Table S1. List of bacterial strains and plasmids used in this study.

Name	Description/genotype	Marker	Reference
Strains			
M. abscessus S	*Mabs sensu stricto*, strain CIP104536^T, smooth	_	Laboratoire de Référence des Mycobactéries
M. abscessus R	*Mabs sensu stricto*, strain CIP104536 ^{T}, rough	_	Laboratoire de Référence des Mycobactéries
M. abscessus S	*Mabs sensu stricto*, strain ATCC 19977, smooth	_	ATCC
CFZ-R1	*M. abscessus* S harboring the L40W mutation in MAB_2299c, co-resistant to CFZ/BDQ	_	This study
CFZ-R3	*M. abscessus* S harboring a c276 deletion leading to a P92 frameshift mutation in MAB_2299c, co-resistant to CFZ/BDQ	_	This study
CFZ-R4	*M. abscessus* S harboring a g541t substitution leading to an early stop codon in position E181 in MAB_2299c, co-resistant to CFZ/BDQ	_	This study
CFZ-R6	*M. abscessus* S harboring a 318a insertion leading to a D106 frameshift mutation in MAB_2299c, co-resistant to CFZ/BDQ	_	This study
CFZ-R7	*M. abscessus* S harboring the L151P mutation in MAB_2299c, co-resistant to CFZ/BDQ	_	This study
CFZ-R9	*M. abscessus* S harboring the G215S mutation in MAB_2299c, co-resistant to CFZ/BDQ	_	This study
Δ*MAB_2299c*	*Mabs MAB_2299c* unmarked deletion mutant obtained by two-step homologous recombination in the smooth parental strain CIP104536^T	_	This study
Δ*MAB_2299c*.C	Δ*MAB_2299c* complemented with pMV261-*MAB_2299c*	Kan	This study
Δ*MAB_2299c* / Δ*MAB_2300-MAB_2301*	Δ*MAB_2299c* in which Δ*MAB_2300-2301* have been deleted by two-step homologous recombination.	_	This study
E. coli XL1 Blue	*endA1 gyrA96 recA1 thi-1 relA1 hsdR17 supE44 lac [F´ proAB lacIqZΔM15 Tn10 (Tetr)].*	Tet	Stratagene
E. coli BL21 (DE3) (pRARE2)	*gal dcm (DE3) F- ompT hsdSB(rB- mB-) transformed by our hand with the pRARE2 vector (CamR)*	Cam	New England Biolabs
Plasmids			

pET32a	Vector for high-level expression of proteins fused with an N-terminal thioredoxin protein and a 6×His tag.	Amp	Novagen
pET32a-*MAB_2299c*	pET32a in which *MAB_2299c* is cloned in fusion with the thioredoxin/His tag sequence and containing a TEV cleavage site before MAB_2299c to remove the tags.	Amp	This work
pET32a-*MAB_2299c_L40W*	pET32a into which the *MAB_2299c_L40W* gene is cloned.	Amp	This work
pUX1	Vector for targeted disruption through homologous integration, containing the brightly red fluorescent tdTomato marker for positive selection.	Kan, Hyg	(1)
pUX1-katG	Variant of pUX1 containing the *katG* gene of *M. tuberculosis* H37Rv as a counter-selectable marker in the presence of isoniazid and used for the generation of unmarked chromosomal alterations.	Kan, Hyg	This work
pUX1-katG-*MAB_2299c*	Variant of pUX1-katG containing adjacently cloned up- and downstream sequences (approximately 1 kb each) of the *MAB_2299c* open reading frame. Used to generate an unmarked deletion of *MAB_2299c* in *M. abscessus*.	Kan	This work
pUX1-katG-*MAB_2300-MAB_2301*	Variant of pUX1-katG containing adjacently cloned up- and downstream sequences (approximately 0.5 kb each) of the *MAB_2300-2301* operon. Used to generate an unmarked deletion of *MAB_2300* and *MAB_2301* in Δ*MAB_2299c*.	Kan	This work
pMV261	Multi-copy vector enabling the cloning into *E. coli* and the overexpression of genes under the control of the strong and constitutive *hsp60* promoter in mycobacteria.	Kan	(2)
pMV261-AflII	pMV261 vector where an AflII restriction site was inserted within the multi-cloning site.	Kan	(1)
pMV261-*MAB_2299c*	pMV261 overexpressing the TetR MAB_2299c under the control of the *hsp60* promoter.	Kan	This work

Amp, ampicillin; Kan, kanamycin; Tet, Tetracycline; Stp, streptomycin ; Cam, chloramphenicol.

References

1. Viljoen A, Gutiérrez, Ana Victoria, Dupont C, Ghigo E, Kremer L. 2018. A simple and rapid gene disruption strategy in Mycobacterium abscessus: on the design and application of glycopeptidolipid mutants. Front Cell Infect Microbiol 8:69.

2. Stover CK, de la Cruz VF, Fuerst TR, Burlein JE, Benson LA, Bennett LT, Bansal GP, Young JF, Lee MH, Hatfull GF. 1991. New use of BCG for recombinant vaccines. Nature 351:456–460.

Table S2: Oligonucleotides used in this study. Fw and Rv stand for forward and reverse, respectively.

Primers	**5’ to 3’ sequence**
Cloning in pET32a	
MAB_2299c_Fw (KpnI) (**TEV Cleavage Site**)	TATAGGTACC**GAGAATCTGTACTTCCAGGGA**GTGGCGCAACTGA CATTCCAACGCG
MAB_2299c_Rv (HindIII)	TCGTAAGCTTTTGGTTCGCCCAGGCGTACACGCGG
Cloning in pMV261	
Compl_MAB_2299c_Fw	CGCAACTGACATTCCAACGCGC
Compl_MAB_2299c_Rv (HindIII)	GAGAGAAAGCTTTTCTGGTGCACGTGAGAGTG
Generation of pUX1-*katG* and derivative constructs	
katG_outer_Fw	TATACCGGACTACGCCGAAC
katG_outer_Rev (AvrII)	GAGAGACCTAGGTGCATGAGCATTATCCCGTA
katG_inner_Fw	CCGGAGCCGCCAGCAACGGCT
katG_inner_Rev	AGCCGTTGCTGGCGGCTCCGG
MAB_2299c_outer_Fw	GAAGAACAGCAGCATCACGA
MAB_2299c_outer_Rev (NheI)	GAGAGAGCTAGCGACACCTTCGTTCACGGTTT
MAB_2299c_inner_Fw	AAAGAAGCGTCGCTCTGACCAGGCTGTTA
MAB_2299c_inner_Rev	GGTCAGAGCGACGCTTCTTTTCGTCAGTCC
MAB_2300-MAB_2301_outer_Fw	ACGCCTTAGCTTTTCCGTTT
MAB_2300-MAB_2301_outer_Rev (NheI)	GAGAGAGCTAGCGCAGATGTCAACAACCGATG
MAB_2300-MAB_2301_inner_Fw	TTGGATCCCGCAGCTCCGATCCGCGTGGT
MAB_2300-MAB_2301_inner_Rev	ATCGGAGCTGCGGGATCCAAGCCCGCTTC
PCR confirmation of *MAB_2299* knock-out	
MAB_2299c_U_scrn_Fw	GAAGATCGCGTAGTCGGTTC
MAB_2299c_U_scrn_Rev	ATCACCACAGATGACCCACA
MAB_2299c_D_scrn_Fw	CGCGTTTCATCAGGATCTTT
MAB_2299c_D_scrn_Rev	CTTTGGAGCATGGGCATATT
MAB_2300-MAB_2301_U_scrn_Fw	ATCACCACAGATGACCCACA
MAB_2300-MAB_2301_U_scrn_Rev	GTGCAGGAATTCTCATGCTG
MAB_2300-MAB_2301_D_scrn_Fw	GGAATCCGATCGGTAGTTGA
MAB_2300-MAB_2301_D_scrn_Rev	CGCTCATGCCGATTTCTTAG
Sequencing	
pMV5’	CGCCCGGCCAGCGTAAGTAGC
pMV3’	GCCTGGCAGTCGATCGTACG
pMV3’ Ext	TTGAGACACAACGTCGCTTT
NheIpUX1	ACGGCATGGACGAGCTGTAC
qRT-PCR	
sigA	Fw: CACATGGTCGAGGTCATCAA Rev: TGGATTTCCAGCACCTTCTC
MAB_3551c (tgs1)	Fw: CACCGTCTACTACGGAATCAAC Rev: TGCGCAGCCTCCAATAAT
MAB_2300	Fw: GAGAAGCATGACCGACGACAAA Rev: CACACCCTTCCTTGTCGATGTAGTT
MAB_2301	Fw: CAGCTCCATCCCATTCCTTATC Rev: TCGGCCTGGACCTTCATA
MAB_2302	Fw: GAGATATTCGGCTCCACCAACATC

	Rev: CACAGATAGTTCACCTCACCCTTTGT
MAB_2303	Fw: ACATTCTCTGTCCCGATCATTC Rev: GTGCTCTTTACCGACCTCTTC
Eletrophoretic Mobility Shift Assay (EMSA)	
Probe A_Fw	GTGCGTAACGCACGCTACCGGGTGGTTCGTTGGCTCGCAAATCAC ACGCCGTGCGTTATCCTCG
Probe A_Rv	CACGCATTGCGTGCGATGGCCCACCAAGCAACCGAGCGTTTAGTG TGCGGCACGCAATAGGAGC
Probe B_Fw	CAGCTCCGATCCGCGTGGTGTCGG
Probe B_Rv	GCTAATTCTCAATGGTGCGATGCC
Probe C_Fw	TCGCCGCTAGTGGTGACGAACGAC
Probe C_Rv	TCAGGGAACTGTACATGTGTCGTC
Probe D_Fw	GCTTGTAGCTAACTAGGCTATTGC
Probe D_Rv	GACTAGTGGGATCCATACCCGCGT
Non-specific probe_Fw	CACTTCGCCATAAGTGGATTGACTCTATCCACTTTTACCCATAGA
Non-specific probe_Fw	TCTATGGGTAAAAGTGGATAGAGTCAATCCACTTATGGCGAAGTG
ΔPalin_Fw	GTGCGGCTACTTACCTACCGGGTGGTTCGTTGGCTCGCAAATCAC ACGCTCGCACGAGTCCTCG
ΔPalin_Rv	CACGCCGATGAATGGATGGCCCACCAAGCAACCGAGCGTTTAGT GTGCGAGCGTGCTCAGGAGC
ΔDR_Fw	GTGCGTAACGCACGCTTGATATACTGCTTAAGGCTCGCAAATCCT GACTTAGTGACCTTCCTCG
ΔDR_Rv	CACGCATTGCGTGCGAACTATATGACGAATTCCGAGCGTTTAGGA CTGAATCACTGGAAGGTCG

[a]Restriction sites are underlined and specified inside brackets.

Table S3. Drug susceptibility profile of *M. abscessus* CIP104536 and its various derivatives to IPM, BDQ, CFZ, INH, EMB or RIF. MIC_{99} (µg/ml) were visually determined on Middlebrook 7H10 agar after 4 days at 37°C.

Strain	MIC (µg/mL)					
	IPM	**BDQ**	**CFZ**	**INH**	**EMB**	**RIF**
CIP104536	4	0.25	2	>256	16	128
ΔMAB_2299c	4	1	8	>256	16	128
ΔMAB_2299c.C	4	0.25	2	>256	16	128
ΔMAB_2299c / *ΔMAB_2300-MAB_2301*	4	0.031	2	ND	ND	ND

IPM, imipenem; BDQ, bedaquiline; CFZ, clofazimine; INH, isoniazid; EMB, ethambutol; RIF, rifampicin. ND, not determined.

Table S4. Percentage of nucleotide or protein sequence identity (ID) between seventeen identified *M. abscessus mmpS-mmpL* couples and the *M. tuberculosis mmpS5-mmpL5* couple after multiple alignment using the MUSCLE algorithm.

MmpS/MmpL	Nucleotide ID[a]	MmpS ID	MmpL ID
Rv0677c-Rv0676c	100	100	100
MAB_0477-MAB_0478	75	35	44
MAB_0988c-MAB_0987c	79	46	57
MAB_1135c-MAB_1134c	80	49	60
MAB_2036-MAB_2037	75	38	52
MAB_2211c-MAB_2210c	73	36	38
MAB_2300-MAB_2301	**80**	**49**	**62**
MAB_2302-MAB_2303	**77**	**47**	**54**
MAB_2572c-MAB_2571c	75	41	20
MAB_2649-MAB_2650	77	41	52
MAB_3149-MAB_3150	80	54	61
MAB_3200-MAB_3201	73	35	42
MAB_3563c-MAB_3562c	77	38	51
MAB_4117c-MAB_4115c[b]	36	48	61, 59[b]
MAB_4262-MAB_4263	72	27	38
MAB_4311c-MAB_4310c	78	50	54
MAB_4383c-MAB_4384c	**82**	**61**	**68**
MAB_4705c-MAB_4703c[b]	35	51	60, 62[b]

[a]For nucleotide alignments and ID calculations, the sequence starting at the start codon of the *mmpS* gene and ending at the stop codon of the *mmpL* gene was used.
[b]*mmpS/mmpL* couples containing an additional mmpL in the operon. For MmpL protein sequence identity to $MmpL5_{Mtb}$ the % ID of the first MmpL is shown first followed by a comma and then the % ID of the second MmpL.

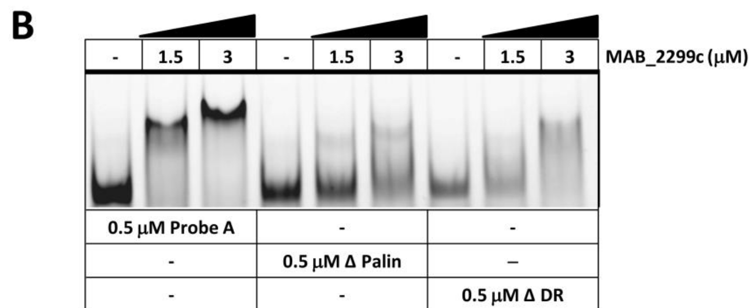
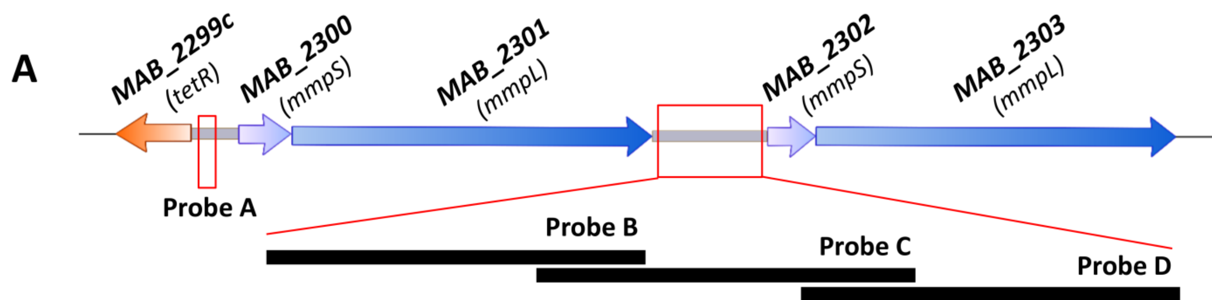


Fig. S1. Binding activity of MAB_2299c to mutated Probe A. (A) DNA sequences of Probe A and its two mutated derivatives, ΔPalin and ΔDR, where the conserved palindromes and the degenerated double repeats were replaced by random nucleotides, respectively. Conserved residues are in color for the palindromes and in bold for the double repeats. **(B)** EMSA using the Probes A, ΔPalin and ΔDR with purified MAB_2299c. Gel Shifts were revealed by fluorescence emission using the corresponding 5’ fluorescein-labeled probes.



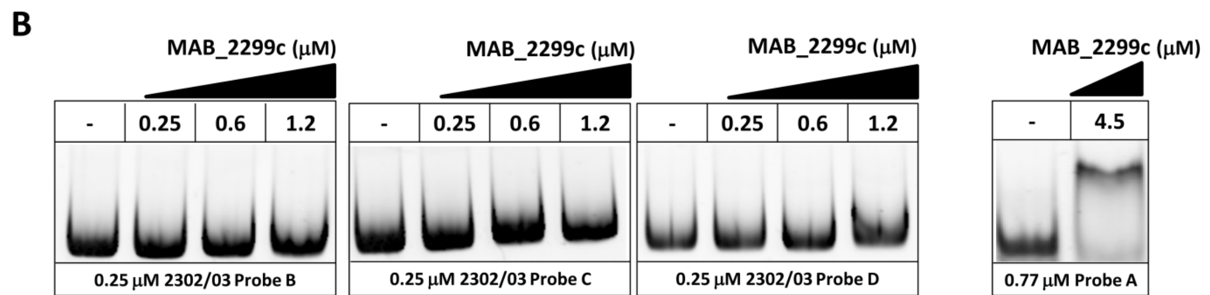


Fig. S2. (A) Schematic representation of the intergenic region $IR_{2302/03}$ present upstream of *MAB_2302/MAB_2303*. Due to its large size, this 893 bp region was divided into three 373 bp overlapping probes (Probes B – C – D). **(B)** EMSA using increasing concentrations of purified MAB_2299c with Probe A (left panel), Probe B (middle panel) or Probe C (right panel). Probe A was also included as a positive control (far right panel). Gel shifts were revealed by fluorescence emission using 5' fluorescein-labeled probes. Experiments were repeated three times with similar results.

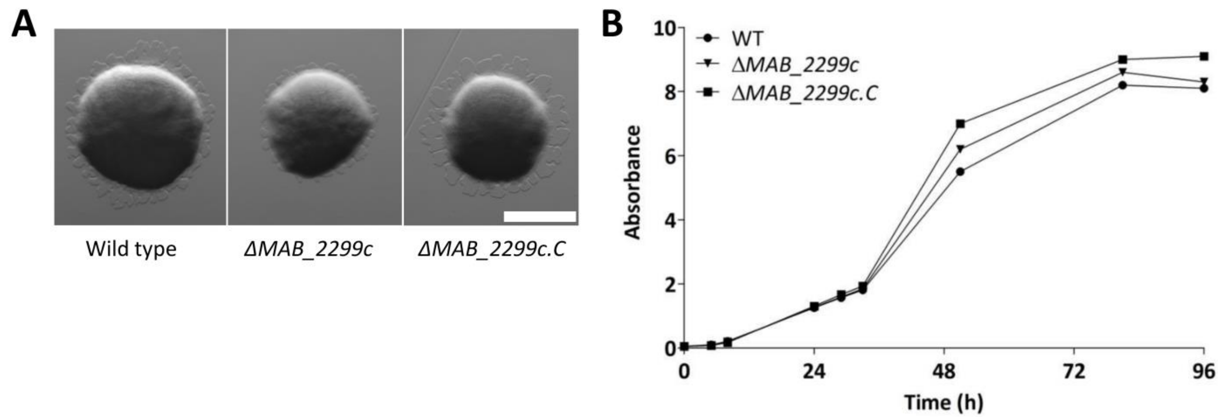


Fig. S3. Colony morphology **(A)** and *in vitro* growth **(B)** of parental *M. abscessus* WT CIP104536^{T}, *ΔMAB_2299c* and its complemented derivative *ΔMAB_2299c.C*. Bacteria were grown in 7H9 broth supplemented with OADC and 0.05% Tween 80. Growth was monitored by determining the OD_{600} at different time points. Scale bar corresponds to 150 µm.