

Extended Materials and Methods

Nucleic Acid Isolation and Sequencing: mRNA was enriched from one microgram total RNA using RiboZero rRNA Removal (Bacteria) Kit. rRNA-depleted mRNA samples were purified by Agencourt AMPure® XP beads and viewed using Agilent High Sensitivity RNA ScreenTape. Double stranded cDNA synthesis was performed using Illumina TruSeq Stranded mRNA Library Prep Kit in accordance with the manufacturer's standard protocol. The amplified libraries were purified using Agencourt AMPure® XP beads. Quality and quantity were assessed using an Agilent DNA1000 ScreenTape and Picogreen assay kit, respectively. Libraries were made single-stranded and diluted to 0.18 pM, as per Illumina manufacturer's instructions. Cluster generation and sequencing was performed on an Illumina NextSeq500 using NextSeq 500/550 High Output v2 kit (75 cycles).

Analysis and Visualization of Genomic Sequence: Following reference assembly and analysis of genomic data, sequences were also inspected visually in order to confirm structural variants using the Integrated Genome Viewer (1). Once confirmed, Muller plot representation of population-level polymorphism data was created using the R library ggmuller (<https://cran.r-project.org/web/packages/ggmuller/index.html>).

Preparation of Samples for GC-MS: Standards were prepared from an 18 amino-acid mixture (AAS-18, Sigma Aldrich), 2-aminoisobutyric acid (AIB, Sigma), and DL-norvaline (NV, Sigma). 20 g/L methoxylamine hydrogen chloride (MOX, Sigma) was prepared weekly in pyridine (Sigma) that had been dried over 4A molecular sieves (JT Baker 2708-1). The samples were hydrolyzed to generate free amino acids by combining 10 µL aliquots of spent media, 10 µL of internal standard solution (4.04 µg of AIB and 4.32 µg of NV), and 75 µL of 7.42 M HCl in polypropylene

vials with screw-caps and o-ring seals (Dot Scientific, Burton, MI). The tubes were purged with nitrogen and incubated for overnight @ 120 °C in an oven flushed continuously with nitrogen. The hydrosylates were dried overnight @ 45 °C in a vacuum centrifuge (LabConco).

T-butyl-dimethylsilyl (TBDMS) derivatives of the amino acids were generated by adding 40 µL of MOX/pyridine to the dried hydrosylates and flushing the tubes' headspaces with nitrogen prior to resealing them. They were shaken at 250 rpm for 90 minutes at 37 °C. The supernatants were combined with 40 µL of N-Methyl-N-tert-butyl-dimethylsilyltrifluoroacetamide + 1% N-tert-butyl-dimethylchlorosilane (MTBSTFA, Restek Inc., Bellefonte, PA) in 250 µL glass inserts (Agilent Technologies). The inserts were placed into 1.5 mL autosampler vials, the vials' headspaces purged with nitrogen, sealed with PTFE-Silicone-PTFE septum crimp caps (Agilent), and heated for 2 hrs @ 120 °C. The TBDMS derivatives were separated on an Agilent 6890/5973 GC-MS equipped with a 30 m long 0.25 mm i.d., 0.25 µm df ZB-5msi column (Phenomenex) using 1.2 mL/min He carrier gas. 1 µL was injected into a 300 °C injector port with a 1:25 split ratio and the oven program: hold 120 °C for 1 min, ramp 10 °C/min to 320°, hold for 0.5 min (21.5 min total); the transfer line was held @ 300 °C. The MS was operated in selected ion monitoring mode according to Table S5.

Supplementary References

1. Thorvaldsdóttir H, Robinson JT, & Mesirov JP (2013) Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Brief Bioinform* 14(2):178–192.

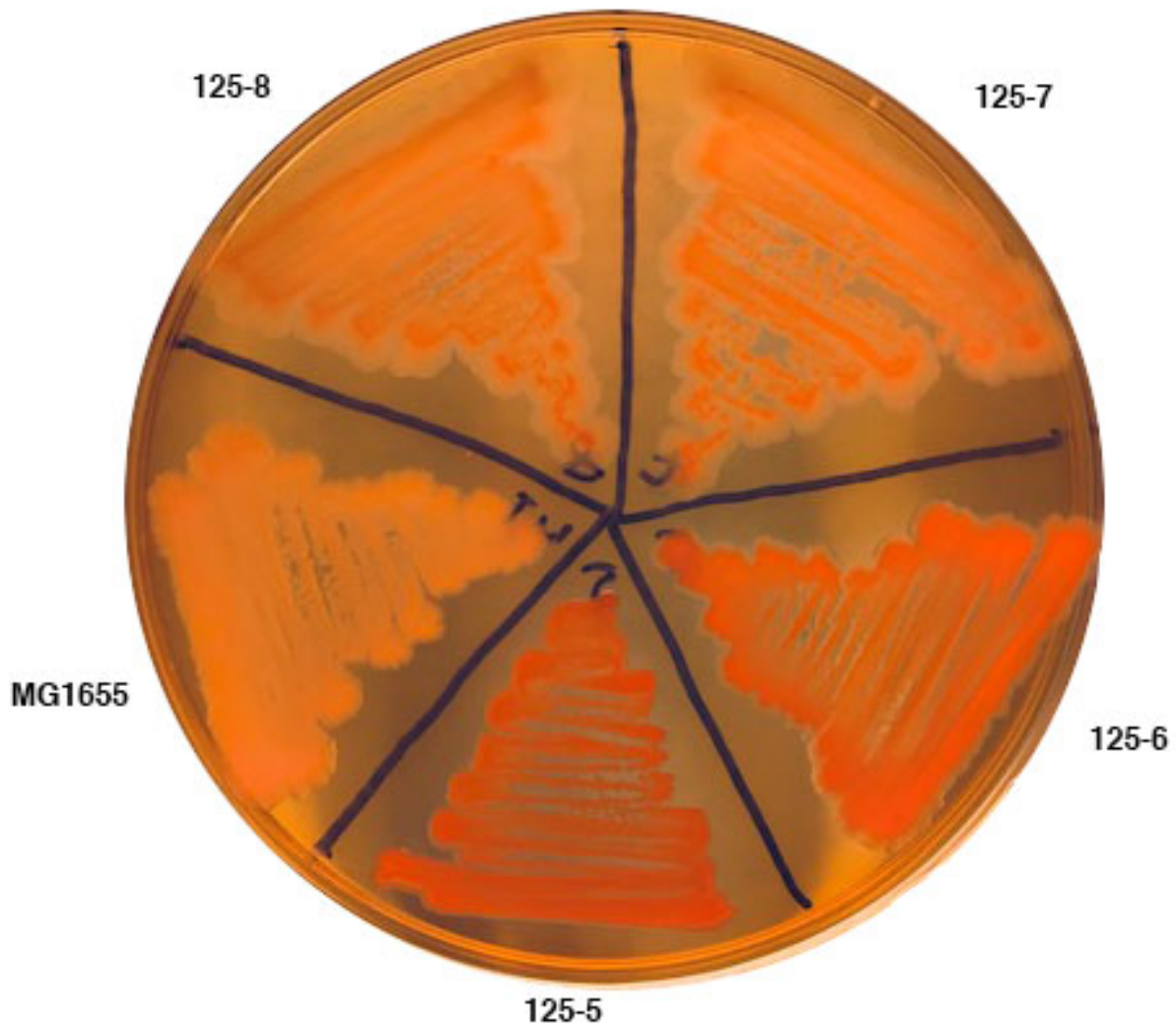


Figure S1. Wild-type progenitor cells (MG1655) and evolved clones (125-5, 125-6, 125-7, 125-8) streaked on Congo Red agar to confirm biofilm production. Cells producing biofilm polysaccharides adopt a red morphology on Congo Red, while cells not producing biofilm polysaccharides appear beige.

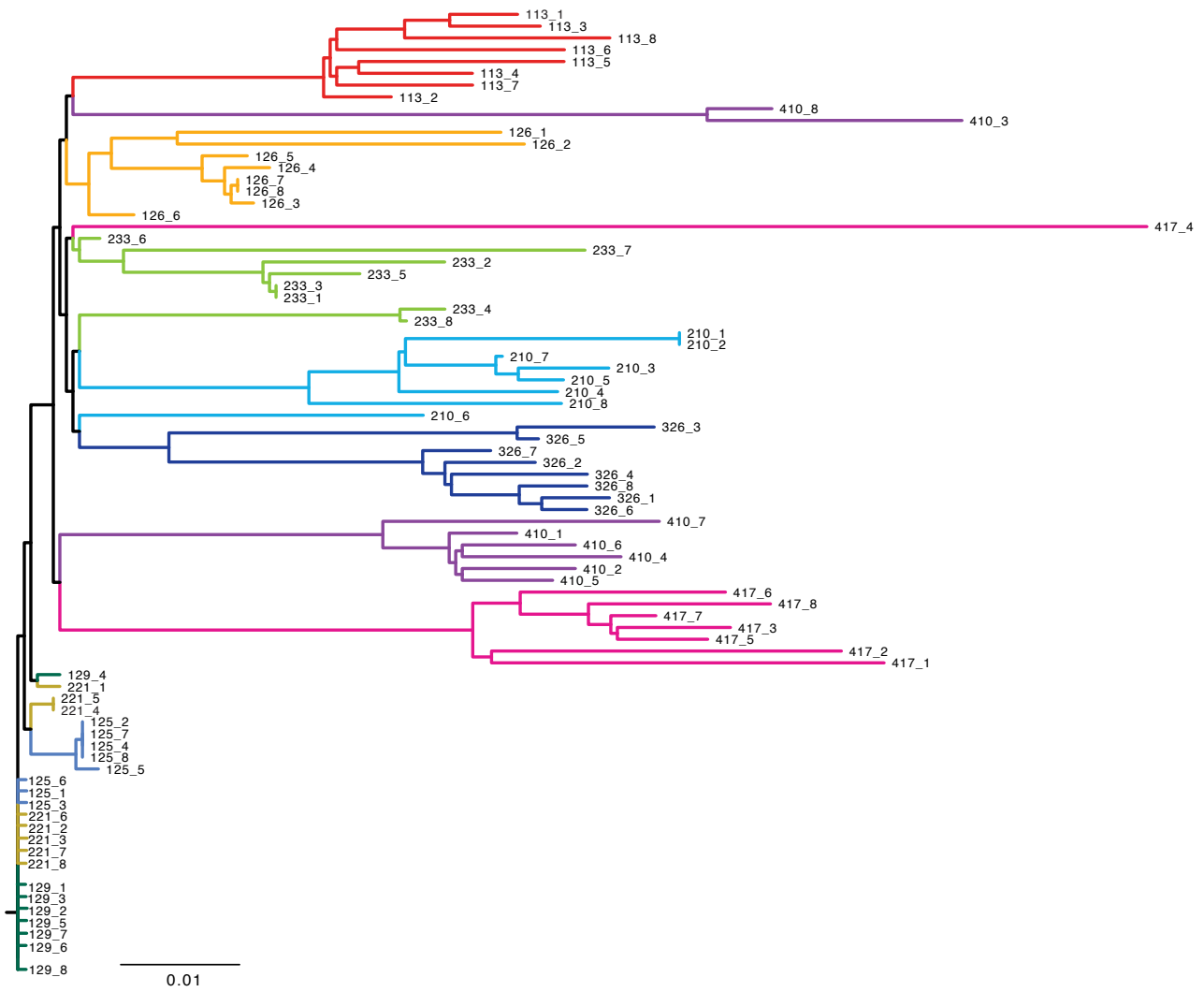


Figure S2: Maximum likelihood phylogenetic relationship of all clonal isolates. Colors represent populations from which individual clones were isolated (113: Red, 125: Lt. Purple, 126: Orange, 129: Dk. Green, 210: Cyan, 221: Gold, 233: Lt. Green, 326: Navy, 410: Dk. Purple, 417: Pink).

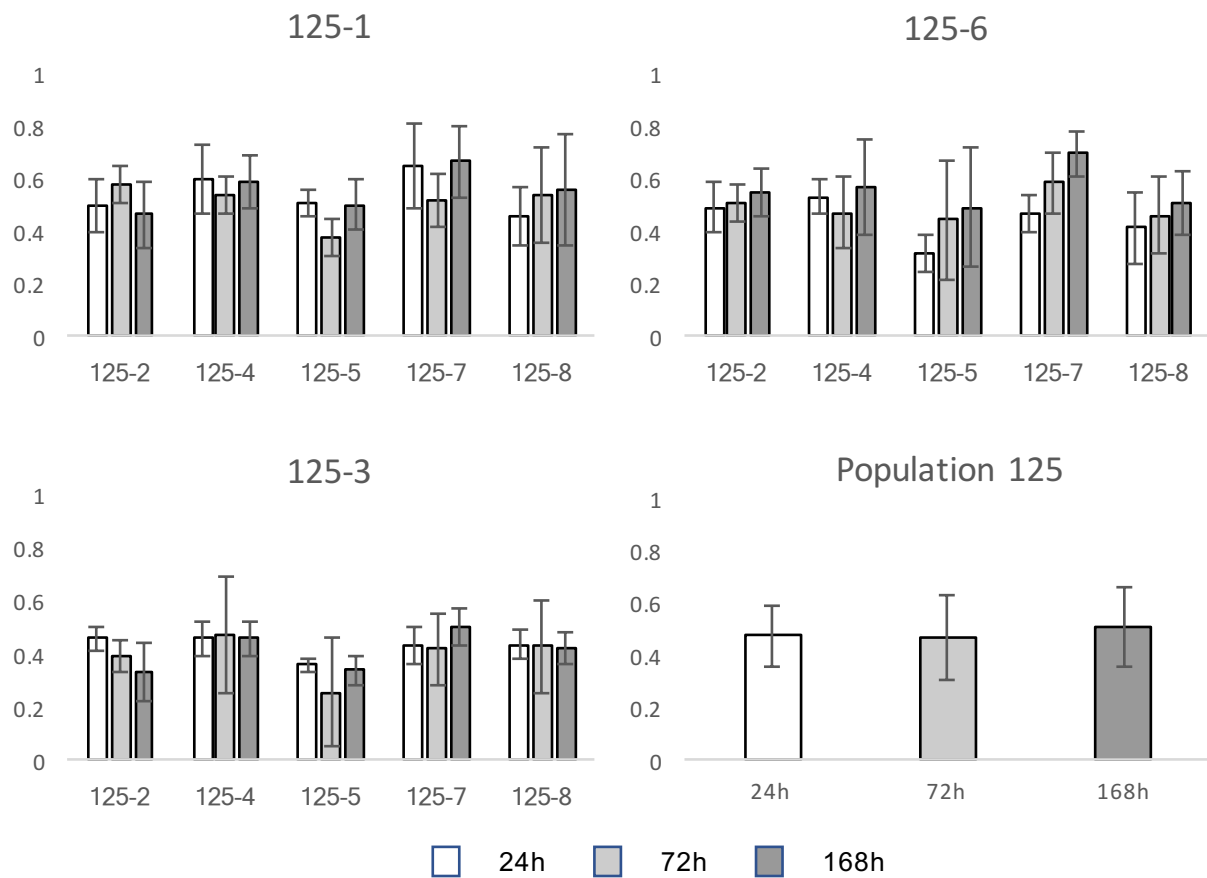


Figure S3: Average proportion of co-culture belonging to Haplotype B (Clones 125-2, 125-4, 125-5, 125-7, or 125-8) vs. Haplotype A (Clones 125-1, 125-3, or 125-6) over 168 hours (7 days).

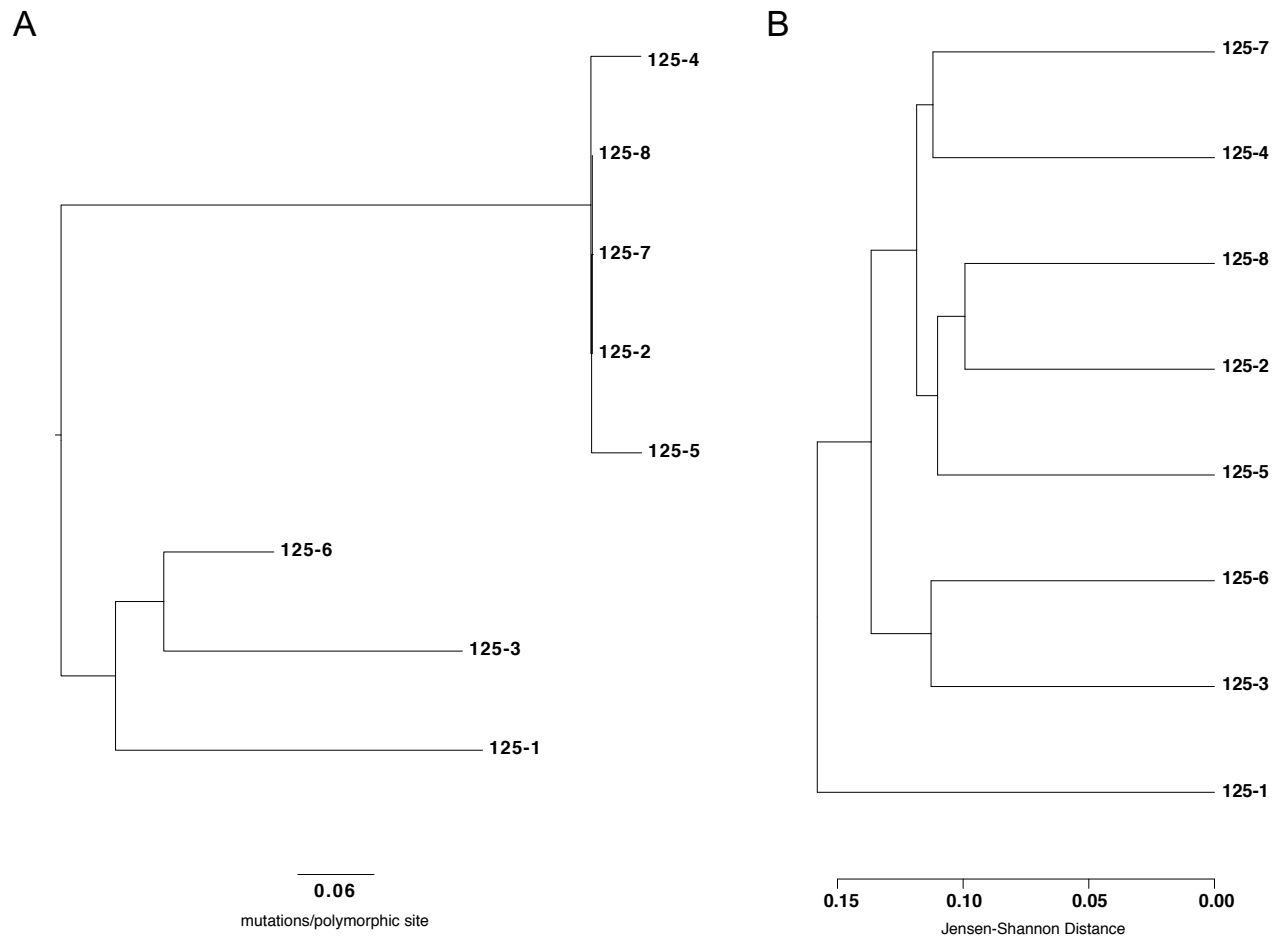


Figure S4: Trees created from genomic and transcriptomic data resemble each other based on organization. A) Maximum-likelihood phylogenetic relationship between individual clones from Population 125. Tree is based on the 25 segregating sites accounting for all the SNPs between the clones. B) Dendrogram visualizing the gene-by-gene differences in FPKM between each clone from population 125.

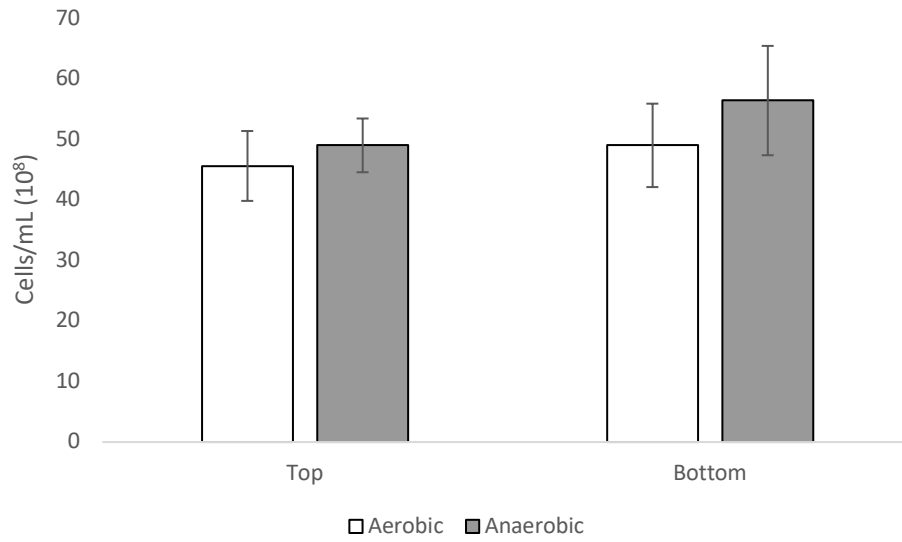


Figure S5: Average cell counts of liquid culture aspirated from the top and bottom halves of the culture tube. Aerobic cells were incubated on LB plates at 37°C with no atmospheric adjustments while anaerobic cells were incubated on LB plated at 37°C in an anaerobic jar treated with a gas generating sachet resulting in an environment containing >1% O₂.

Table S1.

Genes accumulating SNPs or indels in 5 or more populations.

| Gene | # of populations affected | Protein |
|--------------|---------------------------|---|
| <i>adhE</i> | 10 | fused acetaldehyde-CoA dehydrogenase |
| <i>acrB</i> | 9 | multidrug efflux system protein |
| <i>prfB*</i> | 9 | peptide chain release factor RF-2 |
| <i>treB</i> | 9 | fused trehalose(maltose)-specific PTS enzyme |
| <i>cytR</i> | 8 | Anti-activator for CytR-CRP nucleoside utilization regulon |
| <i>dcuS</i> | 8 | sensory histidine kinase |
| <i>ompF</i> | 8 | outer membrane porin 1a |
| <i>rpsG*</i> | 8 | 30S ribosomal subunit protein S7 |
| <i>fimH</i> | 7 | minor component of type 1 fimbriae |
| <i>hsdR</i> | 7 | endonuclease R Type I restriction enzyme |
| <i>aceB</i> | 6 | malate synthase A |
| <i>clpA</i> | 6 | ATP-dependent protease specificity component and chaperone |
| <i>clpB</i> | 6 | protein disaggregation chaperone |
| <i>eptB</i> | 6 | KDO phosphoethanolamine transferase |
| <i>fdnG</i> | 6 | formate dehydrogenase-N, alpha subunit, nitrate-inducible |
| <i>gsiA</i> | 6 | glutathione transporter ATP-binding protein |
| <i>narY</i> | 6 | nitrate reductase 2 (NRZ), beta subunit |
| <i>nlpD</i> | 6 | activator of AmiC murein hydrolase activity |
| <i>potE</i> | 6 | putrescine/ornithine antiporter |
| <i>trkH</i> | 6 | potassium transporter |
| <i>xdhA</i> | 6 | xanthine dehydrogenase |
| <i>yejM</i> | 6 | lipid A production and membrane permeability factor |
| <i>yfeA</i> | 6 | putative diguanylate cyclase |
| <i>yggN</i> | 6 | DUF2884 family predicted periplasmic protein |
| <i>aas</i> | 5 | fused 2-acylglycerophospho-ethanolamine acyl transferase |
| <i>amn</i> | 5 | AMP nucleosidase |
| <i>betT</i> | 5 | choline transporter of high affinity |
| <i>cusA</i> | 5 | copper/silver efflux system |
| <i>dcuD</i> | 5 | putative transporter |
| <i>ecpC</i> | 5 | ECP production outer membrane protein |
| <i>elfC</i> | 5 | putative outer membrane fimbrial subunit export usher protein |
| <i>fadE</i> | 5 | acyl coenzyme A dehydrogenase |
| <i>feoB</i> | 5 | fused ferrous iron transporter |

| | | |
|-------------|---|---|
| <i>fhIA</i> | 5 | formate hydrogenlyase transcriptional activator |
| <i>fhuE</i> | 5 | ferric-rhodotorulic acid outer membrane transporter |
| <i>fiu</i> | 5 | catecholate siderophore receptor Fiu |
| <i>flu</i> | 5 | antigen 43 phase-variable biofilm formation autotransporter |
| <i>gcd</i> | 5 | glucose dehydrogenase |
| <i>glmU</i> | 5 | fused N-acetyl glucosamine-1-phosphate uridyltransferase |
| <i>hyfB</i> | 5 | hydrogenase 4, membrane subunit |
| <i>ileS</i> | 5 | isoleucyl-tRNA synthetase |
| <i>kdpD</i> | 5 | sensory histidine kinase |
| <i>lacI</i> | 5 | transcriptional repressor of the lac operon |
| <i>mreB</i> | 5 | dynamic cytoskeletal protein MreB |
| <i>mscK</i> | 5 | mechanosensitive channel protein |
| <i>mscM</i> | 5 | mechanosensitive channel protein |
| <i>narG</i> | 5 | nitrate reductase 1, alpha subunit |
| <i>paaH</i> | 5 | 3-hydroxyadipyl-CoA dehydrogenase |
| <i>proA</i> | 5 | gamma-glutamylphosphate reductase |
| <i>rhcC</i> | 5 | Rhc family putative polymorphic toxin |
| <i>rseB</i> | 5 | anti-sigma E factor |
| <i>eoti</i> | 5 | SoxR iron-sulfur cluster reduction factor component |
| <i>setB</i> | 5 | lactose/glucose efflux system |
| <i>speG</i> | 5 | spermidine N1-acetyltransferase |
| <i>sstT</i> | 5 | serine / threonine:Na ⁺ symporter |
| <i>tamB</i> | 5 | translocation and assembly module for autotransporter export |
| <i>tolB</i> | 5 | periplasmic protein |
| <i>trkA</i> | 5 | NAD-binding component of Trk potassium transporter |
| <i>trpB</i> | 5 | tryptophan synthase, beta subunit |
| <i>xylB</i> | 5 | xylulokinase |
| <i>yahJ</i> | 5 | putative metallo-dependent hydrolase domain deaminase |
| <i>ydhS</i> | 5 | putative oxidoreductase |
| <i>ydiK</i> | 5 | inner membrane protein |
| <i>yegE</i> | 5 | putative diguanylate cyclase |
| <i>yghJ</i> | 5 | DUF4092 family putative lipoprotein peptidase |
| <i>yhcG</i> | 5 | DUF1016 family protein in the PD-(D/E)XK nuclease superfamily |
| <i>yjgR</i> | 5 | DUF853 family protein with NTPase fold |

**mutation resulted in reversion*

Table S2.

Sampling point at which select parallel mutations were first detected within each population.

| Population | <i>adhE</i> | <i>acrB</i> | <i>prfB</i> | <i>treB</i> | <i>cytR</i> | <i>dcuS</i> | <i>ompF</i> | <i>rpsG</i> | <i>fimH</i> | <i>trkH</i> | <i>fimE/fimS</i> | <i>hns-tdk</i> |
|------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|------------------|----------------|
| 113 | 5 | n/a | 2 | n/a | 3 | 6 | 1 | 1 | 5 | 1 | 1 | n/a |
| 125 | 6 | 3 | 5 | 1 | 6 | n/a | 2 | n/a | n/a | n/a | 1 | 1 |
| 126 | 2 | 1 | 6 | 4 | 4 | 1 | 1 | 2 | 4 | 1 | 1 | n/a |
| 129 | 3 | 2 | n/a | 1 | 6 | 2 | 3 | 6 | n/a | n/a | 1 | 1 |
| 210 | 2 | 2 | 1 | 1 | 3 | 3 | 2 | 3 | 4 | 1 | 1 | 1 |
| 221 | 6 | 6 | 2 | 3 | n/a | n/a | n/a | 4 | n/a | 2 | n/a | 1 |
| 233 | 5 | 1 | 5 | 2 | 1 | 5 | 2 | 3 | 3 | 1 | 1 | 1 |
| 326 | 3 | 1 | 1 | 2 | n/a | 2 | n/a | 5 | 6 | n/a | n/a | 2 |
| 410 | 6 | 1 | 1 | 5 | 5 | 2 | 5 | 2 | 6 | 1 | 1 | 4 |
| 417 | 2 | 2 | 1 | 2 | 2 | 5 | 4 | n/a | 6 | n/a | 1 | n/a |
| Median | 4 | 2 | 2 | 2 | 3.5 | 2.5 | 2 | 3 | 5 | 1 | 1 | 1 |

Table S3.

Summary of structural variants shared by 2 or more populations.

| Locus | Structural Variant | # of Populations Affected |
|-------------------|--------------------|---------------------------|
| <i>gat</i> operon | Deletion | 6 |
| <i>hns-tdk</i> | Transposition | 6 |
| CPS-53 prophage | Deletion | 3 |
| DLP-12 prophage | Deletion | 3 |
| <i>fimE</i> | Transposition | 3 |
| <i>fimE-fimA</i> | Transposition | 3 |
| <i>lptD-djlA</i> | Transposition | 3 |
| <i>metA-aceB</i> | Transposition | 3 |
| <i>nlpD</i> | Transposition | 3 |
| <i>tnaA</i> | Transposition | 3 |
| <i>tnaC-tnaA</i> | Transposition | 3 |
| <i>cra-mraZ</i> | Transposition | 2 |
| <i>cyoA-ampG</i> | Transposition | 2 |
| <i>cytR</i> | Transposition | 2 |
| e-14 prophage | Deletion | 2 |
| <i>glf</i> | Transposition | 2 |
| <i>nlpD</i> | Deletion | 2 |
| <i>tdcA</i> | Transposition | 2 |
| <i>tnaA-tnaB</i> | Transposition | 2 |
| <i>tnaB</i> | Transposition | 2 |
| <i>tomB-acrB</i> | Transposition | 2 |
| <i>yadC</i> | Transposition | 2 |
| <i>ydfJ</i> | Transposition | 2 |
| <i>ynfB</i> | Transposition | 2 |

Table S4

| Gene | Description | Experiment observed | G-Score Rank (mutator) | G-Score Rank (non-mutator) |
|------|--|-------------------------|------------------------|----------------------------|
| cusA | copper/silver efflux system, membrane component | Kinnersley et al., 2014 | 64 | N/A |
| cytR | DNA-binding transcriptional dual regulator | Kinnersley et al., 2014 | 7 | 24 |
| dcuS | sensory histidine kinase in two-component regulatory system with DcuR, regulator of anaerobic fumarate respiration | Kinnersley et al., 2014 | 12 | 6 |
| fdnG | formate dehydrogenase-N, alpha subunit, nitrate-inducible | Kinnersley et al., 2014 | 1660 | N/A |
| fhIA | DNA-binding transcriptional activator | Kinnersley et al., 2014 | 49 | N/A |
| fimH | minor component of type 1 fimbriae | Kinnersley et al., 2014 | 5 | 17 |
| fiu | catecholate siderophore receptor Fiu | Kinnersley et al., 2014 | 788 | N/A |
| rpsG | 30S ribosomal subunit protein S7 | Kinnersley et al., 2014 | 8 | 1 |
| setB | lactose/glucose efflux system | Kinnersley et al., 2014 | 80 | 27 |
| sslE | Putative secreted and surface-associated lipoprotein mucinase | Barrick et al., 2009 | 196 | N/A |
| xylB | xylulokinase | Kinnersley et al., 2014 | 691 | N/A |
| yegE | predicted diguanylate cyclase, GGDEF domain signaling protein | Kinnersley et al., 2014 | 2576 | N/A |

* N/A denotes gene was not mutated in this genetic background

Table S5: Table showing SIM group, SIM ion, dwell time, and retention time information for all amino acids monitored in this study.

| Amino Acid | SIM Group | Ion m/z | Dwell Time (msec) | Ret. Time (min) |
|--------------|-----------|---------|-------------------|-----------------|
| Ala | 1 | 260 | 30 | 6.707 |
| Gly | 1 | 246 | 30 | 6.936 |
| 2-AIB | 1 | 172 | 30 | 7.323 |
| Val | 1 | 186.1 | 30 | 7.99 |
| NV | 1 | 186.1 | 30 | 8.123 |
| Leu | 1 | 200.1 | 30 | 8.436 |
| Ile | 1 | 200.1 | 30 | 8.775 |
| Pro | 1 | 184.1 | 30 | 9.166 |
| Met | 2 | 292.1 | 30 | 11.183 |
| Ser | 2 | 288.1 | 30 | 11.41 |
| Thr | 2 | 303.1 | 50 | 11.716 |
| Phe | 2 | 302.1 | 50 | 12.35 |
| Asp | 2 | 418.1 | 30 | 12.91 |
| Cys* | 2 | 302.1 | 50 | 13.392 |
| Glu | 3 | 330.1 | 30 | 13.956 |
| Arg (as Orn) | 3 | 286.1 | 50 | 14.01 |
| Lys | 3 | 300.1 | 30 | 14.864 |
| His | 3 | 196.1 | 30 | 16.533 |
| Tyr | 3 | 302.0 | 30 | 16.903 |
| Cys-Cys* | 3 | 348.0 | 50 | 20.64 |

*Cysteine and Cystine (Cys-Cys) were both monitored but not quantified as the levels varied greatly due to random disulfide bond formation/reduction.

Dataset S1 (separate file)

Small mutations identified in all sequenced clones.

Dataset S2 (separate file)

Structural variants identified in all sequenced clones.

Dataset S3 (separate file)

G scores for individual genes in wildtype and $\Delta mutL$ populations.

Dataset S4 (separate file)

Ratios of amino acid concentrations for five sequenced clones vs. initial amino acid concentration over a period of six hours.

Dataset 5 (separate file)

Gene Expression Values for genes that were either significantly upregulated or downregulated at mid-log phase.