Supplemental Materials and Methods

Superoxide dismutase confers antibiotic tolerance to stationary phase *Pseudomonas aeruginosa* and requires (p)ppGpp signalling

Dorival Martins[1,2], Geoffrey McKay[2], Gowthami Sampathkumar[1], Malika Khakimova[1], Ann English[4], Dao Nguyen[1,2,3,*]

[1] Department of Microbiology and Immunology, McGill University

[2] Meakins-Christie Laboratories, Research Institute of the McGill University Health Centre

[3] Department of Medicine, McGill University

[4] Department of Chemistry and Biochemistry, Concordia University

[*]Corresponding author: dao.nguyen@mcgill.ca

www.pnas.org/cgi/doi/10.1073/pnas.1804525115

Table S1. Bacterial strains

Strain name (ID)	Description	Source
WT (DN276)	*Pseudomonas aeruginosa* PAO1 wild-type strain	(1)
ΔSR (DN23)	PAO1 Δ*relA* Δ*spoT* with Δ*relA* (Δ181-2019) Δ*spoT* (Δ200-1948) unmarked deletions	(1)
+SR (DN110)	ΔSR with chromosomal complementation of *relA* and *spoT*	(1)
sodA (DN914)	PAO1 *sodA* mutant *sodA::ΩaaC1,* Gm^r	(2)
sodB (DN916)	PAO1 *sodB* mutant *sodB::ΩTc,* Tc^r	(2)
WT *sodB-lacZ* (DN1012)	PAO1 with *sodB-lacZ* transcriptional reporter attTn7::miniTn7-Gm-GW-*sodB-lacZ*, Gm^r	This study
ΔSR *sodB-lacZ* (DN1014)	ΔSR with *sodB-lacZ* transcriptional reporter attTn7::miniTn7-Gm-GW-*sodB-lacZ*, Gm^r	This study
WT +vc (MK318)	PAO1 with vector control attTn7::miniTn7-Gm, Gm^r	(3)
WT +*sodA* (DN871)	PAO1 with *pBAD-sodA* overexpression attTn7::miniTn7-Gm-GW-*araC*-pBAD-RBS-*sodA*	This study
WT +*sodB* (DN872)	PAO1 with *pBAD-sodB* overexpression attTn7::miniTn7-Gm-GW-*araC*-pBAD-RBS-*sodB*	This study
ΔSR +vc (MK320)	ΔSR with vector control attTn7::miniTn7-Gm, Gm^r	(3)
ΔSR +*sodA* (DN898)	ΔSR with *pBAD-sodA* overexpression attTn7::miniTn7-Gm-GW-*araC*-pBAD-RBS-*sodA*	This study
ΔSR +*sodB* (DN870)	ΔSR with *pBAD-sodB* overexpression attTn7::miniTn7-Gm-GW-*araC*-pBAD-RBS-*sodB*	This study
CI-27 (DN380)	Clinical isolate 27 (CI-27) from cystic fibrosis patient	(1)
CI-27 ΔSR (DN379)	Δ*relA* (Δ181-2019) Δ*spoT* (Δ200-1948) unmarked deletion mutant in CI-27 background	(1)
CI-28 (DN386)	Clinical isolate 28 (CI-28) from cystic fibrosis patient	(1)
CI-28 ΔSR (DN385)	Δ*relA* (Δ181-2019) Δ*spoT* (Δ200-1948) unmarked deletion mutant in CI-28 background	(1)
rpoS (DN705)	PAO1 *rpoS* mutant with *rpoS*-B03:: IS*lacZ/hah* allele from PW7151; Tc^r	(4)
ΔSR *rpoS* (DN707)	ΔSR *rpoS::Tn* triple mutant, Tc^R	(3)

Table S2. Plasmids

Plasmids	Description	Source
pDONR211 P1-P5r	Multisite Gateway donor vector, Km^r	Invitrogen
pDONR211 P5-P2	Multisite Gateway donor vector, Km^r	Invitrogen
pUC18 miniTn7T-Gm-GW	Destination vector with pUC18miniTn7 backbone for chromosomal integration into the Tn7 insertion site; Ap^R Gm^R	(5)
pTNS2	T7 transposase expression vector, Ap^R	(5)
pMK131	pDONR221 P1-P5r with an *attL*-flanked, 349-bp fragment encoding the *P. aeruginosa* PAO1 *sodB* promoter region, Km^R	This study
pMK314	pDONR221 P5-P2 with an *attL*-flanked, 3,276-bp fragment encoding an RNase III site and a ribosomal binding site flanking a *lacZ* ORF, Km^R	(3)
sodB-lacZ (pMK398)	*sodB* transcriptional *lacZ* reporter construct pUC18miniTn7T-Gm-GW destination vector containing. Ap^R Gm^R	This study
pJJH187	pDONR221 P1-P5r with an *attL*-flanked, 1,192-bp fragment encoding the *araC* repressor and the *pBAD* promoter, Km^R	(3)
pMK155	pDONR221 P5-P2 with an *attL* flanked,-626-bp fragment encoding a 14-bp synthetic ribosomal binding site and the 612-bp *sodA* ORF from *P. aeruginosa* PAO1, Km^R	This study
pMK169	pDONR221 P5-P2 with an *attL* flanked,-596 bp-bp fragment encoding a 14-bp synthetic ribosomal binding site and the 582-bp *sodB* ORF from *P. aeruginosa* PAO1, Km^R	This study
pBAD-*sodA* (pMK110)	*pBAD-sodA* overexpression construct containing the *araC* regulator, *pBAD* promoter and RBS-*sodA* ORF, pUC18 mini-Tn7T-GW-Gm-*araC*-pBAD-RBS-*sodA* for chromosomal integration, Ap^R Gm^R	This study
pBAD-*sodB* (pMK397)	*pBAD-sodB* overexpression construct containing the *araC* regulator, *pBAD* promoter and RBS-*sodB* ORF, pUC18 mini-Tn7T-GW-Gm-*araC*-pBAD-RBS-*sodB* for chromosomal integration, Ap^R Gm^R	This study

Table S3. Primers

Primers	Description	Source
psodB-F	**GGGGACAAGTTTGTACAAAAAAGCAGGCTAC**TAACGCGGTTTCTGTCTTCC	This study
psodB-R	**GGGGACAACTTTTGTATACAAAGTTGT**GGTGTCTCTCCTATTCTTCC	This study
lacZ-F	**GGGGACAACTTTGTATACAAAAGTTGCATT**ACTAACTAGCGATCCCGAC	(3)
lacZ-R	**GGGGACCACTTTGTACAAGAAAGCTGGGTA**TTATTTTTGACACCAGACCAACTG	(3)
pGLMS-down	GCACATCGGCGACGTGCTCTC	(3)
pTN7-R	CACAGCATAACTGGACTGATTTC	(3)
M13-F	GTAAAACGACGGCCAG	(3)
M13-R	CAGGAAACAGCTATGAC	(3)
sodA-F	**GGGGACCACTTTGTACAAGAAAGCTGGGTA**CTAGGCCAGGGCTTGCG	This study
sodA-R	**GGGGACAACTTTGTATACAAAAGTTGCCAGAGGAGGATATTC**ATGCCCCACGCCTTGCC	This study
sodB-F	**GGGGACAACTTTGTATACAAAAGTTGCCAGAGGAGGATATTC**ATGGCT TTCGAATTGCCGC	This study
sodB-R	**GGGGACCACTTTGTACAAGAAAGCTGGGTA**TCAGGCAGCGAAATTCTTCG	This study

Bold and underlined sequences represent the *att* and target ORF annealing sites.

Supplementary Methods:

Bacterial strains and plasmid construction: All bacterial strains, plasmids and primers used in this study are listed in Tables S1, S2 and S3, respectively. The PAO1 ΔSR, +SR, *sodA* and *sodB* mutants were constructed as previously described in (1) and (2) respectively. The *sodB-lacZ* reporter, *pBAD-sodA* and *pBAD-sodB* overexpression constructs were constructed using the Multisite Gateway® Pro cloning system (Invitrogen) as previously described (3) and as detailed below. These plasmid constructs were chromosomally integrated at the neutral *att*Tn7 site of WT and ΔSR cells using the destination vector pUC18-miniTn7T-Gm-GW. For the *sodB-lacZ* transcriptional fusion, a 349 bp fragment containing the putative promoter sequence of *sodB* was amplified by PCR using the primer pair psodB-F/psodB-R, and recombination of pMK131 and pMK314 entry clones generated the *sodB-lacZ* transcriptional fusion (pMK398). For the overexpression of SodA and SodB, we generated the arabinose inducible expression constructs *pBAD-sodA* (+*sodA*) and *pBAD-sodB* (+*sodB*) by fusing a *pBAD* promoter to a DNA fragment containing the *sodA* or *sodB* ribosomal binding site (RBS) and open reading frame (ORF). The RBS-*sodA* ORF (612 bp) and RBS-*sodB* ORF (582 bp) fragments were amplified by PCR using the primers sodA-F/sodA-R and sodB-F/sodB-R respectively. Recombination of the appropriate *sodA* (pMK155) and *sodB* (pMK169) entry clones with pJJH187 which contains a 1,192-bp fragment encoding the *araC* regulator and the pBAD promoter generated the *pBAD-sodA* and *pBAD-sodB* overexpression constructs. All constructs described above were stably integrated into the WT and ΔSR chromosomes at the *att*Tn7 integration site by electroporation using standard techniques and pTNS2 as helper plasmid. Removal of the mini-Tn7T plasmid backbone from *pBAD-sodB* was performed using the pFLP2 plasmid as described previously (5, 6).

Media and growth conditions. Bacteria were grown in LB Miller medium (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl, Difco) at 37°C. 5 mL cultures (LB medium with antibiotics for selection when appropriate) were inoculated with single colonies and grown in slanted 25 mL tubes for 8 or 16 h. Cultures were then diluted to an initial OD_{600}= 0.05 in 15 mL of LB and grown with high aeration and shaking at 250 r.p.m in 125 mL flasks. Exponential phase cells were grown for ~2 h to an OD_{600}= 0.2, and stationary phase cells for 16 h to an OD_{600}= ~3.5. For SOD expression using the pBAD-*sodA* and pBAD-*sodB* constructs and control vectors, 1% L-arabinose (w/v) was added to the medium at T=0 of the experiment. Gentamicin 75 μg/mL or tetracycline 90 μg/mL were used for selection when appropriate.

Antibiotic killing assays: Exponential or stationary phase cultures (200 μL) were challenged with 50 μg/mL gentamicin (Sigma), 5 μg/mL ofloxacin (Sigma) or 300 μg/mL meropenem (Sigma) without addition of fresh medium, and incubated in 96-well plates at 37 °C with shaking at 250 r.p.m. At specific time points, 30 μL of culture was mixed with an equal volume of activated charcoal (25 mg/mL in PBS) to bind the remaining free drug. Viable colony forming units (CFU) were measured by serial microdilution on LB agar plates after overnight growth at 37 °C. Untreated cells were used as controls.

Preparation of cell lysates: For total protein assays, bacterial cultures in exponential (OD_{600}= 0.2) or stationary (OD_{600}= 3.5) phases were aliquoted, centrifuged and washed twice with PBS (10 mM sodium phosphate, 2.7 mM KCl, 137 mM NaCl, pH 7.4). Pellets were resuspended in 250 μL 2 N NaOH and boiled for 20 min at 95 °C to ensure complete cell lysis. For SOD assays, 1 mL of bacterial cultures was centrifuged at 10,000xg for 5 min at room temperature. Pelleted cells were

washed twice with PBS, resuspended in 0.25 mL PBS and lysed by sonication (20 s at 100 W power output x 6 cycles on ice). The lysates were centrifuged at 12,000 g for 5 min to remove debris, and the supernatants containing soluble proteins were collected and immediately assayed for total protein content and SOD activity.

Total protein assay: The total protein content of exponential or stationary cells was measured in the lysates of washed cells using the Bradford method (Biorad) using bovine serum albumin (BSA) as a standard.

SOD activity assays: Total SOD activity was measured in cell lysates using an in-solution assay with ferricytochrome c and xanthine as described previously (7). Briefly, 5-20 μg of total protein were assayed for SOD activity in a solution containing 50 mM potassium phosphate buffer (pH 7.5), 30 μM horse heart ferricytochrome c and 100 μM xanthine. Upon addition of 0.25 μg/mL xanthine oxidase, the superoxide-mediated reduction of ferri- to ferrocytochrome c was measured via absorbance at 550 nm (Tecan Infinite M1000 microplate reader). One unit of SOD activity inhibits 50% of the rate of ferricytochrome c reduction per mg of protein (U/mg).

In-gel SOD activity was performed as described previously (7). The cell lysates containing 25 μg of soluble proteins were loaded and fractionated by native PAGE at 200 V for 50 min. After washing with Milli-Q water, gels were incubated with 20 mL of the staining solution (1 mM riboflavin, 2.5 mM nitroblue tetrazolium, 20 μL TEMED in 50 mM potassium phosphate buffer, pH 7.8) in the dark for 20 min, then washed with Milli-Q water. Gels were then exposed to room light for 1 h for development of the negatively stained bands containing SOD activity. Densitometry quantification of SOD activity in gels was done using ImageJ by measuring the area under the curve of the relative intensity distribution of each band.

LacZ reporter assays: β-galactosidase activity in strains expressing the *sodB-lacZ* reporters was measured using the Zhang and Bremer method (8) with some minor modifications as previously described (3). The Miller units (MU) used to express β-galactosidase activity were calculated as follows: $M.U.= 1000xA_{420}/(OD_{600}x\ t\ x\ V)$ where "t" is the time of reaction and "V" is the volume of permeabilized cells (mL) added to the reaction vessel.

Paraquat killing assays: Planktonic cultures were grown in LB Miller medium to exponential ($OD_{600}= 0.2$) or stationary phase ($OD_{600}= 3.5$) as described above. At specified time points, 200 μL of bacterial cultures were challenged with 10 mM paraquat without addition of fresh medium, and incubated in 96-well plates at 37 °C with shaking at 250 rpm. At each indicated time point, 30 μL of bacterial cultures were sampled and mixed with an equal volume of 25 mg/mL activated charcoal diluted in PBS for binding of the remaining free drug. Untreated cells were used as controls. Viable bacterial counts were measured by serial microdilution and CFU were counted on LB agar plates after overnight growth at 37 °C.

Respiratory activity assays: Respiration of exponential and stationary phase cultures was estimated using resazurin reduction to resorufin (9). Resazurin was added to the cultures at a final concentration of 0.1 mg/mL, the cultures were incubated at 37 °C for 30 min shaking at 250 r.p.m, diluted 5-fold in PBS and resorufin fluorescence was measured at Ex/Em 550/585 nm (Tecan Infinite M1000 microplate reader). As a negative control, 125 mM Na azide was added to the cultures during the incubation with resazurin, which does not cause loss of viability during the time-frame of the experiment.

Antibiotic killing assays in presence of SOD mimetic: Planktonic stationary phase cultures were diluted to 10^8 CFU/mL in their own conditioned medium obtained from the cell-free supernatant of the same stationary phase culture. This step was done to reduce bacterial density without adding fresh medium to the cultures. The cell permeable SOD mimetic Mn(III)-tetrakis-(1-methyl-4-pyridyl) porphyrin pentachloride (Mn^{III}TMPyP, Cayman Chemicals) was added at 100 μM for 2 min prior to challenge with 50 μg/mL gentamicin, 5 μg/mL ofloxacin or 300 μg/mL meropenem.

DHE and EtBr staining: Cellular superoxide levels were assessed with the dihydroethidium (DHE) probe (10). Exponential or stationary phase cells grown in LB medium were diluted to a cell density of $3x10^8$ CFU/mL in PBS and stained with 15 µM DHE for 1 h at room temperature in the dark. DHE staining was also carried out either in the presence or absence of 100 μM carbonyl cyanide m-chlorophenyl hydrazone (CCCP) to inactivate efflux pumps, which substantially increases the steady-state amount of internalized probe without affecting the relative fold differences between the strains. Cells were fixed with 4% formalin (v/v) and analyzed by flow cytometry (BD Accuri C6, BD Biosciences). Relative fluorescence units (RFU) of individual cells were measured at Ex/Em 490/580 nm, and the median RFU of 10,000 individual cells was used for estimating superoxide levels. Staining with 15 µM EtBr was performed under the same conditions as for DHE with or without 100 μM CCCP as indicated. The RFU of 10,000 individual cells was measured by flow cytometry analysis at Ex/Em 490/585 nm. The DHE/EtBr fluorescence ratio was used as an estimate of relative superoxide levels and was calculated by dividing the DHE fluorescence by the EtBr fluorescence.

β-lactamase leakage assay: To estimate outer membrane permeability, we measured extracellular β-lactamase activity released from cells. Stationary phase bacteria were pelleted, washed twice with PBS and suspended to an OD_{600}= 15 in PBS. Bacterial suspensions were then incubated at 37 °C and sampled every 30 min for 2 h. Aliquots were centrifuged twice at 12,000xg for 10 min and β-lactamase activity was measured in the cell-free supernatant in a solution containing 0.3 mM nitrocefin diluted in 50 mM KPi buffer pH 7.4.

Membrane permeabilization by polymyxin B nonapeptide (PMBN): PMBN (50 μg/mL) was added to undiluted cultures of stationary phase WT cells simultaneously with 5 μg/mL ofloxacin, 50 μg/mL gentamicin or 300 μg/mL meropenem, and to control cells (without antibiotics). Viable bacterial counts were measured by serial microdilution and CFU were counted on LB agar plates after overnight growth at 37 °C. To measure EtBr internalization, a second aliquot of PMBN-treated WT cells was immediately diluted 10-fold in PBS containing 15 μM EtBr and 100 μM CCCP, stained and measured for fluorescence intensity at Ex/Em 490/585 as described under "DHE and EtBr staining".

Drug internalization assays: Texas Red-gentamicin synthesis was carried out as previously described (11). Briefly, 16 mM Texas Red (Invitrogen) dissolved in dimethyl formamide was mixed with gentamicin (21 mM in 100 mM K_2CO_3, pH 8.5) at a 1:33 molar ratio, incubated for 30 min at 4 °C in the dark and used without further purification. Equimolar preparations of unconjugated Texas Red in 100 mM K_2CO_3 was used as negative control. Texas Red-gentamicin 40 μg/mL or equivalent volume of unconjugated probe was incubated with 600 μL of undiluted stationary phase cultures for 45 min at room temperature. Cultures were washed three times and resuspended with PBS, and the fluorescence was measured by plate reader at Ex/Em 595/613 nm (Tecan Infinite M1000 microplate reader).

Ofloxacin uptake was carried out as described previously (12). Briefly, 1mL of stationary phase cultures were pelleted and washed once with 1 mL of 50 mM potassium phosphate buffer (pH 7.0) followed by centrifugation at 10,000xg for 2 min. The washed cells were resuspended in buffer and incubated at 37 °C shaking at 250 r.p.m. for 10 min. Ofloxacin at 5 μg/mL was added to the bacterial suspensions and incubated for 1.5 h at 37 °C shaking at 250 r.p.m. The suspensions were pelleted, washed once with buffer and the washed pellets were incubated in 500 μL 0.1 M glycine (pH 3.0) for 1 h at room temperature to extract intracellular ofloxacin. The samples were diluted in 500 μL PBS and an aliquot was measured at OD_{600}.The bacterial suspensions were centrifuged at 10,000xg for 10 min and the ofloxacin in the supernatant was measured using its intrinsic fluorescence at Ex/Em 292/496 nm (Tecan Infinite M1000 microplate reader). The ofloxacin concentration was calculated using a standard curve of ofloxacin (Sigma), and the results were normalized by the sample OD_{600}.

Meropenem internalization was assayed using FITC-labelled meropenem kindly provided by (13). Briefly, 1 mL stationary phase cultures were washed twice with PBS, and resuspended to an OD_{600}= 3.0 in PBS containing 2 mM clavulanate for 2 h at room temperature to inactivate β-lactamases. The bacterial suspensions were then incubated with 4 μM FITC-meropenem or FITC for 15 min at 37 °C shaking at 250 r.p.m. Cells were fixed with 4% formalin (v/v) and analyzed by flow cytometry (BD Accuri C6, BD Biosciences). Relative fluorescence units (RFU) of individual cells were measured at Ex/Em 490/520 nm and the median RFU of 10,000 cells was used to estimate FITC-meropenem internalization.

Ofloxacin resistance assay: The generation of ofloxacin resistant mutants was carried out as previously described (1) with slight modifications. Briefly, overnight cultures of corresponding strains were diluted to an initial OD_{600}= 0.05 in 25 mL LB in 250 mL flasks and grown at 37 °C with shaking at 250 r.p.m. The LB medium was supplemented with 1% L-arabinose for strains carrying pBAD-*sodB* or vector control. After 16 h, the stationary phase cells were pelleted, resuspended in 2 mL of PBS, and 1 mL of the bacterial suspension (~10^{11} CFU total) was plated onto LB agar supplemented with 12 μg/mL ofloxacin, a concentration equivalent to 3-fold the minimal inhibitory concentration for our strains. Clones that grew on the ofloxacin agar plates were counted daily, and those that arose within the first 72 h were excluded as they might have stemmed from drug resistant cells pre-existing in the initial inoculum. The frequency of ofloxacin resistance was calculated as the number of ofloxacin resistant clones at Day 5 CFU divided by the total input at Day 0.

Statistical analyses: All experiments were carried out with the indicated number of independent biological replicates in at least two independent experiments. Statistical analyses were carried out using the Prism 7 software (GraphPad, CA USA). The two-tailed Student's t-test was used for comparison between two conditions, while one-way analysis of variance (ANOVA) with Tukey multi-comparison post-test was used for comparison between three or more conditions. P values <0.05 were considered statistically significant.

Supplemental figures

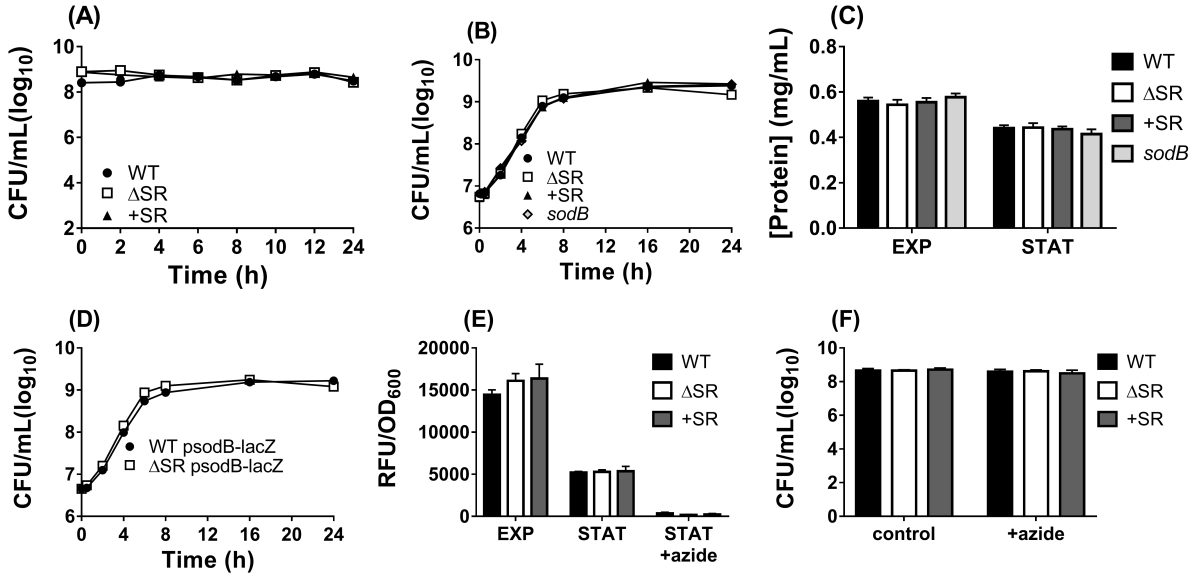

Fig. S1: Bacterial survival in stationary phase, growth curves, total protein content and respiratory activity of the bacterial strains. (A) Survival of WT, ΔSR, +SR in stationary phase. Cultures (initial OD_{600}= 0.05 by diluting an overnight culture) were first grown to late stationary phase (16 h) under high aeration (15 mL in 125 mL flasks) at 37 °C with shaking at 250 r.p.m. To assess bacterial viability in stationary phase conditions similar to the killing assays, 200 µL culture aliquots were then transferred and incubated in 96-well plates at 37 °C with shaking at 250 r.p.m. At indicated times after transfer, viable CFU counts were determined by serial dilution and plating on LB agar. (B) Growth of WT, ΔSR, +SR and *sodB* in LB medium. Cultures were inoculated to an initial OD_{600}= 0.05 by diluting an overnight culture and grown under high aeration (15 mL in 125 mL flasks) at 37°C with shaking at 250 r.p.m. At indicated times, viable CFU counts were determined as above. (C) Total protein content in exponential (EXP) or stationary (STAT) phase cells. (D) Growth of WT and ΔSR±p*sodB-lacZ* used in Fig. 2C of the main text. Cultures were inoculated and assayed as described in (B). (E) Respiratory activity and (F) viability of WT, ΔSR and +SR grown in LB medium to exponential (EXP) or stationary (STAT) phase was assayed after a 30 min of incubation with resazurin ±NaN_3 at 37°C and 250 r.p.m. The data are shown as mean ±SEM (n≥6).

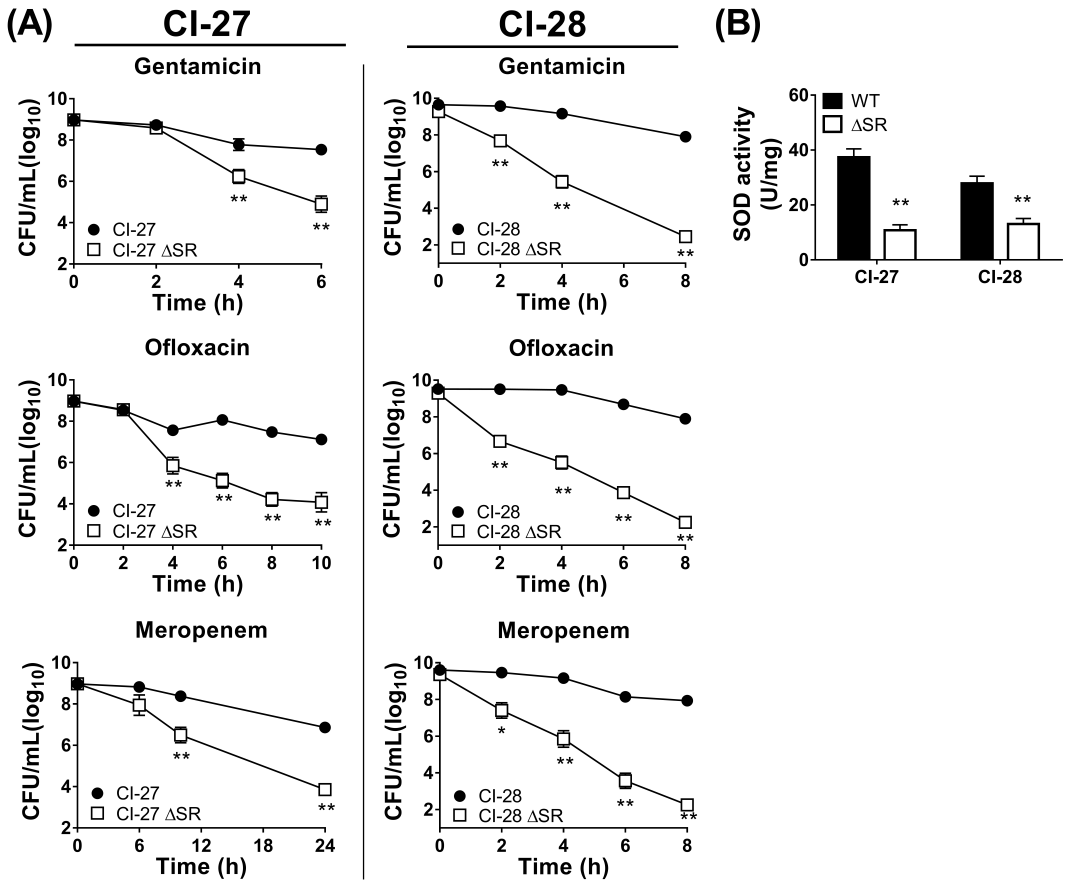

Fig. S2. SR inactivation impairs stationary phase multidrug tolerance and SOD activity in *P. aeruginosa* clinical isolates. (A) CI-27 and CI-28 are two clinical isolates from cystic fibrosis patients. Stationary phase wild-type cells and their respective isogenic (p)ppGpp-null (ΔSR) mutants were challenged 50 μg/mL gentamicin, 5 μg/mL ofloxacin or 300 μg/mL meropenem in antibiotic killing assays. (B) Total SOD activity of stationary phase wild-type clinical isolates CI-27 and CI-28, and respective isogenic ΔSR mutants. Total SOD activity was measured in cell lysates using the in-solution assay. All results are shown as mean ± SEM (n=6). ** P< 0.01 vs. WT.

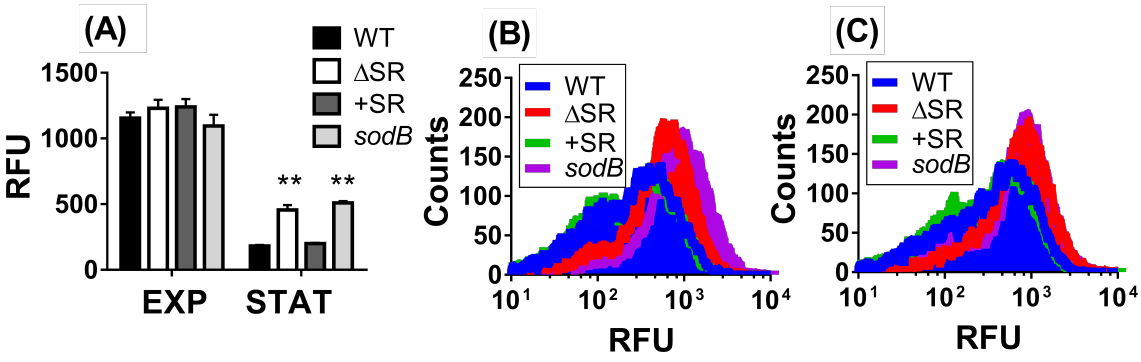

Fig. S3. SR inactivation increases superoxide levels and membrane permeability. (A) Dihydroethidium (DHE) fluorescence of exponential (EXP) or stationary (STAT) phase cells in the presence of CCCP. A representative frequency distribution of stationary phase cells treated with CCCP and stained with 15 μM (B) DHE or (C) ethidium bromide (EtBr) are shown. In A, results are shown as mean ±SEM (n≥6). ** P< 0.01 vs. WT.

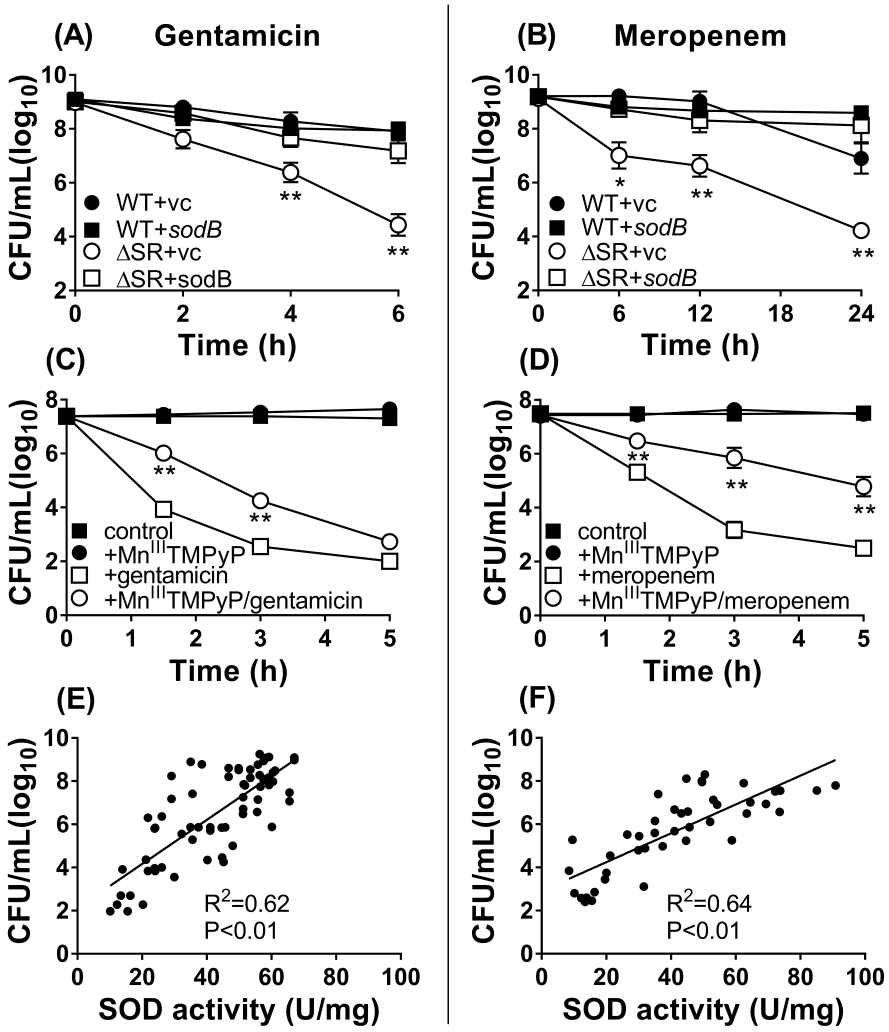

Fig. S4: Complementation of SOD activity, which correlates with antibiotic tolerance, rescues gentamicin and meropenem tolerance in the ΔSR mutant. Stationary phase WT and ΔSR cells carrying the *pBAD-sodB* (+*sodB*) or the vector control (+vc) were assayed for killing with (A) 50 μg/mL gentamicin or (B) 300 μg/mL meropenem. Prior to assaying, bacteria were grown in LB + 1% arabinose for induction of *pBAD-sodB* and the vector control. Stationary phase ΔSR cells were pre-treated for 2 min with and without the SOD mimetic Mn^{III}TMPyP, then challenged with (C) 50 μg/mL gentamicin or (D) 300 μg/mL meropenem. All results are shown as mean ±SEM (n≥6). * for P< 0.05 and ** for P< 0.01 vs. WT +vc (in A and B) or vs. "+antibiotic" (in C and D). Correlation between survival to (E) 50 μg/mL gentamicin for 4 h or (F) 300 μg/mL meropenem challenge and SOD activity. Bacterial viability after challenge h was measured in each culture and plotted against its corresponding SOD activity. Each data point represents an independent replicate (n≥40) and the correlation coefficient R^2 was calculated using a linear regression.

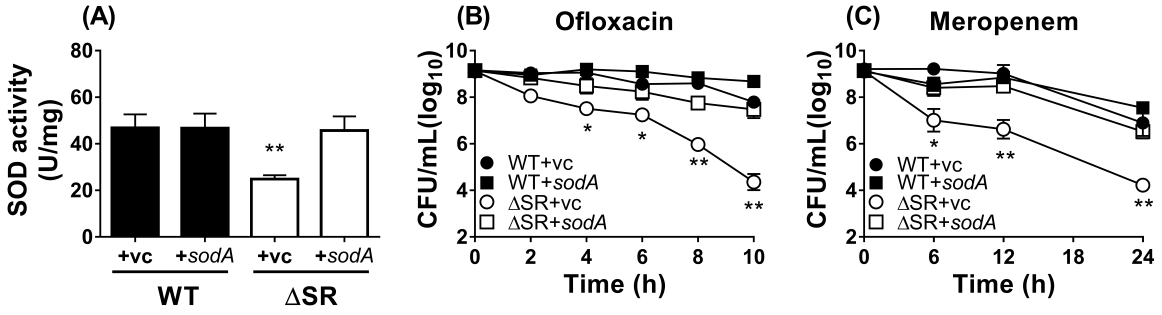

Fig. S5: Overexpression of *sodA* restores SOD activity and antibiotic tolerance to the ΔSR mutant. Stationary phase WT and ΔSR cells carrying the pBAD-*sodA* (+*sodA*) or the vector controls (+vc) were assayed for (A) total SOD activity, or antibiotic killing with (B) 5 μg/mL ofloxacin or (C) 300 μg/mL meropenem. Bacteria were grown in LB with 1% arabinose (wt/v) for induction of *pBAD-sodA* and the vector control. Results are shown as mean ±SEM (n≥6). * P< 0.05 and ** P< 0.01 vs. the corresponding +vc strain.

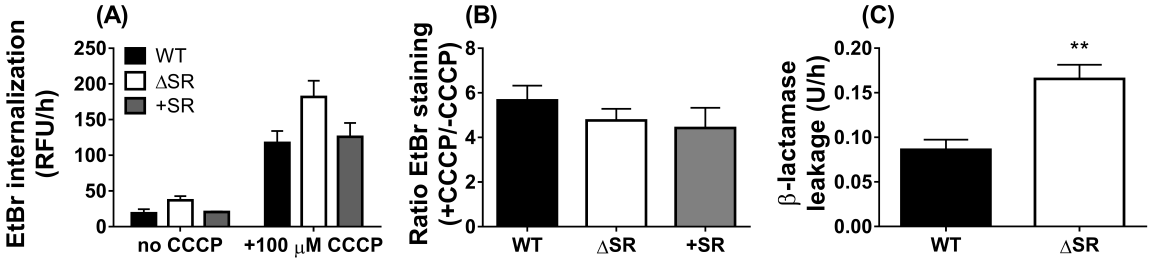

Fig. S6. Inactivation of the SR increases membrane permeability in stationary phase cells. (A) The rate of EtBr internalization in stationary phase cells treated ±100 μM CCCP was calculated as a rate over 2 h from measurement every 30 min. (B) Ratio of EtBr internalization with and without CCCP was calculated as a ratio of values reported in panel A. (C) The rate of extracellular β-lactamase activity released from stationary phase cells. Results are shown as mean ±SEM (n≥6). ** P< 0.01 vs. WT.

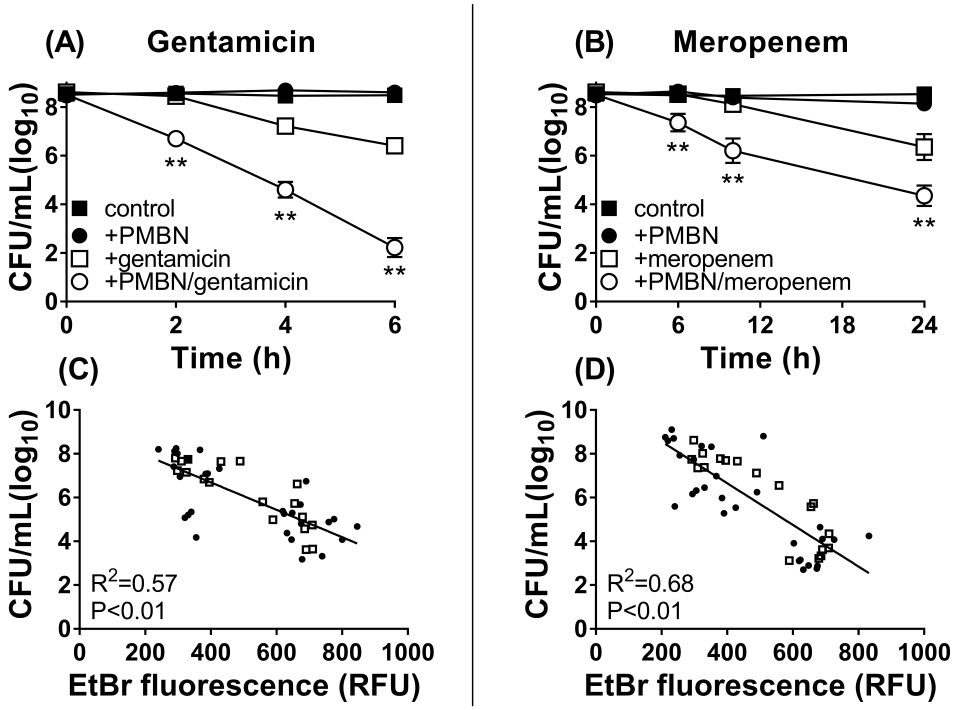

Fig. S7: SR and SODs control membrane permeability which is a critical component of gentamicin and meropenem tolerance. Stationary phase WT ±50 μg/mL PMBN to enhance membrane permeability were challenged with (A) 50 μg/mL gentamicin or (B) 300 μg/mL meropenem. The results are shown as mean ±SEM (n≥6). * for P< 0.05 and ** for P< 0.01 comparing "+drug" alone vs. "+PMBN/drug". The correlation between survival of stationary phase cells to (C) 50 μg/mL gentamicin (x 4 h) or (D) 300 μg/mL meropenem (x 24 h) and membrane permeability (EtBr internalization). Each data point represents an independent replicate of (●) WT, ΔSR and +SR cultures (some carrying the pBAD-*sodA* and pBAD-*sodB* constructs), or (□) WT ±50 μg/mL PMBN. The correlation coefficient R^2 was calculated using a linear regression (n≥40).

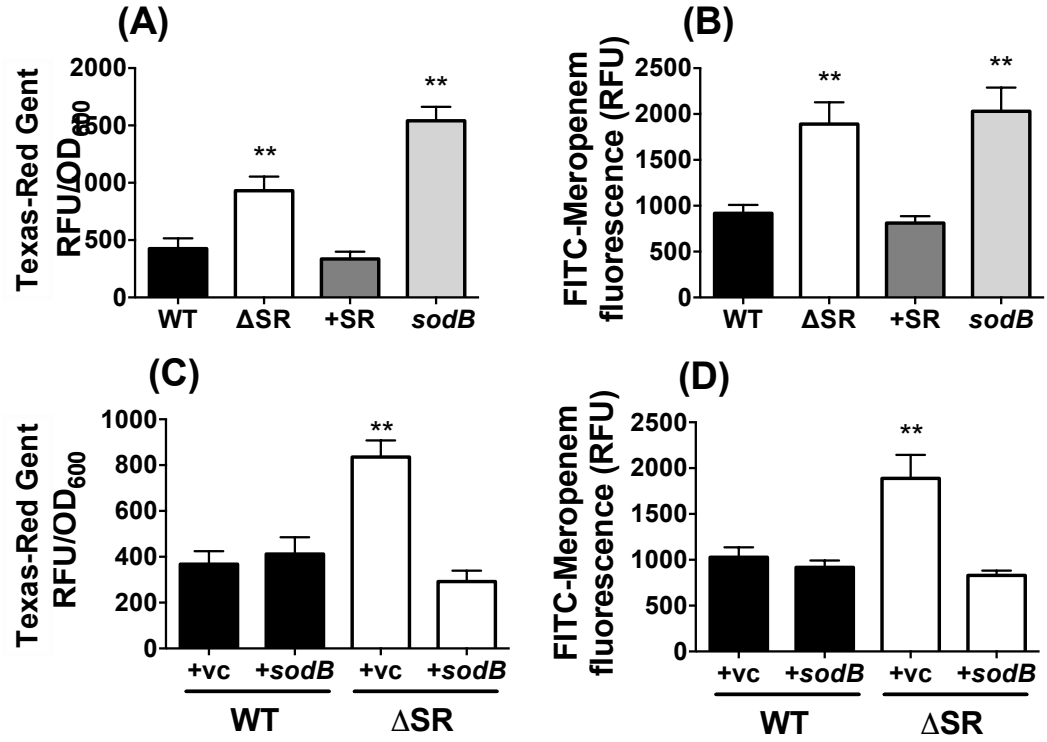

Fig. S8: Loss of (p)ppGpp signalling and SOD activity increases drug internalization. Intracellular drug levels measured in stationary phase cells by fluorescence (RFU) of Texas Red-gentamicin (Ex/Em 595/613 nm) in (A) and (C), and FITC-meropenem (Ex/Em 490/520) in (B) and (D). The +*sodB* and vector control (+vc) strains were grown in LB + 1% L-arabinose prior to the drug internalization assays. Results are shown as mean ±SEM (n≥6). ** P< 0.01 vs WT.

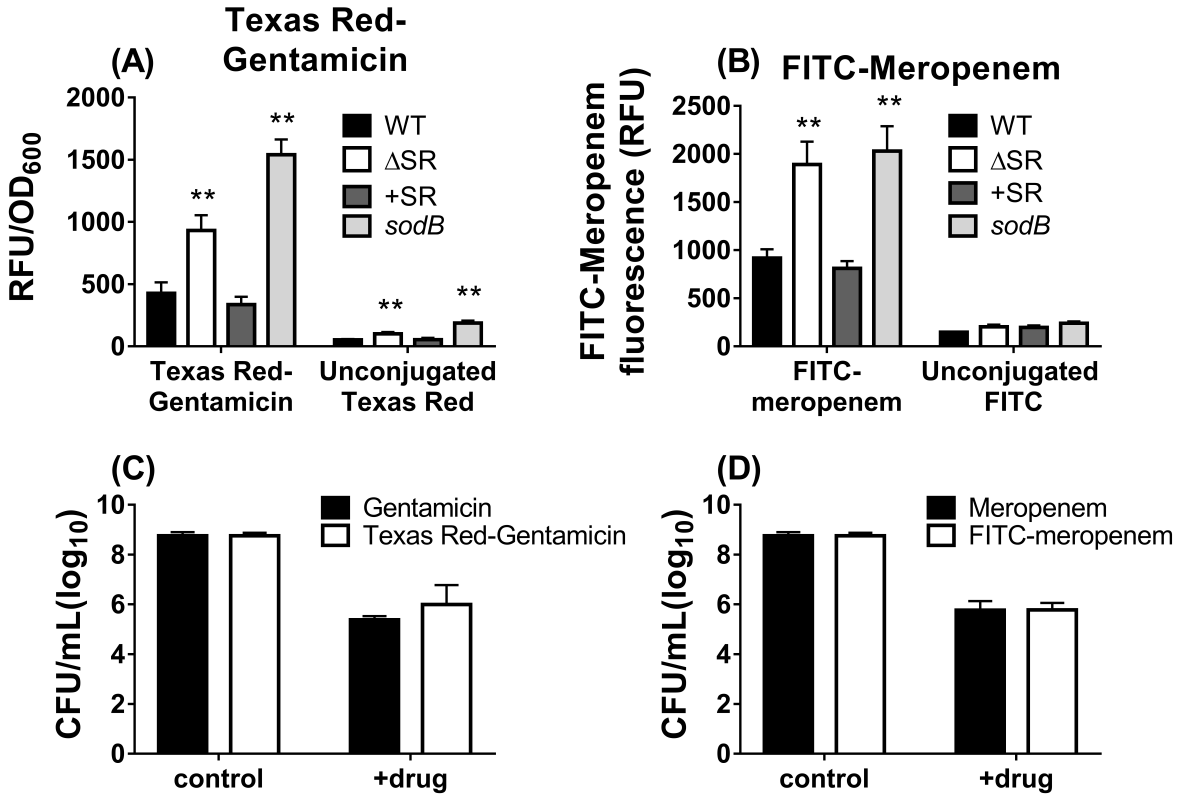

Fig. S9. *P. aeruginosa* largely excludes unconjugated Texas Red and FITC but accumulates Texas Red-gentamicin and FITC-meropenem. Fluorescence (RFU) of (A) Texas Red-gentamicin or unconjugated Texas Red (Ex/Em 595/613 nm), (B) FITC-meropenem or unconjugated FITC (Ex/Em 490/520) in stationary phase cells. The bactericidal activity of (C) 105 μM Texas Red-Gentamicin and (D) 780 μM FITC-meropenem against stationary phase wild-type PAO1 was compared with equivalent molar amounts of untagged gentamicin or meropenem after a 6- or 24-h challenge, respectively. Results are shown as mean ±SEM (n≥6). ** P< 0.01 vs WT.

References

1. Nguyen D, et al. (2011) Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-Llimited bacteria. *Science* 334(6058):982–986.

2. Iiyama K, et al. (2007) Effect of superoxide dismutase gene inactivation on virulence of Pseudomonas aeruginosa PAO1 toward the silkworm, Bombyx mori. *Appl Environ Microbiol* 73(5):1569–1575.

3. Khakimova M, Ahlgren HG, Harrison JJ, English AM, Nguyen D (2013) The stringent response controls catalases in Pseudomonas aeruginosa and is required for hydrogen peroxide and antibiotic tolerance. *J Bacteriol* 195(9):2011–2020.

4. Held K, et al. (2012) Sequence-Verified Two-Allele Transposon Mutant Library for. *J Bacteriol* 194(23):7–10.

5. Choi K-H, Schweizer HP (2006) mini-Tn7 insertion in bacteria with single attTn7 sites: example Pseudomonas aeruginosa. *Nat Protoc* 1(1):153–161.

6. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP (1998) A broad-host-range F1p-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: Application for isolation of unmarked Pseudomonas aeruginosa mutants. *Gene* 212(1):77–86.

7. Weydert CJ, Cullen JJ (2010) Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. *Nat Protoc* 5(1):51–66.

8. Zhang X, Bremer H (1995) Control of the Escherichia coli rrnB P1 promoter strength by ppGpp. *J Biol Chem* 270(19):11181–11189.

9. Hoffman LR, et al. (2006) Selection for Staphylococcus aureus small-colony variants due to growth in the presence of Pseudomonas aeruginosa. *Proc Natl Acad Sci* 103(52):19890–19895.

10. Martins D, Kathiresan M, English AM (2013) Cytochrome c peroxidase is a mitochondrial heme-based H2O2 sensor that modulates antioxidant defense. *Free Radic Biol Med* 65:541–51.

11. Meylan S, et al. (2017) Carbon Sources Tune Antibiotic Susceptibility in Pseudomonas aeruginosa via Tricarboxylic Acid Cycle Control. *Cell Chem Biol* 24(2):195–206.

12. Chapman JS, Georgopapadakou NH (1989) Fluorometric assay for fleroxacin uptake by bacterial cells. *Antimicrob Agents Chemother* 33(1):27–29.

13. June CM, et al. (2015) A fluorescent carbapenem for structure function studies of penicillin-binding proteins, β-lactamases, and β-lactam sensors. *Anal Biochem* 463:70–74.