

(b)



(d)



(f)

Figure S1. Enzymatic activities of *S. mitis* CdaA₁₀₃₋₂₈₅, Pde1₅₃₋₆₆₇ and Pde2. (a) Nucleotide control peaks for ATP, AMP, pApA and c-di-AMP eluted separately with the reversed-phase chromatography method. The x-axis of the chromatogram shows the retention volume in milliliters (mL) and y-axis shows the absorbance at 280 nm in milli-absorbance units (mAU). (b). Pde1₅₃₋₆₅₇ degrading c-di-AMP to pApA and AMP. (c). Pde1₅₃₋₆₅₇ degrading pApA into AMP. (d). Pde2 degrading c-di-AMP directly into AMP (e), and pApA into AMP (f). CdaA₁₀₃₋₂₈₅ synthesis of c-di-AMP from ATP.



Figure S2. Flow cytometry was used to compare size and granularity of WT and mutants. Scatter plots from one representative experiment. (a) WT. (b) $\Delta cdaA$. (c) $\Delta pde1$. (d) $\Delta pde2$. (e) cdaA-KB. (f) pde1-KB. (g) pde2-KB. (h) $\Delta pde1\Delta pde2$.

Table S1. Primers used in this study.

	Primer	Sequence (5' - 3')	Description
0	11.4		

Gene deletion

- cdaA-KO-L for AGGATGGGACAGAAGTGGC
- cdaA-KO--L-rev TATTTTTTCTCATCTGCTGTCTCCTCTGTC
- cdaA-KO-R-for GACAGCAGATGAGAAAAAATAGTCTATATATC
- cdaA-KO-R-rev TCTCGCCAGTTAAAGACAC
- PDE1-KO-L for CCACTGGGCTCACCATC
- PDE1-KO-L-rev AAAGATTACTTTCCAAACCTCTTGGCACC
- PDE1-KO-R-for GAGGTTTGGAAAGTAATCTTTTTAGCAGATGT
- PDE1-KO-R-rev CCATAGTTAGAACTATTCCC
- PDE2-KO-L for CTCACTTACCTCACCAAAG
- PDE2-KO-L-rev CAAGTATTTTAAAATAACCTCAATTCTTTCTCA
- PDE2-KO-R-for TTGAGGTTATTTTAAAATACTTGCCAAACTTTTC
- PDE2-KO-R-rev TGTGCTTGGCGCATCATG
- GHR17 ACAGGACCACCACCTACAAT
- GHR18 CAAGGTCACACGATTAGCTC
- GHR19 CCAATGTGATTTATCGTCGT
- GHR20 TTAATTGCTTTTGCTTCTGC

Amplification of the upstreamflanking DNA region of *cdaA*

Amplification of the downstreamflanking DNA region of *cdaA*

Amplification of the upstreamflanking DNA region of *pde1*

Amplification of the downstreamflanking DNA region of *pde1*

Amplification of the upstreamflanking DNA region of *pde2*

Amplification of the downstreamflanking DNA region of *pde2*

Screen for *cdaA* knock out -Forward

Screen for *cdaA* knock out -Reverse Screen for *pde1* knock out -Forward

Screen for *pde1* knock out -Reverse

GHR21	ATCAACATCAACATCCAAGC	Screen for <i>pde2</i> knock out - Forward
GHR22	ACGGATCAATGGGTAAGTTT	Screen for <i>pde2</i> knock out - Reverse
FP1242	CTTGAGCTGGGCTTCGTAGT	Amplification of Erythromycin cassette Forward
FP1243	ACAGGGGATGTCATGGGTAA	Amplification of

RT-qPCR

GHR7

GHR27	AAGGGCGTGACTAAGGTCAA	Amplification of <i>ldh</i>
GHR28	TATCCTTGTCGGTGATGGTG	
GHR29	CGTTGCAACTTTGGGACCTG	Amplification of <i>pk</i>
GHR30	AGTTGAAACGGAATGTGTTAGCA	
GHR31	GAGACGCCCCTGGTATGAAC	Amplification of pfk-1
GHR32	GTCCCCTACTGAAGCTGCAT	
GHR1	GGGCCAGAGTCATCTGGTAA	Amplification of <i>recA</i>
GHR2	CGTCAATGTTGACACCAAGG	
GHR3	GATGGTCATGGGAACTTTGG	Amplification of <i>gyrA</i>
GHR4	TTCACGTTCATTGGCATCAT	
GHR5	GAAAGCACAGGGATTTCCAA	Amplification of <i>cdaA</i>
GHR6	CGTGCTTGAAAACACCGTTA	

GAACAGATGCCTGTCGGAGT

Amplification of *pde1*

Erythromycin cassette Reverse

GHR8	GATTCCCCACAGAAGCCTTT	
GHR9	CTTGGATGGCTGAGATGGAT	Amplification of
GHR10	CATCATTTGGATGGTGGTCA	μιε
Cloning		
pET28a(+)-Seq-F	CCGGCCACGATGCGTCC	pET28a(+) sequencing
pET28a(+)-Seq-R	ACAGGGCGCGTCCCATTC	primers
pET28a(+)-KpnI- mut-F	AGAAGGAGAGGTACCATGGGCAGCAGCCATCATCATCATCATC AC	Site-directed mutagenesis of a restriction site from NcoI to KpnI
pET28a(+)-KpnI- mut-R	CCCATGGTACCTCTCCTTCTTAAAGTTAAACAAAATTATTTCTAG AGGGGAATTGTT	
DisA-HindIII-R	TATCAAGCTTCTATTTTTTCTCATATTTCCAAC	Full-length <i>cdaA</i>
DisA-N-His-F	GATCGGTACCATGCATCACCATCACCATCACAACTTTCAACAAT TATCC	Full-length <i>cdaA</i> with an N- terminal 6x His- Tag
DisA-T-N-His-F	GATCGGTACCATGCATCACCATCACCATCACCCAGAGATTCGGA CTG	<i>cdaA</i> 94-285 with an N-terminal 6x His-Tag
PDE1-HindIII-R	TATCAAGCTTCTATCATTCTTCTTTCTCCTTTTCCT	Amplification of full-length and truncated <i>pde1</i>
PDE1-N-His-F	GATCGGTACCATGCATCACCATCACCATCACAAAAAATTTTATG TAAGTCCTATTTTC	Full-length <i>pde1</i> PCR and addition of an N- terminal 6x His- Tag – F

PDE1-midseq-F	TTCGTAGTGTGGATCAGGTT	<i>pde1</i> sequencing primer
PDE1_Truncated- N-His-F	GATCGGTACCATGCATCACCATCACCAGAGAGAGACTACT ACTCAAAG	<i>pde1</i> 53-657 with an N-terminal 6x His-Tag - F
PDE2-HindIII-R	TATCAAGCTTCTATCAGTTTTTAAGCAAGTTTTTTAAC	Full-length <i>pde</i> 2 PCR
PDE2-N-His-F	GATCGGTACCATGCATCACCATCACCATCACGACATTTGCCATC AAATTTTAG	Full-length <i>pde2</i> with an N- terminal 6x His- Tag - F
SMITIS_CdaA2-For	CCATCACCATTTGGGAAGGGCGACAGATTTCTTTTCTACTGCTCA AATTAG	Site-directed deletion of <i>cdaA</i> aa residues 94- 102
SMITIS_CdaA2- Rev	CCCTTCCCAAATGGTGATGGTGATGGTGCATGGTATATCTCCTTC TTAAAG	Site-directed deletion of <i>cdaA</i> aa residues 94- 102