Supplemental Table S1. Primers for all the constructs

Wildtype	**NewE-5'**	GGGCCATGGgcGTTCGTTGGACCCTGTGGGACACCCTGGCTTTCCTGCTGCTGC
	NewE-Rev1	GGGATGAACATGATCAGCAGAGACGGCAGCAGCAGAGACAGCAGCAGCAGGAAA GCCAG
	NewE-Fwd2	CTGCTGATCATGTTCATCCCGTCTACCTTCAAACGTCCGGTTTCTTCTTGGAAAGC
	NewE-Rev2	CAGAAGAAGCCATCAGCAGGGTTTTACGCAGGTTCAGAGCTTTCCAAGAAGAAAC CGG
	NewE-Fwd3	CCTGCTGATGGCTTCTTCTGTTCGTCTGAAACCGCTGAACTGCTCTCGTCTGCCGTG
	NewE-Rev3	GTCAGCAGGAAGGTCAGGGTTTCCTGAGCGTAAACGCACGGCAGACGAGAGCAG
	NewE-Fwd4	CCCTGACCTTCCTGCTGACCCAGAAAAAAACCTGCGTTAAAAACTACGTTCGTAAA G
	NewE-3'	GGGGCGGCCGCATTAGTGATGGTGATGGTGATGTTCTTTACGAACGTAGTTTTTAA CGC
E-LS, E-L_N, & E-L_C	**E-LS-5'**	GGTGTGCCATGGgtGTTCG
	E-LS-Rev1	AGCAGCAGCAGCAGCAGAGTGTCCCACAGGGTCCAACGAACACCCATGGCAC
	E-LS-Fwd2	TGCTGCTGCTGCTGCTCTCTCTCCTGCTGCCATCTCTGCTCCTCCTGCTCCTGCC
	E-LS-Rev2	GCGCTTTCCAAGAAGAAACCGGACGTTTGAAGGTAGAAGGCAGGAGCAGGAGGAG
	E-LS-Fwd3	GGTTTCTTCTTGGAAAGCGCTGAACCTCCGTAAAACGCTGCTCATGGCGAGCTCT
	E-LS-Rev3	CACGGCAGACGAGAGCAATTCAGCGGTTTCAGACGAACAGAGCTCGCCATGAGCA
	E-LS-Fwd4	TGCTCTCGTCTGCCGTGCGTTTACGCGCAGGAAACCCTGACCTTCCTCCTCACCC
	E-LS-Rev4	CTTTACGAACGTAGTTTTTAACGCAGGTTTTTTTCTGGGTGAGGAGGAAGGTCAG
	E-LS-Fwd5	GCGTTAAAAACTACGTTCGTAAAGAACACCATCATCACCACCACCTCGAGCACAC
	E-LS-3'	GGTGTGCTCGAGGTGGTGG
	E-L_N-Rev1	TGCTGCTGCTGCTGCTCTCTCTCCTGCTGCCATCTCTGCTCATCATGTTCATCCC
	E-L_N-Fwd2	GCGCTTTCCAAGAAGAAACCGGACGTTTGAAGGTAGAAGGGATGAACATGATGAG CAG
	E-L_C-Fwd2	AGCAGCAGCAGGAAAGCCAGAGTGTCCCACAGGGTCCAACGAACACCCATGGCAC
	E-L_C-Rev2	GGCTTTCCTGCTGCTGCTCTCTCTCCTGCTGCCATCTCTGCTCCTCCTGCTCCTGCC
E_{25-28}	**E-Fwd**	GGTGTGCCATGGgcGTTCATTGGACCCTGTGGGACAC
	E28lyso-Rev	GTTCATACCGCTACCGCTGCCGATGAACATGATCAGCAGAGACGGC
	E27lyso-Rev	GTTCATACCGCTACCGCTGCCGAACATGATCAGCAGAGACGGCAG
	E26lyso-Rev	GTTCATACCGCTACCGCTGCCCATGATCAGCAGAGACGGCAGAAAC
	E25lyso-Rev	GTTCATACCGCTACCGCTGCCGATCAGCAGAGACGGCAGAAACAG
	Lyso-Fwd	GGCAGCGGTAGCGGTATGAACATCTTCGAAATGCTCCGTATCGAC
	Lyso-Rev	GGTGTGGCGGCCGCATTAGTGATGGTGATGGTGATGCAGGTTTTTGTACGCGTCCC AGG
N-terminal deletion	**E_{N9}-Fwd**	GGTGTGCCATGGgaCTGGCTTTCCTGCTGCTGCTGTC
	E_{N10}-Fwd	GGTGTGCCATGGgaGCTTTCCTGCTGCTGCTGTCTCTG
	E_{N11}-Fwd	GGTGTGCCATGGgaTTCCTGCTGCTGCTGTCTCTGCTG
	E_{N26}-Fwd	GGTGTGCCATGGgaTTCATCCCGTCTACCTTCAAACGTCC
	E-Rev	GGTGTGGCGGCCGCATTAGTGATGGTGATG

*Restriction sites are indicated with underline. Nucleotides added to introduce Gly residue at the position 2 are in lower case.

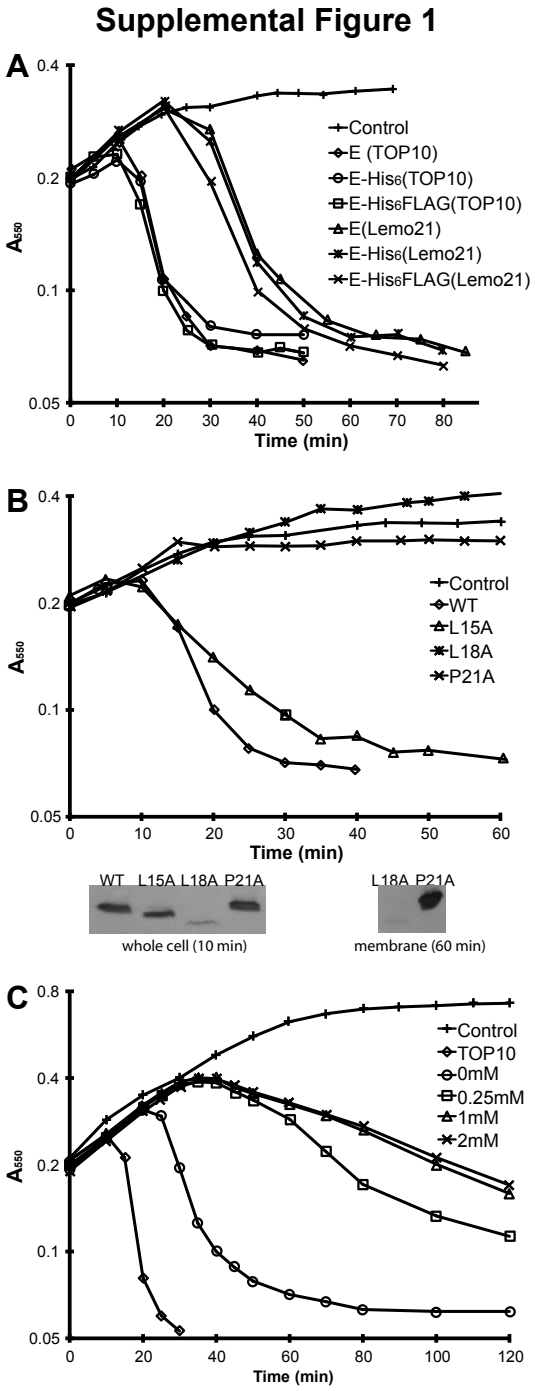

Fig. S1. Effect of the His6-tag and FLAG-tag on E, alanine-scanning in TOP10, and the effect of various L-rhamnose concentraions in lysis onset.

A. Growth curve comparison of wild-type E with His6-tagged or His6-FLAG tagged E.

B. Representative growth curves of alanine-scanning mutants in TOP10 cells. Expression of each E construct was induced at time= 0. Western blot of whole cells after 10 min of induction and E in membrane fractions after 60 min of induction.

C. Growth curves of wild-type in TOP10 and Lemo21 with different concentrations of L-rhamnose.

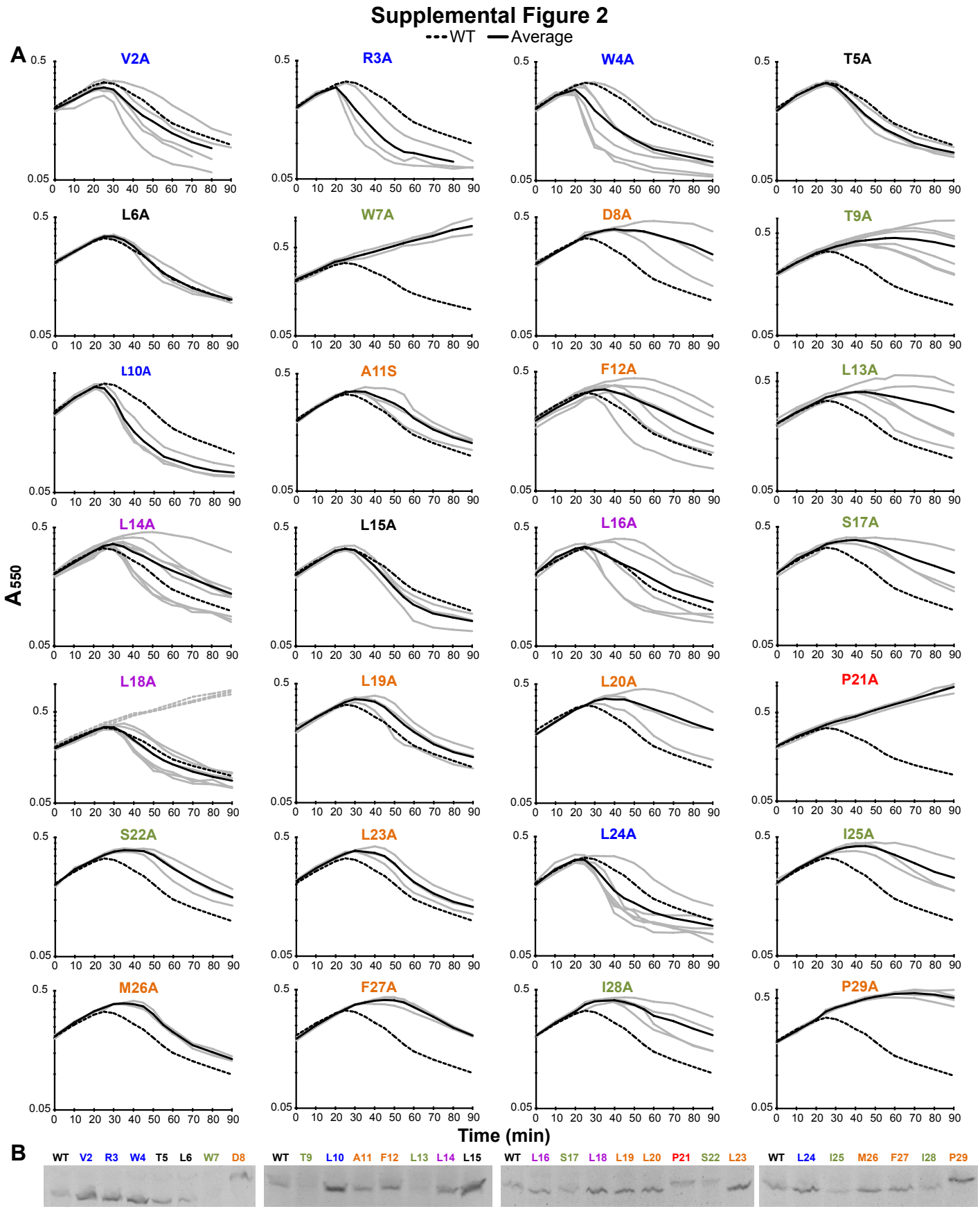

Fig. S2. Alanine-scanning in Lemo21.

A. Raw data of lysis assays of alanine-scanning mutants in Lemo21 cells. At least three replicates growth curves of each mutant were monitored by OD550(grey lines). Average growth curve are shown as solid black lines. For comparison, the averaged wild-type growth curve is shown as a dotted line. Each mutation is categorized by color as in Fig. 2.

B. Western blot of protein levels in whole cells after 20 min of induction.

Supplemental Figure 3

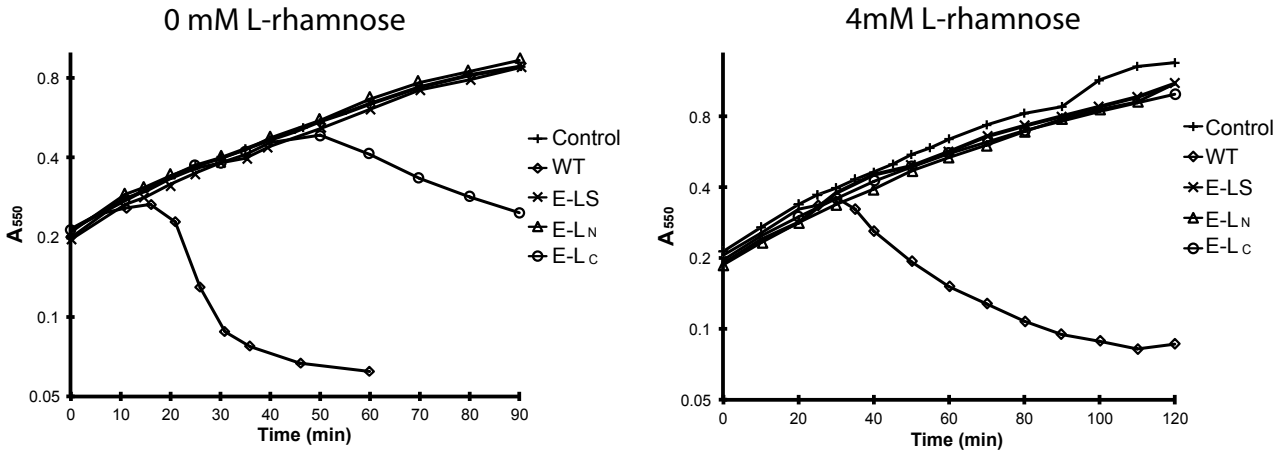

Fig. S3. Growth curves of leucine mutants in Lemo21.
Cultures grown with 0 mM L-rhamnose (left) or 2 mM L-rhamnose (right).

Supplemental Figure 4

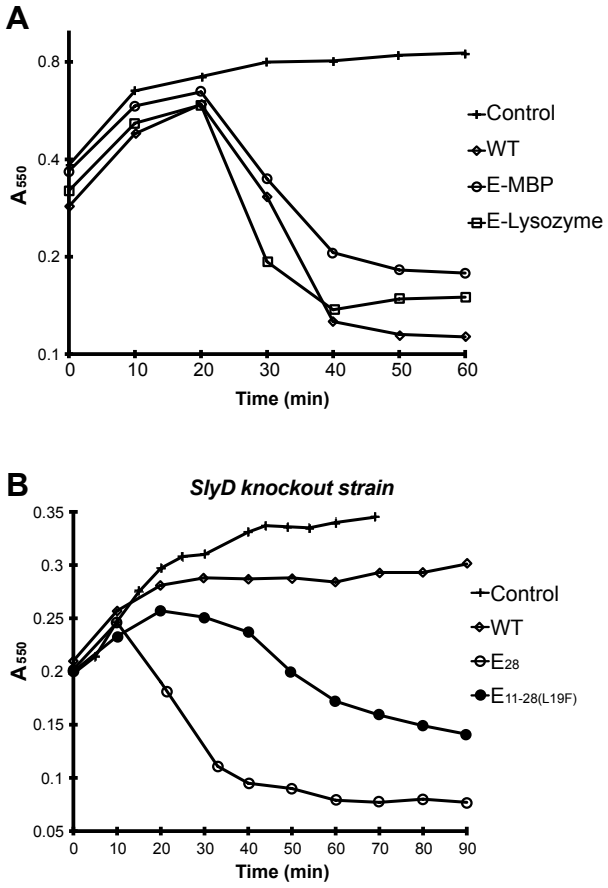

Fig. S4. A. Growth curves of E-MBP and E-lysozyme fusion constructs.
B. Growth curve of E_{28} and $E_{11\text{-}28(L19F)}$ in the *SlyD* knockout strain.

Supplemental Table S2. Predicted apparent free energy difference for insertion of the Phe mutants

Mutants	Sequence of TM	Predicted ΔG
$E_{11\text{-}28}$	MGAFLLLLSLLLPSLLIMFI	-1.470
$E_{11\text{-}28(L13F)}$	MGAFFLLLSLLLPSLLIMFI	-1.351
$E_{11\text{-}28(L14F)}$	MGAFLFLLSLLLPSLLIMFI	-1.303
$E_{11\text{-}28(L15F)}$	MGAFLLFLSLLLPSLLIMFI	-1.343
$E_{11\text{-}28(L16F)}$	MGAFLLLFSLLLPSLLIMFI	-1.368
$E_{11\text{-}28(L18F)}$	MGAFLLLLSFLLPSLLIMFI	-1.297
$E_{11\text{-}28(L19F)}$	MGAFLLLLSLFLPSLLIMFI	-1.359
$E_{11\text{-}28(L19I)}$	MGAFLLLLSLILPSLLIMFI	-1.502
$E_{11\text{-}28(L20F)}$	MGAFLLLLSLLFPSLLIMFI	-1.347
$E_{11\text{-}28(L23F)}$	MGAFLLLLSLLLPSFLIMFI	-1.378
$E_{11\text{-}28(L24F)}$	MGAFLLLLSLLLPSLFIMFI	-1.338

* Values for predicted ΔG were calculated using the prediction of ΔG for TM helix insertion website at http://dgpred.cbr.su.se/index.php?p=TMpred.