1	Characterization of putative DD-carboxypeptidase-encoding genes in Mycobacterium
2	smegmatis
3	
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6	SUPPLEMENTARY INFORMATION
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Table S1. Bacterial strains and plasmids used in this study.

Name	Description	Source/Reference
Strains		
<u>M. smegmatis</u>		
$mc^2 155$	<i>ept-1</i> (efficient plasmid transformation) mutant of mc^26	1
Δ2432	Derivative of $mc^{2}155$ carrying an unmarked, out-of-frame deletion in <i>M. smegmatis</i> Msmeg_2432 (786bp internal racion available)	This work
Δ2433	Derivative of $mc^2 155$ carrying an unmarked, out-of-frame deletion in <i>M. smegmatis</i> Msmeg 2433	This work
	(870bp internal region excised)	
Δ1661	Derivative of mc ² 155 carrying an unmarked, in-frame deletion in <i>M. smegmatis</i> Msmeg_1661 (1158bp	This work
	internal region excised)	
$\Delta dac B$ TetO	Derivative of $mc^{2}155$ in which the native promoter of Msmeg_6113 (<i>dacB</i>) is substituted with	This work
	PmyclitetO. The native promoter regulates expression of a truncated (350 bp) 5' region of <i>dacB</i> ; Hyg'.	
	This strain contains no Anhydrotetracycline-responsive repressor in the L5 attB site thus termed	
	$\frac{1}{2}$	7 T 1' 1
$\Delta dacB$ LetON _s	Derivative of mc ⁻¹⁵⁵ in which the native promoter of Msmeg_6113 (<i>dacB</i>) is substituted with P_{max} table. The native promoter resultance of a transacted (250 km) 5 ² region of d_{max} By Hug ¹	This work
	PmyclitetO. The native promoter regulates expression of a truncated (350 bp) 5 region of <i>adcB</i> ; Hyg.	
	derived from pMC1s	
AdacB TetOFF	Derivative of $mc^{2}155$ in which the native promoter of Msmeg 6113 (<i>dacB</i>) is substituted with	This work
	Pmvc1tetO. The native promoter regulates expression of a truncated (350 bp) 5' region of <i>dacB</i> ; Hyg ^r .	
	This strain contains the "reverse" Anhydrotetracycline-responsive repressor (revtetR) in the L5 attB site	
	derived from pTEK-4S0X.	
∆dacB attB::pMVdacB	Derivative of mc ² 155 carrying native <i>dacB</i> , $\Delta dacB$ and additional copy of native <i>dacB</i> in the L5 <i>attB</i>	This work
_	site. Strain was constructed by electroporation with pMV <i>dacB</i> and p2 Δ <i>dacB</i> g17 vectors; Hyg ^r , Km ^r .	
$\Delta dac B$	Derivative of mc ² 155 carrying native <i>dacB</i> , $\Delta dacB$ and additional copy of homologous operon from <i>M</i> .	This work
attB::pMVRv3627c_operon	tuberculosis in the L5 attB site. Strain was constructed by electroporation with pMVRv3627c_operon	
	and $p2\Delta dacBg17$ vectors; Hyg ^r , Km ^r .	

<u>Plasmids</u>		
p2NIL	<i>E. coli</i> cloning vector, Kan ^r	2
pMV306H	E. coli-Mycobacterium integrating shuttle vector. Integrates in the L5 attB site in mycobacteria.	H. Boshoff
	Derivative of pMV306 (³) carrying a hyg gene; Hyg ^r	
pMV <i>dacB</i>	Derivative of pMV306H carrying full-length dacB (1443 bp) plus 150 bp upstream of start codon	This work
	representative of native promoter sequence; Hyg ^r	
pMVRv3627c_operon	Derivative of pMV306H carrying four gene operon (Rv3627c, Rv3626c, Rv3625c (mesJ) and Rv3624c	This work
	(hpt)) from <i>M. tuberculosis</i> ; Hyg ^r	
pMV1661	Derivative of pMV306H carrying full-length MSEMG_1661 (1194 bp) plus 150 bp upstream of start	This work
	codon representative of native promoter sequence; Hyg ^r	
pMV2432	Derivative of pMV306H carrying full-length MSMEG_2432 and MSMEG_2433 (1788 bp) plus 150 bp	This work
-	upstream of start codon representative of native promoter sequence; Hyg ^r	
pMV2433	Derivative of pMV306H carrying full-length MSMEG_2433 (891 bp) plus 150 bp upstream of start	This work
*	codon representative of native promoter sequence; Hyg ^r	
	Plasmid carrying <i>lacZ</i> and <i>sacB</i> markers as a <i>PacI</i> cassette; Km ^r	2
pGOAL17	Plasmid carrying <i>lacZ-sacB-Hyg^r</i> markers as a <i>PacI</i> cassette; Hyg ^r	2
pGOAL19	Knockout vector for creating ΔMsmeg_2432 mutant. The 1.2 kb upstream region including 18 bp of the	This work
p2∆2432	5' sequence of Msmeg_2432 was PCR-amplified to incorporate HindIII and NdeI restriction sites. The	
1	1.2 kb downstream was similarly obtained but incorporated NdeI and Acc65I restriction sites. The	
	fragments were cloned into p2NIL and the PacI cassette from pGOAL19 was inserted; Km ^r Hyg ^r .	
	Knockout vector for creating ΔMsmeg_2433 mutant. The 1.2 kb upstream region including 18 bp of the	This work
p2Δ2433	5' sequence of Msmeg_2433 was PCR-amplified to incorporate HindIII and NdeI restriction sites. The	
r	1.2 kb downstream was similarly obtained but incorporated NdeI and Acc65I restriction sites. The	
	fragments were cloned into p2NIL and the PacI cassette from pGOAL19 was inserted; Km ^r Hyg ^r .	
	Knockout vector for creating \DeltaMsmeg_1661 mutant. The 1.2 kb upstream region including 18 bp of the	This work
p2A1661	5' sequence of Msmeg_2433 was PCR-amplified to incorporate HindIII and NdeI restriction sites. The	
r	1.2 kb downstream was similarly obtained but incorporated NdeI and Acc65I restriction sites. The	
	fragments were cloned into p2NIL and the PacI cassette from pGOAL19 was inserted; Kmr Hygr.	
$p2\Delta dacB$	Knockout vector for creating ΔMsmeg_6113 (dacB) mutant. The 1.2 kb upstream region including 18 bp	This work
r	of the 5' sequence of Msmeg_2433 was PCR-amplified to incorporate <i>HindIII</i> and <i>NotI</i> restriction sites.	
	The 1.2 kb downstream was similarly obtained but incorporated NotI and Acc651 restriction sites. The	

p2∆ <i>dacB</i> g17	fragments were cloned into p2NIL and the <i>PacI</i> cassette from pGOAL19 was inserted; Km ^r Hyg ^r . Knockout vector for creating Δ Msmeg_6113 (<i>dacB</i>) mutant. The 1.2 kb upstream region including 18 bp of the 5' sequence of Msmeg_2433 was PCR-amplified to incorporate <i>HindIII</i> and <i>NotI</i> restriction sites. The 1.2 kb downstream was similarly obtained but incorporated <i>NotI</i> and <i>Acc651</i> restriction sites. The fragments were cloned into p2NIL and the <i>PacI</i> cassette from pGOAL17 was inserted; Km ^r .	This work
pSE100	Derivative of pMS2 containing Pmyc1tetO carrying a <i>hyg</i> gene; Hyg ^r . pMS2 is a shuttle vector containing origins of replication for <i>Escherichia coli</i> (pMB1) and mycobacteria (pAL500) and a multiple cloning site flanked by two transcriptional terminators.	Sabine Erht
pMC1s	Derivative of pMV306K, which is a single-copy-integrating plasmid which inserts into L5 attB; Km ^{r} . Anhydrotetracycline-responsive repressor (tetR) is not codon-usage adapted for mycobacteria but is expressed from a strong mycobacterial promoter (psmyc = puv15).	Sabine Ehrt
pTEK-4S0X	Kanamycin resistant derivative of pTE-4S0X; Km^{r} . Anhydrotetracycline-responsive repressor (revtetR) is expressed from a strong mycobacterial promoter (psmyc = puv15). High-copy number in <i>E. coli</i> and low-copy number in mycobacteria.	Sabine Ehrt
pS <i>dacB</i>	Derivative of pSE100. Suicide construct from which the mycobacterial ori was excised and the native promoter (15 bp upstream start codon) and 350 bp of the 5' region of <i>dacB</i> cloned directly downstream of the Pmyc1tetO using <i>SphI</i> and <i>MscI</i> ; Hyg ^r .	This work

Region amplified	Primer name	Sequence $(5'-3')^a$	Description of amplicon	Mutation
Msmeg_6113 (<i>dacB</i>)	dacB_UF	5' – CGCG <u>AAGCTT</u> GCGAGCCCCTGGTGAACC	1200 bp of upstream and 21	
upstream region	dacB_UR	5' – CGCG <u>GCGGCCGC</u> GCGCGTCAGCCTATCGCTCAT	bp of the 5'-end of	
			Msmeg_6113 (<i>dacB</i>)	1400 bp unmarked
Msmeg_6113 (dacB)	dacB_DF	5' – CGCG <u>GCCGGCCGC</u> CAGCTGCGGATGCGGCGCATGA	1339 bp of downstream and	deletion in <i>dacB</i>
downstream region	dacB_DR	5' – CGCG <u>GGTACC</u> ATCTCGGCGCCAACACAT	22 bp of the 3'-end of	
			Msmeg_6113 (<i>dacB</i>)	
Msmeg_1661	1661_UF	5' – CGCG <u>AAGCTT</u> GCGAGGCCGTCGAGAGCG	1200 bp of upstream and 18	
upstream region	1661_UR	5' – CGCG <u>CATATG</u> CACCGCGCGTCGCGTCAT	bp of the 5'-end of	1150 hn unmarked
			Msmeg_1661	deletion in
Msmeg_1661	1661_DF	5' – CGCG <u>CATATG</u> CAGCGCCCGCAGCACTGA	1375 bp of downstream and	Mamag 1661
downstream region	1661_DR	5' – CGCG <u>GGTACC</u> ATTCGGGTTGTCGCGGTT	18 bp of the 3'-end of	Wishieg_1001
			Msmeg_1661	
Msmeg_2432	2432_UF	5' – CGCG <u>AAGCTT</u> CCGGGAACGCAGGCAGC	1200 bp of upstream and 21	
upstream region	2432_UR	5' – CGCG <u>CATATG</u> CGCCGCGAACAGTCTTCGCAC	bp of the 5'-end of	796 hn unmarkad
			Msmeg_2432	deletion in
Msmeg_2432 2432_DF		5' – CGCG <u>CATATG</u> GACTACGGCTTCAGCCAGTAG	1200 bp of downstream and	Mamag 2422
downstream region	2432_DR	5' – CGCG <u>GGTACC</u> GGCCGACTTCGACCTGTCG	18 bp of the 3'-end of	Wishieg_2452
			Msmeg_2432	
Msmeg_2433	2433_UF	5' – CGCG <u>AAGCTT</u> ACGATCCCGCGGACCAGG	1200 bp of upstream and 21	
upstream region	2433_UR	5' – CGCGCATATGCCCGAAGGCGTACCTCCACAT	bp of the 5'-end of	970 hn unmarkad
			Msmeg_2433	deletion in
Msmeg_2433	2433_DF	5' – CGCG <u>CATATG</u> GCGAGCATCGGGGGCGCTCTGA	1200 bp of downstream and	Mamog 2422
downstream region	2433_DR	5' – CGCG <u>GGTACC</u> ATCGGCCACGGCCGCATC	21 bp of the 3'-end of	wishieg_2455
			Msmeg_2433	

Table S2. Primers used to generate suicide plasmids for MSMEG_6113 (*dacB*), MSMEG_2432, MSMEG_2433 and MSMEG_1661.

a. Restriction sites are underlined.

Table S3. Primers	used for gene	expression	analysis by q	PCR.

Gene	Primer name	Sequence (5' – 3')	Amplicon size (bp)
Msmeg_6113 (<i>dacB</i>)	<i>dacB_</i> qF	5' – AACGAGTCGGACAACGTGAT	Positions 934-1140 (207 bp)
	dacB_qR	5' – GACCACCTCATCGAGGGTAA	
Msmeg_1661	1661_qF	5' – CCACACCCAGAGCTACCAGT	Positions 732-924 (193 bp)
	1661_qR	5' – GGTTCCCTTGAGCAACACC	
Msmeg_1900	1900_qF	5' – GACAACGCGGTTCAGACCTA	Positions 313-491 (179 bp)
	1900_qR	5' – CAGGATTCGTTGGCGTAGAT	
Msmeg_2432	2432_qF	5' – GCTGAACTATCCGGTGATCG	Positions 567-768 (202 bp)
	2432_qR	5' – GTACATCTGCACCACCACCA	
Msmeg_2433	2433_qF	5' – GCTGGTCAACCAGAACGAGT	Positions 651-803 (153 bp)
	2433_qR	5' – TCTTTCACCAGGCCGTACAT	

Table S4. Primers used for genotyping M. smegmatis mutant strains. Primers F and R2 situated 100 bp upstream and downstream of start and stop codons,
respectively. Primer R1 approximately in the middle of gene targeted for deletion.

Gene	Primer name	Sequence (5' – 3')	Amplicon sizes (bp)
Msmeg_6113 (dacB)	KOdacB_F	5' – ACGACGTCGAACTCCACC	FR1 = 800 bp (wild-type)
	KOdacB_R1	5' – CGTCGATGTCGGCCGGGTC	$FR1 = absent (\Delta)$
	KOdacB_R2	5' – ATGGCCTGGCGGCGCGTG	FR2 = 1643 bp (wild-type)
			$FR2 = 242 \text{ bp } (\Delta)$
Msmeg_1661	KO1661_F	5' – CACACGAGTCACAACTGT	FR1 = 701 bp (wild-type)
	KO1661_R1	5' – TGGCGGCCAGCGTGTTGA	$FR1 = absent (\Delta)$
	KO1661_R2	5' – CGGCATGGTGAACCAGTC	FR2 = 1394 bp (wild-type)
			$FR2 = 236 \text{ bp} (\Delta)$
Msmeg_2432	KO2432_F	5' – GGACCGTTCGGCGAGCAT	FR1 = 500 bp (wild-type)
	KO2432_R1	5' – TGTCGGCGAGCATGTTGG	$FR1 = absent (\Delta)$
	KO2432_R2	5' – CGGATCTGGTGATCCTGC	FR2 = 1028 bp (wild-type)
			$FR2 = 242 \text{ bp } (\Delta)$
Msmeg_2433	KO2433_F	5' – GCAGTCGAATAGTCCACC	FR1 = 500 bp (wild-type)
	KO2433_R1	5' – CCGCGTCGTTGCCCGACG	$FR1 = absent (\Delta)$
	KO2433_R2	5' – AGTGCCGTGCTGAGGGCG	FR2 = 1028 bp (wild-type)
			$FR2 = 242 bp (\Delta)$

Gene	Primer name	Sequence (5' – 3') ^a	Description	Amplicon size (bp)
MSMEG_6113 (<i>dacB</i>)	Comp_ <i>dacB</i> _F Comp_ <i>dacB</i> _R	5'-GCGC <u>ACGCGT</u> TCTCGTGATCCACCTCGT-3' 5'-GCGC <u>CTGCAG</u> TCATGCGCCGCATCCGCA-3'	150 bp upstream of 5'-end amplified with full-length <i>dacB</i> to maintain native promoter sequences (-10 and -35)	1593 bp
MSMEG_1661	Comp_1661_F Comp_1661_R	5'-GCGC <u>ACGCGT</u> GAGAAGTTCGAGTAACGC-3' 5'-GCGC <u>ACGCGT</u> TCAGTGCTGCGGGCGCTG-3'	150 bp upstream of 5'-end amplified with full-length MSMEG_1661to maintain native promoter sequences (-10 and -35)	1364 bp
MSMEG_2432	Comp_2432_F Comp_2432_R	5'-GCGC <u>AAGCTT</u> CGTCCGGGGTGAACACCG-3' 5'-GCGC <u>AAGCTT</u> CTACTGGCTGAAGCCGTA-3'	150 bp upstream of 5'-end amplified with full-length MSMEG_2433 and MSMEG_2432 to maintain native promoter sequences (-10 and -35)	1958 bp
MSMEG_2433	Comp_2433_F Comp_2433_R	5'-GCGCAAGCTTCGTCCGGGGTGAACACCG-3' 5'-GCGC <u>AAGCTT</u> TCAGAGCGCCCCGATGCT-3'	150 bp upstream of 5'-end amplified with full-length MSMEG_2433to maintain native promoter sequences (-10 and -35)	1061 bp
<i>attB</i> (tRNA for glycine)	attL2_F attBS1_R	5' – CTT GGA TCC TCC CGC TGC GC – 3' 5' – ACG TGG CGG TCC CTA CCG – 3'	Site for integration of the complementation vector pMV <i>dacB</i> in the bacterial	282 bp
	attBS2_F attL4_R	5' – ACA GGA TTT GAA CCT GCG GC – 3' 5' – AAT TCT TGC AGA CCC CTG GA – 3'	chromosome	320 bp

a. Restriction sites are underlined.

Gene	Primer name	Sequence (5' – 3') ^a	Description	Amplicon size (bp)
Msmeg_6113	KD_dacB_F	5' – GC <u>GCATGC</u> CCGGGCCAACCTTAGATGAGCGATA	15 bp upstream of	
(dacB)			start codon and 350	
	KD_dacB_R	5' – GCGC <u>CCCGGG</u> CCCGTGTCGGCGTCGGTG	bp of 5'-end	393 bp
			harbouring putative	
			native promoter (-10)	
			sequence	

Table S6. Primers used to generate suicide plasmid for use in constructing Msmeg_6113 (*dacB*) promoter replacement strain in *M. smegmatis*.

a. Restriction sites are underlined and start codons are indicated in bold.

Supplementary Figures

Mtb_dacB1	MAFLRSVSCLAAAVFAVGTGIGLPTAAGEPNAAPA						
Mtb_dacB2 Mtb_By3627c							
MSMEG dacB	MSDRLTRRGGSPRSIESRGSMRPTRWR-RSTHVVVGAAVLAFVAVVVAAAALVIIG						
MSMEG_2432							
MSMEG_2433							
MSMEG_1661 MSMEG_1900		MT-RRAVTAV	GALALLTAPLMTAGVAGAEPGV				
<i>E.coli</i> dacA							
<i>E.coli</i> _dacB							
<i>E.coli_</i> dacC_PBP6							
<i>E.coli_</i> dacD_PBP6b							
Mtb dacB1	ACPYKVSTPPAVDSSEVPAAGE-P-PLPLVVPPTPVGGNALGGCGIITAPGSAPAP						
Mtb_dacB2	MRKLMTATAALCACAVTVSAGAAWADADVQPAGSVPIPDGP						
Mtb_Rv3627c	GHRAGVRAPAPPPRPPTVKAGVVPVADTAATPSAAGVTAALAVVA						
MSMEG_dacB	KHSDAAEAVPPAPPPA'I'	ADPGVVP	VDLSAPTPTRRGLATALAAALAN				
MSMEG_2432 MSMEG_2433	MWRYAFGLVVLVVSGLI	TGPGSLAVP	VARADADIOOVGSVAPPEGP				
MSMEG_1661	DCPYRETTPPAVDASEV	PKPGE-AAPGPLPVPP	KAIGGEALSGCGVITAPNTPPVP				
MSMEG_1900							
E.coli_dacA	MNTIFSARIMKRLA	LTTALC	TAFISAAHADDLNIKTMIPGVP				
E.COLL_daCB E_COLL_daCC_PBP6	MTOYSSLUBGLA	AGSAFI	IAFSVQAANVDEYITQ-LPAGA				
<i>E.coli</i> dacD PBP6b	MKRRLI	IAASLF	VFNLSSGFAAENIPFSPQPP				
Mtb_dacB1	GDVSAEAWLVADLDSGAVI	AARDPHGRHRPASVIK	VLVAMASINTLTLNKSV				
Mtb_dacB2 Mtb_Bv3627c	PDLGKLAGRITDALTGOEL	WORLDDVPLVPASTIK					
MSMEG dacB	PDLGLITGRITDADTGAEL	WEOGARVPMOPASVNK	VLTTAAALLTLDRDARLTTTVVAAD				
MSMEG 2432	AKAWLVADMDTGRVL	ASKDPYGSYA <mark>PAS</mark> TI <mark>K</mark>	PLLAMVVLDHLRPDNFA				
MSMEG_2433	AETWVVADMDTGQIL	AGRGEYVRHA <mark>PAS</mark> TI <mark>K</mark>	TLLALVVLDEVPLDSTI				
MSMEG_1661	EDVSAEAWIVADMDTGDVI	AARDPHGRHR <mark>PAS</mark> II <mark>K</mark>	VLTATAALNELNLNKRV				
MSMEG_1900			MMTSYVICOAMKACKEK				
E.coli dacB	NLALMVOKVGASAPA	IDYHSOOMALPASTOK	VITALAALIOLGPDFRF-TTTLETK				
<i>E.coli</i> dacC PBP6	-SVDARAWILMDYASGKVL	AEGNADEKLD <mark>PAS</mark> LT <mark>K</mark>	IMTSYVVGQALKADKIK				
<i>E.coli_</i> dacD_PBP6b	-EIHAGSWVLMDYTTGQIL	TAGNEHQQRN <mark>PAS</mark> LT <mark>K</mark>	LMTGYVVDRAIDSHRIT				
Mth dacB1		agmar					
Mtb dacB2	VADVAVEGTAVEGTAVEGTAVEGTAVEGTAVEGTAVEGTAVEG						
Mtb Rv3627c	QNPQGPVVLVGAGDPTLSA	APPGQDTWYHGAARIG	DLVEQIRRSGVTPTAVQVDA				
MSMEG_dacB	DQP-GLVVLRGGGDTTLSA	APKGTDTWYKGAARIS	DLADQVRARGIRVTRVRVDT				
MSMEG_2432	RANASHTKVECSCVGLKPGQPYTTR						
MSMEG_2433 MSMEC_1661							
MSMEG 1900	AGTQDDANAEGTKVGVGPGGQYTIN						
<i>E.coli</i> dacA	ETDLVTIGNDAWATGNPVFKGSSLMFLKPGMOVPVS						
<i>E.coli</i> _dacB	GNV-ENGVLKGDLVARFGADPTLK-RQDIRNMVATLKKSGVNQIDGNVLIDT						
E.coli_dacC_PBP6	LTDM	VTVGKDAWATGN	PALRGSSVMFLKP-GDQVSVA				
<i>E.coli_</i> dacD_PBP6b	PDDI	VTVGRDAWAKDN	PVFVGSSLMFLKEGDRVSVR				
Mtb dacB1	QLLHGLLMH <mark>SG</mark>	NDAAYALARQLGGM	PAALEKINLLAAKLGGRDTRVAT				
Mtb_dacB2	QLLDGLLLV <mark>SG</mark>	N DAANTLAHMLGGQ	DVTVAKMNAKAATLGATSTHATT				
Mtb_Rv3627c	SAFSGPTMAPGWDPADIDN	GDIAPIEAAMIDAG	RIQPTTVNSRRSRTPALDAGRELAK				
MSMEG_dacB	SAYSGPTMAPGWDPADIDG	GDIAPMESVMLDGG	RTQPTTVESRRSKSPALDAGKALAA				
MSMEG_2432 MSMEC_2433	QLLDALLMVSG	NDAANMLADMLGGP	KVAVAAMNKKAAAVGARNTRAAS				
MSMEG 1661	DITHCITWASC	NDAANILARMILGGP	DPALOKI.NTI.AAKI.GGRDTRAAT				
MSMEG 1900	AVLTGALTAPA-PSPOIAI	VDNTEALTSSDGSLAD	GQAFSPFDLQQPAIAKLDPRLLA				
<i>E.coli</i> _dacA	QLIRGINLQ <mark>SG</mark>	NDACVAMADFAAG-SQ	DAFVGLMNSYVNALGLKNTHFQT				
<i>E.coli</i> _dacB	SIFASHDKA <mark>PG</mark> WPW	NDMTQCFSA	PPAAAIVDRNCFSVSLYSA				
E.coli_dacC_PBP6	DLNKGVIIÇ <mark>SG</mark> NDACIALADYVAG-SQESFIGLMNGYAKKLGLTNTTF						
<i>E.COLI_</i> dacD_PBP6b	DLSRGLÍVD <mark>SG</mark>	NPACVALADYIAG-GQ	KQFVEMMNNYAEKLHLKDTHFET				

Mtb_dacB1 Mtb_dacB2 Mtb_Rv3627c MSMEG_dacB MSMEG_2432 MSMEG_2433 MSMEG_1661 MSMEG_1900 <i>E.coli_</i> dacA <i>E.coli_</i> dacA <i>E.coli_</i> dacB <i>E.coli_</i> dacC_PBP6 <i>E.coli_</i> dacD_PBP6b	PSGLDGPGMSTSAYDIGLFYRYAWQN-PVFADIVATRTFDFPGHGDHPGYELEND PSGLDGPGGSGASTAHDLVVIFRAAMAN-PVFAQITAEPSAMFPSDNGEQLIVNQ ALGLDPAAVTI-ASAPAGARQLAVVQSA-PLIQRLSQMMNASDNVMAECIGREVAVA ALGVEPESVTLMPSGMRGGTTIAEVQSA-PLIERLRQMMNISDNVMAESIAREVAEA PSGLDGPGWESLTTPHDLAVIFRAALNY-PVIAQILRQTTAQFPGKTLTYQ PSGLDAPGMPFWSTPHDLAVIFRAAMAD-PVFAQITAMPSTVFPAKTGDRVLVNQ PSGLDGPGMSTSAYDIALFYRYAWQN-PVFADIVHTQSYQFPGR-DGGSYPIEND AVQNAANAAAAEGITMTVTSGWRS-PAFQQTLLDNAVQTYGS-LAAAREVVQT VHGLDADGQYSSARDMALIGQALIRDVPNEYSIYKEKEFTFNGIRQLNR PKPGDMAFIRVASYY-PV-TMFSQVRTLPRGSAEAQYCELDVVPG VHGLDAPGQFSTARDMALLGKALIHDVPEEYAIHKEKEFTFNKIRQPNR VHGLDAPGQHSSAYDLAVLSRAIIHGEPEFYHMYSEKSLTWNGITQQNR
Mtb_dacB1 Mtb_dacB2 Mtb_Rv3627c MSMEG_dacB MSMEG_2432 MSMEG_2433 MSMEG_1661 MSMEG_1900 <i>E.coli_</i> dacA <i>E.coli_</i> dacB <i>E.coli_</i> dacC_PBP6 <i>E.coli_</i> dacD_PBP6b	NQLLYNYPGALGGKTG YTDDAGQTFVGAANRD-GRRLMTVLLHGTRQPIPP- DELLQRYPGAIGGKTG YTNAARKTFVGAAARG-GRRLVIAMMYGLVKEGGPT INRPQSFSGAVDAVTSRLNTA HIDTAGAALVDSSGLSLDNRLTARTLDATMQAAAGP LGRPQSFEGAVGAVLTQLRSV GIDTSGAKLVDSSGLSVDNRLTALTLDEVVNAAAGH NELLTRYPGDIGGKTG YTNLARKTYVGAAQRG-NRRLVVVQMYGTGD NELLHRYPGTLGGKTG YTDLARKTFVAAAQRD-GRRLVIAMMYGLVKEGGPT NKLLANYPGALGGKTG YTDDAGQTFVGAAERD-GRRLIAVLLKGTRVPIAP- PTASRHVTGEAVDIGGKTG YTDDAGQTFVGAAERD-GRRLIAVLLKGTRVPIAP- NGLLWDNSLNVDGKTG HTDKAGYNLVASATEG-QMRLISAVMGGRTFKGR DLNRFTLTGCLPQRSEPLPLA FAVQDGASYAGAILKD-ELKQAGITWSGTLLRQTQ- NRLLWSSNLNVDGKTG HTSGAGYNLVASATQG-DMRLISVVLGAKTDRIR NGLLWDKTMNVDGLKTG HTSGAGFNLIASAVDG-QRRLIAVVMGADSAKGR
Mtb_dacB1 Mtb_dacB2 Mtb_Rv3627c MSMEG_dacB MSMEG_2432 MSMEG_2433 MSMEG_1661 MSMEG_1900 <i>E.coli_</i> dacA <i>E.coli_</i> dacB <i>E.coli_</i> dacC_PBP6 <i>E.coli_</i> dacD_PBP6b	-WEQAAHLLDYGFNTPAGTQIGTLIEPDPSLMSTDRNPADRQRVDP-QAAARISAADALP YWDQAATLFDWGFALNPQASVGSLGERFLDAATDQGPAGWLRA <mark>KTG</mark> SLTAINSL- TQPALRPLVDLLPIAGGSGTLSNRYLDTDAGRAAAGWLRA <mark>KTG</mark> SLTGTNAL- LYDQAIRLFDYGFSQ
Mtb_dacB1 Mtb_dacB2 Mtb_Rv3627c MSMEG_dacB MSMEG_2432 MSMEG_2433 MSMEG_1661 MSMEG_1900 <i>E.coli_</i> dacA <i>E.coli_</i> dacB <i>E.coli_</i> dacC_PBP6 <i>E.coli_</i> dacD_PBP6b	VRVGVAVIGALIVFGLIMVARAMNRRPQH
Mtb_dacB1 Mtb_dacB2 Mtb_Rv3627c MSMEG_dacB MSMEG_2432 MSMEG_2433 MSMEG_1661 MSMEG_1900 <i>E.coli_</i> dacA <i>E.coli_</i> dacA <i>E.coli_</i> dacB <i>E.coli_</i> dacD_PBP6b Mtb_dacB1 Mtb_dacB1	TINFQLDGKTIEQRPLVVLQEIPEGNFFGKIIDYIKLMFHHWFG

Mtb_Rv3627c	
MSMEG dacB	
MSMEG 2432	
MSMEG 2433	
MSMEG 1661	
MSMEG ¹⁹⁰⁰	
<i>E.coli</i> dacA	
<i>E.coli</i> dacB	LQGVYNLAGFITTASGQRMAFVQYLSGYAVEPADQRNRRIPLVRFESRLYKDIYQNN
<i>E.coli</i> dacC PBP6	
<i>E.coli</i> _dacD_PBP6b	

Figure S1A. Multiple sequence alignments for DD-CPase homologues. Shown are the DD-CPase homologues from *E. coli*, *M. tuberculosis* (Mtb) and *M. smegmatis* (MSMEG). The alignment was generated using Kalign (http://www.ebi.ac.uk/Tools/msa/kalign/). Conserved domains are highlighted in yellow (100% homologous) and turquoise (>= 75% homologous). The conserved Ser-Xaa-Xaa-Lys (SxxK), Ser-Xaa-Asn (SxN) and Lys-Thr-Gly (KTG) domains are shown in red boxes.

		20		40		60		80	(j
Mtb_Rv3330	M		AFLRSV	SCLAAAVFAV	GTGIGLPT	A	AGEPNAAPAA	- CPYKVSTPP	45
MSMEG_1661	M	1010101010	T-RRAV	TAVGA	LALLT	APLMT	AGVAGAEPGV	DCPYRETTPP	41
Mtb_Rv2911	M		RKLMTA	TAALCACAVT	VSAGA	A	WADADVQPA-		32
MSMEG_2432	M		RRLFAA	AAFALSTAL -	AAATETPV	A	HAEPAAAPA-		34
Mtb_Rv3627c	MG	•••••	PTRWRKST	HVVVGAAVLA	FVAVVVAAAA	LVTTGGHRA-	GVRAPAPPPR	PPTVKAGVVP	59
MSMEG_6113	MSDRLTRRGG	SPRSIESRGS	MRPTRWRRST	HVAVGVAVLA	LVVAVVAAAA	LFT GKHSD	AAEAVPPAPP	PATADPGVVP	78
Consensus	M		RWLRST	XVVXXAAXLA	AAAXAXPT	A	AAEAXAPPA-	- CXYK TXP	
Conservation									ĺ.
0%	Lannanaea	100		120		140		180	Ĝ
Mtb_Rv3330	AVDSSEVPAA	GEP - PLPLVV	PPTPVGGNAL	GGCGIITAPG	SAPAPGDVSA	EAWLVADLDS	GAVIAARDPH	GRHRP / SVIK	124
MSMEG 1661	AVDSSEVPQT	GNP - PMPLAV	PPKPVGGAAL	SCGIVTAPD	TPPVPGDLSA	EAWLVADLNS	GAV I AARDPH	GRHRP AS I I K	134
Mtb_Rv2911				GSVPIPD	GP A	QTWIVADLDS	GOVLAGROON	VAHPPASTIK	72
MSMEG_2433 MSMEG_2432				GAAAVTD	GPA	E TWV V ADMDT	GRULASKOPY	VRHAP ASTIK	77
Mtb_Rv3627c	VADTAATPSA	A		GVTAALA	VVAADPDLGK	LAGRITDALT	GOELWORLDD	VPLVPASTNK	117
MSMEG 6113	VTETAVVPSV	G		GLAAALA	SALADPSLGS	FGGRITDAMT	AKELWQQQDD	MPLVPASANK	117
Consensus	XVDSXEVP	G		GXXXAPX	GPPAPPDLXA	EAWXVADXDT	GXVLAARDPH	XRHRPASTIK	150
Conservation					_0_0_0_0				
0%		180	06mm80_00	200		220		240	1
Mtb Rv3330	VLVAMASINT	LTLNKSV				AGTAD	DAAVEGTKVG	VNTGGTYTVN	166
ML0691	VLLAMASINT	LNLNKSV				AGTAE	DAAAEGTKVG	VHDGGTYTIN	176
MSMEG_1661 Mtb Rv2911	VLIATAALNE	LNLNKRV				VADVA	DTQAECNCVG	VGPGGQYTTN	163
MSMEG_2433	TLLALVVLDE	VPLDSTI				VADET	DTDVECNCAG	VAPGRTYTAR	119
MSMEG_2432 Mtb Rv3627c	PLLAMVVLDH	LRPDNFA	VVAGGONPOG	PVVLVGAGDP	TLSAAPPGOD	TWYHGAARIG	DLVEQIRRSG		116
	LLTAAAALLT	LDRQTRISTR	VVAAGPNAQG	PVVLVGAGDP	TLSAASPDQS	TWYRGAPRIS	DLVEQVRRSG	VTPTAVQVDT	197
MSMEG_6113 Consensus	VLTAAALLI		VVAADDQP-G	LVVLRGGGDT	TLSAAPKGTD	TWYKGAARIS	DLADQVRARG	VXPCXXXTDX	215
Concention									2
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Mth Du2220		CNDAAXA	LADOL	COMPANIERI	NULLAAKLOOD		CLOCRCMS	TRAVDICIEN	024
MLD_RV3550 ML0691	QLLHGLLM	GNDAAHS	LAMQL	GGMQQALQKI	NVLAAKLGGQ	DT RVATPS	GLDGPGMS	TSAYDIGLEY	244
MSMEG_1661	DLLHGLLM	GNDAAHA	LAMQL	GGWDPALQKL	NTLAAKLGGR	DT RAATPS	GLDGPGMS	TSAYDIALFY	231
MSMEG_2433	QLLEAALL/S	GNDAANT	LARML	GGPEAAVAKM	NAKAAQLGAR	DT NVVTPS	GLDAPGMPFW	STPHDLAVIE	189
MSMEG_2432		GNDAANM	LADML	GGPRVAVAAM	NRKAAAVGAR	NT RAASPS	GLDGPGWESL	TTPHDLAVIE	186
ML0211	SLFTGPTMAQ	GWDPADVDNG	YTAPIESAMI	DAGRIOPTTV	KSRRSRTPAL	DAGRELAKAL	GVAPDAVTIV	-KASSGARQL	276
MSMEG_6113	SAYSGPTMAP	GWDPADIDGG	DIAPMESVML	DGGRTQPTTV	ESRRSKSPAL	DAGKALAAAL	GVEPESVTLM	PSGMRGGTTI	295
Consensus 100%	QLLHGLLMAS	GNDAAXX	LAXML	GGGRXAXXKX	NSXAAKLGAR	DI RXAIPS	GLDGPGMS	ISAXDXAXXX	
Conservation									ĺ.
		340 		360 1		380 		400 	e e e e e e e e e e e e e e e e e e e
Mtb_Rv3330	RYAWONPSES	DIVATRTEDE	PGHGDHPGYE	LENDNOLLYN	YPGALGOKTG	YTDDAGQTEV	GAANRDG	RRLMTVL	308
MSMEG_1661	RYAWQNPVFA	DIVHTOSYOF	PGR - DGGSYP	IENDNKLLAN	YPGALGOKTG	YTDDAGQTEV	GAAERDG	RRLIAVL	304
Mtb_Rv2911 MSMEG 2433	RAAMANPVFA	QITAEPSAME	PS DNGEQL	IVNODELLOR	YPGAIGGKTG	YTNAARKTEV	GAAARGG	RRLVIAN	256
MSMEG_2432	RAALNYPVIA	QILROTTAGE	PGKT	LTYQNELLTR	YPGDIGEKTG	YTNLARKTYV	GAAORGN	RRLVVVC	254
Mtb_Rv3627c ML 0211	AVVQSAPLIQ AVVQSAPLVQ	RESEMMONSO	N /MAECIGRE	VAVAINRPOS	FSGAVDAVTS	RENTAHIDTA	GAAL VDSSGL	SUDNRLTART	356
MSMEG_6113	AEVQSAPLIE	RLRQMMNESD	NMAESIARE	VAEALGRPQS	FEGAVGAVLT	QLRSVGIDTS	GAKLVDSSGL	SVDNRLTALT	375
Consensus	RXAXXNPVFA	XIVAMMXASF	PGDXXGRE	LXNXNELLQX	YPGALGGKTG	YTDDAGXTEV	GAALRDG	RRLXAVX	
Conservation				nononananan					ĺ.
		420 I		440 1		480 I		480 1	l.
Mtb_Rv3330	LHGTROPIPP	WEQAAHLL	DYGENTPAGT	QIGTLIEPDP	SLMSTDRNPA	DRORVDP	QAAARISAAD	ALPVRVGVAV	383
MSMEG_1661	LKGTRVPIAP	WEQAARLL	DYGFATPPGT	KVGTLVDPDP	SLA	PKQAEEPSPA	QAASVLPPAD	AMPVRVGVAV	375
Mtb_Rv2911	MYGLVKEGGP	TYWDQAATLF	DWGFALNPQA	SVGSL					291
MSMEG_2432	MYGT GD	LY - DQAIRLF	DYGFSQ	STGAL					275
Mtb_Rv3627c	LOATMQAAAG	PDOPALRPLL	DL LP IAG	GSGTL	GERFLDA	ATDOGPAGWL	RAKTGILTAI	NSLVGVLTDR	425
MSMEG_6113	LDEVVNAAAG	HTQPALRPLV	DL LP IAG	GSGTL	SNRYLDT	DAGRAAAGWL	R/KTG LTGT	NALAGIVTDR	444
Consensus	LYGTVQXXAP	- YWXQAAXLL	DYGFALPIXX	GXGTL	A	DA	XAXIGSLIAD	N - XVXVGXXX	1
Conservation					00-800-8e	80000000000			
0%		500	0						
Mtb_Rv3330	IGALIVEGLI	MVARAMNRRP	Q	H 405					
MSMEG_1661	VGALIVEGLI	LGARQLNORP	Q	H 411					
Mtb_Rv2911				291					
MSMEG_2433 MSMEG_2432				275					
Mtb_Rv3627c	SCRVLTFAFI	SNEAGPNGRN	AMDALATKLW	FCGCTT 461					
MSMEG_6113	SGRVLTFALI	SNNAGPTGRT	AIDALAAVLR	SCGCGA 480					
Consensus	SGRVXXFALI	SN-XGPNGRP	Χ	H					
Conservation									

Figure S1B. Multiple sequence alignments for mycobacterial DD-CPase homologues. Shown are the DD-CPase homologues from *M. tuberculosis* (Mtb), *M. smegmatis* (MSMEG) and *M. leprae* (ML). The alignment was generated using CLC sequence viewer (Version 7.8). Conserved domains are highlighted in red boxes and correspond to the SxxK, SxN and KTG motifs required for DD-CPase activity.



Figure S2. Homology trees for mycobacterial DD-CPase homologues. (A) Homology between four *M. smegmatis* mc2155 homologues. (B) Homology between three *M. tuberculosis* H37Rv homologues. In both cases, one homologue is located on a distinct branch in the phylogenetic tree (MSMEG_DacB and Rv3627c). Trees were compiled using DNAMAN software (version 4.13; Lynnon Biosoft).



Figure S3A. Genotypic characterization of the MSMEG_2432 deletion mutant by PCR and Southern blot analysis. The genomic map of the relevant locus is shown for the wild-type and mutant strain. Also shown on the left is the PCR confirmation of the genotype and Southern Blotting is shown on the right. For PCR confirmation, chromosomal DNA was used to amplify the MSMEG_2432 alleles from the wild-type and mutant strains using the primer pairs described in Table S4 and indicated as red arrows above. The expected sizes of the amplicons are as follows: MSMEG_2432, 1028 bp and $\Delta 2432$, 236 bp. For the Southern blot analysis chromosomal DNA from the SCO, parental and mutant strain was digested with *Mlu*I [M]. The probe used for hybridization is shown as a solid green box and the expected sizes are indicated with orange text. The figures are not drawn to scale.



Figure S3B. Genotypic characterization of the MSMEG_2433 deletion mutant by PCR and Southern blot analysis. The genomic map of the relevant locus is shown for the wild-type and mutant strain. Also shown on the left is the PCR confirmation of the genotype and Southern Blotting is shown on the right. For PCR confirmation, chromosomal DNA was used to amplify the MSMEG_2433 alleles from the wild-type and mutant strains using the primer pairs described in Table S4 and indicated as red arrows above. The expected sizes of the amplicons are as follows: MSMEG_2433, 1091 bp and Δ 2433, 236 bp. For the Southern blot analysis chromosomal DNA from the SCO, parental and mutant strain was digested with *Not*I [N]. The probe used for hybridization is shown as a solid green box and the expected sizes are indicated with orange text. The figures are not drawn to scale.



Figure S3C. Genotypic characterization of the MSMEG_1661 deletion mutant by PCR and Southern blot analysis. The genomic map of the relevant locus is shown for the wild-type and mutant strain. Also shown on the left is the PCR confirmation of the genotype and Southern Blotting is shown on the right. For PCR confirmation, chromosomal DNA was used to amplify the MSMEG_1661 alleles from the wild-type and mutant strains using the primer pairs described in Table S4 and indicated as red arrows above. The expected sizes of the amplicons are as follows: MSMEG_1661, 1394 bp and $\Delta 1661$, 236 bp. For the Southern blot analysis chromosomal DNA from the SCO, parental and mutant strain was digested with *NcoI* [N]. The probe used for hybridization is shown as a solid green box and the expected sizes are indicated with orange text. The figures are not drawn to scale.

 $mc^{2} 155$ $\Delta MSMEG_{2} 2432$ $\Delta MSMEG_{2} 2433$ $\Delta MSMEG_{1} 661$ $MSMEG_{1} 661$

Α



Figure S4. *In vitro* **assessment of phenotypes in single deletion mutants.** (A) Colony morphology of wild-type and single deletion mutants. Strains were grown to OD_{600nm} of 0.3 and 10 µl were subsequently spotted onto 7H10 solid media. Plates were incubated at 37 °C for 5 days and growth was monitored daily. (B) Representative SEM micrographs wild-type and mutant strains. Quantitative representation of distribution of cell lengths in wild-type and mutant strains. Number of cells counted for each strain (n) = 166, 203, 238 and 179, respectively.



Figure S5. Assessment of changes in cell wall stability. (A) SDS killing (0.2%, w/v) of wild-type and single deletion mutant strains. Cultures were grown to OD_{600nm} of 0.3 following the addition of SDS at time 0. At 3 and 6 hours, 1 ml of sample was removed to prepare 10-fold serial dilutions for plating on 7H10 solid media. The data are the average of three independent experiments plotted with standard errors. (B) Assessment of biofilm formation in DD-CPase mutant strains. Biofilm formation was measured by the formation of a floating pellicle at the liquid-air interface in Sauton's minimal media (pH 7) at 37 °C for 5 days. Images are representative of three biological repeats.



Figure S6. Fluorescence microscopy and quantitative assessment of peptidoglycan localization in DD-CPase deletion mutants. (A) BODIPY-vancomycin localization in *M. smegmatis*: (I) Wildtype, (II) $\Delta 2432$, (III) $\Delta 2433$, (IV) $\Delta 1661$ mutants. Strains were grown to OD_{600nm} of 0.3 and then stained with BODIPY-vancomycin, three representative images are shown for each strain. Scale bar = 5 µm. (B) Quantitative assessment of the distribution of staining patterns. Data is representative of one biological repeat where *n* (number of cells counted for each strain) = 77, 84, 90 and 223 for wildtype, $\Delta MSMEG_2432$, $\Delta MSMEG_2433$ and $\Delta MSMEG_1661$ respectively.



Figure S7A. Southern blot to confirm upstream and downstream SCOs for *dacB* **mutant.** Shown are the genomic maps for the wild-type, upstream (US) single crossover (SCO) and downstream (DS) SCO. Chromosomal DNA was isolated from wild-type (1), US SCO (2) and DS DSCO (3) and digested with *Bam*HI. The probe used for hybridization is shown as a solid green box and the expected sizes are indicated in orange text.

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Figure S7B. Attempts to delete *dacB* in a merodiploid strain containing an additional copy of *dacB*, inserted at the *attB* site. Shown are genomic maps corresponding to wild-type, $\Delta dacB$, the upstream single crossover (SCO) and resulting merodiploid strain. Southern hybridizations were performed by digesting 2 µg of chromosomal DNA isolated from the wild-type (1), the merodiploid strain (2) and possible double crossover recombinants isolated from counter selection of the merodiploid strain (3-6), was digested with *Bam*HI and probed with a region homologous to 1.2 kb upstream of *dacB* (green box). Expected fragment sizes are indicated in orange text. Counter selection yielded the wild-type (3705 bp) and not the mutant (2312 bp) band. Figure not drawn to scale.

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Figure S8. Genotypic characterization of the MSMEG_6113 (*dacB*) promoter replacement mutant by PCR and Southern blot analysis. The genomic map of the relevant locus is shown for the wild-type and mutant strain. Also shown on the left is the PCR confirmation of the genotype and Southern blotting is shown on the right. For PCR confirmation, chromosomal DNA was used to yield an amplicon specific for *dacB* directly downstream of Pmyc1tetO from the mutant strains (absent in wild-type) using the primer pairs described in Table S4, shown as red arrows. The expected sizes of all amplicons are 984 bp. For the Southern blot analysis chromosomal DNA from the parental and mutant strains were digested with *SmaI* [S]. The probe used for hybridization is shown as a solid green box and the expected sizes are indicated with orange text. The figures are not drawn to scale.



wild-type



dacB TetONs - repressed





dacB TetONs - repressed



dacB TetOFF - repressed





dacB TetOFF - repressed

Figure S9. Representative images of PG staining patterns observed in *dacB* **knockdown strains.** Cells corresponding to the wild-type (A), *dacB* TetON_s 0 ng/ml aTc (repressed) (B) and *dacB* TetOFF 100 ng/ml aTc (repressed) (C) were stained with BODIPY-vancomycin during late logarithmic growth. Images were captured in the DIC and FITC channels and staining patterns were quantitated as either bipolar (yellow arrows) or monopolar (blue arrows). Scale bars depicted in each image.





References

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- 2. Parish, T. & Stoker, N. Use of a flexible cassette method to generate a double unmarked Mycobacterium tuberculosis tlyA plcABC mutant by gene replacement. *Microbiology* **146**, 1969–75 (2000).