Characterization of putative DD-carboxypeptidase-encoding genes in *Mycobacterium smegmatis*

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SUPPLEMENTARY INFORMATION

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Running title: Putative DD-carboxypeptidases in mycobacteria

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Supplementary Tables

Table S1. Bacterial strains and plasmids used in this study.

Name	Description	Source/Reference
Strains		
M. smegmatis		
mc^2155	*ept-1* (efficient plasmid transformation) mutant of mc^26	[1]
Δ2432	Derivative of mc^2155 carrying an unmarked, out-of-frame deletion in *M. smegmatis* Msmeg_2432 (786bp internal region excised)	This work
Δ2433	Derivative of mc^2155 carrying an unmarked, out-of-frame deletion in *M. smegmatis* Msmeg_2433 (870bp internal region excised)	This work
Δ1661	Derivative of mc^2155 carrying an unmarked, in-frame deletion in *M. smegmatis* Msmeg_1661 (1158bp internal region excised)	This work
Δ*dacB* TetO	Derivative of mc^2155 in which the native promoter of Msmeg_6113 (*dacB*) is substituted with Pmyc1tetO. The native promoter regulates expression of a truncated (350 bp) 5' region of *dacB*; Hyg^r. This strain contains no Anhydrotetracycline-responsive repressor in the L5 attB site thus termed repressor-less.	This work
Δ*dacB* $TetON_s$	Derivative of mc^2155 in which the native promoter of Msmeg_6113 (*dacB*) is substituted with Pmyc1tetO. The native promoter regulates expression of a truncated (350 bp) 5' region of *dacB*; Hyg^r. This strain contains the "strong" Anhydrotetracycline-responsive repressor (tetR) in the L5 attB site derived from pMC1s.	This work
Δ*dacB* TetOFF	Derivative of mc^2155 in which the native promoter of Msmeg_6113 (*dacB*) is substituted with Pmyc1tetO. The native promoter regulates expression of a truncated (350 bp) 5' region of *dacB*; Hyg^r. This strain contains the "reverse" Anhydrotetracycline-responsive repressor (revtetR) in the L5 attB site derived from pTEK-4S0X.	This work
Δ*dacB attB*::pMV*dacB*	Derivative of mc^2155 carrying native *dacB*, Δ*dacB* and additional copy of native *dacB* in the L5 *attB* site. Strain was constructed by electroporation with pMV*dacB* and p2Δ*dacB*g17 vectors; Hyg^r, Km^r.	This work
Δ*dacB attB*::pMVRv3627c_operon	Derivative of mc^2155 carrying native *dacB*, Δ*dacB* and additional copy of homologous operon from *M. tuberculosis* in the L5 *attB* site. Strain was constructed by electroporation with pMVRv3627c_operon and p2Δ*dacB*g17 vectors; Hyg^r, Km^r.	This work

Plasmids		
p2NIL	*E. coli* cloning vector, Kan^r	[2]
pMV306H	*E. coli*-Mycobacterium integrating shuttle vector. Integrates in the L5 *attB* site in mycobacteria. Derivative of pMV306 ([3]) carrying a *hyg* gene; Hyg^r	H. Boshoff
pMV*dacB*	Derivative of pMV306H carrying full-length *dacB* (1443 bp) plus 150 bp upstream of start codon representative of native promoter sequence; Hyg^r	This work
pMVRv3627c_operon	Derivative of pMV306H carrying four gene operon (Rv3627c, Rv3626c, Rv3625c (mesJ) and Rv3624c (hpt)) from *M. tuberculosis*; Hyg^r	This work
pMV1661	Derivative of pMV306H carrying full-length MSEMG_1661 (1194 bp) plus 150 bp upstream of start codon representative of native promoter sequence; Hyg^r	This work
pMV2432	Derivative of pMV306H carrying full-length MSMEG_2432 and MSMEG_2433 (1788 bp) plus 150 bp upstream of start codon representative of native promoter sequence; Hyg^r	This work
pMV2433	Derivative of pMV306H carrying full-length MSMEG_2433 (891 bp) plus 150 bp upstream of start codon representative of native promoter sequence; Hyg^r	This work
pGOAL17	Plasmid carrying *lacZ* and *sacB* markers as a *PacI* cassette; Km^r	[2]
pGOAL19	Plasmid carrying *lacZ-sacB-Hyg^r* markers as a *PacI* cassette; Hyg^r	[2]
p2Δ2432	Knockout vector for creating ΔMsmeg_2432 mutant. The 1.2 kb upstream region including 18 bp of the 5' sequence of Msmeg_2432 was PCR-amplified to incorporate *HindIII* and *NdeI* restriction sites. The 1.2 kb downstream was similarly obtained but incorporated *NdeI* and *Acc65I r*estriction sites. The fragments were cloned into p2NIL and the *PacI* cassette from pGOAL19 was inserted; Km^r Hyg^r.	This work
p2Δ2433	Knockout vector for creating ΔMsmeg_2433 mutant. The 1.2 kb upstream region including 18 bp of the 5' sequence of Msmeg_2433 was PCR-amplified to incorporate *HindIII* and *NdeI* restriction sites. The 1.2 kb downstream was similarly obtained but incorporated *NdeI* and *Acc65I r*estriction sites. The fragments were cloned into p2NIL and the *PacI* cassette from pGOAL19 was inserted; Km^r Hyg^r.	This work
p2Δ1661	Knockout vector for creating ΔMsmeg_1661 mutant. The 1.2 kb upstream region including 18 bp of the 5' sequence of Msmeg_2433 was PCR-amplified to incorporate *HindIII* and *NdeI* restriction sites. The 1.2 kb downstream was similarly obtained but incorporated *NdeI* and *Acc65I r*estriction sites. The fragments were cloned into p2NIL and the *PacI* cassette from pGOAL19 was inserted; Km^r Hyg^r.	This work
p2Δ*dacB*	Knockout vector for creating ΔMsmeg_6113 (*dacB*) mutant. The 1.2 kb upstream region including 18 bp of the 5' sequence of Msmeg_2433 was PCR-amplified to incorporate *HindIII* and *NotI* restriction sites. The 1.2 kb downstream was similarly obtained but incorporated *NotI* and *Acc65I r*estriction sites. The	This work

	fragments were cloned into p2NIL and the *PacI* cassette from pGOAL19 was inserted; Km^r Hyg^r.	
p2Δ*dacB*g17	Knockout vector for creating ΔMsmeg_6113 (*dacB*) mutant. The 1.2 kb upstream region including 18 bp of the 5' sequence of Msmeg_2433 was PCR-amplified to incorporate *HindIII* and *NotI* restriction sites. The 1.2 kb downstream was similarly obtained but incorporated *NotI* and *Acc65I r*estriction sites. The fragments were cloned into p2NIL and the *PacI* cassette from pGOAL17 was inserted; Km^r.	This work
pSE100	Derivative of pMS2 containing Pmyc1tetO carrying a *hyg* gene; Hyg^r. pMS2 is a shuttle vector containing origins of replication for *Escherichia coli* (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 *E. coli* and low-copy number in mycobacteria.	Sabine Ehrt
p*SdacB*	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 *dacB* cloned directly downstream of the Pmyc1tetO using *SphI* and *MscI*; Hyg^r.	This work

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

Region amplified	Primer name	Sequence (5' – 3')[a]	Description of amplicon	Mutation
Msmeg_6113 (*dacB*) upstream region	dacB_UF	5' – CGCGAAGCTT GCGAGCCCCTGGTGAACC	1200 bp of upstream and 21 bp of the 5'-end of Msmeg_6113 (*dacB*)	1400 bp unmarked deletion in *dacB*
	dacB_UR	5' – CGCGGCGGCCGCGCGCGTCAGCCTATCGCTCAT		
Msmeg_6113 (*dacB*) downstream region	dacB_DF	5' – CGCGGCGGCCGCCAGCTGCGGATGCGGCGCATGA	1339 bp of downstream and 22 bp of the 3'-end of Msmeg_6113 (*dacB*)	
	dacB_DR	5' – CGCGGGTACCATCTCGGCGCCAACACAT		
Msmeg_1661 upstream region	1661_UF	5' – CGCGAAGCTTGCGAGGCCGTCGAGAGCG	1200 bp of upstream and 18 bp of the 5'-end of Msmeg_1661	1158 bp unmarked deletion in Msmeg_1661
	1661_UR	5' – CGCGCATATGCACCGCGCGTCGCGTCAT		
Msmeg_1661 downstream region	1661_DF	5' – CGCGCATATGCAGCGCCCGCAGCACTGA	1375 bp of downstream and 18 bp of the 3'-end of Msmeg_1661	
	1661_DR	5' – CGCGGGTACCATTCGGGTTGTCGCGGTT		
Msmeg_2432 upstream region	2432_UF	5' – CGCGAAGCTTCCGGGAACGCAGGCAGC	1200 bp of upstream and 21 bp of the 5'-end of Msmeg_2432	786 bp unmarked deletion in Msmeg_2432
	2432_UR	5' – CGCGCATATGCGCCGCGAACAGTCTTCGCAC		
Msmeg_2432 downstream region	2432_DF	5' – CGCGCATATGGACTACGGCTTCAGCCAGTAG	1200 bp of downstream and 18 bp of the 3'-end of Msmeg_2432	
	2432_DR	5' – CGCGGGTACCGGCCGACTTCGACCTGTCG		
Msmeg_2433 upstream region	2433_UF	5' – CGCGAAGCTTACGATCCCGCGGACCAGG	1200 bp of upstream and 21 bp of the 5'-end of Msmeg_2433	870 bp unmarked deletion in Msmeg_2433
	2433_UR	5' – CGCGCATATGCCCGAAGGCGTACCTCCACAT		
Msmeg_2433 downstream region	2433_DF	5' – CGCGCATATGGCGAGCATCGGGGCGCTCTGA	1200 bp of downstream and 21 bp of the 3'-end of Msmeg_2433	
	2433_DR	5' – CGCGGGTACCATCGGCCACGGCCGCATC		

a. Restriction sites are underlined.

Table S3. Primers used for gene expression analysis by qPCR.

Gene	**Primer name**	**Sequence (5' – 3')**	**Amplicon size (bp)**
Msmeg_6113 (*dacB*)	*dacB*_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*)	KO*dacB*_F	5' – ACGACGTCGAACTCCACC	FR1 = 800 bp (wild-type)
	KO*dacB*_R1	5' – CGTCGATGTCGGCCGGGTC	FR1 = absent (Δ)
	KO*dacB*_R2	5' – ATGGCCTGGCGGCGCGTG	FR2 = 1643 bp (wild-type)
			FR2 = 242 bp (Δ)
Msmeg_1661	KO1661_F	5' – CACACGAGTCACAACTGT	FR1 = 701 bp (wild-type)
	KO1661_R1	5' – TGGCGGCCAGCGTGTTGA	FR1 = absent (Δ)
	KO1661_R2	5' – CGGCATGGTGAACCAGTC	FR2 = 1394 bp (wild-type)
			FR2 = 236 bp (Δ)
Msmeg_2432	KO2432_F	5' – GGACCGTTCGGCGAGCAT	FR1 = 500 bp (wild-type)
	KO2432_R1	5' – TGTCGGCGAGCATGTTGG	FR1 = absent (Δ)
	KO2432_R2	5' – CGGATCTGGTGATCCTGC	FR2 = 1028 bp (wild-type)
			FR2 = 242 bp (Δ)
Msmeg_2433	KO2433_F	5' – GCAGTCGAATAGTCCACC	FR1 = 500 bp (wild-type)
	KO2433_R1	5' – CCGCGTCGTTGCCCGACG	FR1 = absent (Δ)
	KO2433_R2	5' – AGTGCCGTGCTGAGGGCG	FR2 = 1028 bp (wild-type)
			FR2 = 242 bp (Δ)

Table S5. Primers used to amplify Msmeg_6113 (*dacB*) for complementation and for construction and confirmation of merodiploid strain in *M. smegmatis*.

Gene	Primer name	Sequence (5' – 3')[a]	Description	Amplicon size (bp)
MSMEG_6113 (*dacB*)	Comp_*dacB*_F Comp_*dacB*_R	5'–GCGCACGCGTTCTCGTGATCCACCTCGT–3' 5'–GCGCCTGCAGTCATGCGCCGCATCCGCA–3'	150 bp upstream of 5'–end amplified with full-length *dacB* to maintain native promoter sequences (-10 and -35)	1593 bp
MSMEG_1661	Comp_1661_F Comp_1661_R	5'–GCGCACGCGTGAGAAGTTCGAGTAACGC–3' 5'–GCGCACGCGTTCAGTGCTGCGGGCGCTG–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'–GCGCAAGCTTCGTCCGGGGTGAACACCG–3' 5'–GCGCAAGCTTCTACTGGCTGAAGCCGTA–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'–GCGCAAGCTTTCAGAGCGCCCCGATGCT–3'	150 bp upstream of 5'–end amplified with full-length MSMEG_2433to maintain native promoter sequences (-10 and -35)	1061 bp
attB (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*dacB* in the bacterial chromosome	282 bp
	attBS2_F attL4_R	5' – ACA GGA TTT GAA CCT GCG GC – 3' 5' – AAT TCT TGC AGA CCC CTG GA – 3'		320 bp

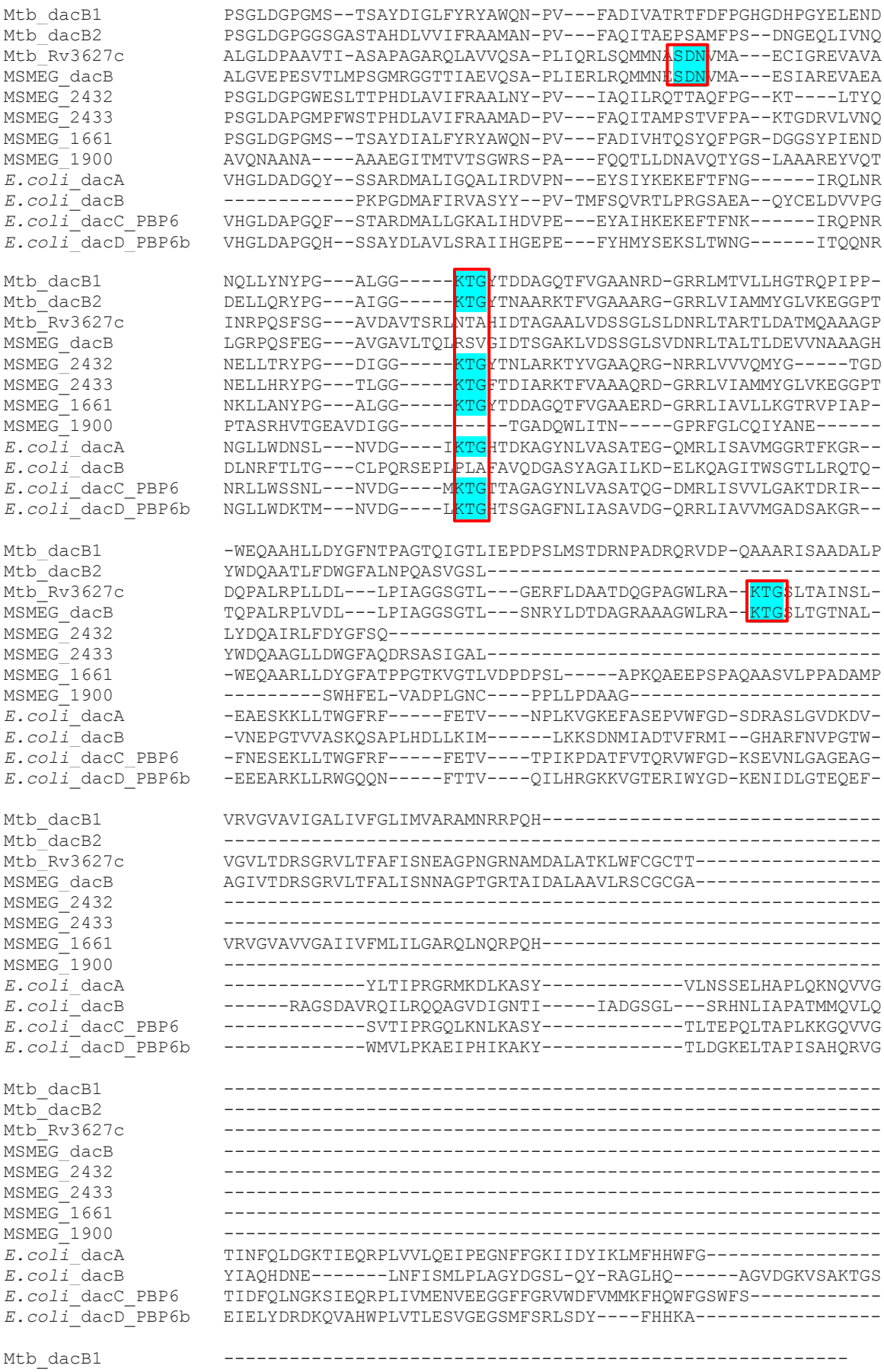
a. Restriction sites are underlined.

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

Gene	**Primer name**	**Sequence (5' – 3')**[a]	**Description**	**Amplicon size (bp)**
Msmeg_6113 (*dacB*)	KD_*dacB*_F	5' – GCGCATGCCCGGGCCAACCTTAG**ATG**AGCGATA	15 bp upstream of start codon and 350 bp of 5'–end harbouring putative native promoter (-10) sequence	393 bp
	KD_*dacB*_R	5' – GCGCCCCGGGCCCGTGTCGGCGTCGGTG		

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

Supplementary Figures


Mtb_dacB1 PSGLDGPGMS--TSAYDIGLFYRYAWQN-PV---FADIVATRTFDFPGHGDHPGYELEND
Mtb_dacB2 PSGLDGPGGSGASTAHDLVVIFRAAMAN-PV---FAQITAEPSAMFPS--DNGEQLIVNQ
Mtb_Rv3627c ALGLDPAAVTI-ASAPAGARQLAVVQSA-PLIQRLSQMMNASDNVMA---ECIGREVAVA
MSMEG_dacB ALGVEPESVTLMPSGMRGGTTIAEVQSA-PLIERLRQMMNISDNVMA---ESIAREVAEA
MSMEG_2432 PSGLDGPGWESLTTPHDLAVIFRAALNY-PV---IAQILRQTTAQFPG--KT----LTYQ
MSMEG_2433 PSGLDAPGMPFWSTPHDLAVIFRAAMAD-PV---FAQITAMPSTVFPA--KTGDRVLVNQ
MSMEG_1661 PSGLDGPGMS--TSAYDIALFYRYAWQN-PV---FADIVHTQSYQFPGR-DGGSYPIEND
MSMEG_1900 AVQNAANA----AAAEGITMTVTSGWRS-PA---FQQTLLDNAVQTYGS-LAAAREYVQT
E.coli_dacA VHGLDADGQY--SSARDMALIGQALIRDVPN---EYSIYKEKEFTFNG------IRQLNR
E.coli_dacB ------------PKPGDMAFIRVASYY--PV-TMFSQVRTLPRGSAEA--QYCELDVVPG
E.coli_dacC_PBP6 VHGLDAPGQF--STARDMALLGKALIHDVPE---EYAIHKEKEFTFNK------IRQPNR
E.coli_dacD_PBP6b VHGLDAPGQH--SSAYDLAVLSRAIIHGEPE---FYHMYSEKSLTWNG------ITQQNR
Mtb_dacB1 NQLLYNYPG---ALGG-----KTGYTDDAGQTFVGAANRD-GRRLMTVLLHGTRQPIPP-
Mtb_dacB2 DELLQRYPG---AIGG-----KTGYTNAARKTFVGAAARG-GRRLVIAMMYGLVKEGGPT
Mtb_Rv3627c INRPQSFSG---AVDAVTSRLNTAHIDTAGAALVDSSGLSLDNRLTARTLDATMQAAAGP
MSMEG_dacB LGRPQSFEG---AVGAVLTQLRSVGIDTSGAKLVDSSGLSVDNRLTALTLDEVVNAAAGH
MSMEG_2432 NELLTRYPG---DIGG-----KTGYTNLARKTYVGAAQRG-NRRLVVVQMYG-----TGD
MSMEG_2433 NELLHRYPG---TLGG-----KTGFTDIARKTFVAAAQRD-GRRLVIAMMYGLVKEGGPT
MSMEG_1661 NKLLANYPG---ALGG-----KTGYTDDAGQTFVGAAERD-GRRLIAVLLKGTRVPIAP-
MSMEG_1900 PTASRHVTGEAVDIGG----------TGADQWLITN-----GPRFGLCQIYANE------
E.coli_dacA NGLLWDNSL---NVDG----IKTGHTDKAGYNLVASATEG-QMRLISAVMGGRTFKGR--
E.coli_dacB DLNRFTLTG---CLPQRSEPLPLAFAVQDGASYAGAILKD-ELKQAGITWSGTLLRQTQ-
E.coli_dacC_PBP6 NRLLWSSNL---NVDG----MKTGTTAGAGYNLVASATQG-DMRLISVVLGAKTDRIR--
E.coli_dacD_PBP6b NGLLWDKTM---NVDG----LKTGHTSGAGFNLIASAVDG-QRRLIAVVMGADSAKGR--
Mtb_dacB1 -WEQAAHLLDYGFNTPAGTQIGTLIEPDPSLMSTDRNPADRQRVDP-QAAARISAADALP
Mtb_dacB2 YWDQAATLFDWGFALNPQASVGSL----------
Mtb_Rv3627c DQPALRPLLDL---LPIAGGSGTL---GERFLDAATDQGPAGWLRA--KTGSLTAINSL-
MSMEG_dacB TQPALRPLVDL---LPIAGGSGTL---SNRYLDTDAGRAAAGWLRA--KTGSLTGTNAL-
MSMEG_2432 LYDQAIRLFDYGFSQ----------
MSMEG_2433 YWDQAAGLLDWGFAQDRSASIGAL----------
MSMEG_1661 -WEQAARLLDYGFATPPGTKVGTLVDPDPSL-----APKQAEEPSPAQAASVLPPADAMP
MSMEG_1900 ---------SWHFEL-VADPLGNC----PPLLPDAAG----------
E.coli_dacA -EAESKKLLTWGFRF-----FETV----NPLKVGKEFASEPVWFGD-SDRASLGVDKDV-
E.coli_dacB -VNEPGTVVASKQSAPLHDLLKIM------LKKSDNMIADTVFRMI--GHARFNVPGTW-
E.coli_dacC_PBP6 -FNESEKLLTWGFRF-----FETV----TPIKPDATFVTQRVWFGD-KSEVNLGAGEAG-
E.coli_dacD_PBP6b -EEEARKLLRWGQQN-----FTTV----QILHRGKKVGTERIWYGD-KENIDLGTEQEF-
Mtb_dacB1 VRVGVAVIGALIVFGLIMVARAMNRRPQH----------
Mtb_dacB2 ----------
Mtb_Rv3627c VGVLTDRSGRVLTFAFISNEAGPNGRNAMDALATKLWFCGCTT----------
MSMEG_dacB AGIVTDRSGRVLTFALISNNAGPTGRTAIDALAAVLRSCGCGA----------
MSMEG_2432 ----------
MSMEG_2433 ----------
MSMEG_1661 VRVGVAVVGAIIVFMLILGARQLNQRPQH----------
MSMEG_1900 ----------
E.coli_dacA -------------YLTIPRGRMKDLKASY-------------VLNSSELHAPLQKNQVVG
E.coli_dacB ------RAGSDAVRQILRQQAGVDIGNTI-----IADGSGL---SRHNLIAPATMMQVLQ
E.coli_dacC_PBP6 -------------SVTIPRGQLKNLKASY-------------TLTEPQLTAPLKKGQVVG
E.coli_dacD_PBP6b -------------WMVLPKAEIPHIKAKY-------------TLDGKELTAPISAHQRVG
Mtb_dacB1 ----------
Mtb_dacB2 ----------
Mtb_Rv3627c ----------
MSMEG_dacB ----------
MSMEG_2432 ----------
MSMEG_2433 ----------
MSMEG_1661 ----------
MSMEG_1900 ----------
E.coli_dacA TINFQLDGKTIEQRPLVVLQEIPEGNFFGKIIDYIKLMFHHWFG----------
E.coli_dacB YIAQHDNE-------LNFISMLPLAGYDGSL-QY-RAGLHQ------AGVDGKVSAKTGS
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E.coli_dacD_PBP6b EIELYDRDKQVAHWPLVTLESVGEGSMFSRLSDY----FHHKA----------
Mtb_dacB1 ----------
Mtb_dacB2 ----------

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Mtb_Rv3627c          ------------------------------------------------------------
MSMEG_dacB           ------------------------------------------------------------
MSMEG_2432           ------------------------------------------------------------
MSMEG_2433           ------------------------------------------------------------
MSMEG_1661           ------------------------------------------------------------
MSMEG_1900           ------------------------------------------------------------
E.coli_dacA          ------------------------------------------------------------
E.coli_dacB          LQGVYNLAGFITTASGQRMAFVQYLSGYAVEPADQRNRRIPLVRFESRLYKDIYQNN
E.coli_dacC_PBP6     ------------------------------------------------------------
E.coli_dacD_PBP6b    ------------------------------------------------------------
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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 (S*xx*K), Ser-Xaa-Asn (S*x*N) and Lys-Thr-Gly (KTG) domains are shown in red boxes.

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 S*xx*K, S*x*N and KTG motifs required for DD-CPase activity.

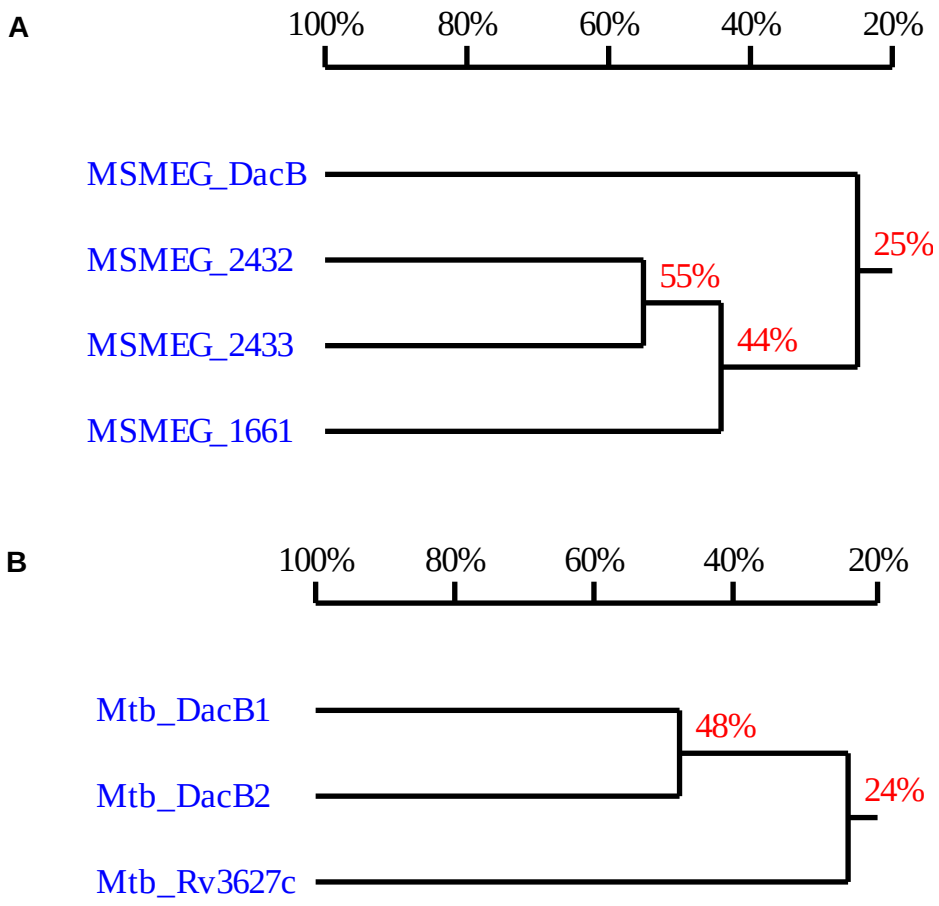


Figure S2. Homology trees for mycobacterial DD-CPase homologues. (A) Homology between four *M. smegmatis* mc2155 homologues. (B) Homology between three *M. tuberculosis* H37Rv homologues. In both cases, one homologue is located on a distinct branch in the phylogenetic tree (MSMEG_DacB and Rv3627c). Trees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

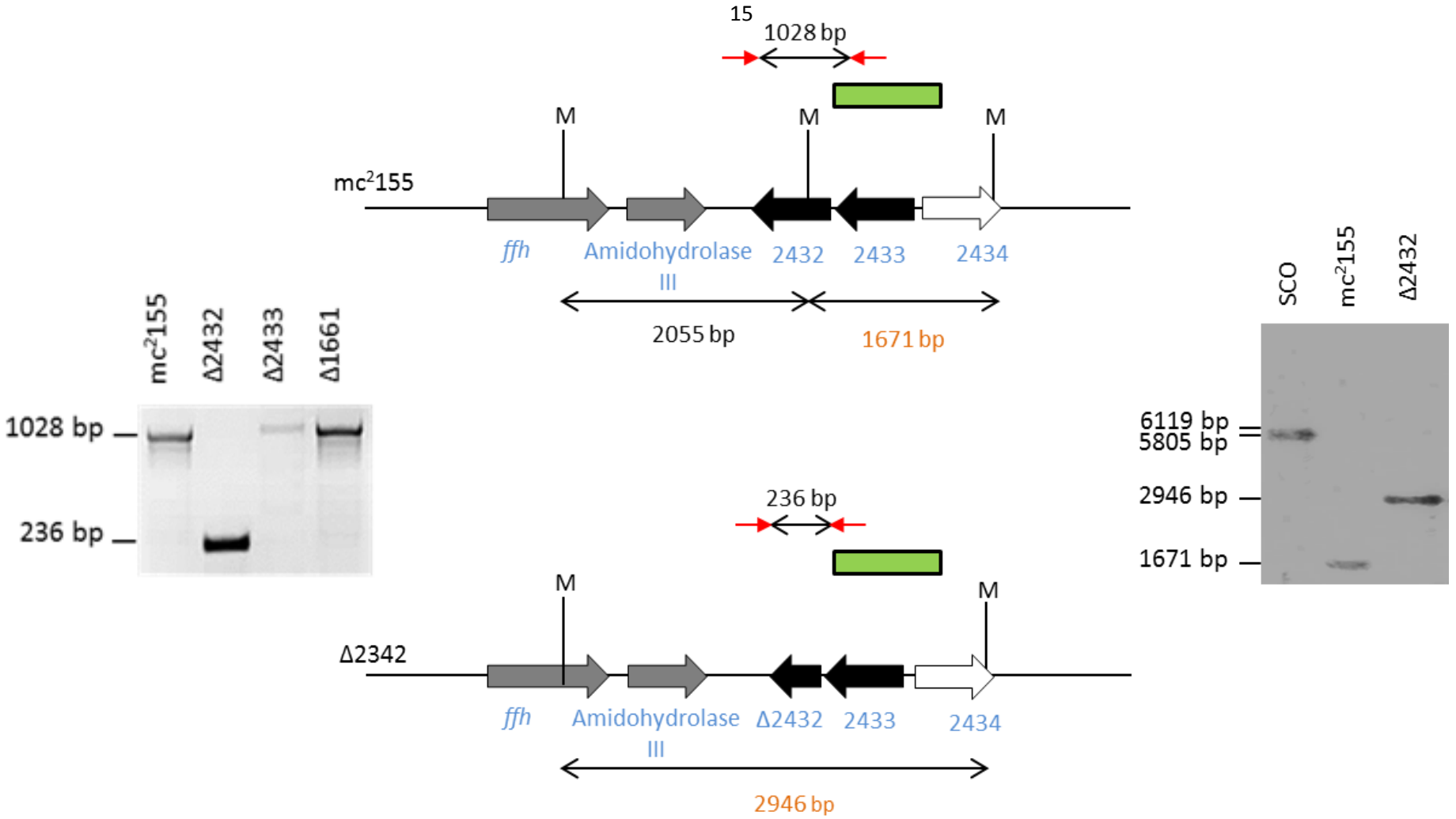


Figure S3A. Genotypic characterization of the MSMEG_2432 deletion mutant by PCR and Southern blot analysis. The genomic map of the relevant locus is shown for the wild-type and mutant strain. Also shown on the left is the PCR confirmation of the genotype and Southern Blotting is shown on the right. For 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 Δ2432, 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.

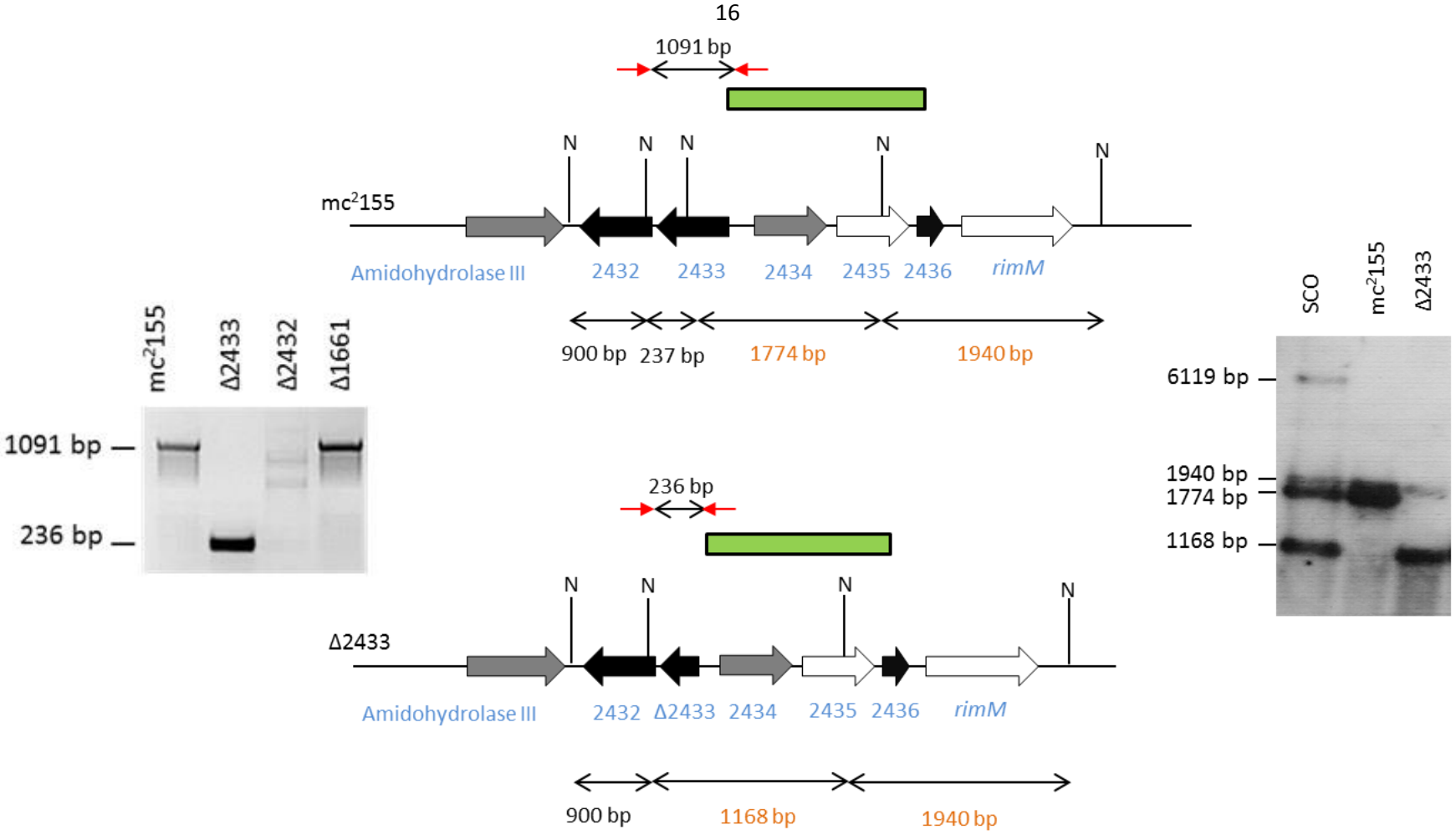


Figure S3B. Genotypic characterization of the MSMEG_2433 deletion mutant by PCR and Southern blot analysis. The genomic map of the relevant locus is shown for the wild-type and mutant strain. Also shown on the left is the PCR confirmation of the genotype and Southern Blotting is shown on the right. For 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 Δ2433, 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.

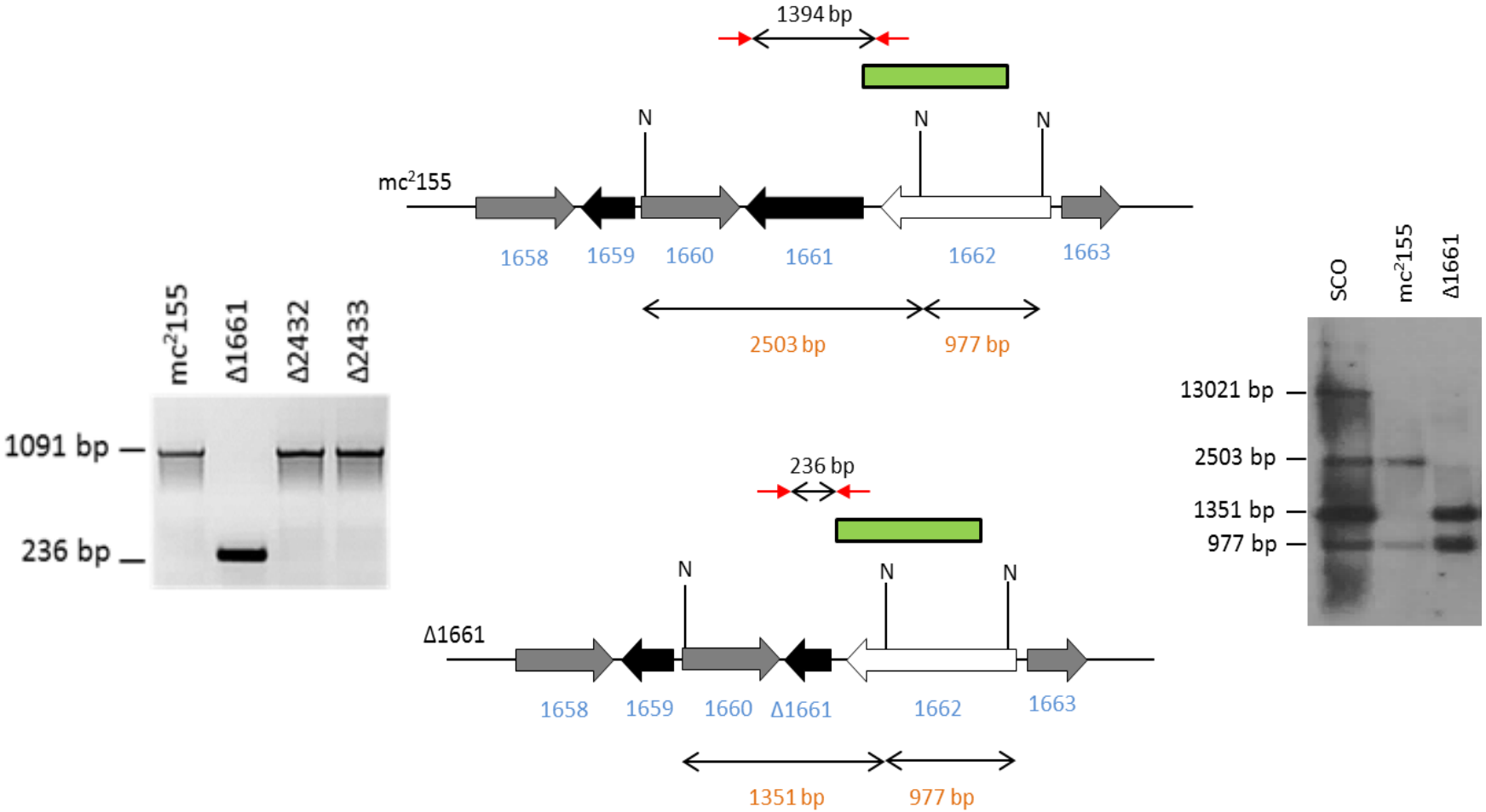


Figure S3C. Genotypic characterization of the MSMEG_1661 deletion mutant by PCR and Southern blot analysis. The genomic map of the relevant locus is shown for the wild-type and mutant strain. Also shown on the left is the PCR confirmation of the genotype and Southern Blotting is shown on the right. For 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 Δ1661, 236 bp. For the Southern blot analysis chromosomal DNA from the SCO, parental and mutant strain was digested with *Nco*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.

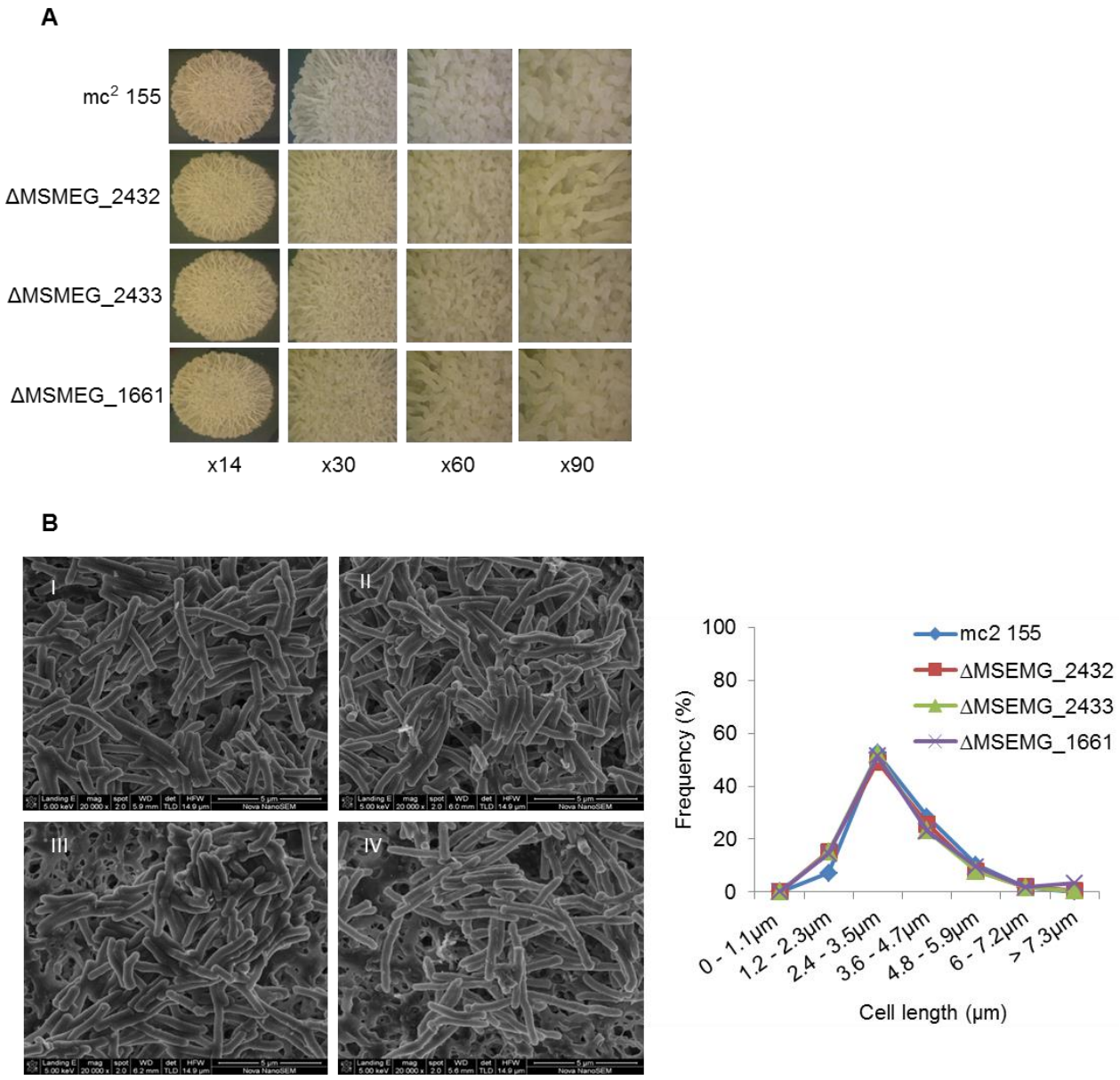


Figure S4. ***In vitro*** **assessment of phenotypes in single deletion mutants.** (A) Colony morphology of wild-type and single deletion mutants. Strains were grown to OD_{600nm} of 0.3 and 10 µl were subsequently spotted onto 7H10 solid media. Plates were incubated at 37 °C for 5 days and growth was 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.

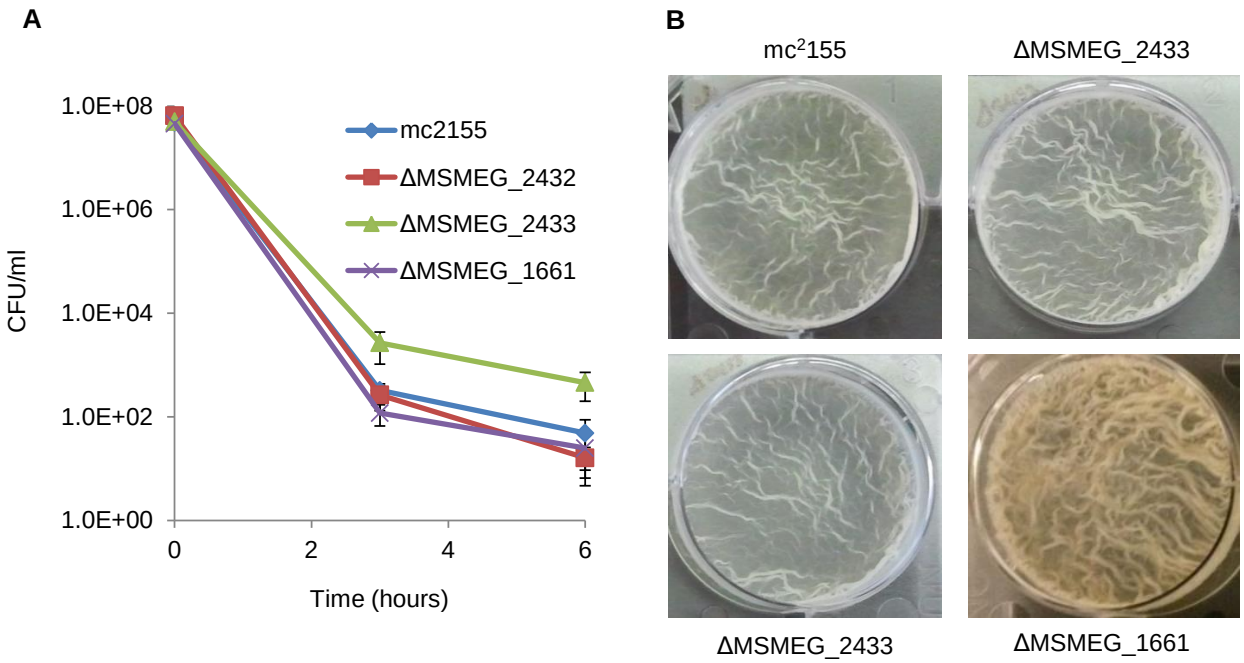


Figure S5. Assessment of changes in cell wall stability. (A) SDS killing (0.2%, w/v) of wild-type and single deletion mutant strains. Cultures were grown to OD_{600nm} of 0.3 following the addition of SDS at time 0. At 3 and 6 hours, 1 ml of sample was removed to prepare 10-fold serial dilutions for 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.

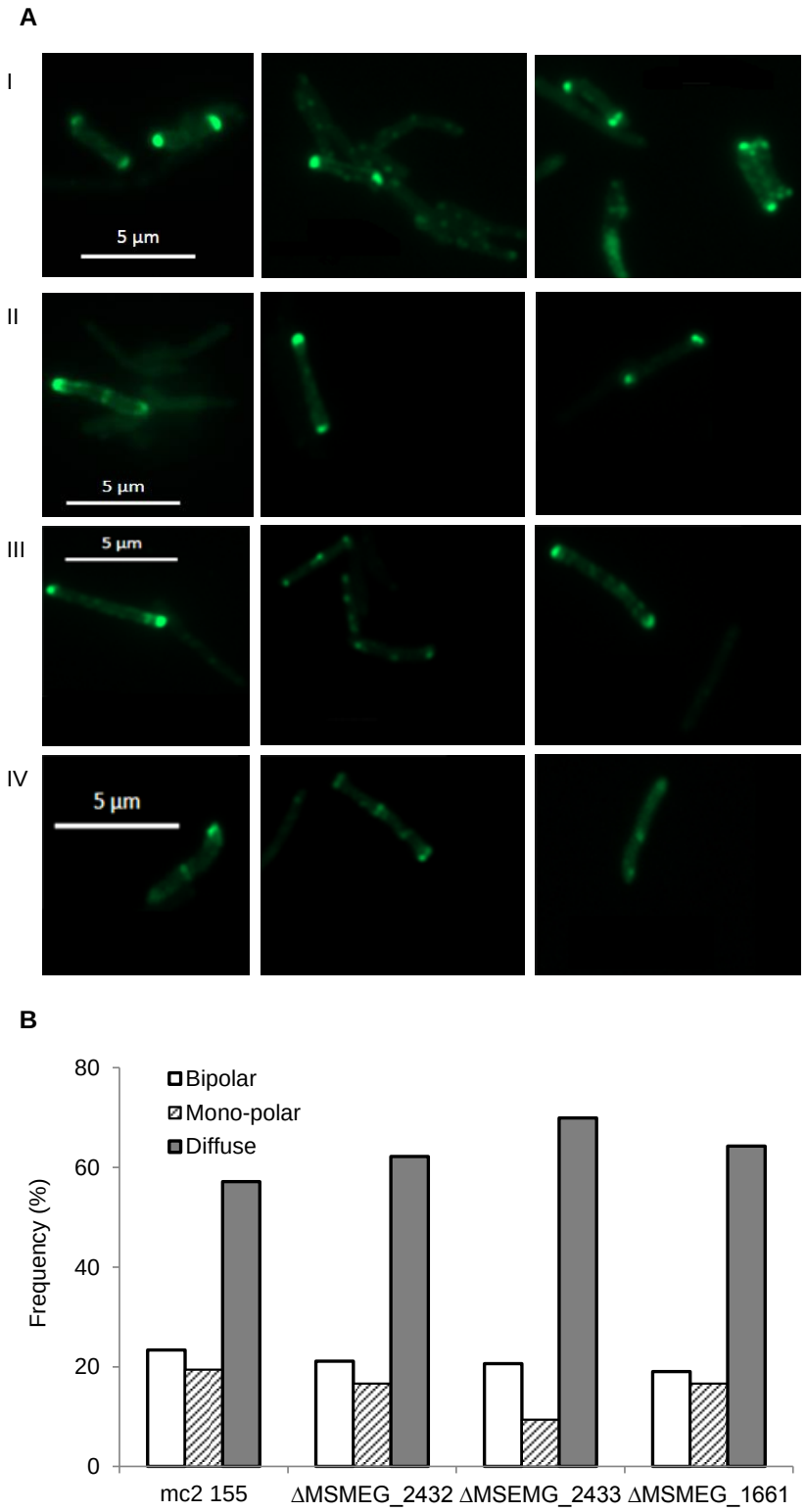


Figure S6. Fluorescence microscopy and quantitative assessment of peptidoglycan localization in DD-CPase deletion mutants. (A) BODIPY-vancomycin localization in *M. smegmatis*: (I) Wild-type, (II) Δ2432, (III) Δ2433, (IV) Δ1661 mutants. Strains were grown to OD_{600nm} of 0.3 and then stained with BODIPY-vancomycin, 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 wild-type, ΔMSMEG_2432, ΔMSMEG_2433 and ΔMSMEG_1661 respectively.

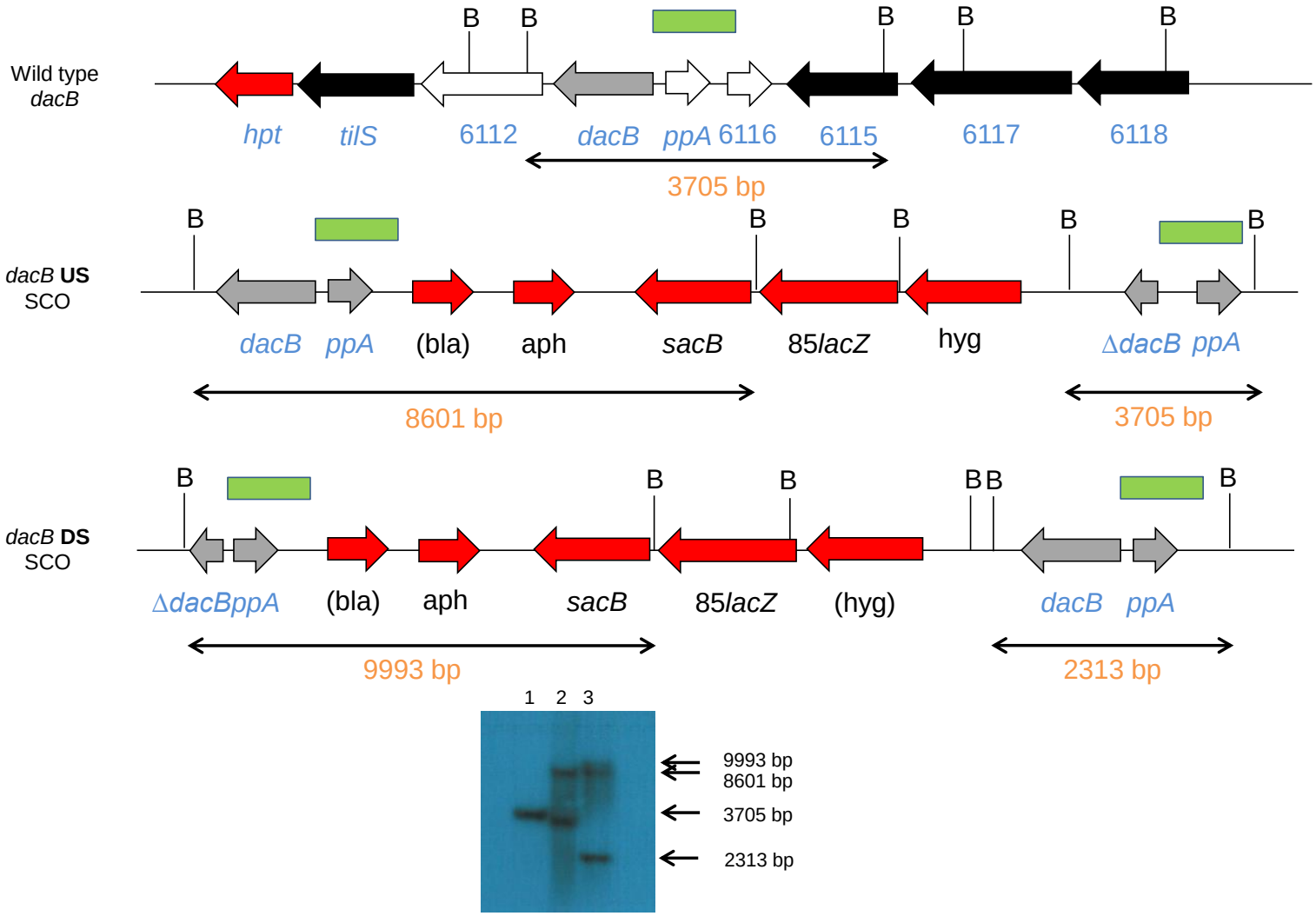


Figure S7A. Southern blot to confirm upstream and downstream SCOs for *dacB* mutant. Shown are the genomic maps for the wild-type, upstream (US) single crossover (SCO) and downstream (DS) SCO. Chromosomal DNA was isolated from wild-type (1), US SCO (2) and DS DSCO (3) and digested with *Bam*HI. The probe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

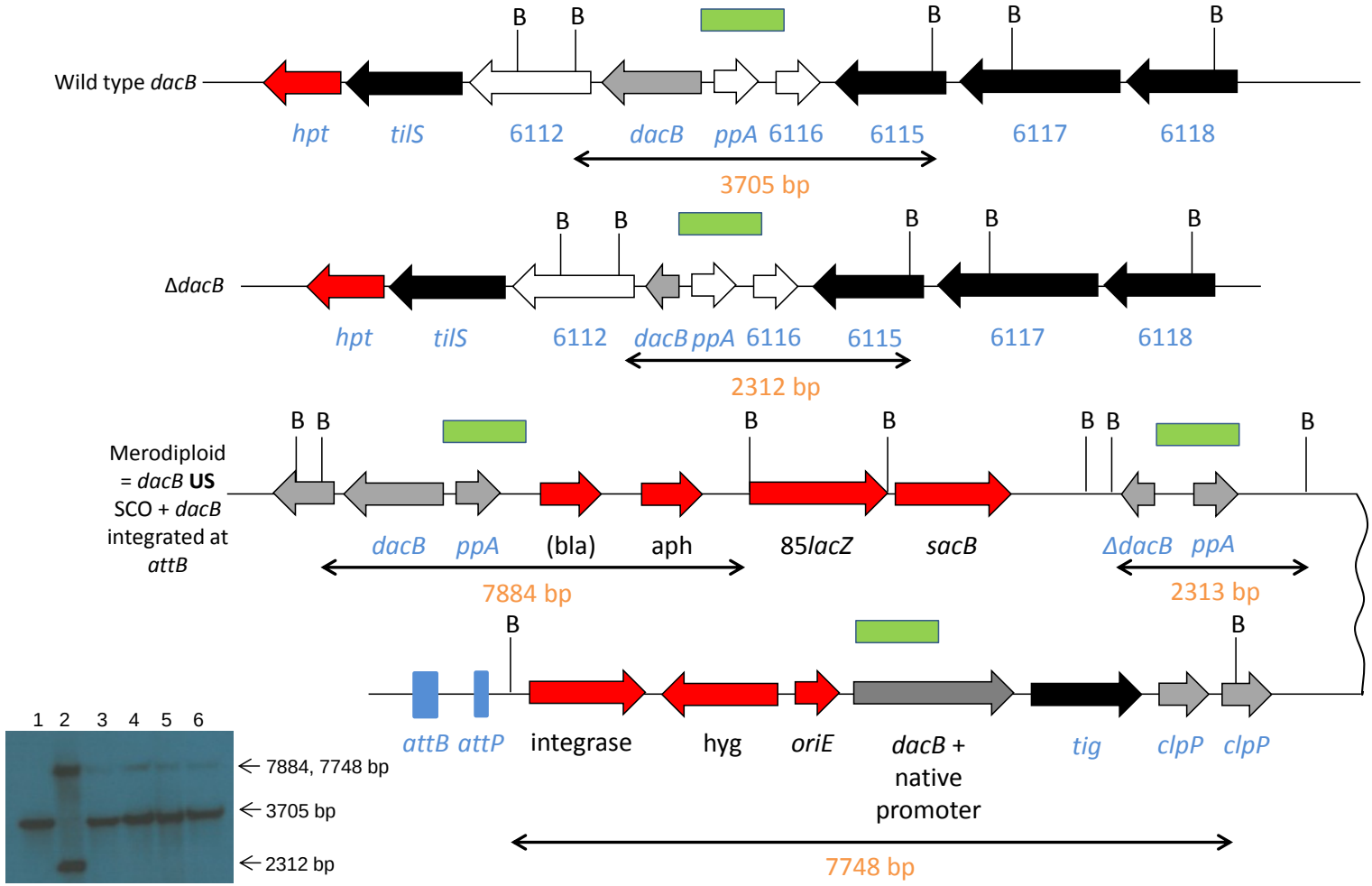


Figure S7B. Attempts to delete *dacB* in a merodiploid strain containing an additional copy of *dacB*, inserted at the *attB* site. Shown are genomic maps corresponding to wild-type, Δ*dacB*, the upstream single crossover (SCO) and resulting merodiploid strain. Southern hybridizations were performed 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.

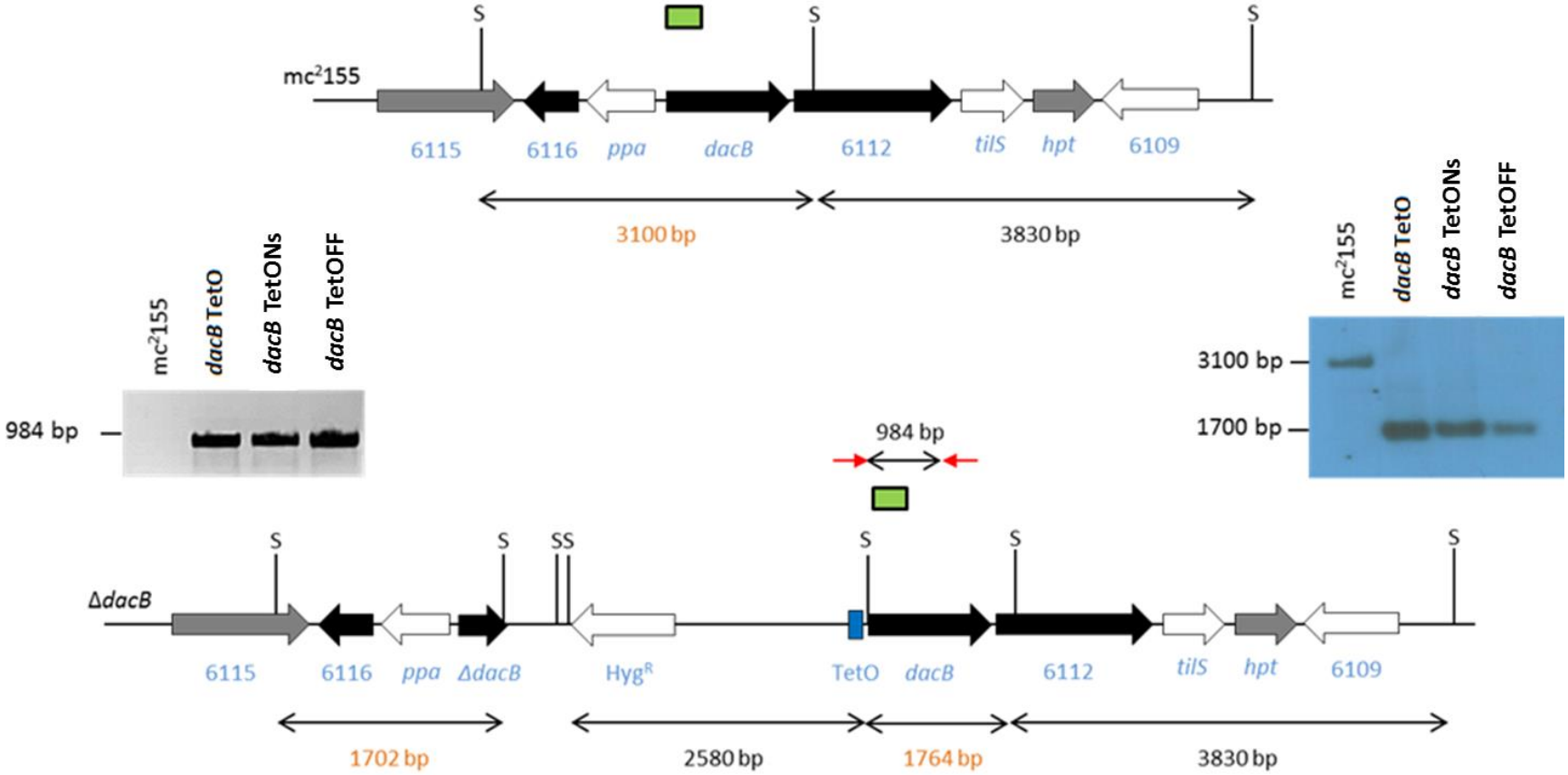
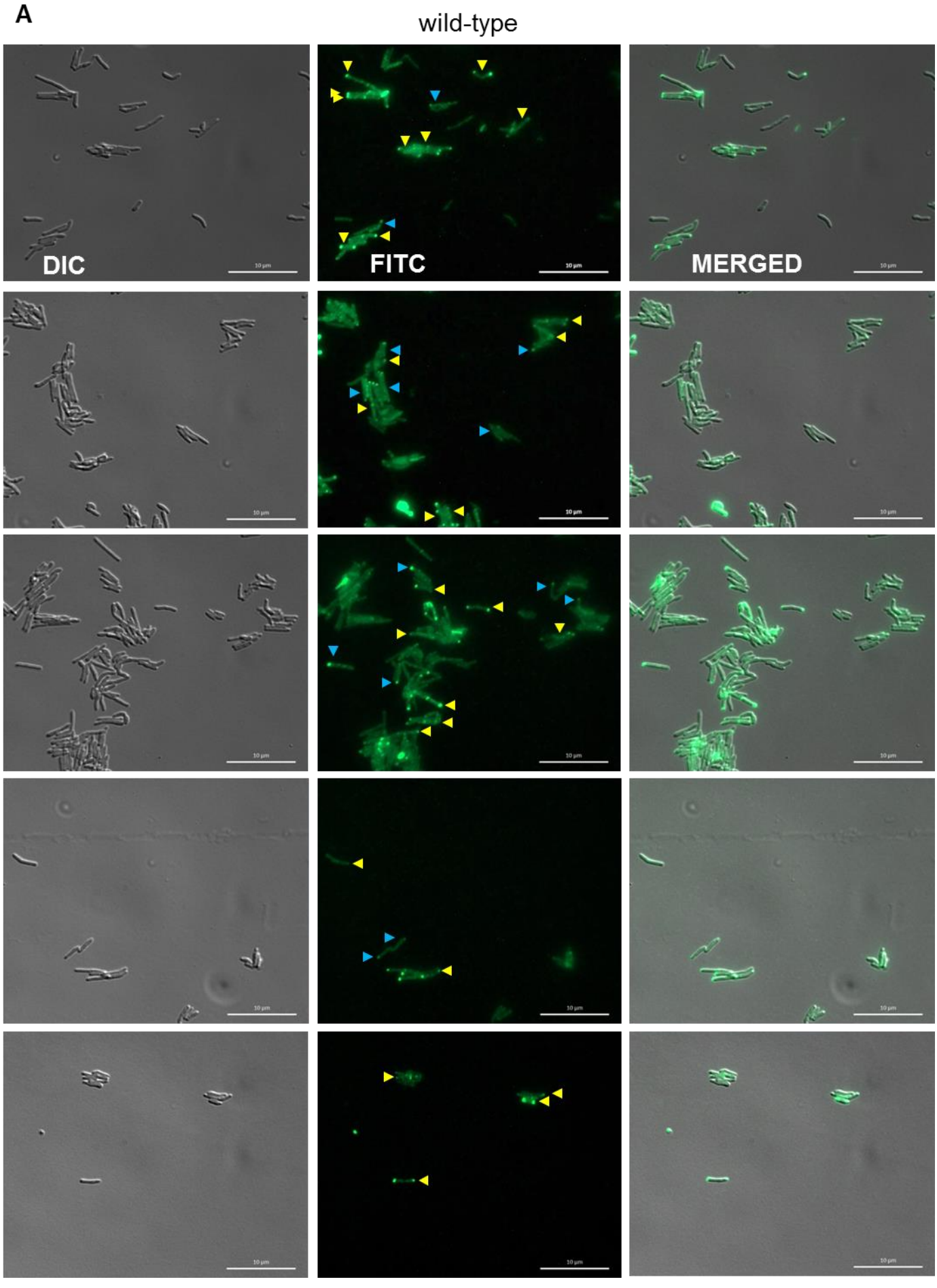
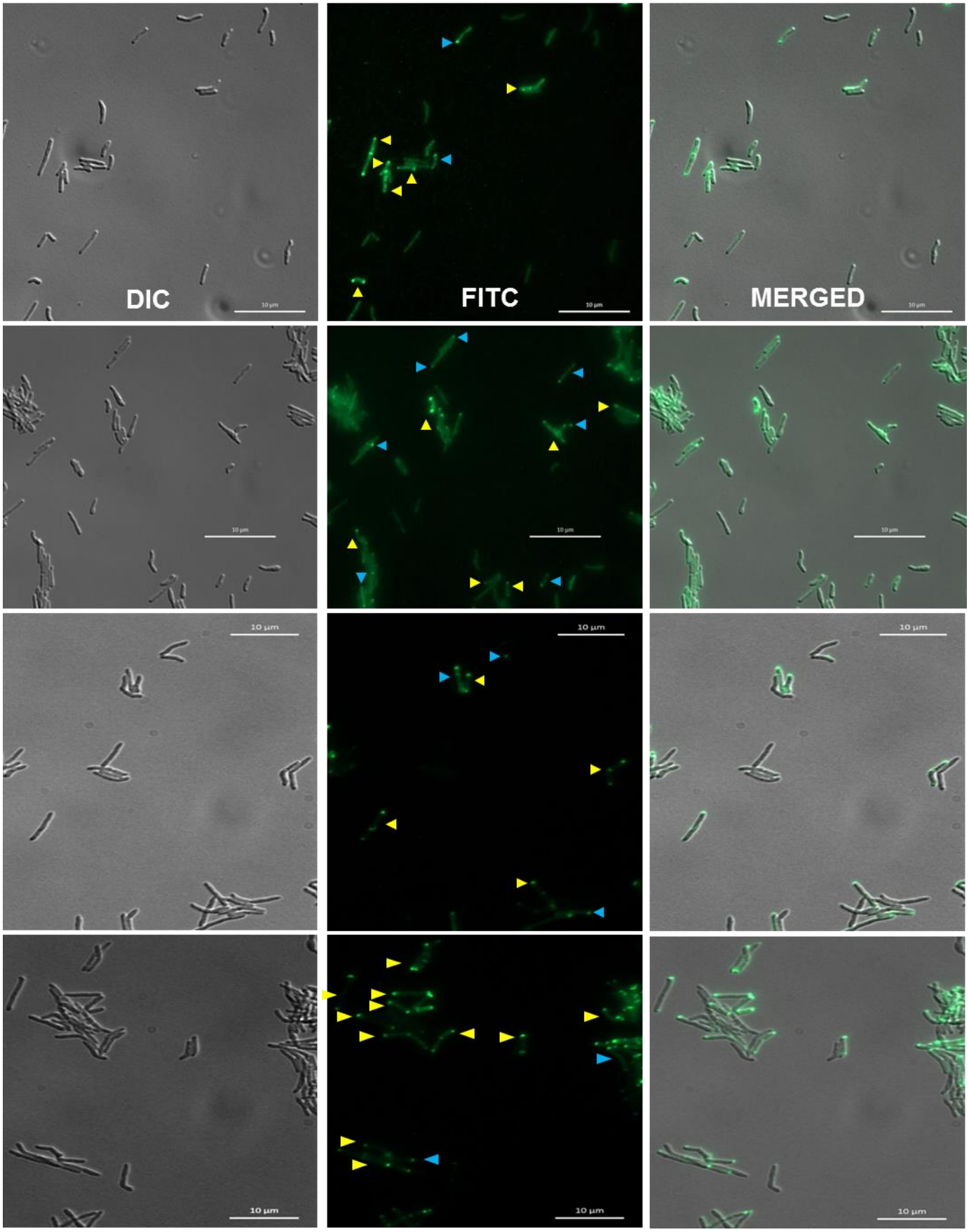
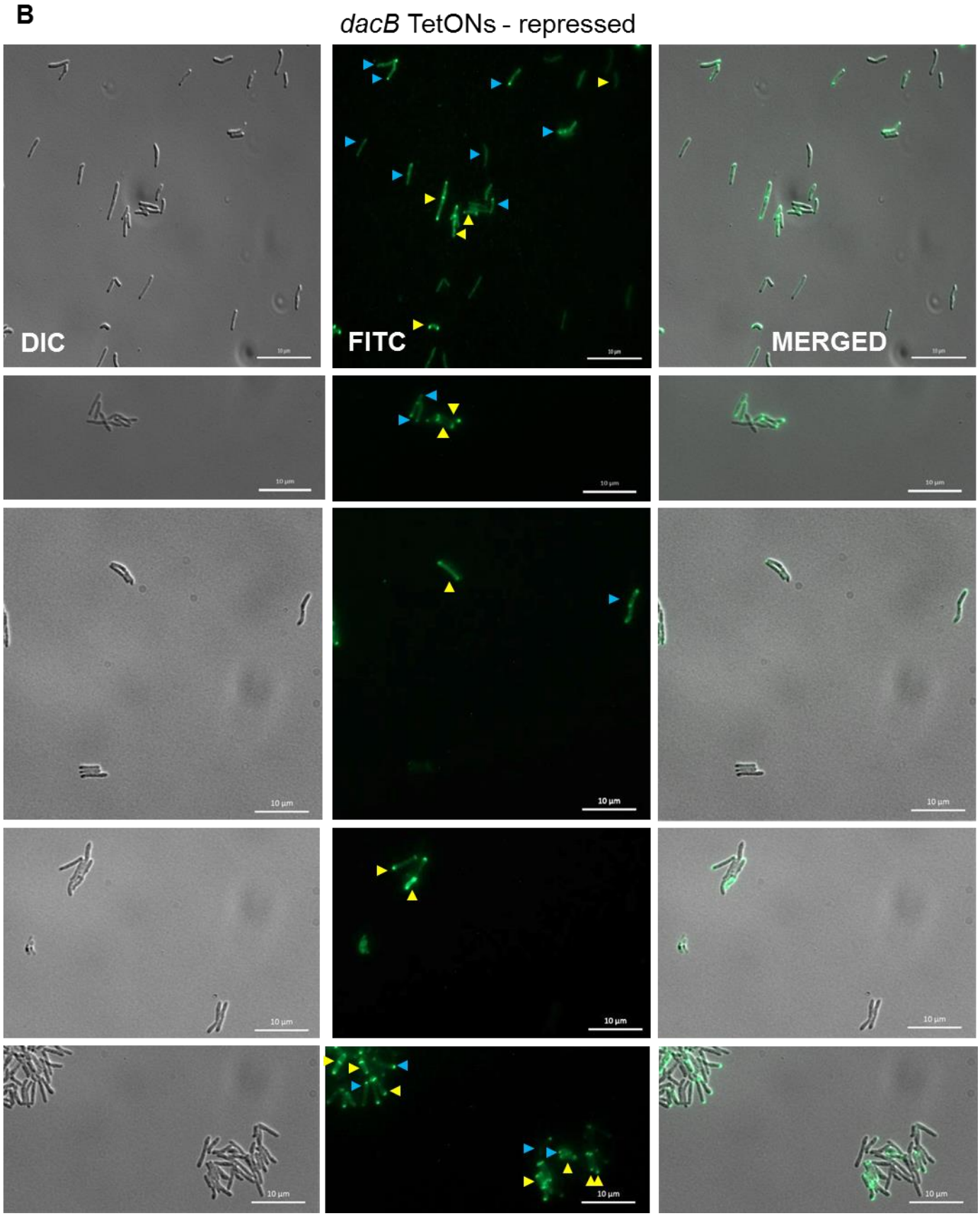


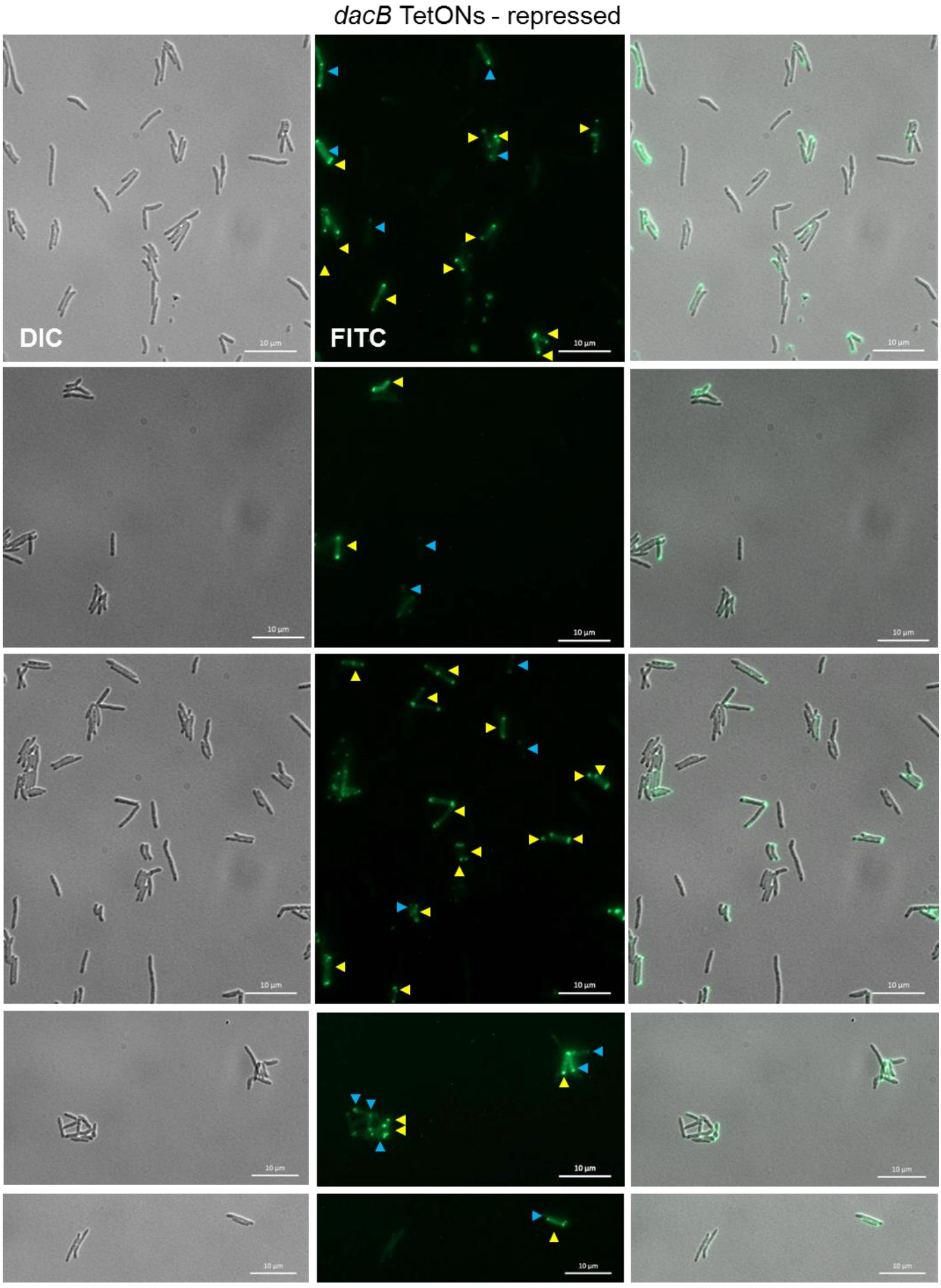
Figure S8. Genotypic characterization of the MSMEG_6113 (*dacB*) promoter replacement mutant by PCR and Southern blot analysis. The genomic map of the relevant locus is shown for the wild-type and mutant strain. Also shown on the left is the PCR confirmation of the genotype and Southern blotting is shown 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 *Sma*I [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.

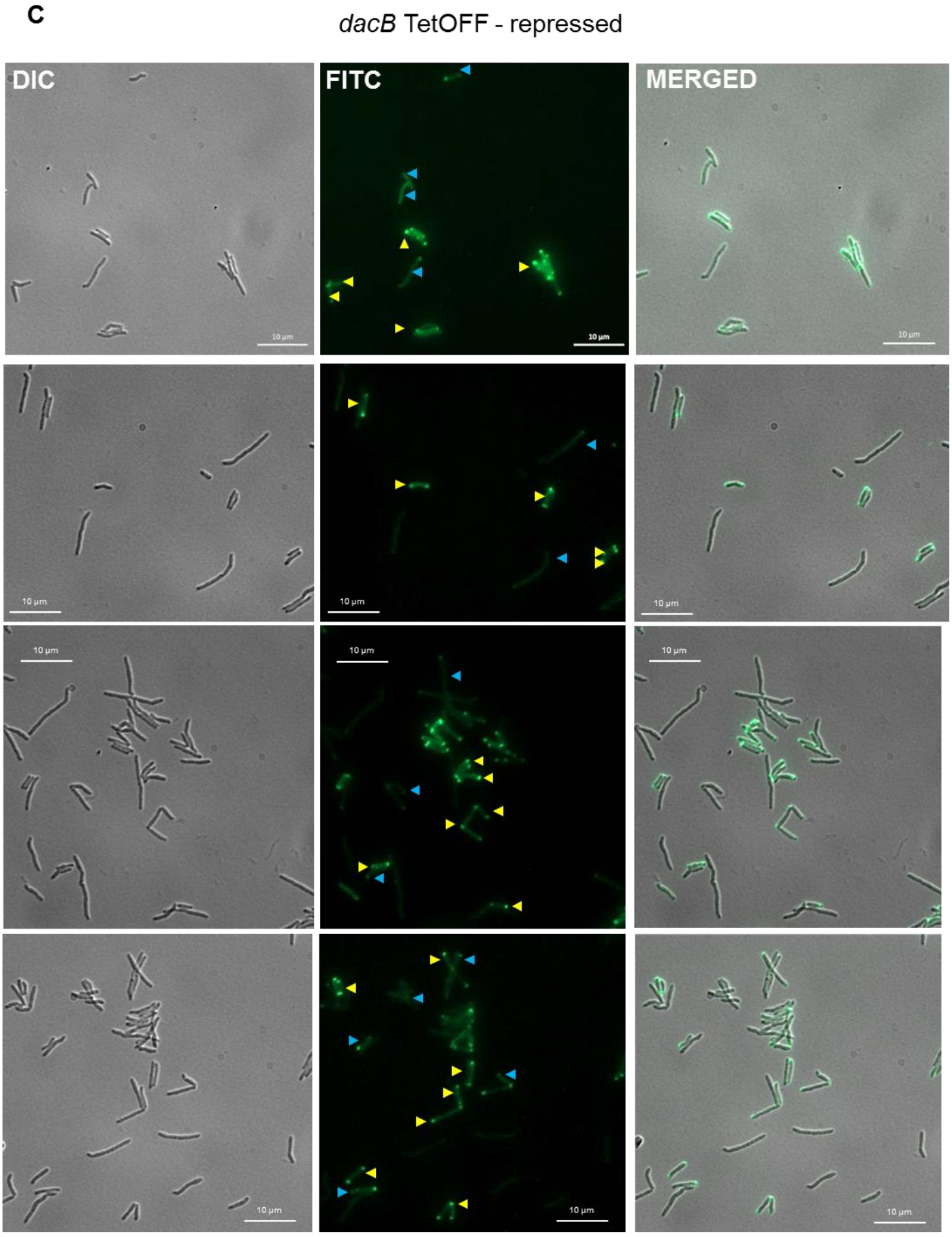

A
wild-type
DIC
FITC
MERGED

wild-type




B
dacB TetONs - repressed
DIC
FITC
MERGED
10 µm


dacB TetONs - repressed
DIC
FITC
10 µm


C
dacB TetOFF - repressed
DIC
FITC
MERGED
10 µm

dacB TetOFF – repressed

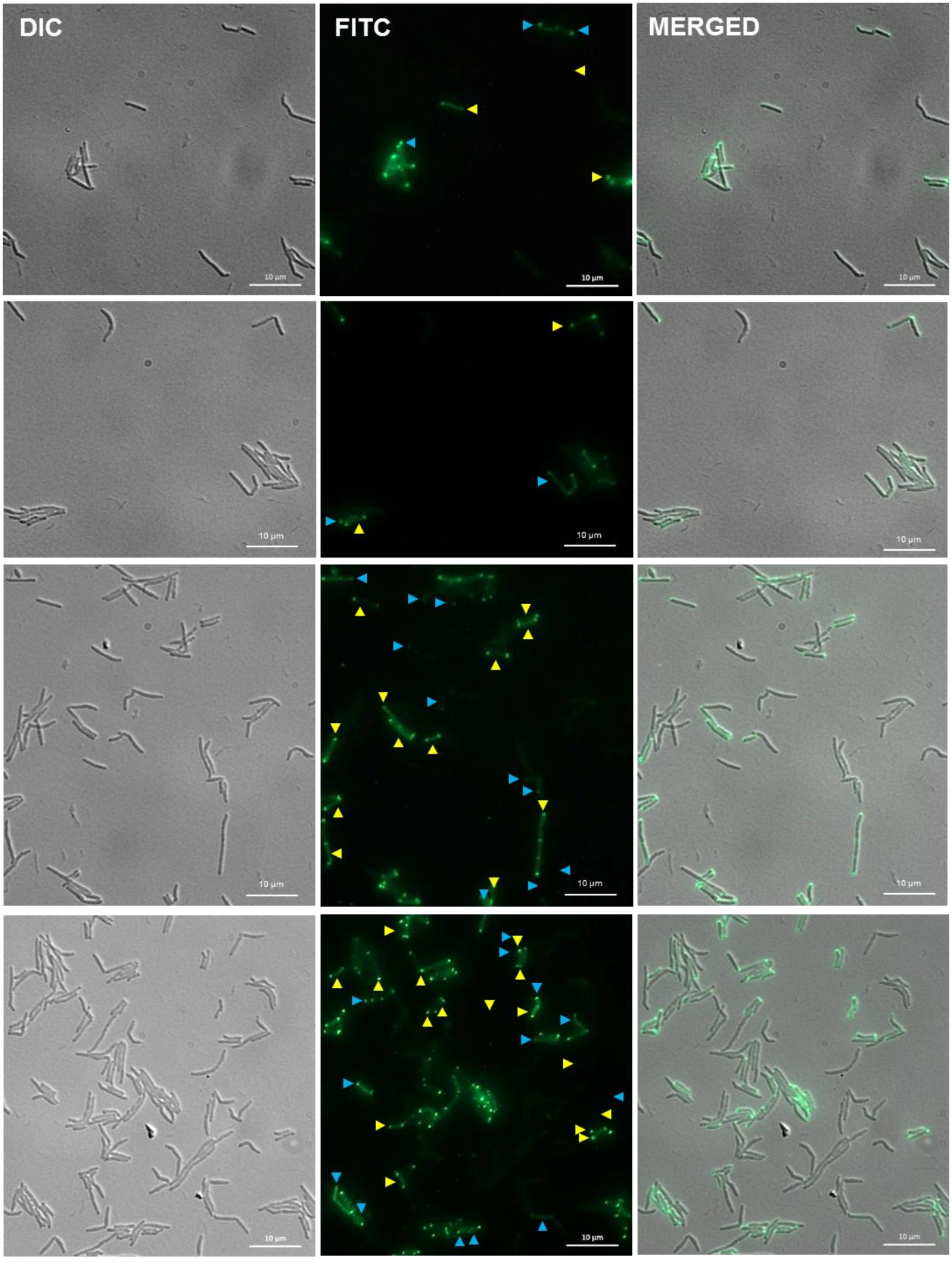


Figure S9. Representative images of PG staining patterns observed in *dacB* knockdown strains. Cells corresponding to the wild-type (A), *dacB* $TetON_s$ 0 ng/ml aTc (repressed) (B) and *dacB* TetOFF 100 ng/ml aTc (repressed) (C) were stained with BODIPY-vancomycin during late logarithmic growth. Images 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.

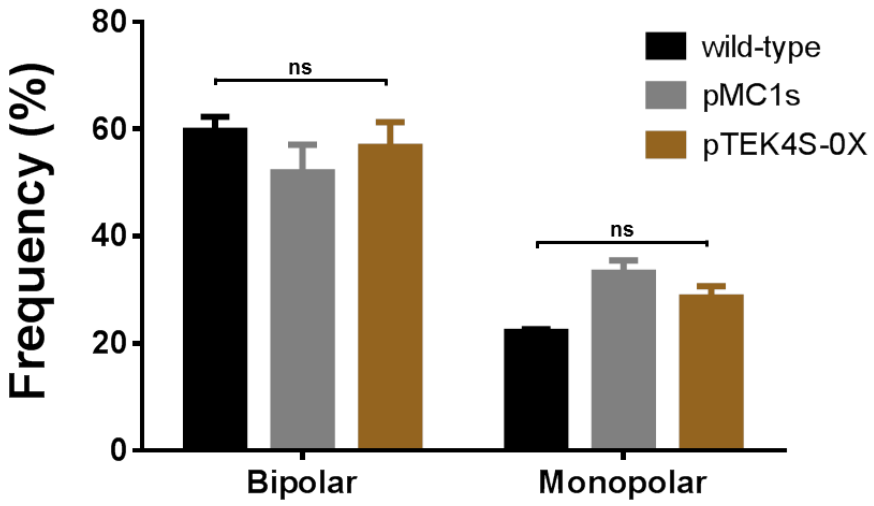


Figure S10. BODIPY-vancomycin staining of wild-type and repressor only (pMC1s or pTEK4S-0X) strains. Strains were sampled during late logarithmic growth and stained. Staining patterns were viewed under bright-field (DIC) and FITC-channels and scored as bipolar or monopolar. Data are represented as the average of three independent biological repeats with error bars depicting standard error from the mean. No statistically (ns) significant differences were detected, comparing strains in each staining type.

References

1. Snapper, S., Melton, R., Mustafa, S., Kieser, T. & Jacobs Jr., W. Isolation and characterization of efficient plasmid transformation mutants of Mycobacterium smegmatis. *Mol Microbiol* **4,** 1911–9 (1990).

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).