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ANALYSIS OF BACTERIAL FACTORS ASSOCIATED WITH PATHOLOGICAL OR CLINICAL MANIFESTATIONS OF *MYCOBACTERIUM AVIUM* DISEASE BASED ON GENOME ANALYSIS

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Abstract [Background] Infectious disease caused by *Mycobacterium avium* shows diverse pathological and clinical manifestations. This is possibly due to both host factors and bacterial factors, but many questions remain answered regarding these manifestations. [Methods] To assess the relationship between the different pathological and clinical manifestations of *M.avium* disease and bacterial factors, we performed comparative genome analysis using clinical isolates from patients with various symptoms. [Results] We determined the complete genome sequence of the previously unreported *M.avium* strain TH135 isolated from a patient with pulmonary *M.avium* disease, and further demonstrated the presence of a novel plasmid, pMAH135, encoding proteins involved in the pathogenicity and antimicrobial resistance of mycobacteria. Our analysis also showed that *M.avium* strains, which cause pulmonary and disseminated disease, have genetically distinct features, and isolates from patients with pulmonary disease were more resistant to seven antibiotics, including clarithromycin, than isolates from patients with disseminated disease. Comparative genome analysis of 79 *M.avium* strains comprising four subspecies revealed the presence of genetic elements specific to each lineage, which are thought to be acquired via horizontal gene transfer during the evolutionary process. Moreover, the analysis identified potential genetic determinants associated with not only the progression of pulmonary disease but also the host range characteristics of *M.avium*. Notably, this analysis indicated an association between the progression of pulmonary *M.avium* disease and several virulence genes including pMAH135. [Conclusion] These results suggest that bacterial factors play an important role in the diverse pathological and clinical manifestations of *M.avium* disease.

Key words: *Mycobacterium avium* disease, Pathological manifestation, Clinical manifestation, Bacterial factors, Genome analysis

INTRODUCTION

Nontuberculous mycobacteria (NTM) are ubiquitous in the environment, including natural water, soil, and household dust[1)2)], and can cause significant disease in humans and animals[3)]. The incidence of NTM infection is increasing annually in many countries, including the United States and Japan[4)–7)]. In Japan, the causative NTM strain for pulmonary disease with the highest incidence is *Mycobacterium avium* (approximately 60%), followed by *M.intracellulare*, *M. kansasii*, and *M.abscessus*, and the incidence per 100,000 population has increased remarkably from 5.7 in 2007 to 14.7 in 2014[6)8)].

Among NTM species, *M.avium* is the most clinically significant species in humans and animals and comprises four subspecies that have specific pathogenic and host range characteristics as follows: *M.avium* subsp. *avium* (MAA) and *M.avium* subsp. *silvaticum* (MAS) are avian pathogens; *M.avium* subsp. *paratuberculosis* (MAP) causes John's disease in ruminants; and *M.avium* subsp. *hominissuis* (MAH) infects mainly pigs and humans[9)–11)]. MAH is the causative pathogen of two main types of disease in humans: disseminated disease in immunocompromised hosts such as individuals infected with human immunodeficiency virus (HIV), and pulmonary disease in individuals without systemic immunosuppression[3)]. However, the genetic differences among the four subspecies are still unknown.

Pulmonary disease caused by NTM, which is both intractable and infectious, has variable clinical manifestations. Although some patients remain stable without treatment,

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others have deteriorating symptoms despite drug therapy, demonstrating the disease's diverse clinical course[3)12)]. This is possibly the result of host factors as well as bacterial factors. The guidelines for antibiotic treatment of pulmonary MAH disease recommend macrolide-based multidrug therapy, comprising macrolides such as clarithromycin or azithromycin, in combination with rifampicin and ethambutol. In addition, aminoglycosides, such as streptomycin or amikacin, are recommended for patients with severe disease[13)]. However, drug therapy is associated with two issues. Firstly, there are possible adverse effects of long-term treatment and of drug toxicity in patients, and secondly, the efficacy of the drug therapy above certain levels is unknown[13)-15)]. Consequently, predicting the clinical course of pulmonary MAH disease is a highly useful clinical indicator for determining the appropriate treatment approach. Furthermore, the timing of treatment initiation influences outcomes and is thus considered important. However, no clear criteria for determining the timing of treatment are currently available.

Plasmids have been shown to contain important genes that determine bacterial virulence and resistance to antimicrobial agents including antibiotics. With regard to mycobacterial plasmids, previous studies isolated pAL 5000[16)] and pJAZ38[17)] from *M.fortuitum* and pMSC 262[18)] from *M.scrofulaceum*. In addition, two types of plasmids were isolated from *M.avium*, pVT2[19)] and pLR7[20)], the latter of which was from a strain isolated from HIV-positive patients and has no homology to pVT2. Because of their relatively small size of 4.8−16 kb and the absence of virulence genes, the significance of these plasmids is currently unknown. Stinear et al. isolated pMUM001, a 174-kb giant plasmid containing virulence genes, from *M.ulcerans*[21)]. pMUM001 contains genes that are involved in the synthesis of a macrolide toxin, called mycolactone, which exhibits cytotoxic, analgesic, and immunosuppressive activities. Furthermore, plasmids isolated from *M.marinum* and *M.abscessus* contain mercury resistance genes[22)23)].

To assess the relationship between the different pathological and clinical manifestations of MAH disease and bacterial factors, we mainly performed comparative genome analysis using clinical isolates from patients with various symptoms.

1. Complete genome sequence of strain TH135 from patient with pulmonary MAH disease

To explore the bacterial factors that affect the establishment of pulmonary disease caused by *M.avium* subsp. *hominissuis* (MAH), we determined the complete genome sequence of the previously unreported strain TH135 isolated from a HIV-negative patient with pulmonary MAH disease at Higashinagoya National Hospital of the National Hospital Organization in Japan[24)]. The complete chromosome sequence of strain TH135 has been deposited in DDBJ/EMBL/GenBank under accession no. AP012555. The genome was composed of a single circular chromosome of 4,951,217 bp with an average G+C content of 69.32%, 4,636 predicted coding sequences (CDSs), 46 tRNA genes, and a single rRNA operon with the typical order of 16S, 23S, and 5S rRNA genes.

2. A novel plasmid, pMAH135, derived from strain TH135

Genomic sequencing of strain TH135 demonstrated the presence of a plasmid, designated pMAH135[25)]. To confirm the presence of pMAH135, we carried out pulsed-field gel electrophoresis analysis by treatment with S1 nuclease, which converts supercoiled plasmids into full-length linear DNA molecules. A band was observed close to 194 kb, which closely matched the size of pMAH135 as determined by sequence analysis. The complete sequence of pMAH135 was deposited in DDBJ/EMBL/GenBank under accession no. AP012556. This circular plasmid was composed of 194,711 bp with an average G+C content of 66.5%, 164 predicted CDSs, 1 tRNA gene, and 6 IS elements. This G+C content was characteristically low compared with that of the chromosome (69.3%), suggesting that the plasmid had been transformed into the cell at some point during the evolutionary process. pMAH135 was unique in terms of homology to other mycobacterial plasmids. BLAST analysis revealed that 47.8% of the protein CDSs in pMAH135 showed the highest homology to proteins coded by the *M. parascrofulaceum* chromosome, and 5.5% and 4.9% of the protein CDSs in pMAH135 were homologous to proteins in *M.* sp. MOTT36Y and *M.indicus pranii*, respectively.

Of the pMAH135 CDSs, attention must be paid to those encoding proteins involved in mycobactin biosynthesis and the type VII secretion system, both of which are important to the virulence of mycobacteria as well as to proteins with putative conserved domains of the multidrug efflux transporter.

2-1. ESX-5 system encoded by pMAH135

Pathogenic mycobacteria carry the type VII secretion system membrane complex, termed the ESX system, to transport virulence factors across their cell envelope, and to date, five types of ESX systems, ESX-1 to ESX-5, have been reported[26)27)]. ESX-1 is responsible for secreting 6-kDa early secreted antigenic target (ESAT-6), which can disturb the activation of macrophages, induce apoptosis, and subvert host immunity, and its protein partner the 10-kDa culture filtrate protein-10, thereby contributing to the virulence of pathogenic mycobacteria[26)28)29)]. On the other hand, ESX-3 plays a role in iron transport and is thus essential for bacterial viability[30)31)]. ESX-5, which is the most-recently evolved type VII secretion system, mediates the secretion of ESAT-6-like protein EsxN and mycobacteria-specific proteins with conserved N-terminal domains containing prolyl-glutamic acid (PE) or prolyl-prolyl glutamic acid (PPE) motifs[32)-34)]. The *M.marinum* ESX-5 system is involved in inducing cell

death of infected macrophages and modulating the immune response[26,35]. Comparative analysis revealed that pMAH135 contained CDSs with a 33.3−91.5% sequence identity to ESX-related proteins encoded by the *esx-5* locus in the *M. tuberculosis* H37Rv genome (GenBank accession no. NC_000962). As described above, because ESX-5 is involved in the virulence of pathogenic mycobacteria, it is likely that the ESX-5-related proteins encoded by pMAH135, together with those in the chromosome, contribute to the pathogenicity of strain TH135.

2-2. Mycobactin encoded by pMAH135

Iron is an essential nutrient for almost all organisms. Like many bacterial pathogens, mycobacteria synthesize siderophores to capture iron, which is present in limited concentrations in living hosts[36]. Pathogenic mycobacteria, including *M.tuberculosis* and *M.avium*, utilize two forms of siderophores with a 2-hydroxyphenyloxazoline moiety; these are termed carboxymycobactin and mycobactin and differ according to the length of their alkyl substitution[37]. The loci involved in iron acquisition via siderophores comprise the siderophores biosynthesis genes *mbtA-N* and *irtAB* that encode iron-regulated transporters[36,38]. De Voss et al. reported that an *M.tuberculosis* mutant lacking the *mbtB* gene, which encodes a non-ribosomal peptide synthetase, interrupted the biosynthesis of siderophores and impaired the growth of macrophages[39]. Thus, siderophores can be regarded to play a significant role in *M.tuberculosis* pathogenicity. The chromosome of strain TH135 contains CDSs with a 46.9−82.2% sequence homology to proteins encoded by the *mbtA-N* and *irtAB* genes in the *M.tuberculosis* H37Rv genome. In addition to these CDSs, pMAH135 contains 5 CDSs (MAH_p49, MAH_p44, MAH_p43, MAH_p47, and MAH_p48) with a 34.9−51.3% sequence identity to the MbtB to MbtF proteins of *M.tuberculosis* H37Rv, which are involved in the synthesis of the siderophore core. These results suggest that the MAH strains harboring these genes can take up iron more efficiently and may therefore be important for the onset of pathogenicity.

2-3. Multidrug efflux transporters encoded by pMAH135

Bacterial multidrug efflux transporters are classified into the following five groups according to their primary structure and mode of energy-coupling[40]: major facilitator superfamily (MFS); small multidrug resistance family; resistance nodulation cell division family; ATP-binding cassette superfamily; and multidrug and toxin extrusion (MATE) family. MATE transporters have a 12-membrane helix topology and utilize H^+ or Na^+ transmembrane gradients to drive substrate export[41]. Interestingly, protein BLAST analysis of pMAH 135 CDSs identified a CDS (MAH_p85) with putative conserved domains of MATE family proteins similar to NorM from *V.cholerae*. On the other hand, the TH135 chromosome does not encode MATE family protein homologues. Amino acid sequence alignment of MAH_p85 and *V.cholerae* NorM showed that of the 10 amino acid residues constituting the cation-binding site in NorM[41], 4 were identical while 3 were conservative substitutions in MAH_p85. Furthermore, MAH_p59 has a 54.4% sequence identity to the MFS transporter EmrB of *M.tuberculosis* H37Rv and a 57.4% sequence identity to MFS-like transporter MAH_0637 encoded by the TH135 chromosome. It is possible that both MAH_p85 and MAH_p59 encoded by pMAH135 greatly influence the resistance of strain TH135 to antimicrobial agents including antibiotics.

3. Genetic diversity in MAH strains that cause pulmonary and disseminated disease

To explore the bacterial factors that affect the pathological state of disease caused by MAH, we carried out comparative analysis between genomes of strain TH135 and strain 104 derived from an acquired immunodeficiency syndrome patient with MAH disease[42]. The chromosome size of strain TH135 is 524,274 bp shorter than that of strain 104 (5,475,491 bp)[24]. Although both strains belong to the same subspecies, insertion sequence (IS) content is very different between the strains, and the strain 104 genome carries more IS elements than the strain TH135 genome. On the other hand, it is noteworthy that strain TH135 harbors five IS*Mav6* genes (MAH_0649, MAH_1321, MAH_2272, MAH_2945, and MAH_3485) that have 60 point mutations compared with a subspecies differentiation marker IS*901*, which is on the genomes of different subspecies —*M.avium* subsp. *avium* and *M.avium* subsp. *silvaticum*[43]. IS elements are thought to be one of the major players in prokaryote genome plasticity[44]. A greater number of IS elements indicates that the genome has undergone further structural variation during strain evolution.

Whole-genome alignment of both strains was carried out using Mauve software[24]. Although high conservation in both the sequence and gene order of strain TH135 and 104 was observed, there were gene insertions and two large inversions. On strain-specific regions of over 10,000 bp in length, strain TH135 has 10 loci (specific region (SR)-1 to SR-10) and strain 104 has 11 loci (SR-11 to SR-21). Interestingly, many of these regions have low G+C content compared with the mean G+C content of the corresponding chromosome, which is an added sign of foreign origin. Furthermore, such specific regions are flanked by genes which encode integrases of phage origin and/or transposases derived from transposons. Taken together, these regions are likely to be inserted into chromosomes via horizontal gene transfer during strain evolution.

To investigate the importance of genes in strain-specific regions, we screened 35 clinical isolates (including strain TH135) from the sputa of HIV-negative patients with pulmonary MAH disease and 29 clinical isolates (including strain 104) from the blood of HIV-positive patients with disseminated MAH disease for these genes. Screening of

clinical isolates for genes located in the strain-specific regions revealed that the detection rates of strain TH135-specific genes were generally high in clinical isolates from pulmonary MAH disease patients. On the other hand, the detection rates of strain 104-specific genes were generally high in clinical isolates from HIV-positive patients. These results suggest that MAH strains that cause pulmonary and disseminated disease possess genetically distinct features, and the genes located in the strain-specific regions have a strong influence on the pathological manifestations of MAH disease.

3-1. Antibiotic susceptibility of MAH strains that cause pulmonary and disseminated disease

As described above, we have showed genetic differences between strain TH135 isolated from a patient with pulmonary MAH disease and strain 104 obtained from a HIV-positive patient by comparing the genomes of the strains[24]. Such genetic differences may affect not only the pathological manifestation of MAH infection but also various phenotypes of MAH, such as antibiotic susceptibility. Therefore, we examined the characteristics of antibiotic susceptibility of MAH isolates from different hosts by measuring the MICs of eight drugs (clarithromycin, rifampicin, ethambutol, streptomycin, kanamycin, amikacin, ethionamide, and levofloxacin) for 46 isolates from the sputa of HIV-negative patients who were diagnosed with pulmonary MAH disease but received no antibiotic treatment, as well as 30 isolates from blood of HIV-positive patients with disseminated MAH disease by the broth dilution method[45]. Interestingly, isolates from pulmonary MAH disease patients were more resistant to seven drugs except for rifampicin compared with isolates from HIV-positive patients. This suggests an association between drug susceptibility and types of MAH infection.

3-2. VNTR genotype and drug susceptibility in MAH isolates from different origins

We performed variable-number tandem-repeats (VNTR) typing analysis using 15 *M.avium* tandem repeats (MATR) loci to examine VNTR genotypes of isolates from hosts with different types of MAH infection[45]. The strains examined in this study were roughly classified into three clusters: cluster I, cluster II, and cluster III. The proportion of isolates from pulmonary MAH disease patients and that of isolates from HIV-positive patients in each cluster was 6.5% (3/46) and 36.7% (11/30) for cluster I, 41.3% (19/46) and 36.7% (11/30) for cluster II, and 52.2% (24/46) and 26.7% (8/30) for cluster III, respectively. The ratio of isolates from HIV-positive patients to those from pulmonary MAH disease patients was significantly higher in cluster I compared with the other clusters ($p=0.0017$), indicating that a group of isolates from HIV-positive patients have a unique VNTR genotype. Moreover, the genetic distance from a reference strain 104 in isolates from pulmonary MAH disease patients was statistically different from that in isolates from HIV-positive patients ($p=0.0018$), suggesting that MAH strains that cause pulmonary and disseminated disease have genetically distinct features.

Next, we analyzed the association between VNTR genotype and drug susceptibility in strains within each cluster, revealing a significant difference in $\log_2$ MICs of seven drugs except for ethambutol[45]. Furthermore, intergroup comparisons revealed that strains in cluster I had the lowest MIC values and those in cluster III tended to have the highest values. These results are related to the proportions of isolates from pulmonary MAH disease patients and those of isolates from HIV-positive patients in each cluster. In good agreement with above, the proportion of isolates from HIV-positive patients was highest in cluster I and lowest in cluster III. Taken together, these results suggest that an association between types of MAH infection, drug susceptibility, and VNTR genotypes and the properties of MAH strains associated with the development of pulmonary disease are involved in higher antibiotic resistance.

4. Cause of progression of pulmonary MAH disease

Pulmonary disease caused by MAH has a variable clinical course. Although this is possibly the result of not only host factors, but also bacterial factors, many questions remain to be answered regarding these manifestations. To examine the cause of the progression of pulmonary MAH disease, we enrolled patients with pulmonary disease caused by MAH, from nine National Hospital Organization hospitals in Japan between July 2008 and September 2009 and collected the corresponding MAH isolates and clinical data[46,47].

4-1. Characterization of subjects

Of the patients diagnosed with pulmonary MAH disease corresponding to the diagnostic criteria of the American Thoracic Society and the Infectious Diseases Society of America[13], those who started clarithromycin-based multidrug treatment within 18 months, based on decisions made by the corresponding physician-in-charge because of deterioration in the patients' condition, were classed as the progressive disease group (n=17). Those who did not receive treatment because their condition was stable were classed as the stable disease group (n=29). During the observation period, the condition of each patient was evaluated several times a year based on chest radiograph findings (including chest computed tomographic images), clinical symptoms, and/or microbiological findings. We compared clinical characteristics between the two groups, but parameters of age, sex, type of pulmonary disease, and the presence of underlying disease were not significantly different between these groups[46].

4-2. Relationship between progression of pulmonary MAH disease and bacterial factors

To assess the relationship between the progression of pulmonary MAH disease and bacterial factors, we performed

MATR-VNTR analysis using 46 isolates from pulmonary MAH disease patients with different clinical courses as described above, and furthermore, examined the association between disease progression and the pMAH135 plasmid[46]. MATR-VNTR analysis showed that 46 isolates were classified roughly into three clusters: cluster I, cluster II, and cluster III (including strain TH135). Clusters I, II, and III accounted for 17.6% (3/17), 29.4% (5/17), and 52.9% (9/17) of the isolates from the progressive disease group, respectively, showing that the proportion of cluster III isolates was significantly larger than that from the stable disease group ($p=0.019$). Furthermore, to examine relationships between VNTR genotype and disease progression in pulmonary MAH disease, we compared the genetic distances of clinical isolates from the progressive and the stable disease group as previously described[48]. The genetic distance from a reference strain TH135 in isolates from the progressive disease group was statistically different from that in isolates from the stable disease group ($p=0.035$), suggesting that MAH isolates from the progressive and the stable disease group have genetically distinct features. In previous studies, MATR-VNTR analysis of isolates from patients with pulmonary MAH disease demonstrated that isolates from progressive disease cases are grouped in a specific cluster[48], and we revealed that many of the isolates from both groups are classified into the same cluster. These findings suggest that strains in this cluster are highly virulent.

Next, to examine the relationship between the progression of pulmonary MAH disease and pMAH135, we screened 46 clinical isolates for 6 CDSs (MAH_p01, MAH_p47, MAH_p49, MAH_p59, MAH_p85, and MAH_p143) located in pMAH135[46]. The pMAH135 genes were found in 35.3−47.1% of isolates from the progressive disease group compared with 10.3−13.8% of isolates from the stable disease group. In particular, the detection rate of MAH_p47, MAH_p49, and MAH_p143 was significantly higher in isolates from the progressive disease group than in those from the stable disease group. These findings show that pMAH135 genes were more prevalent in isolates from the progressive disease group than in those from the stable disease group.

5. Comparative genome analyses of 79 *M. avium* strains

To improve our understanding of the genetic landscape and diversity of *M.avium* and its role in disease, we performed a comparative genome analysis of 79 *M.avium* strains[49]. This analysis included the genomes of 46 MAH isolates from 17 patients with progressive disease and 29 patients with stable disease that were sequenced in this study and 32 additional *M. avium* genomes that are publicly available. These 32 genomes include all *M.avium* subspecies: fifteen MAH strains isolated abroad (United States, Belgium, and Germany), six MAA strains, seven MAP strains, one MAS strain, and three *M. avium* strains of unknown subspecies.

5-1. Phylogenetic analysis based on single nucleotide variants

Phylogenetic analysis based on single nucleotide variants (SNVs) showed that the *M.avium* strains were roughly classified into three clusters: cluster I, cluster II, and cluster III. Furthermore, it was shown that each cluster has a distinctive subcluster (cluster Ia, cluster IIb or cluster IIIb) comprised of strains with genetic distances that are clearly different from those of the others[49]. Cluster I contained 93.5% (43/46) of the MAH genome sequenced in this study, whereas cluster II contained 80% (12/15) of MAH strains isolated abroad. Using VNTR analysis, Iwamoto et al. and Ichikawa et al. demonstrated a geographical difference in the genetic diversity of MAH[50,51]. In agreement with this, we found that MAH strains isolated in Japan formed a cluster (cluster I) that differs from the cluster (cluster II) containing MAH strains isolated abroad, indicating that they have different genomic features. This may be one of the reasons for the high incidence of pulmonary MAH disease in Japan[6]. Furthermore, all MAP strains belonged to cluster IIIb, and cluster IIb was formed specifically by MAA strains of avian origin and MAS strain. This suggests that strains in cluster IIIb have genomic feature associated with John's disease and that MAA and MAS strains share genomic features that enable them to infect birds.

Next, we examined the phylogenetic relationships among 46 MAH isolates from patients with either progressive or stable disease. Of the 46 isolates, 43 (93.5%) were grouped in cluster I, while only 3 were in cluster II. It is worth mentioning that 41.2% (7/17) of isolates from the patients with progressive disease and 10.3% (3/29) of those from the patients with stable disease were in cluster Ia, a subcluster of cluster I with a distinctively different genetic distance. The ratio of isolates from patients with progressive disease to those from patients from stable disease was significantly higher in cluster Ia than in other subclusters ($p=0.025$). These results indicate a specific genotype of MAH is associated with the progression of pulmonary MAH disease.

Interestingly, isolates in cluster Ia fully corresponded with those in the specific cluster described above obtained by MATR-VNTR analysis examining an identical set of isolates[46]. This result indicates that genotypes based on SNVs overlap with VNTR genotypes. Taken together, these results suggest that the isolates in cluster Ia have unique genomic features associated with the progression of pulmonary MAH disease, and demonstrate that MATR-VNTR analysis can distinguish isolates from progressive disease patients simply. Therefore, this analysis is a clinically useful approach.

5-2. Genomic region specific to cluster consisting of many isolates from progressive disease patients

By analyzing the noncore regions, we identified genomic element (locus 1) specific to cluster Ia consisting of many MAH isolates from progressive disease patients[49]. This

genomic element harbors virulence genes that account for the progression of pulmonary MAH disease. On locus 1, SR-2, which was previously identified as one of the specific regions on strain TH135 chromosome[24], is present and carries virulence-associated *mce* family genes and *mmpL* gene. Although the precise mechanisms of Mce proteins remain unclear, they are thought to be mainly involved in the entry of mycobacteria into mammalian cells and their subsequent survival[52,53]. MmpL and MmpS proteins are reported to mediate the transport of lipid metabolites for the biosynthesis of cell wall lipids in mycobacteria[54-56]. The high content of lipids, such as mycolic acids, in the cell walls plays a pivotal role in host survival[57]. Furthermore, locus 1 contains CDSs that are encoded on pMAH135 and involved in mycobactin biosynthesis and the type VII secretion system. De Voss et al. reported that a *M.tuberculosis* mutant lacking the *mbtB* gene interrupts the biosynthesis of mycobactin and impairs the growth of macrophages[39], suggesting that mycobactin plays a significant role in the pathogenicity of mycobacteria. ESX-5, which is similar to the ESX-related proteins encoded on pMAH135, mediates the secretion of ESAT-6-like proteins EsxN and EsxP, and is involved in inducing cell death in infected macrophages and modulating the immune response[35]. Thus, pMAH135 is thought to be involved in MAH pathogenicity. Interestingly, Ummels et al. reported that pMAH135 is a conjugative plasmid in slow-growing mycobacteria species, including *M.avium*[58]. Taken together, cluster Ia strains acquired genetic regions (e.g. SR-2 and pMAH135) encoding virulence genes via horizontal transfer during the evolutionary process, thereby acquiring pathogenicity resulting in disease progression. It will be intriguing in the future to discover how such virulence factors are involved in pathogenicity.

CONCLUSION

1. The genome of strain TH135 isolated from a serious case with worsening pulmonary MAH disease consists of a single circular chromosome of 4,951,217 bp with an average G+C content of 69.32%, 4,636 predicted CDSs, 46 tRNA genes, and a single rRNA operon with the typical order of 16S, 23S, and 5S rRNA genes.
2. A novel plasmid, pMAH135, derived from strain TH135 consists of 194,711 nucleotides and encodes 164 CDSs. This circular plasmid contains genes associated with the pathogenicity and antimicrobial resistance of MAH.
3. The MAH strains that cause pulmonary and disseminated disease have genetically distinct features, which may influence the pathological manifestations of MAH disease. Furthermore, MAH isolates from patients with pulmonary disease were more resistant to seven antibiotics, including clarithromycin, than isolates from patients with disseminated disease.
4. The progression of pulmonary MAH disease is associated with specific VNTR genotypes in MAH. In addition, we showed an association between the progression of pulmonary disease and pMAH135.
5. Comparative genome analysis of 79 *M.avium* strains comprising four subspecies revealed the presence of genetic elements specific to each lineage, which are thought to be acquired via horizontal gene transfer during the evolutionary process. The analysis identified potential genetic determinants associated with not only the progression of pulmonary MAH disease but also the host range characteristics of *M.avium*.

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