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Method Article

Environmental *Mycobacterium avium* subsp. *hominissuis* have a higher probability to act as a recipient in conjugation than clinical strains

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ABSTRACT

Mycobacterium avium subsp. *hominissuis* (MAH) is a widespread opportunistic pathogen that can be isolated from environment (dust, soil and water) and patients with lung or lymphnode infection. In our previous research we revealed the pronounced genetic diversity in MAH by identifying eight different types of a newly described genomic island. In order to identify mechanisms of such horizontal gene transfer we now analyzed the ability of 47 MAH isolates to inherit the conjugative plasmid pRAW from *M. marinum*. A higher percentage of environmental isolates (22.7%) compared to clinical isolates (8%) had the capacity to function as recipient in conjugal plasmid transfer. Genetic analysis showed additionally that environmental isolates contained more genes homologous to genes present on conjugative mycobacterial plasmids than clinical isolates. Comparative analysis of the genomes of the isolates pointed to a possible association between the ability to act as recipient in conjugation and the structure of a genomic region containing the *radC* gene and a type I restriction/modification system. Finally we found that uptake of pRAW decreased the resistance against various antibiotics.

1. Introduction

In the last two decades, the worldwide increase of infections caused by nontuberculous mycobacteria (NTM) has raised interest in this group of bacteria (Johnson and Odell, 2014; Prevots and Marras, 2015). NTM can cause severe and hard-to-treat lung infections in pre-disposed patients with underlying diseases such as Cystic Fibrosis, COPD (Stout et al., 2016) or immune defects. In addition, also apparently healthy immune-competent persons can endure *M. avium* infection, as in the case of the lung disease known as “Lady Windemere Syndrome” (Rao, 2016). Finally, in young children, *M. avium* is a frequent cause of lymphadentitis (Hazra et al., 1999).

The species *M. avium* is subdivided into four subspecies: *M. avium* subsp. *avium* (which infects birds), *M. avium* subsp. *silvaticum* (which also infects birds), *M. avium* subsp. *paratuberculosis* (which causes Johne's disease in cattle) and finally *M. avium* subsp. *hominissuis* (MAH) (Mijs et al., 2002). The latter is the clinically most important subspecies, causing lung infections and lymphadenitis in humans (Kolb et al., 2014).

MAH displays a high degree of genetic diversity (Rindi and Garzelli, 2014). The presence of genetic markers linked to increased virulence has so far not been reported for MAH. Also, genetic differences between clinical and environmental strains are so far not described. In order to define the clinical relevance of this genetic diversity, we have previously conducted a study comparing genomes from clinical and environmental MAH isolates which led to the identification of a new genomic island divided into eight types that considerably adds to the genetic diversity in MAH (Sanchini et al., 2016). Sequence homologies between the genomic islands and DNA from other mycobacterial species suggests that horizontal gene transfer was involved. Therefore we are interested in revealing the underlying mechanisms of horizontal gene transfer, including conjugation.

While in the other bacterial taxa, conjugative plasmids were given much attention because of their contribution to the spread of antibiotic

Abbreviations: T4SS, Type IV secretion system; T7SS, Type 7 secretion system; MAH, *Mycobacterium avium* subsp. *hominissuis*; OD600, Optical density at 600 nm; ADC, Modified Albumin-dextrose-catalase enrichment; bp, Base pair; COPB, Chronic Obstructive Pulmonary Disease

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resistance genes, plasmid exchange was until recently considered only of little significance for mycobacteria. In 2002 Kirby and colleagues published the presence of a gene encoding a conjugative relaxase in the *M. avium* plasmid pVT2 implicating that pVT2 is a conjugative plasmid (Kirby et al., 2002). In 2014, conjugal transfer of the plasmid pRAW (114.229 bp) from *M. marinum* to the slow-growing mycobacteria *M. tuberculosis* and *M. bovis* was demonstrated (Ummels et al., 2014). Interestingly, the plasmid could not be transferred into rapid growing mycobacteria. The plasmid pRAW contains genes encoding components of a type IV-like secretion system, a relaxase gene and genes belonging to a new type VII secretion system. Both, the type IV (T4SS) and type VII secretion systems (T7SS) were shown to be involved in transfer of pRAW by using pRAW derivatives with mutations in *virB4* (part of T4SS) and in $eccC_{P1}$ (part of T7SS). Both mutations impeded conjugation, which could be restored by complementation with the intact genes (Ummels et al., 2014). These experiments confirmed conjugation and excluded transformation and transduction as mechanism of transfer.

Interestingly, the mechanism of plasmid conjugation in mycobacteria is distinct but not unrelated to conjugation of chromosomal fragments in mycobacteria, known as distributive conjugal transfer (DCT) (Gray et al., 2013). Both systems depend on components of T7SS secretion system, also known as ESX secretion system. Five ESX-systems with different functions are known in mycobacteria (ESX-1 to ESX-5) (Bitter et al., 2009). While ESX-1 is crucial for virulence of *M. tuberculosis* (Pym et al., 2003), it is also involved in conjugation in *M. smegmatis* (Coros et al., 2008; Gray et al., 2013). Conjugative recipient activity in *M. smegmatis* is provided by the ESX-4 system (Gray et al., 2016).

Conjugative activity in *M. avium* still awaits investigation. Plasmids exhibiting extended homology to the conjugative plasmid pRAW such as pTH135 have been described in MAH (Ummels et al., 2014), however the conjugation mechanisms have not been investigated.

M. avium is lacking the ESX-1 system (McNamara et al., 2012) that is required for conjugation in *M. smegmatis*. This could suggest that alternative proteins could be involved in conjugation in rapid and slow-growing mycobacteria.

Our aim is the analysis of the contribution of conjugation to genetic diversity of MAH. We therefore compared a collection of 47 MAH strains isolated from the environment and patients with respect to their ability to act as conjugative recipient and searched for genes affecting conjugation capacity of MAH strains.

2. Results

2.1. MAH strains show pronounced heterogeneity in their conjugation proficiency

Our aim was to characterize and compare the conjugation capacity of MAH from different niches or habitats. For this purpose, we performed filter matings to challenge the ability of 22 environmental isolates (11 from dust, two from water, nine from soil) and 25 clinical isolates (19 from infected lymph nodes of children, four from lungs of adults without cystic fibrosis (CF), two from lungs from CF patients) to accept the conjugative plasmid pRAW from *M. marinum* (Ummels et al., 2014). Whole genome sequences were available for all isolates (see accession numbers in Sanchini et al., 2016 and Supplementary Table S1). Our results showed that, with 22.7% (five isolates out of 22), a higher percentage of the environmental isolates were able to act as conjugative recipient and take up the pRAW plasmid, as compared to clinical isolates with only 8% (two isolates out of 25). Supplementary Table S1 of the supplementary material shows the results of the conjugation experiments for all strains. The conjugation efficiencies (transconjugant per recipient cells) were in the range from 0.1 to 0.2.

Confirmation of successful conjugation was achieved by performing PCR targeted at the pRAW-specific gene *vir*B4. Fig. 1 shows amplification of the corresponding parts of *vir*B4 in five transconjugants while the recipients did not generate PCR products.

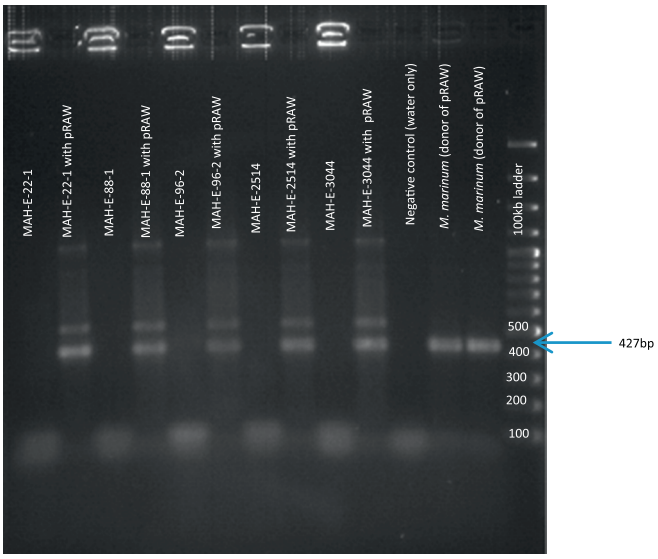

Fig. 1. Confirmation of transfer of pRAW DNA by PCR. The transfer of DNA from pRAW was confirmed by PCR using primers specific for the gene *vir*B4 from pRAW. PCR products were visualized by running a 1.5% agarose gel and staining the gel with GelRed.

2.2. Uptake of pRAW can influence the antibiotic resistance

In order to identify possible phenotypic effects resulting from the uptake of pRAW by MAH, we compared the antibiotic resistance patterns of two recipient/transconjugant pairs. To this aim we measured the minimal inhibitory concentration (MIC) for 13 antibiotics of an environmental isolate from water (MAH E-2514) and a clinical isolate (MAH P-833, lymph node isolate) with the Sensititre system. We omitted aminoglycoside antibiotics offered by the Sensititer plates (amikacin and streptomycin) from the analysis. As shown in Fig. 2 A and B, the transconjugants of both strains exhibited reduced resistance compared to the recipient strains. At least 2 fold lower MIC values in both transcojugants and all three repetitions were found for the antibiotics ciprofloxacin, linezolid and moxifloxacin. Additionally, in strain MAH P-883, the MIC values for clarithromycin, doxycycline and rifampin were also reduced by at least by a factor of two in all experiments after uptake of pRAW. No consistent influence on resistance was observed for the antibiotics ethambutol, ethionamide, isoniazid and trimethoprime/sulfamethoxazole. Overall, these results indicate that pRAW does not transfer antibiotic resistance genes, but in fact seems to increase sensitivity for some antibiotics.

2.3. Environmental MAH isolates contain more genes homologous to conjugative plasmids than clinical isolates

To estimate the presence of conjugative plasmids in our isolates, we performed blast analysis with the genome sequences of our isolates and the three conjugative mycobacterial plasmids that have been fully sequenced, i.e. pRAW (Ummels et al., 2014), pMA100 (da Saliva Rabello et al., 2012) and pMAH135 (Uchiya et al., 2015) and calculated the percentage of identical nucleotides (% of plasmid sequence covered/% identical sites in alignment $\times 100\%$). The mycobacterial plasmids pMA100 and pMAH135 were identified as the ones that exhibited the highest degree of homology to our strain collection. This analysis revealed higher frequencies of plasmid sequences in environmental isolates as compared to clinical isolates. While none of the clinical isolates reached levels of 20% or more, values of up to 58% were found in environmental isolates (Supplementary Table S2). We then performed a gene-wise homology search with all genes present in the plasmids

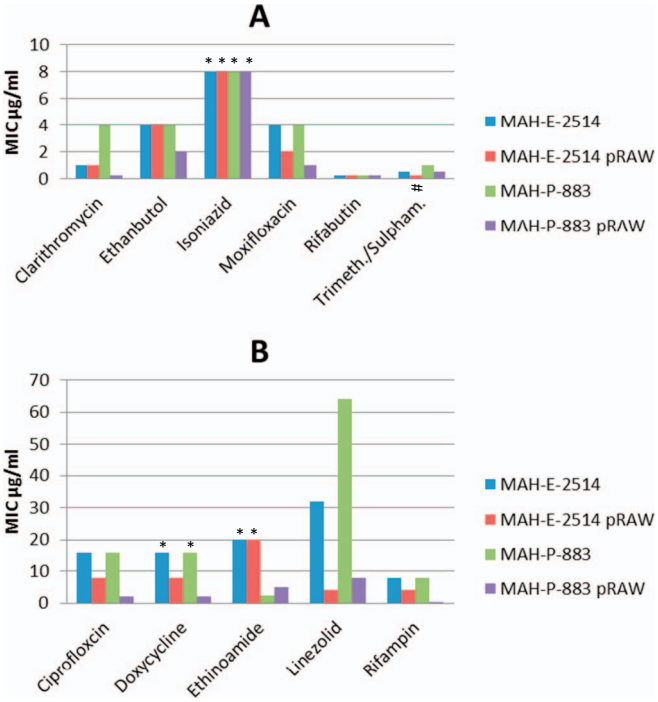

Fig. 2. Median MIC values (three independent measurements) of the strains MAH-E-2514 and MAH-P-833 with and without pRAW. Panel A shows the results for antibiotics with MICs up to 8 µg/ml, panel B shows the results for antibiotics with higher MICs. *: MICs were above the highest concentrations offered by the Sensititre plates. #: value for trimethoprime only.

pMA100 and pTH135 as well as pRAW by Blast analysis (blast N, minimal query coverage 70% and minimal identity 70%). Of the 98 genes annotated in the sequence of the pRAW plasmid (according to accession NZ_HG917973), 34 were present in at least one of the environmental isolates while only 18 were present in at least one of the clinical isolates. The percentages for all three plasmids are shown in Fig. 3 and confirm higher abundance of plasmid-related genes in environmental isolates. Details on the presence of genes in the MAH collection homologous to pRAW, pMA100 and pMAH135 genes are presented in Supplementary Tables S3, S4 and S5 in the supplementary materials. Tables S3 to S5 also illustrate that the seven isolates which had the ability to uptake pRAW were not distinguished from isolates without the ability by the possession of specific genes also present on the conjugative plasmids pRAW, pMA100 or pMAH135.

2.4. The radC gene and the neighboring type I restriction/modification system genes are differently organized in environmental and clinical isolates

Extraction of genes specific for the group of conjugating versus non-conjugating strains was achieved by comparing the gene annotations in the two groups followed by statistical correlation tests. As shown in the Supplementary Table S6, in total 58 genes were identified that were differentially represented in the two groups when applying a P value < 0.05 (hypergeometric test). As shown in Fig. 4 genes belonging to the COG categories “replication, recombination and repair” and “secondary metabolites, biosynthesis, transport and catabolism” were over-represented. The gene with the best statistical significance ($P = 0.007$) was annotated as β-glucanase, followed by two genes ($P = 0.009$) annotated as HNH endonuclease domain protein and type II restriction enzyme. One gene which is annotated as DNA binding protein *radC* was absent in all of the conjugating isolates according to the annotations. We therefore decided to investigate the structure of the corresponding genomic region in more detail and found a type I restriction/modification system located downstream of *radC*. A Blast

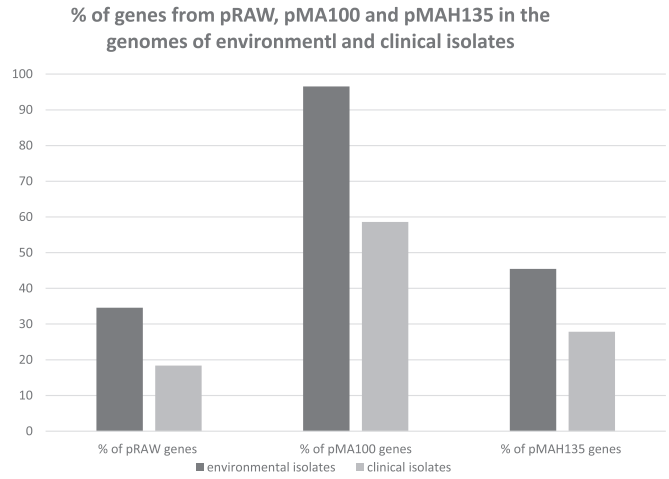

Fig. 3. Presence of genes in the collection of environmental and clinical MAH strains homologous to genes from pRAW, pMA100 and pMAH135.

analysis of a 21 kb genomic region from strain MAH 104 (accession CP00479) and all investigated isolates revealed important difference between MAH 104 and our isolates on the one hand and between clinical and environmental and isolates on the other hand (Supplementary Fig. S1). Fig. 5 demonstrates that the *radC* genes present in the environmental isolates are shorter than their clinical counterparts.

The genomic region contains seven transposase genes in two segments that are separated by two coding sequence (GntR-family transcriptional regulator CDS and Sulfatase modifying factor 1 precursor CDS). The predicted *radC* gene in the different strains are of different sizes ranging from 201 to 405 nucleotides. Downstream from *radC,* a type I restriction/modification system with the three subunits (RMS) is present. The S subunit gene overlaps by four nucleotides with *radC* gene. It was striking that, none of the conjugating isolates contained both, a full-length *radC* together with a complete type I restriction/modification system.

3. Material and methods

3.1. Bacterial strains and culture conditions

The characteristics of bacterial isolates and the plasmids that were analyzed in this study are listed in Table 1 and Table 2. All patient isolates were isolated from either respiratory samples or lymph nodes from diseased persons. The patient isolates were either obtained from the National Reference Center for Mycobacteria (Borstel, Germany) or isolated from sputum from patients with Cystic Fibrosis. The environmental isolates were isolated from soil samples, dust samples (from vacuum cleaners) or water samples and either isolated as described in Lahiri et al., 2014 or provided by Roland Schulze-Röbbecke (University Hospital Düsseldorf, Germany) or Petra Möbius (Friedrich Löffler Institute, Jena, Germany). *M. marinum* and MAH were grown in Middlebrook 7H9 liquid medium (BD Biosciences) along with 0.05% Tween 80 and supplemented with 10% modified ADC (2% glucose, 5% BSA. 0.85% NaCl) without shaking or on Middlebrook 7H11 agar supplemented with 10% modified ADC and 0.5% glycerol and incubated at 30 °C for *M. marinum* or 37 °C for MAH.

3.2. Conjugation experiments

The donor *M. marinum* (kanamycin-resistant mutant $M^{USA}4$ from strain *M. marinum* M^{USA} (pSMT3 dsRed) (pRAW) and the recipients MAH were freshly grown to an optical density at 600 nm (OD 600 nm) between 1.5 and 2. Cells of 1 ml of culture were harvested by centrifugation (3000 rpm/5 min) and resuspended in 1 ml of 7H9 broth

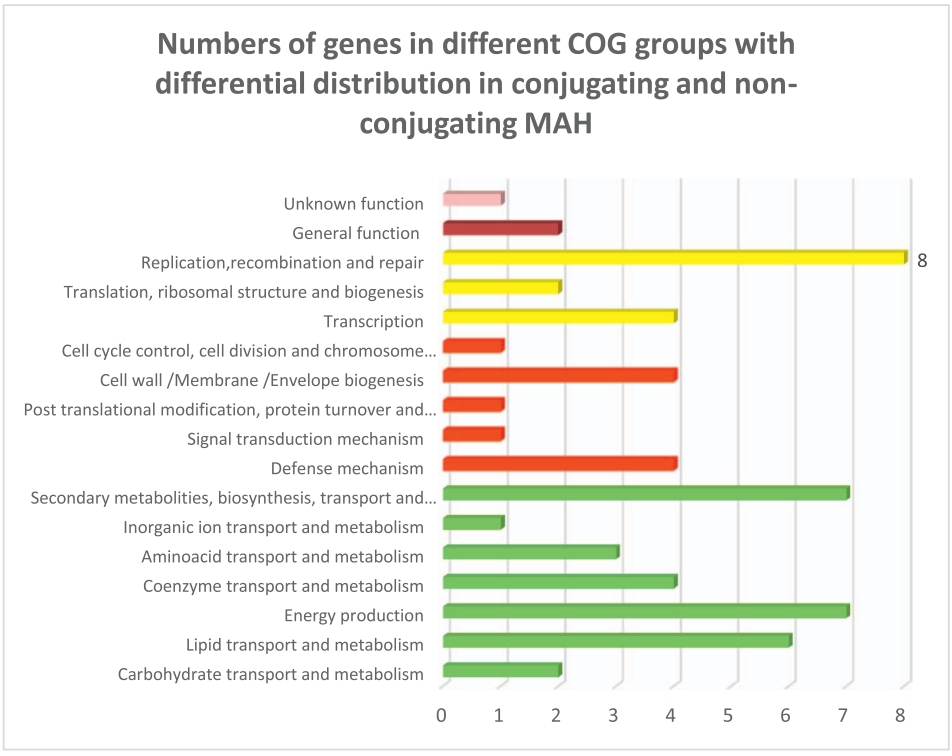

Fig. 4. COG function of annotated genes that were differently distributed in the groups of conjugating versus non-conjugating isolates.

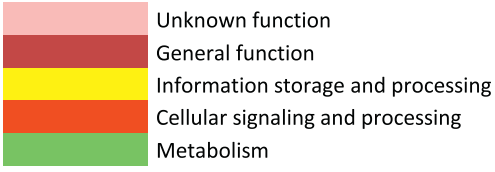

Fig. 5. Difference in *radC* gene size in the environmental versus clinical isolates. The nucleotid alignment of *radC* in clinical isolates (numbers 1–16) and environmental isolates (numbers 17–31) is shown. The small blue rectangle in sequence number 20 indicates a T to C base exchange. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Characteristics of bacterial strains and isolates:

Name of strain/isolate	Source[a]	Reference
M. marinum M^{USA} (pSM128/lacZ)		Ummels et al., 2014
M. marinum M^{USA} (pSMT3 dsRed) (pRAW), mutant $M^{USA}4$		Ummels et al., 2014
MAH-E-82-7	dust	Sanchini et al., 2016
MAH-E-83-1	dust	Sanchini et al., 2016
MAH-E-88-1	dust	Sanchini et al., 2016
MAH-E-89-1	dust	Sanchini et al., 2016
MAH-E-27-1	dust	Sanchini et al., 2016
MAH-E-57-3	dust	Sanchini et al., 2016
MAH-E-61-1	dust	Sanchini et al., 2016
MAH-E-63-1	dust	Sanchini et al., 2016
MAH-E-101-6	dust	Sanchini et al., 2016
MAH-E-104-1	dust	Sanchini et al., 2016
MAH-E-108	dust	Sanchini et al., 2016
MAH-E-128	soil	Sanchini et al., 2016
MAH-E-14	soil	This study
MAH-E-22-1	soil	Sanchini et al., 2016
MAH-E-96-2	soil	Sanchini et al., 2016
MAH-E-106	soil	Sanchini et al., 2016
MAH-E-118	soil	Sanchini et al., 2016
MAH-E-149-2	soil	Sanchini et al., 2016
MAH-E-149-3	soil	Sanchini et al., 2016
MAH-E-149-7	soil	Sanchini et al., 2016
MAH-E-2514	water	Sanchini et al., 2016
MAH-E-3044	water	Sanchini et al., 2016
MAH-P-11082/03	human lymph node	Sanchini et al., 2016
MAH-P-1620/04	human lymph node	This study
MAH-P-10091/06	human lymph node	Sanchini et al., 2016
MAH-P-10203/06	human lymph node	Sanchini et al., 2016
MAH-P-2721/04	human lymph node	Sanchini et al., 2016
MAH-P-9036/04	human lymph node	This study
MAH-P-528/08	human lymph node	Sanchini et al., 2016
MAH-P-589/08	human lymph node	Sanchini et al., 2016
MAH-P-709/08	human lymph node	Sanchini et al., 2016
MAH-P-772/08	human lymph node	Sanchini et al., 2016
MAH-P-2014/08	human lymph node	Sanchini et al., 2016
MAH-P-2630/08	human lymph node	This study
MAH-P-3269/08	human lymph node	This study
MAH-P-3449/08	human lymph node	Sanchini et al., 2016
MAH-P-3646/08	human lymph node	This study
MAH-P-4023/08	human lymph node	Sanchini et al., 2016
MAH-P-4557/08	human lymph node	This study
MAH-P-883	human lymph node	Sanchini et al., 2016
MAH-P-7673/04	human lymph node	Sanchini et al., 2016
MAH-P-9060/06	human lung, no CF	Sanchini et al., 2016
MAH-P-10058/06	human lung, no CF	Sanchini et al., 2016
MAH-P-9268/06	human lung, no CF	This study
MAH-P-8933/06	human lung, no CF	Sanchini et al., 2016
MAH-P-04/13	human lung, CF	This study
MAH-P-09/13	human lung, CF	This study

[a] CF: Cystic Fibrosis.

Table 2
Description of the used plasmids

Plasmid	Accession number	source	Size	No. of annotated genes
pRAW	NZ_HG17973	*M. marinum* E11	114.229 bp	98
pMA100	KR997898	MAH strain 88Br	116.415 bp	116
pMAH135	AP012556	MAH TH135	194.711 bp	165

without Tween 80. Then the mixed suspension as well as pure unmixed suspension of donor and recipient cells were pressed through 0.2 µm cellulose acetate filters in syringe filter holders (Sartorius, Stedim Biotech, Germany), placed on 7H11 agar supplemented with 10% modified ADC and 0.5% glycerol and incubated at 30 °C for 48 h to allow growth of both recipient and donor species. The bacteria were washed from the filters and suspended in 2 ml of 7H9 broth supplemented with 0.05% Tween 80 and 10% modified ADC. Different dilutions of the suspensions were plated on 7H11 agar plates with kanamycin (25 µg/ml) and incubated for 10–14 days at 37 °C to allow growth of the transconjugants only. The suspensions from the filters with both recipients and donor were also plated on agar plates without kanamycin and incubated at 37 °C to be able to calculate conjugation efficiencies. Ability of MAH wild type recipients to grow on plates with kanamycin concentrations used for selection was tested beforehand. All conjugations were repeated three times.

3.3. PCR

Confirmation of transfer of plasmid pRAW was obtained by PCR using boiled cultures (15 µl) and primers specific for the gene *vir*B4 (see Table 3 for description of primers) and the DreamTaq PCR kit from Thermo Fischer Scientific according to the manufacturer's recommendations. Gel products were visualized in 1.5% agarose gels stained with GelRed (Biotrend).

Table 3
Description of the used primers

Primer name	Sequence	Target	Product size	Reference
*vir*B4 Fw	GGCATAGGATTTGCCGTATC	virB4 from pRAW	427 bp	Ummels et al., 2014
*vir*B4 Rev	AGCTTGAAGCCAACATCGAC			

3.4. Antibiotic resistance testing

MIC concentrations were recorded by using the Sensititre TM SLOMYCOI plates (Thermo Scientific) according to the manufacturer's instructions. The growth of the mycobacteria was evaluated after ten days.

3.5. Bioinformatics analysis

Nucleotide sequence homologies were determined by NCBI Blast analysis or by using Geneious 10.0.5 software program. Contigs were annotated using RAST (Aziz et al., 2008). Coding sequences exhibiting at least 70% query coverage and at least 70% identity were considered to represent same genes. Statistical analysis for probability distribution was done by Hypergeometric test (significance level: $P < 0.05$). The Clusters of Orthologous Groups (COG) category of genes was assigned by screening each gene in a COG database (Huerta-Cepas et al., 2016). For those hypothetical proteins which we do not have assigned functions in COG we applied protein modelling by using the I-TASSER server (Roy et al., 2010) and protein putative function prediction using the DALI server (Holm and Rosenström, 2010).

4. Discussion

An outstanding feature of MAH is its high degree of genetic diversity. Our previous study had shown the presence of highly heterogeneous genomic islands (GIs) in MAH that had resulted from horizontal gene transfer events (Sanchini et al., 2016). Our aim was to find explanations for this genetic diversity and we therefore compared our strain collection that is composed of isolates from different sources with respect to their ability to take up conjugative plasmids. In many bacterial species, plasmid conjugation is an important mediator of horizontal gene transfer. However in mycobacteria, conjugative plasmids have only rarely been reported (da Silva Rabello et al., 2012; Ummels et al., 2014; Uchiya et al., 2015). Interestingly, the slow-growing and highly pathogenic *M. tuberculosis* so far was not reported to contain natural conjugative plasmids, although in an experimental setting the conjugative plasmid pRAW from *M. marinum* could be transferred to *M. tuberculosis* by conjugation (Ummels et al., 2014). The conjugation proficiency of MAH that also belongs to the slow growing Mycobacteria is until now not known and was investigated in the present study by measuring the conjugative recipient activity of a collection of 22 environmental and 25 clinical MAH isolates from Germany during incubation with *M. marinum* containing the conjugative plasmid pRAW (Ummels et al., 2014). The plasmid pRAW was selected for these experiments because its transfer to other slow-growing mycobacteria such as *M. tuberculosis* and *M. bovis* had been shown before and this plasmid contains a selectable marker (Ummels et al., 2014).

Suprisingly, the ability to take up the plasmid pRAW strongly varied between the tested isolates. The plasmid pRAW was transferred to only 14.89% of the isolates, with differing percentages in environmental (22.7%) and clinical (8%) isolates.

In *M. smegmatis,* the Esx-1 and Esx-4 secretion systems were identified as being essential for conjugative plasmid uptake (Gray et al., 2013). While *M. tuberculosis* as well as *M. smegmatis* contains both secretion systems, ESX-1 and ESX-4, MAH is lacking ESX-1. Contrary to *M. smegmatis,* it contains the secretion systems ESX-2 and ESX-5. A plasmid-type version of ESX-5 is present on the conjugative plasmid pRAW and might be involved in conjugation.

As plasmid incompatibility can be an exclusion factor for conjugation we searched for sequences in the investigated isolates homologous to pRAW, pMA100 (da Silva Rabello et al., 2012) and pMAH153 (Uchiya et al., 2015). The latter two plasmids were chosen because they are similar to pRAW and were shown to be conjugative (da Silva Rabello et al., 2012, Uchiya et al., 2015, Ummels et al., 2014). The presence or absence of plasmid genes in the strain collection did not correlate with ability to conjugate. It was, however, very surprising that the amount of plasmid genes was by far higher in the environmental isolates compared to clinical ones.

The relatively high number of plasmid genes in some of the recipients is also of interest regarding surface/entry exclusion (Garcillán-Barcia and de la Cruz, 2008). This together with the absence of TraT (involved in surface exclusion) and TraS (involved in entry exclusion) on the plasmid pRAW or the pRAW-like plasmids pMAH135 and pMA100 argues against a role of surface/entry exclusion in transfer of pRAW-like plasmids. This was further supported by the transfer of pRAW into MAH TH135 (data not shown), which contains the plasmid pMAH135 carrying the same T4SS and T7SS as pRAW.

A major finding of this study is the demonstration of different distribution of genes homologous to genes from conjugative plasmids in clinical compared to environmental isolates. Differing genetic structure of environmental versus clinical isolates was until now not reported and it challenges the basic assumption that the infection source of MAH is environmental and that transmission does only exceptionally occur between patients. Either different lineages of MAH exist that prefer different habitats – environmental or host tissues – or plasmids are disadvantageous during infection and as a consequence lost. The conjugative plasmids analyzed in this study are large plasmids with sizes of 114.229 bp (pRAW), 116.415 bp (pMA100) and 194.711 bp (pMAH135). *M. avium* genomes have sizes of around 5 Mb. Uptake of the above-mentioned plasmids thus increases genome size by around 2% to 4%. In case of copy numbers of more than one the amount of additional DNA would further increase. The carriage of such huge plasmids exerts a considerable physiologic burder for host cells (for plasmid replication, repair, transcription, translation). Calculation of the fitness cost of newly acquired plasmids of 80–200 kb in *Enterococcus* strains demonstrated a reduction in host finess of around 25% (Starikova et al., 2013). Similarly, uptake of the megaplasmid pMPPla107 by certain *Pseudomonas syringae* strains induces a fitness reduction of 20% (Romanchuk et al., 2014). The cost of plasmid carriage may therefore favor loss of this type of plasmids in case of stress condition such as for example nutrient limitation in infection conditions. It is an interesting aspect that *M. tuberculosis*, which has adapted to the host environment by evolutionary genome reduction, in general also is devoid of conjugative plasmids (Bachrach et al., 2000; Stinear et al., 2004). In this study we discovered another possible reason for the lack of plasmids in clinical *M. avium*, namely the increased antibiotic susceptibility of the pRAW transconjugants as compared to the plasmid-free strains, which was observed for antibiotics acting on replication, transcription and translation but not those acting on the cell wall synthesis. The clinical isolates more probably were subject to antibiotic exposure than environmental isolates, and adverse effects of this type of conjugative plasmid on antibiotic resistance may be one of the reasons for less frequent occurrence of plasmid-derived genes in the clinical isolates. Another possible reason for reduced presence of genes occurring in conjugative plasmids in the clinical isolates may be the immune response of the host, which can be directed against pilus proteins. Pilus components can be highly immunogenic, as was shown for example for pili from *Burkholderia mallei* (Fernandes et al., 2007). However, this still does not explain why clinical isolates were less active in conjugation in the filter mating experiments.

In order to identify genes influencing the conjugative recipient

capacity, we compared the gene annotations in whole genome sequences in the group of all conjugating isolates versus the group of all non-conjugating isolates. 58 genes were found to be differentially distributed in these two groups. The statistically most significant were genes encoding a beta-glucanase, a HNH endonuclease domain protein and a Type II restriction enzyme (*Pae*R7I).

One gene, *radC*, was found to be present in none of the conjugating isolates and exhibited significant differential distribution in the two groups. RadC was annotated as DNA repair protein based on studies performed with *E. coli* mutants (Felzenszwalb et al., 1984). However, Attaiech et al. (2008) experimentally challenged the DNA repair function of the protein from *Streptococcus pneumoniae*. Their results did not support any DNA repair function of RadC and they concluded the name to be misleading. Bioinformatic function analysis of RadC from MAH classifies it as a DNA-binding protein. The *radC* gene in MAH is located downstream and in opposite orientation of three genes (specificity subunit, modification subunit, restriction subunit) encoding a type I restriction/modification system and overlaps by 4 bp with the gene for the specificity subunit. This genetic organization points to a possible role of RadC in the regulation of expression of the type I restriction system.

When comparing the region containing *radC* in our strain collection, we found striking heterogeneity among the isolates together with the presence of several mobile elements (transposons). One of these transposons is located adjacent to the *radC* gene and may be responsible for the variations of the structure of this gene in the strain collection, which was either present in a full length version (405 bp), a shorter version (381 bp to 207 bp) or even absent. Also the type I restriction enzyme encoding genes were differently organized in the isolates. It was striking that none of the isolates acting as conjugative recipient contained both a complete *radC* gene together with a complete type I restriction/modification system suggesting a role of this type I restriction/modification system in persistence of the transferred DNA in the recipients. However, absence of the full-length *radC*/type I restriction/modification system cannot be the only determinant of conjugation capacity, since some strains were not able to take up pRAW although they did not contain this region at all. We conclude that conjugative recipient activity of MAH is multifactorial and propose the genomic region containing the *radC*/type I restriction/modification system to contribute to conjugation activity. An impact of type I restriction/modification systems on the conjugation efficiency has also been shown in other bacteria such as *Escherichia coli* (Roer et al., 2015).

Moreover, the group of environmental versus clinical isolates differed with respect to the organization of the genomic region containing the *radC*/type I restriction/modification system. While 56% (14 out of 25) of the patient isolates contained a full-length *radC* gene, only 4.5% (1 out of 22) of the environmental isolates did so and only one environmental isolate contained a complete *radC*/type I restriction/modification system. The diversity in this genomic region was probably driven by transposons located near *radC*. Genetic variation in this genomic region may reflect evolutionary events that created MAH branches adapted to conditions present either in the environmeor in host tissue.

5. Conclusion

We conclude from our study that conjugation in MAH is strain-dependent and multifactorial and propose that the *radC*/type I restriction/modification system contributes to conjugation efficiency. Our future work will concentrate on confirming a role of this region in conjugation by investigating the effect on conjugation of mutagenesis and recombinant expression of these genes in MAH. Furthermore, our study for the first time reveals differences in the genetic structure between environmental and clinical MAH, which may in the future help to define infection sources and identify markers differentiating clinical from environmental MAH.

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