Supplemental Material

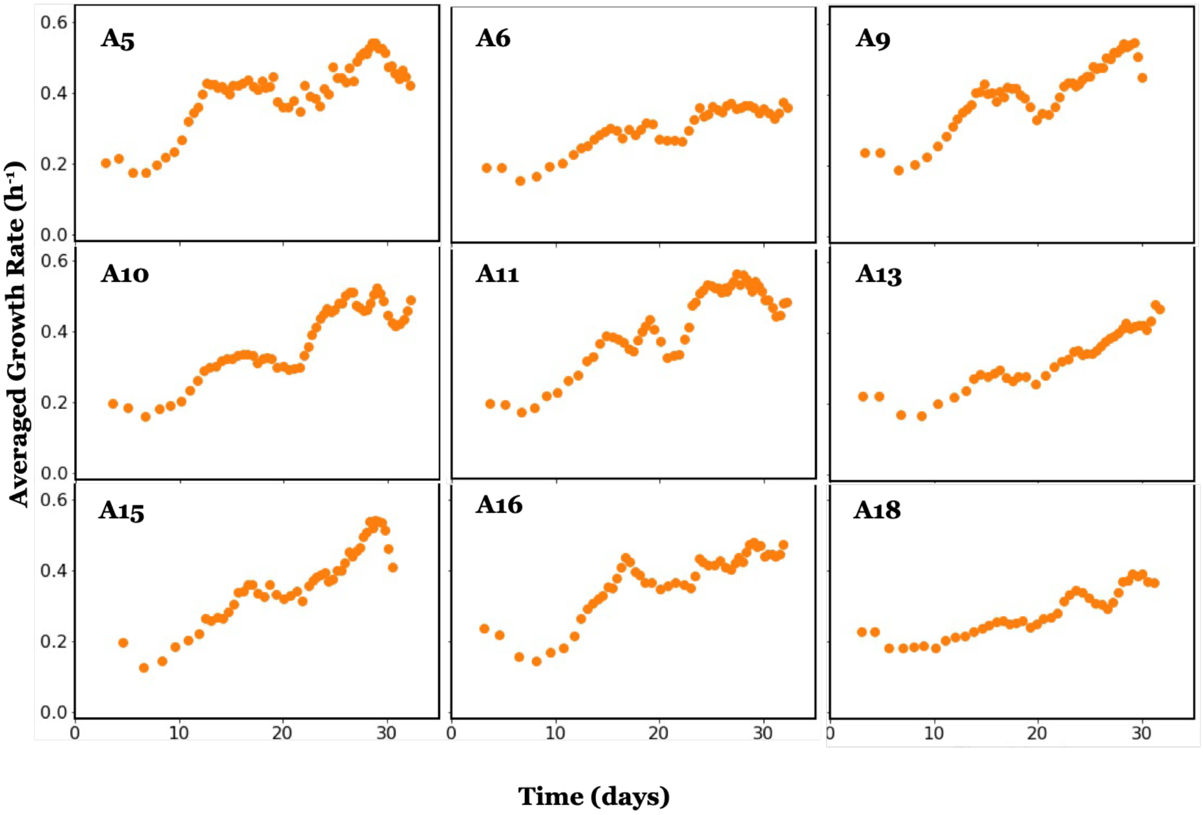


Fig. S1. Fitness trajectories of other replicates for the ALE experiment on SMM. A5, A6, A9 and A10 are biological replicates of *S. aureus* USA300 LAC. A11, A13, A15, A16 and A18 are biological replicates of USA300 TCH1516. All strains demonstrated fitness increase over the course of the experiment.

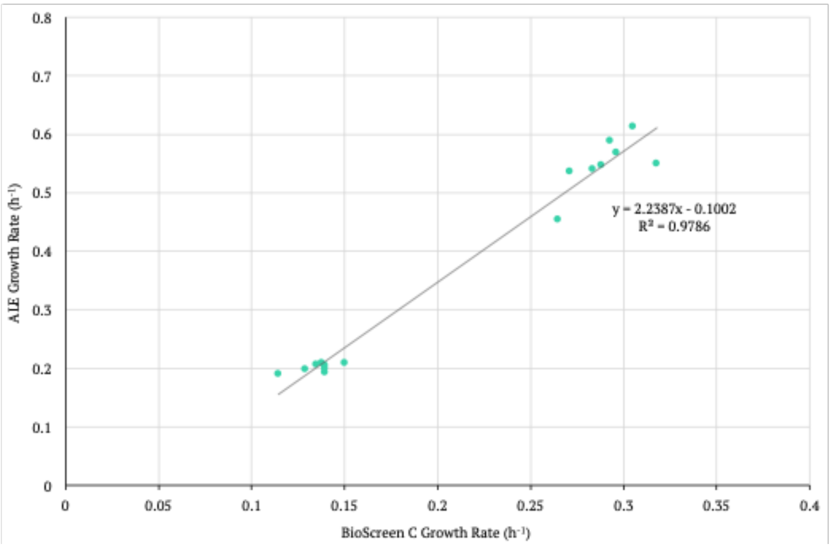


Fig. S2. Correlation of growth rates calculated from growth curves performed using the ALE machine and the BioScreen C (A). The aeration is higher in the ALE machine compared to the BioScreen C.

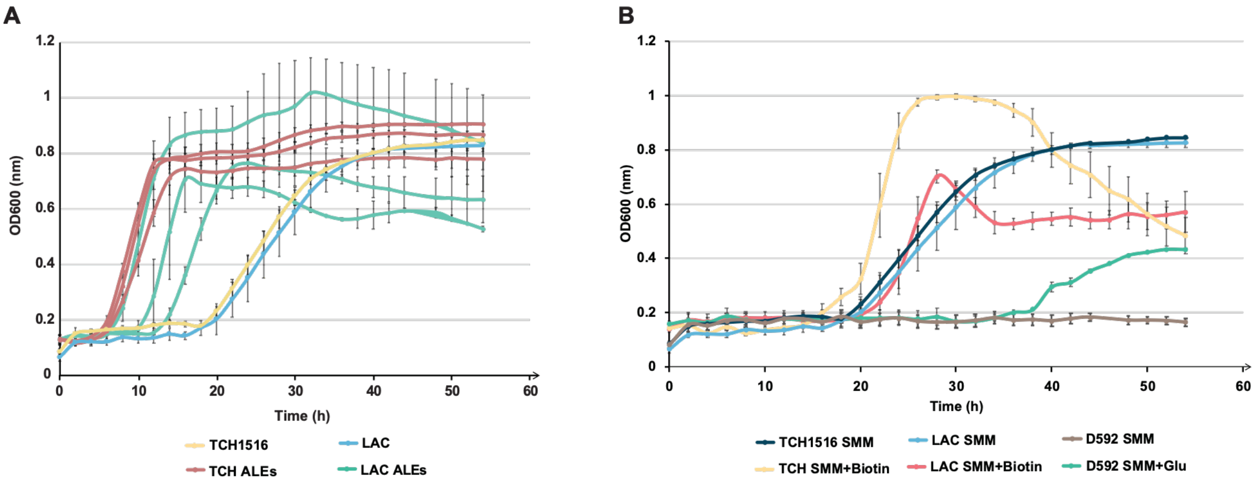
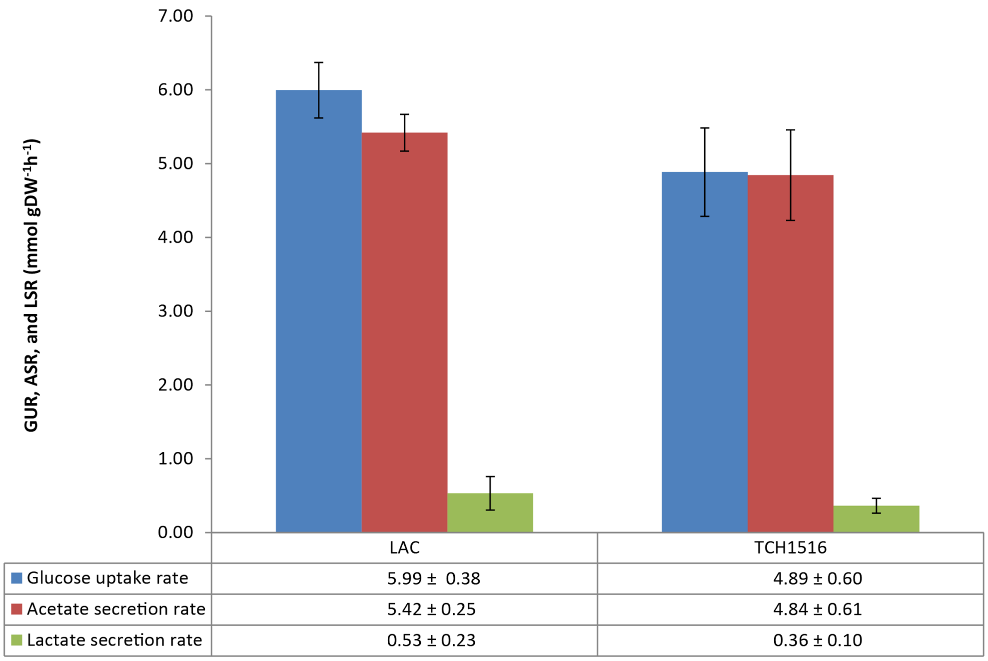


Fig. S3. Growth curves of wild-type and evolved strains in SMM media (A). Growth curves of wild type strains in SMM supplemented with glutamic acid (SMM + Glu; 250 mg/L) or biotin (SMM + Biotin; 0.1 mg/L).



	LAC	TCH1516
🟦 Glucose uptake rate	5.99 ± 0.38	4.89 ± 0.60
🟥 Acetate secretion rate	5.42 ± 0.25	4.84 ± 0.61
🟩 Lactate secretion rate	0.53 ± 0.23	0.36 ± 0.10

Fig. S4. Phenotypic characteristics of the evolved LAC and TCH1516 clones. All values shown are calculated by averaging all replicates of the same strain (LAC and TCH1516). The glucose uptake rates (GURs) and the acetate secretion rates (ASRs) are roughly equivalent across both strains. The lactate secretion rates (LSRs) are relatively low compared, but detectable.

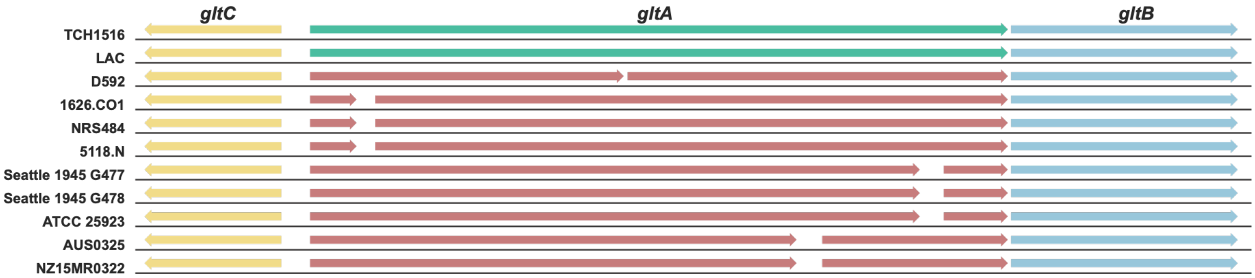


Fig. S5. Alignment of the *glt* operon of *Staphylococcus aureus* studied strains (TCH1516, LAC, and D592) and other strains identified to also have a disrupted glutamate synthase gene *(gltA)*.

Table S1. Strains used in this study. Y - yes, phenotypically characterized; N - no, not phenotypically characterized.

Strain	Description	# Flask	Phenotypically characterized (growth/MIC)	Biosample Accession number
LAC	Wild type	-	Y/Y	
TCH1516	Wild type	-	Y/Y	SAMN00253845
D592	Wild type	-	N/N	
LAC A3	LAC strain evolved in SMM media	51	Y/N	SAMN10712247
LAC A4	LAC strain evolved in SMM media	52	Y/N	SAMN10712248
LAC A5	LAC strain evolved in SMM media	70	N/N	SAMN10712249
LAC A6	LAC strain evolved in SMM media	51	N/N	SAMN10712250
LAC A7	LAC strain evolved in SMM media	69	Y/Y	SAMN10712251
LAC A9	LAC strain evolved in SMM media	62	N/N	SAMN10712252
LAC A10	LAC strain evolved in SMM media	62	N/N	SAMN10712253
TCH1516 A11	TCH1516 strain evolved in SMM media	-	N/N	-
TCH1516 A13	TCH1516 strain evolved in SMM media	48	N/N	SAMN10712254
TCH1516 A14	TCH1516 strain evolved in SMM media	60	Y/N	SAMN10712255
TCH1516 A15	TCH1516 strain evolved in SMM media	40	N/N	SAMN10712256
TCH1516 A16	TCH1516 strain evolved in SMM media	58	N/N	SAMN10712257
TCH1516 A18	TCH1516 strain evolved in SMM media	48	N/N	SAMN10712258
TCH1516 A19	TCH1516 strain evolved in SMM media	57	Y/Y	SAMN10712259
TCH1516 A20	TCH1516 strain evolved in SMM media	58	Y/N	SAMN10712260
D592 A25	D592 strain evolved in SMM media	19	Y/N	SAMN10712261
D592 A30	D592 strain evolved in SMM media	8	N/N	SAMN10712262
D592 A35	D592 strain evolved in SMM media	16	N/N	SAMN10712263

Table S2. Key mutations identified in TCH1516 evolved strains, their products/function and occurrences.

ALE identification numbers are reported and when followed by ‘p’, indicate evidence of the mutation only in respective ALE populations and not in clones.

dtpT - POT family proton (H+)-dependent oligopeptide transporter; *fliA* - DNA-directed RNA polymerase sigma subunit FliA; *ecfT* - ABC superfamily ATP binding cassette transporter, ABC protein.

Gene	Position	Mutation Type	Sequence Change	Details	#ALE (p - population)
spoVG **(stage V sporulation protein G)**	532,912	SNP	A→T	R7* (AGA→TGA)	A15
	532,916	SNP	T→A	L8H (CTT→CAT)	A14
	532,968	INS	+T	coding (75/303 nt)	A11
	533,042	SNP	A→T	K50I (AAA→ATA)	A19
	533,074	SNP	G→C	A61P (GCG→CCG)	A16
	533,080	SNP	C→A	P63T (CCT→ACT)	A20
	533,090	SNP	C→A	S66* (TCA→TAA)	A13
USA300HOU_0709 (MarR family transcriptional regulator)	760,322	SNP	C→A	R92L (CGT→CTT)	A13p
	760,340	SNP	C→G	R86P (CGT→CCT)	A19
	760,376	SNP	T→A	K74I (AAA→ATA)	A14
	760,404	SNP	C→A	D65Y (GAT→TAT)	A11p
	760,466	SNP	A→T	L44* (TTA→TAA)	A16
	760,470	SNP	C→A	V43F (GTC→TTC)	A15

dtpT	804,025	SNP	T→A	I351F (ATT→TTT)	A11p, A16p, A19, A20
fliA	2,185,439	SNP	C→A	R241L (CGA→CTA)	A18
	2,185,713	DEL	Δ3 bp	coding (446-448/771 nt)	A15
	2,186,106	SNP	A→C	W19G (TGG→GGG)	A16
rsbU **(sigma factor B regulator)**	2,187,358	DEL	Δ1 bp	coding (705/1002 nt)	A11p
	2,187,404	SNP	G→C	A220G (GCT→GGT)	A13p
	2,187,658	SNP	A→T	Y135* (TAT→TAA)	A14
	2,187,720	SNP	G→A	Q115* (CAA→TAA)	A19
ecfT	2,357,045	SNP	C→T	E2E (GAG→GAA)	A15
	2,357,046	SNP	T→A	E2V (GAG→GTG)	A14
bioW **(6-carboxyhexanoate-CoA ligase)**	2,546,229	INS	+C	coding (678/678 nt)	A18
	2,546,230	SNP	T→C	*226W (TAG→TGG)	A16
	2,546,231	SNP	A→T	*226K (TAG→AAG)	A11p, A15p, A19
bioD**, USA300HOU_2412 (dethiobiotin synthase, ABC membrane protein)**	2,551,111	SNP	T→A	intergenic (-55/+397)	A15, A16, A20
	2,551,111	SUB	2 bp→AT	intergenic (-55/+396)	A19
	2,551,118	SNP	C→A	intergenic (-62/+390)	A20
	2,551,125	SNP	T→G	intergenic (-69/+383)	A13, A14
USA300HOU_2595 (APC family amino acid-polyamine-organocation transporter)	2,739,781	SNP	G→A	G91D (GGT→GAT)	A19
	2,739,840	SNP	T→A	W111R (TGG→AGG)	A14
	2,740,512	SNP	G→A	A335T (GCA→ACA)	A15
	2,740,561	SNP	C→A	S351* (TCA→TAA)	A16

Table S3. Key mutations identified in LAC evolved strains, their products/function and occurrences. ALE identification numbers are reported and when followed by ‘p’, indicate evidence of the mutation only in respective ALE populations and not in clones. § denotes possibility of being present in the starting strain, therefore not being an adaptive key mutation.

dtpT - POT family proton (H+)-dependent oligopeptide transporter; *fliA* - DNA-directed RNA polymerase sigma subunit FliA.

Gene	Position	Mutation Type	Sequence Change	Details	#ALE
polA_2 (DNA polymerase I)	514,525	SNP	G→T	R616I (AGA→ATA)	A4, A9, A10p
***ebpS*, LAC_H_00573 (Elastin-binding protein, Glucosaminate ammonia-lyase)**	559,049	SNP	C→T	intergenic (+77/-325)	A4, A9, A10p
***slyA_1*, LAC_H_01002 (Transcriptional regulator SlyA/hypothetical protein)**	1,052,459	SNP	A→T	intergenic (-14/-139)	A9p
	1,052,460	SNP	C→A	intergenic (-15/-138)	A4, A5, A9
§***leuA_1 (2-isopropylmalate synthase)***	1,129,558	SNP	A→T	N20K (AAT→AAA)	A3, A4, A5, A6, A7, A9, A10
§***nrdI, ribN (Protein NrdI, Riboflavin transporter)***	1,302,769	INS	+A	intergenic (-505/-295)	A3, A4, A5, A6, A7, A9, A10
dtpT	1,304,808	SNP	C→G	S6S (TCC→TCG)	A5
	1,305,841	SNP	A→T	I351F (ATT→TTT)	A5, A9, A10
rpoC (DNA-directed RNA polymerase subunit beta)	1,512,105	SNP	G→A	A1177V (GCT→GTT)	A5
	1,514,860	SNP	G→C	R259G (CGT→GGT)	A5
	1,514,895	SNP	C→T	G247E (GGA→GAA)	A10

ftsH (ATP-dependent zinc metalloprotease FtsH)	1,561,825	DEL	Δ1 bp	coding (2063/2094 nt)	A3
	1,561,827	SNP	T→A	K687N (AAA→AAT)	A3
	1,562,920	SNP	G→A	P323L (CCA→CTA)	A6
	1,563,782	SNP	T→A	T36S (ACA→TCA)	A6p
spoVG (Putative septation protein SpoVG)	1,578,164	SNP	G→T	P63T (CCT→ACT)	A10
	1,578,208	SNP	G→A	P48L (CCA→CTA)	A4
	1,578,209	SNP	G→T	P48T (CCA→ACA)	A5
	1,578,275	INS	+A	coding (76/303 nt)	A9
yhdG_2 (putative amino acid permease YhdG)	2,244,291	SNP	G→A	R410C (CGT→TGT)	A7p
	2,244,313	SNP	T→A	L402F (TTA→TTT)	A6
	2,244,413	SNP	A→G	L369P (CTT→CCT)	A7p
	2,244,426	SNP	C→T	A365T (GCA→ACA)	A6
	2,244,610	SNP	C→T	W303* (TGG→TGA)	A9
	2,244,998	SNP	G→T	S174* (TCA→TAA)	A3
	2,245,301	SNP	G→A	A73V (GCT→GTT)	A4
LAC_H_02272, *bioD1* *(putative ABC transporter ATP-binding protein, ATP-dependent dethiobiotin synthetase BioD1)*	2,433,905	SNP	A→C	intergenic (+383/-69)	A4, A7, A9
	2,433,912	SNP	G→T	intergenic (+390/-62)	A10
	2,433,919	SNP	A→T	intergenic (+397/-55)	A10
	2,433,926	SNP	A→T	intergenic (+404/-48)	A5, A10p
bioW (6-carboxyhexanoate-CoA ligase)	2,438,797	DEL	Δ3 bp	coding (689-691/693 nt)	A3
	2,438,799	SNP	T→A	*231K (TAG→AAG)	A10p

	2,438,800	INS	+G	coding (692/693 nt)	A5p
	2,438,800	SNP	A→G	*231W (TAG→TGG)	A7p
rhaS (HTH-type transcriptional activator RhaS)	2,571,420	SNP	G→T	R377I (AGA→ATA)	A5, A7
ecfA1 (Energy-coupling factor transporter ATP-binding protein EcfA1)	2,627,985	SNP	G→A	E2E (GAG→GAA)	A7p
	2,628,048	SNP	C→G	F23L (TTC→TTG)	A5p
rsbU (Phosphoserine phosphatase RsbU)	2,797,241	SNP	C→T	Q92* (CAA→TAA)	A6
	2,797,625	SNP	G→A	A220T (GCT→ACT)	A3
	2,797,930	INS	+T	coding (963/1002 nt)	A7

Table S4. Key mutations identified in D592 evolved strains, their products/function and occurrences.

§ denotes possibility of being present in the starting strain, therefore not being an adaptive key mutation.

Gene	Position	Mutation Type	Sequence Change	Details	#ALE
codY (GTP-sensing transcriptional pleiotropic repressor CodY)	783,648	SNP	C→T	G239S (GGT→AGT)	A35
	783,817	DEL	Δ60 bp	coding (487-546/774 nt)	A30
	784,076	SNP	G→A	T96I (ACA→ATA)	A25
§KJJPFECL_00960 (Lipoate-protein ligase 1)	1,023,420	SNP	C→G	L22V (CTT→GTT)	A25, A30, A35
§patA_2 (Peptidoglycan O-acetyltransferase)	1,127,647	SNP	T→A	I334F (ATC→TTC)	A25, A30, A35
norA (multidrug efflux MFS transporter)	1,321,058	DEL	Δ60 bp	coding (839-898/1167 nt)	A25
	1,321,658	DEL	Δ129 bp	coding (170-298/1167 nt)	A30
	1,321,906	DEL	Δ1 bp	coding (50/1167 nt)	A35
gltA_2 (Glutamate synthase [NADPH] large chain)	1,579,069	SNP	A→T	*675K (TAA→AAA)	A25
	1,579,069	SNP	A→G	*675Q (TAA→CAA)	A30, A35
§pflB (Formate acetyltransferase)	1,810,242	SNP	T→C	E341G (GAA→GGA)	A25, A30, A35
§KJJPFECL_01783 (hypothetical protein)	1,893,832	INS	+TT	coding (7/183 nt)	A25, A30, A35
§yfmJ (Putative NADP-dependent oxidoreductase YfmJ)	2,613,600	SNP	A→C	D282A (GAT→GCT)	A25, A30, A35

Table S6. Evaluation of growth of transposon mutants in SMM and SMM supplemented with glutamic acid (SMM + Glu; 250 mg/L) or biotin (SMM + Biotin; 0.1 mg/L). Strains were grown for 72 h and growth was called when OD was above 0.3. Growth and no-growth are indicated with plus (+) and minus (-) symbols, respectively; n.d. - non determined. JE2 is the wild type strain used in the construction of the mutants, it is a LAC strain curated of its plasmids.

Strain	KO Gene	SMM	SMM + Biotin	SMM + Glu
JE2	-	+	+	+
NE777	*bioD*	-	+	n.d.
NE999	*bioW*	-	+	n.d.
NE390	*gltA*	-	n.d.	+