Defining the genes for the final steps in biosynthesis of the complex polyketide antibiotic mupirocin by *Pseudomonas fluorescens* NCIMB10586

Jack A. Connolly^[a], Amber Wilson^[a], Malgorzata Macioszek^{[a][c]}, Zhongshu Song^[b], Luoyi Wang^[b], ¹, Hadi H. Mohammad^[a], Mukul Yadav^[a], Maura di Martino^{[a][d]}, Claire E. Miller^[a], Jo Hothersall^[a], Anthony S. Haines^[a], Elton R. Stephens^[a], Matthew P. Crump^[b], Christine L. Willis^[b], Thomas J. Simpson^[b], Peter J. Winn^[a] and Christopher M. Thomas^{*[a]}.

Supplementary Methods

Bacterial Strains	Source/Reference	Notes
Escherichia coli DH5α	Thermofisher	Cat#18263-012
<i>E. coli</i> S17-1 (RP4 <i>tra</i>)	Ref. 31	N/A
E. coli ER2925 (Dam ⁻ and Dcm ⁻)	New England Biolabs	Cat#E4109
Bacillus subtilis 1604/168	ATCC23857	Mupirocin sensitive
Pseudomonas fluorescens SBW25	Ref. 33	WT Non-producer
Pseudomonas fluorescens NCIMB10586	NCIMB ref. 8	WT mupirocin producer
P. fluorescens NCIMB10586 ΔmupI xylE::mupA	Ref. 21	Reporter <i>xyIE</i> gene in <i>mupA</i>
P. fluorescens NCIMB10586 ΔmupZ-mupI	Claire Miller, this work	Lacks the mup cluster
P. fluorescens NCIMB10586 ΔmacpA	Ref. 18	No production
P. fluorescens NCIMB10586 ΔmacpB	Ref. 18	No production
P. fluorescens NCIMB10586 ΔmacpC	Ref. 18	Produced Mupirocin H and mupiric acid
P. fluorescens NCIMB10586 ΔmacpD	Ref. 18	No production
P. fluorescens NCIMB10586 ΔmacpE	Ref. 18	Produces PA-B
P. fluorescens NCIMB10586 ΔmmpA	Ref. 17	Produces mupircin acid
P. fluorescens NCIMB10586 $\Delta mmpB$	Jo Hothersall, this work	Produces mupircin acid
P. fluorescens NCIMB10586 mmpE∆OR	Ref. 17	Produces PA-C
P. fluorescens NCIMB10586 ΔmupA	Jo Hothersall, this work	Produces mupiric acid
P. fluorescens NCIMB10586 $\Delta mupB$	Jo Hothersall, this work	No PA-A production
P. fluorescens NCIMB10586 $\Delta mupC$	Ref. 18	Produces Mupirocin C
P. fluorescens NCIMB10586 ΔmupD	Ref. 18	No PA-A production
P. fluorescens NCIMB10586 ΔmupE	Ref. 18	No PA-A production
P. fluorescens NCIMB10586 ΔmupF	Ref. 18	Produces Mupirocin F
P. fluorescens NCIMB10586 ΔmupG	Ref. 18	Produces Muprocin H
P. fluorescens NCIMB10586 ΔmupH	Ref. 18	Produces Muprocin H
P. fluorescens NCIMB10586 Δmupl	Ref. 21	No production
P. fluorescens NCIMB10586 ΔmupJ	Ref. 18	Produces Muprocin H
P. fluorescens NCIMB10586 ΔmupK	Ref. 18	Produces Muprocin H
P. fluorescens NCIMB10586 ΔmupL	Ref. 18	N o production
P. fluorescens NCIMB10586 ΔmupM	Ref. 18	No production
P. fluorescens NCIMB10586 ΔmupN	Ref. 18	No production
P. fluorescens NCIMB10586 ΔmupO	Ref. 15	Produces PA-B
P. fluorescens NCIMB10586 ΔmupP	Ref. 18	Produces Mupirocin P
P. fluorescens NCIMB10586 $\Delta mupR$	Ref. 21	No production
<i>P. fluorescens</i> NCIMB10586 Δ mupQ, Δ mupS, Δ macpD,	Mukul Yadav, this work	Produces Mupirocin H
AmmpF	Dof 15	and mupiric acid
Γ. IIUUIESCEIIS NUIVIBIU386 ΔΠΙΔΟ	nel. 15	FIDUUCES PA-B

Table S1. Bacterial strains used and constructed in this work.

<i>P. fluorescens</i> NCIMB10586 Δ <i>mupW</i>	Ref. 15	Produces Mupirocin W
<i>P. fluorescens</i> NCIMB10586 Δ mupW, Δ mupT	Jo Hothersall, this work	Produces Mupirocin W
P. fluorescens NCIMB10586 $\Delta mupX$	Ref. 15	Reduced PA-A production
P. fluorescens NCIMB10586 mupV Y167F	Jack Connolly, this work	Produces PA-B
P. fluorescens NCIMB10586 mupV V581A H631A	Jack Connolly, this work	Produces PA-B
P. fluorescens NCIMB10586 mupZ C25X (TERM)	Hadi Mohammed, this work	No PA-A production

Plasmid	Source/Reference	Notes
pAKE604	Ref. 21	Km ^H Ap ^H pMB1 replicon, <i>oriT</i> _{RK2}
pJC70	Jack Connolly, this work	Km ^R Ap ^S derivative of pAKE604
pGEM-T Easy	Promega Cat#A1360	Ap ^R pMB1 replicon linear T-tailed vector
pJH2	Ref. 18	pJH10 expressing mupR
pJH10	Ref. 9	Tc ^H IncQ vector with <i>tac</i> p <i>lacf</i> ⁴ expression
pAW1	This work	pJC70 derivative for <i>mupV</i> domain 1 Y167F mutagenesis
pJC102	This work	pJC70 derivative for <i>mupV</i> domain 2 V581A, H631A mutagenesis
pHHMZC25	This work	pAKE604 derivative for <i>mupZ</i> mutagenesis
pMY21	This work	pAKE604 derivative to delete mupQ mupS macpD mmpF
pSC∆T	Ref. 15	pAKE604 derivative to delete mupT
pMMG1	This work	pGEM-T derivative with <i>mupO</i>
pMMG2	This work	pGEM-T derivative with macpE
pMMG12	This work	pGEM-T derivative with <i>mupO, macpE</i>
pMMG34	This work	pGEM-T derivative with <i>mupU, mupV</i>
pMMG1234	This work	pGEM-T derivative with <i>mupO</i> , <i>macpE</i> , <i>mupU</i> , <i>mupV</i>
pMMG5	This work	pGEM-T derivative with <i>mupC</i>
pMMG6	This work	pGEM-T derivative with <i>mupF</i>
pMMG56	This work	pGEM-T derivative with <i>mupC, mupF</i>
pMMG66	This work	pGEM-T derivative with <i>mupO</i> , <i>macpE</i> , <i>mupU</i> , <i>mupV</i> , <i>mupC</i> ,
pMMH6	This work	n H10 derivative expressing mup() macpE mup() mup() mup()
		mupF
pJC124	This work	pJH10 derivative expressing mupO, mupP, macpE, mupU, mupV,
10.100		mupC, mupF
pJC132	This work	pJH10 derivative expressing mupO, mupP, macpE, mupU, mupV, mupC, mupF, mupM, mupN
pJC133	This work	pJH10 derivative expressing mupO, mupP, macpE, mupU, mupV,
		mupC, mupF, mupL, mupM, mupN
pJC134	This work	pJH10 derivative expressing <i>mupO</i> , <i>mupP</i> , <i>macpE</i> , <i>mupU</i> , <i>mupV</i> ,
		παρο, παρε, παρε-π230Α, παρινι, παριν

Table S2. Plasmids used and constructed in this work.

Table S3. Oligonucleotides used in this work.

Oligonucleotides: Name and sequence	Source	Purpose
AW1 AGACAATTGAGCAGGCCCGTCATTTAC	This work	<i>mupV</i> mutagenesis, pAW1
AW2 TGGTTTCTTCGAAATAGTTATTGAATTCAGTGC	This work	<i>mupV</i> mutagenesis, pAW1
AW3 CAATAACTATTTCGAAGAAACCAAATGTGCG	This work	<i>mupV</i> mutagenesis, pAW1
AW4 AGAGGATCCGTTGTGGATGTAGGGGTTGTAAAAC	This work	<i>mupV</i> mutagenesis, pAW1
JC1 GGAATCGTCGACAAAGCACATCTGATCGGCT	This work	mupV mutagenesis, pJC102
JC2 CAAACCAAATGCATCCATGCCAGCGATTTC	This work	mupV mutagenesis, pJC102
JC3 GGCATGGATGCATTTGGTTTGTATAACCACCTGG	This work	mupV mutagenesis. pJC102
JC4 GCACTGGAATTCTGACGACAACTGTTGTAGTAGGC	This work	mupV mutagenesis, pJC102
	This work	Deletion of mupS Q macpD mmpF
	This work	Deletion of mupS Q macpD, mmpF
	This work	Deletion of mupS () macpD, mmpF
	This work	Deletion of mupS, Q macpD, mmpF
	This work	munZmutaganagia nLILIMZCOE
		mupz mutagenesis, pHHMZC25
HHMZ2F GCG1A11GAGCCG1G111A1C	This work	mup2 mutagenesis, pHHM2C25
HHMZ2R GAGTCTAGAGAGTGACGCCGCCATTAC	This work	<i>mupZ</i> mutagenesis, pHHMZC25
FAbla TCCGAGCTCCCAGTTCGATGTAACCCACTC	This work	Deletion of <i>bla</i> from pAKE604
RAbla GAAGAGCTCAGGCAACTATGGATGAACG	This work	Deletion of <i>bla</i> from pAKE604
FmupO GGTACCGGAGAGCAACGATGACATCGTGGGAAAGAGA	This work	mupO amplification
RmupO ACCGGTTACCGGGCGATTCCGTGG	This work	mupO amplification
FmacpE ACCGGTCACAGGAGGCGTAGATGC	This work	macpE amplification
RmacpE	This work	macpE amplification
ACTAGTTCTAGACTTAAGTCACTGCTCACGTTGGGC		
FmupUV CTTAAGGGCAAGGGAATGTGAGATG	This work	mupUV amplification
RmupUV	This work	mupUV amplification
	This work	munC amplification
	This work	
	This work	mup- amplification
	This work	mupOP amplification
	This work	mupOP amplification
EmacnE2	This work	macpE amplification
ACAGGAGCACCACCAGGAGGCGTAGATGC		maop_ ampinoation
RmacpE2 AGACTTAAGTCACTGCTCACGTTGGGC	This work	macpE amplification
FmupLMN	This work	mupLMN amplification
CCACTCTAGACAGTGAAGAGAGGGACACACAGC		
Rmup(L)MN GGTCTCAGGCCGCGATCCAAACCTATAAGCACTG	This work	mupLMN and mupMN amplification
FmupMN	This work	mupMN amplification
TAGCTCTAGACTGACAGGTGTGACTGATGAGTACG		
	This work	Mutation of <i>mupL</i>
Bmunl-H256A	This work	Mutation of <i>munl</i>
AGCGTGTAGGCCCCCCAATCGTCCATGATG		

Construction of plasmids created during this work

Construction of mutagenesis plasmids

All plasmid constructs reported in this paper were checked by suitable restriction digests and Sanger sequencing of inserted DNA.

pAW1 – mupV domain 1 Y167F

Primer pairs AW1 plus AW2 and AW3 plus AW4 were used to amplify 555 bp and 502 bp segments of the NCIMB10586 chromosome, respectively. Primers AW2 and AW3 were designed to generate a 23 bp overlap, which included the *mupV* Y167F mutation (DNA codon TAC to TTC). The products were spliced by overlap PCR using primers AW1 and AW4, and cloned using Mfel/BamHI digestion and ligation with pAKE604 digested with *Eco*RI/*Bam*HI to yield mutagenesis vector pAW1.

pHHMZC25 – mupZ C25X (TERM)

Primers HHMZ1F plus HHMZ1R and HHMZ2F plus HHMZ2R were used to amplify 546 bp and 582 bp fragments from the NCIMB10586 chromosome. Primers HHMZ1R and HHMZ2F were designed to generate a 21 bp overlap which contained the base to be mutated from C to A, generating a TGA stop codon. The products were spliced by overlap PCR using primers HHMZ1F and HHMZ2R, and cloned using BamHI/Xbal digestion and ligation with pAKE604 digested with *Eco*RI/*Bam*HI to yield mutagenesis vector pHHMXC25.

pJC70 – derivative of suicide plasmid pAKE604 lacking the *bla* gene that confers Ap^R

To allow easy selection of transconjugants after transfer of suicide mutagenesis plasmids from *E. coli* to *P. fluorescens* (which is naturally Ap^{R}) the *bla* gene was deleted. Primers F Δ bla and R Δ bla were used to amplify the 6574 bp segment of pAKE604 that excludes the *bla* gene, recircularised by ligation after cutting with SacI and introduced into *E. coli* DH5 selecting Km^R. A correct clone not conferring Ap^{R} was designated pJC70.

pJC102 - mupV domain 2 V581A, H631A

Primer pairs JC1 plus JC2 and JC3 plus JC4 were used to amplify 546 bp and 500 bp segments of the NCIMB10586 chromosome, respectively. Primers JC2 and JC3 were designed to generate a 21 bp overlap, which included the *mupV* H631A mutation (DNA codon CAT to GCA). The products were spliced by overlap PCR using primers JC1 and JC4, and then A-tailed and ligated into pGEM-T Easy (Promega). Sequencing revealed a secondary, unintended point mutation (V581A), which was deemed acceptable as it was also in domain 2 of *mupV*. The DNA fragment was cloned using *Sall/Eco*RI digestion and ligation into pJC70 to yield mutagenesis vector pJC102.

pMY21 – deletion of mupQ mupS macpD and mmpF

Primers QDA1F plus QDA1R and QDA2F plus QDA2R were used to amplify 547 bp and 504 bp fragments from the NCIMB10586 chromosome. Primers QDA1R and QDA2F were designed to incorporate an *Xba*l site allowing the two arms to be joined to an in-frame deletion starting in *mupQ* and ending in *mmpF* and cloned using *Eco*RI/*Bam*HI digestion and ligation with pAKE604 digested with *Eco*RI/*Bam*HI to yield mutagenesis vector pMY21.

Construction of expression plasmids

Initial constructions were designed when we predicted *mupO*, *macpE*, *mupU*, *mupV*, *mupC* and *mupF* to be sufficient for the PAB to PAA conversion. A series of intermediate plasmids were constructed using AT-cloning by the protocol in the pGEM-T Easy manual prior to generation of combinations of genes and then cloning into the broad host-range expression vector pJH10. Primers were designed to include the native NCIMB10586 ribosome binding site (RBS) for each gene. The template DNA for all initial PCRs was the *P. fluorescens* NCIMB10586 chromosome.

pMMG1 - intermediate carrying mupO

Primers FmupO and RmupO were used to amplify a 1.4 kb fragment, which was AT-cloned into pGEM-T Easy.

pMMG2 – intermediate carrying macpE

Primers FmacpE and RmacpE were used to amplify a 257 bp fragment, which was AT-cloned into pGEM-T Easy.

pMMG12 - intermediate carrying mupO and macpE

A 269 bp *macpE*-containing fragment was released by *Agel/Xba*l digest of pMMG2, and cloned into pMMG1 digested *Agel/Xba*l.

pMMG34 – intermediate carrying *mupU* and *mupV*

Primers FmupUV and RmupUV were used to amplify a 3.6 kb fragment encoding both *mupU* and *mupV*, which was AT-cloned into pGEM-T Easy.

pMMG1234 – intermediate carrying *mupO*, *macpE*, *mupU* and *mupV*

The 3.6 kb *mupU* - *mupV* fragment from pMMG34 was released by *AflII/Xba*I digestion and cloned into *AflII/Xba*I-cut pMMG12.

pMMG5 – intermediate carrying mupC

Primers FmupC and RmupC were used to amplify a 1.3 kb fragment, which was AT-cloned into pGEM-T Easy.

pMMG6 – intermediate carrying mupF

Primers FmupF and RmupF were used to amplify a 1.0 kb fragment, which was AT-cloned into pGEM-T Easy.

pMMG56 – intermediate carrying mupC and mupF

The 1.0 kb *mupF* fragment from pMMG6 was released by *AvrII/SpeI* digestion and cloned into *AvrII/SpeI*-cut pMMG5.

pMMG66 - intermediate carrying mupO, macpE, mupU, mupV, mupC and mupF

The 2.4 kb *mupC - mupF* fragment from pMMG56 was released by *HindIII/Xba*I digestion and cloned into *HindIII/Xba*I-cut pMMG1234.

pMMH6 – expression plasmid carrying *mupO*, *macpE*, *mupU*, *mupV*, *mupC* and *mupF*

The 7.6 kb fragment from pMMG66 containing all six genes was released by *Kpnl/Xba*l digestion and cloned into broad host-range expression vector pJH10 cut with *Kpnl/Xba*l.

pJC124 – expression plasmid carrying *mupO*, *mupP*, *macpE*, *mupU*, *mupV*, *mupC* and *mupF*

Primers FmupOP and RmupOP were used to amplify a 2.4 kb fragment including genes *mupO* and *mupP*. Primers FmacpE2 and RmacpE2 were used to amplify a 278 bp fragment carrying *macpE*. Primers RmupOP and FmacpE2 were designed to overlap. The products were spliced by overlap PCR with primers FmupOP and RmacpE2, to give a 2.6 kb fragment that was digested with *Kpnl/Afl*II and inserted into pMMH6 to replace the *mupO-macpE* segment, yielding pJC124.

pJC132 – expression plasmid carrying *mupO*, *mupP*, *macpE*, *mupU*, *mupV*, *mupC*, *mupF*, *mupM* and *mupN*

Primers FmupMN and RmupMN were used to amplify a 4.1 kb fragment including genes *mupM* and *mupN*. This product was AT-cloned to pGEM-T Easy. The DNA insert was released by *Xbal/Bsal* digest, and inserted into *Xbal/Not*I-digested pJC124 yielding pJC132.

pJC133 – expression plasmid carrying *mupO*, *mupP*, *macpE*, *mupU*, *mupV*, *mupC*, *mupF*, *mupL*, *mupM* and *mupN*

Primers FmupLMN and RmupMN were used to amplify a 5.1 kb fragment including genes *mupL*, *mupM* and *mupN*. This product was AT-cloned to pGEM-T Easy. The DNA insert was released by *Xbal/Bsal* digest and inserted into *Xbal/Not*l-digested pJC124 yielding pJC133.

pJC134 – expression plasmid pJC133 with point mutation H256A in MupL

Primers FmupLMN and RmupL-H256A were used to amplify an 809 bp N-terminal segment of *mupL* while primers FmupL-H256A and RmupMN were used to amplify a 4.3 kb fragment with the C-terminal part part of *mupL* plus *mupM* and *mupN*. The overlap in primers FmupL-H256A and RmupL-H256A included the *mupL* H256A mutation (DNA codon CAC to GCC) allowing overlap PCR using outer primers FmupLMN and RmupMN, to generate a 5.1 kb fragment including *mupL*H256A, *mupM*

and *mupN*. This product was AT-cloned into pGEM-T Easy and then released by *Xbal/Bsa*l digest before insertion into *Xbal/Not*l-digested pJC124 yielding pJC134.

Supporting Information

Figure S1. Genetic map of the mupirocin cluster



Figure S2. XyIE assay: the crude PA-B extract contains the homoserine lactone product of Mupl, and activates the *mupA* promoter in 10586 Δ*mupl xyIE::mupA*. XyIE catalyses conversion of catechol to the yellow compound 2-hydroxymuconic semialdehyde. Representative plate shown, n=9.



Figure S3. Amino acid alignment of MupV domain 2, His631 is conserved.

		0.	26				
Mup	νŪ		GADYSEIAGM	DHFGLYNHLG	LL <mark>G</mark> VV <mark>T</mark> AYI <mark>K</mark>	NLSPVMPGSQ	ADA
αi	503699206	ref WP 013933282.	DAOYKIFDRW	GHYNLFTDTA	EVCEDLILKA	G	
άi	557381884	irefiWP_023402309.	DAEYKTFANW	GHYNLET DTA	EVCEDLILNG	H	
σi	820895882	rof WP_046802015	ASREVTAGC	GHTPCLEOPL	AYTOAASTEL	KTLPEH	
9±	91939/400	rof WD 05202013.	ASHEVT ACC	CHIPCIFORI			
91	919395450	TCT WF_052520041.	COBRETERCA		AT LOAT TREV		
ĞТ	947200117	[rel WP_055972512.	GORENILUGA	GRIPCVERPD			
gı	832308903	[ref]WP_04/701002.	DALLHVESNG	SHLAFFESTH	RSKQTAFRFL	ESTLESVAV-	
gi	751317467	ref WP_041024121.	DALLHVESNG	SHLAFF <mark>EST</mark> H	HSKQTAFRFL	ESTLESVAV-	
gi	939983614	gb KPW11279.1	DATLHVERDG	SHLAFFA <mark>SS</mark> H	QS <mark>K</mark> QTAFSFL	<mark>EE</mark> VL <mark>QP</mark> AVA-	
gi	499587035	rcf WP 011267818.	DATLHVERNG	SHLAFFA <mark>SSQ</mark>	QS <mark>K</mark> QTAFSFL	EEVLQPAVA-	
ği	333982465	qb EGH80563.1	DATLHVERDG	SHLAFFA <mark>SS</mark> H	QS <mark>KQTAF</mark> SFL	EEVLQPAVA-	
qi	591140130	[gb]AHL29301.1]	GATLHIEKDG	SHLAFF <mark>ER</mark> AA	ES <mark>KK</mark> TAF <mark>EFI</mark>	DATLELAEV-	
σi	643814111	ref WP 025260246	GARLHVEENG	SHLAFFEHAP	RSCATAFHFM	KELEEPALT	
αi	981413155	Iref WP 059624560	GARLEVEVGG	SHPKLERASP	VSAARALKEL	AGHA	
ai	981716584	1rof/WP_059311230	GARLEVEAGG	SHOKLEDASD	ASACRALOFI.	λΡΗΛ	
31	497807285	$1 \times 10^{-1} \text{MP} = 0.000011 = 0.000011 = 0.0000000000000$	CARLEVEACC	SHARLERADA	ASTSIALPER		
91	976140675	1 mof WD_0E0200232	MART CARDEC	DUTCLEVICC			
9÷	970140070	[[[]]] [] [] [] [] [] [] []	NAILSVEPIG	DHISLERLGG	REGELALNEL		PGGRAAPR
gi.	9/2351269	[rei]WP_059J05309.	NATLEVEFIG	DHISLFTDGA	RLAGPALDFL		PAGAAARD
gι.	503052063	[rei]wP_013287039.	GSVLHVARHG	DHISTFHARE	SLVDLAAREV	AGDDALGTRS	PARAGREVPP
gı	517613690	[ref WP_018783898.	G <mark>SVLHVA</mark> GHG	DHLSLFHAEE	SLVDLAARFV	AGEVALGTRS	PAEAGRRVPP
gi	504914902	ref WP_015102004.	GATLTVLPHG	DHLSLF <mark>R</mark> AEP	HVV <mark>D</mark> LAL <mark>R</mark> FL	ADPRTPGV	PPEEHHPLCE
gi	973807235	gb KUM78476.1	HGQLRVEPHG	DHISLFTADD	SLL <mark>R</mark> VA <mark>ED</mark> FM	ARH	PRPAPI
gi	513768114	ref WP 019925403.	NARLRVEPHG	DIIIALFRADD	ALV <mark>Q</mark> VAI D FI	ADC	PRFL
qi	973832444	qb KUN02865.1	RAELRTMPDG	DHLTAFDGGH	ELTALAAEFL	HTVTA PDRKG	T
qi	558544681	ab EST35553 1	RAELRTMPDG	DHLTAFDGGG	ELVALA <mark>R</mark> EFL	HSVTVPERKG	T
σi	926335235	fref WP 053668419.	RADLRMLPDG	DHLGAFDAGP	ELVRLAEEFL	HDVSAPHRKD	T
σi	663341636	lref WP_030340763	RARLVTRPSG	DHLTAFDAGP	EVVRLAERFL	SDVLEDA	-
αi	926382215	lref WP_053711128	BARLVTRPSG	DHUTAFDAGP	EVVRLAEREL	SDVLEDA	
ai.	852466786	ab 3KN74581 1	BARLVTRPTG	DHLTAFDAGP	EVVRTAERET.	SDVLEDA	
<u>a</u> i	517512741	1 rof WD 018682040	PAHT HPT PHC	DOLTAYDAAD	DITDIATCET	AATVAMMERS	
9± mi	91719/059	ref WP 051790770	RSVI.HMEPHC	DHLSVEFACE	ALTRVAACEL	KEVCHC	
91	01/10-030	1 201 WD 052657993					
91	920323333	1 maflWD 05057003.	CARVERCOM	NILLDAP NATA	MURINECVID		
gT.	973313103	1161 ML_02010001'	GALIKE SGM	CUEDUXX DCC		NER SEPARE	
дŤ.	937061363	[rel]WP_054545555.	NGREEMEEDS	GULLAUTOS	LADSITUFVA		A
gi	182108559	rei WP 045613185.	HGRLLMLENS	GHFAHIILSK	TAESTIQEVG	QINALRESVS	A
дī	499814783	rei wP_011495517.	NSELVMLENA	GHFAQYYDHH	VANLVADEVS	RIKTHSROAL	<u>CQQ</u>
gī	946897890	rei WP_055320676.	NSSIVIEPEG	DHYELLRDA-	TRVMGVTR	DFLTGAYEGV	A
qi	495413405	ref WP_008138103.	GSQLYIDFLG	DHYGLIRQN-	STTLEYIS	SYLSMELSNV	<u></u>
gi	736062577	ref WP_034205959.	NAEQHVVVPG	DHYEILRGR-	NAVNDSIV	EFFGGRGAA-	
gi	981755134	ref WP_059947822.	GATLETIDDA	GHFGVYTSRA	L <mark>QER</mark> VAAFLD	GAGRPAGERL	SDKE
gi	755822516	ref WP_042585689.	GATLETIDDA	GHFGVYTSRA	L <mark>QER</mark> VAAFLD	GAGRPAGERL	SDKE
gi	738018432	ref WP 035979103.	<mark>G</mark> AAL <mark>QIV</mark> EAA	GHFAVHTSRD	LH <mark>ER</mark> IAAFL <mark>K</mark>	D <mark>a</mark> geagg <mark>kr</mark> h	LG <mark>KE</mark>
qi	746142252	ref WP_039205345.	GAALQIV<mark>E</mark>AA	GHFAVHTSRD	LH <mark>ER</mark> IAAFL <mark>K</mark>	D <mark>A</mark> GEAGGKRH	LGKE
gi	941151686	gb ALK30672_1	GAALQTVEAT	GHFGVHTSRD	L <mark>QER</mark> IAAFL <mark>K</mark>	DAGEAGGKRL	LG <mark>K</mark> E
qi	737300203	ref WP 035283227.	NARHVRIDGA	THYVMHDRPD	LVATIVDGFL	RGHERPLDRA	GGVHWAGSPA
ģi	26541530	gb AAN85517.1 AF48	GARHERLAGA	THHSMYDRPR	EVARLLTDFF	TEAVRPRSIV	SGAAR
āi	754800482	ref WP 042163776.	AGRHGELVGA	THYTMIDRPE	VVAAVCGAFL	DDPSGGAPLA	TGPAEIRWAP
σi	739946650	ref WP_037795935	TAGHGLLVGA	THYSMLDRPF.	VVAAVCGAFL	DDPRPGPPAV	GGPREVEWST
āī	819030940	Ire[WP_046713660	DGRYVEVHAG	THYCLYDRPA	LVAEVMERFF	ADPROVDGLS	GEVEBVT
σi	111222887	abiKJK59199 1	HARHVELLCA	THYCLHDRAD	LVARLIGGEV	REPGCPGADG	EELRWER
9∸ αi	919125977	r_{pol}	HARHVELLCA	THYCLHDRAD	LVARLIGGEV	REPGCPGADG	EELRWER
91 01	663330374	1 rof WD 030257054		SHYLMYDDAF	LVAAVVCDEV	RDPAZCADPA	
94	503220244	1 xoflWD 020207000.	NENVTOLDCA			KVVCKNTTCZ	
91	101752754	1101 WF_020515960.	NCNVCDIDCA		DUMEDIEQUM	NTIONNIISN VVV2VNITCV	
9±	431133130	ITELIMP UU4010001.	NON I OR LEGA			TI I SKINI I SK	
gı	023193382	rei WP 040/54916.	HAVETQRKKG	DHASLFDAPK	ELQQVFADF1	EIVA	



Figure S4. Bioassay activity of 10586 mupV point mutants vs Bacillus subtilis, n=2.





Figure S5. HPLC of 10586 *mupV* point mutants, n=3, metabolite mass confirmed by LC-MS.

Figure S6. A, LCMS and B, ¹H-NMR of 10586 *mupV* point mutants showing that point mutations in MupV domain 1 and domain 2 produce PA-B just like a $\Delta mupV$ mutant.

S6A.







Figure S7. HPLC: testing for complementation of 10586 Δ*mupL*, 10586 Δ*mupM* and 10586 Δ*mupN* by pJC132 and pJC133.

