum* (13 bp) and *M. malmoense* (17 bp).

Commercial molecular diagnostic assays offer a user-friendly, rapid method of classifying mycobacteria and are typically based on the ribosomal operon. The first such available assay was the AccuProbe test (GenProbe, Inc., San Diego, CA), developed for the most common mycobacteria in human clinical samples, including the MTBC, MAC, *M. kansasii*, and *M. gordonae*. In addition to the pan-MAC probe, species-specific probes against *M. avium* and *M. intracellulare* are also available. However, cross-reaction of other mycobacterial species with the MAC probe is not uncommon (72, 247), and only one probe can be tested at a time. Two kits based on the technology of reverse hybridization and on line probe assays have recently been developed which can identify several mycobacterial species at once, with the number increasing with new kit versions. The Inno-LiPA Mycobacteria test (Innogenetics, Belgium) is based on the 16S-23S ITS region (146, 256, 273), and the GenoType Mycobacteria test (Hain Lifescience GmbH, Nehren, Germany) is based on the 23S rRNA gene (217, 229). Both of these contain probes that identify *M. avium* and *M. intracellulare*, while the Inno-LiPA kit contains additional probes for the "MAIS complex" and *M. intracellulare* II. Isolates which hybridize to the MAIS complex probe but not the species-specific probes are common and may pose a diagnostic dilemma, but they do emphasize the complexity of strains that resemble MAC organisms and prevent their undue assignment to either species (147). None of these systems was designed to distinguish between the subspecies of *M. avium* based on the targets chosen.

The 16S-23S ITS is a highly variable genetic region that has been used extensively to study the variability within MAC organisms. To date, 35 MAC sequevars have been identified, including MavA to -H for the species *M. avium*, MinA to -D for *M. intracellulare*, and MAC-A to -X for strains which could not be assigned to either species (70, 92, 176, 186) (GenBank no. AY701784 to -86 [unpublished]). Mav and Min sequevars vary by only 1 to 4 bp, while the MAC sequevars present with significant variability and are candidates for new species. To date, three such new species have been described. *M. chimaera*, characterized by the MAC-A sequevar, and *M. colombiense*, characterized by the MAC-X sequevar, are genetically related to the MAC and are considered as such in the clinical setting. In contrast, the species *M. parascrofulaceum*, characterized by the MAC-G sequevar, is a distant species from MAC organisms and should therefore not be considered as such. With that being said, most clinical MAC isolates present with a MavA, MavB, or MinA sequevar (70, 92, 188), and *M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum*, and *M. avium* subsp. *paratuberculosis* belong to the MavA sequevar (277). Therefore, subspecies of *M. avium* cannot be distinguished from each other by this method, and the majority of the many ITS sequevars are rare and of unknown epidemiological significance.

The *hsp65* gene. Housekeeping genes offer a higher level of sequence variation than do ribosomal genes but are nonetheless useful for taxonomic purposes due to the relative sequence conservation imposed to maintain function. In this category, the stress protein gene *hsp65* is a preferred target for mycobacterial identification to the species level, having routinely been used in diagnostics since the development of a rapid PCR-restriction enzyme analysis (PRA) method using a

441-bp section of the ~1,600-bp gene (262). However, the PRA method, which is dependent on band size interpretation, shows poor interlaboratory correlation in band size designations. Also, since protein-encoding genes generally have higher mutation rates, as little as one SNP in a restriction enzyme site can result in a different PRA pattern, complicating interpretation (145). With more access to sequencing technology, some laboratories perform *hsp65* gene sequencing in the same manner as that done for 16S rRNA gene sequencing (170). The use of this target as an epidemiological tool for closely related mycobacteria, including MAC organisms, has been investigated (72, 86, 190, 288, 289, 303). Single SNPs in this region exist among various subsets of *M. avium* subsp. *hominissuis*, and great variability can be observed in *M. intracellulare* (246). However, like the case for the ITS, the fragment targeted cannot distinguish between *M. avium* subsp. *avium* (and *M. avium* subsp. *silvaticum*) isolates, *M. avium* subsp. *paratuberculosis* isolates, and a great proportion of *M. avium* subsp. *hominissuis* isolates. Conversely, the *hsp65* sequence outside the Telenti fragment offers unique sequence signatures that can help to identify the various subspecies of *M. avium*. PRA analysis of a 960-bp fragment was shown to differentiate *M. avium* subsp. *avium* from *M. avium* subsp. *paratuberculosis* (86). Based on this, comparative sequencing of the nearly complete *hsp65* gene was performed on a large panel of isolates, revealing that polymorphisms in the 3′ end, beyond the Telenti region, can unambiguously distinguish between *M. avium* subsp. *avium* strains, *M. avium* subsp. *paratuberculosis* strains of bovine and ovine types, and six sequevars of *M. avium* subsp. *hominissuis* (277).

Other housekeeping genes. Housekeeping genes other than the *hsp65* gene have been evaluated, though to a lesser extent, for mycobacterial identification to the species level. Unfortunately, these studies typically do not include all members of the MAC, omitting at least one of the main subtypes from test panels, and therefore the true utility of these sequence-based tools, at least in the detection and epidemiology of MAC organisms, remains unknown. The manganese superoxide dismutase gene (*sodA*) revealed several distinct sequevars among MAC organisms, including a unique sequevar for *M. avium* subsp. *paratuberculosis* (29), compared to human *M. avium* isolates. However, SNPs present among three sequences submitted to GenBank representing the identical type strain of *M. avium* subsp. *avium* (X81384, U11550, and AY544802) make it difficult to establish whether avian strains do or do not have a unique *sod* sequevar.

A 236-bp fragment of the *dnaJ* gene has been reported to produce variable sequences across a panel of MAC strains, but the utility of this target requires further evaluation (179). The reference panel of isolates tested included the 28 MAC serotypes and a set of clinical strains but did not include any samples of *M. avium* subsp. *paratuberculosis*. Notably, the degree of genetic diversity observed among *M. avium* isolates was relatively limited compared to that for strains of *M. intracellulare* (179). Therefore, further study of this gene target across a broader sample, and perhaps including a larger fragment, is required to determine the utility of *dnaJ* variability for characterization of MAC organisms.

Several other genes have been assessed for their diagnostic potential for mycobacteria, including *gyrB* (132), *recA* (21), the 32-kDa protein gene (247), *rpoB* (98, 136), and a combination of these in a multigene approach (74). However, these studies generally evaluated only a few strains belonging to the MAC, often limited to the type strains of *M. avium* subsp. *avium* and *M. intracellulare*. Therefore, the utility of these genes in distinguishing between epidemiologically important subsets of MAC is largely unknown.

The observed variability in a number of genes across MAC isolates suggests that a multilocus approach may provide greater discrimination than analysis of each target on its own. For other pathogenic bacteria, such as *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*, the combination of high-throughput sequencing technologies and recognized variations in housekeeping genes has enabled the emergence of multilocus sequence typing (MLST) (154, 155) as a powerful tool for typing and taxonomic purposes. An important advantage of this method is the existence of more than 30 (and increasing) curated MLST sequence databases freely available on the Internet, permitting direct comparisons with existing data (154). MLST has not formally been initiated in mycobacteriology to date, but given the number of variable genes noted above, this method could easily be implemented and serve as an epidemiologic or phylogenetic tool to characterize MAC organisms on the subspecies, geographic, or ecotype level.

COMPARATIVE GENOMICS OF THE MAC

Genetic Variability in the Pregenomic Era

Prior to the availability of genome sequence data, and hence the capacity to perform microarray studies, genetic methods such as restriction mapping, Southern blotting, suppression subtractive hybridization (SSH), and representational difference analysis (RDA) were employed to identify regions of difference among study strains. These methods and others were applied to MAC organisms in several studies, as described below.

The earliest genetic variability studies of the MAC set out to identify a genetic basis for differences in GPL composition between rough colony variants of *M. avium* and their smooth counterparts (15, 16, 81). Belisle et al. (15) performed Southern blot analysis against morphological variants of an *M. avium* serovar 2 strain, using restricted fragments from a plasmid probe containing the complete *ser2* gene cluster responsible for biosynthesis of the serovar 2-specific sugar residue characterized earlier (17). Striking differences were observed between rough morphotypes and their parent smooth strains, and it was determined that this was due to genetic deletions, one of which resulted from IS element-mediated recombination causing the loss of the complete *ser2* cluster (81). Some genetic differences were also observed between the two serotype 2 strains, i.e., TMC724 from a bird and *M. avium* 2151, isolated from human sputa (15) and later characterized as having multicopy IS*1245* and therefore being *M. avium* subsp. *hominissuis* (140). Part of the *ser2* gene cluster was also identified by RDA and characterized in an independent study (30, 268), although the association with the *ser2* GPL cluster was not confirmed until recently (80). The region was designated "GS," was described as "genetic island-like" with a lower G+C content, and was

found in *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *silvaticum*, and some *M. avium* subsp. *avium* strains of serotype 2 but not in other strains of *M. avium* subsp. *avium*. Although the following was not specifically addressed, MAC strains not containing the GS element could be inferred to be *M. avium* subsp. *hominissuis*. In an attempt to identify genetic differences between a virulent *M. avium* strain (*M. avium* subsp. *avium* strain 724) and a less virulent human strain of *M. avium* (*M. avium* subsp. *hominissuis* strain A5), RDA analysis was performed and also revealed genes belonging to the *ser2* GPL cluster in strain 724 (141).

SSH of *M. avium* subsp. *paratuberculosis* against strains of *M. avium* subsp. *avium* identified 42 short genetic regions, 24 of which were deemed specific for *M. avium* subsp. *paratuberculosis* on the basis that they also revealed low sequence similarity by BLAST searching of genome sequence data from *M. avium* strain 104 (138). To identify genetic differences between *M. avium* subsp. *paratuberculosis* variants of type I/S/ovine and type II/C/bovine (75), the avian type strain of *M. avium* subsp. *avium* (ATCC 25291) served to identify *M. avium* subsp. *paratuberculosis*-specific sequences by RDA, and one each of the *M. avium* subsp. *paratuberculosis* bovine and ovine types was tested against the other to uncover their unique genetic signatures. Three small genetic regions were identified as unique to *M. avium* subsp. *paratuberculosis* versus *M. avium* subsp. *avium*. Also, three genetic regions present in type I but not type II strains were also present in *M. avium* subsp. *avium*, suggesting that type I *M. avium* subsp. *paratuberculosis* is an evolutionary intermediate between *M. avium* subsp. *avium* and bovine *M. avium* subsp. *paratuberculosis*. No genetic regions unique to type II strains and not present in type I strains were identified in the study. In contrast, Marsh and Whittington did uncover an 11.5-kb region missing from the S/I type and present in the C/II type by RDA (162), although comparison with other *M. avium* strains was not carried out. RDA analysis also uncovered a 7-kb section which was further characterized as part of a putative 38-kb pathogenicity island specific for *M. avium* subsp. *paratuberculosis* (253). Described as an ABC transporter operon (*mpt*), no similarity was found in sequences available from GenBank or in the sequenced genome of *M. avium* strain 104 from TIGR. Since the driver used in the RDA experiments was *M. avium* subsp. *avium* (ATCC 25291^T), it is likely that this region is also not found in avian strains.

While this review is focused on chromosomal genetics, it is important that many strains of MAC organisms are known to harbor a variety of plasmids (58, 60, 163, 171). This aspect of MAC research is significantly understudied at present but has revealed important findings in terms of strain epidemiology (171), virulence (95, 96), adaptability and resistance (91, 94, 205), and other mechanisms attributed to the presence of plasmids (61). While most *M. avium* strains isolated from AIDS patients in the United States were found to contain plasmids (59, 114, 180), the sequenced strain of *M. avium* 104 is not known to contain any and was chosen in part due to its rare ability among *M. avium* isolates to be genetically manipulated (197, 271). Furthermore, to our knowledge, no plasmids have been discovered or identified in strains of *M. avium* subsp. *paratuberculosis*.

Genomes of Strains *M. avium* subsp. *paratuberculosis* K-10 and *M. avium* 104

MAC genetics and genomics have advanced exponentially in the past decade, with but one paragraph on this topic in the last comprehensive MAC review (124). The turn of the century brought us the first genome sequences of MAC organisms, for (i) *M. avium* strain 104 from the blood of an AIDS patient (TIGR), a representative of *M. avium* subsp. *hominissuis*; and (ii) *M. avium* subsp. *paratuberculosis* strain K-10, isolated from a cow with Johne's disease (152). Several other MAC genome sequencing projects are under way and are expected to generate more data in the coming 3 to 5 years (John Bannantine and Vivek Kapur, personal communication).

The K-10 genome is 4.83 Mb long, has a G+C content of 69.3%, and contains 4,350 open reading frames (ORFs) (152). For comparison, *M. avium* 104 has an additional ~700 kb of DNA, for a total genome size of 5.48 Mb, but has a similar G+C content of 69.0%. Comparison of genes orthologous between the two genomes reveals 98 to 99% sequence identity. Approximately 75% of the K-10 genome has homologs in the published *M. tuberculosis* H37Rv genome sequence (48, 152). Since little is known about the actual virulence mechanisms of *M. avium* subsp. *paratuberculosis*, the representation of H37Rv genes associated with virulence was investigated (152). The K-10 genome has significantly fewer PE/PPE genes (i.e., with Pro Glu and Pro Pro Glu motifs) (1% versus 10% of total genes in H37Rv). As well, K-10 has additional mammalian cell entry (*mce*) gene homologs or operons but also a lack of some of the specific *mce* genes described for H37Rv. Additionally, K-10 has a larger number of genes possibly involved in lipid metabolism. K-10 is notably lacking two important operons, for polyketide and mycocerosic acid synthesis, that together result in the production of the cell wall component phthiocerol dimycocerosate. However, additional genes were identified which may possibly play a role in phthiocerol dimycocerosate synthesis. Since several of these are also found in *M. avium* 104, they alone cannot explain the pathogenic nature of *M. avium* subsp. *paratuberculosis*.

The raw genome sequence of *M. avium* 104 has been available from TIGR since about 2003. However, the TIGR annotation was just released in late 2006 (GenBank accession no. CP000479). In the interim, publications on the *M. avium* 104 genome were based on the in-house annotation efforts of individual groups (240, 304). These predicted the presence of between 4,480 (240) and 4,987 (304) ORFs. The present TIGR annotation includes 5,313 genes, 5,120 of which code for proteins. The differences in gene content reflect differences in annotation methods and improvements in the identification of short genes. A formal published annotation of *M. avium* 104 will be a useful resource for helping to resolve these differences and opening the door to further postgenomic study of the MAC.

Comparative Genomics of the MAC

Prior to the completion of the two MAC genome sequences, comparative analyses using contig fragments representing the partial genome of K-10 already revealed 27 genes unique to it compared to *M. avium* strain 104 (6). Some of these were

found to be present in other mycobacteria, such as *M. intracellulare* and other strains of *M. avium*, when screened by PCR, emphasizing caution in the interpretation of what is truly subset specific and foreshadowing the genetic variability in the complex. Additionally, examination of the origin of replication (*oriC*) site of *M. avium* subsp. *paratuberculosis* compared to that of *M. avium* subsp. *avium* as well as random genomic regions beyond the *oriC* site did not reveal any notable differences that could explain their divergent phenotypes. Nucleotide identity values were no different from those observed for other bacteria belonging to the same species and with identical phenotypes (8).

Based on the availability of genome sequence data, three groups have assembled DNA microarrays, two based on the genome sequence of *M. avium* 104 (240, 304) and one starting with the *M. avium* subsp. *paratuberculosis* K-10 genome as a template (201). These microarrays then served to evaluate genomic variability in the MAC as a whole. The different groups employed similar experimental approaches, beginning with a relatively small panel of MAC strains of different types to identify large sequence polymorphisms (LSPs) or regions of difference by microarray analysis and then confirming the presence/absence of these regions by PCR and sequencing across a larger panel of isolates. In part due to an unawareness of the taxonomic issues detailed above, discrimination between *M. avium* subsp. *hominissuis* and "true" *M. avium* subsp. *avium* isolates was not taken into consideration, which may have resulted in greater complication and/or simplification in the interpretation of results from these experiments.

The first of these experiments used the first available genome data set, albeit not an annotated set, namely, that of *M. avium* 104 from TIGR (240), and included 93% of the putative ORFs, representing a first-generation array. Microarray analyses were performed against strains representative of other *M. avium* subsets, including *M. avium* subsp. *paratuberculosis* K-10 (bovine type) and LN20 (ovine type) and the type strain of *M. avium* subsp. *silvaticum*. Fourteen LSPs, defined as representing six or more contiguous ORFs missing in test strains, were identified as present in strain 104 but not in the others. These were first labeled LSP1 to LSP14 and subsequently renamed LSP^A1 to LSP^A14 to distinguish them from the LSP^Ps described in a subsequent study as present in *M. avium* subsp. *paratuberculosis* K-10 but missing from *M. avium* 104 (236). LSP^As were 21 kb to 197 kb long, for a total of 727 kb (13.5% of the strain 104 genome). Only LSP^A11, representing part of the *mce2* operon, appeared to be variably present in *M. avium* subsp. *paratuberculosis*. Upon further investigation, it was determined to be missing specifically from an ovine strain of a specific IS*900* RFLP type (unpublished data), which happened to have been the only ovine strain tested by microarray in this study. Notably, only 3 of the 14 LSP^As were observed as uniformly present in non-*M. avium* subsp. *paratuberculosis* MAC strains, indicating that a small minority of the variability observed represented differences between *M. avium* subsp. *hominissuis* and *M. avium* subsp. *paratuberculosis*. Additionally, no LSP^A could serve to distinguish *M. avium* subsp. *silvaticum* from strains designated *M. avium* subsp. *avium*. Principal observations made from this comparative analysis included a high conservation of PE/PPE genes in all tested strains and variability in the distribution of *mce* genes. Genes of the mycobactin synthesis operon (*mbtA* to *mbtJ*), which is characterized for *M. tuberculosis* (212), are all present in strains 104 and K-10. However, *mbtA*, believed to be the initiator of mycobactin synthesis, is truncated in *M. avium* subsp. *paratuberculosis* strain K-10 (240), as confirmed by others (152). The hypothesis that this may be the cause of mycobactin dependency in *M. avium* subsp. *paratuberculosis* remains to be confirmed formally by functional studies and may be technically hampered by the existence of a number of smaller mutations in other genes of the *mbt* operon (C. Y. Turenne and M. A. Behr, unpublished).

More recently, another group took a similar approach where smaller regions of deletion, defined as three or more consecutive ORFs, were considered in comparative analyses. Twenty-four LSPs were identified as missing from *M. avium* subsp. *paratuberculosis* strains, which included most of those described by Semret et al. plus an additional 96 ORFs distributed among 11 LSPs. Altogether, these ranged from 3 to 196 kb long, totaling 846 kb (17% of the strain 104 genome) (304). In addition to *mce* operons, genome plasticity was also observed in TetR transcriptional regulators. Finally, three large genetic inversions were described between the 104 and K-10 genomes.

Working in the converse sense, the availability of the *M. avium* subsp. *paratuberculosis* K-10 genome has facilitated in silico (236, 304) and microarray-based (201) approaches to determine which genes are present in K-10 but missing from *M. avium* 104 and other MAC organisms. Semret et al. identified 17 LSP^Ps spanning 230 kb of sequence in sections of 3 to 66 kb (236). Comparably, Wu et al. identified 18 LSP^Ps (GI MAP-1 to -18) spanning 240 kb, 16 of which were perfectly shared in both studies, with the only differences being due to short genetic regions spanning a few genes (304). Paustian et al. presented their microarray data according to any individual genes differentially present in MAC strains versus K-10 (201), not restricted to runs of genes. Not surprisingly, many of these consisted of transposase genes specific to *M. avium* subsp. *paratuberculosis*. Also, they identified seven large regions of difference that corroborated with the larger LSP^Ps described in the other two studies and several single genes or genes in small groups that corresponded mostly to the smaller LSP^Ps.

Studies to date have found that little genomic variability exists among *M. avium* subsp. *paratuberculosis* strains. However, the level of variability between *M. avium* subsp. *paratuberculosis* and the other MAC organisms is >1 log greater than that observed through nearly a decade of genomic studies on the MTBC. In common with efforts for the MTBC, though different labs find various numbers of elements, LSPs noted across different papers are typically recognizable as the same genomic regions, providing valuable independent confirmation for the findings presented.

Genetic Variability in the Postgenomic Era

Evolutionary events among the MAC organisms can be speculated upon by the use of LSP analysis. LSPs can be the result of (i) horizontal gene transfer (HGT) or genetic insertion events or (ii) deletion events. Which event occurred is not always evident by simple comparative genomics of two strains against each other. Events in intergenic regions reveal no directionality by themselves. One can best assume an insertion event if the flanking regions represent a single gene split in

two. Conversely, a deletion event can be inferred if a gene of known function or homology to a closely related species is truncated or if both the stop codon of one gene and the start codon of another are deleted, resulting in a chimeric gene (26). Additionally, different organisms lacking the same element via a deletion should share precisely the same truncation point, as revealed by sequencing across the deletion (183). Another approach, which requires investigation beyond direct comparative genomics, involves determining the distribution of variable genetic elements in closely related strains, in this case restricted to the MAC complex. Ideally, such an approach would serve to inform *M. avium* genomic studies if the sequence of an *M. intracellulare* strain were determined. However, since this is not the case, and few nontuberculous mycobacterium genome sequences exist, the tendency is perforce to perform comparative genomics with what is available, mainly the MTBC. HGT in *M. avium* subsp. *paratuberculosis* K-10 has been evaluated by this approach (157). However, without taking into consideration other members of the MAC apart from *M. avium* strain 104, HGT remains difficult to ascertain.

The availability of the genome sequences provides the capacity to build whole-genome arrays of strains 104 and K-10, separately or combined, for screening against other MAC species. Of particular interest is the assessment of genomic variability within *M. avium* subsp. *paratuberculosis* as a whole or between the two predominant types, i.e., S and C. Two independent studies identified several different LSPs between the C and S types based on microarray analyses. Firstly, two LSPs spanning clusters of several genes were found to be present in the C type, not the S type. One of these, called LSP^{A}20 (239) or deletion 1 (160), spans from MAP1484c to MAP1491 and contains putative pyruvate dehydrogenase genes. The region is also present in *M. avium* 104, suggesting that the LSP may represent a true deletion in the S lineage. The second region not present in S-type versus C-type strains, which is 20 kb long and referred to as deletion 2 (160), spans from MAP1728c to MAP1744 and includes mycobacterial membrane protein genes (*mmpL5* and *mmpS5*) previously identified by RDA (162). Comparison with the *M. avium* 104 genome, however, reveals a complex event. The middle section of deletion 2 is missing from strain 104 and overlaps with the LSP-P9 fragment (236), while the two end clusters of genes found present in 104 are separated by a 203-kb sequence corresponding to the previously designated LSP1 (now LSP^{A}1) (240). Further analysis will be required to establish the sequence of events in that region.

In addition, the use of a combined strain 104 and K-10 genome array uncovered two LSPs that were present in *M. avium* 104 and *M. avium* subsp. *paratuberculosis* S-type strains but deleted from C-type strains (239). The first, LSP^{A}18, is a 16-kb sequence that is replaced by an IS*900* element in strain K-10. The second LSP, designated LSP^{A}4-II, is of particular interest because it consists of a 26-kb section of the 197-kb sequence previously designated LSP4 (or LSP^{A}4) that is present in *M. avium* 104 but not in strain K-10. Interestingly, the *mbtA* gene is intact in the S type due to the presence of the LSP^{A}4-II sequence, whereas the absence of this element in C-type strains results in the truncation of the *mbtA* gene in K-10 (239). This finding provides strong evidence that the ovine form is ancestral to bovine *M. avium* subsp. *paratuberculosis* and related to the domestication of sheep predating that of cattle (45). Moreover, this genetic finding challenges the mycobactin-dependent phenotype ascribed to *M. avium* subsp. *paratuberculosis*, which has been examined more rigorously for C-type strains. Again, functional studies will be required to address this point, and since S-type strains are so fastidious, mycobactin dependence may not be readily quantified. Certainly, other factors are implicated in the painstakingly slow in vitro growth rate of ovine *M. avium* subsp. *paratuberculosis*. The evidence obtained from comparative genomics of the C and S types does suggest that the *M. avium* subsp. *paratuberculosis* S-type strains are intermediary between *M. avium* and C-type strains. However, an important limitation of microarray analysis is the inability to uncover an extra genomic region in these strains. Using RDA, fragments of the regions LSP^{A}18 and LSP^{A}4-II had been identified, but no fragments unique to *M. avium* subsp. *paratuberculosis* S-type strains were reported (75). Since RDA is only able to detect relatively short genetic sequences and is subject to significant interexperiment variability (162), the ultimate determination of whether ovine strains of *M. avium* subsp. *paratuberculosis* contain additional genetic material will come from a complete genomic sequence.

In addition to the study of genetic variability within *M. avium* subsp. *paratuberculosis*, microarray analysis of other MAC species has been initiated. A 6-kb region designated LSP^{A}17, spanning from MAP1375c to MAP1381c, was found to be missing from all IS*901*$^{+}$ strains tested, i.e., *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum*, but was present in all *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *hominissuis* strains (with the exception of those represented by a code 3 *hsp65* sequence) (239). The significance of the commonality between the avian strains and a specific subset of *M. avium* subsp. *hominissuis* strains is unknown but does emphasize the importance of including a representative panel of isolates to avoid bias in the interpretation of comparative genomic data. The directionality of this event, like that for many others, remains to be determined by further analysis.

Work on the *ser2* GPL cluster progressed further with the availability of the genome sequence of *M. avium* 104. A pathway for the biosynthesis of the serovar-specific GPL in MAC strains of serovar 2 was ultimately proposed based on a combination of previous data with sequence comparison with *M. avium* 104 (serovar 1) and analysis of strain 104 rough morphotype mutants (80). An additional strain, A5 of serotype 4 from an AIDS patient, was evaluated in similar comparative analyses of the GPL cluster (141), providing further insight into the high level of genomic variation, including insertions-deletions and possible rearrangements, within members of *M. avium*.

Diagnostics and Taxonomy Based on LSPs

There are unique regions of diagnostic interest in *M. avium* subsp. *paratuberculosis* which may help to overcome many of the sensitivity and/or specificity hurdles that continue to hinder *M. avium* subsp. *paratuberculosis* diagnostics. Comparative genomics of strains 104 and K-10 have allowed for the characterization of genetic regions unique to each, but only in relation to each other. The K-10 genome revealed ~161 "unique" sequences (152), or approximately 200 kb of se-

quence data was found in K-10 only, not in strain 104. However, among the 17 LSPs constituting the 200 kb, 10 were also found present in other MAC species (236). The LSPs specific to *M. avium* subsp. *paratuberculosis* (designated LSP^P2, -4, -11, -12, and -14 to -16) contained, not surprisingly, the previously described *hspX* gene (in LSP^P12; gene MAP2182c), F57 (in LSP^P4; gene MAP0865), and the 38-kb pathogenicity island or ABC transporter operon (in LSP^P14; MAP3725 to -3764). Whether these regions are present in all *M. avium* subsp. *paratuberculosis* strains remains to be clarified. In at least two studies using PCR-based screening, certain elements were reported as variably present (236, 304); however, employing Southern blot analysis, we instead found that these elements are consistently present across *M. avium* subsp. *paratuberculosis* strains (Turenne and Behr, unpublished observations). Regarding LSP^P14, the surface-exposed MptD protein (encoded by gene MAP3732c) was subsequently used as an *M. avium* subsp. *paratuberculosis*-specific target for the development of a peptide-mediated diagnostic assay for detection in bulk milk samples (252). Additionally, *M. avium* subsp. *paratuberculosis*-specific DNA sequences previously identified by SSH (138) correspond to sections within LSP^P2, -4, -11, -14, and -16.

Strains can also be identified by what they do not contain, as applied to "brand" lineages of the MTBC by their genomic deletion profiles (185, 198, 257). For example, LSP^A8 is a region that shares homology with elements of the MTBC and is present in all MAC species except *M. avium* subsp. *paratuberculosis*, where it has been deleted (239). Presumably, this deletion involved the loss of genes no longer selected for in the bacterium's host-adapted environment. In the practical sense, this region was used to design a three-primer PCR that could distinguish unambiguously between *M. avium* subsp. *paratuberculosis* and non-*M. avium* subsp. *paratuberculosis* strains, which is useful not only for rapid identification to the subspecies level but also for the detection of mixed cultures or infections (239).

While there is great genetic variability among MAC organisms, very little genomic variation seems to exist among *M. avium* subsp. *paratuberculosis* strains. The principle differences documented to date involve variability between ovine and bovine subtypes. Epidemiological tests also reflect the clonal characteristics of the species, but further comparative genomic studies will be required to determine if unrecognized variability exists within *M. avium* subsp. *paratuberculosis*.

Immunodiagnostics of *M. avium* subsp. *paratuberculosis*

The search for *M. avium* subsp. *paratuberculosis*-specific antigenic proteins is ongoing, as their discovery would have great potential for the design of diagnostic methods for detection of *M. avium* subsp. *paratuberculosis*. Not surprisingly, however, most genes identified as coding for *M. avium* subsp. *paratuberculosis* antigenic proteins are also present in other mycobacteria or subspecies of the MAC (reviewed in references 112 and 200). Their nonspecificity may therefore translate into false-positive results in serodiagnostic assays, as most hosts for which a diagnosis of *M. avium* subsp. *paratuberculosis* infection is being investigated are likely to be exposed to environmental strains of *M. avium*. For the antigenic proteins known to be *M. avium* subsp. *paratuberculosis* specific, their utility in immunodiagnostics is uncertain: the HspX protein can be recognized by a small portion of *M. avium* subsp. *paratuberculosis*-infected cattle sera only (7). The differential expression of antigenic proteins in *M. avium* subsp. *paratuberculosis* versus other mycobacterial species may offer additional options for immunodiagnostics (192, 193), although much work is needed to investigate this possibility.

With the completion of the *M. avium* 104 and *M. avium* subsp. *paratuberculosis* K-10 genome sequences, Paustian et al. focused on a selection of 13 ORFs deemed specific to *M. avium* subsp. *paratuberculosis* based on BLAST searches against public databases and PCR screening against other MAC organisms and mycobacteria (200). Five of these were cloned, expressed in *Escherichia coli*, purified in adequate amounts, and tested for reactivity against sera from animals experimentally immunized or naturally exposed to *M. avium* subsp. *paratuberculosis*. Variable results were observed across the five genes, but the protein from MAP0862 showed great promise, as all serum samples from cattle in the clinical stages of disease but none of the uninfected sera were reactive.

In contrast to efforts in building one protein at a time, an alternative approach recently advanced was to generate protein extracts from the surfaces of the relevant bacteria and then determine whether cross-reactivity was observed. This report suggested the generation of a highly specific preparation for Johne's disease immunodiagnostics (82), although further validation and independent verification are needed.

Comparative Genomics: Current Appreciation and Diagnostic Implications

With the exclusion of *M. leprae* and the MTBC, both of which are considered strictly host-restricted pathogens, for most other mycobacteria living in the soil or water there should be plenty of opportunity for gene exchange. However, since the most-studied mycobacterial species is the MTBC, documentation of HGT in mycobacteria has been rare. Few genomic studies have been done within the genus outside the MTBC, which is in great part due to the fact that the earliest and most abundant *Mycobacterium* genome sequences available were for strains of the MTBC.

General observations from comparative genomic analyses of the MAC have been presented in this review and indicate the tremendous degree of genomic variability, the presence of both vertical and horizontal forms of genetic variability, and the potential to exploit these findings for laboratory identification. While further work is clearly indicated to completely catalogue differences between MAC organisms, there are now a number of bacterial targets for identification to the species level and the first examples of protein targets offering the possibility of specific immunodiagnosis. A tabulation of some of these elements and their distribution across MAC organisms for diagnostic purposes is presented in Table 3.

UNRESOLVED ISSUES

Does *M. avium* subsp. *silvaticum* Really Exist?

The existence of *M. avium* subsp. *silvaticum* as a unique subspecies depends upon the interpretation of phenotypic assays, including mycobactin dependence upon primary isolation

TABLE 3. Distributions of genetic elements and gene targets across MAC organisms

Genetic target	IS family[j]	Presence of target[g]						Comment	Reference(s) for distribution within subspecies
		104[a]	MAH[b,h]	K-10[c]	MAP[d,i]	MAA[e,h]	MI[f]		
IS*900*	IS*110*	−	−	+	+	−	−	SNPs present in S type (238)	10, 49, 51, 84
IS*901*	IS*110*	−	−	−	−	+	−		10, 22, 219
IS*1245*	IS*256*	+	+	−	−	+	−		10, 49, 84, 219
IS*1311*	IS*256*	+	+	+	+	+	−	SNPs present among MAC subsets (296)	49, 73
IS*Mav1*	IS*21*	+	+/?	−	?	+/?	?	Same as IS*1612* and IS*2534*	30, 80
IS*666*	IS*256*	+	+ (21%)	−	−	−	−		227
IS*1601*	IS*256*	+	+/?	−	?	?	?		81
IS*Mav2*	IS*481*	−	?	+	+	−	−/?		243, 254
IS*999*	IS*3*	+	+/?	−	−/?	−/?	?		144
IS*Mpa1*	IS*110*	−	+/?	+	+	−/?	−/?	Same as IS_MAP12 (152)	191
IS*1110*	IS*110*	−	+/?	−	?	?	?		116
IS*1141*	IS*3*	−	?	−	?	?	?	GenBank accession no. L10239	
IS*1626*	IS*110*	−	−/?	−	?	?	?		210
IS*1613*	IS*110*	−	?	−	?	?	?	GenBank accession no. AJ011837	
hspX	NA	−	−	+	+	−	−	Present in LSP[P]12 (236)	83, 84
F57	NA	−	−	+	+	−	−	Present in LSP[P]4 (236)	206, 258, 282
DT1	NA	−	−/?	−	−	+/?	+	Found in other species related to the MAC	72, 264, 265
DT6	NA	+	+	+	+	+	−		72, 264, 265
mptD	NA	−	−	+	+	−	−	Present in LSP[P]14 (236)	252

[a] Sequenced strain *M. avium* 104, which is an *M. avium* subsp. *hominissuis* strain.
[b] *M. avium* subsp. *hominissuis* strains in general.
[c] Sequenced strain *M. avium* subsp. *paratuberculosis* K-10.
[d] *M. avium* subsp. *paratuberculosis* strains in general.
[e] *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum* strains in general.
[f] *M. intracellulare*.
[g] ?, presence unknown; +, present; −, absent; +/? (or −/?), present (or absent) in one or some strains, but distribution throughout is variable or unknown.
[h] While the distinction between the two subsets has not always been taken into consideration, extrapolation could generally be made by the use of known strains or by assumptions.
[i] Presence in both bovine and ovine forms, unless stated otherwise.
[j] NA, not applicable.

(267). Results to date of genetic and genomic studies have failed to reveal a distinct molecular profile for *M. avium* subsp. *silvaticum* strains compared to that for *M. avium* subsp. *avium* strains (79, 239, 277). This discordance raises the issue of whether the phenotypic uniqueness of these organisms is sufficient to merit the designation of a distinct subspecies, and by extension, whether the assays so used have sufficient reliability to serve in this role. The phenotype of mycobactin dependency upon primary isolation is difficult to determine in a controlled manner, particularly if it is dependent on the bacterial burden. Successful culture of these organisms from acid-fast test-positive wood pigeon or roe deer tissue was sparse and required 4 to 5 months for visible growth, even in the presence of mycobactin (131, 249). In one study, wood pigeon mycobacteria grew better in the presence of mycobactin but could also be cultured without mycobactin if a large number of bacilli were present (165). At best, this phenotype might be mycobactin-enhanced growth rather than mycobactin dependence. Barclay and Ratledge found that once mycobactin-dependent *M. avium* strains lost this phenotype, they were able to produce mycobactin in small amounts, implying a regulatory effect as opposed to a genetic defect (9). Finally, in another study, wood pigeon mycobacteria grew exclusively in 7H11 medium, not on media containing mycobactin, after 6 months (128).

Another important problem in determining the taxonomic status of *M. avium* subsp. *silvaticum* is that a small number of strains have been described in the literature, and these isolates have then been passed around from lab to lab for testing by novel methods, contributing to laboratory-generated selection of colonies that replicate best under in vitro conditions. Also, since few *M. avium* subsp. *silvaticum* isolations have been reported in the past several decades, it is possible that fastidious nutritional requirements have resulted in underdetection of these organisms. Alternatively, in an era where molecular testing has largely eliminated detailed phenotypic study, organisms previously called *M. avium* subsp. *silvaticum* may simply be classified as IS*901*$^+$ *M. avium* subsp. *avium* in the modern era.

At the molecular level, doubt regarding the validity of this species first emerged from a comprehensive study evaluating IS*901* and IS*1245* RFLP patterns across a large number of *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum* isolates from birds, pigs, cattle, humans, and a few odd hosts and reference strains (79). The *M. avium* subsp. *silvaticum* strains revealed two IS*901* profile types: the first was identical to that from strains of *M. avium* subsp. *avium*, and the other profile revealed a single band shift from the pattern for the avian strains. On this basis, the authors emphasized that identification of *M. avium* subsp. *silvaticum* could not be made on the basis of mycobactin J dependency and IS*901* positivity alone. In other genetic studies, the GS region characterized in *M. avium* subsp. *silvaticum* (268) was found to be identical in sequence, including that in the IS*Mav1* site, to the syntenous region in the avian type strain, TMC724 (80). PCR results for GS-specific genes in other MAC strains of bird origin were also indistinguishable from those for *M. avium* subsp. *silvaticum* (30). Finally, in a sequence-based evaluation of the nearly

complete *hsp65* genes of MAC isolates, *M. avium* subsp. *silvaticum* was found to have an identical sequevar to that of *M. avium* subsp. *avium*, and all strains in both groups were IS*901*$^+$. Moreover, no IS*901*-negative MAC strains presented with that particular sequevar (277). Overall, no genetic data exist to distinguish *M. avium* subsp. *silvaticum* from *M. avium* subsp. *avium*. Comparative tests have been done, but only against *M. avium* subsp. *paratuberculosis* and/or *M. avium* subsp. *hominissuis* (under the label of *M. avium* subsp. *avium*). With the availability of new genomic tools and the vast amount of information obtained, nothing more has been noted as strikingly distinguishable in *M. avium* subsp. *silvaticum* since its description in 1990.

Sequencing the genome of *M. avium* subsp. *silvaticum* may provide some answers. However, a genome sequence is not yet available for *M. avium* subsp. *avium* or, for that matter, any IS*901*$^+$ strain. By habit, the *M. avium* subsp. *avium* designation is still used by many to denote both the bird pathogen and environmental strains that cause opportunistic infections of humans. Since the sequenced strain *M. avium* 104 belongs to the *M. avium* subsp. *hominissuis* subset, a future genome sequencing project for the MAC should include at minimum the type strain of *M. avium* subsp. *avium* (TMC724 or ATCC 25291) or another well-characterized IS*901*$^+$ strain typical of the pathogenic bird isolates. Sequencing of an *M. avium* subsp. *silvaticum* strain, preferably the type strain, ATCC 49884, could be done in tandem or thereafter. Should an *M. avium* subsp. *silvaticum* genome sequence be determined, it will be important to not consider unique attributes to be specific to *M. avium* subsp. *silvaticum* without first determining if these genetic variants are shared with IS*901*$^+$ *M. avium* subsp. *avium* strains. If there are truly very few differences between the genome sequences of *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum*, as suspected, it may provide closure to the confusion and result in the demise of the latter name.

Unique genetic regions in an IS*901*-positive strain compared to *M. avium* 104 have already been identified as part of a study characterizing the integration sites of the IS*901* element in a cervine strain (126). A gene encoding the p40 protein which is present in the genomes of many mycobacteria, including members of the MAC, was found to be expressed only in IS*901*$^+$ strains (1, 125). Additional interest in sequencing an avian strain stems from the view that this organism is a professional pathogen, like *M. avium* subsp. *paratuberculosis*, that has an unknown capacity to replicate in the environment. Perhaps *M. avium* subsp. *silvaticum* does represent a specific host-associated (wood pigeon) subset of the *M. avium* subsp. *avium* branch (that can infect a variety of birds), akin to caprine and bovine variants of *M. bovis* described for the MTBC. With that being said, genetic markers that can make this distinction do not yet exist, and it will be interesting to see what future genomic data can provide.

Is *M. avium* subsp. *hominissuis* the Only True Environmental *M. avium* Subspecies?

While the appropriate designation of a MAC isolate may seem unimportant to the molecular biologist, veterinarian, or physician, this distinction is critical for understanding the epidemiology and capacity to cause disease. For example, where efforts have been applied to distinguish between *M. avium* subsp. *avium* and *M. avium* subsp. *hominissuis*, studies have consistently revealed that human MAC isolates are generally in the latter category (178, 263), indicating either a reduced exposure or a reduced risk of disease due to the former. Thus, when attempting to determine the source of exposure for a patient with *M. avium* subsp. *hominissuis* disease, there is little a priori reason to consider birds or environments that they could contaminate, and consistent with this, when a link between human disease and birds was investigated, it could not be demonstrated (219, 263). A possible explanation is that birds are infected by a specific subset of *M. avium* strains that are obligate pathogens of birds, in the same fashion that *M. avium* subsp. *paratuberculosis* is an obligate pathogen of ruminants. Supporting this notion, avian MAC infection does not seem to affect the bird population on a large scale, as would be expected if this *M. avium* subset was prevalent in the environment. If this is true, then the vast majority of the biomass of *M. avium* subsp. *avium* will be found in birds, not the environment, explaining why humans are generally not infected by avian strains. In this case, the majority of MAC and related species in the environment are simply *M. avium* subsp. *hominissuis* and *M. intracellulare*, and the general statement that *M. avium* is an environmental organism is misleading.

The highly variable protective efficacy of *M. bovis* BCG has prompted scientists to evaluate the impact of environmental mycobacteria on mycobacterial vaccination and/or infection (196). Brandt et al. showed that presensitization of mice by environmental mycobacteria resulted in a rapid immune response upon subsequent vaccination which inhibited the multiplication of BCG, thereby diminishing the capacity of BCG to provide protection. This was first tested by presensitization at 2-week intervals with three different ATCC strains and species, including *M. avium* ATCC 15769, which in actuality is an IS*901*$^+$ bird strain rarely found in the environment (24). The second experiment evaluated the BCG-blocking capacity of two soil and two sputum isolates of rapidly growing mycobacteria as well as two sputum isolates of *M. avium*. Of these, only the *M. avium* strains prevented multiplication of BCG, but the true species or subspecies designation of these strains was not provided. Concurrently, Buddle et al. evaluated the efficacy of BCG and newly attenuated *M. bovis* strains in calves with strong responses to avian PPD, and hence exposure to "environmental" mycobacteria, and found that the protective effect of BCG in PPD-positive animals was also diminished (27). In a follow-up study, de Lisle et al. performed similar experiments in guinea pigs but methodically compared the effects on vaccine efficacy of presensitization with IS*901*$^+$ *M. avium* versus IS*901*$^-$ *M. avium* strains, based on observations that *M. avium* strains that caused lesions in farmed deer and cattle were IS*901*$^+$, while strains that did not cause lesions were IS*901*$^-$ and genetically distinct from the IS*901*$^+$ strains (68). They found that animals presensitized orally with IS*901*$^+$ strains received little protection from BCG, whereas presensitization with IS*901*$^-$ strains did not impair BCG-induced protection. These studies show that not all environmental mycobacteria or *M. avium* strains impair protection by BCG equally. Furthermore, the impact of selecting an IS*901*$^+$ strain as representative of an environmental isolate for PPD testing is uncertain

while IS*901*$^+$ organisms have not been detected in the environment.

Considerations in Exploring *M. avium* subsp. *paratuberculosis* as a Human Pathogen

The hypothesized link between *M. avium* subsp. *paratuberculosis* and CD originally dates to similarities noted between the pathology of Johne's disease and that of human cases subsequently classified as CD (64). Unfortunately, this possibility remains a source of debate nearly a century later. One of the critical issues remains the exceptionally slow growth of *M. avium* subsp. *paratuberculosis* and its specific growth requirements that are typically not part of routine clinical microbiology protocols. It appears that the best chance at detecting and culturing *M. avium* subsp. *paratuberculosis* from human samples, although not assured, is with tremendous patience, experience, and tailored culture media. Despite this, *M. avium* subsp. *paratuberculosis* has very seldom been cultured from human specimens (42, 43). One case described the isolation of *M. avium* subsp. *paratuberculosis* from 8 of 21 specimens obtained from an AIDS patient over a 3-year period (218). Histology revealed many acid-fast bacteria, but mycobactin was not incorporated into the media at first, and therefore growth was very poor in liquid, and no growth was observed on solid media. Only after 2 years was *M. avium* subsp. *paratuberculosis* considered a possibility, and then culture was achieved on Middlebrook medium with mycobactin J. This culture was then determined to be positive for the IS*900* element. The authors noted that limited growth of *M. avium* subsp. *paratuberculosis* may occur in liquid media if the organism is abundant in clinical specimens, but evidently this is a rare case, because large series of disseminated MAC disease in AIDS patients failed to show the presence of *M. avium* subsp. *paratuberculosis* organisms (275, 287, 308). Additionally, this type of disease presentation in a case of AIDS does not reflect the burden of organisms one might expect in CD, where by definition, pathogenic organisms have not been detected. Therefore, if humans with CD have *M. avium* subsp. *paratuberculosis* infection, the microbiologic status must be paucibacillary, providing a further technical challenge to its detection amid large numbers of rapidly growing enteric flora.

One hypothesis for the difficulties in isolating *M. avium* subsp. *paratuberculosis* from CD tissue is that it may be present in a cell wall-deficient (CWD) form in tissue. *M. avium* subsp. *paratuberculosis* in a CWD form was first documented for cultures derived from CD patients after 8 to 15 months of incubation on Herrold's egg yolk medium slants (43). Subsequently, others have cultured CWD cells, or "spheroplasts," from CD tissue, on the basis that organisms recovered were non-acid-fast or variably acid-fast coccoid or pleomorphic cells and, following a long incubation phase in vitro, eventually adopted the standard acid-fast mycobacterial phenotype (99, 156). In specimens where only the CWD form would exist upon culture, species identification could not be reliably confirmed. CWD forms were also observed from ulcerative colitis patients and control samples (31).

To overcome the noted problems in culturing *M. avium* subsp. *paratuberculosis*, and as a means of addressing the possibility of CWD forms, an alternative advanced in the past decade has been to apply molecular methods instead of conventional microbiologic assays. The number of papers existing that incorporate the terms "PCR" and "IS*900*" currently rivals the number of mycobacterial species ($n = 130$). In the context of CD, results run the gamut from (i) no evidence of *M. avium* subsp. *paratuberculosis* DNA in CD samples (5, 18, 35, 39, 77, 222) to (ii) *M. avium* subsp. *paratuberculosis* DNA found primarily in CD samples (4, 28, 69, 224, 226, 233, 235) and (iii) *M. avium* subsp. *paratuberculosis* DNA found in CD patient samples, ulcerative colitis patient samples, and healthy controls, in various proportions (53, 187, 255). The controversy will likely continue, and it is not our intention to recapitulate the many arguments presented for and against the hypothesis; 20% of publications containing the terms "Crohn" and "paratuberculosis" are review articles, and the following references address some of these issues (13, 36, 37, 40, 105, 106, 111, 115, 213, 230, 242, 281, 286).

What is certain is that if *M. avium* subsp. *paratuberculosis* does infect humans, it is not an easy task to prove that this is the case. A recent study by Jeyanathan et al. set out to evaluate the sensitivities and specificities of in situ detection methods for *M. avium* subsp. *paratuberculosis*, employing a murine model where culture-positive paucibacillary *M. avium* subsp. *paratuberculosis* infection had been established (129). One simple observation from analysis of these tissues was that diseased tissue may or may not contain visible mycobacteria, but mycobacteria were not seen in histopathologically normal tissue. This observation suggests that the threshold number of *M. avium* subsp. *paratuberculosis* organisms needed to elicit a host response is lower than the number required for microscopic detection. Furthermore, to observe acid-fast forms, whether by Ziehl-Neelsen or auramine-rhodamine staining, required searching many fields at a magnification of ×1,000. Considering that the threshold of detection by microscopy is on the order of 10,000 bacilli per ml of sputum or per gram of tissue, it is logical to assume that a low burden of infection (<10 bacilli per mg), akin to that in tuberculoid leprosy or Johne's disease in sheep, will be exceptionally hard to detect and typically reported as "negative" by staining. Another pertinent observation was that *M. avium* subsp. *paratuberculosis* cells are significantly smaller than MTBC organisms and present a coccobacillary form, contributing further to the difficulty in their detection (129). Interestingly, methods previously reported as more sensitive, such as in situ hybridization (121, 234) and in situ PCR (228) of the IS*900* element, were no more sensitive than cell wall staining methods but were prone to false-positive results in uninfected control animals and *M. tuberculosis*-infected hosts (129). These results provided no evidence for the CWD hypothesis, as stains targeting nucleic acids did not result in more mycobacterial forms than stains for the cell wall. Notably, while CWD bacteria have been described during the isolation of *M. avium* subsp. *paratuberculosis* and other mycobacteria from CD patients (31, 99), to date the existence of *M. avium* subsp. *paratuberculosis* in a CWD form has yet to be proven within human tissue. Until the existence of CWD forms of *M. avium* subsp. *paratuberculosis* is formally demonstrated, diagnostic strategies based solely on this concept will be difficult to reconcile with discordant results obtained using conventional microbiologic methods.

FUTURE RESEARCH

Experience from MTBC genomics demonstrates the rapid conversion of genomic data into insights about pathogenesis that have subsequently been applied to the clinical laboratory. First, the *M. tuberculosis* H37Rv genome provided the cornerstone of future research (48). Next, comparative genomic tools permitted the demonstration of variable regions across otherwise closely related organisms (14, 102, 153). The distribution of these regions was then shown to be specific to certain subsets of the MTBC, which was of immediate use in the clinical microbiology laboratory (198, 257). The ability to accurately delineate subspecies and to compare their genomic profiles permitted investigators to derive a phylogenetic scenario for MTBC evolution (25, 183). This scenario then provided a framework for understanding phenotypic variability among MTBC members. An in vitro phenotype that benefited from this framework was the demonstration that *M. bovis* requires the addition of pyruvate to the culture medium for optimal growth because of a mutated pyruvate kinase gene, *pykA*, that has resulted in an inactive pyruvate kinase in *M. bovis* but not *M. bovis* BCG (133). The most compelling in vivo phenotype spurred on by comparative genomic studies has been the demonstration that the RD1 region, which is absent from BCG strains, in part explains the attenuation of BCG vaccines from between 1908 and 1921 (151, 211). Moreover, the determination that the RD1 region encodes virulence antigens recognized by the infected host has been exploited in the development of immunodiagnostic tests, such as the QuantiFERON-TB Gold test (Cellestis Limited, Carnegie, Australia) and the T-SPOT.TB test (Oxford Immunotec, Abingdon, United Kingdom), which are designed to detect latent tuberculous infection but not sensitization by previous BCG vaccination (195).

The preceding sequence of events can serve as a template for MAC research, using postgenomic findings to both directly bolster diagnostic capacity and guide functional studies of eventual applicability to clinical laboratories. Already, genome sequence data serve as the foundation for comparative sequencing and genomic studies (152, 304). Results of these approaches are already applicable to diagnostic laboratories and serve to brand MAC lineages for further study (239). The concordance of the *hsp65*-based phylogeny of the MAC with lineage-specific IS and a genomic deletion specific to *M. avium* subsp. *paratuberculosis* provides confidence that a phylogenetic framework is now at hand to guide functional studies (Fig. 1). The current challenge, therefore, is to distill the considerable genomic diversity within the MAC into tangible phenotype-genotype associations that can then lend themselves to formal experimental evaluation.

Some outstanding questions about MAC variability include verifying whether all *M. avium* subsp. *paratuberculosis* strains are mycobactin dependent, understanding the basis of the fastidious growth of *M. avium* subsp. *paratuberculosis* in vitro, and determining why ovine *M. avium* subsp. *paratuberculosis* strains are particularly slow-growing in the media currently used. The principal in vivo question is to accurately catalogue virulence, however defined, across MAC organisms in order to determine whether there are particularly robust patterns, such as a greater pathogenicity of bird strains of *M. avium* or *M. avium* subsp. *paratuberculosis*. Since livestock are undoubtedly exposed to environmental mycobacteria, such as *M. avium* subsp. *hominissuis*, but only develop classical Johne's disease from *M. avium* subsp. *paratuberculosis*, it is intuitive to consider the latter more inherently pathogenic. Despite this, to our knowledge, there is no standardized in vivo or ex vivo model that demonstrates a greater infectivity, persistence, or pathogenicity by *M. avium* subsp. *paratuberculosis* than that by *M. avium* subsp. *hominissuis*. While comparative genomics of the MTBC identified the absence of RD1 in BCG, it should be remembered that the subsequent experimentation was based on decades-old observations of decreased virulence of BCG strains in mouse, guinea pig, and other higher animal models (33, 76) that could readily be recapitulated in the *M. tuberculosis* ΔRD1 strain (151). To this end, an important bottleneck in functional genomic study of the MAC may not be the genomic data or even the tools for genetic manipulations (34, 113), but rather a robust virulence phenotype to guide studies trying to understand why certain MAC organisms have profoundly different lifestyles and capacity to cause disease from those of others. As a start, we encourage efforts to generate observational data from macrophage and small-mammal infections on the sequenced strains *M. avium* 104 and *M. avium* subsp. *paratuberculosis* K-10 and also to contrast other pairs of environmental and pathogenic *M. avium* organisms. If it can be documented that certain MAC organisms have an inherently different biology that impacts their capacity to cause disease (20) or to be detected by the clinical laboratory, then the results of these functional studies may translate into either improved laboratory methodologies (such as media that enhance the growth of ovine *M. avium* subsp. *paratuberculosis*) or more tailored diagnostic approaches targeting specific pathogenic subsets.

While we have described, to the best of our ability, what we believe to constitute the different members of the MAC, we accept that additional sequencing or other genetic studies may provide information to further delineate the complex. It is not unreasonable to contemplate that specific MAC types exist that are more pathogenic to specific hosts, with currently available methods unable to formally address this concept. This idea has previously been advanced for humans with different forms of mycobacterial susceptibility by showing differences between isolates from AIDS patients and those from non-AIDS patients (62, 169, 175, 215). Moreover, the latter group of patients, who are often grouped together, comprises distinct age- and sex-specific scenarios, such as lymph node disease in children (302), lung disease in immunocompetent middle-aged males with predisposing lung conditions (148, 221, 290, 301), and lung disease in a particular group of elderly white women without predisposing lung conditions (118, 127, 148, 166, 209, 216, 290). In these cases, disease may therefore involve the interaction of strain-specific properties of the bacterium as well as both genetic and environmentally determined host factors. Since MAC disease in immunocompetent persons is rare overall and disease due to MAC organisms likewise does not affect the majority of birds, pigs, and cattle, factors that determine the outcome of exposure, including whether infection is established and progresses to disease, are in need of further investigation.

CONCLUSION

In an editorial comment published in 1998, Telenti noted that while lab resources may be best directed towards TB research, publications on nontuberculous mycobacteria are abundant: "If we are going to do the work anyway, let's at least do it properly by calling things by a useful name" (261). This issue remains at large in MAC research nearly a decade later, as certain species names carry little information, while other terms, such as MAC and *M. avium*, obscure the existence of distinct subsets of organisms. Several examples have been provided in this review to underscore how flawed interpretation can follow when not all members of the MAC are considered in the experimental design. Most notably, this issue is likely to be critical in pursuing the functional genomics of MAC organisms and understanding the relative capacities of different organisms to cause infection and disease based on their natural reservoirs and their pathogenicities. Advances in evolutionary studies and taxonomy of the MTBC have changed the definition of the species in the last few years. Similarly, we now have the tools to confidently type MAC organisms and to define precisely which organisms are the subjects of study, providing valuable strain information to other laboratories aiming to replicate and build upon published findings. *M. avium* is genomically very heterogenous (more like *E. coli* than *M. tuberculosis*), and it is important to be cognizant of the various subsets to study them appropriately. Therefore, providing only the host type or source of *M. avium* subspecies is unlikely to adequately brand an organism, given that the distribution of various subtypes is not uniform or, more commonly, is unknown. At the other extreme, the degree of genetic similarity between phenotypically different organisms is often not recognized and used to focus study towards specific differences. For instance, to understand why *M. avium* subsp. *paratuberculosis* is a pathogen, one should consider parallel control experiments with *M. avium* subsp. *hominissuis* to increase the chance of determining which specific features of *M. avium* subsp. *paratuberculosis* translate into the host phenotype (disease). If an *M. avium* subsp. *paratuberculosis* virulence factor is also found in *M. avium* subsp. *hominissuis*, it may be true that the absence of the gene confers an attenuated phenotype, but it cannot readily be advanced that the presence of the gene explains why *M. avium* subsp. *paratuberculosis* is a pathogen. With this in mind, we encourage a convergence of studies related to *M. avium* subsp. *paratuberculosis* and of those related to the other subspecies of *M. avium*. Mycobacteria, whether saprophytic or pathogenic, have many similar characteristics that characterize the genus, but it is setting apart what makes one different from another that will help us to truly understand the biological differences between different species and subspecies of the MAC.

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