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Supplementary Information for

Alarmone Ap4A is elevated by aminoglycoside antibiotics and enhance their bactericidal activity

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SUPPLEMENTARY FIGURES and TABLES

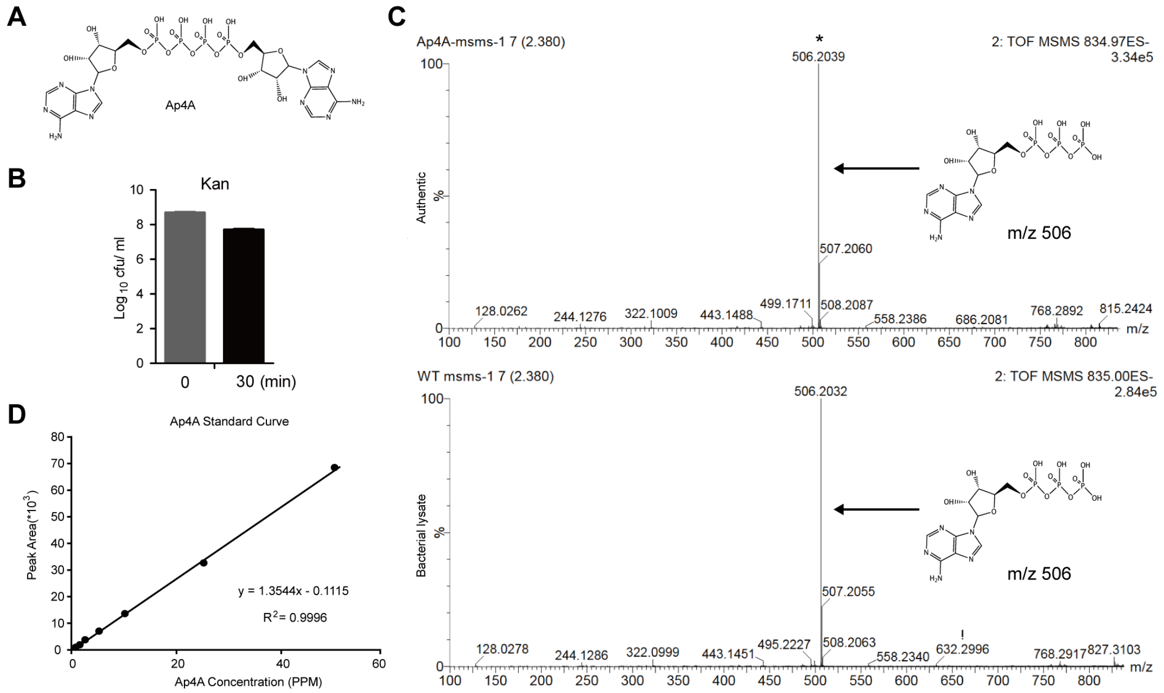


Fig. S1. Characterization of Ap4A. (A) Chemical structure of diadenosine tetraphosphate (Ap4A). (B) Survival of *E. coli* WT upon 100 µg/ml kanamycin treatment for 30 min. (C) The tandem mass spectrometry (MS/MS) fragmentation of authentic Ap4A (upper panel) and the identified ion (m/z=835.0794) in the lysate of bacteria treated with 100 µg/ml kanamycin (lower panel) produced the same corresponding ion at m/z = 506. The predicted chemical structure corresponding to m/z= 506 from Ap4A is shown. (D) The standard curve built with authentic Ap4A by UPLC/ESI-Q-TOF/MS for intracellular Ap4A quantification.

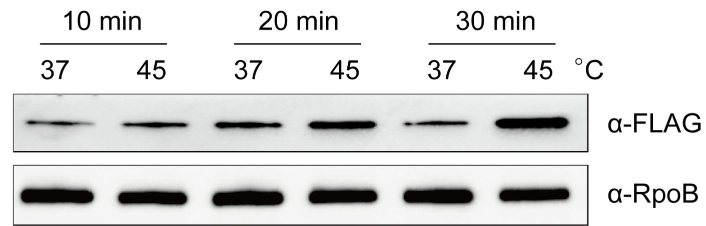
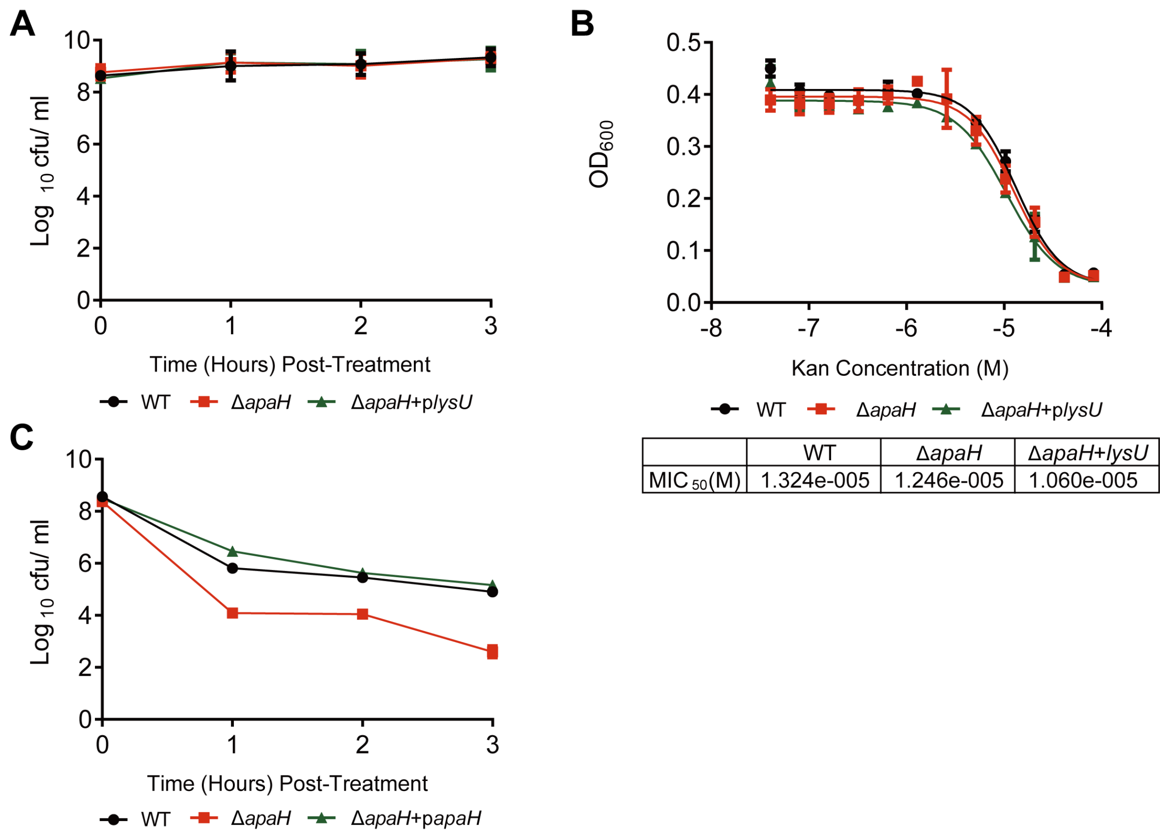


Fig. S2. Heat-shock stress induces the expression of LysU. Chromosomally expressed LysU was tagged with FLAG epitope in *lysU::flag* and the expression of the fusion protein was examined with anti-FLAG antibody. The expression of LysU was shown to be induced by heat-shock stress as a control for Fig. 1C. *lysU::flag* was treated at 45℃ for 10, 20 and 30 min before protein extraction.



	WT	ΔapaH	ΔapaH+lysU
MIC 50(M)	1.324e-005	1.246e-005	1.060e-005

Fig. S3. Ap4A contributes to aminoglycoside killing of *E. coli*. (A) Growth curves for WT, Δ*apaH* and Δ*apaH*+p*lysU* as measured by CFU counting. (B) Growth inhibition of WT, Δ*apaH* and Δ*apaH*+p*lysU* caused by kanamycin. The minimum inhibitory concentration (MIC_{50}) of each strain is shown. (C) Survival of WT, Δ*apaH,* and its complement (Δ*apaH*+p*apaH*) upon treatment with 100 µg/ml kanamycin. Sample aliquots were harvested at 1, 2, and 3 hours for CFU enumeration. WT and Δ*apaH* both contain the pBAD24 empty plasmid. Each point represents the mean ± SD from one representative experiment in triplicate. At least three biological replicates were performed.

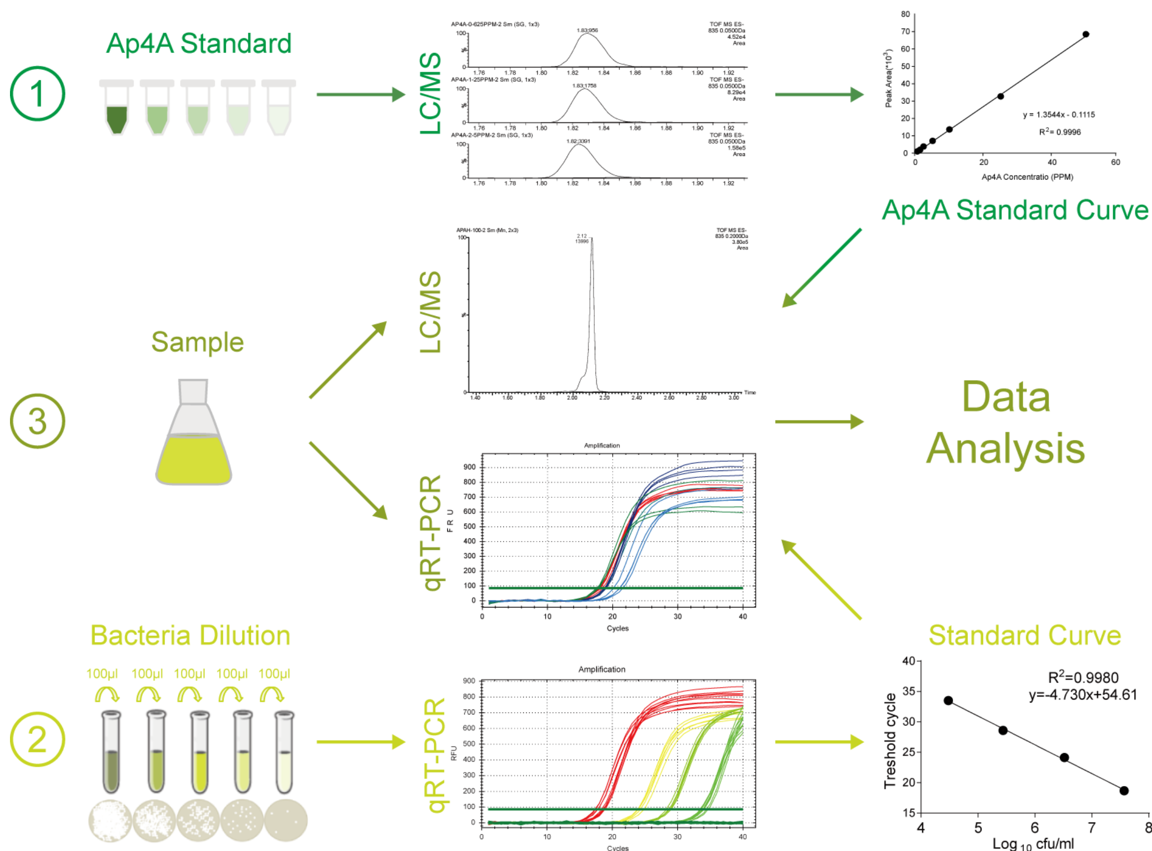


Fig. S4. Schematic illustration of the procedure to normalize the measured Ap4A concentration in each bacterial sample. ① An Ap4A standard curve is built by plotting the area under the curve measured by UPLC/ESI-TOF/MS against its concentration. A linear relationship over a concentration of 0.125 µM to 50 µM is shown. The regression equation of Ap4A was y = 1.3544x - 0.1115, and the correlation coefficient (R^2) was 0.9996, indicating a good linearity. ② *E. coli* WT were grown in LB medium at 37℃ to OD_{600} = 0.5. Ten-fold serial dilutions were conducted and divided into two parts: 1 ml sample was used to measure the level of *rpoB* gene by qRT-PCR; another 1 ml of the same sample was used for CFU counting (diluted accordingly if necessary). The Ct value for each sample was then used to build a stand curve of the CFU number of each samples. ③ The measured Ct value of *rpoB* gene in the antibiotic treated bacterial sample was matched to the standard curve to estimate the CFU of the samples. The Ap4A concentration from UPLC/ESI-TOF/MS was normalized based on the estimated bacterial CFU.

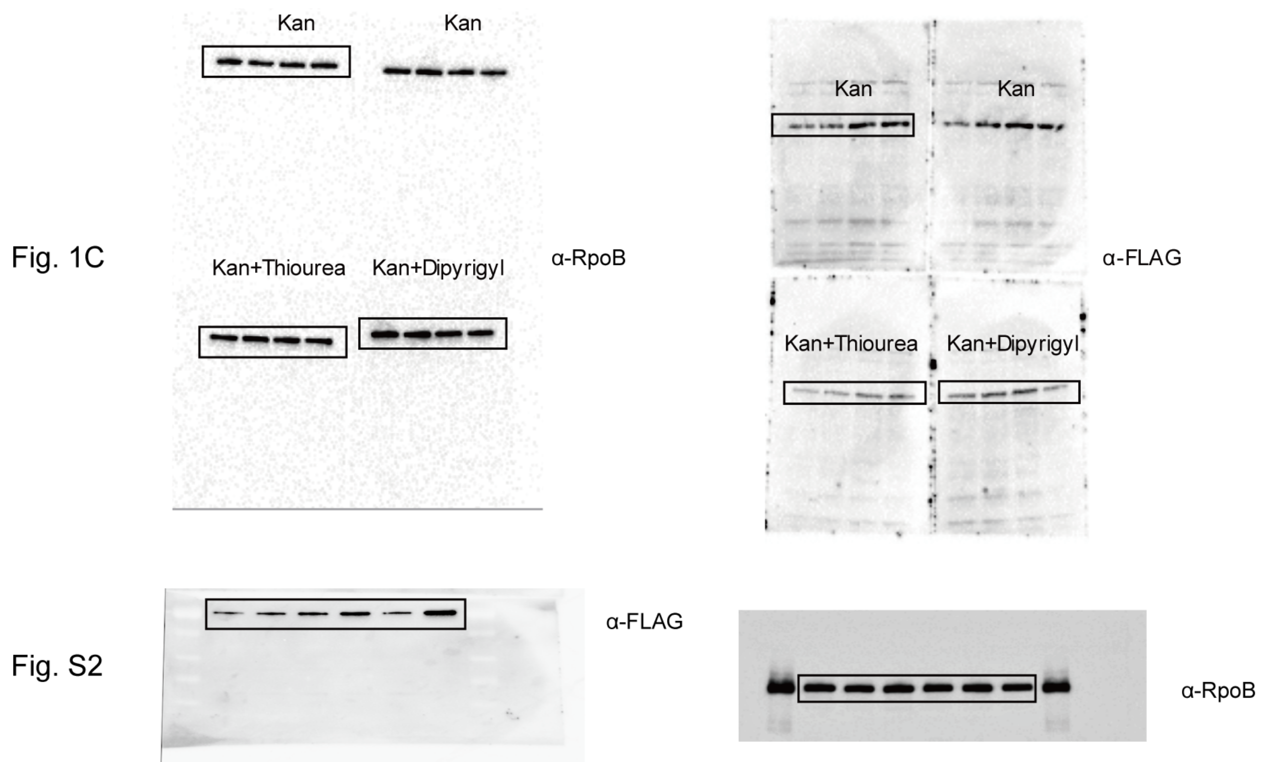


Fig. S5. Original image of the Western blot analysis in this study.

Table S1. The 26 ions with the most prominent increase or decrease in abundance in *E. coli* treated with kanamycin

Metabolite No.	Metabolite Matched	MS (Da)	RT (min)	VIP	Increase or decrease
M1	Acetyl-CoA	808.1458	2.07	28.9433	↓
M2	Coenzyme A	766.1304	1.99	27.6813	↓
M3	Glycyl-D-tyrosyl-D-phenylalanyl-D-phenylalanyl-D-tyrosine	730.2789	2.05	18.1724	↑
M4	Succinyl-CoA	866.1411	1.98	17.1395	↓
M5	NADH	664.1287	1.62	15.2196	↓
M6	Unknown	809.1478	2.06	15.092	↓
M7	(Methylenecyclopropyl)acetyl-CoA	860.2475	1.74	13.0732	↑
M8	Unknown	786.1920	1.97	12.997	↑
M9	NADP	742.1007	1.74	12.0936	↑
M10	2,3-(Di-glutathion-S-yl)-1,4-naphthoquinone	767.1369	1.92	11.4878	↓
M11	FAD	784.1832	2.09	11.3095	↑
M12	Unknown	731.2823	2.05	10.7593	↑
M13	Ap4A	835.0794	2.04	10.6579	↑
M14	PS(O-20:0/22:0)	860.7498	1.75	10.5657	↑
M15	UDP-N-acetylmuraminate	678.1247	1.81	10.3167	↑
M16	Unknown	810.1467	2.07	9.91576	↓
M17	Unknown	720.1571	1.71	8.92531	↓
M18	Dephospho-CoA	686.1665	1.93	8.69693	↑
M19	Unknown	866.1612	1.75	7.79572	↑
M20	Unknown	665.1322	1.62	7.55813	↓
M21	L-Asparaginyl-L-tyrosyl-L-tyrosyl-L-tryptophyl-L-serine	730.2592	1.79	7.08896	↓
M22	Unknown	867.1478	1.93	6.75062	↓
M23	Unknown	687.1701	1.93	4.64844	↑
M24	Unknown	768.1457	1.64	3.40754	↑
M25	Unknown	766.5044	1.57	3.12319	↑
M26	Unknown	788.1308	1.67	2.96498	↑

Note: MS. Molecular Mass; RT: retention time; VIP, Variable importance in the projection; ↓ denote decrease and ↑ denote increase in respective metabolite.

Table S2. Bacterial strains used in this study

Name	Description	Reference/Source
MG1655	Wild type *Escherichia coli*, WT	ATCC 700926
MC1061 λpir	*thi thr-1 leu-6 proA2 his-4 argE2 lacY1 galK2 ara-14 xy15 supE44 pir*	(1)
DH5α	*fhuA2* Δ(*argF-lacZ*)*U169 phoA glnV44 Φ80* Δ(*lacZ*)*M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17 pir*	(2)
WT+p*apaH*	MG1655 with p*apaH*	This study
Δ*apaH*	MG1655; in-frame deletion of *apaH* (deleting amino acids from 1 to 280)	This study
Δ*apaH*+p*lysU*	*ΔapaH* with p*lysU*	This study
Δ*apaH*+p*apaH*	*ΔapaH* with p*apaH*	This study
lysU::flag	MG1655, LysU in-frame tagged with FLAG tag	This study
Δ*apaH*+p*E264Q*	Δ*apaH* with p*E264Q*	This study
WT_AB	*Acinetobacter baumannii* ATCC17978, wild type	ATCC17978
*ΔapaH*_AB	*A. baumannii* ATCC17978, in-frame deletion of *apaH* (deleting amino acids from 1 to 280)	This study
WT_PA	*Pseudomonas aeruginosa* PAO1, wild type	(3)
*ΔapaH*_PA	*P. aeruginosa* PAO1, in-frame deletion of *apaH*	This study

Table S3. Plasmids used in this study

Name	Description	Source
pDS132	pCVD442 modified suicide plasmid, *pir* dependent, *sac*B, Cm^R,	(4)
pEXG2	ColE1 mob+, *sacB* suicide vector (Gm^R)	(5)
pBAD24	pBR322/ColE1 modified expression plasmid, araC, Amp^R	(6)
pPSV37	*colE1* origin, gentR, PA origin, *ori*T, *lac*UV5 promoter, *lacI*^{q}lacI^q	(7)
pDS132Δ*apaH*	pDS132 with flanking regions of *apaH* for deleting *apaH* of *E. coli* from amino acids 1 to 280	This study
pDS132-*lysU*::*flag*	pDS132 with a fragment for construction of LysU:: FLAG	This study
p*apaH*	pBAD24 with wild-type *apaH*	This study
p*lysU*	pBAD24 with wild-type *lysU*, arabinose inducible	This study
p*E264Q*	pBAD24 with E264Q mutant of LysU	This study
pEXG2Δ*apaH*_AB	pEXG2 with flanking regions of *apaH* for deleting *apaH* of *A. baumannii* from amino acids 1 to 280	This study
pEXG2Δ*apaH*_PA	pEXG2 with flanking regions of *apaH*, deleting *apaH* of *P. aeruginosa*	This study

Table S4. Primers used in this study

Name	Sequence (5'→3')	Description
RT-*rpoB*-for	CCTGCGCAAAGGTATGCCAA	For qRT-PCR of *rpoB*
RT-*rpoB*-rev	TACCGGACGCTCGAACTGTT	
lysU-flag-CH1-for	GTCAAACGTTTGTTGTCCG	For confirmation of *lysU::flag*
lysU-flag-CH1-rev	GTCGTCATCGTCTTTGTAGTC	
lysU-flag-CH2-for	CAAAGACGATGACGACAAG	
lysU-flag-CH2-rev	GCAAATCGCTGAAATTGTC	
lysU-flag-for	GGATCGATCCTCTAGGAAACCCCGATGATGCAG	For construction of *lysU::flag*
lysU-flag-rev	ATGCGGTACCTCTAGATGTCGATGAAAGCCTGA	
lysU-flag-int-rev	TTACTTGTCGTCATCGTCTTTGTAGTCTTTCTGTGGG CGCATCG	
lysU-flag-int-for	GACTACAAAGACGATGACGACAAGTAAATTTCACTT TAATGAACGAAGC	
del*apaH*-for	GGATCGATCCTCTAGACCTGCCTTATAACATCTCC	For construction of *apaH* deletion mutant
del*apaH*-int-rev	GCTGTGTTTACGCCATATTCTTTTAATGAA	
del*apaH*-int-for	GAATATGGCGTAAACACAGCCTGATATAGG	
del*apaH*-rev	ATGCGGTACCTCTAGTTAAGTGAGATCATTACCGA	
del*apaH*-CH1-for	GCCGAGAAAATGGGTCAG	For confirmation of *apaH* deletion
del*apaH*-CH1-rev	TCTTATCCGGCCTTCCTA	
del*apaH*-CH2-for	CTTTCAGCATCGACATTC	
del*apaH*-CH2-rev	ACAGTATTGTGTCTGCCA	
BAD*apaH*-for	GAGGAATTCACCATGGCGACATACCTTATTGGC	For construction of p*apaH*
BAD*apaH*-rev	GCAGGTCGACTCTAGTGTTTAAGACGCCGCCGCTT	
BAD*lysU*-for	GAGGAATTCACCATGTCTGAACAAGAAACAC	For construction of p*lysU*
BAD*lysU*-rev	GCAGGTCGACTCTAGTTATTTCTGTGGGCGCA	
E264QlysU-int-rev	ACCTTGATTACGGAAGTTACG	For construction of LysU 264 amino acid point mutant pE264Q
E264QlysU-int-for	ATCAAGGTATTTCTGTTCGC	
Del*apaH*AB-for	CATAAATGTAAAGCAATGTCGCTGTCGATTTAGC	For construction of *apaH* deletion mutant in *A. baumannii*
Del*apaH*AB-int-rev	CTTTTTTTTAGTCACCAGCAAACCAGATAA	
Del*apaH*AB-int-for	TGCTGGTGACTAAAAAAAAGCCGCTGAAACGG	
Del*apaH*AB-rev	TTAAGGTACCGAATTTATGCTTTGCCAGCATTAGT	
Del*apaH*AB-CH1-for	GGGTTTTGGTGAAGCAATCA	For confirmation of *apaH* deletion in *A. baumannii*
Del*apaH*AB-CH1-rev	TAATCAGATCTGGATTCGCC	
Del*apaH*AB-CH2-for	AACTCTTGCAGAGTTTGTGG	
Del*apaH*AB-CH2-rev	GGTTGCTTTAGCCATACCTT	
DelapaH-3'fragment_for	CCCGTGGAAATTAATTAAGGTACCGTGGCGCCCTTT CGCATC	For construction of *apaH* deletion mutant in *P. aeruginosa*
DelapaH-3'fragment_rev	AACTCGAGCCGCAAGCATGCTGAAGTAGACCGCCA TCAGTGCAGC	
DelapaH-5'fragment_for	TTCAGCATGCTTGCGGCTCGAGTTACGCCCGCATGA ATCCCCGGC	

DelapaH-5'fragment_rev	GAGCCGGAAGCATAAATGTAAAGCAGGCTCGCCGATCGCC	
DelapaH_for	TCGGCAGCATGCGCGGCAGCT	For confirmation of *apaH* deletion in *P. aeruginosa*
DelapaH_rev	ACCACCTGGGCGCCGTTTTCG	

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