**Cytosine Deaminase-Assisted Mutator for Genome Evolution in *Cupriavidus necator***

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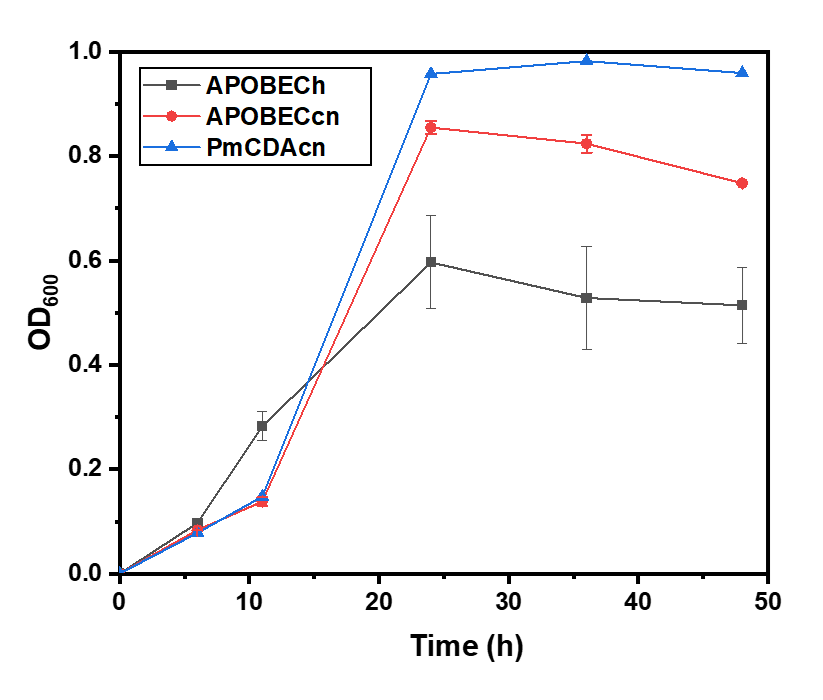
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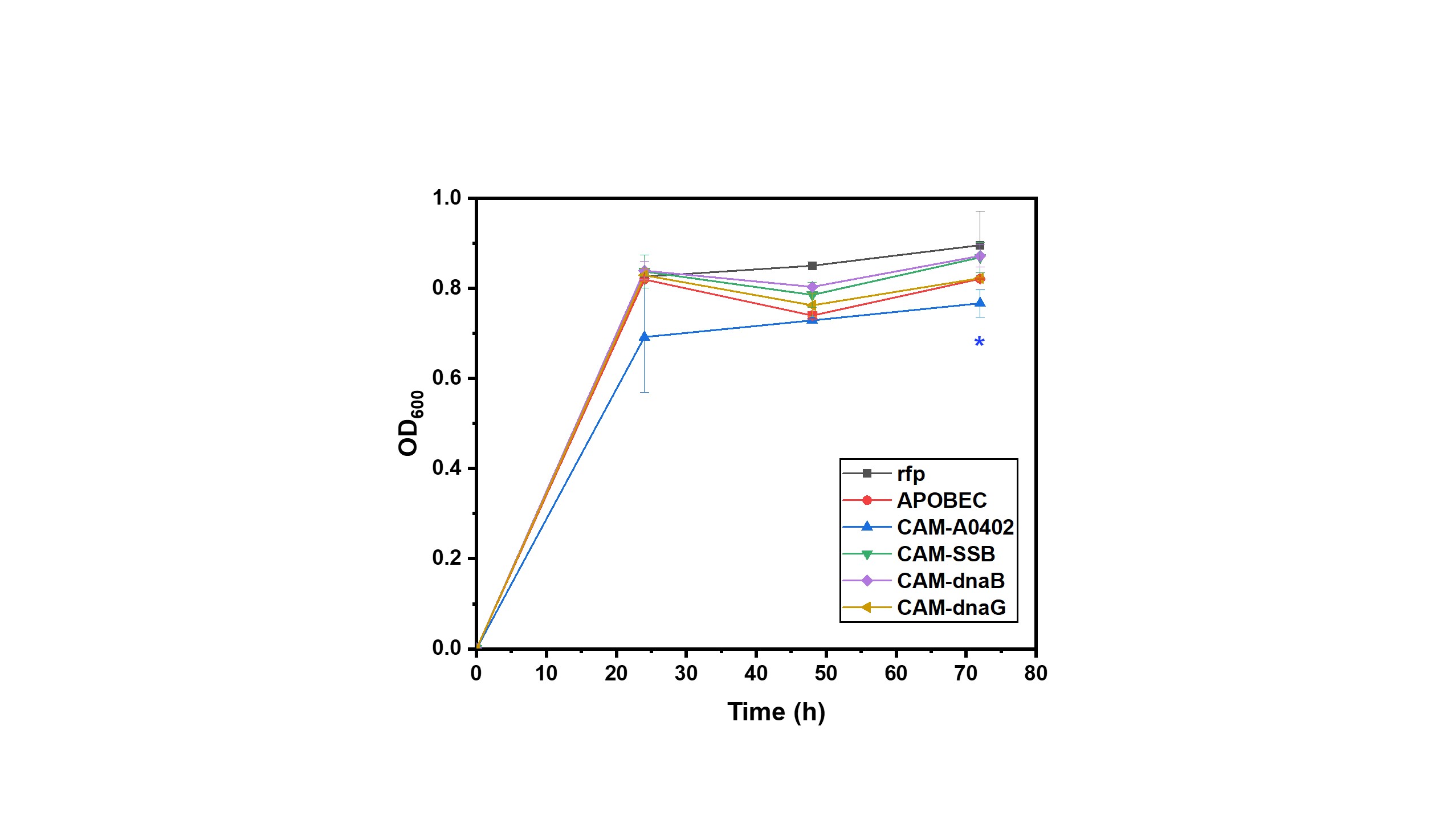
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**Supplementary Figures**

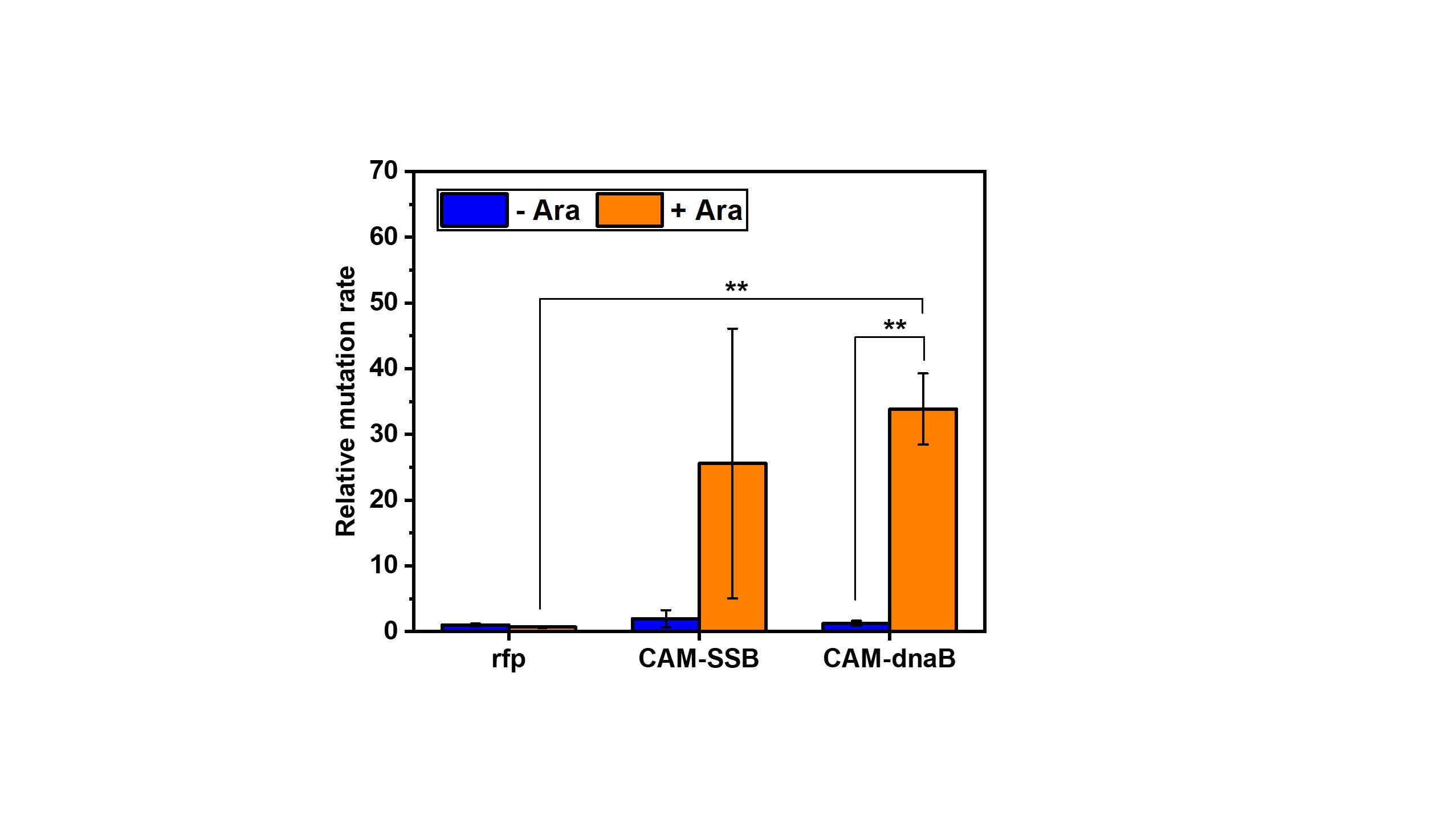
**Supplementary Fig. S1 Growth curves of *C. necator* bearing different cytosine deaminases.** Error bars represented the mean ± s.d. of biological triplicates (n=3).



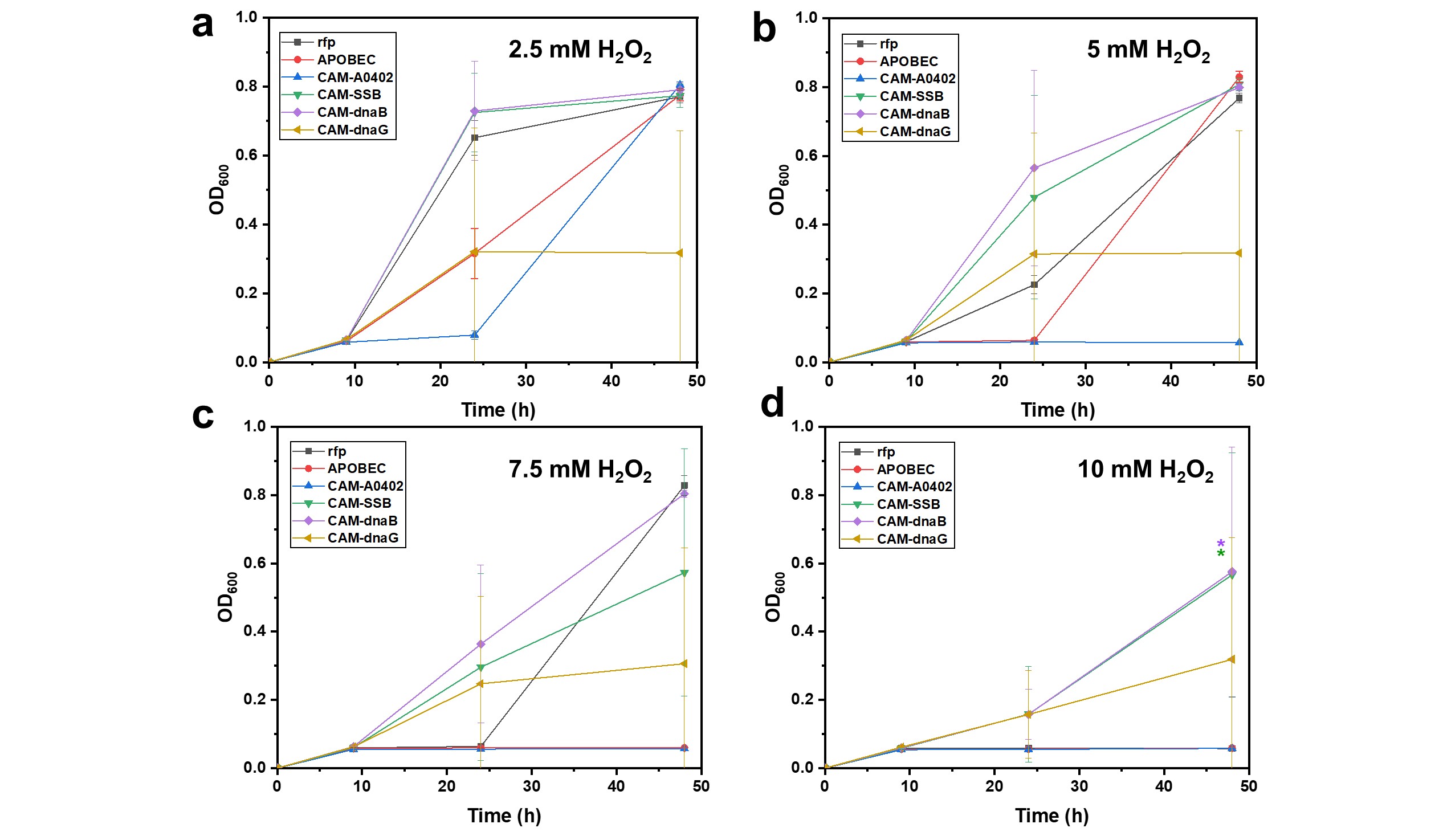
**Supplementary Fig. S2 Growth curves of various CAM strains and control strains under non-stressed condition.** Error bars represented the mean ± s.d. of biological triplicates (n=3). Statistical analysis was performed using a two-sided Student’s t test, with p-value versus the control C5/rfp. Statistical designations with different colors refered to the corresponding data points with the same color. \* P < 0.05.



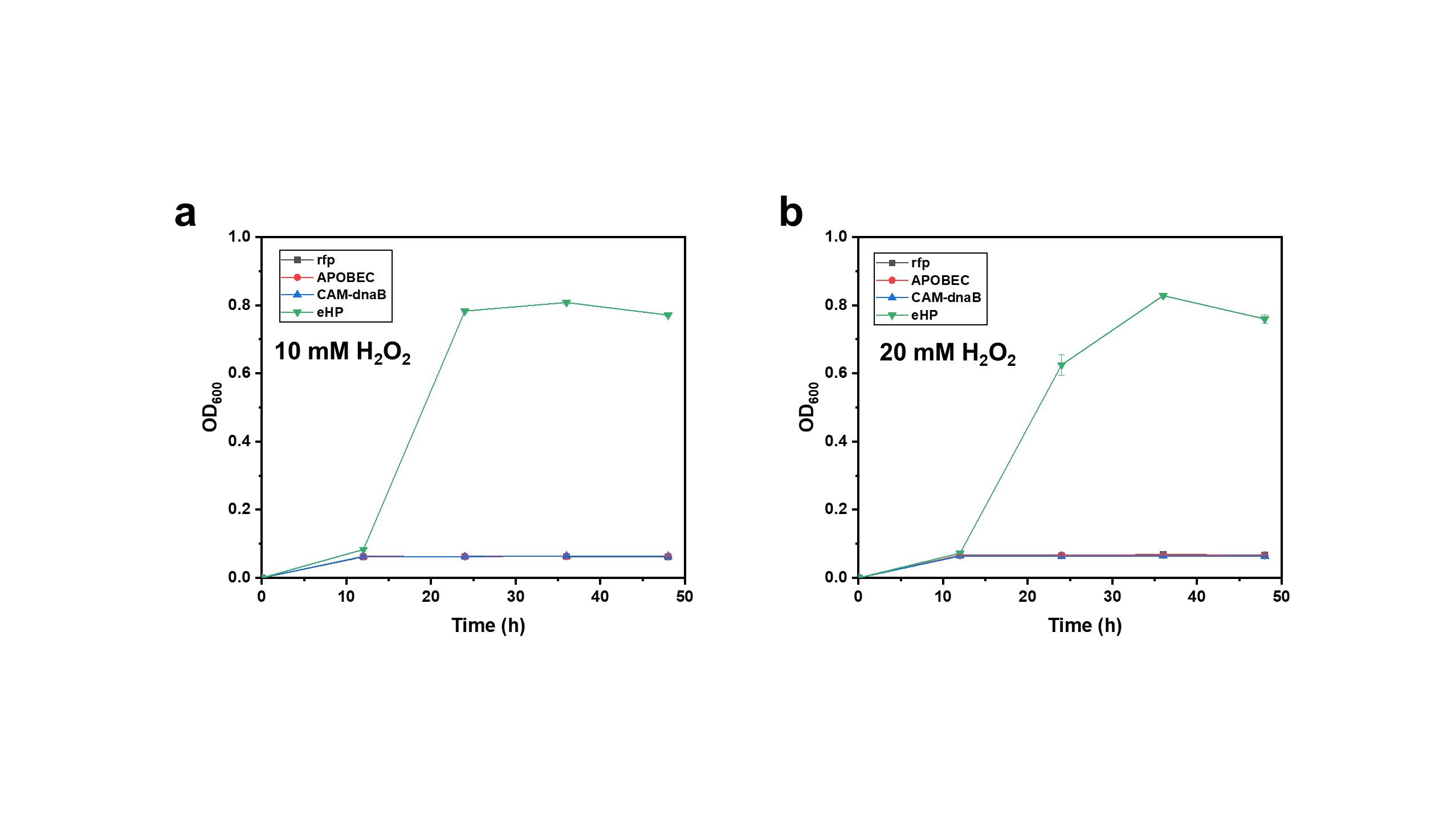
**Supplementary Fig. S3 Inducibility of CAM system.** Error bars represented the mean ± s.d. of biological triplicates (n=3). Statistical analysis was performed using a two-sided Student’s t test. Selected comparisons were shown. \*\* P < 0.01.



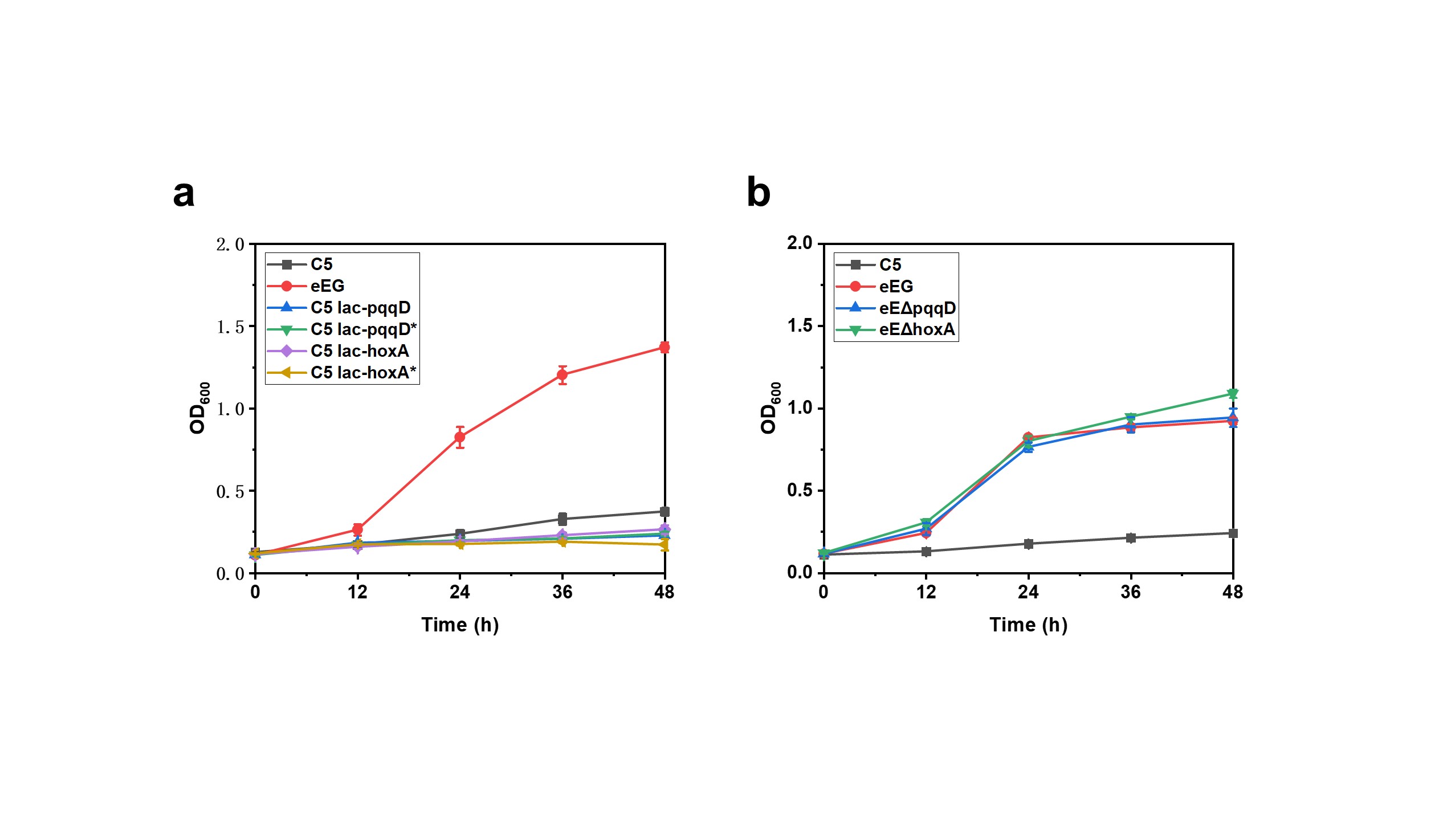
**Supplementary Fig. S4 Evaluation of various CAMs for improving H2O2 resistance.** Growth curves of CAM strains in LB medium containing H2O2 with a concentration of 2.5 (a), 5 (b), 7.5 (c), and 10 (d) mM, respectively. C5/rfp and C5/APOBEC were included as negative controls. Error bars represented the mean ± s.d. of biological triplicates (n=3). Statistical analysis was performed using a two-sided Student’s t test, with p-value versus the control C5/rfp. Statistical designations with different colors refered to the corresponding data points with the same color. \* P < 0.05.



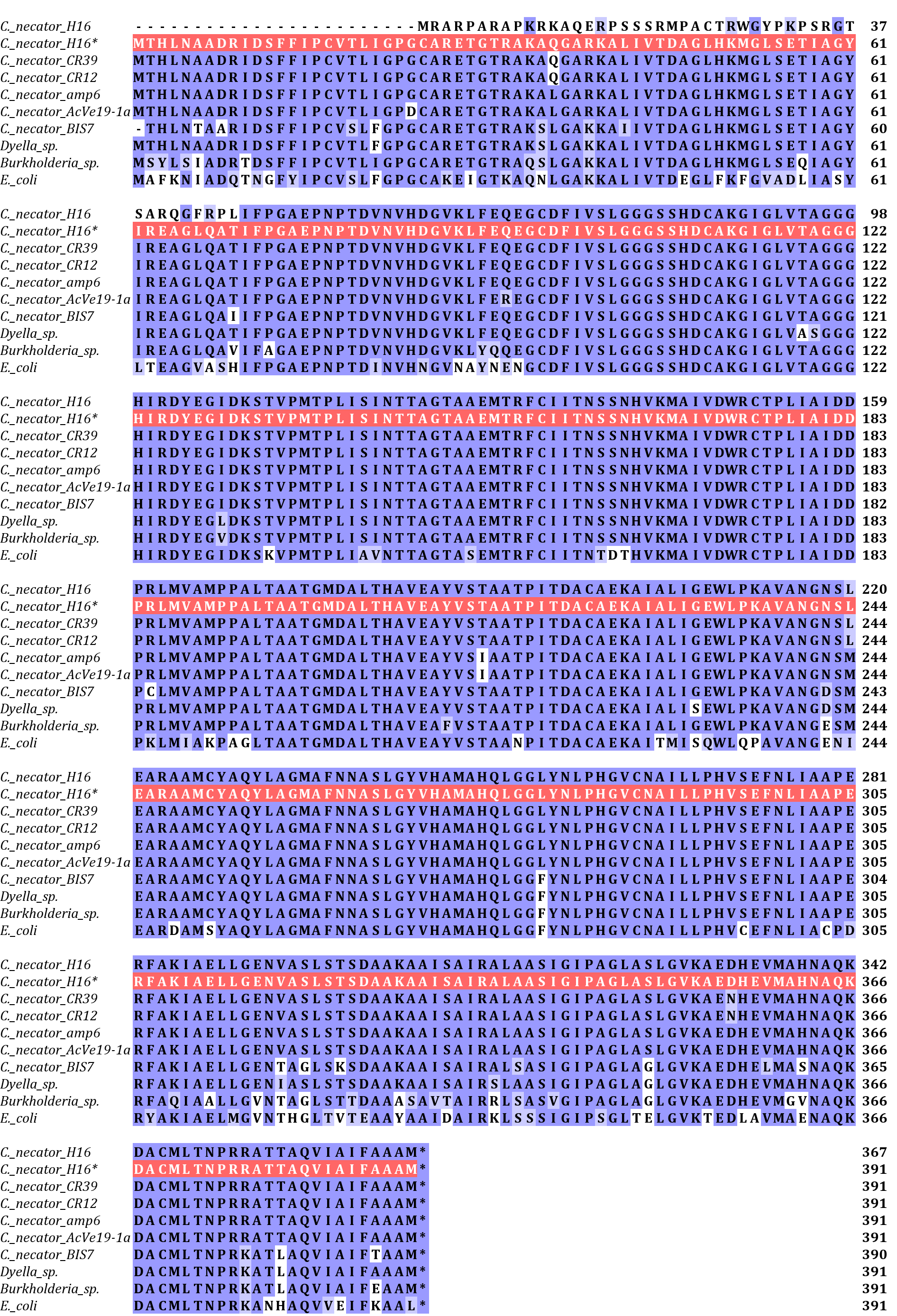
**Supplementary Fig. S5 DnaB-mediated continuous evolution of H2O2 tolerance.** After 12 rounds of evolution, three individual dnaB-evolved clones (eHP1, eHP2, and eHP3) were isolated and evaluated in LB medium containing 10 (a) and 20 (b) mM H2O2, with C5/rfp, C5/APOBEC, and un-evolved C5/CAM-dnaB included as negative controls. Error bars represented the mean ± s.d. of biological triplicates (n=3).



**Supplementary Fig. S6** **Mutation analysis of the evolved strain eEG.** The contribution of the identified mutations to EG utilization was evaluated by gene overexpression (a) or gene deletion (b). Error bars represented the mean ± s.d. of biological triplicates (n=3).



**Supplementary Fig. S7** **Protein-BLAST analysis of A0861w\* between *C. necator* H16 and other *C. necator* species as well as *E. coli*.**



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

|  |  |  |
| --- | --- | --- |
|  | Description | Source |
| ***Strains*** |  |  |
| *Escherichia coli* DH5α | Host for cloning | Our lab |
| *Escherichia coli* S17 | Host for conjugation | Our lab |
| *Cupriavidus necator* C5 | H16∆*H16\_A0006*∆*H16\_A0008-9* | 1 |
| *C. necator* eHP1 | Evolved for higher resistance to H2O2 | This work |
| *C. necator* eHP2 | Evolved for higher resistance to H2O2 | This work |
| *C. necator* eHP3 | Evolved for higher resistance to H2O2 | This work |
| *C. necator* eEG1 | Evolved for enhanced EG utilization | This work |
| *C. necator* eEG2 | Evolved for enhanced EG utilization | This work |
| *C. necator* eEG3 | Evolved for enhanced EG utilization | This work |
| *C. necator* C5Δ*pqqD* | H16∆*H16\_A0006*∆*H16\_A0008-9*Δ*pqqD* | This work |
| *C. necator* C5Δ*hoxA* | H16∆*H16\_A0006*∆*H16\_A0008-9*Δ*hoxA* | This work |
| *C. necator* C5Δ*B1607* | H16∆*H16\_A0006*∆*H16\_A0008-9*Δ*B1607* | This work |
| *C. necator* C5Δ*A0861* | H16∆*H16\_A0006*∆*H16\_A0008-9*Δ*A0861* | This work |
|  |  |  |
| ***Plasmids*** |  |  |
| pBBR1MCS-2 | KmR, broad host range vector | Addgene#85168 |
| pBBR-P*ara*-*rfp* | KmR, rfp was expressed by P*ara* | This work |
| pBBR-P*ara*-*APOBEC*h | KmR, APOBECh was expressed by P*ara* | This work |
| pBBR-P*ara*-*APOBEC*cn | KmR,APOBECcn was expressed by P*ara* | This work |
| pBBR-P*ara*-*PmCDA*cn | KmR, PmCDAcn was expressed by P*ara* | This work |
| pBBR-P*ara*-*APOBEC*cn-*A0204* | KmR, CAM-A0204 was expressed by P*ara* | This work |
| pBBR-P*ara*-*APOBEC*cn-*SSB* | KmR, CAM-SSB was expressed by P*ara* | This work |
| pBBR-P*ara*-*APOBEC*cn-*dnaB* | KmR, CAM-dnaB was expressed by P*ara* | This work |
| pBBR-P*ara*-*APOBEC*cn-*dnaG* | KmR, CAM-dnaG was expressed by P*ara* | This work |
| pBBR-P*lacRBS*-*pqqD* | KmR, pqqD was expressed by P*lacRBS* | This work |
| pBBR-P*lacRBS*-*pqqD\** | KmR, pqqD\* was expressed by P*lacRBS* | This work |
| pBBR-P*lacRBS*-*hoxA* | KmR, hoxA was expressed by P*lacRBS* | This work |
| pBBR-P*lacRBS*-*hoxA\** | KmR, hoxA\* was expressed by P*lacRBS* | This work |
| pBBR-P*lacRBS*-*B1607* | KmR, B1607 was expressed by P*lacRBS* | This work |
| pBBR-P*lacRBS*-*B1607\** | KmR, B1607\* was expressed by P*lacRBS* | This work |
| pBBR-P*lacRBS*-*A0861* | KmR, A0861 was expressed by P*lacRBS* | This work |
| pBBR-P*lacRBS*-*A0861\** | KmR, A0861\* was expressed by P*lacRBS* | This work |
| pBBR-P*A0861*-*A0861w* | KmR, A0861w was expressed by P*A0861* | This work |
| pBBR-P*A0861*-*A0861w\** | KmR, A0861w\* was expressed by P*A0861* | This work |
| pBBR-P*lacRBS*-*A0861w* | KmR, A0861w was expressed by P*lacRBS* | This work |
| pBBR-P*lacRBS*-*A0861w\** | KmR, A0861w\* was expressed by P*lacRBS* | This work |
| pK19mobSacB | KmR, vector for conjugation | Our lab |
| pK19mobSacB-*pqqD* | KmR, for knocking out *pqqD* | This work |
| pK19mobSacB-*hoxA* | KmR, for knocking out *hoxA* | This work |
| pK19mobSacB-*B1607* | KmR, for knocking out *B1607* | This work |
| pK19mobSacB-*A0861* | KmR, for knocking out *A0861* | This work |

Note: All plasmids files can be accessed at benchling via <https://benchling.com/gaomujun/f_/KPFqkAWa-cam/>.

**Supplementary Table S2.** Primers used in this study

|  |  |
| --- | --- |
| **Name** | **Sequence (5’🡪3’)** |
| PmCDACn-F | tttcgactgagcctttcgttttatttgcttacacggccggcgacttggtg |
| PmCDACn-R | gatcttttaagaaggagatatacatgatgaccgacgccgagtacg |
| APOBECh-F | tttcgactgagcctttcgttttatttgcttatttcaacccggtggcccag |
| APOBECh-R | atcttttaagaaggagatatacatgatgagctcagagactggccc |
| CAM-APOBECCn-F | cttttaagaaggagatatacatgatgtcgtcggagacggg |
| CAM-APOBECCn-R | cagaactttcgggtgtggcggactctgaggtcccgggagtctcgctgccgctagaaccaccagaagaaccaccagacttcaggccggtggccc |
| CAM-ssb-F | ccgccacacccgaaagttctggtggttcttctggtggttctgcatccgtgaacaaagtg |
| CAM-ssb-R | ctgagcctttcgttttatttgctcagtaccgcggcgcgcc |
| CAM-A0402-F | ccgccacacccgaaagttctggtggttcttctggtggttctgcatcggtcaacaaagtc |
| CAM-A0402-R | tgagcctttcgttttatttgctcagaacgggatatcgtcg |
| CAM-dnaB-F | ccgccacacccgaaagttctggtggttcttctggtggttctaacgcgcccgtcgccgac |
| CAM-dnaB-R | ctgagcctttcgttttatttgctcaggtatcgttgtcgaagaaag |
| CAM-dnaG-F | cgccacacccgaaagttctggtggttcttctggtggttctattccgcaatcctttattc |
| CAM-dnaG-R | actgagcctttcgttttatttgctcagccaagttggcgcc |
| pqqD-down-F | tcggccagcagctcgaggggttggcttgctggatggc |
| pqqD-down-R | acgttgtaaaacgacggccagtgccgggcatggcatcgaggtggcgta |
| pqqD-up-F | caagcttgcatgcctgcaggtcgactccaaggaaggtggtgccccag |
| pqqD-up-R | gccaacccctcgagctgctggccgagctgacctac |
| pqqD-v-F | tgcgtggcgacattgggcagcgcca |
| pqqD-v-R | caacaataccaacccggtgctggtggaaga |
| hoxA -down-F | cttgagccctcgagacgcgcctgtatgtaccggtc |
| hoxA -down-R | acgttgtaaaacgacggccagtgccgcctgggtacccttgcatcccatg |
| hoxA -up-F | acaagcttgcatgcctgcaggtcgactctggtgctgctggggggagag |
| hoxA -up-R | tacaggcgcgtctcgagggctcaagatcgtttccccgcaag |
| hoxA -v-F | gtactgggaagacgattccggcgcgggcctggtcggatac |
| hoxA -v-R | ccgcgggtacaggaaccctctccgttccatag |
| B1607-down-F | cgagccgaagctcgaggctggagcgcaatgcggcacaaag |
| B1607-down-R | cgttgtaaaacgacggccagtgccgcggcccgggtaattgggagcg |
| B1607-up-F | caagcttgcatgcctgcaggtcgacttacgggatgagccttgaactcgaacgac |
| B1607-up-R | cgctccagcctcgagcttcggctcgaccaccgcgcc |
| B1607-v-F | gatgaagtcgcgccaccatttcatcagctc |
| B1607-v-R | ggtggtcggacggggctacctcgaactgac |
| A0861-down-F | gcataccttactagtggcgtctccaatattgtagtgatgaggtg |
| A0861-down-R | cgttgtaaaacgacggccagtgccggcggcgctcgatatcctcgg |
| A0861-up-F | caagcttgcatgcctgcaggtcgactctagaaaaaaacaggtcgaggcatcatcc |
| A0861-up-R | ttggagacgccactagtaaggtatgccccctccgacgaac |
| A0861-v-F | tgacctgctcaacgatttccgggaactcat |
| A0861-v-R | gcaaggcctttacgggcgaattgacggaac |
| PlacRBS-pqqD-F | ggtaccgggccccaaaggaggaagtgatgacagccagcacgcaagacctc |
| PlacRBS-pqqD-R | cgactgagcctttcgttttatttgctcagaccagccagccgcgttcgatcgcatgtttg |
| PlacRBS-hoxA-F | gggtaccgggccccaaaggaggaagtgatgtctgacaagcaggccactgttcttg |
| PlacRBS-hoxA-R | ttcgactgagcctttcgttttatttgctcatttctcctccaagccaaagcgcaac |
| PlacRBS-B1607-F | gggtaccgggccccaaaggaggaagtgatgacttcgcaggcccgctcctc |
| PlacRBS-B1607-R | tcgactgagcctttcgttttatttgcctaacgcgcgagtaacgcccgc |
| PlacRBS-A0861-F | ggtaccgggccccaaaggaggaagtggtgcgcgcgagaccggcac |
| PlacRBS-A0861-R | tcgactgagcctttcgttttatttgcttacatcgctgcagcgaagatagcgatgac |
| PlacRBS-A0861w-F | ggtaccgggccccaaaggaggaagtgatgacccacctgaacgccgc |
| PlacRBS-A0861w-R | tcgactgagcctttcgttttatttgcttacatcgctgcagcgaagatagcgatgac |
| PA0861-A0861-F | cggtggcggccgctctagaactagtgagaccgatgccgatgccccc |
| PA0861-A0861-R | acaaaagctgggtaccgggccccccccgcacaaggtgcgtgacaagg |

**Supplementary Table S3.** Genes used in this study

|  |  |
| --- | --- |
| **Name** | **Sequence (5’🡪3’)** |
| *APOBEC*h | atgagctcagagactggcccagtggctgtggaccccacattgagacggcggatcgagccccatgagtttgaggtattcttcgatccgagagagctccgcaaggagacctgcctgctttacgaaattaattgggggggccggcactccatttggcgacatacatcacagaacactaacaagcacgtcgaagtcaacttcatcgagaagttcacgacagaaagatatttctgtccgaacacaaggtgcagcattacctggtttctcagctggagcccatgcggcgaatgtagtagggccatcactgaattcctgtcaaggtatccccacgtcactctgtttatttacatcgcaaggctgtaccaccacgctgacccccgcaatcgacaaggcctgcgggatttgatctcttcaggtgtgactatccaaattatgactgagcaggagtcaggatactgctggagaaactttgtgaattatagcccgagtaatgaagcccactggcctaggtatccccatctgtgggtacgactgtacgttcttgaactgtactgcatcatactgggcctgcctccttgtctcaacattctgagaaggaagcagccacagctgacattctttaccatcgctcttcagtcttgtcattaccagcgactgcccccacacattctctgggccaccgggttgaaa |
| *APOBEC*cn | atgtcgtcggagacgggcccggtggccgtggacccgaccctgcgccgccgcatcgaaccgcacgagttcgaggtgttcttcgacccgcgcgagctgcgcaaggagacgtgcctgctgtacgagatcaactggggcggccgccactcgatctggcgccacacctcgcagaacaccaacaagcacgtggaggtgaacttcatcgaaaagttcaccaccgaacgctacttctgcccgaacacccgctgctcgatcacctggttcctgagctggtcgccgtgcggcgagtgctcgcgcgccatcaccgaattcctgtcgcgctacccgcacgtgaccctgttcatctacatcgcccgcctgtaccaccacgccgacccgcgcaaccgccagggcctgcgcgacctgatctcgtcgggcgtgaccatccagatcatgaccgagcaggagtcgggctactgctggcgcaacttcgtgaactacagcccgtcgaacgaagcccactggccgcgctacccgcacctgtgggtgcgcctgtacgtgctggaactgtactgcatcatcctgggcctgccgccgtgcctgaacatcctgcgccgcaagcagccgcagctgaccttcttcaccatcgccctgcagtcgtgccactaccagcgcctgccgccgcacatcctgtgggccaccggcctgaag |
| *PmCDA*cn | atgaccgacgccgagtacgtgcgcatccacgagaagctggacatctacaccttcaagaagcagttcttcaacaacaagaagtcggtgtcgcaccgctgctacgtgctgttcgagctgaagcgccgcggcgagcgccgcgcctgcttctggggctacgccgtgaacaagccgcagtcgggcaccgagcgcggcatccacgccgaaatcttctcgatccgcaaggtggaggagtacctgcgcgacaacccgggccagttcaccatcaactggtactcgtcgtggtcgccgtgcgccgactgcgccgaaaagatcctggagtggtacaaccaggagctgcgcggcaacggccacaccctgaagatctgggcgtgcaagctgtactacgagaagaacgcccgcaaccagatcggcctgtggaacctgcgcgacaacggcgtgggcctgaacgtgatggtgtcggagcactaccagtgctgccgcaagatcttcatccagtcgtcgcacaaccagctgaacgaaaaccgctggctggagaagaccctgaagcgcgccgagaagcgccgcagcgagctgtcgatcatgatccaggtgaagatcctgcacaccaccaagtcgccggccgtg |

**Supplementary Table S4.** Colony-forming units (CFU)

**Fig. 2a**

|  |  |  |  |
| --- | --- | --- | --- |
| **Biological replicates** | **1** | **2** | **3** |
| **APOBECh (diluted 1/10)** | 5600 | 6400 | 12800 |
| **APOBECcn (diluted 1/4)** | 2800 | 1000 | 1200 |
| **PmCDAcn (no dilution 1/1)** | 114 | 100 | 110 |

Amount of plasmid: 0.4 μg

**Fig. 2b**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Biological replicates** | **1** | | **2** | | **3** | |
|  | -Cm | +Cm | -Cm | +Cm | -Cm | +Cm |
| **rfp** | 57 | 109 | 12 | 135 | 100 | 750 |
| **APOBEC** | 6 | 91 | 155 | 400 | 250 | 200 |
| **CAM-A0402** | 400 | 700 | 121 | 800 | 219 | 1100 |
| **CAM-SSB** | 17 | 50 | 220 | 500 | 4000 | 2000 |
| **CAM-dnaB** | 280 | 28 | 8000 | 2000 | 1400 | 760 |
| **CAM-dnaG** | 320 | 800 | 5 | 350 | 7 | 400 |
| **Biological replicates** | **4** | | **5** | | **6** | |
|  | -Cm | +Cm | -Cm | +Cm | -Cm | +Cm |
| **rfp** | 5 | 63 | 4 | 35 | 19 | 73 |
| **APOBEC** | 7 | 221 | 23 | 68 | 16 | 88 |
| **CAM-A0402** | 69 | 55 | 200 | 58 | 51 | 45 |
| **CAM-SSB** | 105 | 66 | 100 | 50 | 400 | 55 |
| **CAM-dnaB** | 1400 | 103 | 300 | 81 | 460 | 68 |
| **CAM-dnaG** | 22 | 69 | 21 | 55 | 58 | 77 |

**Supplementary Fig. S3**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Biological replicates** | **1** | | **2** | | **3** | |
|  | -Cm | +Cm | -Cm | +Cm | -Cm | +Cm |
| **rfp (-Ara)** | 63 | 280 | 85 | 288 | 52 | 312 |
| **rfp (+Ara)** | 90 | 592 | 114 | 576 | 67 | 496 |
| **CAM-SSB (-Ara)** | 150 | 360 | 53 | 496 | 100 | 120 |
| **CAM-SSB (+Ara)** | 2700 | 216 | 1900 | 752 | 2440 | 960 |
| **CAM-dnaB (-Ara)** | 54 | 312 | 96 | 296 | 110 | 296 |
| **CAM-dnaB (+Ara)** | 2900 | 432 | 4100 | 432 | 4800 | 680 |

**Supplementary References**

1. Xiong, B.; Li, Z.; Liu, L.; Zhao, D.; Zhang, X.; Bi, C., Genome editing of *Ralstonia eutropha* using an electroporation-based CRISPR-Cas9 technique. *Biotechnol. Biofuels* **2018,** *11* (1), 1-9.