**Supplemental Data**

# RND transporters protect *Corynebacterium glutamicum* from antibiotics by assembling the outer membrane

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**Table S1. Strains and plasmids used in this study**

| Strain/plasmid | Description | Source |
|---|---|---|
| *E. coli* | | |
| DH5α | *supE44 ΔlacU169 hsdR17 recA1 gyrA96 thi-1 relA1* | |
| S17-1 | *recA pro hsdR RP4-2*-Tc::Mu-Km::Tn7 | |
| *C. glutamicum* | | |
| WT | Wild type *C. glutamicum* ATCC13032 | ATCC |
| *⊿pks13* | ATCC13032 *⊿pks13::km* | (Portevin *et al.*, 2004) |
| LY100 | Δ*cmpL1* (*Ncgl2769*) | This study |
| LY101 | Δ*cmpL2* (*Ncgl0887*) | This study |
| LY102 | Δ*cmpL3* (*Ncgl0599*) | This study |
| LY103 | Δ*cmpL4*(*Ncgl0228*) | This study |
| LY104 | Δ*cmpL1* Δ*cmpL3* | This study |
| LY105 | Δ*cmpL2* Δ*cmpL3* | This study |
| LY106 | Δ*cmpL2* Δ*cmpL4* | This study |
| LY107 | Δ*cmpL3* Δ*cmpL4* | This study |
| LY108 | Δ*cmpL1* Δ*cmpL2* Δ*cmpL3* | This study |
| LY109 | Δ*cmpL2* Δ*cmpL3* Δ*cmpL4* | This study |
| LY110 | $Tc^R$; Δ*cmpL1* Δ*cmpL4 (*pML1-Tc) | This study |
| LY111 | $Tc^R$; Δ*cmpL1* Δ*cmpL4 (*pML4-Tc) | This study |
| LY112 | $Tc^R$; Δ*cmpL1* Δ*cmpL2* Δ*cmpL3* Δ*cmpL4* (pML1-Tc) | This study |
| LY113 | $Tc^R$; Δ*cmpL1* Δ*cmpL2* Δ*cmpL3* Δ*cmpL4* (pML4-Tc) | This study |
| LY114 | Δ*cmpL1* Δ*cmpL2* Δ*cmpL3* Δ*cmpL4 ppc*::*lacI*-$P_{tac}$-*cmpL1* | This study |
| LY115 | Δ*cmpL1* Δ*cmpL2* Δ*cmpL3* Δ*cmpL4 ppc*::*lacI*-$P_{tac}$-*cmpL4* | This study |
| LY116 | $Tc^R$; Δ*cmpL1* Δ*cmpL2* Δ*cmpL3* Δ*cmpL4* (pML4-Tgt-Tc) | This study |
| *Plasmids* | | |
| pAN6 | $Kan^R$; *C. glutamicum*/*E. coli* shuttle vector | (Frunzke *et al.*, 2008) |
| pK19*mob-sacB* | $Kan^R$; vector for allelic exchange in *C. glutamicum* (pK18*ori*$V_{E.c.}$*sacB lacZ*α) | (Schafer *et al.*, 1994) |
| pML1 | $Kan^R$; pAN6 derivative containing $P_{tac}$-*cmpl1* | This study |
| pML3 | $Kan^R$; pAN6 derivative containing $P_{tac}$-*cmpl3* | This study |
| pML4 | $Kan^R$; pAN6 derivative containing $P_{tac}$-*cmpl4* | This study |
| pDG1514 | $Tc^R$; plasmid contains a tetracycline cassette | (Guerout-Fleury *et al.*, 1995) |
| pML1-Tc | $Tc^R$; pML1 with tetracycline cassette | This study |
| pML4-Tc | $Tc^R$; pML4 with tetracycline cassette | This study |
| pML4-Tgt-Tc | $Tc^R$; pAN6 derivative containing $P_{tac}$-*cmpL4-tgt* (N*cgl0228-0229*) | This study |

**Table S2. Antibiotic susceptibility of *C. glutamicum* cells lacking *cmpLs*.**

| Strain | MIC (μg/ml) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | CAR | CHL | RIF | LIN | NOR | VAN | NOV | GM | TOB | SPE | EB |
| WT | 3-6 | 3 | 0.0125 | 6.25 | 1.5-3 | 0.38 | 6.25-12.5 | 0.19 | 0.1-0.2 | 31.25-62.5 | 3.125-6.25 |
| LY100 | 3-6 | 3 | 0.0125 | 6.25 | 1.5-3 | 0.38 | 6.25-12.5 | 0.19 | 0.1-0.2 | 31.25-62.5 | 1.56-3.125 |
| LY103 | 3-6 | 3 | 0.0125 | 6.25 | 1.5-3 | 0.38 | 6.25-12.5 | 0.19 | 0.1-0.2 | 31.25-62.5 | 1.56-3.125 |
| LY104 | 3-6 | 3 | 0.0125 | 6.25 | 1.5-3 | 0.38 | 6.25-12.5 | 0.19 | 0.1-0.2 | 31.25-62.5 | 1.56-3.125 |
| LY106 | 3-6 | 3 | 0.0125 | 6.25 | 1.5-3 | 0.38 | 6.25-12.5 | 0.19 | 0.1-0.2 | 31.25-62.5 | 1.56-3.125 |
| LY107 | 3-6 | 3 | 0.0125 | 6.25 | 1.5-3 | 0.38 | 6.25-12.5 | 0.19 | 0.1-0.2 | 31.25-62.5 | 1.56-3.125 |
| LY108 | 3-6 | 3 | 0.0125 | 6.25 | 1.5-3 | 0.38 | 6.25-12.5 | 0.19 | 0.1-0.2 | 31.25-62.5 | 1.56-3.125 |
| LY109 | 3-6 | 3 | 0.0125 | 6.25 | 1.5-3 | 0.38 | 6.25-12.5 | 0.19 | 0.1-0.2 | 31.25-62.5 | 1.56-3.125 |

CAR, carbenicillin; CHL, chloramphenicol; RIF, rifampiicin; VAN, vancomycin;, LIN, lincomycin; NOR, norfloxacin; NOV, novobiocin; GM, gentamycin; TOB, tobramycin; SPE, spectinomycin; EB, ethidium bromide

**Table S3. Antibiotic susceptibility of *C. glutamicum* mutant cells overproducing various CmpL proteins.**

| Strains | IPTG (mM) | Diameters (mm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | CAR | NOR | SPE | TOB | EB | NOV |
| WT(pAN6) | - | 25.9±0.2 | 26.8±0.3 | 13.9±0.7 | 27.5±0.4 | 28.0±0.7 | 21.1±0.7 |
| | 0.1 | 25.8±0.3 | 27.3±0.3 | 13.8±0.3 | 28.5±0.5 | 28.5±0.5 | 21.8±0.3 |
| LY108(pML1) | - | 25.6±0.4 | 26.4±0.4 | 13.4±0.4 | 28.5±0.5 | 27.8±0.2 | 21.8±0.3 |
| | 0.01 | 27.6±1.1 | 26.9±1.1 | 13.4±0.4 | 28.4±0.4 | 27.9±0.2 | 22.7±0.9 |
| LY108(pML3) | - | 25.5±0.5 | 26.5±0.5 | 14.0±0.8 | 27.8±0.3 | 27.3±0.5 | 22.4±0.4 |
| | 0.01 | 25.8±0.7 | 26.8±0.6 | 13.4±0.4 | 28.3±0.5 | 27.7±0.7 | 22.3±0.6 |
| LY109(pML4) | - | 25.5±0.4 | 27.5±0.3 | 13.8±0.6 | 28.3±0.3 | 28.0±0.8 | 21.8±0.2 |
| | 0.1 | 25.9±0.7 | 27.7±0.5 | 14.1±1.1 | 30.0±0.8 | 27.7±0.5 | 22.3±0.6 |

**Table S4 Primers used in this study**

| Name | Sequences(5'-3') |
|---|---|
| *Primers used for crossover PCR* | |
| Cmpl1-del-1 | ACTGAATTCTCCTTCTAGTGTGGGACGCTC |
| Cmpl1-del-2 | CCCATCCACTAAACTTAAACAAGCTTGGTGCCGAAAATGAC |
| Cmpl1-del-3 | TGTTTAAGTTTAGTGGATGGGCTCAACGACGATGAGGAAGTCACC |
| Cmpl1-del-4 | ATTGAATTCTTTTCGACGAAGCCCCACTCCC |
| Cmpl2-del-1 | TACGAGGTCTGCGGTGGCAATG |
| Cmpl2-del-2 | CCCATCCACTAAACTTAAACACCGCCAGCGTCGTGATACCTATGAG |
| Cmpl2-del-3 | TGTTTAAGTTTAGTGGATGGGAAGGAAAATGTAGGTGTCGG |
| Cmpl2-del-4 | ACTGAATTCAATGCGACTCCATCTTCTATG |
| Cmpl3-del-1 | ACTGCATGCCCAATGAGAGCAGCGGCAATT |
| Cmpl3-del-2 | CCCATCCACTAAACTTAAACAGGGCAGAAAGATTCTCATCAAGCG |
| Cmpl3-del-3 | TGTTTAAGTTTAGTGGATGGGTGGCCGTCAAAATTGTCCAATCAG |
| Cmpl3-del-4 | ACTTCTAGATCTCTAGCCGTTTTTTGC |
| Cmpl4-del-1 | ACTGTCGACTCCCGTACTTGTTCAGGATTAG |
| Cmpl4-del-2 | CCCATCCACTAAACTTAAACAGAATAGCAATTTCGCCACGG |
| Cmpl4-del-3 | TGTTTAAGTTTAGTGGATGGGCGGGCGTGTTTTTCGATGCTTTC |
| Cmpl4-del-4 | TTCGTCGACAAGCGCCATGC |
| *Primers used to verify mutants* | |
| Cmpl1-mut-f | CAATCCCACTGACAAAGAAGGC |
| Cmpl1-mut-r | CGGTGAGCGAGACCGAGC |
| Cmpl2-mut-f | CATGATTGCGCGTTGTCG |
| Cmpl2-mut-r | TTCTTAATCCAGGCGCATGC |
| Cmpl3-mut-f | GTTAATTTTCTGGAATGCC |
| Cmpl3-mut-r | TGAGTCGGCTAAAAACAG |
| Cmpl4-mut-f | GCTAACAAGGTTCTGGGTG |
| Cmpl4-mut-r | CAGATTTGAAATCCACGC |
| *Primers used for construction of expression plasmids* | |
| Cmpl1-Exp-f | TAATCCACATATGTTTTCTAAATGGGGCCACTTTG |
| Cmpl1-Exp-r | ATATTCGCGCTAGCATCTAGATCCTCAAGCCTG |
| Cmpl3-Exp-f | GGCGGCATATGTCTACTAGCATCACAACAGAG |
| Cmpl3-Exp-r | GGATGTCTAGATAGCTGAGGCTGCTTCTG |
| Cmpl4-Exp-f | GAAGAACCATATGGCGAAATTGCTATTCAGG |
| Cmpl4-Exp-r | TAATAATAGCTAGCACGTGCAGCCTGCTTCTC |
| Cmpl4+Tgt-Exp | TCCTGATA GCTAGCTGAAACCTTCGACGCGTAG |
| *Primers for amplification of tetracycline cassette* | |
| Tc cassette-f | CAAGCCCGGGAACATGAGAATTCCTG |
| Tc cassette-r | TTCGCCCGGGTCTGCAGATAGTGTAC |

## References


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Guerout-Fleury, A.M., K. Shazand, N. Frandsen & P. Stragier, (1995) Antibiotic-resistance cassettes for Bacillus subtilis. *Gene* 167: 335-336.

Portevin, D., C. de Sousa-D'Auria, C. Houssin, C. Grimaldi, M. Chami, M. Daffé & C. Guilhot, (2004) A polyketide synthase catalyzes the last condensation step of mycolic acid biosynthesis in mycobacteria and related organisms. *Proceedings of the National Academy of Sciences* 101: 314-319.

Schafer, A., A. Tauch, W. Jager, J. Kalinowski, G. Thierbach & A. Puhler, (1994) Small mobilizable multi-purpose cloning vectors derived from the Escherichia coli plasmids pK18 and pK19: selection of defined deletions in the chromosome of Corynebacterium glutamicum. *Gene* 145: 69-73.

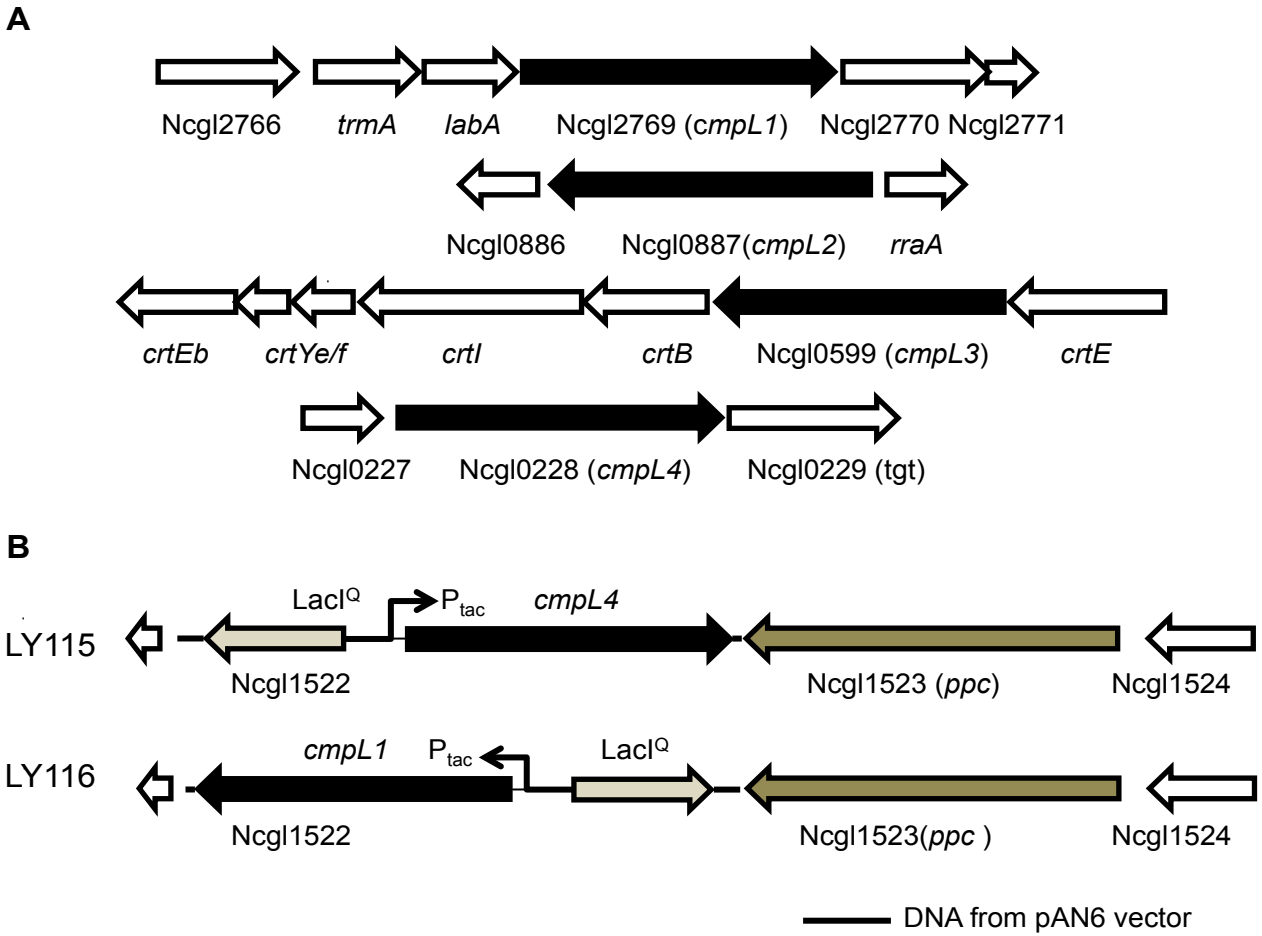


Fig. S1. (A) Genetic contexts of four *cmpL* genes encoded on *C. glutamicum* chromosome. (B) Localization and orientation of *cmpL1* and *cmpL4* genes expressed under the IPTG -inducible Ptac promoter in LY115 and LY116.

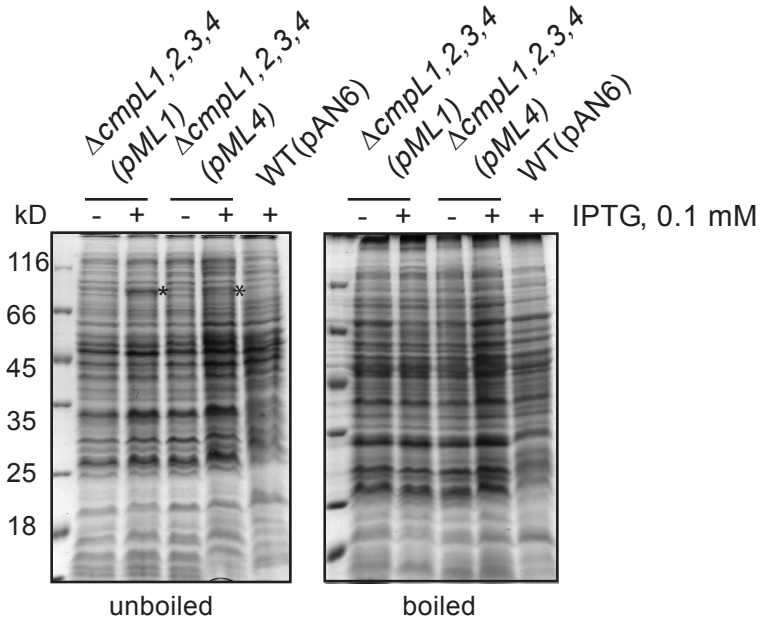


Fig. S2. Protein composition of membranes isolated from LY112 (Δ*cmpl1*,*2,3,4* (pML1-Tc)) and LY113 (Δ*cmpl1*,*2,3,4* (pML4-Tc)) in the presence and absence of IPTG. Membrane fractions were isolated by ultracentrifugation and analyzed by 12% SDS-PAGE. Overproduced CmpL1 and CmpL4 can be seen in unboiled samples (marked with asterisk).

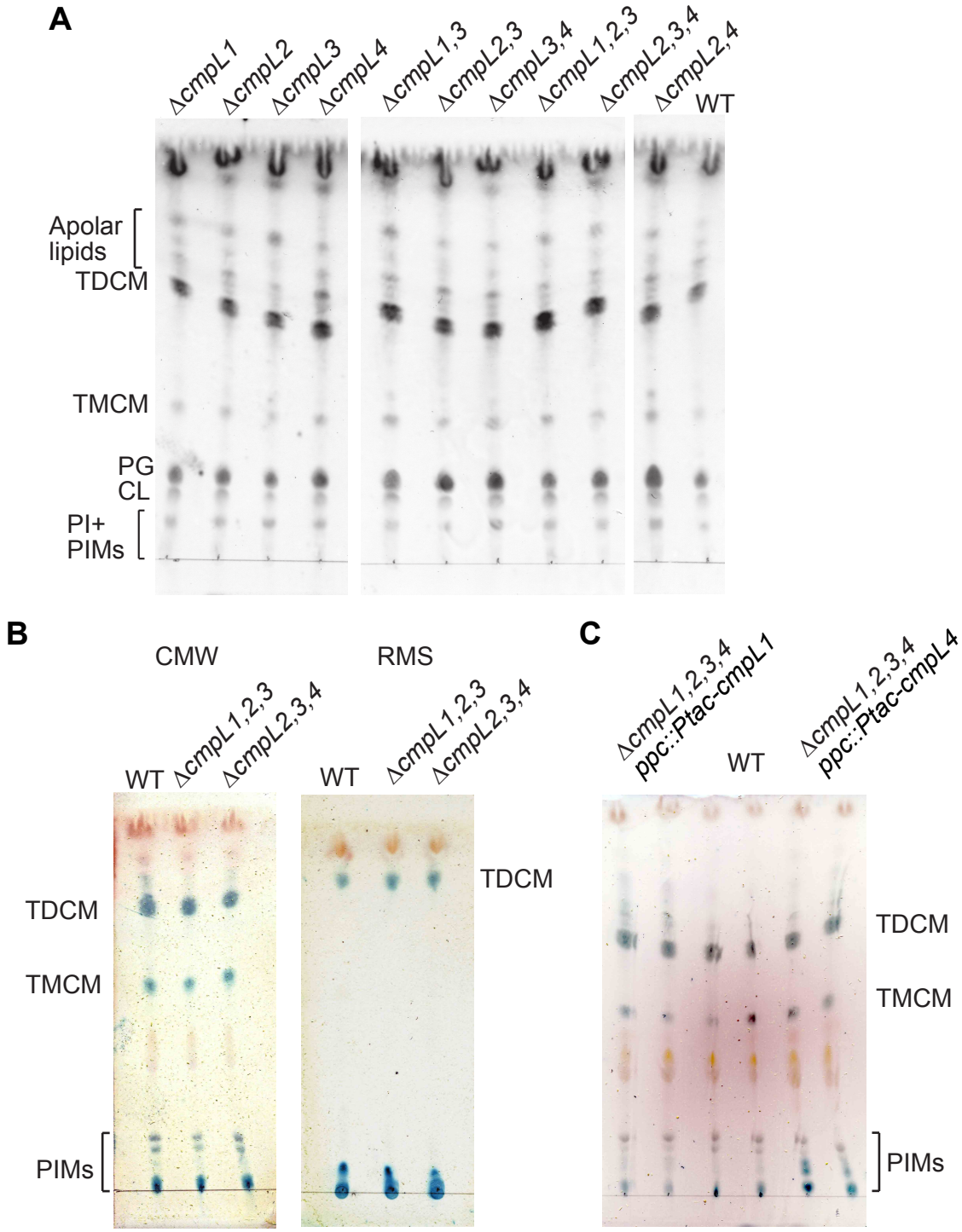


Fig. S3. Composition of lipids extracted from *C. glutamicum* mutants. (A) Cells were grown in the minimal medium and noncovalently associated lipids extracted with CMW mixture. TLC was carri out with C:M:W 30:8:1 as the mobile phase and lipids were stained with 5% phosphomolybdic solution. (B) Mutant cells were grown in minimal medium. Lipids were extracted either with CMW panel) or RMS (right) mixtures, separated on TLC plates with C:M:W 30:8:1 as the mobile phase stained with anthrone. (C). Glycolipid composition of LY114 and LY115 knock-in mutant strains. Lipids were extracted from cells grown in minimal medium with CMW and analyzed as in (B).

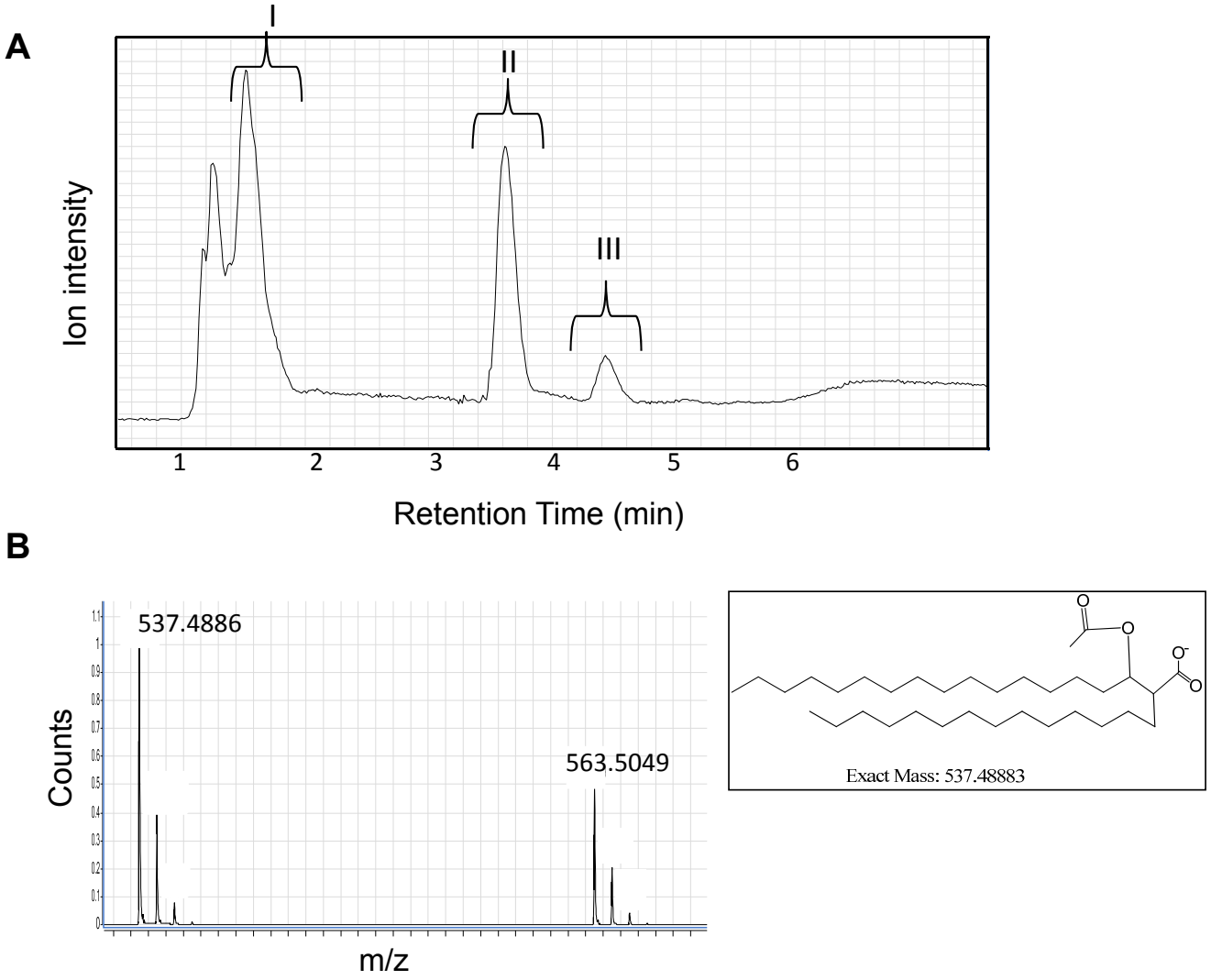


Fig. S4. (A) LC/MS chromatogram of Lipid X. Component I which barely stuck to the reversed phase LC column was not further analyzed and may or may not be part of the Lipid X TLC spot. Component III is like component II except it is based on a C-34 fully saturated corynomycolate. (B) The mass spectrum of component II consistent with acetylated C-32 fully saturated corynomycolate (m/z = 537.4886) whose structure is shown on the right) and monounsaturated C-34 corynomycolate (m/z = 563.5049).

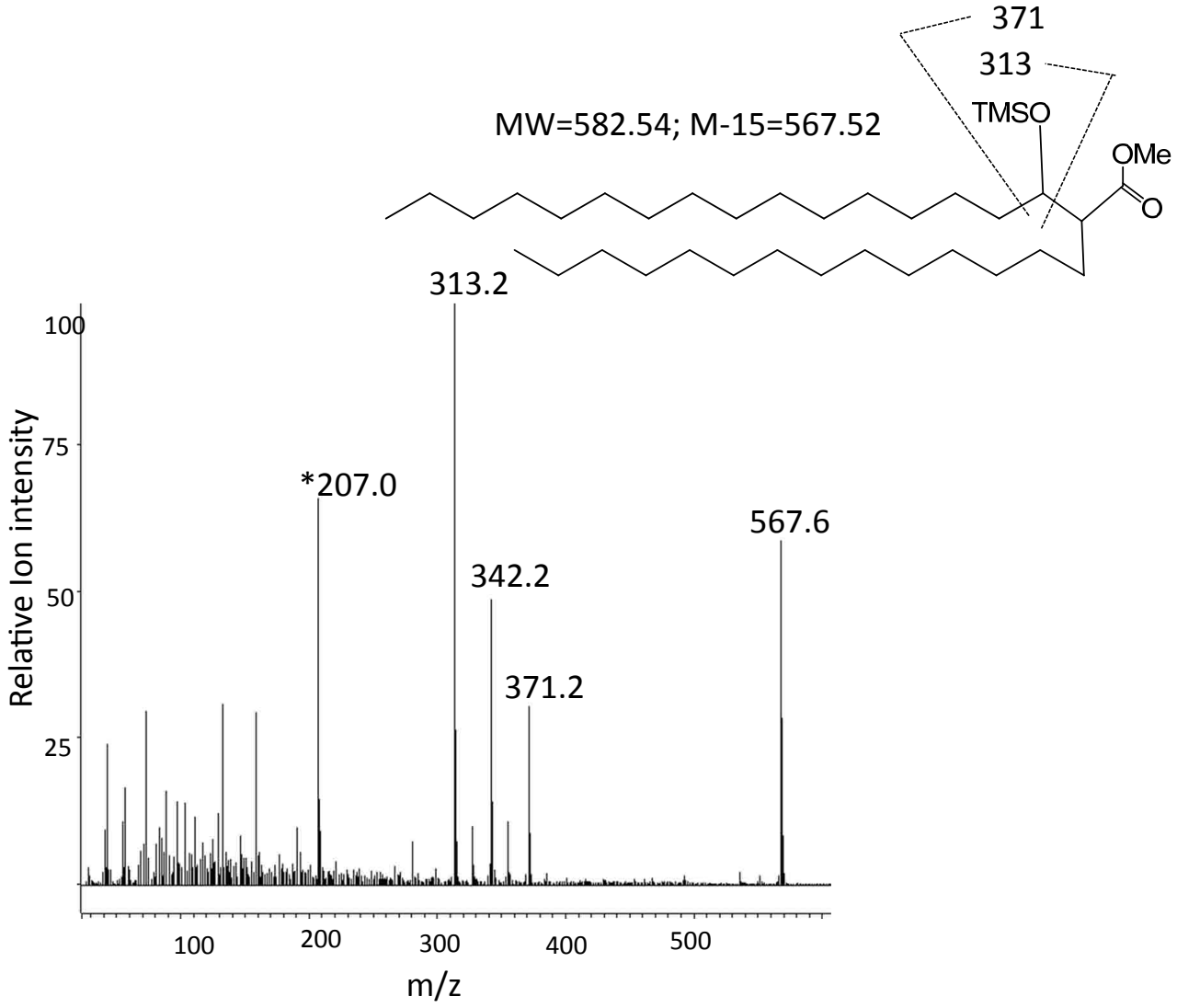


Fig. S5. The structure of the methyl ester trimethylsilyl ether of C-32 corynomycolate as determined by GC/MS after formation of the methyl ester and trimethylsilylation of the alcohol. The m/z 207 ion (asterisk) is from column bleed. M-15 is 567.6. The original LC/MS analysis also had a complex early eluting peak containing ions at m/z 485.3854, 489.3359, and 521.3622 and the GC/MS had many components in addition to the corynomycolates. These possible other components have not been investigated.