1 **Supplemental Material**

2 Two DD-carboxypeptidases from *Mycobacterium smegmatis* affect cell surface 3 properties through regulation of peptidoglycan cross-linking and 4 glycopeptidolipids

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Figure S1: The structure of the *M. smegmatis* non-serovar specific GPL.

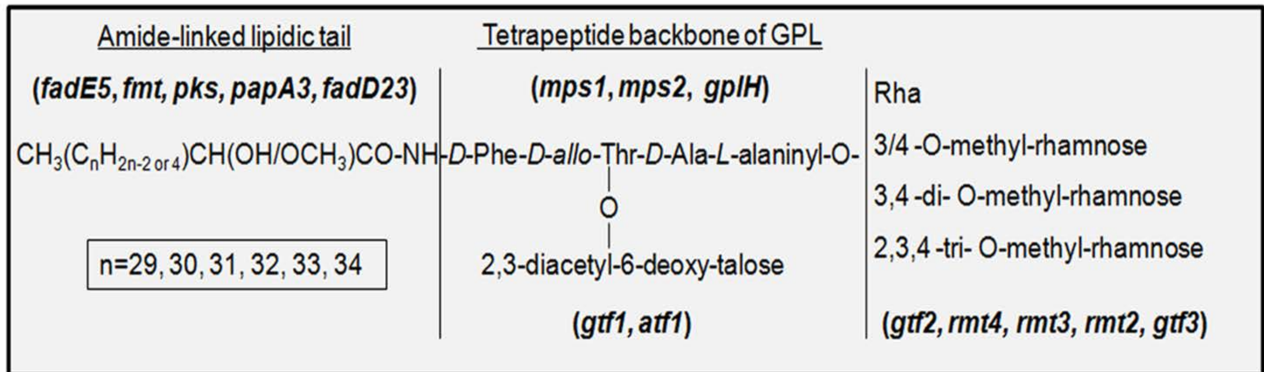

Fig. S1. The structure of the *M. smegmatis* non-serovar specific GPL. Genes involved in the biosynthesis of respective parts shown in bold italicize letters. Expansion of genes are as follows; *mps* (mycobacterial peptide synthetase), *gplH* (glycopeptidolipid *mbtH*-like gene), *pks* (polyketide synthase), *fad* (fatty acid desaturase), *fmt* (fatty acid O-methyltransferase), *pap* (polyketide associated protein), *gtf* (glycosyltransferase), *rmt* (rhamnosyl methyltransferase), *atf* (acetyltransferase), *tmtpC* (MmpL family transmembrane protein) and *gap* (GPL addressing protein).

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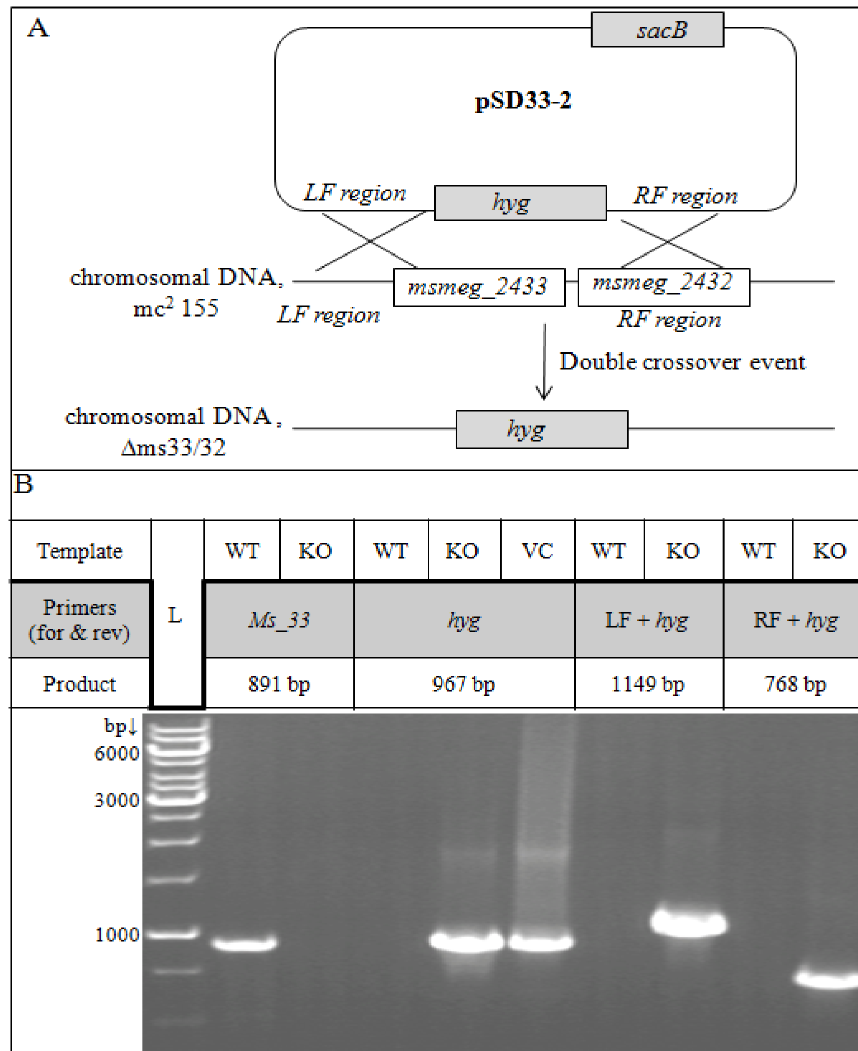
35 **Figure S2. Deletion of *msmeg_2433* from *M. smegmatis* mc^{2}155 chromosome.**



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37 **Fig. S2.** Deletion of *msmeg_2433* from *M. smegmatis* mc^{2}155 chromosome. (A) Schematic
38 representation of double-crossover event during homologous recombination. The non-replicative
39 plasmid pSD33-2 was constructed from a mycobacterial suicide vector, pSMT100 bearing *sacB*
40 and hygromycin cassette, by stepwise cloning of left flanking (LF) and right flanking (RF)
41 region of *msmeg_2433,* on both side of the hygromycin cassette. The pSD33-2 was then

transformed into electrocompetent *M. smegmatis* mc^{2}155, and knockout mutant Δ*ms33/32* was selected on Middlebrook 7H11 plate containing hygromycin and 10% sucrose. The expression of levansuccrase (from SacB) is lethal therefore, single crossed over strain cannot grow on the plate (1). Later, it was found to be a double mutant of *msmeg_2433* and *msmeg_2432* due to the polar effect (Fig. 1). (B) Confirmation of the mutant strain by PCR using various set of primers (refer Table S1). The WT (mc^{2}155), KO (Δ*ms33/32*) and VC (vector control; pSMT100 harboring hygromycin, used as positive control) were used as templates for the respective primer pairs (shown in the figure). The expected size of amplicons is shown in 1% agarose gel. 'L' represents DNA ladder.

Figure S3. Multiple sequence alignment of protein sequence of DacB2 (Rv2911) of *M. tuberculosis*, MSMEG_2433 and MSMEG_2432 of *mc*^{2}*155* using CLUSTALW.

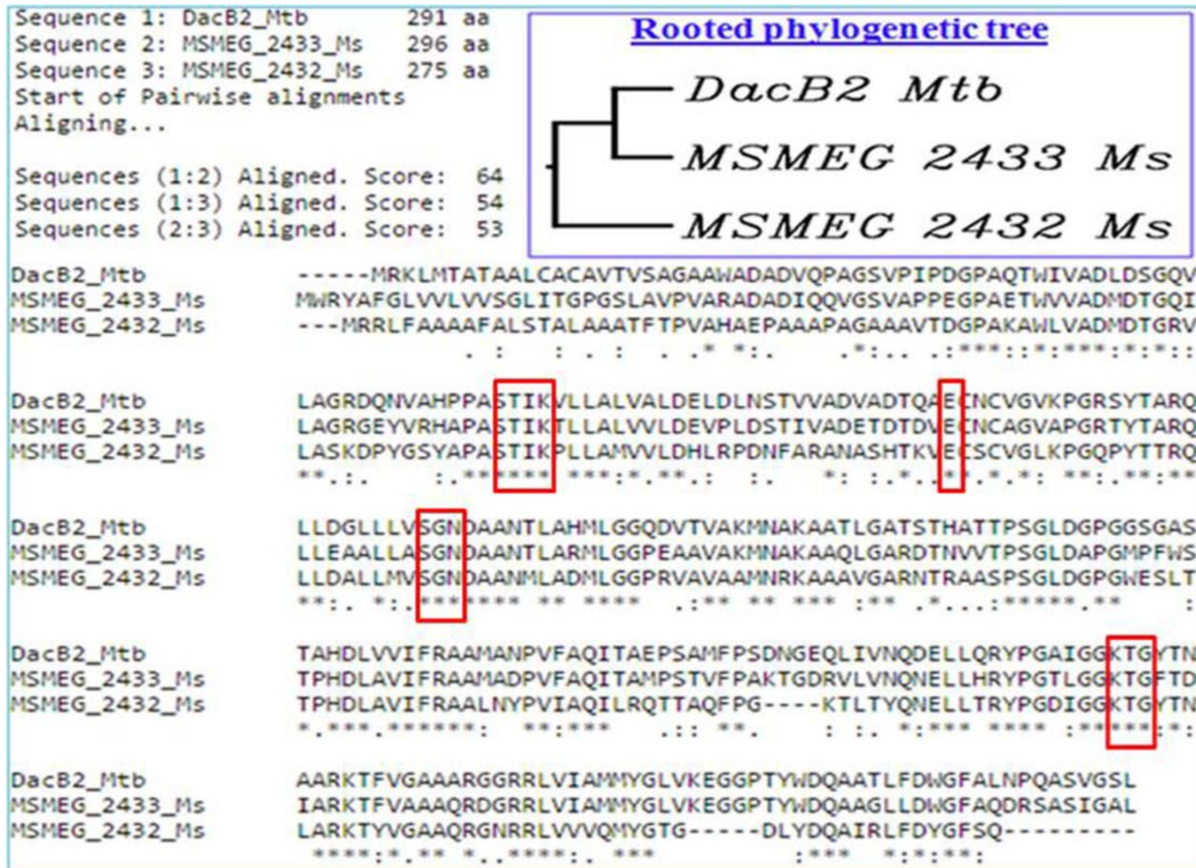

Fig. S3. Multiple sequence alignment of protein sequence of DacB2 (Rv2911) of *M. tuberculosis*, MSMEG_2433 and MSMEG_2432 of *M. smegmatis* using CLUSTALW. The red highlighted rectangles STIK, SGN and KTG shows the signature motifs of low molecular mass penicillin-binding proteins (LMM-PBPs) (2). DacB2 and MSMEG_2433 are characterized as DD-CPases (3, 4) whereas highlighted 'E' in the Ω-like loop of MSMEG_2433 is reported to be responsible for the additional β-lactamase activity (3). Alignment scores are indicated in the figure and the inset represents the rooted phylogenetic relations of the three proteins.

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73 **Figure S4. Membrane localization of MSMEG_2433 and Rv2911 (DacB2) and β-lactamase**

74 **assay.**

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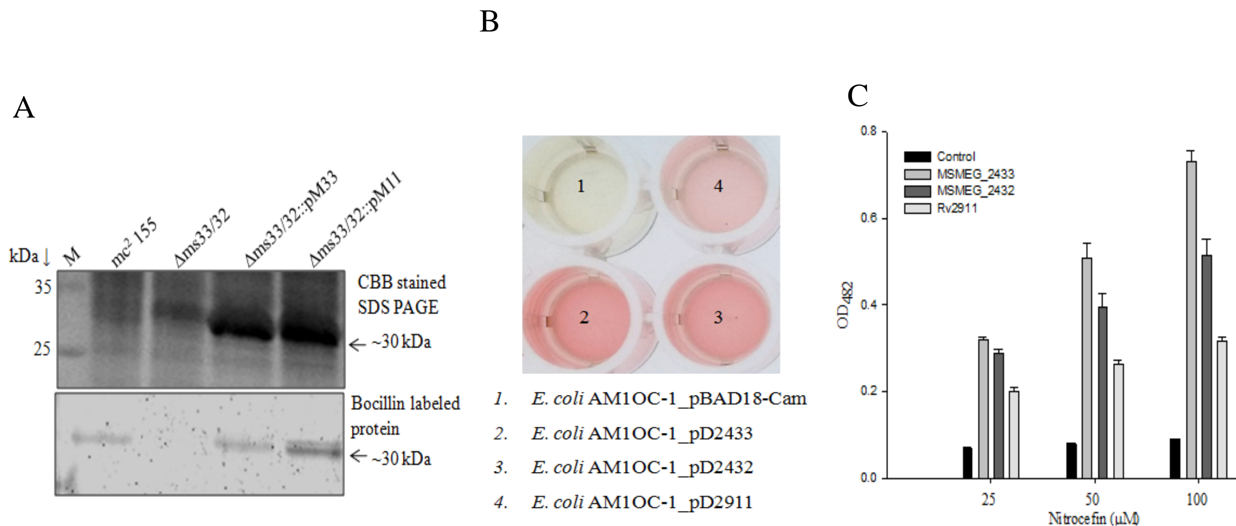

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81 **Fig. S4**. Membrane localization of MSMEG_2433 and Rv2911 and β-lactamase assay. Panel

82 (A) Upper panel, the coomassie brilliant blue (CBB) stained 15% SDS PAGE gel showing

83 membrane proteins isolated from the 4-mycobacterial strains, and the panel below indicates the

84 bocillin-FL (fluorescent tagged penicillinV; binds only to the PBPs) labeled proteins scanned

85 using Typhoon (refer Methods for detail). M, represents the protein molecular weight marker.

86 Panel (B) shows the hydrolysis of nitrocefin in presence of MSMEG_2433, MSMEG_2432 and

87 Rv2911to prove the β-lactamase activities of these proteins that we claimed a reason of disparity

88 in the band intensities of panel (A)'s CBB stained and bocillin labeled protein bands. We chose

89 *E. coli* AM1OC-1 strain (Δ*ampC*) that has no β-lactamase activity as negative control (number

90 1). The strain AM1OC-1 was complemented with MSMEG_2433, MSMEG_2432 and Rv2911

91 using arabinose inducible vector, pBAD18-Cam to generate AM1OC-1_pD2433 (number 2),

92 AM1OC-1_pD2432 (number 3) and AM1OC-1_pD2911 (number 4), respectively. The β-
93 lactamase activity of the complemented strains with ectopically expressed proteins were tested
94 and compared against the negative control. The β-lactamase activity of MSMEG_2433 has been
95 reported earlier (3) so that can be considered as positive control. The Whole cell lysates (2 µg) of
96 all the strains were treated with 50 µM of nitrocefin and incubated at RT for 30 min before
97 picture was shot. Panel (C), graph shows the nitrocefin reduction assay of whole cell lysates
98 from panel B treated with 25, 50 and 100 µM of nitrocefin in triplicates incubated at RT for 30
99 min. Absorbance was measured at 482 nm.

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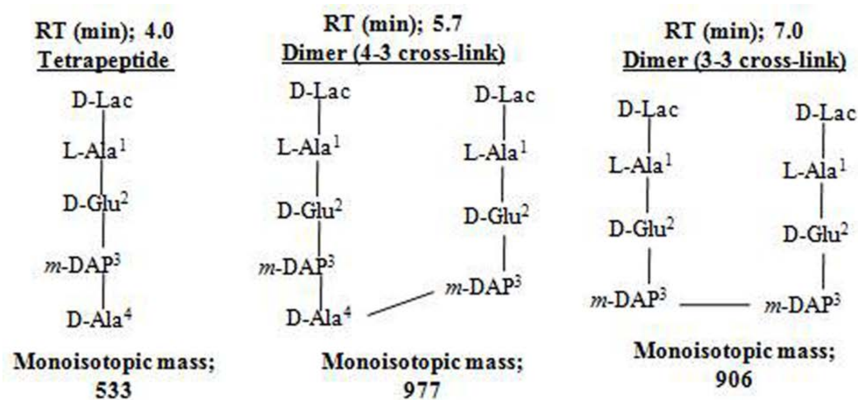
101 **Figure S5. Structure of PG muropeptides, monomer and dimers of *Mycobacterium*.**



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103 **Fig. S5.** Structure of PG muropeptides, monomer and dimers of *Mycobacterium*. Figure shows
104 the structures and monoisotopic masses (calculated MW) of the tetrapeptide, 4-3 and 3-3
105 crosslinked dimers, of mycobacterial PG muropeptides. 'RT' indicates, retention time.

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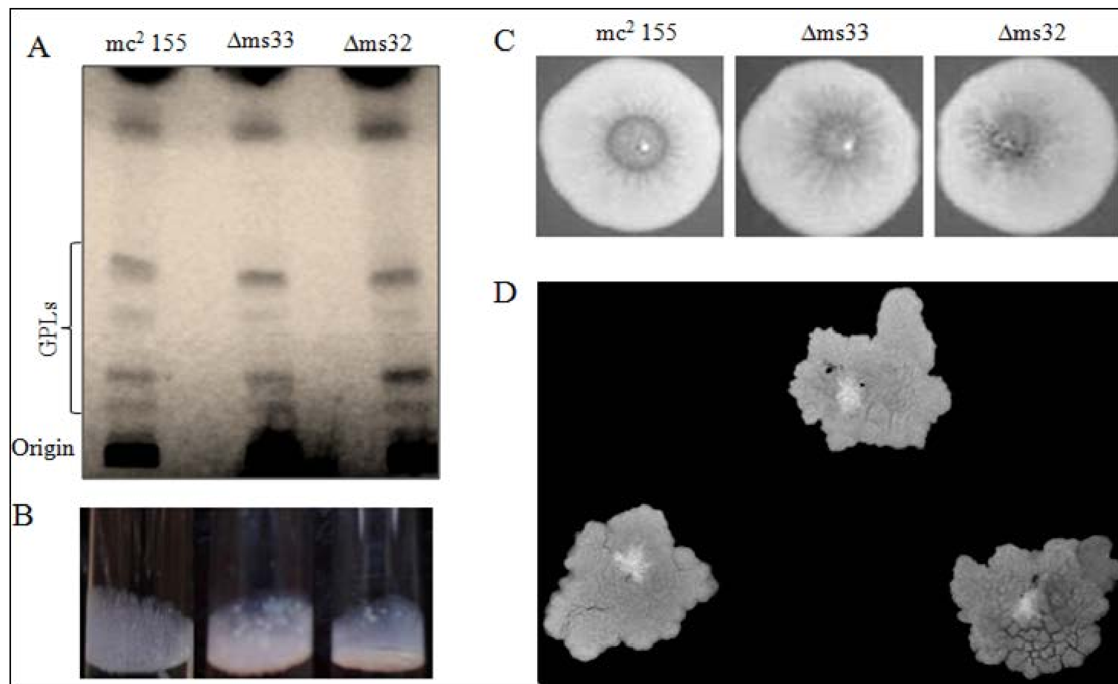
110 **Figure S6. Phenotypic evaluation of single gene deleted knockout mutants of *M. smegmatis*.**



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112 **Fig. S6.** Phenotypic evaluation of single gene deleted knockout mutants, Δ*ms33* and Δ*ms33* of
113 *M. smegmatis.* Panel (A) shows the surface glycopeptidolipids (GPLs) isolated from $mc^2$155,
114 $mc^2$155.Δ*msmeg_2433* (Δ*ms33*) and $mc^2$155.Δ*msmeg_2432* (Δ*ms32*). Panel (B) shows the
115 pellicle formation of the three strains on the liquid-air surface. Panel (C) and (D) represent the
116 colony morphology of the three strains on M63 medium in presence of 0.8 % and 0.3% agarose,
117 respectively where panel D represents the motility of the strains. Single knockout mutants show
118 no significant difference in any of these phenotypic properties. Please refer Methodology for the
119 experimental detail.

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123 **Table S1. Synthesized primers used in this study.**

Deletion of double (*msmeg_2433/32)* and single genes, *msmeg_2433 or msmeg_2432* from *M. smegmatis* chromosome		
Primer name	Primer sequence (5' – 3')	PCR product length (bp)
LFF_*Bam*HI LFR_ *Spe*I	ATT AG*G GAT CC*T CCT TCT TCG GCG CCT TC GGA T*AC TAG T*GC CTG GAG ATC CAG CAA C	1009
RFF_*Xba*I LFR_ *Pst*I	ATG T*TC TAG A*GC CCT ACC GTC CGT AAC GTC ATT AGT *CTG CAG* TAG CGC GTG AGC AAC TCG	689
Ms33_F Ms33_R	GTAGGGATCCCTCGGCCAGGCTCAACATGTCTCGATTCAACACGACTA GGCTGGCCGGCCATAGAGGTCCGCTGTGACACAA (BamHI) TAGTCTGCAGCAGCGGCACCTCGTCGAGAACCACGAGCGCCAGCAGG GTCTTGATGGTGCTCACAGTTCCTCCGGATCGGTGAAG (PstI)	1070
Ms32_LF_F Ms32_LF_R	ATTAG*GGATCC*CGACATGGACACCGGCCAGAT GGAT*ACTAGT*GCAGCGGCTTGATGGTGCTCG	1031
Ms32_RF_F Ms32_RF_R	GGGT*TCTAGA*GGCAAGACCCTGACCTATCAG ATAGT*CTGCAG*AGGATCTGCCGCGCATCATCC	1061
Primers for the PCR - based screening of the *msmeg_2433* deleted strain; Δms33/32		
Ms33_for Ms33_rev	CTC TCT *GCT AGC* AGG AGG CTC TCT CTA TGT GGA GGT ACG CCT TCG GGC TGG T CTC TCT *AAG CTT* TCA GAG CGC CCC CGA TGC TCG CCG AA	891
*hyg*_for *hyg*_rev	ATA GAG GTC CGC TGT GAC ACA A CAG TTC CTC CGG ATC GGT GAA G	967
LF+*hyg*_for LF+*hyg*_rev	ATT AGG GAT CCT CCT TCT TCG GCG CCT TC TTG TGT CAC AGC GGA CCT CTA T	1149

RF+*hyg*_for RF+*hyg*_rev	CTT CAC CGA TCC GGA GGA ACT G ATT AGT *CTG CAG* TAG CGC GTG AGC AAC TCG	768
Primers for complementing Δms33/32 with *msmeg_2433* and *rv2911* to generate Δms33/32::pM33 and Δms33/32::pM11		
pM33_for pM33_rev	CTC TCT *CAT ATG* AGG AGG CTC TCT ATG TGG AGG TAC GCC TTC GGG CTG G CTC TCT *AAG CTT* TCA GAG CGC CCC CGA TGC TCG CCG AA	891
pM32_for pM32_rev	CAATATCATATGAGGAGGCTCTCTGTGCGAAGACTGTTCGCG CTCTCTAAGCTTCTACTGGCTGAAGCCGTAGTCGAA	828
pM11_for pM11_rev	CTC TCT *CAT ATG* AGG AGG CTC TCT CTA TGC GAA AGC TCA TGA CCG CGA CCG CTC TCT *AAG CTT* TCA GAG CGA GCC GAC GCT GGC CT	876
Primers for RT-PCR (semi quantitative) to confirm polar effect of deletion on *msmeg_2432*		
X_for Y_rev	CAC CGA CAT CGC GCG CAA GA ATG TCG GCG ACC AGC CAC GCC TT	402
X1_for (with Y)	CTT CAC CGA TCC GGA GGA ACT G	316
X2_for (with Y)	CAA TAT CAT ATG AGG AGG CTC TCT GTG CGA AGA CTG TTC GCG	155
16SrRNA_F 16SrRNA_R	GTG GAC TAC CAG GGT ATC TAA TCC T GGG TCT CTG GGC AGT AAC TG	76

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131 **Table S2. Muropeptide peaks, their retention time and relative abundance (peak area)**

132 **obtained through RP-HPLC analysis**

Peak	mc^2155		Δms33/32	
	Retention Time (min)	Relative abundance (%)	Retention Time (min)	Relative abundance (%)
1	3.6	7.4	3.6	5.7
2	**4.0**	**16.4**	**4.0**	**6.2**
3	4.2	4.7	4.1	2.8
4	4.7	27.6	4.4	0.4
5	5.1	6.5	4.7	27.3
6	5.3	9.2	5.1	9.7
7	5.5	4.1	5.4	1.5
8	**5.7**	**1.1**	5.6	5.8
9	6.0	9.4	**5.7**	**21.1**
10	6.2	8.2	6.2	12.2
11	**7.0**	**3.8**	**7.0**	**0.4**
12	8.6	1.3	7.2	0.8
13	11.3	0.6	7.5	2.6
14	-	-	8.9	2.5
15	-	-	9.4	1.1

133 Numerals in 'bold' shows the similar retention time from both the strain with >2.5-fold

134 difference in the relative abundance, were selected for the MALDI-ToF analysis. Values of the

135 retention time and peak area percent are shown as per the instrument, RP-HPLC display and
136 rounded off to the nearest total.

137 **References:**

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