Supporting Information

Desai and Miller 10.1073/pnas.1007559107

SI Materials and Methods

Expression and Purification of MurA. The *murA* gene was PCR amplified from *E. coli* strain MG1655 and cloned between the *NdeI* and *XhoI* sites of pET-22b so that a hexa-histidine tag was added to the C terminus. This construct was transformed into BL21 (DE3) and protein was produced by inoculating 0.5 L of LB with ampicillin (100 μg/mL) and growing the culture to an OD_{600} of 0.8 at 37 °C. IPTG was then added to 0.5 mM, growth was continued for 2 h and cells were pelleted by centrifugation. The cell pellet was resuspended in buffer A [50 mM Tris-HCl pH 8.0, 300 mM NaCl, 4 mM dithiothreitol (DTT) and 40 mM imidazole] and extracts were prepared by passage through a French Press followed by centrifugation. The supernatant was loaded onto a nickel-nitrilotriacetic acid column that had been equilibrated with buffer A. The column was washed with 10 column volumes of buffer A, followed by 10 column volumes of buffer A containing 65 mM imidazole. The enzyme was eluted in buffer A containing 250 mM imidazole. Covalently bound PEP was removed by incubation of MurA with 1 mM UDP-GlcNAc for 10 min at 25 °C. Purified enzyme was dialyzed against 2×1 L changes of dialysis buffer (25 mM triethanolamine-HCl pH 8.0, 1 mM TCEP and 1 mM EDTA). The concentration of MurA was determined from absorbance readings at 280 nm using a calculated extinction coefficient of 18,825 $M^{-1}\,cm^{-1}$ (ExPASy).

Expression and Purification of MurB. The plasmid encoding MurB was isolated from the ASKA collection and transformed into MC4100. MurB was produced by inoculating 0.5 L of LB containing chloramphenicol and induced as described for MurA. MurB was also purified as described for MurA except that buffer A consisted of 50 mM Tris-HCl pH 8.0, 0.5 mM DTT and 40 mM imidazole. Purified MurB was dialyzed overnight against 2 L of 50 mM Tris-HCl pH 8.0 with 0.5 mM DTT. The concentration of MurB was calculated with an extinction coefficient of 11,700 $M^{-1}\,cm^{-1}$ using absorbance readings at 463 nm from bound FAD (1).

Detection of Alkylated MurA. MurA (20 μM) was incubated in 200 μL of dialysis buffer containing bromoacetate (400 μM) at 25 °C for 1 h. An identical control incubation mixture was prepared but not exposed to bromoacetate. Incubation mixtures were desalted by applying 50 μL to a Sephadex G-50 spin column. Desalted MurA was prepared for tryptic digestion by bringing the mixture to 25 mM ammonium bicarbonate, 1 M urea and 1 mM DTT. Trypsin (500 ng) was added and the mixture was incubated overnight at 37 °C. Digestions were desalted with C_{18} Zip-Tips and eluted with 50% acetonitrile/0.1% trifluoroacetic acid containing α-cyano-4-hydroxycinnamic acid (10 mg/mL). Mass spectra were acquired on a Shimadzu Axima CFR plus in positive reflectron mode.

Inactivation Assays. Assays were performed in 1 mL mixtures of 100 mM triethanolamine-HCl pH 8.0 containing various amounts of bromoacetate and MurA (5 μM) at 25 °C. Control experiments revealed that, in the absence of bromoacetate, the activity of MurA remained constant in the assay mixture over the time frame of the experiment. Studies that tested the effect of UDP-GlcNAc on the rate of inactivation included this molecule at 1 mM. At various time intervals, 100 μL aliquots were assayed for residual activity in a continuous assay using MurB as the coupling enzyme, essentially as previously described (2). The assay mixture contained 50 mM Tris-HCl pH 8.0, 20 mM KCl, 200 μM NADPH, 1 mM PEP, 1 mM UDP-GlcNAc, 1 mM DTT, 20 mM glucose, 20 units of glucose oxidase, and 1 μM MurB. Percent residual activity, $f(t)$, was plotted as a function of incubation time (t) and data were fit to the single-exponential equation, $f(t) = 100 \times e^{-k_{obs}t}$, where k_{obs} is the observed first-order rate constant of inactivation at a given bromoacetate concentration. The second-order inactivation rate (k_{inact}) was obtained by plotting k_{obs} values vs. bromoacetate concentrations (x) and fitting data to $f(x) = k_{inact}x$, where $f(x)$ is k_{obs}.

Uncatalyzed Rate Assays. Assays were performed in 10 mL mixtures at 25 °C containing 100 mM HEPES pH 7.0, 0.5 mM L-glutathione and either 29 mM or 58 mM bromoacetate. At various time points 1 mL was removed and quenched by the addition of *N*-ethylmaleimide to 20 mM. An assay was also prepared that contained bromoacetate but not glutathione, serving as the blank to correct for the hydrolysis of bromoacetate over the time course of the experiment. After reactions had been quenched at all of the required time points, the bromide concentration was determined, as described above, and is equal to the concentration of glutathione that reacted with bromoacetate. Percent glutathione remaining, $f(t)$, was plotted as a function of incubation time (t) and data were fit to the single-exponential equation, $f(t) = 100 \times e^{-k_{obs}t}$, where k_{obs} is the observed first-order rate constant of the uncatalyzed conjugation of glutathione to bromoacetate at a given bromoacetate concentration. The second-order uncatalyzed rate constant (k_{uncat}) was obtained by plotting the observed first-order rate constants (k_{obs}) vs. the bromoacetate concentration (x) and fitting data to $f(x) = k_{uncat}x$, where $f(x)$ is k_{obs}. The kinetic values reported are the mean values from two separate experiments with the errors representing the deviation from the mean.

1. Benson TE, Marquardt JL, Marquardt AC, Etzkorn FA, Walsh CT (1993) Overexpression, purification, and mechanistic study of UDP-N-acetylenolpyruvylglucosamine reductase. *Biochemistry* 32:2024–2030.

2. Schönbrunn E, Eschenburg S, Krekel, F, Luger, K, Amrhein N (2000) Role of the loop containing residue 115 in the induced-fit mechanism of the bacterial cell wall biosynthetic enzyme MurA. *Biochemistry* 39:2164–2173.

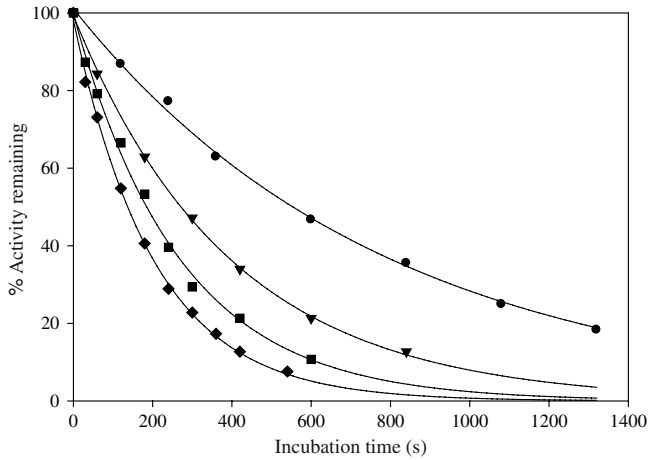

Fig. S1. Time-dependent loss of MurA activity in the absence of UDP-GlcNAc. Bromoacetate concentrations were 0.4 mM (•), 0.8 mM (▼), 1.2 mM (■), and 1.6 mM (◆). Data were fit to $f(t) = 100 \times e^{-kobst}$, where t is time and k_{obs} is the observed first-order rate constant of inactivation at a given bromoacetate concentration.

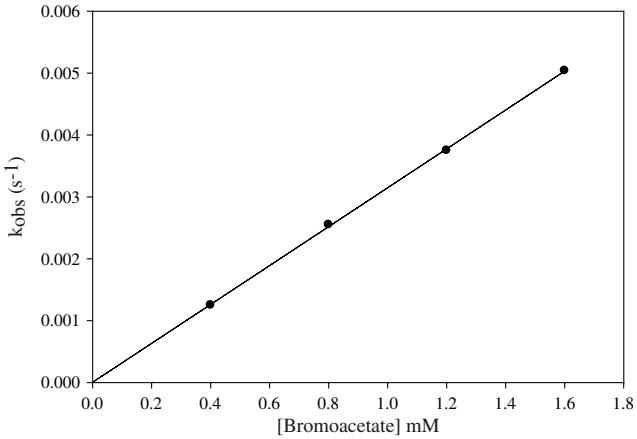

Fig. S2. Replot of the k_{obs} values vs. bromoacetate concentration. Data were fit to $f(x) = k_{inact}x$, where x is the bromoacetate concentration and k_{inact} is the second-order rate constant of inactivation.

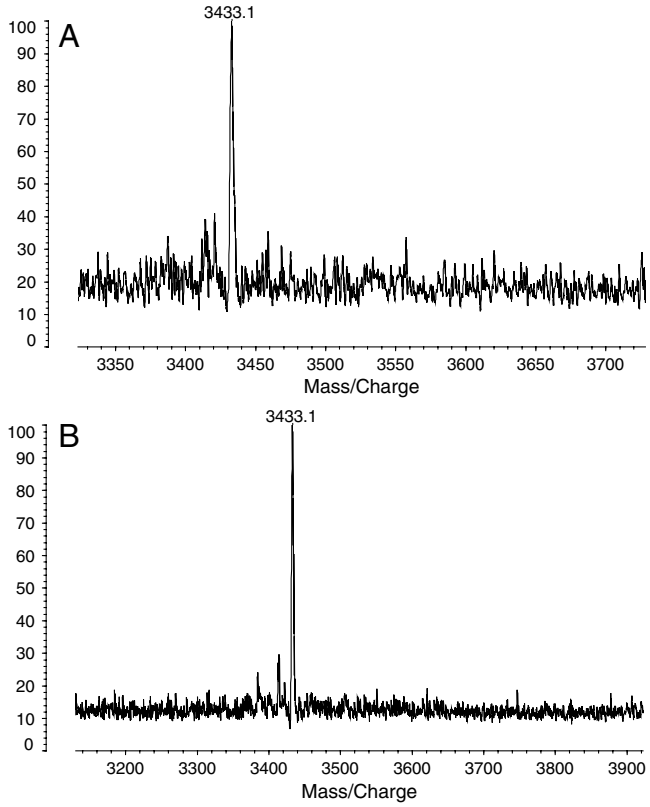

Fig. S3. MALDI mass spectrometry of MurA C115D tryptic digest. (*A*) The mass peak from the peptide containing D115 (expected mass 3433.9 Da). (*B*) The mass peak from the peptide containing D115 after the protein was incubated with bromoacetate.

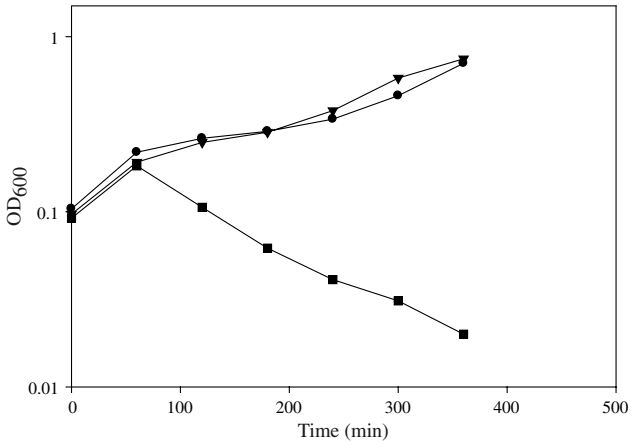

Fig. S4. Growth curves of *E. coli* strain MC4100 overproducing wild-type (•), C115D (▼) or C115A (■) MurA. Overnight cultures were diluted to an OD_{600} of 0.1 in LB media supplemented with bromoacetate (0.5 mM), IPTG (0.1 mM) and chloramphenicol (30 μg/mL). The growth of duplicate cultures was determined by monitoring the OD_{600} every 60 min.

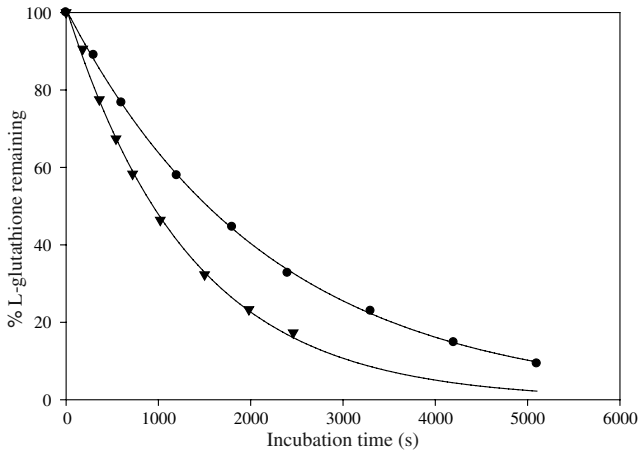

Fig. S5. Time-dependent uncatalyzed conjugation of glutathione to bromoacetate. The L-glutathione concentration was 0.5 mM and the bromoacetate concentrations were 29 mM (•) and 58 mM (▼). Data were fit to $f(t) = 100 \times e^{-kobst}$, where t is time and k_{obs} is the observed first-order rate constant of the uncatalyzed conjugation of glutathione to bromoacetate at a given bromoacetate concentration.

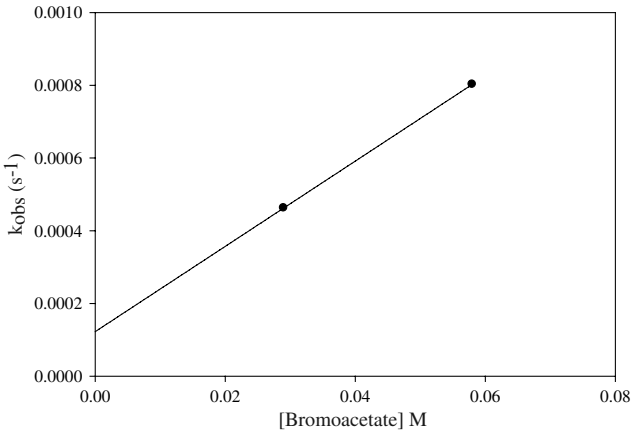

Fig. S6. Replot of the observed first-order uncatalyzed rate constants (k_{obs}) vs. the bromoacetate concentration. Data were fit to $f(x) = k_{uncat}x$, where x is the bromoacetate concentration and k_{uncat} is the second-order uncatalyzed rate constant.

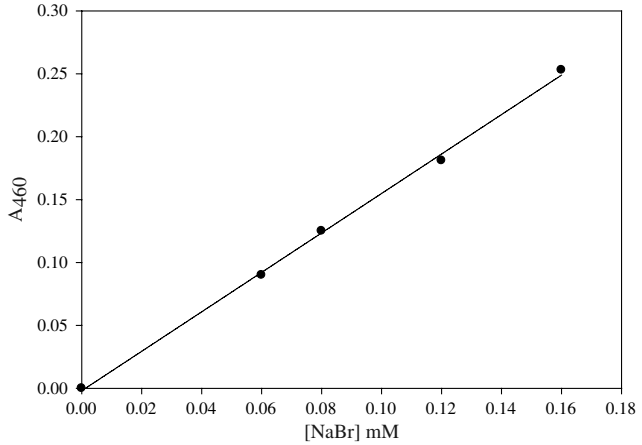

Fig. S7. Bromide assay calibration curve with NaBr.

Table S1. Keio mutants hypersensitive to bromoacetate that are also sensitive to at least 1 of 22 antibiotics tested (1, 2)

Gene	Class	Gene product description
gshA	1	glutamate-cysteine ligase
aceF	2	pyruvate dehydrogenase/lipoate acetyltransferase
ackA	2	acetate kinase
nuoM	2	subunit of NADH:ubiquinone oxidoreductase
rpiA	2	ribose-5-phosphate isomerase A
sucD	2	subunit of succinyl-CoA synthetase
ubiG	2	ubiquinone biosynthetic enzyme
bamB	3	subunit of outer membrane protein biogenesis complex
cpxA	3	sensor kinase-phosphotransferase
mrcB	3	penicillin-binding protein 1B
pal	3	peptidoglycan-associated lipoprotein
recA	4	DNA recombinase
cysB	5	transcriptional dual regulator
fis	5	DNA binding transcriptional dual regulator
rfaH	5	transcriptional antiterminator
hfq	6	RNA-binding postranscriptional regulator
nfuA	6	iron-sulfur cluster scaffold protein
ybdN	7	conserved protein

1 Tamae C et al. (2008) Determination of antibiotic hypersensitivity among 4,000 single-gene-knockout mutants of *Escherichia coli*. *J Bacteriol* 190:5981–5988.

2 Liu A et al. (2010) Antibiotic sensitivity profiles determined with an *Escherichia coli* gene knockout collection: Generating an antibiotic bar code. *Antimicrob Agents Ch* 54:1393–1403.