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REVISED VERSION

Beyond phenotype: The genomic heterogeneity of co-infecting *Mycobacterium abscessus* smooth and rough colony variants in cystic fibrosis patients

Running title: Heterogeneity in *M. abscessus* infection

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22 Number of Tables: 1

23

24 **Highlights**

25 - *Mycobacterium abscessus* is an opportunistic pathogen in cystic fibrosis patients
26 - Two unrelated CF patients were colonized with two rough/smooth colony variants
27 - WGS was used to identify the heterogenic isolates and determine resistance profile
28 - We identify novel SNPs in one patient that could be involved in S to R morphology
29 - Unexpectedly, one rough *M. abscessus* clone that did not lose glycopeptidolipids

30

Summary

Two unrelated cystic fibrosis patients were co-infected with *Mycobacterium abscessus* smooth and rough phenotypes. Smooth *M. abscessus* is proposed as the infecting form, and the subsequent loss of glycopeptidolipids in the host leads to a rough phenotype. Whole-genome sequencing (WGS) diagnosed two different *M. abscessus* strains in patient N°1 but only one strain in patient N°2. In patient N°1, rough isolate had novel mutations potentially involved in smooth-to-rough morphology changes. In patient N°2, four genes were present in only the smooth isolate. In addition, we obtained different susceptibility profiles in the four clinical isolates. We revealed a new paradigm describing a cystic fibrosis patient infected with two different clones, including a rough isolate, and identifying a rough *M. abscessus* clone that did not lose glycopeptidolipids. We propose WGS for the identification of heterogenic isolates and genetic determinants of antimicrobial resistance, which we believe will positively influence treatment prognosis.

Key words: *Mycobacterium abscessus;* cystic fibrosis; smooth variant; rough variant; Whole genome sequencing

48 **1. INTRODUCTION**

49 *Mycobacterium abscessus* (*Mabs*) has emerged as one of the most difficult-to-
50 manage opportunistic pathogen responsible for pulmonary infections, especially in
51 cystic fibrosis (CF) patients [1]. *Mabs* exhibits smooth or rough colony morphotypes
52 characterized by the presence or absence of surface-associated glycopeptidolipids
53 (GPL), respectively. Within the GPL locus in *Mabs* (CIP104536T), the *MAB_4099c*
54 (*msp1*), *MAB_4098c* (*msp2*), *MAB_4097c* (*gap*), *MAB_4117c* (*mmpS4*), *MAB_4116c*
55 (*mmpL4a*) and *MAB_4115c* (*mmpL4b*) genes [2] have shown to play an important
56 role in the GPL production. Indels and SNPs in this locus have also been related to
57 the loss of production of GPL leading to rough morphotype in clinical isolates [3]. The
58 rough morphotype is associated with higher virulence and persistent infections [4] .
59 Previous studies have shown a low frequency of this mixed phenotype in *Mabs*
60 pulmonary infections and more recently seems to become a new concern due to
61 different resistance profiles between isolates [5,6]

62 Here, we describe two cases of respiratory tract co-infection with *Mabs*
63 smooth and rough phenotypes, in two unrelated CF patients. We used whole
64 genome sequencing (WGS) to precisely identify the isolates and describe potential
65 genetic differences associated with the clonality of smooth-to-rough transition.

66

67 **2. LABORATORY INVESTIGATIONS**

68 **2.1. *Mabs* isolates.**

69 A sputum sample collected in a 34-year-old CF patient N°1 yielded *Mabs* rough
70 colonies (P9527) and smooth colonies (P9528) (Figure 1; Supplementary Files). Both
71 isolates exhibited the same antibiotic susceptibility profile except for clarithromycin-

72 resistance in P9527 and clarithromycin-susceptibility in P9528 (Table 1). Further, a
73 sputum sample collected in a 25-year-old CF patient N°2, yielded *Mabs* rough
74 colonies (P9529) and smooth colonies (P9530) (Figure 1). P9529 exhibited a high-
75 resistant level to clarithromycin whereas we observed an inducible resistance to
76 clarithromycin at day 14 in P9530 isolate (Table 1).

77 **2.2. Lipid analysis.**

78 Apolar lipids were extracted from smooth and rough colonies of reference *Mabs*
79 CIP104536^T and from our four isolates and analysed by thin-layer chromatography
80 (TLC) as previously described [3]. Rough P9527 isolate lacked bands corresponding
81 to GPL whereas this lipid was surprisingly intact in rough P9529 isolate (Figure 2).

82 **2.3. WGS bioinformatic analysis.**

83 Illumina MiSeq sequences (Illumina Inc., San Diego, USA) were obtained in a paired-
84 end strategy, assembled using Spades [8] and annotated using Prokka [9].Genome
85 sequences have been deposited into the EMI database under the following
86 accession numbers: P9527 (ERS3527803), P9528 (ERS3527804), P9529
87 (ERS3527805), and P9530 (ERS3527806).The Roary pipeline
88 (https://usegalaxy.org.au./) generated pangenome using default parameters. *In silico*
89 DNA-DNA hybridization yielded 94·6% identity between P9527 and P9528
90 suggesting that patient N°1 was infected by two different *Mabs* clones; and 99·8%
91 identity between P9529 and P9530 suggesting that patient N°2 may be infected by
92 one *Mabs* clone (Table S1). We calculated the single-nucleotide polymorphisms
93 (SNPs) distance of the four genomes identified 21,825/4,162,736-bp SNPs between
94 P9527 and P9528 but 1,313/4,162,736-bp between P9529 and P9530. In order to
95 genotypically associate the possible smooth-to-rough conversion in patient N°2,

96 SNPs were identified manually in the GPL locus using MUSCLE in Unipro UGENE
97 v1·32·0 [10] (Supplementary Files). Although no indels were detected, a comparison
98 between the smooth P9530 and rough P9529 strain identified a discrete amount of
99 nonsynonymous SNP (Table S2) including *mmpL4b* (A212C), *mps1* (C2369G), *mps2*
100 (A1400G, C1534G, G2041C, G6194T, G7534C) and *gap* (G11A, G50T). Using
101 Phandango online tool [9], four genes were found only in smooth isolates (Figure
102 S1). Nucleotide BLAST alignment showed the following: (1) a hypothetical protein
103 presenting a partial match with *MAB_3888* and corresponding to a putative RNA
104 polymerase sigma-70 factor, (2) a PE family immunomodulator, PE5, having a
105 complete match with *MAB_0046*, (3) a putative PPE family protein, PPE2, showing a
106 complete match with *MAB_0809c* and (4) a hypothetical protein presenting a partial
107 match with *MAB_4691c* and corresponding to a probable non-ribosomal peptide
108 synthetase PstA (Table S3). Further analysis was performed in another promising
109 area of the pangenome showing a differential pattern between the smooth and rough
110 morphotypes, however any genetic trait linked to a specific phenotype was observed.

111

112 **2.4. Genotypic characterization of *erm (41)* and *rrl* genes.**

113 None of the four clinical isolates had mutations at positions 2058 and 2059 in the *rrl*
114 gene [12]. As for the *erm(41)* gene [13,14], only P9528, which showed a
115 clarithromycin-sensitive phenotype, carried a non-functional Erm(41) (C28 sequevar),
116 while the three other isolates clarithromycin-resistant after 14-day incubation, were
117 identified as T28 sequevar. An additional mutation (V80I) found in all isolates (Table
118 S5), has been previously associated with clarithromycin resistance in Korea [10].

119 **3. DISCUSSION**

120 Smooth/rough mixed pulmonary infection are rarely reported in *Mabs* and *M. bolletii*
121 and never for *M. massiliense* [5]. In two CF patients here reported, *Mabs*
122 rough/smooth colony variants were firmly identified by WGS analysis.

123 Smooth non-cording and rough cording morphotype differences rely on the
124 presence/absence of GPL, respectively [12]. In *M. abscessus,* the *msp1, msp2, gap,*
125 *mmpS4, mmpL4a* and *mmpL4b* genes drive GPL production: A CG insertion in *msp1*
126 arrests transcription of the *msp1-msp2-gap* operon [6]. Additional indels (insertions
127 and deletions) and SNPs in *msp1, msp2, gap, mmpS4, mmpL4a* and *mmpL4b* also
128 induce loss of production of GPL and a rough morphotype in clinical isolates [7].
129 Although these previously reported insertions were not found in P9527, this isolate
130 however harboured multiple novel SNPs potentially linked to GPL loss and rough
131 morphology. Moreover, a recent report explained the effect of aminoglycosides in the
132 smooth-to-rough transition in *Mabs* without depletion of GPL, cells exposed to
133 subinhibitory concentrations of amikacin showed a rough phenotype and an
134 increased expression of *MAB_3508c*, homologous to *M. tuberculosis whiB7*
135 responsible of mediate antibiotic resistance [13]. Patient N°2 was treated with
136 tobramycin, this may have influence in the smooth-to-rough transition of P9530 strain
137 which did not present any change in the GPL profile. A second GPL-like locus of two
138 non-ribosomal peptide synthase *pst*A genes (*MAB_4690c-MAB_4691c*) similar to
139 *mps1* and *mps2*, may be implicated in GPL production and the smooth-to-rough
140 transition [6]. Here, we identified four genes specific for the smooth clinical isolates,
141 one of them encoding a hypothetical protein homologous to MAB_4691c, opening the
142 possibility of new markers for the smooth-to-rough transition.

143 Our genetic analysis suggests colonization by two distinct strains in one patient,
144 while in the second patient, both the smooth and rough strains were closely related,

145 suggesting morphology conversion. It has been proposed that the smooth-to-rough
146 conversion occurs in the host by clonal diversification. In a follow-up study in patients
147 chronically infected with *Mabs*, initial isolation of the smooth morphotype and later
148 isolation of the rough morphotype was observed [7]; nevertheless, whether this event
149 could be due to initial colonization with smooth morphotype and a consequent re-
150 colonization with a secondary rough strain is not being considered. Despite having
151 multiple *ex vivo* and *in vivo* reports that relate the severity of the infection to
152 morphology [14], it is still not clear how this change from a smooth to rough
153 morphology could affect a patient's prognosis.

154 *Mabs* is a difficult-to-treat opportunistic pathogen and clarithromycin has been
155 used in monotherapy before description of inducible resistance to macrolides [14]. In
156 addition to acquired macrolide resistance conferred by mutated *rrl* gene encoding the
157 peptidyltransferase domain of the 23S rRNA, inducible resistance to macrolides
158 attributed to *erm(41)* was described; this methylase is proposed to monomethylate
159 adenine at position 2058 in the 23S rRNA, conferring resistance [15]. Loss of function
160 of *erm(41)* is linked to a clarithromycin-sensitive phenotype, as described for the
161 T28C mutation [16]. Here, we describe a patient coinfected with two different *Mabs*
162 strains, P9527/P9528, each genotype concordant with the clarithromycin-
163 resistant/sensitive phenotype, respectively. This diversification can have implications
164 on the susceptibility profile, increasing the difficulty of selecting the proper therapy,
165 which could impact the disease outcome. Another unexplored possibility is co-
166 infection by multiple *M. abscessus* complex strains without differences in colony
167 morphology that go unnoticed.

168 We propose systematic WGS to guarantee the identification of different *Mabs*
169 isolates and their antibiotic susceptibility profile, to guide the most appropriate
170 treatment in CF patients.

171 Previous studies using *ex vivo* and *in vivo* models suggested that rough morphotype
172 exhibited increased virulence as compared to smooth morphotype,[8,9] yet these
173 experimental observations poorly translated in clinical observations and the actual
174 characterization of clinical isolates. Such clinical isolates have not been genome
175 sequenced in order to more deeply interpret phenotypic data. Future studies could
176 focus on the influence of Whib7 on phenotype and antibiotic resistance.

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183 **Transparency declaration**

184 MD is a co-founder and shareholder of the start-up Culture-Top that is cited in this
185 manuscript. The other authors declare that they have no competing interests.

186 **Authors' contributions**

187 SB and MDr designed the study and drafted and revised the manuscript.

188 MRG performed medical examinations.

189 AVG, FSS, and JS performed microbiology analyses.

190 AVG performed SNP identification on the GPL locus and *erm(41)* and *rrl* genes,

191 performed the lipid extraction and analysis, and wrote the paper.

192 JS performed the WGS and analyses.

193 All authors have read and approved the final manuscript.

194

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266 Table 1. Results of broth microdilution susceptibility testing of the two morphotypes of
267 each species. Minimum Inhibitory Concentration were interpreted using CLSI M24-2
268 recommendations.

	P9527	**P9528**	**P9529**	**P9530**
Amikacin	16 (R)	32 (R)	16 (R)	32 (R)
Clarithromycin (D4)	16 (R)	0.25 (S)	16 (R)	0.5 (S)
Clarithromycin (D14)	>256 (R)	0.5 (S)	>256 (R)	4 (I)
Ofloxacin	16 (R)	16 (R)	8 (R)	>16 (R)
Bactrim	>16 (R)	>16 (R)	>16/304 (R)	32 (R)
Tigecycline	1 (S)	1 (S)	1 (S)	<1 (S)
Doxycycline	>16 (R)	>16 (R)	>16 (R)	>16 (R)

269 S, susceptible; R, resistant; I, intermediate. The results are expressed in $\mu g \cdot mL^{-1}$.

270

271 **Figure legends:**

272 **Figure 1.** Morphologic appearance of four clinical isolates of *M. abscessus* and
273 rough (R) and smooth (S) reference strains after 4-day incubation on Middlebrook
274 7H10 agar at 37°C.

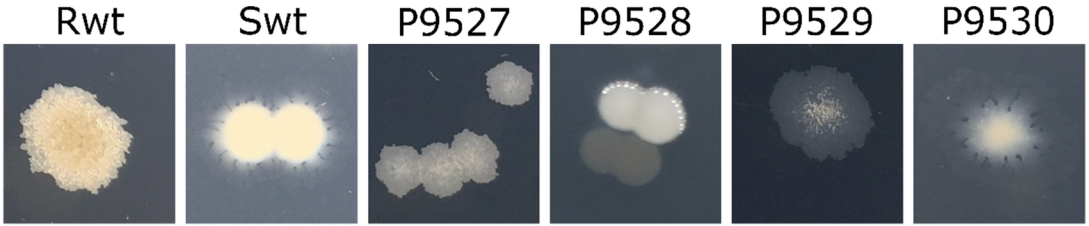
275

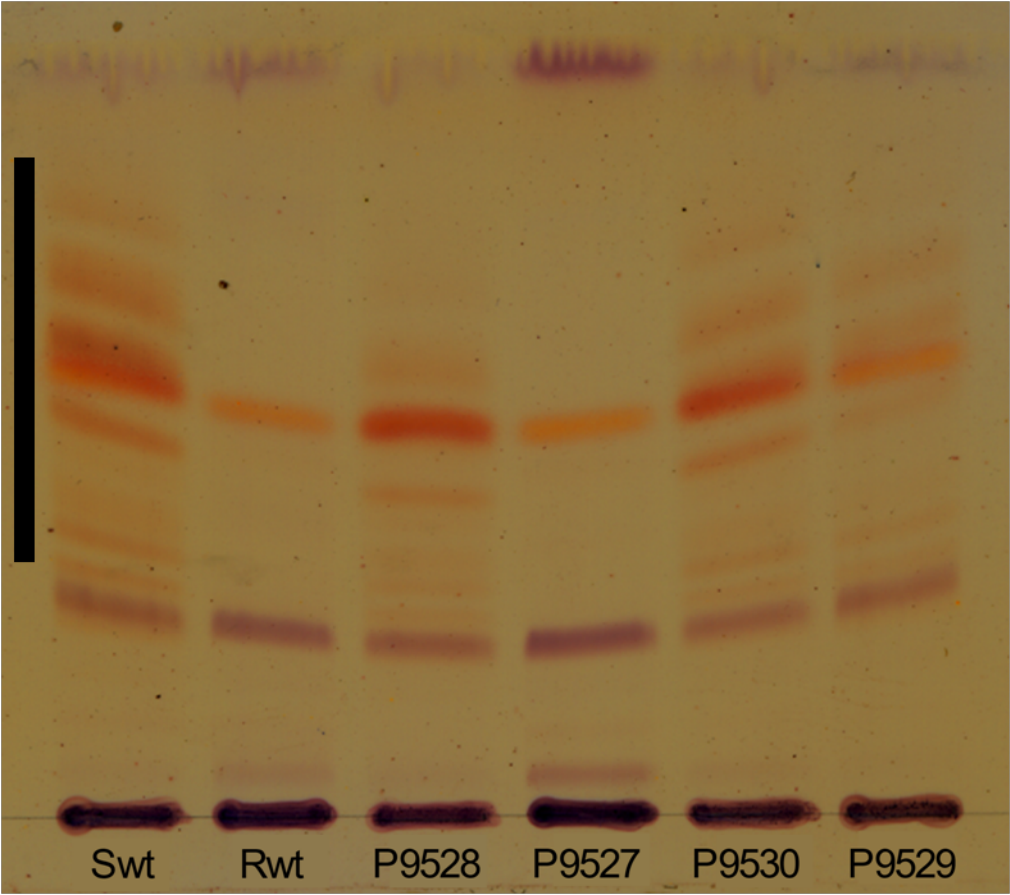
276 **Figure 2.** Lipid profile of four *M. abscessus* isolates, showing the P9527 strain as the
277 only one with a deficient glycopeptidolipid (GPL) profile. Equal weights of total lipid
278 extracts from the *M. abscessus* smooth (Swt), rough (Rwt), P9527, P9528, P9529
279 and P9530 strains were analysed by thin-layer chromatography using
280 chloroform/methanol/water (9:10:1, v/v/v). GPLs are indicated with a black box.

281

282

283


Rwt
Swt
P9527
P9528
P9529
P9530


Swt
Rwt
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P9529