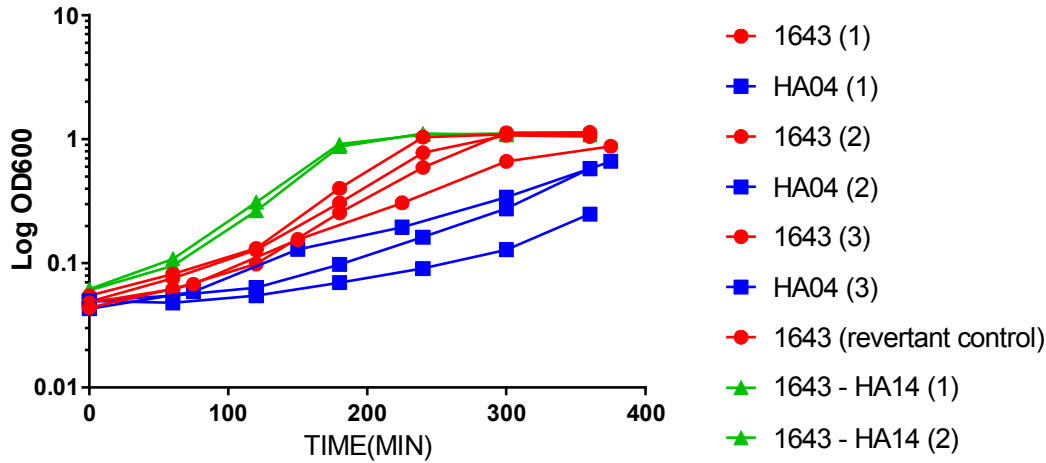
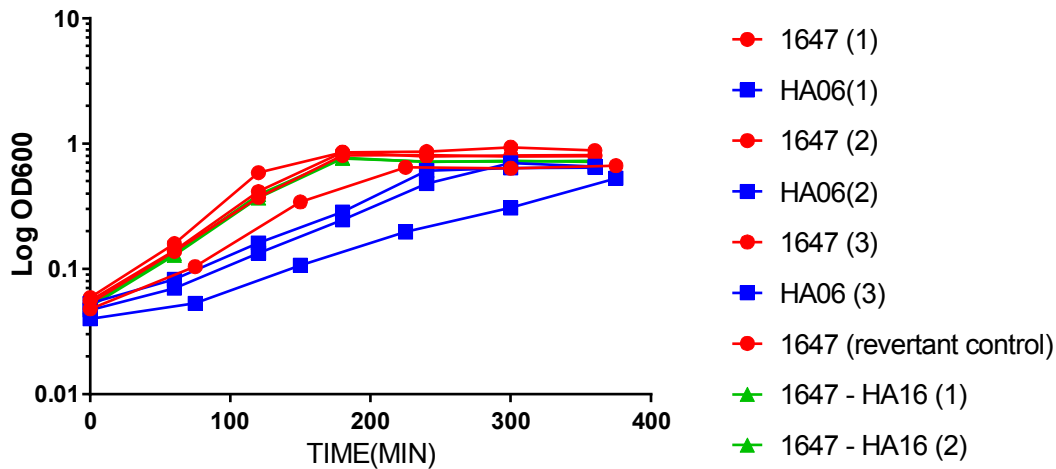


1643/HA04/HA14



1647/HA06/HA16



1648/HA08/HA18

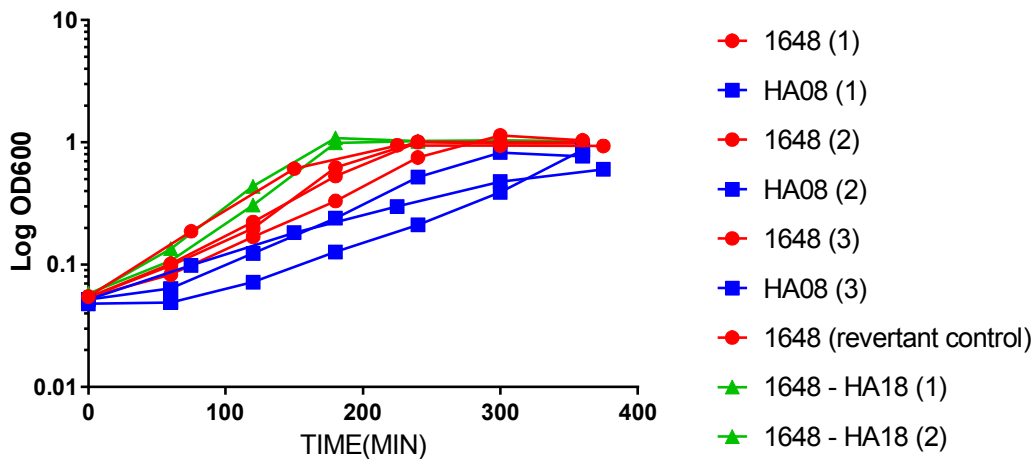
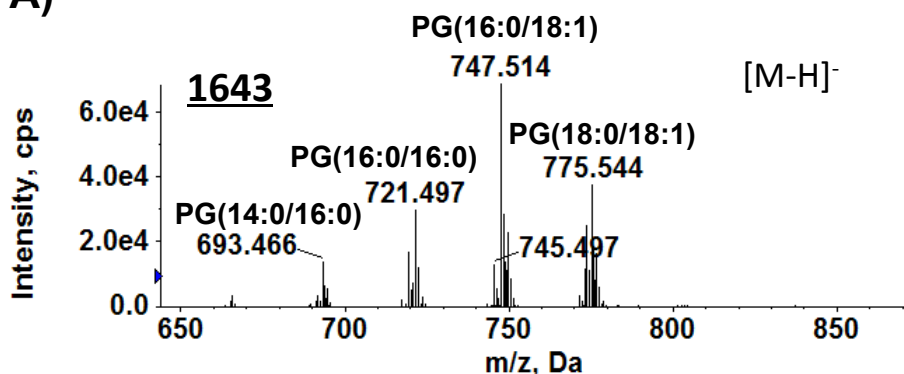


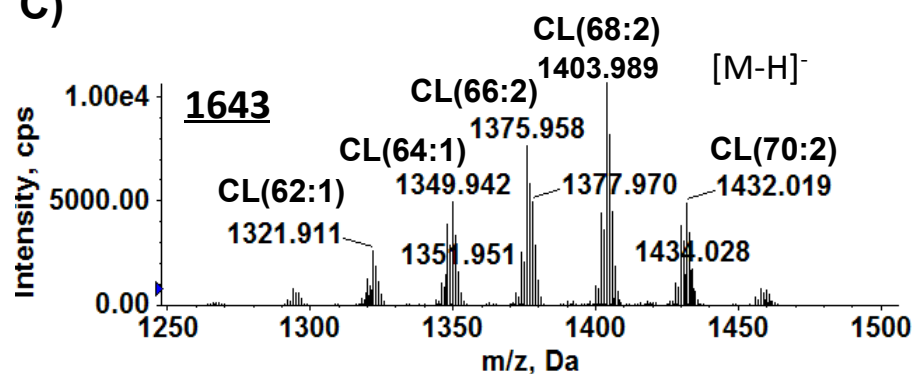
Figure S1. Growth curves for parental strains, their DAP^R derivatives, and DAP^S revertants. *S. mitis* 1643, DAP^R derivative 1643-HA04, and DAP^S revertant 1643-HA14 are displayed top; *S. oralis* 1647, DAP^R derivative 1647-HA06, and DAP^S revertant 1647-HA16 are displayed middle; *S. oralis* 1648, DAP^R derivative 1648-HA08, and DAP^S revertant 1648-HA18 are shown bottom. Red lines represent parental strains, blue lines represent resistant strains, and green lines represent revertant strains. Cultures were grown overnight in BHI and diluted to an OD₆₀₀ of 0.05 in fresh BHI. OD₆₀₀ readings were taken either every hour or every 75 minutes for six hours.

Figure S2: Representative negative ion ESI mass spectra of PG and CL species in a DAP sensitive/resistant strain pair.

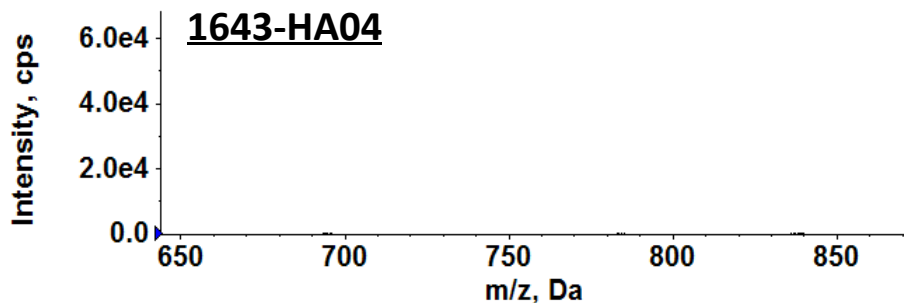
A)



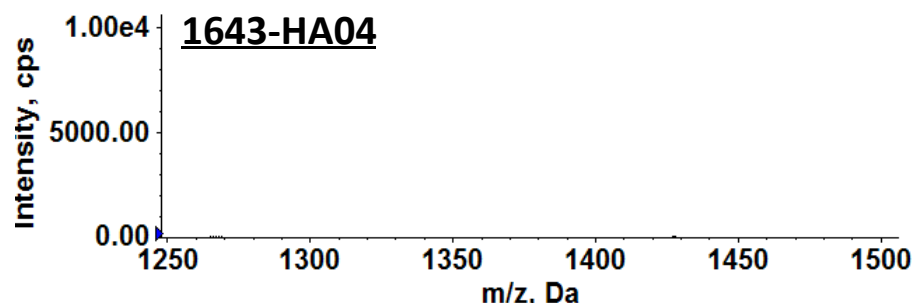
C)



B)



D)



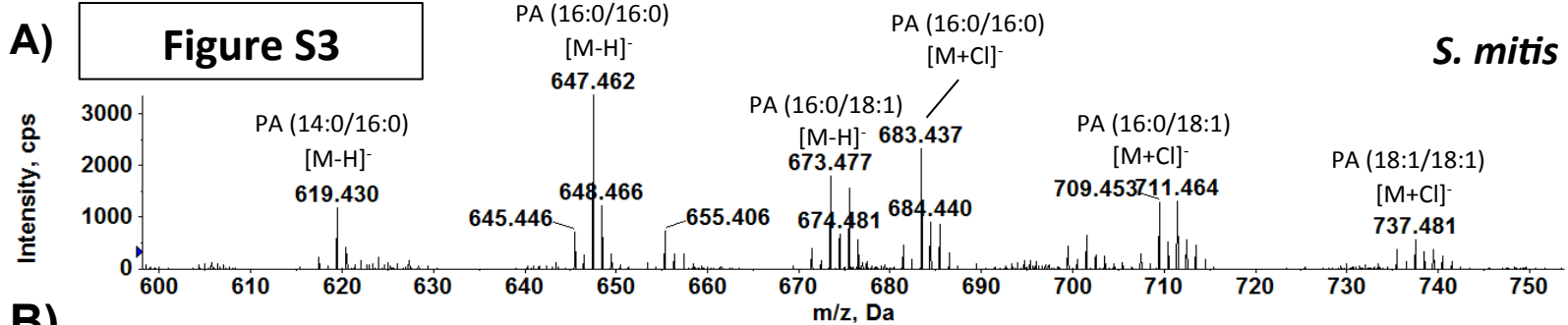
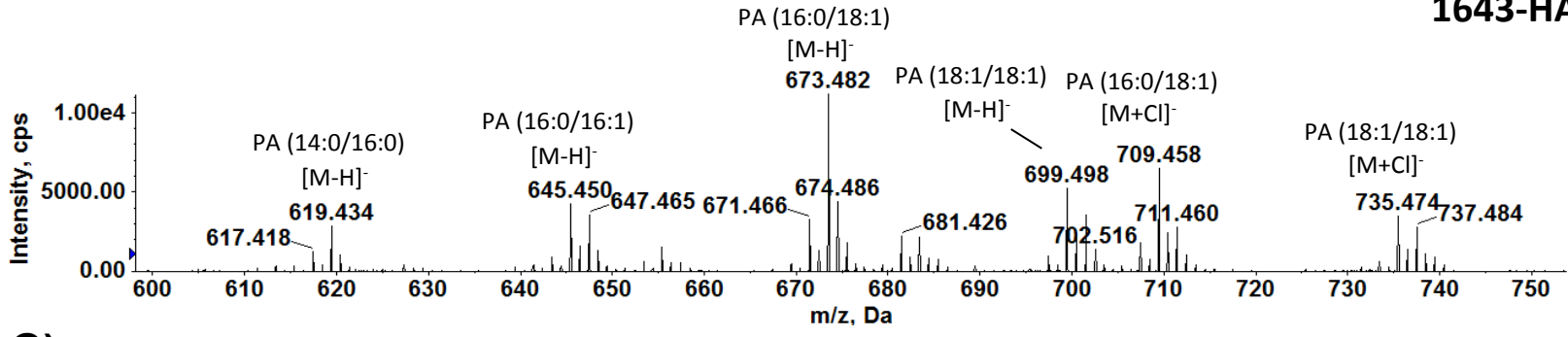
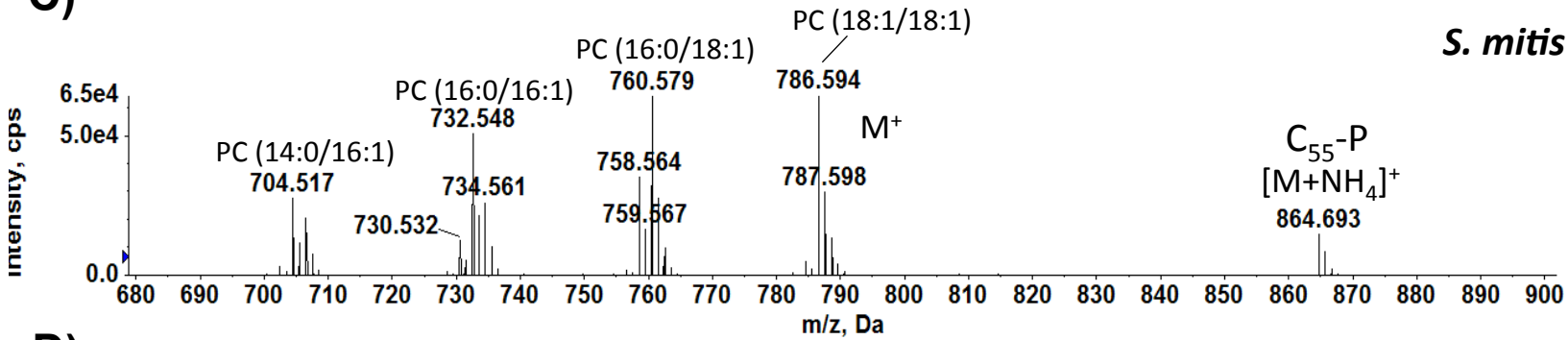
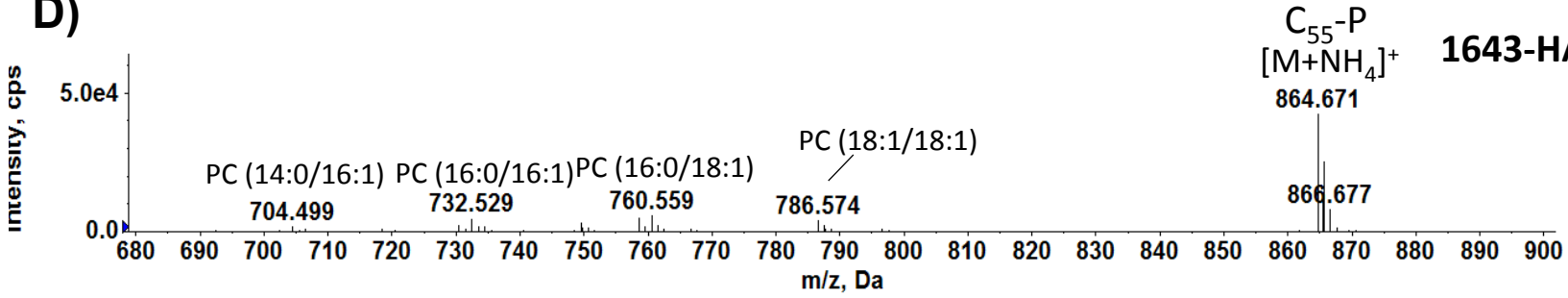
A)**Figure S3*****S. mitis* 1643****B)****1643-HA04****C)*****S. mitis* 1643****D)****1643-HA04**

Fig S3. Mass spectra of PAs, PCs, and undecaprenyl phosphate (C_{55} -P) in (A), (C) DAP-sensitive and (B), (D) DAP-resistant strains. The mass spectra shown are averaged from spectra acquired by negative ion ESI/MS during the 20.2-21.2 min window. Both deprotonated $[M-H]^-$ and chloride adduct $[M+Cl]^-$ ions of PA are detected. The level of PC is significantly lower in the DAP-resistant strain compared to the parental DAP-sensitive strain.

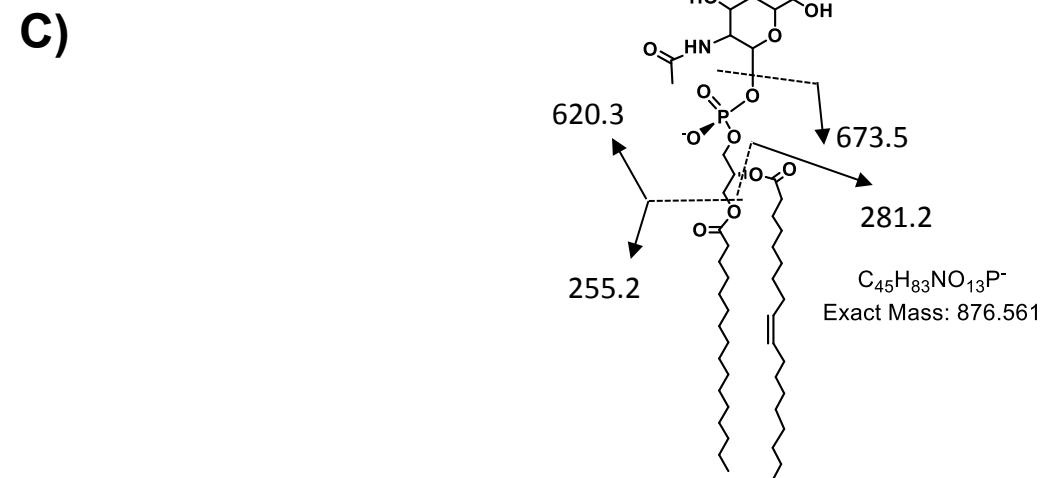
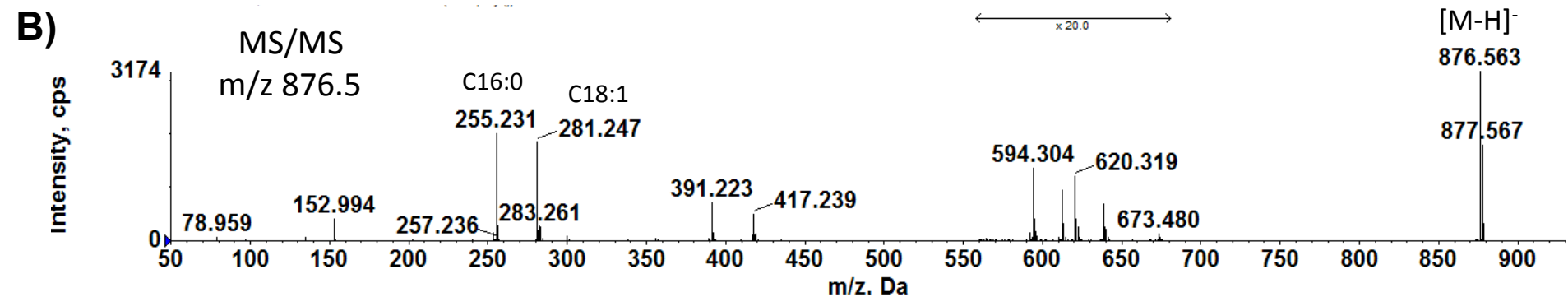
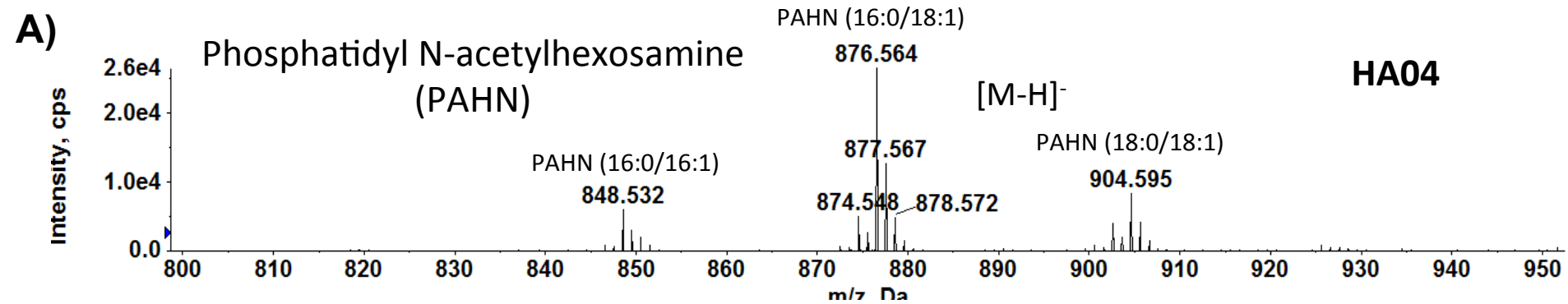


Fig S4. Identification of phosphatidyl N-acetylhexosamine (PAHN) from strain HA04.

Fig S4. Identification of phosphatidyl N-acetylhexosamine (PAHN) from strain HA04.

- A. Negative ion ESI mass spectrum showing the $[M-H]^-$ ions of the PAHN molecular species emerging at 14.8-15.3 min.
- B. Collision-induced dissociation (CID) MS/MS spectrum of the $[M-H]^-$ ion PAHN (16:0/18:1) at m/z 876.5. The arrows indicating x20 reflect magnification of the product ion peaks in the corresponding region of the m/z values on the mass spectrum.
- C. Chemical structure of PAHN (16:0/18:1) and the fragmentation scheme for the observed product ions in B.

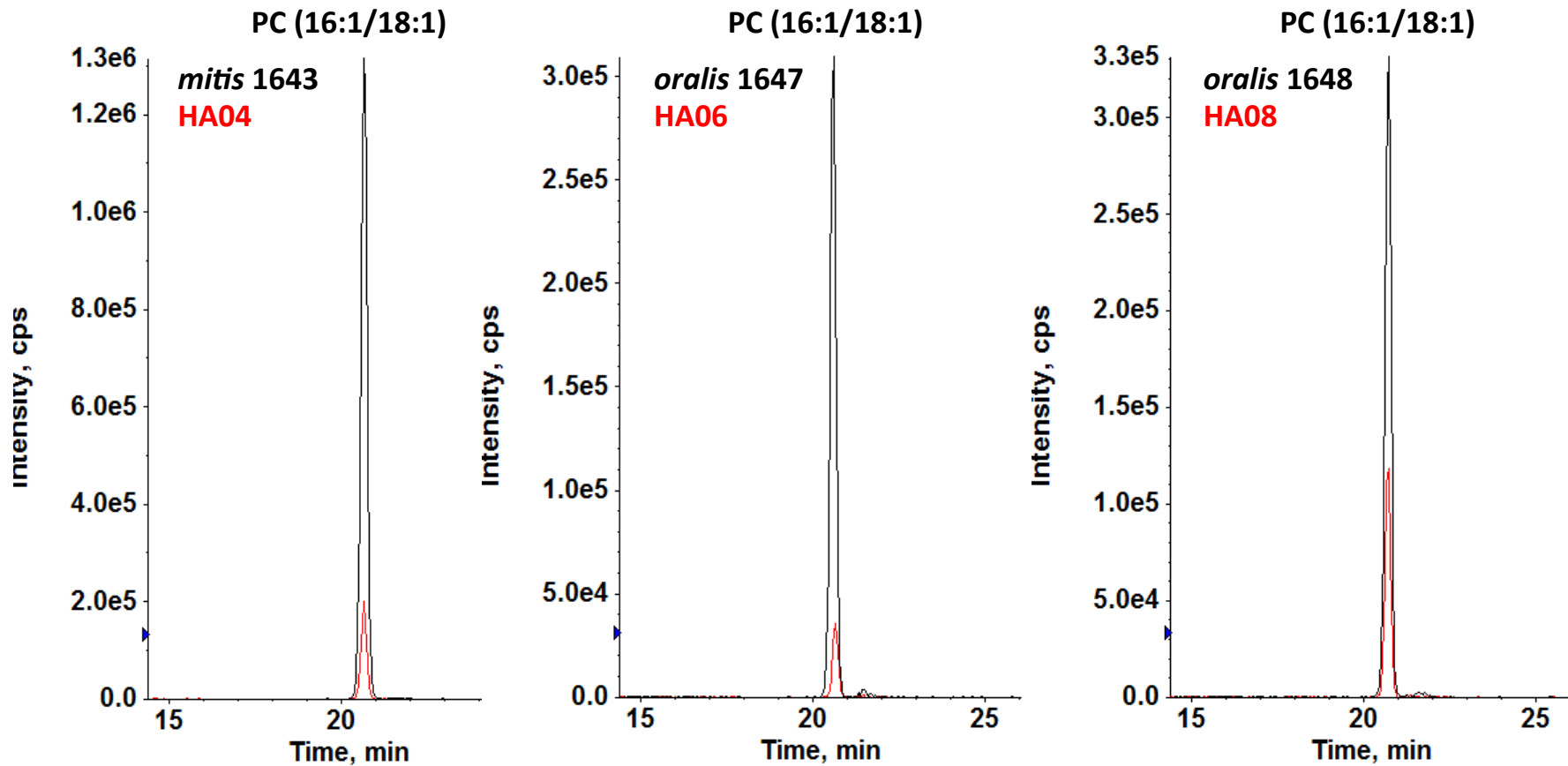


Fig S5. Comparison of the level of PC (16:1/18:1) in the DAP-resistant and DAP-sensitive pair strains. Extracted ion chromatograms of m/z 758.5 for the M^+ ion of PC (16:1/18:1), a major PC species in *S. mitis* 1643 (black) and *S. mitis* 1643-HA04 (red).

Table S1. De novo assembly data for parental strains.

Strain	# reads	# contigs	Min contig size	Max contig size	Average contig size	Contig N50 (bp)	Genome Size (Mbp)
1643	12,173,871	119	211	253,142	15,805	66,153	1,880,744
1647	14,577,054	158	202	224,156	12,380	53,890	1,956,052
1648	14,503,619	199	205	153,663	9,413	48,229	1,873,268

Table S2. Primers used in this study.

Primer Name	Sequence
1643_IntGen_19_F	CCAGTGTAACCTTTACTGCA
1643_IntGen_19_R	CGAGCTGACTGAAGCAGAC
1643_CellWall_F	GCCTGACTTAACTAATCTGG
1643_CellWall_R	CCACGAAGTCGAATCGTTAC
1643_Hypothetical_F	GGAGATTTAGAGGGGTGTC
1643_Hypothetical_R	CGGAACTATATCCAATCTGTC
1643_SerThr_Kinase_F	GCGTCAGATAAGTGAAGTGG
1643_SerThr_Kinase_R	CCCATCCACGAAGAAAACAT
1643_IntGen_32_F	GTGCCGTCAGTTTTGACTTG
1643_IntGen_32_R	GGACTACTGTAGCTGACAC
1643_Ribo_S5p_F	GCCAGATGGCGTCAAAGT
1643_Ribo_S5p_R	GGCTAGTGATGTCCGAGAG
1643_cdsA_F	GCAATTGGGAAAGCGGCA
1643_cdsA_R	CCGTACGAGTGGTGAGTTG
1647_IntGen_13_F	GCTACAAAAGCGCAGGCTTG
1647_IntGen_13_R	GGCTTACTATGGCTTTGAC
1647_DAG_Kinase_F	CGGCTCCTGCCGCCATGT
1647_DAG_Kinase_R	GCCTACATTGGGGAACTTTTC
1647_IntGen_17_F	CGAAATCAGATAGACTTGTC
1647_IntGen_17_R	GATTTGGCTCTTTTACCATAC
1647_cdsA_F	GGGCCCCATACCAATGGC
1647_cdsA_R	GCGCAGCCTACCTGACAG
1647_Ribo_Mtase_F	CCAGCAGTCGCCTCATAG
1647_Ribo_Mtase_R	GCCCTAAACACTGTGCCA
1647_ppGpp_Synthetase_F	GGACTATGCTACCGAACG
1647_ppGpp_Synthetase_R	CTCCTCCGCCAGATAGTT
1648_cdsA_F	GAGGAAGGATTCTGAATGGT
1648_cdsA_R	GGCTCCGAGAGTTTCTCC
1648_IntGen_27_F	GCTCACCAATCCCAAACAC
1648_IntGen_27_R	GGAACGCGTTCTCAGAAAG
1648_ppGpp_Synthetase_F	CCTCTGGGCCAACACCG
1648_ppGpp_Synthetase_R	GCAATCCAGTGCCGGGTG
1648_Ribo_S5U_Mtase_F	CCAGTTGACTAGACTCGTC
1648_Ribo_S5U_Mtase_R	CGAAGACAGAGATAAGCTAG
1648_Helicase_F	GGAAGCATGGCTTTTAGGATG
1648_Helicase_R	CGTGAAGCGTTTCGTCTC
Full_cdsA_F	GGTTAGCAATCCAATCATTC
Full_cdsA_R	CCATCGCAATCGCCGTTTTG
CdsA_promoter_F	GCCTCCTAGAGGAAGGAT
CdsA_promoter_R	GGGACGGTATACTAGGTTC
CdsA_Sequence_1	GCCTTGCAGGAAGCTATTG

Table S3. Major lipid species in wild-type, *cdsA* mutant, and revertant *S. mitis* detected by negative ion ESI/MS.

Lipid class	[M – H]⁻	Molecular species^a	WT	CdsA	Revertant
DAG	547.42 ^b	DAG (26:0)	+	+	+
	573.43 ^b	DAG (28:1)	+	+	+
	575.44 ^b	DAG (28:0)	+	+	+
	601.46 ^b	DAG (30:1)	+	+	+
	603.47 ^b	DAG (30:0)	+	+	+
	627.47 ^b	DAG (32:2)	+	+	+
	629.49 ^b	DAG (32:1)	+	+	+
	657.52 ^b	DAG (34:1)	+	+	+
FA	225.19	FA (14:1)	+	+	+
	227.2	FA(14:0)	+	+	+
	253.22	FA (16:1)	+	+	+
	255.23	FA (16:0)	+	+	+
	281.25	FA (18:1)	+	+	+
	283.26	FA (18:0)	+	+	+
MHDAG	709.46 ^b	MHDAG (26:0)	+	+	+
	735.48 ^b	MHDAG (28:1)	+	+	+
	737.49 ^b	MHDAG (28:0)	+	+	+
	763.50 ^b	MHDAG (30:1)	+	+	+
	765.51 ^b	MHDAG (30:0)	+	+	+
	791.54 ^b	MHDAG (32:1)	+	+	+
	819.57 ^b	MHDAG (34:1)	+	+	+
DHDAG	871.52 ^b	DHDAG (26:0)	+	+	+
	897.53 ^b	DHDAG (28:1)	+	+	+
	899.54 ^b	DHDAG (28:0)	+	+	+
	925.56 ^b	DHDAG (30:1)	+	+	+
	927.57 ^b	DHDAG (30:0)	+	+	+
	953.58 ^b	DHDAG (32:1)	+	+	+
	981.62 ^b	DHDAG (34:1)	+	+	+

PG	693.47	PG(14:0/16:0)	+	—	+
	719.49	PG(16:0/16:1)	+	—	+
	721.5	PG(16:0/16:0)	+	—	+
	773.53	PG(16:0/18:1)	+	—	+
	775.54	PG(18:0/18:1)	+	—	+
CL	1321.91	CL(62:1)	+	—	+
	1349.94	CL(64:1)	+	—	+
	1375.96	CL(66:2)	+	—	+
	1403.99	CL(68:2)	+	—	+
	1432.02	CL(70:2)	+	—	+
PAHN	848.53	PAHN(16:0/16:1)	+	+	+
	874.55	PAHN(16:1/18:1)	+	+	+
	876.56	PAHN(16:0/18:1)	+	+	+
	902.58	PAHN(18:1/18:1)	+	+	+
	904.6	PAHN(18:0/18:1)	+	+	+
PA	619.43	PA(14:0/16:0)	+	+	+
	645.45	PA(16:0/16:1)	+	+	+
	647.46	PA(16:0/16:0)	+	+	+
	673.48	PA(16:0/18:1)	+	+	+
	699.49	PA(18:1/18:1)	+	+	+
	701.50	PA(18:0/18:1)	+	+	+
PC	740.50 ^b	PC(14:0/16:0)	+	+	+
	766.51 ^b	PC(16:0/16:1)	+	+	+
	792.53 ^b	PC(16:1/18:1)	+	+	+
	794.54 ^b	PC(16:0/18:1)	+	+	+
	820.56 ^b	PC(18:1/18:1)	+	+	+

^aThe individual or total numbers of acyl chain carbon atoms and double bonds are shown in parentheses.

^bDAG, MHDAG, DHDAG, and PC values are for the [M + Cl]⁻ ions.

Abbreviations: WT, *S. mitis* 1643; CdsA, *S. mitis* 1643-HA04; Revertant, *S. mitis* 1643-HA14; DAG, diacylglycerol; MHDAG, monohexosyldiacylglycerol; DHDAG, dihexosyldiacylglycerol; FA, fatty acid; CL, cardiolipin; PA, phosphatidic acid; PAHN, phosphatidyl N-acetylhexosamine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine.

Table S4. Mutations in *cdsA* identified in daptomycin spontaneous resistance experiments.

Strain ID	Nucleotide Change	Amino Acid Change
LJ35	G35A	A12E
LJ39	T264A	Y88*
LJ310	T264A	Y88*
LJ311	T264A	Y88*
LJ713	C351A	S117R
LJ72	G361T	G121C
LJ76	G361T	G121C
LJ313	T366del	F122fs
LJ510	G394-T645del	V132-S215 del
LJ72	G421A	A141T
LJ54	T428-T695del	F143-F232delfs
LJ34	T431ins	I144fs
LJ56	G445A	D149N
LJ514	C450G	S150R
LJ714	C455T	A152V
LJ76	C459G	Y153*
LJ52	A481T	R161*
LJ53	A499T	R167*
LJ31	C506T	S169F
LJ51	C506T	S169F
LJ712	G516C	K172N
LJ33	G527C	G176A
LJ37	G527C	G176A
LJ711	T531del	F177fs
LJ32	C551A	A184E
LJ59	C551A	A184E
LJ36	G679C	A227P
LJ512	C688T	R230C
LJ312	A731C	H244P
LJ314	A731C	H244P
LJ55	G734T	G245V

S. mitis 1643/RA04

>1643_Hypothetical

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R

>HA04_Hypothetical

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>HA04_Ser_Thr_Kinase

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N

>HA04_Ribosomal_alanineacyltransferase

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N

>1643_cdsA

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FGLF

>HA04_cdsA

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***S. oralis* 1647/RA06**

>1647_cdsA

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FGLF

>HA06_cdsA

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FGLF

>1647_Intergenic_Contig_13

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>HA06_Intergenic_Contig_13

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>1647_Intergenic_Contig_17

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>HA06_Intergenic_Contig_17

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***S. oralis* 1648/RA08**

>1648_cdsA

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>HA08_cdsA

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>1648_ppGpp_synthetase

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>HA08_ppGpp_synthetase

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FEEIYDLIAIRCILDTSQSDVYAMLGYVHELWKPMMPGRFKDYIANRKANGYQSIHTTVYGPKGPIE
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MDKRHMDEVLQKTSYKTEEALYAAIGFGEIGAITVFNRLTEKERREEERAKAKAEAEELVKGGE
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>1648_Ribosomal_SSU_MTase

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PCSGIGLLRRKPDIKYNKETADFTSLQEIQLEILGSVCQTLRKGGIITYSTCTIVSEENFQVVEAFL
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>HA08_Ribosomal_SSU_MTase

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PCSGIGLLRRKPDIKYNKETADFTSLQEIQLEILGSVCQTLRKGGIITYSTCTIVSEENFQVVEAFL
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>1648_Helicase_PriA

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RAKKKAELRDYLLSHPKSAPLASLLESYSREQVNFVEQGAVSIVQKEVQRSAAAYFEGIEASQP
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>HA08_Helicase_PriA

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