Supplementary Materials for

Cell death from antibiotics without the involvement of reactive oxygen species

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Materials and Methods

Reagents.

β-mercaptoethanol, bovine liver catalase, diethylenetriaminepentaacetic acid (DTPA), deferoxamine mesylate (DFO), dipyridyl, ferric chloride, type II horseradish peroxidase, 30% hydrogen peroxide, *o*-nitrophenyl β-D-galactopyranoside (ONPG), $K_3Fe(CN)_3$, thiourea, dithionite, 6-phosphogluconate, lactate dehydrogenase, NADH, $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$, dithiothreitol (DTT), cysteine, potassium D-gluconate, ampicillin, kanamycin, and norfloxacin were purchased from Sigma. KNO_3 and D-glucose were obtained from Fisher ChemAlert® Guide. Albumin standard and Coomassie protein assay reagent were purchased from Thermo Scientific. 3′-(*p*-hydroxyphenyl) fluorescein (HPF) and Amplex Red were purchased from Invitrogen. Standard reagents for RNA work were obtained from Ambion. iQ™ SYBR® Green Supermix was from BioRad, and real-time PCR plates were purchased from Eppendorf.

Strain construction.

The strains used in this study are listed in Table S1; they are all congenic derivatives of MG1655. Deletions were constructed by the Red recombination deletion method (*30*), were transferred to other strains by P1 transduction, and were confirmed by PCR and/or phenotype analyses (*31*). The antibiotic-resistance cassettes were subsequently removed by FLP-mediated excision (*30*). Gene fusions between the *katG* upstream region (-160 bp to +16 bp) and *lacZ* were created by standard methods and integrated into the lambda phage attachment site (*32*), preserving the wild-type *katG* gene at its native chromosomal locus.

Bacterial Growth

All cultures were grown in LB (10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter). Experimental protocols were designed to ensure that measurements were performed upon exponentially growing cells. Overnight cultures were diluted into fresh media and grown for 5 generations to early log phase (OD_{600nm} of 0.10 to 0.20) before treatment with antibiotics. All cultures were grown at 37 °C in the dark. Aerobic LB medium was made one day previously and stored in the dark to avoid the photochemical generation of H_2O_2. Anaerobic medium was transferred immediately after autoclaving to an anaerobic chamber (Coy Laboratory Products), where it was stored under an atmosphere of 5% CO_2/10% H_2/85% N_2 for at least one day prior to use. Where indicated, 40 mM KNO_3, 0.2% gluconate, or 0.2% glucose was included in the

medium. When used, dipyridyl was made fresh and added into the media at a final concentration of 500 μM before inoculation.

Most experiments included in this report employed fixed concentrations of antibiotics (5 μg/ml ampicillin, 250 ng/ml norfloxacin, and 5 μg/ml kanamycin) designed to match doses used by other workers (*4*). However, a higher dose of kanamycin (30 μg/ml) was used in presenting the effects of nitrate upon killing so that the impact of nitrate was clear. In the Edd assay, a higher dose of kanamycin (20 μg/ml) was used because supplementation with 0.2% gluconate decreased sensitivity of the cells to kanamycin. The *Δfur* mutant was also more resistant to kanamycin, therefore 10 μg/ml kanamycin was used to show the killing.

Cell viability

To determine the cell viability, cultures were grown to 0.1-0.2 OD_{600nm} (aerobic cultures) or 0.05 OD_{600nm} (anaerobic cultures) prior to the addition of antibiotics. The lower density of anaerobic cultures ensured that they did not approach stationary phase during antibiotic treatment. Cell samples were collected at designated time points and washed twice with PBS (50 mM potassium phosphate with 0.9% NaCl, pH 7.2) and then serially diluted, mixed with LB top agar, and spread onto the surface of LB plates. Colonies were allowed to grow aerobically for 24 h, and plates that had 30~300 colonies were selected for counting. At least three replicates of the survival-curve experiments were performed. Day-to-day variations in medium composition shifted the curves slightly, but the sample-to-sample comparisons cited in the text were invariable. Therefore, representative survival curves are presented.

O_2 consumption

Cells were cultured in LB and treated with antibiotics as described above. At designed time points samples were collected and diluted in air-saturated LB to 0.05~0.15 OD_{600nm}, and respiration was measured with a Digital Model 10 Clark-type oxygen electrode (Rank Brothers Ltd) at 37 °C. The machine was calibrated by air-saturated LB medium and sodium dithionite.

Real-time PCR

Cells were cultured in LB as described above. For the H_2O_2 control, aliquots of cultures were treated with 250 μM H_2O_2 at room temperature for 10 min. RNA was harvested by the hot phenol method and cleaned up using the RNeasy mini Kit (Qiagen). The DNA-free RNA samples were generated by DNase I digestion (Biolabs) and converted to cDNA by SuperScript III

Reverse Transcriptase (Invitrogen). The following primers were used to analyze expression of *ahpC*, *katG*, *yaaA*, and the housekeeping gene *gapA*: *ahpC* forward, 5′-GACCCGTAACTTCGACAACA-3′; *ahpC* reverse, 5′-ATGCCTTCAGCGGTAACTTC-3′; *katG* forward, 5′-TACTGGGTGCCAACTTCGAT-3′; *katG* reverse, 5′-GGTCGCTTTCCACTCGTAAC-3′; *yaaA* forward, 5′-GCTGTTAGACAATTCCCAGCAG-3′; *yaaA* reverse, 5′-AAGCCGGTGTAGACATCACCTT-3′; *gapA* forward, 5′-CGTTCTGGGCTACACCGAAGATGACG-3′; *gapA* reverse, 5′-AACCGGTTTCGTTGTCGTACCAGGA-3′. The 25 μl real-time PCR mixture contained 50 ng of cDNA, 12.5 μl of SYBR® Green Supermix (BioRad), and 400 nM of *ahpC, gapA* or *yaaA* primers, or 1 μM of *katG* primers. Thermal cycling was performed using a Mastercycler® ep *realplex* (Eppendorf) for 40 cycles in 3 steps: 95 °C for 20 s, 58 °C for 20 s and 72 °C for 20 s. Amplification efficiencies were determined for each primer set (0.9 to 1.1) and were used in calculating the fold change. The melting curves were checked for all samples at the end.

β-Galactosidase activity

Samples were collected at designated time points, and cells were centrifuged, washed and resuspended in ice-cold 50 mM Tris·HCl (pH 8.0), followed by lysis with a French pressure cell (SIM Aminco FA-003). Cell debris was removed by centrifugation, and β-galactosidase activity in the supernatant was measured by standard procedures (*31*). Protein concentrations were determined by the Bradford assay (Thermo Scientific) using bovine serum albumin as the standard.

H_2O_2 production measurement

The rate of H_2O_2 formation by antibiotic-treated cells was measured with Hpx^- (catalase/peroxidase-null) mutants as described (*17*). Anaerobic overnight cultures of Hpx^- cells were diluted and grown at least five generations in aerobic LB broth to 0.1~0.2 OD_{600nm}. They were then treated with antibiotics as described above. At time points the cells were centrifuged, suspended in fresh tryptone/NaCl (10 g tryptone and 10 g NaCl per liter) at an OD_{600nm} of 0.05~0.1, and incubated aerobically at 37 °C with vigorous aeration. At intervals cells were removed by centrifugation, and H_2O_2 concentrations were measured in the supernatants using the Amplex Red/horseradish peroxidase assay (*17*) in a Shimadzu RF Mini-150 fluorometer.

Electron paramagnetic resonance (EPR) measurement of intracellular unincorporated iron

The pool of intracellular chelatable iron was quantified by established EPR methods (*33*). The cells were cultured aerobically in 1 L of LB as described above. At time points during antibiotic treatment, cells were centrifuged and resuspended at 100-fold higher cell density in LB medium containing 10 mM DTPA (pH 7.0) to block further iron import, and 20 mM DFO (pH 8.0) to facilitate oxidation of intracellular unincorporated ferrous iron to EPR-detectable ferric iron. The suspension was incubated at 37 °C aerobically with shaking for 15 min. The cell pellet was washed once with 5 ml of ice-cold 20 mM Tris·HCl-10% glycerol (pH 7.4) and finally re-suspended in 200 μl of the same buffer before freezing. The EPR analysis was performed as described previously (*33*). Different concentrations of $FeCl_3$ in 20 mM Tris·HCl-10% glycerol-1 mM DFO (pH 7.4) were used to make an EPR signal standard curve. The measured EPR signals were normalized to cell density and converted to intracellular concentrations using the following conversion: 1 ml of *E. coli* culture at OD_{600nm} of 1 comprises 0.52 μl of intracellular volume (*34*).

6-phosphogluconate dehydratase (Edd) assay

Cells were cultured in LB with 0.2% gluconate as described above. Overnight cultures of SOD-deficient mutants were grown in anaerobic media to avoid the selection of suppressor mutations; log-phase cells were then diluted and grown in aerobic media for 3 hours (to 0.2 OD_{600}) prior to harvesting. Samples were collected at designated time points, washed, and resuspended in anaerobic ice-cold 50 mM Tris-Cl (pH 7.65) buffer. Cell extracts were prepared by anaerobic sonication. The activity of Edd was determined by incubating the cell lysate with 6-phosphogluconate for 2, 4, 6 and 8 min, then the amount of pyruvate produced was determined by monitoring NADH consumption catalyzed by lactate dehydrogenase (*35*). In order to repair the damaged Fe-S clusters, cell lysates were incubated with 0.5 mM $Fe(NH_4)_2(SO_4)_2$ and 5 mM DTT at room temperature anaerobically for 45 min before measuring the activity. Typically a minor fraction of the enzyme population has [3Fe-4S] clusters that can be reactivated; this becomes the great majority during oxidative stress (*36*). The addition of IscS protein and cysteine to the repair reaction (*35*) did not further increase the activity, indicating that no apoprotein was present.

H_2O_2 killing assay

At designed time points, cell samples were diluted to an OD_{600nm} of 0.05 in fresh LB and treated with 2.5 mM H_2O_2 at 37 °C. At intervals, aliquots were harvested and immediately mixed with

2500 U/ml catalase (final concentration) in order to terminate the H_2O_2 stress. Cells were serially diluted, mixed with top agar, and then spread on LB plates. CFUs were determined after aerobic incubation at 37 °C for 24 h.

HPF assay

One millimolar H_2O_2 was added into 0.1 M sodium phosphate buffer, pH 7.4, containing 100 μM $Fe(NH_4)_2(SO_4)_2$ and 10 μM 3′-(*p*-hydroxyphenyl) fluorescein (HPF) (*25*). The resultant fluorescent signal was measured in a Shimadzu RF Mini-150 fluorometer using 490/520 nm as excitation/emission wavelengths. To scavenge the oxidant generated by the Fenton reaction, thiourea or ethanol was added to the mixture before H_2O_2 was added. In other experiments, different amounts of $K_3Fe(CN)_3$ were mixed with 10 μM HPF in 0.1 M sodium phosphate buffer, and the fluorescent signal was recorded after 20 min incubation. 100 μM H_2O_2 and 0.2 μg/ml horseradish peroxidase (HRP) were mixed with 10 μM HPF, and the fluorescent signal was monitored over time.

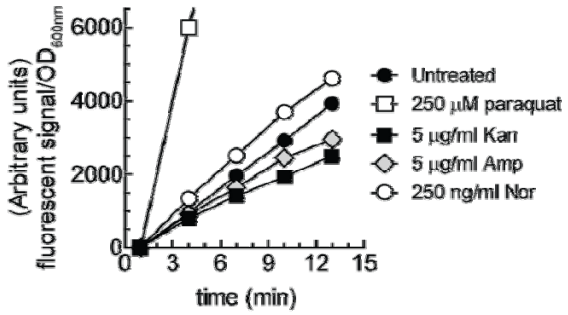

Fig. S1. Representative H_2O_2 production time course. Naïve and 1 h antibiotic-treated Hpx^- cells (AL427) were collected for H_2O_2 measurement. The redox-cycling compound paraquat was included as a positive control. The suspension of untreated Hpx^- cells at 0.05 OD_{600nm} excreted 16 nM H_2O_2/min into growth medium. The line disappearing off-scale for the paraquat curve points toward the next data point.

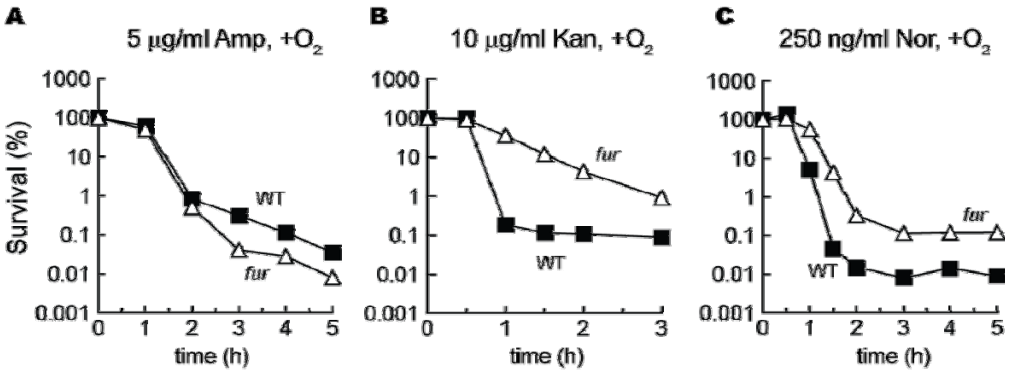

Fig. S2. *Δfur* mutants were invariably more resistant to kanamycin and norfloxacin. *Δfur* mutants were treated with ampicillin (A), kanamycin (B) or norfloxacin (C) aerobically.

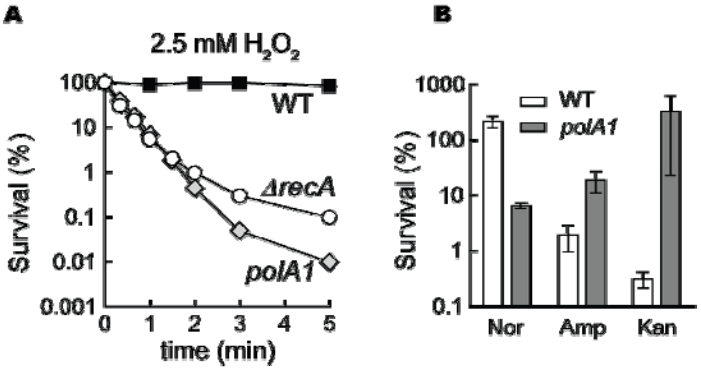

Fig. S3. *polA1* mutants are sensitive to exogenous H_2O_2 but resistant to ampicillin and kanamycin. (A) *ΔrecA* mutants and *polA1* mutants are very sensitive to H_2O_2 challenge. (B) Aerobic log-phase wild type cells and *polA1* mutants were treated with 100 ng/ml norfloxacin for 1 hour or with 5 μg/ml ampicillin or 5 μg/ml kanamycin for 2 hours. The error bars represent the standard error of the mean (SEM). WT, MG1655; *ΔrecA*, AL486; *polA1*, AL410.

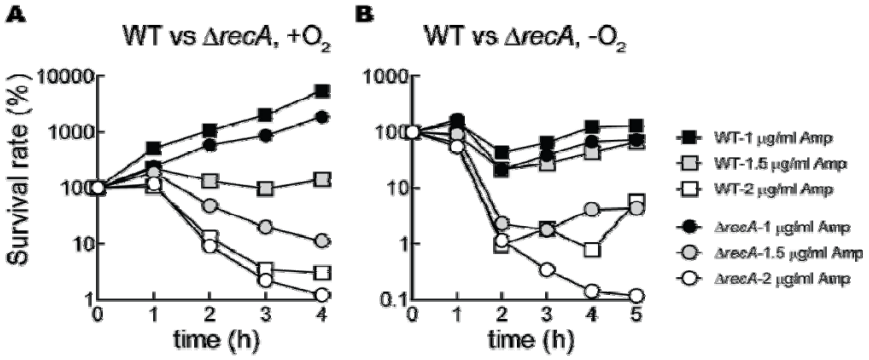

Fig. S4. A *ΔrecA* mutant is slightly sensitive to doses of ampicillin that are bacteriostatic to wild-type cells, under both aerobic and anaerobic conditions.

Aerobic (A) or anaerobic (B) exponential-phase wild type cells and *ΔrecA* mutants were treated with 1, 1.5 and 2 μg/ml ampicillin, and survival was monitored over time. Data are representative of at least three independent experiments.

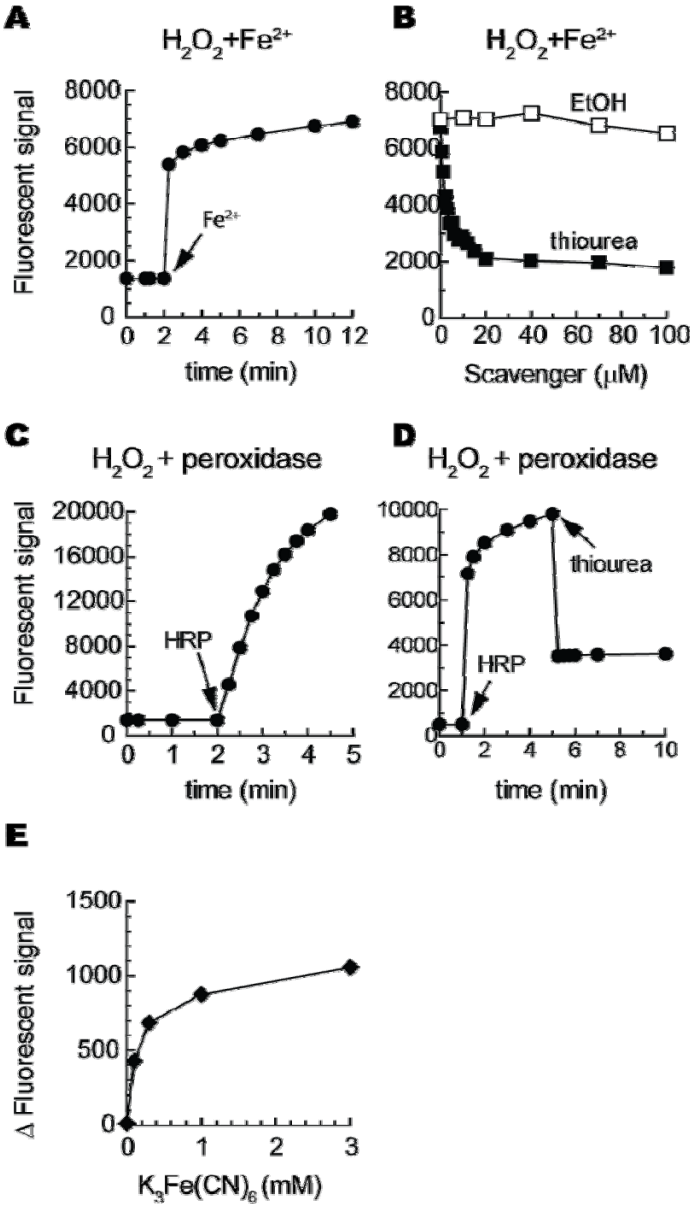

Fig. S5. HPF can be oxidized by oxidants other than hydroxyl radical. (A) HPF dye was oxidized in vitro by a Fenton system consisting of 100 μM ferrous iron plus 1 mM H_2O_2. (B) Thiourea inhibited the oxidation of the dye, but ethanol did not. (C) Horseradish peroxidase (HRP) plus 100 μM H_2O_2 rapidly oxidized HPF. (D) The HRP-oxidized fluorescence (2 μM HPF, 20 μg/ml HPR) was immediately suppressed by the subsequent addition of 100 mM thiourea, added after the completion of the reaction. (E) $K_3Fe(CN)_3$ directly oxidized HPF. The signals were recorded after 20 min incubation.

Data from single experiments are shown; they are representative of at least three independent replicates.

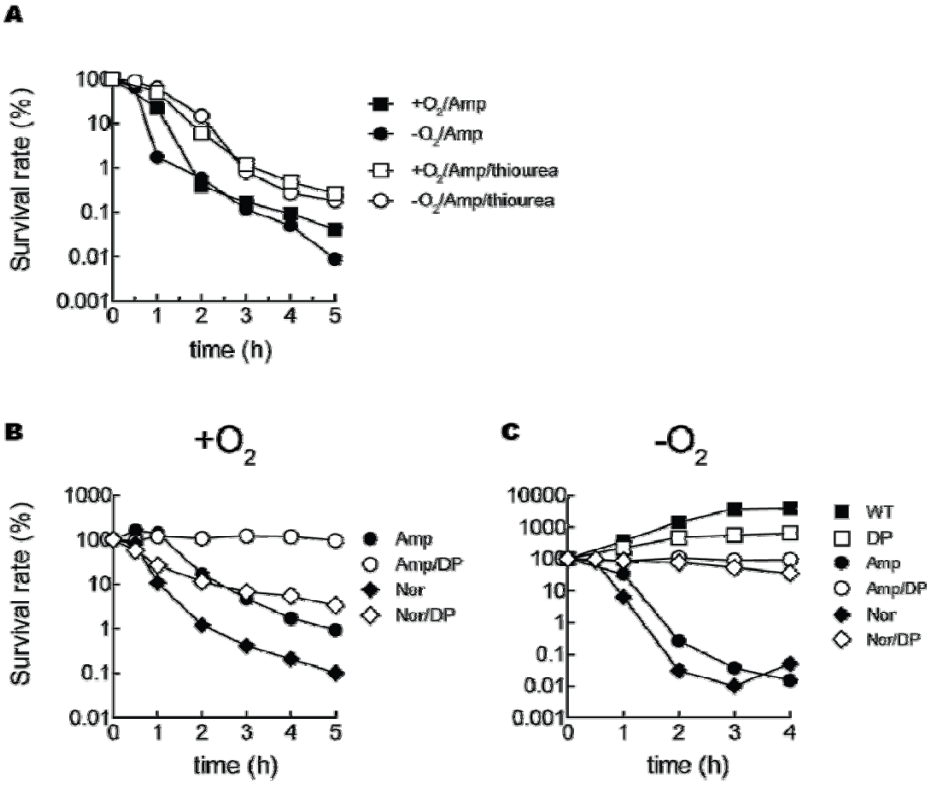

Fig. S6. The protective effects of thiourea and dipyridyl persist when cells are treated with antibiotics in the absence of oxygen. (A) Exponential-phase wild type cells were incubated with 150 mM thiourea and 5 μg/ml ampicillin, under both anoxic and aerated conditions. (B, C) Aerobic (B) or anaerobic (C) wild-type cells were incubated with 5 μg/ml ampicillin or 250 ng/ml norfloxacin. Where indicated, 500 μM dipyridyl (DP) was included. Thiourea and dipyridyl concentrations matched those used in previous studies (*4, 10*).

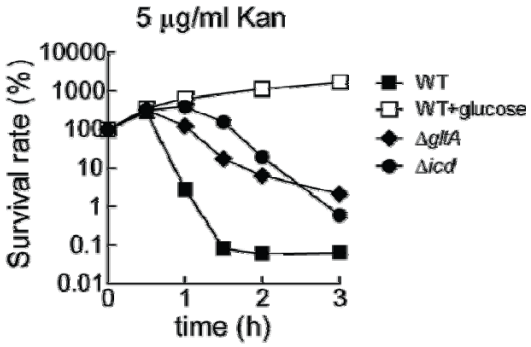

Fig. S7. Diminution of TCA-cycle flux protects cells from kanamycin. Wild-type cells and TCA-cycle mutants *(ΔgltA* and *Δicd*, lacking citrate synthase and isocitrate dehydrogenase, respectively) were treated with 5 μg/ml kanamycin, and survival was monitored over time. Where indicated, 0.2% glucose was included in the LB medium. In response to glucose, the expression of TCA-cycle enzymes is repressed. The results suggest that a shift towards fermentative metabolism suppresses kanamycin toxicity.

Table S1. Strains and plasmids used in this study.

Name	Genotype/characteristics
Strains	
AA16	As MG1655 plus *ΔgltA*::c*at*~Tn*10*
AA98	As MG1655 plus *Δicd1*::c*at*
AL410	As MG1655 plus *polA1~zih-102::*Tn*10*
AL427	As MG1655 plus *Δ*(*ahpCF::cat)1 Δ*(*katG17*::Tn*10*)*1 Δ*(*katE12*::Tn*10*)*1*
AL441	As MG1655 plus *Δ*(*lacZ1*::*cat*)*1 attλ*::[pSJ501:: *katG′-lacZ*$^+$ *cat*$^+$]
AL486	As MG1655 plus *Δ*(*recA774*::*kan*)*1*
AL494	As MG1655 plus *Δ*(*ahpC-ahpF'*) *kan::′ahpF Δ*(*katG17*::Tn*10*)*1 Δ*(*katE12*::Tn*10*)*1 Δ*(*lacZ1*::*cat*)*1* *attλ*::[pSJ501:: *katG′-lacZ*$^+$ *cat*$^+$]
JEM913	As MG1655 plus *Δ*(*lacZ1*::*cat*)*1 Δ*(*fur-731*::*kan*)*1*
KI232	As MG1655 plus *(sodB-kan)1-Δ2 Δ(sodA::cat)1*
LEM17	As MG1655 plus *recA56 srl300*::Tn*10*
MG1655	Wild type

Supplementary references.

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