Superoxide dismutase activity confers (p)ppGpp-mediated antibiotic tolerance to stationary-phase Pseudomonas aeruginosa

Dorival Martins,a,b Geoffrey McKayb, Gowthami Sampathkumara, Malika Khakimova,a Ann M. Englishc, and Dao Nguyena,b,d,1

aDepartment of Microbiology and Immunology, McGill University, Montreal, QC H3A 0G4, Canada; bMeakins-Christie Laboratories, Research Institute of the McGill University Health Centre, Montreal, QC H4A 3J1, Canada; cDepartment of Chemistry and Biochemistry, Concordia University, Montreal, QC H3G 1M8, Canada; and dDepartment of Medicine, McGill University, Montreal, QC H4A 3J1, Canada

Edited by Caroline S. Harwood, University of Washington, Seattle, WA, and approved August 8, 2018 (received for review March 15, 2018)

Metabolically quiescent bacteria represent a large proportion of those in natural and host environments, and they are often refractory to antibiotic treatment. Such drug tolerance is also observed in the laboratory during stationary phase, when bacteria face stress and starvation-induced growth arrest. Tolerance requires (p)ppGpp signaling, which mediates the stress and starvation stringent response (SR), but the downstream effectors that confer tolerance are unclear. We previously demonstrated that the SR is linked to increased antioxidant defenses in Pseudomonas aeruginosa. We now demonstrate that superoxide dismutase (SOD) activity is a key factor in SR-mediated multidrug tolerance in stationary-phase P. aeruginosa. Inactivation of the SR leads to loss of SOD activity and decreased multidrug tolerance during stationary phase. Genetic or chemical complementation of SOD activity of the ΔrelA spoT mutant (ΔSR) is sufficient to restore antibiotic tolerance to WT levels. Remarkably, we observe high membrane permeability and increased drug internalization upon ablation of SOD activity. Combined, our results highlight an unprecedented mode of SR-mediated multidrug tolerance in stationary-phase P. aeruginosa and suggest that inhibition of SOD activity may potentiate current antibiotics.

(p)ppGpp stringent response | Pseudomonas aeruginosa | antibiotic tolerance | superoxide dismutase | stationary phase

Treatment of chronic bacterial infections in the clinic often results in failure or relapses. A drug refractory state, commonly referred to as antibiotic tolerance, occurs even when the infecting organisms harbor no genotypic (heritable) resistance to the antibiotic, and renders many chronic infections difficult to eradicate (1–3). Medically important examples include chronic Pseudomonas aeruginosa lung infections in individuals with the genetic disease cystic fibrosis. Tolerance can also promote the emergence of genotypic drug resistance, thereby posing a major public health challenge (4). Bacteria develop drug tolerance during growth-limiting conditions when they adopt a slow or nonreplicating state, and a fraction of the population survives bactericidal drugs (5). In fact, a large proportion of microbes found in natural environments and in vivo during chronic human infections are likely metabolically quiescent (2, 6).

Laboratory stationary-phase bacteria provide a valuable window into the metabolically quiescent organisms widely observed in nature. The physiology of exponentially growing bacteria change remarkably as they enter stationary phase, yet little is known about the survival strategies of slow or nongrowing cells (7, 8). Stationary-phase bacteria must respond and adapt to a variety of growth-limiting stress and starvation cues (e.g., nutrient exhaustion, pH changes, oxidative or nitrosative stress) through processes regulated by the alternative σ-factor RpoS and (p)ppGpp signaling in Escherichia coli and P. aeruginosa (9, 10). The alarmone (p)ppGpp accumulates upon stress and starvation, leading to a global reorganization of cellular and metabolic functions that promote stress adaptation and cell survival, a process termed the stringent response (SR) (11, 12).

Antibiotic tolerance among metabolically quiescent bacteria is widely attributed to the notion that drug targets are unavailable or inactive when cellular replication and macromolecule synthesis are shut down. Although antibiotic killing typically correlates with bacterial growth rate (13, 14), the lack of replication alone in the absence of (p)ppGpp signaling and downstream adaptive responses is often insufficient to confer tolerance (15–17). The downstream cellular processes that protect against antibiotic toxicity remain poorly understood. We previously observed that SR inactivation in the (p)ppGpp-null ΔrelA spoT mutant of P. aeruginosa (ΔSR) impairs multidrug tolerance in nutrient-limited, biofilm and stationary-phase bacteria (16, 18). Notably, the ΔSR mutant exhibited impaired superoxide dismutase (SOD) and catalase activities, leading us to propose that SR-mediated multidrug tolerance is linked to enhanced antioxidant defenses (16, 18).

Superoxide radicals are by-products of aerobic metabolism and a primary source of intracellular oxidative stress (19). Superoxide causes toxicity through direct damage of iron-containing enzymes, and indirectly through highly reactive hydroxyl radicals generated by Fenton chemistry (20). SODs rapidly disproportionate superoxide to oxygen and hydrogen peroxide, and the latter is detoxified by catalases and peroxidases. P. aeruginosa encodes two different SODs, SodA and SodB. The Fe-cofactored enzymatic conversion of superoxide to oxygen and hydrogen peroxide, and the latter is detoxified by catalases and peroxidases. P. aeruginosa encodes two different SODs, SodA and SodB. The Fe-cofactored enzymatic conversion of superoxide to oxygen and hydrogen peroxide.

Significance

Antibiotic tolerance causes antibiotic treatment failure and promotes the emergence of genotypic resistance in chronic infections, such as those caused by the pathogen Pseudomonas aeruginosa. Laboratory stationary-phase bacteria exhibit a slow growing and metabolically quiescent state associated with high levels of multidrug tolerance likely analogous to the in vivo environment during chronic infection. We demonstrate that superoxide dismutases confer multidrug tolerance in stationary-phase bacteria, and identify a link between (p)ppGpp-mediated stress responses, superoxide metabolism, and membrane permeability to antibiotics. Inhibition of superoxide dismutase activity may overcome multidrug tolerance and potentiate current bactericidal antibiotics in the treatment of P. aeruginosa chronic infections.

Author contributions: D.M. and D.N. designed research; D.M., G.M., G.S., and M.K. performed research; A.M.E. contributed new reagents/analytic tools; D.M. and D.N. analyzed data; and D.M., A.M.E., and D.N. wrote the paper.

This article is a PNAS Direct Submission. The authors declare no conflict of interest.

This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

1To whom correspondence should be addressed. Email: dao.nguyen@mcgill.ca.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1804525115/-/DCSupplemental.

Published online September 10, 2018.
Our recent observation that killing at an OD of 0.2, and stationary-phase cells for 16 h to an OD expression is 2.2- to 2.8-fold lower in stationary

Martins et al. Exponential or stationary-phase cultures were challenged with 50 μg/mL gentamicin, 5 μg/mL oxolinic, or 300 μg/mL meropenem in antibiotic killing assays. Note the different time scale in A and B. Results are mean ± SEM (n = 6). **P < 0.01 vs. WT.

SodB is the most abundant in iron-replete conditions, while the Mn-factorated SodA is under iron-dependent repression and only expressed under iron limitation (21, 22). In this study, we demonstrate that SOD activity is a critical effector of SR-mediated multidrug tolerance in stationary-phase P. aeruginosa, and that SOD activity is correlated with membrane permeability and drug internalization. Our data demonstrate a link between antioxidant defenses, drug permeability, and SR-mediated drug tolerance when P. aeruginosa are metabolically quiescent.

Materials and Methods
Experimental details can be found in the SI Appendix.

Bacterial Strains and Plasmids. All strains and plasmids used are listed in SI Appendix, Tables S1 and S2. The P. aeruginosa laboratory strain PA01 is the parental WT strain. The (pppGpp-null isogenic ΔSR mutant carries unmarked deletions of both (pppGpp synthetases relA and spoT, and the ΔSR mutant in the ΔSr mutant complemented for the relA and spoT genes (16).

Media and Growth Conditions. Bacteria were grown in LB Miller medium as described in SI Appendix. Exponential phase cells were grown for ~2 h to an OD600 = 0.2, and stationary-phase cells for 16 h to an OD600 = 3.5.

SOD Activity Assays. Total SOD and Sod-specific activities of cell lysates were measured using an in-solution and an in-gel SOD activity assay as described previously (23).

Antibiotic Killing Assays. Exponential or stationary-phase cultures were challenged with antibiotics without addition of fresh medium, and incubated in 96-well plates at 37 °C with shaking at 250 rpm. At specific time points, cells were mixed with 1:1 activated charcoal to bind free drug, and viable bacteria were measured by serial microdilution and overnight growth of CFU on LB agar plates.

Results
(pppGpp Signaling Required for the Multidrug Tolerance of Stationary-Phase P. aeruginosa. Stationary-phase bacteria are highly drug tolerant compared with their exponentially growing counterparts. To examine the contribution of (pppGpp signaling in P. aeruginosa stationary-phase drug tolerance, we challenged the (pppGpp-null ΔSR mutant to multiple distinct classes of bactericidal antibiotics and compared it to its WT isogenic parental strain. Stationary-phase ΔSR mutant cells are highly impaired for tolerance compared with WT (Fig. 1A), with 3–4 log10 greater antibiotic killing by the aminoglycoside gentamicin (5.9– vs. 2.9-log10 killing of ΔSR vs. WT, respectively, at t = 6 h), the fluoroquinolone ofloxacin (4.9– vs. 1.9-log10 killing at t = 10 h), and the β-lactam meropenem (6.0– vs. 2.9-log10 killing at t = 24 h). In contrast, exponential phase WT and ΔSR bacteria are equally susceptible to all three drugs and undergo rapid killing (Fig. 1B). Multidrug tolerance is fully restored to WT levels upon complementation of the ΔSR mutant with the relA and spoT genes (+SR), confirming that the loss of tolerance is attributable to relA and spoT mutations. Notably, the bacterial viability in stationary phase and growth rate in exponential phase are similar between the WT, ΔSR, and +SR strains (SI Appendix, Fig. S1A and B). Deletion of relA and spoT in two additional P. aeruginosa clinical strains to abrogate (pppGpp clinical strains to abrogate the (pppGpp synthesis also results in loss of stationary-phase multidrug tolerance, although the magnitude of this effect differs in different P. aeruginosa genetic backgrounds (SI Appendix, Fig. S2A).

SOD Activity Induced During Stationary Phase and Requires (pppGpp Signaling. Our recent observation that ΔSR mutant biofilms exhibit low SOD activity led us to examine whether SOD activity requires (pppGpp signaling in planktonic stationary-phase bacteria. We first note a fourfold increase in SOD activity upon transition of the WT from exponential to stationary phase. However, this induction is largely abrogated in the ΔSR mutant and the SOD activity in stationary-phase ΔSR cells is reduced to 35% of WT levels (40 vs. 14.5 U/mg, P < 0.01) (Fig. 2A). We also observed similar effects upon inactivation of the relA and spoT genes in two P. aeruginosa clinical isolates (SI Appendix, Fig. S1B). Notably, differences in SOD activity are not due to disparities in bacterial growth rate, viability, or total cellular protein content (SI Appendix, Fig. S1 B and C). In addition, complementation of the ΔSR mutant with the relA and spoT genes restores SOD activity to WT levels, and confirms that the SOD defect is attributable to the relA and spoT mutations. These observations thus indicate that (pppGpp signaling is required for full SOD activity during stationary phase.

SodB is the Dominant SOD in Stationary Phase and Is Regulated by the SR and RpoS. We showed by in-gel SOD activity assay that SodB confers all SOD activity in stationary-phase WT, ΔSR, and +SR cells, while SodA activity is undetectable under these conditions (Fig. 2B). Mirroring SodB activity, sodB expression, as measured by a sodB-lacZ transcriptional reporter, is induced in WT cells but not in the ΔSR mutant once cells enter stationary phase (t = 8 h). For example, sodB expression is 2.2- to 2.8-fold lower in stationary phase ΔSR compared with WT cells (Fig. 2C) in the absence of any differences in growth rate (SI Appendix, Fig. S1D).

The SR and the alternative σ-factor RpoS regulatory networks significantly overlap to control gene expression during stationary...
and DSI Appendix
Inactivation of the SR is associated with increased superoxide levels and paraquat killing. (A) Relative intracellular superoxide levels using the DHE/EtBr fluorescence ratio. (B) Paraquat (PQ) killing of exponential (EXP) or stationary-phase (STAT) cells, calculated as percent bacterial survival after 6-h challenge with 10 mM PQ, compared with similar conditions without PQ. Results are shown as mean ± SEM (n ≥ 6). **P < 0.01 vs. WT.

Fig. 3. Inactivation of the SR is associated with increased superoxide levels and paraquat killing. (A) Relative intracellular superoxide levels using the DHE/EtBr fluorescence ratio. (B) Paraquat (PQ) killing of exponential (EXP) or stationary-phase (STAT) cells, calculated as percent bacterial survival after 6-h challenge with 10 mM PQ, compared with similar conditions without PQ. Results are shown as mean ± SEM (n ≥ 6). **P < 0.01 vs. WT.

Stationary-Phase ΔSR Cells Have Increased Superoxide Levels and Lower Paraquat Tolerance Than WT Cells. Because of reduced SOD activity, we predicted that ΔSR cells would be impaired in superoxide detoxification, leading to elevated superoxide levels and greater susceptibility to paraquat, a superoxide-generating compound. We therefore monitored relative intracellular superoxide levels using dihydroethidium (DHE), a cell-permeable probe that, when oxidized by superoxide, is converted to the fluorescent product 2-hydroxyethidium (25). DHE fluorescence intensity is ∼2.5-fold higher in the ΔSR and sodB mutants compared with WT and +SR strains in stationary, but not exponential phase (SI Appendix, Fig. S3 A and B). To account for potential differences in DHE probe loading, we used structurally similar ethidium bromide (EtBr) as a loading control and calculated DHE/EtBr fluorescence ratios as an estimate of relative superoxide levels. The DHE/EtBr ratio is 1.4-fold higher in the ΔSR and sodB mutants compared with the WT and +SR in stationary phase, but no differences are seen in exponential phase (Fig. 3A). Because aerobic respiration is a major source of superoxide (26), we also estimated the respiratory activity using resazurin reduction (27) and found no differences between the strains (SI Appendix, Fig. S1E). This leads us to infer that the excess accumulation of superoxide in the ΔSR mutant is likely attributable to its SOD defect, although other nonrespiratory sources of superoxide cannot be excluded. Additionally, we tested susceptibility to paraquat, a superoxide-generating compound, and found that killing by paraquat correlated with the SOD activity profiles across strains and growth phases (Fig. 3B). Combined, these results suggest that inactivation of the SR impairs protection against superoxide-mediated toxicity during stationary phase.

Genetic or Biochemical Restoration of SOD Activity Rescues Stationary-Phase Multidrug Tolerance. We first expressed pBAD-sodB in the ΔSR mutant (ΔSR +sodB), which restores its SOD activity (Fig. 4A) and DHE fluorescence levels (Fig. 4B) to WT levels. Notably, sodB expression in the ΔSR mutant also restores ofloxacin (Fig. 4C), gentamicin, and meropenem tolerance (SI Appendix, Fig. S4 A and B) to WT levels. Expression of sodA, the Mn-dependent SOD, similarly restores SOD activity and drug tolerance (SI Appendix, Fig. S5). Next, we chemically complemented the ΔSR mutant with 100 μM Mnβ3TMPyP, a cell-permeable SOD mimic that catalytically dismutates superoxide at rates ~10-fold lower than Mn-SodA and Fe-SodB (4 × 107 M−1 s−1 vs. 7 × 107 M−1 s−1) (28, 29). Mnβ3TMPyP confers protection against antibiotic killing, with 2- to 3-log10 higher viable CFU counts following challenges with ofloxacin (Fig. 4D), gentamicin, and meropenem (SI Appendix, Fig. S4 C and D). These results thus suggest that restoring SOD activity in the ΔSR mutant is sufficient to rescue its stationary-phase tolerance defect.

SOD Activity Correlates with Multidrug Tolerance in a Dose-Dependent Manner. We exploited the biological variability of SOD activity in independent replicates of batch cultures to assess the dose-dependent relationship between SOD activity and antibiotic tolerance. We measured SOD activity of a culture before antibiotic challenge and its bacterial viability following antibiotic challenge, and found a strong positive correlation with ofloxacin (R2 = 0.60, P < 0.01) (Fig. 4E), gentamicin (R2 = 0.62, P < 0.01), and meropenem tolerance (R2 = 0.64, P < 0.01) (SI Appendix, Fig. S4E and F).

Stationary-Phase ΔSR Cells Have Increased Membrane Permeability, Which Correlates with Antibiotic Killing. Internalization of EtBr, which was used as a loading control for the DHE probe, also provides a well-established measure of global membrane permeability (30, 31). As expected, we first observed that EtBr internalization is ∼10-fold higher in exponential vs. stationary phase for all strains (Fig. S4 and SI Appendix, Fig. S3C). However, EtBr internalization is ∼twofold higher in the ΔSR and sodB mutants compared with WT and +SR strains during stationary phase, while no differences are seen during exponential phase. Furthermore, genetic restoration of SOD activity with pBAD-sodB expression in the ΔSR mutant restores its EtBr internalization to WT levels (Fig. 5B). Given that EtBr is a substrate for efflux pumps, which are highly expressed in P. aeruginosa, we wanted to confirm that differences in EtBr internalization were not due to differential efflux. To estimate of efflux activity, we measured the ratio of EtBr fluorescence with and without CCCP, a proton ionophore that inactivates efflux pumps, and found no differences between WT, ΔSR, and +SR cells (SI Appendix, Figs. S6 A and B). Finally, to further assess the outer membrane permeability, we carried out a periplasmic β-lactamase leakage assay (32) and found that ΔSR cells have leakier outer membranes than WT cells (SI Appendix, Fig. S6C), consistent with the EtBr results. Together, our results demonstrate that membrane permeability decreases in the WT...
upon transition from exponential to stationary phase, and this process is impaired in the ∆SR and sodB mutants.

To further validate the contribution of membrane permeability to drug tolerance, we permeabilized stationary-phase cells using a chemical approach with polymyxin B nonapeptide (PMBN). PMBN is a cationic peptide that binds outer membrane lipopolysaccharides, leading to permeabilization of the outer membrane without intrinsic bactericidal activity (33). Stationary-phase WT cells challenged with 50 μg/mL PMBN alone show an ∼twofold increase in EtBr fluorescence (Fig. 5B) without loss of viability (Fig. 5C). Importantly, pretreatment of stationary-phase WT cells with PMBN significantly enhances ofloxacin killing (Fig. 5C) (4.4- vs. 1.4-log10 killing at t = 10 h), as well as gentamicin and meropenem killing (SI Appendix, Fig. S7A and B).

Finally, we observed a significant linear correlation between EtBr fluorescence and tolerance to all three drugs (Fig. 5D and SI Appendix, Fig. S7 C and D), suggesting that membrane permeability is a major determinant of SR- and SOD-dependent antibiotic tolerance in stationary-phase P. aeruginosa.

**Drug Penetration IsEnhanced by the ∆SR and sodB Mutant Permeability Defect.** Membrane permeability is a major determinant of drug uptake and internalization. We thus directly assessed intracellular drug levels in stationary-phase cells using ofloxacin (which is intrinsically fluorescent) (SI Appendix, Fig. S6 A and B), Texas Red-labeled gentamicin (SI Appendix, Fig. S8 A and B), and FITC-labeled meropenem (SI Appendix, Fig. S8 C and D). Stationary-phase ∆SR and sodB cells exhibit 2- to 2.5-fold higher drug uptake than WT or +SR complemented cells for all three drugs: ofloxacin (Fig. 6A), Texas Red gentamicin (SI Appendix, Fig. S84), and FITC-meropenem (SI Appendix, Fig. S8C). Furthermore, sodB expression in the ∆SR mutant restores drug accumulation to WT levels (Fig. 6B and SI Appendix, Fig. S8 B and D). As controls, we confirmed that uptake of unconjugated Texas Red and FITC fluorophores is minimal and that the bactericidal activity of labeled drugs is similar to that of unconjugated ones (SI Appendix, Fig. S9).

Taking these data together, we demonstrate that ablation of (p)pGpp signaling or deletion of sodB compromises membrane permeability specifically during stationary phase, leading to enhanced drug penetration and thus drug killing. Because restoration of SOD activity in the ∆SR mutant is sufficient to rescue both membrane impermeability and drug accumulation, this suggests that SOD activity is critical to SR-mediated membrane impermeability and multidrug tolerance in stationary-phase cells.

**Loss of SOD Activity Abrogates the Emergence of Drug Resistance.** Genotypically resistant mutants likely arise from tolerant bacterial populations that survive sustained antibiotic exposure (1, 4). Hence, the loss of tolerance should abrogate the emergence of genotypic resistance. To test this, we measured the emergence of drug-resistant colonies from cell suspensions (~10^11 CFU per plate) of stationary-phase bacteria spread and incubated for 5 d on agar plates containing ofloxacin. We enumerated newly emerging ofloxacin-resistant colonies after 72-h drug exposure to exclude those stemming from preexisting resistant cells. Consistent with our previous observations (16), we find that ablation of (p)pGpp signaling all but eliminates the emergence of ofloxacin resistance, and this defect is restored in the complemented +SR strain (Fig. 6C). The development of ofloxacin resistance is also abrogated in sodB cells, while sodB expression in the ∆SR mutant restores the rate of ofloxacin-resistant mutants to WT levels (Fig. 6D). Hence, perturbations in SOD activity are sufficient to supress the emergence of ofloxacin resistance.

**Discussion**

Our group and others have shown that the SR and (p)pGpp signaling mediate antibiotic tolerance, likely through several different mechanisms (15, 16, 34, 35). Among these, the most extensively studied mechanism is the formation of specialized drug-tolerant cells termed persister cells (36). In *E. coli*, (p)pGpp signaling is central to the regulation of toxin–antitoxin module-dependent pathways involved in persister formation (15, 36). In *P. aeruginosa*, Verstraeten et al. (34) reported that the Ogb GTPhase induces generation of aminoglycoside-tolerant cells, but through mechanisms yet unclear in *P. aeruginosa*. Although such recent studies provide important insights into the molecular basis of persister formation, it remains unclear to what degree persister mechanisms overlap or differ from those implicated in stationary-phase tolerance.

*P. aeruginosa* remains viable in a nonreplicating state for prolonged periods of time (8) and displays a high level of multidrug resistance to drug exposure (4). The contact with our previous observation (16), we find that ablation of (p)pGpp signaling all but eliminates the emergence of ofloxacin resistance, and this defect is restored in the complemented +SR strain (Fig. 6C). The development of ofloxacin resistance is also abrogated in sodB cells, while sodB expression in the ∆SR mutant restores the rate of ofloxacin-resistant mutants to WT levels (Fig. 6D). Hence, perturbations in SOD activity are sufficient to supress the emergence of ofloxacin resistance.
tolerance during stationary phase. The SR acts as a major global regulator of bacterial stationary-phase physiology and stress responses that exerts widespread direct and indirect effects on gene transcription (11, 12). We show here that the SR modulates sodB transcription and total SOD activity in stationary-phase P. aeruginosa primarily through RpoS, which underscores the significant overlap between SR- and RpoS-dependent gene regulation, as we and others have previously noted (18, 37, 38). The link between the SR and SOD regulation is not widely conserved across different bacterial species (39–41) but SodB expression in E. coli is SR-dependent (42), and carbon starvation induces both sodA and SodB synthesis (43).

Despite the pleiotropic effects of the SR, genetic and chemical complementation of SOD activity is sufficient to restore multidrug tolerance of the ΔSR mutant to WT levels. This suggests that SOD activity is protective against antibiotic toxicity and plays a key role in (p)ppGpp-dependent multidrug tolerance during stationary phase. We observed a strong correlation between SOD activity and antibiotic survival to all three classes of drugs, across different isogenic strains constructed in the PA01 genetic background. The role of the SR and SOD appears conserved in other P. aeruginosa clinical strains, although the magnitude of their effect likely varies based on the genetic background.

We recognize that our measurements of SOD activity and stationary-phase survival following antibiotic challenge reflect population rather than single-cell responses. Gene expression is highly heterogeneous during stationary phase (8), and phenotypic heterogeneity allows bacterial populations to persist in the face of fluctuating environments and lethal stress, including antibiotics (17). Future studies with single-cell analyses would be informative in demonstrating whether variations in SOD activity confers survival to antibiotic stress at the individual cell level.

We used DHE to probe superoxide levels and acknowledge its lack of specificity because it can also react with hydroxyl radicals (44). However, in our experiments DHE fluorescence intensity varied with SR retention (Fig. 2A) and in the sodB expression constructs (Fig. 4B), suggesting that the DHE signal reflects relative superoxide levels.

Although superoxide radicals are considered a primary source of intracellular oxidative stress and likely contribute to the lethal effects of antibiotics (45–47), the biological consequences of elevated superoxide are not fully understood. Whether SOD activity confers tolerance directly through its ability to detoxify superoxide radicals, or indirectly through downstream cellular processes responsive to SOD activity remains to be determined. The relationship between SOD activity, SOD-dependent effects, and superoxide metabolism is complex. SODs appear to have moonlighting functions in eukaryotic cells. For example, SOD1 in the yeast Saccharomyces cerevisiae also functions in signal transduction as its SOD activity stabilizes kinases involved in oxidative and metabolic responses (48), and it can act as a nuclear transcription factor (49). Whether such unorthodox SOD functions exist in bacteria remains unknown.

To date, studies that examined the role of SODs in antibiotic lethality in various bacteria report divergent conclusions. SodB mutants showed increased susceptibility to bactericidal antibiotics in Campylobacter jejuni (50), Staphylococcus aureus (51), and Enterococcus faecalis (52), but not Acinetobacter baumannii (53). A sodA sodB mutant of E. coli is reportedly no more susceptible than WT to ampicillin, gentamicin, and norfoxacin killing (54, 55). Furthermore, expression of sodA or sodB did not mitigate ampicillin and ofloxacin killing of E. coli (56). However, several important biological and experimental differences likely account for the different observations on the relationship between SOD and antibiotic susceptibility. First, many antibiotic and antioxidant responses differ between E. coli and P. aeruginosa, including RpoS (57) and SoxR (58), particularly during growth arrest (8). Second, experimental conditions to assess antibiotic lethality differ significantly between the different studies. Most notably, antibiotic killing assays in the E. coli studies (54–56) were carried out on actively replicating (exponential phase) cells, or cells that exit stationary phase to resume growth upon dilution into fresh medium. In contrast, we challenged stationary-phase cells under nongrowing conditions where new nutrients are not supplied, and our results demonstrate that SOD-mediated tolerance is growth phase-specific. Others have also reported that stationary-phase sodA sodB mutant E. coli is more susceptible to gentamicin killing when maintained in a nongrowing state (59). Combined, these results suggest that SOD is particularly important to antibiotic survival during stationary phase.

Interestingly, Dukan et al. (43) previously observed that SOD-deficient E. coli mutants exhibit increased protein oxidation, but only in stationary-phase cultures. They proposed that superoxide stress was a hallmark of respiring but nonreplicating stationary-phase cells (60). More recently, studies in E. coli and S. aureus reported that the antibiotic-tolerant stationary-phase cells and persister cells have low ATP levels, which suggests that increase ATP levels also enhance antibiotic killing (61–63). Although increased respiration increases both ATP levels and superoxide generation, it is not known if SOD activity in turn alters ATP levels. Our results show that the differences in SOD activity and tolerance cannot be attributable to differences in respiration.

Here, we show that the P. aeruginosa cell envelope becomes less permeable during stationary phase, thus limiting drug penetration, and this process is SR- and SOD-dependent. Loss of cell permeability likely represents a common adaptive strategy for bacteria to survive under growth-limiting conditions (2). Alterations in envelope composition and permeability have been described in bacteria that transition into growth-limiting conditions, and those in stationary phase (64, 65). For example, the cell wall structure of stationary-phase S. aureus displays reduced cross-linking and increased peptidoglycan mass (66). Nutrient-starved and nonreplicating Mycobacterium tuberculosis have altered cell walls that display decreased permeability to chemically distinct classes of drugs, including fluoroquinolones (67). How SOD activity directly or indirectly contributes to the alterations in the cell envelope remains to be determined.

Although measured intracellular drug concentrations, which result from the net effect of drug uptake and efflux. Because our results showed comparable efflux activity in the ΔSR and sodB mutants, this implies that increased drug accumulation is likely due to increased internalization. In Gram-negative bacteria, the outer membrane is widely recognized as the primary barrier to drug internalization, although the molecular mechanisms of drug uptake are often still poorly understood (68). We tested gentamicin, ofloxacin, and meropenem, three distinct classes of bactericidal antibacterial agents that target the bacterial ribosomal translational machinery, DNA gyrase, and peptidoglycan cell wall synthesis, respectively. We recognize that these chemically distinct drugs are likely internalized through different pathways. Aminoglycosides, such as gentamicin, require active uptake through a proton-gradient dependent process (69), while meropenem and ofloxacin likely diffuse passively through the membrane bilayer via outer membrane porins, such as oprD (70) and ompF (71), respectively. The mechanisms by which SR- and SOD-dependent tolerance influence these uptake processes remain unknown.

Our study uncovers an unprecedented link between SOD activity, SR-mediated multidrug tolerance, membrane permeability, and antibiotic internalization in stationary-phase P. aeruginosa. We also demonstrate that deletion of SOD-dependent pathways enhances antibiotic lethality and abrogates the emergence of genotypic resistance in stationary-phase P. aeruginosa. Thus, targeting the (p)ppGpp and SOD-dependent pathways may improve bactericidal activity against slow-growing bacteria and prevent the emergence of drug resistance in chronic infections.

ACKNOWLEDGMENTS. We thank Dr. Joe Harrison (University of Calgary) for the Gateway vectors; Prof. Kazuhiro Iiyama (Kyushu University) for the Gateway vectors; Prof. David Leonard (Grand Valley State University) for providing FITC-meropenem; and Drs. Pradeep K. Singh, Dianne Newman, and Lucas Hoffman for helpful discussions and review of our manuscript. This research was funded by the Burroughs Wellcome Fund (Award 1006827.01 to D.N.) and Canadian Institutes of Health Research (Grant MOP102727 to D.N.). D.N. is a Cystic Fibrosis Canada and Fonds de Recherche du Quebec, Sante Scholar, and D.M. is supported by a Canadian Institutes of Health Research and Cystic Fibrosis Canada fellowship.
1. Levin BR, Deeny DE (2006) Non-inherited antibiotic resistance. Nat Rev Microbiol 4: 55–62. 11:57:53-548.

2. Rittgershaus E, Baek S-H, Sasseti CM (2013) The normality of dormancy: Common themes in microbial quiescence. Cell Host Microbe 13:643–651.

3. Meylan S, Andrews IW, Collins JJ (2018) Targeting antibiotic tolerance, pathogen persistence, and survival. J Bacteriol 184:1220–1230.

4. Levin-Reisman I, et al. (2017) Antibiotic tolerance facilitates the evolution of resistance. Science 355:826-830.

5. Brauner A, Fridman G, Oefen F, Balaban NQ (2016) Distinguishing between resistance to antimicrobial treatment and antibiotic tolerance. Nat Rev Microbiol 14:320–330.

6. Kopf SH, et al. (2016) Trace incorporation of heavy water reveals slow and heterogeneous growth path rates in cystic fibrosis sputum. Proc Natl Acad Sci USA 113: E110–E116.

7. Doi A, Kato K, Ito T (1992) Life after log. J Bacteriol 174:345–348.

8. Bergkessel M, Basta DW, Newman DK (2016) The physiology of growth arrest: Uniting molecular and environmental microbiology. Nat Rev Microbiol 14:549–562.

9. Chang DE, Vodisek D, Venkatesh DV (2004) Pseudomonas aeruginosa relA mutant cells. J Bacteriol 186:4539–4548.

10. Navarro Llorens JM, Tormo A, Martinez-García E (2010) Stationary phase in gram-negative bacteria. FEMS Microbiol Rev 34:476–495.

11. Braeken K, Moris M, Daniels R, Vanderleyden J, Michiels J (2006) New horizons for (p)ppGpp in bacterial and plant physiology. Trends Microbiol 14:32–42.

12. Traxler MF, et al. (2008) The global, ppGpp-mediated stringent response to amino acid starvation. J Bacteriol 190:2741–2751.

13. Tuomanen E, Cozens R, Tosch W, Zak O, Tomasz A (1986) The rate of killing of Escherichia coli by beta-lactam antibiotics is strictly proportional to the rate of bacterial growth. J Bacteriol 167:1228–1234.

14. Hassett DJ, et al. (1996) Ferric uptake regulator (Fur) mutants of Pseudomonas aeruginosa. Antimicrob Agents Chemother 40:229–236.

15. Navarro Llorens JM, Tormo A, Martinez-Garcia E (2010) Stationary phase in gram-negative bacteria. FEMS Microbiol Rev 34:476–495.

16. Nicas TI, Hancock RE (1983) 9802

17. Manina G, Dhar N, McKinney JD (2015) Stress and host immunity amplify bet-hedging strategy that leads to antibiotic tolerance. Proc Natl Acad Sci USA 112:14717–14722.

18. Reddy AR, Culotta VC (2013) SOD1 integrates signals from oxygen and glucose to repress cell respiration. Cell 152:224–235.

19. Tsang CK, Liu Y, Thomas J, Zhang Y, Zheng XFS (2014) Superoxide dismutase 1 acts as a nuclear transcription factor to regulate oxidative stress resistance. J Bacteriol 196:849–856.

20. Johansson I, Park H, Rosenqvist M, Varga Z (2003) The Enterococcus faecalis superoxide dismutase is essential for its tolerance to vancomycin and penicillin. J Antimicrob Chemother 51:1196–1202.

21. Heindorf M, Kadar M, Heider C, Skiebe E, Willmarth G (2014) Impact of Acinetobacter baumannii superoxide dismutase on motility, virulence, oxidative stress resistance and susceptibility to antibiotics. J Cell Sci 127:453–463.

22. Wang X, Zhao X (2009) Contribution of oxidative damage to antibiotic lethality. Antimicrob Agents Chemother 53:1395–1402.

23. Erazly B, et al. (2013) Fe-S cluster biosynthesis controls uptake of aminoglycosides in a ROS-less death pathway. Science 340:1583–1587.

24. Orman MA, Bryndissen MP (2015) Inhibition of stationary phase respiration impairs persister formation in E. coli. Nat Commun 6:7983.

25. Durfee T, Hansen AM, Zhi H, Blattner FR, Jin DJ (2008) Transcription profiling of the stationary growth phase of Pseudomonas aeruginosa 112:21969.

26. Ladjouzi R, et al. (2013) Analysis of the adaptive response. Biochemistry 181:3890–3897.

27. Palma M, et al. (2005) Pseudomonas aeruginosa SoxR does not conform to the archetypal paradigm for SoxR-dependent regulation of the bacterial oxidative stress adaptive response. Infect Immun 73:2955–2966.

28. Wang JH, et al. (2014) Sigma S-dependent antioxidant defense protects stationary-phase Escherichia coli against bactericidal antibiotic gentamycin. Antimicrob Agents Chemother 58:5964–5975.

29. Dukan S, Nystöm T (1998) Bacterial senescence: Stasis results in increased and differential expression of cytoplasmic proteins leading to developmental induction of the heat shock regulon. Genes Dev 12:3431–3440.

30. Conlon BP, et al. (2016) Persister formation in Staphylococcus aureus is associated with ATP depletion. Nat Microbiol 1:16051.

31. Lobritz MA, et al. (2015) Antibiotic efficacy is linked to bacterial cellular respiration. Proc Natl Acad Sci USA 112:8173–8180.

32. Adolphsen KJ, Bryndissen MP (2015) Furlie cycling increases sensitivity toward oxidative stress in Escherichia coli. Metab Eng 29:26–35.

33. Kjellberg S, Hermansson M, Márden P, Jones GW (1987) The transient phase between growth and nongrowth of heterotrophic bacteria, with emphasis on the manganocline environment. Ann Rev Microbiol 41:21–49.

34. Cronan JE, Jr (1968) Phospholipid alterations during growth of Pseudomonas aeruginosa. J Biol Chem 243:7475–7480.

35. Zhou X, Cegelski L (2012) Nutrient-dependent structural changes in the outer membrane permeability barrier of Pseudomonas aeruginosa. Mol Microbiol 85:512–522.