Supporting Information

Cell surface remodeling of *Mycobacterium abscessus* under cystic fibrosis airway growth conditions

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Text S1: Methods

Table S1: Monosaccharidic composition of total extractable lipids (A) and delipidated cells (B) prepared from *Mmas* 1239, *Mmas* 184, *Mmas* 604 and *Mabs* 1091 grown in 7H9-ADC-Tween-80, SCFM2 complete medium or SCFM2 devoid of mucin.

Table S2A-G **[see separate Excel file]**: RNA-seq dataset of differentially expressed genes in all comparisons.

A. Differentially expressed *Mmas* 1239 genes in SCFM2 vs 7H9-ADC-Tween 80
B. Differentially expressed *Mmas* 184 genes in SCFM2 vs 7H9-ADC-Tween 80
C. Differentially expressed *Mmas* 604 genes in SCFM2 vs 7H9-ADC-Tween 80
D. Differentially expressed *Mabs* 1091 genes in SCFM2 vs 7H9-ADC-Tween 80
E. Differentially expressed *Mmas* 1239 genes in SCFM2 vs 20% CF sputum

F. Comparison of the transcriptional response of *Mmas* 1239 in CF sputum versus SCFM2. Differentially expressed genes in *Mmas* 1239 grown in CF sputum and SCFM2 compared to *Mmas* 1239 cells grown in 7H9-ADC-Tween 80 were defined as ≥2 Log_2-fold change in expression, with a false discovery rate adjusted *p*-value <0.05. Similarly colored genes denote gene clusters.
G. *Mmas* 1239 genes expressed at higher or lower levels in CF sputum compared to SCFM2 (relative to 7H9-ADC-Tween 80). Genes showing opposite expression trends in SCFM2 and CF sputum relative to 7H9-ADC-Tween 80 are highlighted in red. Expressed at a significantly higher level in SCFM2 compared to CF sputum was the cluster of genes of unknown function encompassing *MAB_4742c* to *MAB_4748c* which was one of the most strongly upregulated in *Mmas* 1239 grown in SCFM2 [see Table 1 and Figure S5].

Table S3: MIC values against a diversity of drug-susceptible and drug-resistant *Mabs* and *Mmas* clinical isolates in cation-adjusted Mueller Hinton II broth and SCFM2 devoid of mucin.

Figure S1: Growth characteristics of isolates *Mmas* 184 and *Mmas* 604 in 7H9-ADC-Tween-80 and SCFM2 at 37°C.

Figure S2: Thin-layer chromatography analysis of GPLs produced by *Mmas* 1239, *Mmas* 184, *Mabs* 1091 and *Mmas* 604.

Figure S3: Amino alcohol content of GPLs produced by *Mmas* CIP 108297.

Figure S4: Extractable lipid weight and mycolic acid analysis from MABSC isolates grown in 7H9-ADC-Tween-80, SCFM2 complete medium and/or SCFM2 without mucin.

Figure S5: Transcriptional profiling of *Mmas* 1239 grown in different media.

Figure S6: Gene replacement at the *mmpL4b* and *BCKADH* loci of *Mmas* CIP 108297.

Text S1: Methods

Bacterial strains - Reference strains *Mabs* ATCC 19977 and *Mmas* CIP 108297 were obtained from the ATCC and CIP collections, respectively. *Mabs* 1091, a rough clustered isolate; *Mmas* 184, a smooth non-clustered isolate; and *Mmas* 1239, a smooth clustered isolate were clinical isolates from the UK. *Mmas* 604, a smooth non-clustered isolate, was from a patient in Denmark. All these isolates were characterized by whole genome sequencing[1].
Mabs clinical isolates 1334, OM128, OM200, OM91 and 1339; and *Mmas* clinical isolates 1335, 1337, OM194, OM130, and OM69 were from CF patients at the Royal Papworth Hospital, Cambridge, UK. All isolates displayed a smooth morphotype with the exception of OM128 and OM200 which were rough. The patients from which these isolates originated all had American Thoracic Society-defined disease except for the patient infected with *Mmas* OM69.

Culture conditions **-** MABSC strains were grown in Middlebrook 7H9 (BD Biosciences) supplemented with 0.5% glycerol, 10% albumin-dextrose-catalase (ADC), and 0.05% Tween-80; on Middlebrook 7H10 agar supplemented with 10% ADC; in SCFM2[2], in minimal M63 medium supplemented with 1 mM $MgSO_4$, 0.05% tyloxapol, and 0.2% glucose; in M63 medium supplemented with 1 mM $MgSO_4$, 0.05% tyloxapol and 10% or 20% CF sputum (see below); or in minimal medium (50 mM MOPS, 0.085% NaCl, 50 μM $FeCl_3$, 0.59 μM $MnSO_4$, 3.5 μM $ZnSO_4$, 4.5 μM $CaCl_2$, 20 mM Asn, 1.6 mM $MgSO_4$, 2.5 mM Na_2HPO_4/KH_2PO_4, 0.05%, pH 7) supplemented with 0.05% tyloxapol and various concentrations of branched-chain amino acids. Bovine submaxillary gland mucin (Alfa Aesar) was used in the preparation of complete SCFM2. Cation-adjusted Mueller Hinton II Broth (BD Biosciences) was used for MIC testing.
CF sputum was collected from six patients receiving treatment at Royal Papworth Hospital, Cambridge, UK, and 14 patients receiving treatment at National Jewish Health in Denver, Colorado, USA. Sputum collection at NJH was performed independent of underlying disease severity and without regard to existing infections. These studies were approved by the National Jewish Health Institutional Review Board (NJH IRB), and written informed consent approved by the NJH IRB was obtained from all sputum donors. The study was conducted in accordance with the Declaration of Helsinki. Sputum collection at Royal Papworth Hospital, Cambridge, UK, was from consented adults with CF not infected with NTM in accordance with Royal Papworth Hospital Tissue Bank Ethic Board Approval. The sputa were sterilized by γ-irradiation delivering a dose of 2.5 megarads. Samples from National Jewish Health patients or Papworth University Hospital patients were pooled, centrifuged (10 min at 3000 x *g*) to remove debris, diluted to a concentration of 10% or 20% vol/vol in minimal M63 medium and finally filtered through a 0.45 μm filter to remove any remaining insoluble material.

Minimum Inhibitory Concentration (MIC) determinations - The susceptibility of MABSC isolates to antibiotics was determined in 96-well microtiter plates in cation-adjusted Mueller Hinton II broth, SCFM2 or SCFM2 without mucin using the resazurin blue test[3] and visually scanning for growth. MIC was determined at day 5 or 10 (for macrolides) by reading the well with the lowest concentration of antibiotic showing no visible growth.

Lipid, sugar, mycolic acids and fatty acid analyses - Total lipids were extracted from MABSC cells with $CHCl_3/CH_3OH$ (1:2, v/v) overnight followed by two overnight extractions with $CHCl_3/CH_3OH$ (2:1, v/v). Lipids resuspended in $CHCl_3/CH_3OH$ (2:1, v/v) were either analyzed

by TLC or by LC-MS in positive and negative ion modes following the method described by Sartain *et al.*[4] on a high-resolution Agilent 6220 TOF mass spectrometer interfaced to a LC.

Mycolic acid methyl esters for TLC analysis were prepared from delipidated cells by incubation with 15% tetrabutylammonium hydroxide (Sigma-Aldrich) overnight at 100°C followed by methylation with iodomethane (Sigma-Aldrich) for 4 h at room temperature and extraction with dichloromethane. The preparation of mycolic acids from delipidated cells for LC-MS analysis followed the procedure described by Bhamidi *et al.*[5]. LC/MS analysis of mycolic acids followed the procedure described by Grzegorzewicz *et al*.[6].

Fatty acid methyl esters were prepared from extractable lipids by incubation overnight with 3M methanolic HCl (Sigma-Aldrich) at 80°C. GC/MS analyses of fatty acid methyl esters were carried out using a TRACE 1310 gas chromatograph (Thermo Fisher) equipped with a TSQ 8000 Evo Triple Quadrupole in the electron impact mode and scanning from m/z 70 to m/z 1000 over 0.8 s. Helium was used as the carrier gas with a flow rate of 1 mL min^{-1}. The samples were run on a ZB-5HT column (15 m x 0.25mm i.d.) (Zebron). The injector (splitless mode) was set for 300°C. The oven temperature was held at 60°C for 2 min, programmed at 20°C min^{-1} to 375°C, followed by a 10 min hold. The data analyses were carried out on a Thermo Scientific Chromeleon Chromatography Data System.

Determination of the monosaccharide composition of extractable lipids and delipidated cells followed earlier procedures[7]. Alditol acetates were analyzed by GC/MS on a Thermo Scientific TRACE 1310 Gas Chromatograph paired with a Thermo Scientific TSQ 8000 Evo Triple Quadrupole GC-MS/MS. Samples were run on a Zebron ZB-5HT Inferno 30 m x 0.25 mm x 0.25 µm capillary column (Phenomenex) at an initial temperature of 100°C. The temperature was increased to 150°C at a ramp rate of 20°C min^{-1}, then to 240°C at a ramp rate of 5°C min^{-1} and was held at this temperature for 3 min to be finally increased to 300°C at a rate of 30°C min^{-1} and held at the final temperature for 5 min. Data handling was carried out using the Chromeleon software.

To determine the amino alcohol and amino acid content of GPLs, surface lipids were first extracted from whole MABSC cells for 2 h with water-saturated 1-butanol as described by Morita *et al*.[8]. The amino alcohol and amino acid contents were then determined using a slightly modified procedure from that described by Simek *et al.*[9]. Briefly, an aliquot of GPLs hydrolyzed in 6 M HCl were dried under nitrogen and subjected to silylation using 100 µl of neat *N*-tert-Butyldimethylsilyl (TBDMS)-*N*-methyltrifluoroacetamide followed by 100 µl of acetonitrile. After 1 min of thorough vortexing and 3 min of sonication, the samples were incubated at room temperature for 15 min. The derivatized samples were analyzed by GC/MS as described for alditol acetates scanning for masses from *m/z* 60 to *m/z* 520 over 0.2 s starting at 2.5 min. Samples were run split (1:20) with helium as a carrier gas with a flow rate of 1 mL min^{-1}. The oven temperature was held at 155°C for 2 min, increased to 250°C at a ramp rate of 10°C min^{-1}, and held at the final temperature for 5 min. Data analysis was carried out on a Chromeleon data station.

Generation of *mmpL4b* and *BCKADH* knockout mutants of *Mmas* CIP 108297 - Recombineering was used to inactivate the *mmpL4b* and *bkdA-bkdB-bkdC* genes of *Mmas* CIP 108297 by allelic replacement. To this end, the Gp60 and Gp61 recombineering proteins from mycobacteriophage Che9c were expressed from the replicative plasmid pJV53-XylE under control of an acetamide-inducible promoter[10-11]. Acetamide-induced *Mmas* CIP 108297 harboring pJV53-XylE were electro-transformed with ~ 500 ng of linear allelic exchange substrates encompassing the *mmpL4b* and and *MAB_4918c-MAB_4915c* loci and double-crossover mutants were isolated

on streptomycin-containing medium. The linear allelic substrate used to delete the *mmpL4b* and *BCKADH* loci were generated by bracketing the streptomycin-resistance cassette from pHP45Ω with approximately 1,000-bp of upstream and downstream DNA sequence flanking the two loci. pJV53-XylE was cured from the knock-out mutants by subculturing in the absence of kanamycin in the culture medium.

Cell infections **-** Adenocarcinomic human alveolar basal epithelial A549 cells and acute monocytic leukemia monocyte-derived THP-1 cells were infected with *Mmas* 1239 and *Mmas* 184 isolates grown in different culture media. A549 and THP-1 cells were maintained in complete RPMI medium. THP-1 cells were differentiated to macrophages by incubating for 3 days with 100 nM phorbol 12-myristate 13-acetate (PMA). $1x10^5$ THP-1 or A549 cells were infected at a multiplicity of infection (MOI) of 10. Infected cells were incubated at 37°C in a 5-6% CO_2 incubator for two hours, then washed 3 times with PBS to remove extracellular bacteria. Intracellular bacteria (CFUs) were quantified 2 hours, one day and two days post-infection. To this end, the medium was removed from the infected cell cultures and replaced with sterile lysis buffer containing 0.05% Tween-80 in double-distilled H_2O, followed by incubation at 37°C for 20 minutes. The plate wells were then scraped and the solution was removed, diluted, and plated on 7H11-OADC agar plates. CFUs were counted after 3-4 days incubation at 37°C.

RNA extraction - MABSC RNA was extracted from 5-ml cultures grown to early exponential phase using the Direct-zol™ RNA Miniprep kit with on-column deoxyribonuclease I (DNAse) (Zymo Research) per the manufacturer's instructions. Total RNA was treated with a second round of DNAse (Ambion, DNAse Turbo™, ThermoFisher) and subsequently checked by PCR to verify the absence of genomic contamination (data not shown).

Reverse transcription and qRT-PCR – Reverse transcription reactions were carried out using the Superscript IV First-Strand Synthesis System (Thermo Fisher) and qRT-PCRs were run using the SYBR Green PCR Master Mix (Sigma-Aldrich) as per the manufacturers' protocols and analyzed on a CFX96 real-time PCR machine (Biorad). PCR conditions: 98°C (30 s; enzyme activation), followed by 40 cycles of 98°C (10 s; denaturation) and 60°C (30 s; annealing/extension). Mock reactions (no reverse transcription) were done on each RNA sample to rule out DNA contamination. The target cDNA was normalized internally to the *sigA* cDNA levels in the same sample. The following primers were used: MAB_2157_fwd (5'-AGGAAAACCTCGAACGGCAT-3') , MAB_2157_rev (5'-CTCCGGTTTCCAGTCCTCA-3'); MAB_4407c_fwd (5'-GGCCTCGGGAGTCAGAATG-3'), MAB_4407c_rev (5'-CTCGCAAAGTCAGCTCTGG-3'); MAB_4916c_fwd (5' GCCAATTCGACCATCGCCT-3'), MAB_4916c_rev (5'-CCGAGGCGGATCTGATTTC-3'); MAB_4742c_fwd (5'-CGAACGCGGTAATGAGGTC-3'), MAB_4742c_rev (5'-GATTTCTCGGACGAGGCGA-3'); MAB_4743c_fwd (5'-GGTTTGCATGGTTTCCGCAT-3'), MAB_4743c_rev (5'-GCACCACCGTAAGCTTTGAC-3'), and *sigA*_fwd (5'-CGTTCCTGGACCTGATTCAG-3'), *sigA_rev* (5'-GTACGTCGAGAACTTGTAACCC-3').

RNA-seq library preparation **-** RNA was quantified using a Qubit HS RNA spectrophotometer (Thermo Fisher) and sample quality was assessed using an Agilent High Sensitive RNA Screentape on an Agilent Tapestation, according to the manufacturer's recommendations. All RNA had an RNA Integrity Number (RIN) of greater than 6, indicating sufficient RNA quality for

sequencing. One μg of total RNA was treated with RiboZero rRNA Removal Kit (Gram-positive bacteria) (Illumina Inc.) to deplete ribosomal RNA followed by a 1.8X bead clean-up (AMPure RNA) according to the manufacturers' protocols. Libraries were next generated using the KAPA RNA Hyperprep kit (Roche) according to the manufacturer instructions. Briefly, depleted RNA was fragmented 6 min at 85°C following by the 1st and 2nd strand synthesis. Ligation was performed with 1.5 μM of Kapa Dual-Indexed Adapter (Roche). After the final amplification step, libraries were quantified using Qubit dsDNA BR Assay Kit (Thermo Fisher Sc., USA, MA), and the fragment size was assessed on an Agilent Tapestation using the D1000 Screen tape. Libraries were multiplexed in three sequencing runs at equimolar concentrations. Libraries were sequenced using single-end or pair-end reads on an Illumina NextSeq instrument using the high-output 75 cycles or mid-output 150 cycles.

RNA-seq data analysis – RNA-seq reads were trimmed for quality score greater than 20 and length greater than 50 using Skewer (version 0.2.2) automatically detecting adapters[12]. Reads were mapped to the *M. abscessus* subsp. *abscessus* ATCC 19977 genome (NC_010397.1) using Bowtie 2 (version 2.3.5) end-to-end alignment with default parameters[13]. Count tables were constructed from sorted BAM files using HTSeq-count (version 0.11.1)[14] set to non-stranded, intersection_nonempty using the gff3 file for NC_010397.1 and counting reads on gene_id. Gene expression and differential expression analysis was completed in R (version 3.6.0) using DESeq2 (version 1.26.0)[15]. KEGG Orthology categories were assigned with KEGG Mapper, using organism mab. Venn diagrams were constructed with http://bioinformatics.psb.ugent.be/webtools/Venn/. The sequencing data described in this publication have been submitted to the NCBI gene expression omnibus (GEO) under BioProject # PRJNA602697.

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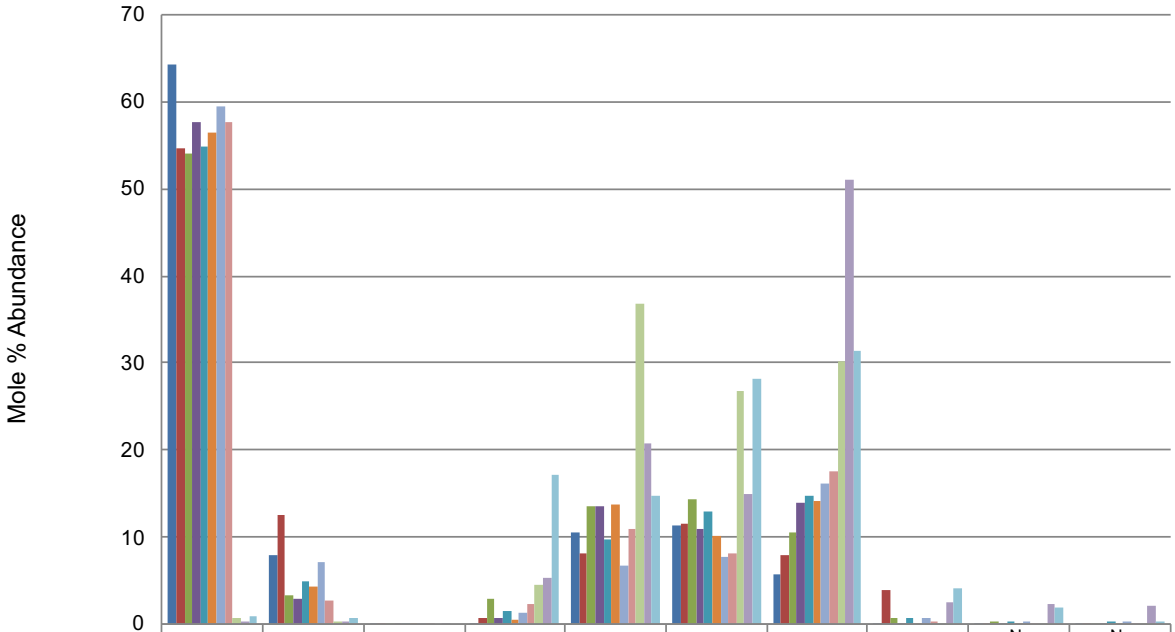
<u>Table S1</u>: Monosaccharidic composition of total extractable lipids (A) and delipidated cells (B) prepared from *Mmas* 1239, *Mmas* 184, *Mmas* 604 and *Mabs* 1091 grown in 7H9-ADC-Tween-80, SCFM2 complete medium or SCFM2 devoid of mucin.

The results for *Mmas* 1239 are representative of two independent experiments (biological duplicates).

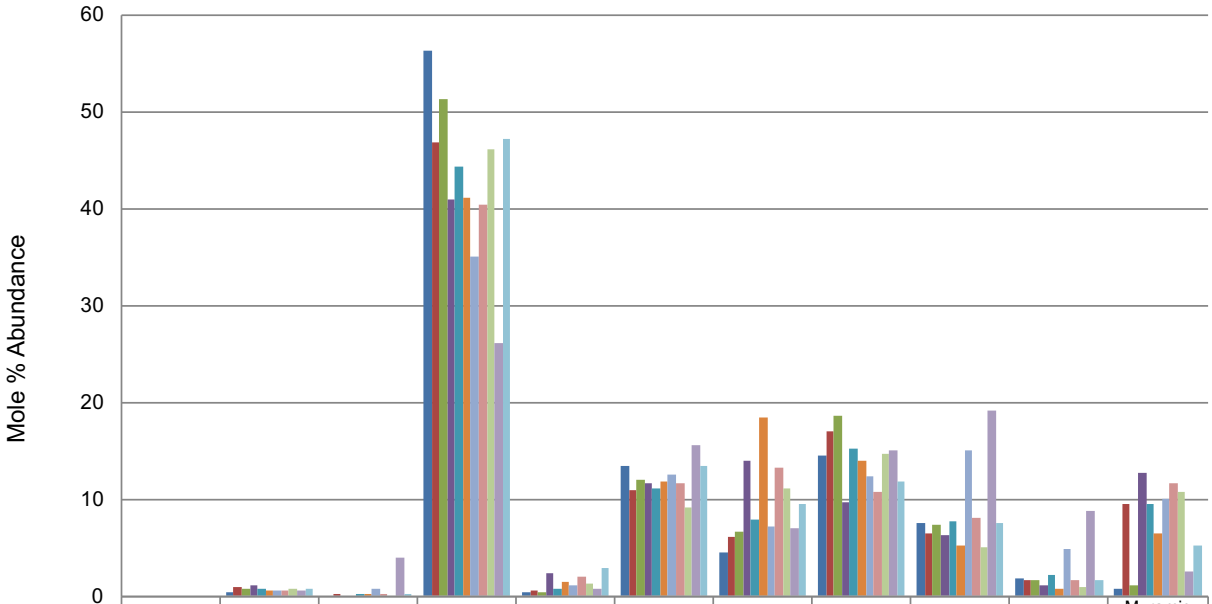
Cell envelope monosaccharides were analyzed by GC-MS as their alditol acetate derivatives from two separate fractions: One corresponding to the total extractable (glyco)lipids from the cells [Table S1A] and the other from the delipidated cells [Table S1B]. The latter mainly consist of the cell wall core (mycolyl-arabinogalactan-peptidoglycan complex) and lipoglycans (i.e., lipomannan and lipoarabinomannan) remaining upon lipid extraction. The results revealed very similar sugar contents in all isolates with the exception of *Mabs* 1091 which is essentially devoid of GPLs and thus presented considerably reduced levels of rhamnose and 6-deoxytalose in its extractable lipids. This led to higher relative abundance of other sugars in this fraction for *Mabs* 1091. Consistent with the presence of more triglycosylated forms of GPLs in the isolates grown in the presence of mucin, more rhamnose was found in isolates *Mmas* 184, 1239 and 604 cultured in complete SCFM2. We also noted the presence of slightly more *N*-acetylgalactosamine, *N*-acetylglucosamine and fucose in delipidated cells from *Mabs* 1091 and *Mmas* 604 grown in complete SCFM2 medium. We attribute this to mucin carryover in these samples. Analysis of the sugar composition of the mucin used in the preparation of SCFM2 indeed showed a high abundance of *N*-acetylgalactosamine (47%) and *N*-acetylglucosamine (35%), with fucose (9%), galactose (8%) glucose (6%), ribose (4%), mannose (2%) and arabinose (1%) being present in lower quantities.

(A)



	6-deoxy talose	Rhamnose	Fucose	Arabinose	Myo Insoitol	Mannose	Glucose	Galactose	N-acetylglucosamine	N-acetylgalactosamine
1239 extractable lipids 7H9-ADC-Tween-80	64.18	7.96	0.01	0.03	10.62	11.37	5.70	0.05	0.04	0.03
1239 extractable lipids SCFM2	54.58	12.66	0.03	0.82	8.18	11.62	7.92	3.96	0.13	0.09
1239 extractable lipids SCFM2 no mucin	54.00	3.42	0.02	3.04	13.61	14.32	10.53	0.68	0.26	0.12
184 extractable lipids 7H9-ADC-Tween-80	57.64	2.86	0.01	0.70	13.56	10.92	14.04	0.15	0.09	0.02
184 extractable lipids SCFM2	54.75	4.97	0.05	1.47	9.70	12.88	14.79	0.66	0.32	0.40
604 extractable lipids 7H9-ADC-Tween-80	56.49	4.33	0.01	0.62	13.83	10.23	14.25	0.12	0.08	0.04
604 extractable lipids SCFM2	59.37	7.15	0.07	1.42	6.78	7.66	16.15	0.71	0.36	0.32
604 extractable lipids SCFM2 no mucin	57.61	2.83	0.03	2.28	10.88	8.16	17.59	0.37	0.21	0.05
1091 extractable lipids 7H9-ADC-Tween-80	0.76	0.41	0.00	4.52	36.80	26.86	30.24	0.16	0.19	0.05
1091 extractable lipids SCFM	0.31	0.42	0.15	5.31	20.84	14.91	51.06	2.50	2.27	2.23
1091 extractable lipids SCFM2 no mucin	0.87	0.75	0.00	17.15	14.85	28.26	31.47	4.22	2.01	0.40

(B)



	6-deoxy talose	Rhamnose	Fucose	Arabinose	Myo Insoitol	Mannose	Glucose	Galactose	N-acetylglucosamine	N-acetylgalactosamine	Muramic acid glucosamine
1239 cell wall 7H9-ADC-Tween-80		0.49	0.00	56.33	0.39	13.52	4.50	14.51	7.57	1.85	0.83
1239 cell wall SCFM2		0.87	0.01	46.81	0.63	10.92	6.07	17.07	6.43	1.69	9.50
1239 cell wall SCFM2 no mucin		0.80	0.00	51.41	0.34	12.03	6.60	18.71	7.30	1.74	1.08
184 cell wall 7H9-ADC-Tween-80		1.12	0.00	40.96	2.31	11.64	14.07	9.64	6.29	1.20	12.77
184 cell wall SCFM2		0.84	0.29	44.38	0.83	11.09	7.84	15.32	7.69	2.16	9.56
604 cell wall 7H9-ADC-Tween-80		0.64	0.04	41.15	1.49	11.76	18.39	13.95	5.20	0.83	6.56
604 cell wall SCFM2		0.60	0.84	35.17	1.10	12.61	7.22	12.36	15.09	4.94	10.06
604 cell wall SCFM2 no mucin		0.63	0.00	40.54	1.93	11.59	13.24	10.71	8.13	1.64	11.59
1091 cell wall 7H9-ADC-Tween-80		0.77	0.00	46.11	1.32	9.23	11.17	14.68	5.03	0.92	10.77
1091 cell wall SCFM2		0.54	4.04	26.19	0.77	15.65	7.04	15.13	19.25	8.82	2.57
1091 cell wall SCFM2 no mucin		0.73	0.03	47.17	2.98	13.43	9.51	11.79	7.53	1.63	5.21

Table S3: **MIC values against a diversity of drug-susceptible and drug-resistant *Mabs* and *Mmas* clinical isolates in cation-adjusted Mueller Hinton II broth and SCFM2 devoid of mucin.**

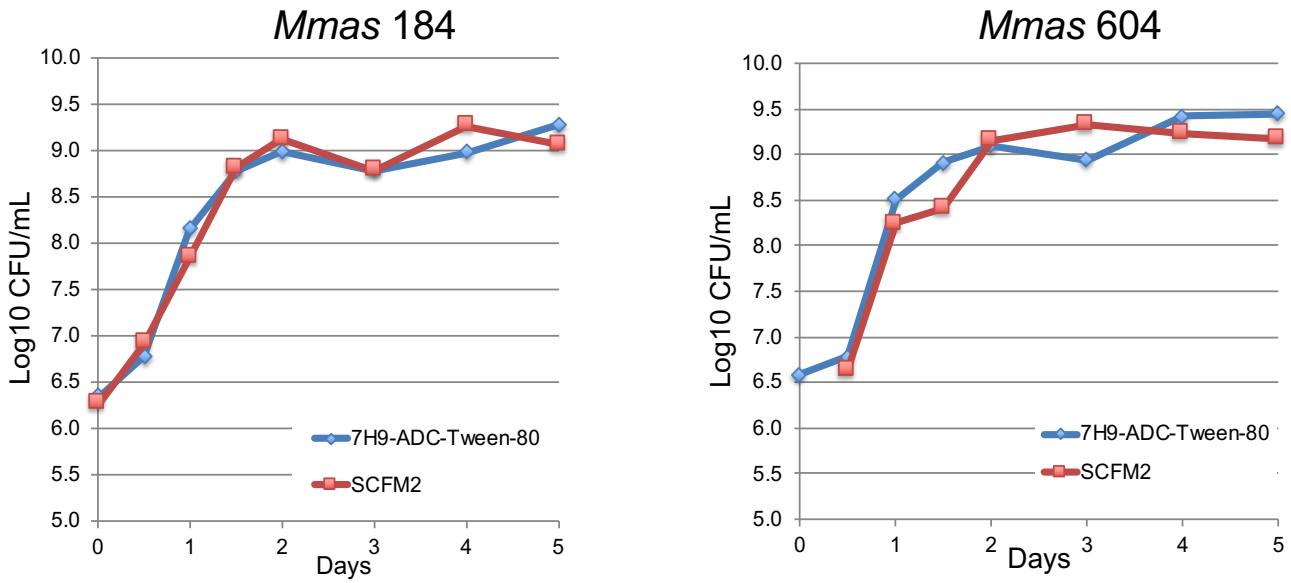
MIC values are in μg/mL. Greater than 4-fold changes in MIC values between the two media are highlighted in green. nd, not determined. MIC determinations were performed on two to three independent culture batches.

M. abscessus subsp. *abscessus* isolates

	M. abscessus subsp. *abscessus* ATCC 19977		*M. abscessus* subsp. *abscessus* 1334		*M. abscessus* subsp. *abscessus* OM 91		*M. abscessus* subsp. *abscessus* 1339		*M. abscessus* subsp. *abscessus* OM 200	
	MHII	SCFM2	MHII	SCFM2	MHII	SCFM2	MHII	SCFM2	MHII	SCFM2
Amikacin	16	8	64	128	64	128	64	64	8	8
Apramycin	32	32	32	16	8	8	8	8	8	8
Azithromycin	>160	>160	>160	>160	>160	>160	0.625	1.25	>160	>160
Clarithromycin	160	160	80	80	>160	>160	0.625	1.25	40	40
Erythromycin	>256	>256	>256	>256	>256	>256	32	64	>256	>256
Kanamycin	16	8	256	64	128	64	32	16	64	64
Ethambutol	160	80	160	160	80	80	160	320	80	160
Rifampicin	>320	>320	>320	>320	>320	>320	>320	320	5	2.5
Streptomycin	128	64	256	>256	256	256	256	>256	256	128
Cefoxitin	100	50	50	50	100	50	50	25	25	25
Tobramycin	64	32	256	128	256	256	nd		64	128
Linezolid	32	64	64	64	128	128	128	128	2	1
Tetracycline	>160	>160	>160	>160	>160	>160	>160	>160	5	5
Imipinem	64	64	64	64	128	128	128	256	128	64
Ciprofloxacin	32	64	32	32	16	16	16	16	>256	>256

M. abscessus subsp. *massiliense* isolates

	M. abscessus subsp. *massiliense* CIP108297		*M. abscessus* subsp. *massiliense* 1335		*M. abscessus* subsp. *massiliense* OM 130		*M. abscessus* subsp. *massiliense* 1337		*M. abscessus* subsp. *massiliense* OM69		*M. abscessus* subsp. *massiliense* OM 194	
	MHII	SCFM2	MHII	SCFM2	MHII	SCFM2	MHII	SCFM2	MHII	SCFM2	MHII	SCFM2
Amikacin	128	64	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
Apramycin	4	4	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
Azithromycin	1.25	2.5	>160	>160	>160	>160	>160	>160	>160	>160	>160	>160
Clarithromycin	0.078	0.156	>160	>160	>160	>160	>160	>160	>160	>160	>160	>160
Erythromycin	8	8	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
Kanamycin	8	8	>256	>256	>256	>256	>256	>256	>256	>256	320	320
Ethambutol	160	160	160	160	320	320	160	320	160	160	>320	>320
Rifampicin	>320	>320	>320	>320	>320	>320	80	40	>320	>320	>320	>320
Streptomycin	8	4	>256	256	>256	>256	128	128	256	128	>256	>256
Cefoxitin	50	50	50	50	50	50	25	25	50	50	50	50
Tobramycin	16	16	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
Linezolid	8	16	64	64	64	64	1	2	32	64	64	64
Tetracycline	20	20	>160	>160	>160	>160	20	20	>160	>160	>160	>160
Imipinem	128	64	64	64	64	64	nd		64	64	64	64
Ciprofloxacin	64	64	>256	>256	>256	>256	32	32	64	32	>256	>256



<u>Figure S1</u>: Growth characteristics of isolates *Mmas* 184 and *Mmas* 604 in 7H9-ADC-Tween-80 and SCFM2 at 37°C. No growth curve is shown for isolate *Mabs* 1091 which displays a rough morphotype and aggregates extensively in liquid broth.

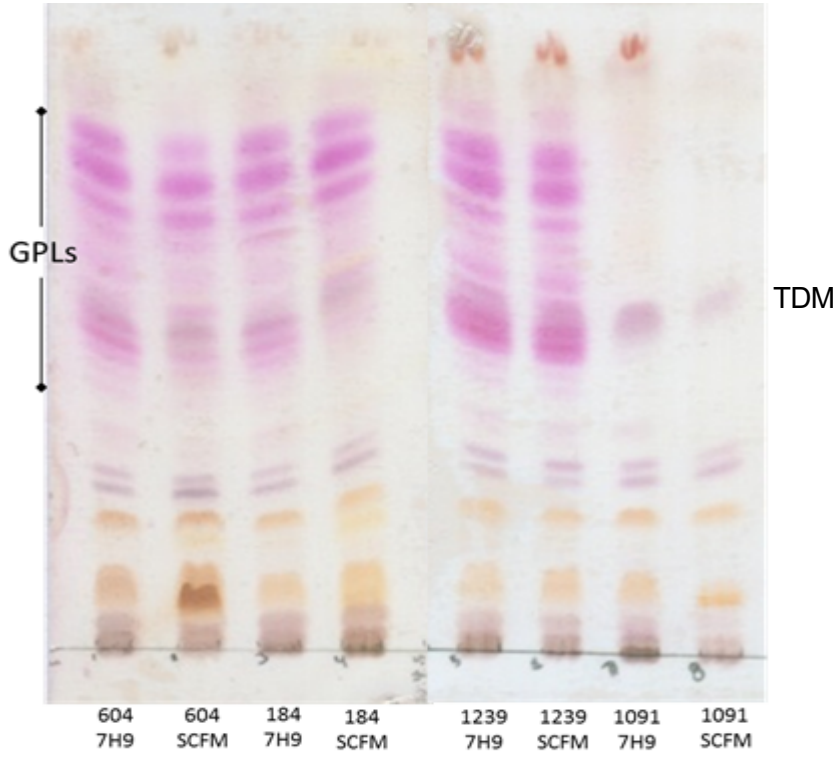
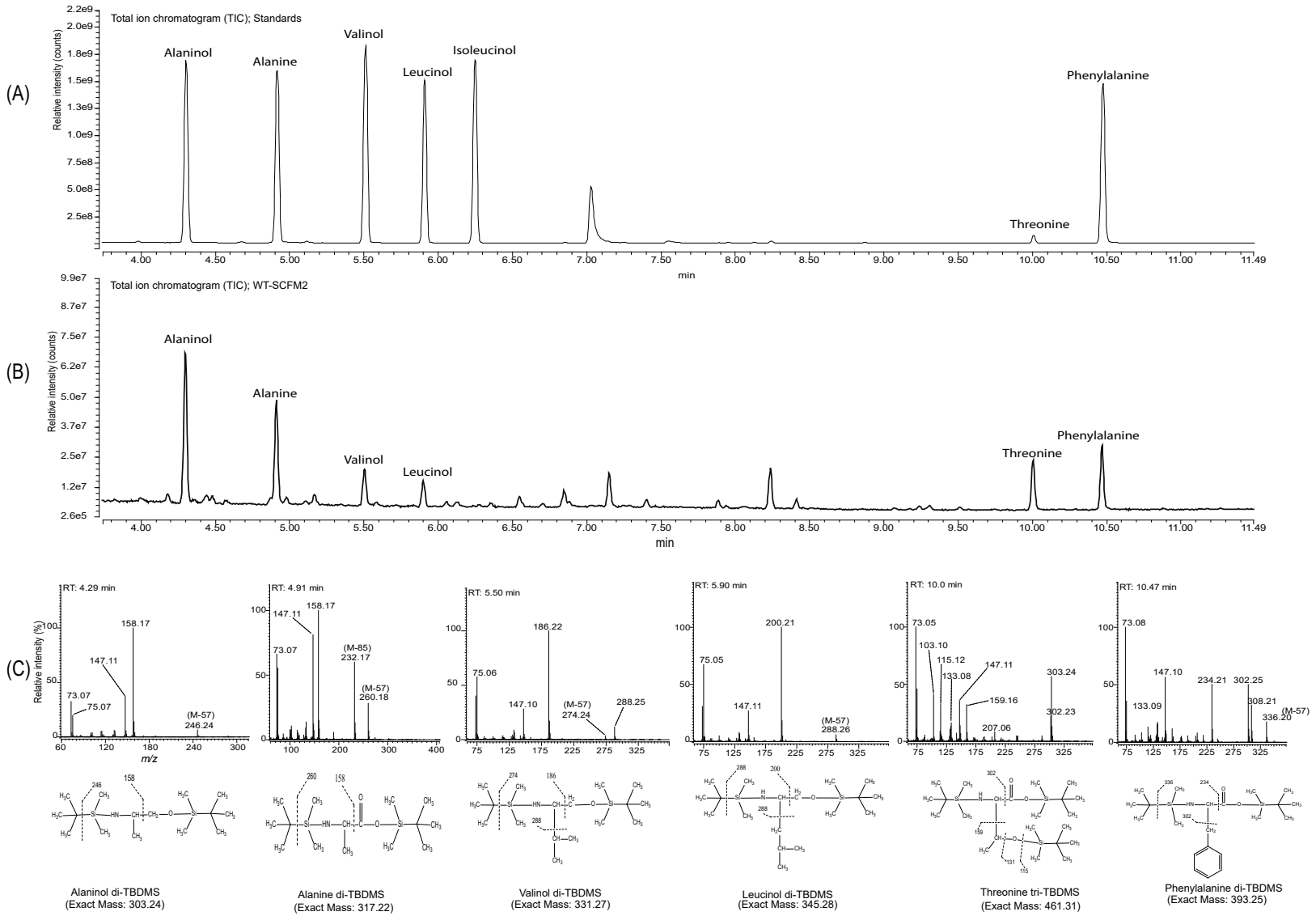


Figure S2: Thin-layer chromatography analysis of GPLs produced by *Mmas* 1239, *Mmas* 184, *Mabs* 1091 and *Mmas* 604.

Thin layer chromatography analysis of total lipids extracted from *Mmas* 1239, *Mmas* 184, *Mabs* 1091 and *Mmas* 604 grown in 7H9-ADC-Tween-80 or SCFM2 complete medium. Lipids extracted from equivalent amounts of bacterial cells were analyzed on silica gel 60-precoated TLC plates F254 (Merck) developed in the solvent system chloroform:methanol:water (90:10:1, v/v/v). The plate was revealed by spraying with α-naphthol and heating. The rough isolate, *Mabs* 1091, does not present any GPL.
TDM, trehalose dimycolates.



<u>Figure S3</u>: Amino alcohol content of GPLs produced by *Mmas* CIP 108297.

(A) GC-MS chromatogram of a mixture of the TBDMS-derivatized standard amino acids (alanine, threonine and phenylalanine) and amino alcohols (alaninol, valinol, leucinol and isoleucinol); (B) GC-MS chromatogram of 6 M HCl-hydrolyzed, TBDMS-derivatized, GPLs from WT *Mmas* CIP 108297 grown in SCFM2. (C) Mass spectra for alanine, alaninol, leucinol, valinol, threonine and phenylalanine.

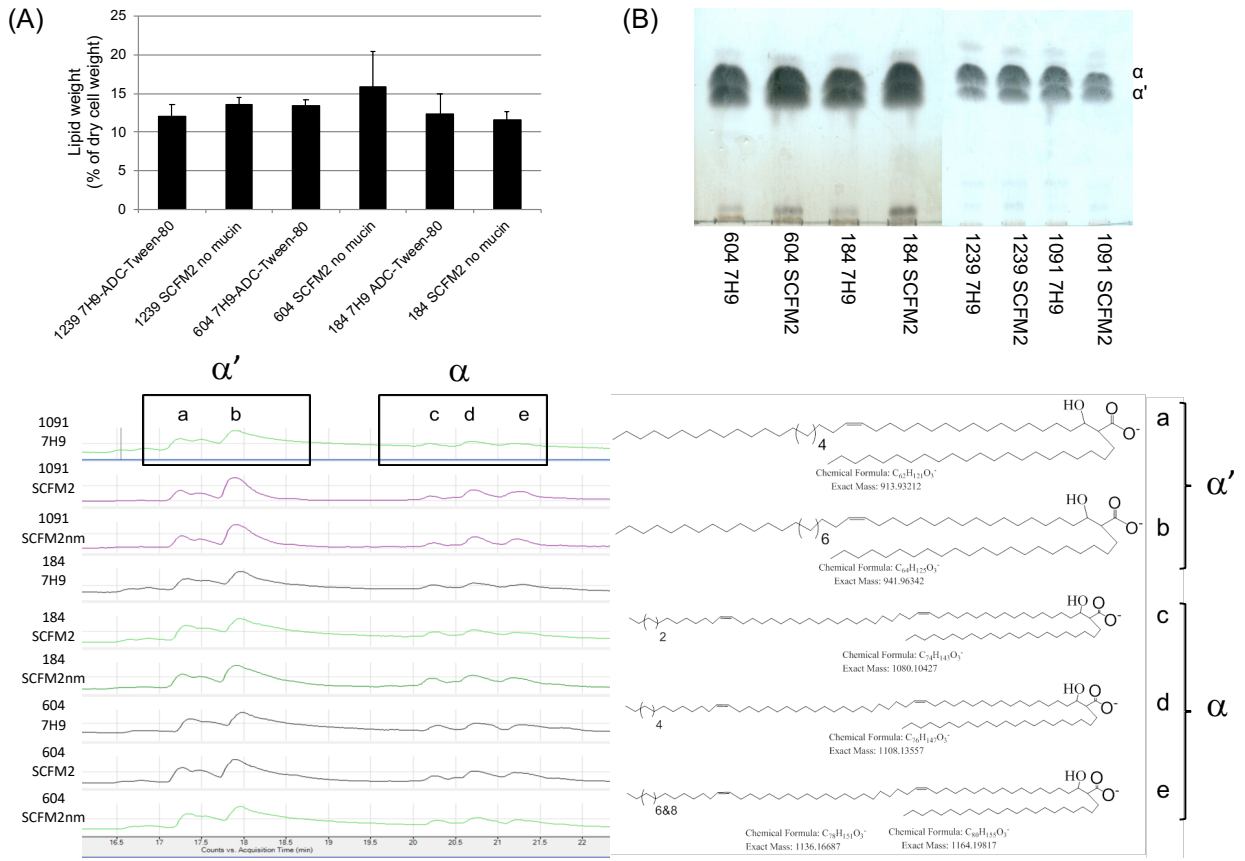
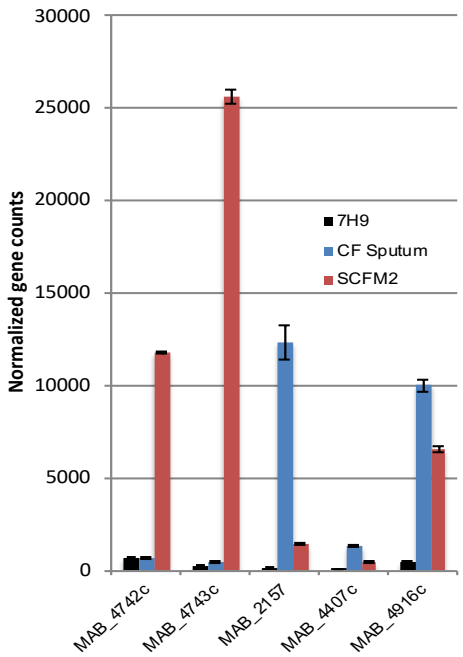


Figure S4: Extractable lipid weight and mycolic acid analysis from MABSC isolates grown in 7H9-ADC-Tween-80, SCFM2 complete medium and/or SCFM2 without mucin.

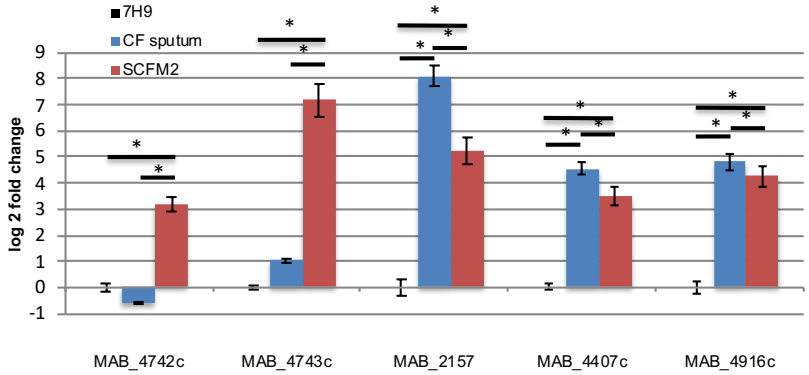
(A) Extractable lipid weight of *Mmas* 1239, *Mmas* 184 and *Mmas* 604 grown in 7H9-ADC-Tween-80 and SCFM2 without mucin. Lipid weights are expressed as percentages of dried cell weights. Lipid weights could not accurately be assessed in isolates grown in complete SCFM2 medium due to extensive mucin carryover. **(B)** Mycolic acid analysis from *Mmas* 1239, *Mmas* 184, *Mabs* 1091 and *Mmas* 604 cells grown in 7H9-ADC-Tween-80, SCFM2 complete medium and SCFM2 without mucin. *Upper right panel*: TLC analysis of mycolic acid methyl esters prepared from delipidated cells of *Mmas* 1239, *Mmas* 184, *Mabs* 1091 and *Mmas* 604 grown in 7H9-ADC-Tween-80 or SCFM2 complete medium. Mycolic acid methyl esters were prepared from equivalent amounts of delipidated cells and analyzed on silica gel 60-precoated TLC plates F254 (Merck) developed three times in the solvent system *n*-hexane(s)/ethyl acetate (95:5, v/v). The plate was revealed by spraying with cupric sulfate (10% CuSO4 in a 8% phosphoric acid solution) and heating. α, alpha-mycolates; α', alpha prime-mycolates. All isolates present the expected α and α' mycolates in apparent similar ratios. *Lower panel*: HPLC-MS total ion chromatograms of mycolic acids prepared from isolates *Mmas* 184, *Mabs* 1091 and *Mmas* 604 grown in 7H9-ADC-Tween-80, SCFM2 complete medium or SCFM2 without mucin (traces labeled "SCFM2nm"). Structures and chemical formulas of the major peaks labeled with small letters on the chromatograms are identified on the right half of the figure. No significant qualitative or quantitative differences between strains or growth conditions are noticeable.

(A)

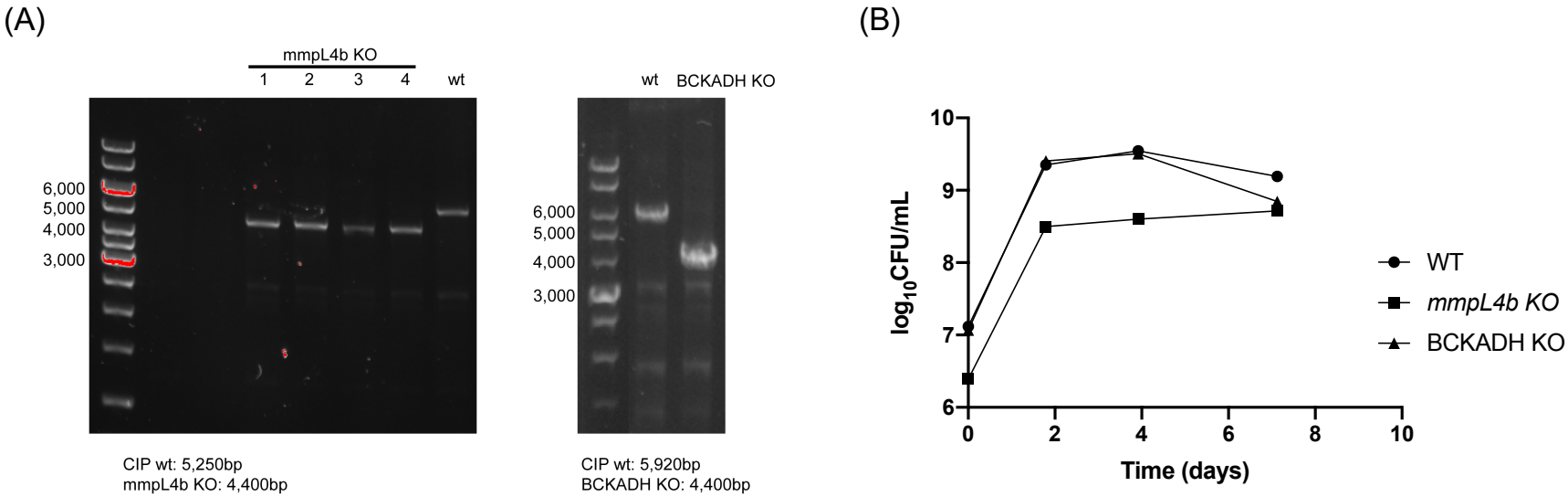


Log 2 Fold Change by RNAseq			
	SCFM2 vs 7H9	CF Sputum vs 7H9	SCFM2 vs CF Sputum
MAB_4742c	4.11	NS	4.1
MAB_4743c	6.78	NS	5.95
MAB_2157	3.69	6.8	NS
MAB_4407c	2.75	4.2	NS
MAB_4916c	3.77	4.4	NS

(B)



<u>Figure S5</u>: Transcriptional profiling of *Mmas* 1239 grown in different media.
RNA-seq (normalized gene counts and $\log_2$ fold-change) (A) and quantitative reverse transcription-PCR (qRT-PCR) (B) show five genes expressed at higher level in *Mmas* 1239 cultured in CF sputum and/or SCFM2 compared to 7H9-ADC-Tween 80 grown cells: *MAB_2157* (a putative fatty acid desaturase), *ahpD* (*MAB_4407c*), *bkdC* (*MAB_4916c), MAB_4742c* and *MAB_4743c*. Ratios of *genes*/*sigA* mRNA are means ± standard deviations (n = 3 RNA extractions and qRT-PCR reactions). Asterisks denote statistically significant differences between media pursuant to the Student's *t*-test ($P < 0.05$).



<u>Figure S6</u>: Gene replacement at the *mmpL4b* and *BCKADH* loci of *Mmas* CIP 108297.
(A) Allelic replacement at the *mmpL4b* and *bkdA-bkdB-bkdC* loci was confirmed by PCR using sets of primers located outside the linear allelic exchange substrates. The expected sizes of the products for the WT and various mutants are indicated under each gene. The *mmpL4b* deletion mutant was confirmed to be deficient in GPL production by TLC analysis of total lipid extracts (data not shown).
(B) Growth of WT *Mmas* CIP108297, the BCKADH-deficient mutant and the GPL-deficient mutant in SCFM2 containing mucin and 0.25% Tween 80 (to facilitate CFU counting). The results presented are representative of at least two independent experiments in SCFM2 with and without mucin using different culture batches.