**Supplementary Figures and Tables**

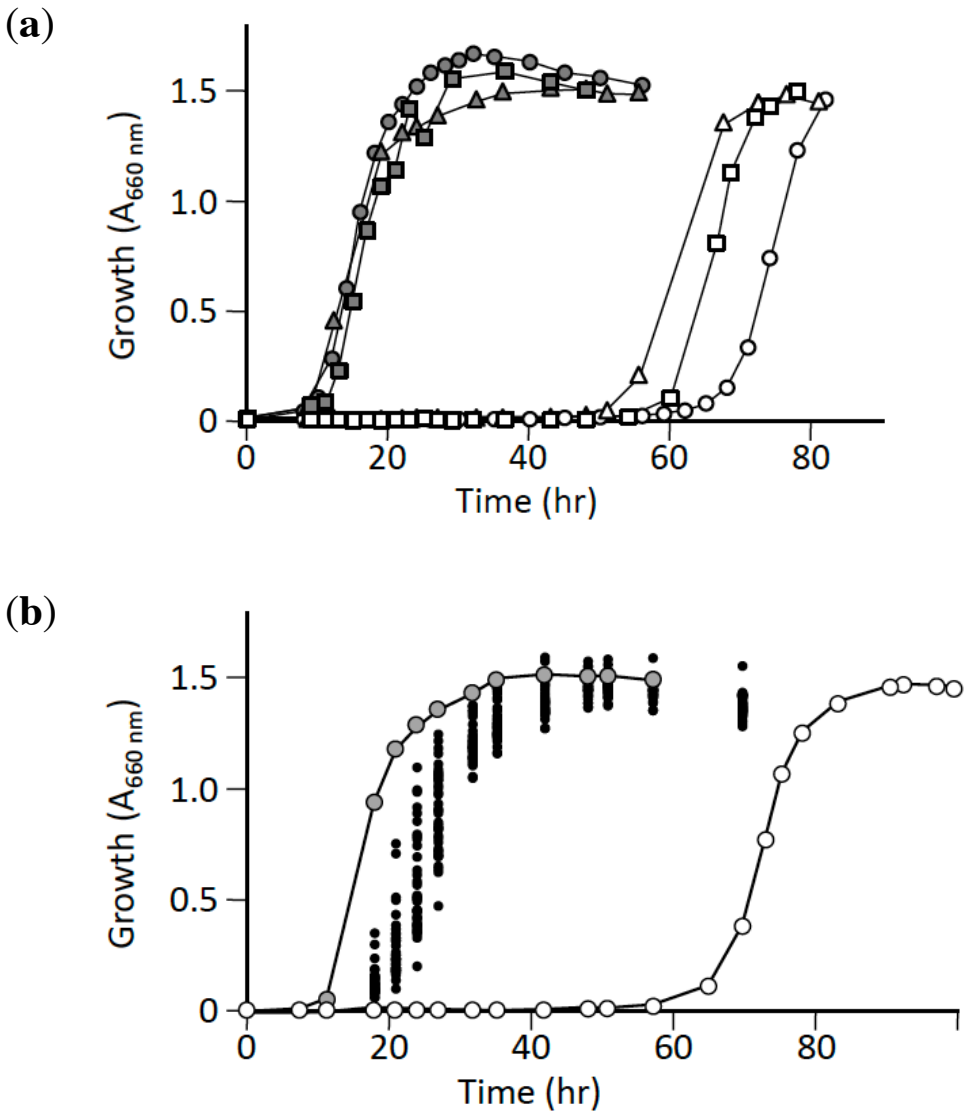



**Figure S1.** Emergence of L-alanine prototrophic mutants from L-alanine auxotroph HYE032. (**a**) Growth of L-alanine auxotroph HYE032 (open symbols) and its parent strain W3110 (grey symbols) in minimal liquid medium. Results of 3 independent experiments are presented. (**b**) Growth of L-alanine prototrophic suppressor mutants obtained in minimal liquid medium. When the growth of HYE032 cells reached the late-log phase in minimal liquid medium, clones were isolated on L-agar medium and each clone was purified once on the same rich medium. Subsequently, the growth of each L-alanine prototrophic mutant was determined in minimal liquid medium. Symbols: large open circles, HYE032; large grey circles, W3110; small closed circles, L-alanine prototrophic mutants.

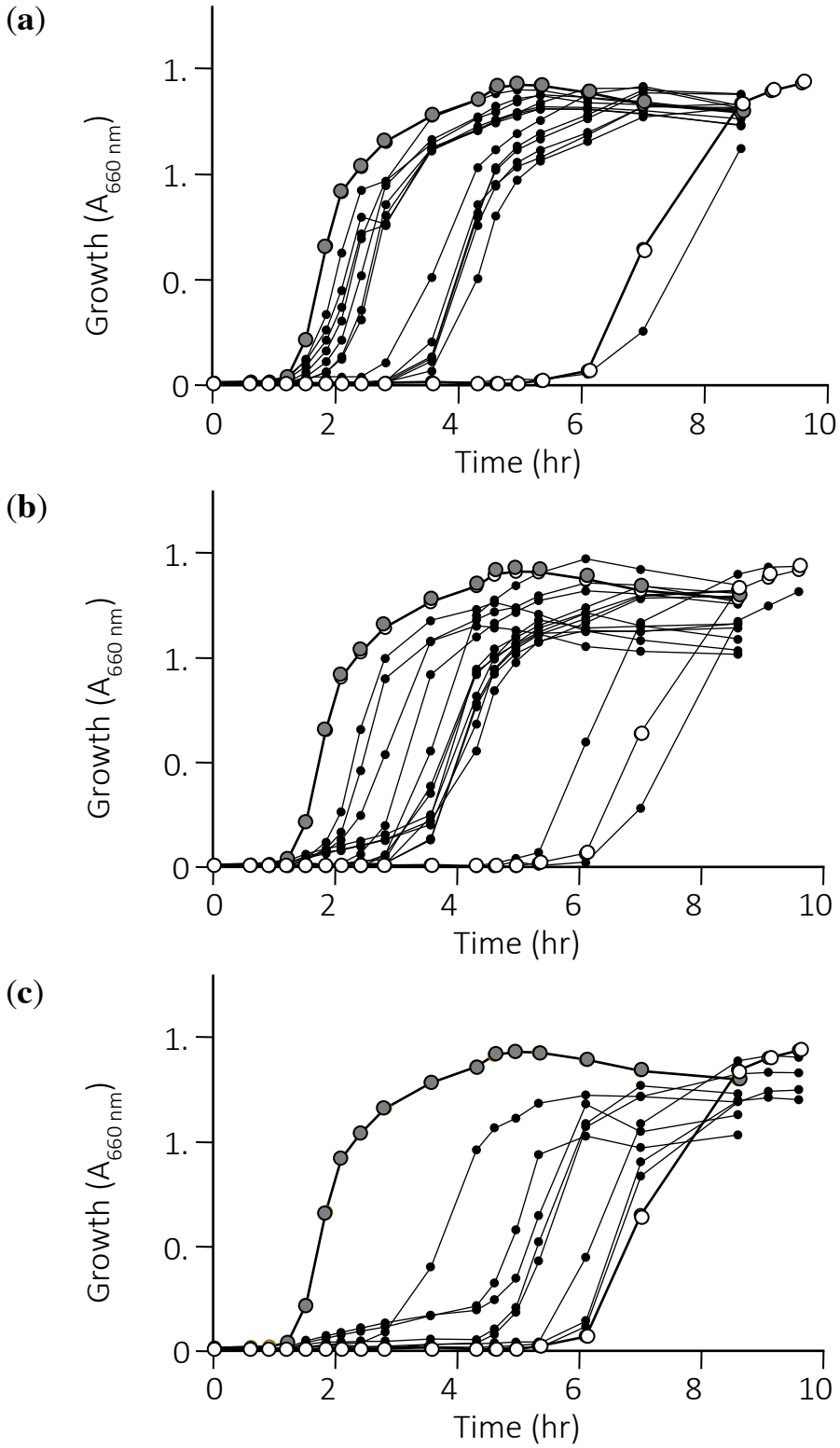



**Figure S2.** Growth of L-alanine prototrophic suppressor mutants obtained on minimal agar medium. L-alanine prototrophic suppressor mutants were obtained after incubation at 37°C for 2 days (**a**), 3 days (**b**), and 4 days (**c**) on minimal agar medium. Subsequently, growth of individual suppressor mutants in minimal liquid medium without L-alanine supplementation was measured. Symbols: large open circles, HYE032; large grey circles, W3110; small closed circles, L-alanine prototrophic mutants.

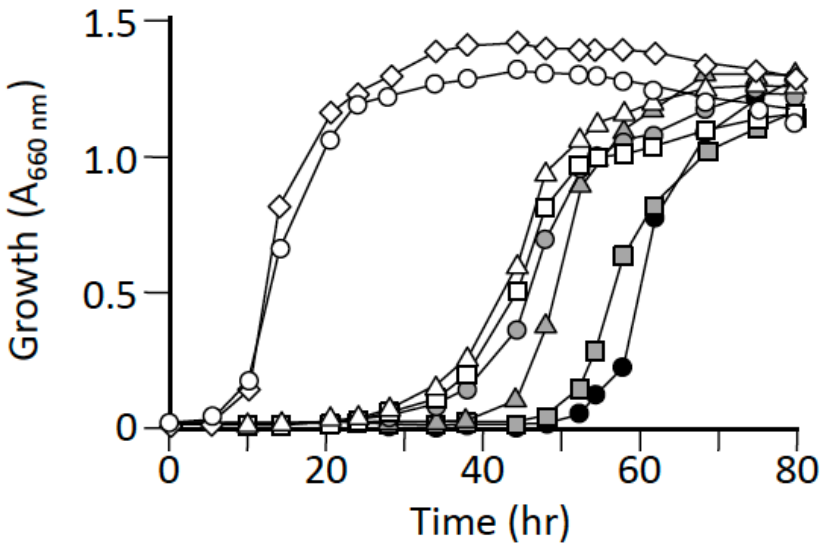



**Figure S3.** Effect of L-glutamic acid on growth of L-alanine auxotroph HYE032. HYE032 cells were inoculated into minimal liquid medium containing various amount of L-glutamic acid (10–2,000 μg/mL), and growth was monitored by measuring the absorbance at 660 nm. Symbols: open diamonds, W3110 with no supplementation; open circles, HYE032 with 100 μg/mL L-alanine; open triangles, HYE032 with 2,000 μg/mL L-Glutamic acid; open squares, HYE032 with 1,000 μg/mL L-Glutamic acid; grey circles, HYE032 with 500 μg/mL L-Glutamic acid; grey triangles, HYE032 with 100 μg/mL L-Glutamic acid; grey squares, HYE032 with 10 μg/mL L-Glutamic acid; closed circles, HYE032 with no supplementation. The figure shows representative results from two experiments.

**Table S1.** Primers used for sequencing of candidate genes.

| Primer name | gene | Nucleotide sequence (5' to 3') |
|---|---|---|
| aceE-Rev3 | *aceE* | catctAGATGTGGCCCTGGAAGTAAACC |
| aceE-Fwd3 | | CGTGATCAAAGTGATGTGGG |
| aceE-Fwd | | TAACCACTTCTTCCGTGCACG |
| aceE-Fwd2 | | AAAAGACCTCGAACTGGGC |
| aceE-Rev2 | | TCAGCTGGATCAGTTTACCG |
| aceE-Fwd1 | | tgtctagAACAGAAGTCGTGAAGAGAGCC |
| aceF-Fwd | *aceF* | agtctaGACTTCAGCAAGTTTGGTG |
| aceF-Rev | | actctagaGTAATACCCTAACCACCACC |
| lpd-Fwd | *lpd* | gctctagaGCAGCTGACAAAGACATCG |
| lpd-Rev | | gttctagaAACCCATTTCGATTGCCAGG |
| pta-Fwd1 | pta | TTCCGTGTTCTTCATGCTGC |
| pta-Rev1 | | GATGACGAGATTACTGCTGC |
| pta-Fwd2 | | TGGTTCGTGAACTGTCTCTG |
| pta-Rev2 | | AGTTTGCGACGATCTCTTCC |
| pta-Fwd3 | | ATCAGCTGACTGAACTTGCG |
| pta-Rev3 | | ATTTCTGCCAGCTGTTCAGC |
| ackA-Fwd | *ackA* | TTTCTGCTATCCGCAACG |
| ackA-Rev | | TTGGTTGGGATAACCACCG |
| cyaA-Fwd | *cyaA* | CAATCAGGTGGTGCATTTCG |
| cyaA-Rev | | AGAACTGGATGATCCCTTCG |
| flgJ-Fwd | *flgJ* | AACGCAGATCGATTTACGCC |
| flgJ-Rev | | TCATCGTAGTTACGTGGCAC |
| gspE-Fwd | *gspE* | CATTCCTGCTTTCGTCTTCC |
| gspE-Rev | | AAGCGTCAGTTCACTATGGC |
| cpxP-Fwd | *cpxP* | ATGATGCGCATAGTTACCGC |
| cpxP-Rev | | ACTACTGGGAACGTGAGTTG |
| yhbO-Fwd | *yhbO* | GATCATGGGGATTAAACACC |
| yhbO-Rev | | ACACCGGTTTGCCACTATTC |
| plsB-Fwd | *plsB* | GCACGTCAATGTGCTTTACC |
| plsB-Rev | | TCTCTCTGATTACCCTTCGC |
| ydhK-Fwd | *ydhK* | ATGAACGCATCGTCATGGTC |
| ydhK-Rev | | TGCCAGAGTAAACTGGCATG |
| entE-Fwd | *entE* | TATTTCCAGCTTTCCGGTGG |
| entE-Rev | | AGTGGATTTCCTTCGGCATC |
| flhC-Fwd | *flhC* | GACGATTACTCAGTTGACGC |
| flhC-Rev | | AACAAACCGCACCAATGTCC |
| ynbB-Fwd | *ynbB* | ATGGTGATTTCAGGTCCACG |
| ynbB-Rev | | CCTTCCAATGTTTTTCCCGG |
| glnS-Fwd | *glnS* | TTACGCACTCTCTGTGTACG |
| glnS-Rev | | AGCACCAGACGTTTGTACTG |
| yhjG-Fwd | *yhjG* | TATTCCCGAAGTCACGATGG |
| yhjG-Rev | | AAAGCAACACGGGTGTTACC |
| ftsI-Fwd | *ftsI* | CGATTACCAAAGTTGACCCC |
| ftsI-Rev | | ACAGCTACAAAGAGATCGCC |
| yhjX-Fwd | *yhjX* | AAACCAGCAATGGTGTGGTG |
| yhjX-Rev | | AGTGAGGCGATAATCGAACC |

Nucleotides written in lowercase were added for cloning purposes.

**Table S2.** Summary of suppressor mutants in terms of numbers of genes harboring mutation(s).

| No. of genes with mutation(s) in each suppressor mutant | Days of isolation | No. of suppressor mutants | Suppressor clone |
|---|---|---|---|
| 1 | 2 | 15 | #4-2, #5-1, #8-2, #11-2, #12-2, #13-3, #16-2, #18-1, #19, #20, #21, #24, #25, #27, #29 |
| | 3 | 12 | #31-1, #33-1, #35-1, #37-1, #39-2*, #40-2, #43-1, #44-1, #45-1, #46-1, #51, #57 |
| | 4 | 3 | #64-1, #65-2, #70 |
| | | (total 30) | |
| 2 | 2 | 5 | #3, #10-1, #15-1, #17-2, #23 |
| | 3 | 3 | #32-1, #48-1, #54 |
| | 4 | 1 | #68-1 |
| | | (total 9) | |
| 3 | 2 | 1 | #14-1 |
| | 3 | 0 | - |
| | 4 | 0 | - |
| | | (total 1) | |

* Suppressor mutant #39-2 harbors two distinct point mutations in *aceE*.

**Table S3.** Summary of suppressor mutants in terms of genes mutated.

| Mutation occured | Mutation gene | No. of suppressor mutants | No. of suppressor mutants with one, two or three mutation gene(s) | | |
|---|---|---|---|---|---|
| | | | one | two[*1] | three[*2] |
| Pta-AckA pathway | *pta* | 17 | 14 | 3 | - |
| | *ackA* | 1 | 1 | - | - |
| | | (Total 18) | | | |
| PDH complex | *aceE* | 11 | 5 | 5 | 1 |
| | *aceF* | 2 | 2 | - | - |
| | *lpd* | 3 | 3 | - | - |
| | | (Total 16) | | | |
| Others | *cyaA* | 4 | 2 | 2 | - |
| | *entE* | 1 | 1 | - | - |
| | *ynbB* | 1 | 1 | - | - |
| | *yhjG* | 1 | 1 | - | - |
| | *gspE* | 1 | - | 1 | - |
| | *plsB* | 1 | - | 1 | - |
| | *ydkK* | 1 | - | 1 | - |
| | *flgJ* | 1 | - | 1 | - |
| | *cpxP* | 1 | - | - | 1 |
| | *yhbO* | 1 | - | - | 1 |
| | *yhiX* | 1 | - | 1 | - |
| | *flhC* | 1 | - | 1 | - |
| | *ftsI* | 1 | - | 1 | - |
| | *glnS* | 1 | - | 1 | - |

[*1] Suppressor mutants #15-1, #17-2, and #23, which carry the *pta* mutation, concurrently harbor a second mutation in *gspE*, *plsB*, and *ydhK*, respectively. Suppressor mutants #3, #10-1, #32-1, #48-1, and #54, which carry the *aceE* mutation, concurrently harbor a second mutation in *flgJ*, *cyaA*, *yhiX*, *flhC*, and *ftsI*, respectively. Suppressor mutant #68-1 harbors mutations in *cyaA* and *glnS*.
[*2] Suppressor mutant #14-1 carries three mutations, in *aceE*, *cpxP*, and *yhbO*.