Supporting Information

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SI Materials and Methods

Strains, Media, and Growth Conditions. *Escherichia coli* strain DH5α-E (Invitrogen) was used for all experiments. Strains were propagated in LB medium supplemented with chloramphenicol (34 $\mu g \cdot mL^{-1}$) at 37 °C, unless noted otherwise.

Library-on-Library Screen. The 5,272 plasmids from the ASKA (A Complete Set of *Escherichia coli* K-12 ORF Archive) library had been pooled previously (1) and were used to transform *E. coli* DH5α-E. The same strain was transformed with the empty vector, pCA24N-NoIns, to yield a negative control. The ASKA pool and the negative control were grown to midlog phase (OD_{600} ~0.5), before protein overexpression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration, 50 μM). After induction for 2 h, cells were harvested by centrifugation. The cell pellet was resuspended to OD_{600} ~0.2 in sterile water, and 100 μL of this cell suspension was added to IF-10 medium (Biolog Inc.) supplemented with 50 μM IPTG, to a density of $\sim 5 \times 10^5$ cells$\cdot mL^{-1}$. The cell suspension was starved by incubating at room temperature for 1 h. Aliquots of each culture (100 μL) were used to inoculate the wells of phenotype microarray (PM) plates PM11 to PM20 (Biolog Inc.). The PM plates were incubated at 37 °C for 7 d. Growth in each well, as indicated by purple color development, was monitored visually and scored daily. Two independent replicates of the complete screen were performed.

Serial Enrichment to Identify Resistance Genes. For conditions in which the ASKA pool had reproducibly out-grown the negative control, fresh aliquots of the pool were now used to inoculate the corresponding wells of new PM plates. The plates were incubated at 37 °C. At the first sign of purple color development in a well (usually 24–72 h), a 2-μL aliquot of the culture from that well was mixed with 98 μL IF-10 medium (supplemented with 50 μM IPTG) and transferred to the corresponding well of a new plate. After a second transfer (carried out in an identical fashion), cells from the final passage were spread on LB-chloramphenicol plates. The universal pCA24N-specific primers pCA24N.for and pCA24N.rev (1) were used to amplify the ASKA ORFs from at least eight of the resulting colonies. PCR products were sequenced to reveal each ORF that was responsible for enhanced growth.

Relative Fitness Assays. First, the negative control strain (*E. coli* DH5α-E harboring pCA24N-NoIns) was marked with the *lacZ* gene (and a gentamicin resistance cassette), which was achieved using a mini-Tn7 system that integrates the marker downstream of the chromosomal *glmS* locus (2). The presence of a functional copy of *lacZ* allowed blue/white colony screening, where blue colonies were the marked negative control and white colonies were the competitor. The relative fitness (W) of the *lacZ*-marked negative control was shown to be identical to the unmarked negative control strain ($W = 1.00 \pm 0.01$, mean ± SE; $n = 8$ replicates; $P = 0.93$).

Twenty-five ASKA plasmids that conferred increased growth in the presence of antibiotics were purified from individual clones and used to transform fresh aliquots of *E. coli* DH5α-E. We measured the fitness of each of these strains, in the absence of antibiotics and in competition with the *lacZ*-marked negative control (which carried pCA24N-NoIns). In each experiment, single colonies of the ASKA strain and of the negative control were used to inoculate separate 5 mL aliquots of the competition medium (2.0 $g \cdot L^{-1}$ tryptone, 1.0 $g \cdot L^{-1}$ yeast extract, 1.0 $g \cdot L^{-1}$ NaCl, 34 $\mu g \cdot mL^{-1}$ chloramphenicol, and 50 μM IPTG). This medium was used because it accurately represents growth conditions in the PM plates (3). The cultures were grown at 37 °C for 24 h, so that the two competing strains were preconditioned to the medium (4). Equal amounts of the two competing strains (~10^6 cfu per strain) were then mixed together in a fresh aliquot of the competition medium (5 mL), and the strains were grown in competition for another 24 h. The initial ($t = 0$ h) and final ($t = 24$ h) densities of each competitor were measured by spreading diluted aliquots of the culture on LB agar plates supplemented with chloramphenicol (34 $\mu g \cdot mL^{-1}$) and X-gal (40 $\mu g \cdot mL^{-1}$). The relative fitness, W, of the ASKA strain was estimated as the ratio of its number of doublings in 24 h, divided by the number of doublings of the *lacZ*-marked negative control (4). Eight replicates were carried out to estimate the mean relative fitness of each ASKA strain.

Antibiotic Susceptibility Testing. Next, the retransformed ASKA strains and the unmarked negative control strain from the fitness assays were grown to midlog phase before IPTG (final concentration, 50 μM) was added to induce protein overexpression for 2 h. Minimum inhibitory concentrations for aztreonam, cefuroxime, cephalothin, ciprofloxacin, erythromycin, nalidixic acid, nitrofurantoin, penicillin G, rifampicin, tobramycin and trimethoprim were determined using E-test strips (AB bioMérieux). A lawn of ~10^6 cells was spread on Mueller-Hinton agar plates supplemented with IPTG (50 μM). The relevant E-test strip was placed on top of the lawn and the agar plates were incubated at 37 °C for 24 h. MICs for benzethonium chloride, enoxacin, lincomycin, oxytetracycline, puromycin, sisomicin, spiramycin, sulfadiazine, and vancomycin were determined in Mueller-Hinton broth, using a microdilution method (5). Reported minimum inhibitory concentrations values are the average of at least two independent experiments.

1. Patrick WM, Quandt EM, Swartzlander DB, Matsumura I (2007) Multicopy suppression underpins metabolic evolvability. *Mol Biol Evol* 24:2716–2722.
2. Choi KH, et al. (2005) A Tn7-based broad-range bacterial cloning and expression system. *Nat Methods* 2:443–448.
3. Zhou L, Lei XH, Bochner BR, Wanner BL (2003) Phenotype microarray analysis of *Escherichia coli* K-12 mutants with deletions of all two-component systems. *J Bacteriol* 185:4956–4972.
4. Lenski RE, Rose MR, Simpson SC, Tadler SC (1991) Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2000 generations. *Am Nat* 138: 1315–1341.
5. Wiegand I, Hilpert K, Hancock REW (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat Protoc* 3:163–175.

Table S1. Genes that confer increased resistance to more than one toxic compound

Gene	Gene product	Frequency*	Toxic compounds	Mechanism of resistance
galE	UDP-glucose 4-epimerase	7	Amitriptyline, chlorpromazine, nalidixic acid, orphenadrine, pridinol, thioridazine, trifluoperazine	Envelope/capsule biosynthesis
bcr	Bcr multidrug transporter	6	Cefoperazone, oxytetracycline, rolitetracycline, sulfachloropyridazine, sulfathiazole, sulfisoxazole	Efflux/transport
ycgZ	Small protein induced by YcgF	6	Aztreonam, cefmetazole, cefuroxime, moxalactam, penimepicycline, sulfamethazine	Stress response
ydaC	Uncharacterized protein from Rac prophage	6	Caffeine, chelerythrine, erythromycin, phenethicillin, plumbagin, sanguinarine	Prophage gene
marA	MarA transcriptional activator	5	Ciprofloxacin, ofloxacin, penimepicycline, proflavine, tylosin	Regulatory effect
mdtM	MdtM multidrug transporter	5	5-Nitro-2-furaldehyde, crystal violet, furaltadone, puromycin, tinidazole	Efflux/transport
cpdA	cAMP phosphodiesterase	4	Dihydrostreptomycin, hydroxyurea, josamycin, L-aspartic-β-hydroxamate	Regulatory effect
csiE	Stationary phase-inducible protein	4	3,4-Dimethoxybenzyl alcohol, antimony (III) chloride, blasticidin S, guanidine hydrochloride,	Stress response
rbsR	Ribose transcriptional repressor	4	Aztreonam, lincomycin, sulfamethazine, sulfamonomethoxine	Regulatory effect
ycbS	Uncharacterized outer membrane β-barrel protein	4	9-Aminoacridine, FCCP, methyl viologen, sanguinarine	Efflux/transport
ydfW	Uncharacterized protein from Qin prophage	4	Chelerythrine, erythromycin, oleandomycin, spiramycin,	Prophage gene
bdm	Biofilm-dependent modulation protein	3	Cefazolin, cephalothin, nafcillin	Biofilm formation & regulation
cmr	MdfA/Cmr multidrug transporter	3	Dodecyltrimethyl ammonium bromide, lincomycin, sodium dichromate	Efflux/transport
yejG	Uncharacterized protein	3	Apramycin, sisomicin, tobramycin	Unknown
folA	Dihydrofolate reductase	2	2,4-Diamino-6,7-diisopropyl-pteridine, trimethoprim	Catalytic promiscuity and/or substrate ambiguity; over-expression of drug target
hcaR	Hca operon transcriptional activator	2	Fusidic acid, sodium *m*-arsenite	Regulatory effect
tusE	Sulfurtransferase	2	Rifampicin, spiramycin	Catalytic promiscuity and/or substrate ambiguity
yfdO	Uncharacterized protein from CPS-53 [KpLE1] prophage	2	Lidocane, nalidixic acid	Prophage gene

*The number of PM wells from which the ASKA-encoded gene was isolated.

Other Supporting Information Files

Dataset S1 (XLS)
Dataset S2 (XLSX)