A Dysfunctional Tricarboxylic Acid Cycle Enhances Fitness of *Staphylococcus epidermidis* During β-Lactam Stress

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ABSTRACT A recent controversial hypothesis suggested that the bactericidal action of antibiotics is due to the generation of endogenous reactive oxygen species (ROS), a process requiring the citric acid cycle (tricarboxylic acid [TCA] cycle). To test this hypothesis, we assessed the ability of oxacillin to induce ROS production and cell death in *Staphylococcus epidermidis* strain 1457 and an isogenic citric acid cycle mutant. Our results confirm a contributory role for TCA-dependent ROS in enhancing susceptibility of *S. epidermidis* toward β-lactam antibiotics and also revealed a propensity for clinical isolates to accumulate TCA cycle dysfunctions presumably as a way to tolerate these antibiotics. The increased protection from β-lactam antibiotics could result from pleiotropic effects of a dysfunctional TCA cycle, including increased resistance to oxidative stress, reduced susceptibility to autolysis, and a more positively charged cell surface.

IMPORTANCE *Staphylococcus epidermidis*, a normal inhabitant of the human skin microflora, is the most common cause of indwelling medical device infections. In the present study, we analyzed 126 clinical *S. epidermidis* isolates and discovered that tricarboxylic acid (TCA) cycle dysfunctions are relatively common in the clinical environment. We determined that a dysfunctional TCA cycle enables *S. epidermidis* to resist oxidative stress and alter its cell surface properties, making it less susceptible to β-lactam antibiotics.

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The tricarboxylic acid (TCA) cycle has traditionally been considered a crucial metabolic hub in aerobic organisms and is heavily involved in the production of anabolic biosynthetic intermediates and reducing potential (1). However, bacterial pathogens also modulate the TCA cycle following diverse environmental stresses, to bring about appropriate metabolic changes that can drive stress tolerance efforts. Indeed, such tolerance mechanisms can be activated following stress-induced diminution of the TCA cycle activity (2). For example, both iron limitation and ethanol stress reduce TCA cycle activity in *Staphylococcus epidermidis*, resulting in the activation of virulence factors, biofilm formation, and long-term survival processes (2).

Multiple studies have suggested that the bactericidal effects of antibiotics are dependent on their ability to induce oxidative stress and damage (3–5). Additionally, Kohanski et al. identified TCA cycle-dependent upregulation of respiration as a significant source of antibiotic-induced oxidative stress (4). In such a scenario, excess superoxide radicals generated as by-products of respiration liberate iron from iron-sulfur cluster-containing enzymes, which facilitates the generation of hydroxyl radicals by Fenton chemistry. Thus, cell death due to antibiotics would be a consequence of oxidative damage (mediated by superoxide and hydroxyl radicals) to cellular macromolecules like DNA and proteins. More recently, this hypothesis has been rigorously contested and as such remains to be further clarified (6, 7). In the current study, we address the antibiotic-mediated oxidative damage hypothesis from an alternate perspective (8), using the opportunistic human pathogen *Staphylococcus epidermidis* 1457 (wild type [WT]) and an isogenic *S. epidermidis* 1457Δ*citCZacnA* mutant (here referred to as the ΔTCA mutant). The latter strain, containing three mutations in the oxidative branch of the TCA cycle (citrate synthase, isocitrate dehydrogenase, and aconitase), was constructed by phage 71 (Φ71)-mediated transduction of the Δ*citCZ*::*ermB* allele into the *S. epidermidis* 1457 Δ*acnA*::*tetM* background. This mutant was preferred in the current study over any single mutation in an effort to ameliorate toxicity resulting from the accumulation of TCA cycle intermediates (e.g., citrate) (9). Yet, relative to the WT strain and consistent with earlier studies (9), the ΔTCA mutant exhibited a modest reduction in growth rate ($\mu \cdot h^{-1}$; $WT_{\mu} = 0.75 \pm 0.02$ versus $\Delta TCA_{\mu} = 0.64 \pm 0.02$) when cultured in tryptic soy broth (TSB).

We reasoned that if antibiotics mediate their cytotoxic effects

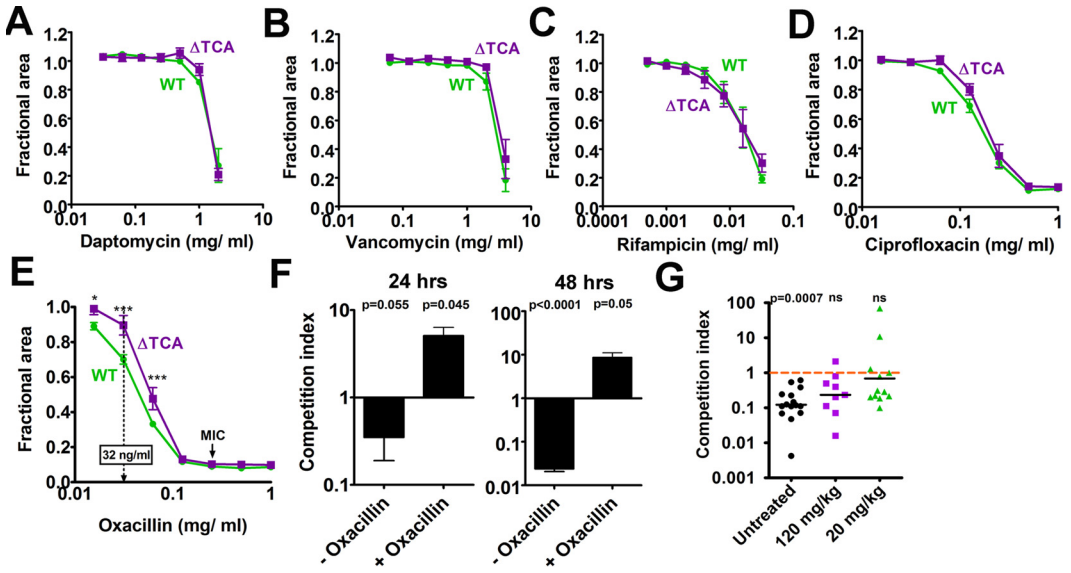

FIG 1 TCA cycle activity imposes a fitness cost during antibiotic stress. The ability of strains to tolerate various concentrations of daptomycin (A), vancomycin (B), rifampin (C), ciprofloxacin (D), and oxacillin (E) was determined by monitoring growth (OD_{600}) according to a previously published method (10). Experiments were performed 4 times. The dotted line denotes sub-MIC concentration utilized in experiments (oxacillin, 32 ng/ml). (F) Competitive fitness of the WT relative to the ΔTCA mutant was determined following coculture of equal concentrations of both strains in TSB. Competitive indices were calculated as the ratio of the ΔTCA mutant and WT in the output normalized to the same ratio in the initial inoculums. Competitive outcomes were determined at 24 h and 48 h of growth. Horizontal lines indicate the arithmetic mean; experiments were performed three times. (G) *In vivo* experiments were conducted in male Sprague Dawley rats that underwent B cell ablation by two intraperitoneal cyclophosphamide injections spaced 3 days apart. Rats were infected with bacteria (both WT and ΔTCA mutant = 10^9 CFU/ml) 24 h after the last cyclophosphamide treatment. Three and four days postinfection, rats were challenged with various doses of oxacillin, and on the fifth day they were sacrificed. Viable bacterial counts from the liver were determined by plating on TSA and TSA containing erythromycin selection. Competitive indices were calculated as the ratio of the ΔTCA mutant and the WT in the output normalized to the same ratio in the initial inoculum. Horizontal lines indicating medians were compared to a hypothetical value of 1 (i.e., equal growth; dashed lines) to determine statistical significance. *P* values were determined by the Wilcoxon signed-rank test. Unless otherwise indicated, data were analyzed using the unpaired Student *t* test ($n \geq 3$; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ns, not significant).

through the TCA cycle, the ΔTCA mutant should more effectively tolerate antibiotics than the parent strain. Accordingly, we tested the effects of five different classes of bactericidal antibiotics (daptomycin, vancomycin, rifampin, ciprofloxacin, and oxacillin) on the growth of both the WT and the ΔTCA mutant. In contrast to previous observations in *Escherichia coli* (4), time-kill studies using antibiotic doses above their MIC did not reveal any significant differences in viability between the WT and the ΔTCA mutant (see Fig. S1A to E in the supplemental material). Therefore, the effects of a range of subinhibitory concentrations of these antibiotics on growth were measured as a function of the area under the bacterial growth curve (optical density at 600 nm [OD_{600}]/time) as previously described (10). The relative amount of growth (fractional area) of both the WT and the ΔTCA mutant was calculated from the ratio of the test (subinhibitory concentrations of antibiotic) area to that of the corresponding control (no antibiotic) and displayed as a function of antibiotic concentration. This approach affords precise comparisons between strains, as it takes into account the growth defect observed in the ΔTCA mutant. Relative to the WT, the ΔTCA mutant was significantly more resistant to sub-MIC concentrations of oxacillin but not the other four tested bactericidal antibiotics (Fig. 1A to E). Notably, corroborating our earlier time-kill studies, no detectable differences were noted between the WT and the ΔTCA mutant in oxacillin concentrations at or above the MIC (Fig. 1E).

Although an active TCA cycle may enhance the fitness of bacteria under natural circumstances, our results suggested that its activity during periods of β-lactam stress may result in a fitness cost. To test this hypothesis, we performed an *in vitro* competition assay of the WT and the ΔTCA mutant in TSB growth medium either in the presence or absence of sub-MIC oxacillin and monitored cell viability after 24 and 48 h of growth. We used an oxacillin dose of 32 ng/ml in competition assays, because maximum differences in growth between the WT and the ΔTCA mutant were observed at this concentration (Fig. 1E). In the absence of oxacillin challenge, the ΔTCA mutant exhibited a fitness defect at 24 h (~3-fold) when cocultured with the WT (Fig. 1F). This defect significantly surged over 40-fold by 48 h, suggesting a significant biological role for the TCA cycle in maintaining the competitive fitness of cells in the stationary phase (Fig. 1F). However, consistent with the decreased susceptibility of the ΔTCA mutant to oxacillin, we observed a significant increase in competitive ability of this strain over the wild-type both at 24 (5-fold) and 48 (~9-fold) h of growth in the presence of sub-MIC oxacillin (Fig. 1F). To ascertain the significance of this observation in the context of an infected host, we determined the competitive indices of the ΔTCA mutant relative to those of the WT in rats challenged with two doses of oxacillin (120 mg/kg of body weight and 20 mg/kg, intraperitoneal inoculation, every 12 hours) and compared them to a control group (no antibiotic). The peak and trough oxacillin serum concentrations of the 120-mg/kg dose was predicted to be 27 μg/ml and 3 μg/ml, whereas for the 20-mg/kg dose, they were 4.5 μg/ml and 0.5 μg/ml, respectively (11). As the liver is the primary organ responsible for the detoxification of oxacillin (12,

13), we predicted that *S. epidermidis* was more likely to be exposed to the targeted sub-MIC of oxacillin, particularly with the 20-mg/kg dose (oxacillin MIC of the WT, 0.25 μg/ml) in this organ. Competitive indices in the liver confirmed ~10-fold outcompetition of the WT over the ΔTCA mutant (Fig. 1G). But, notably, this competitive advantage significantly decreased in rats treated with oxacillin (Fig. 1G). Collectively, these observations are consistent with the ability of oxacillin to at least partially mediate its toxic effects via a functional TCA cycle.

Why does TCA cycle activity elicit a fitness cost in the presence of oxacillin? Sub-MIC oxacillin (32 ng/ml) challenge resulted in decreased viability and lysis of post-exponential-phase bacteria in a TCA cycle-dependent manner (Fig. 2A and B). We directly tested the arguments that these defects following oxacillin treatment may have resulted from the generation of TCA cycle-dependent oxidative stress (14). Accordingly, following oxacillin challenge, bacteria were grown aerobically (10:1 flask-to-volume ratio, 250 rpm, at 37°C) in TSB for 24 h before they were washed and resuspended in KDD buffer [99 mM NaCl, 4.69 mM KCl, 2.5 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM $MgSO_4 \cdot 7H_2O$, 25 mM $NaHCO_3$, 1.03 mM KH_2PO_4, 5.6 mM d-(+)-glucose, 20 mM Na-HEPES, 25 μM deferoxamine, and 5 μM diethyldithiocarbamate] containing the cyclic hydroxylamine spin probe, CMH (0.2 mM). Oxygen radicals were subsequently detected using electron paramagnetic resonance (EPR) spectroscopy. We detected a significant increase (~4-fold) in oxygen radicals (primarily superoxide; see Fig. S2A in the supplemental material) following oxacillin challenge of the wild type but not the ΔTCA mutant (Fig. 2C), supporting observations from earlier studies (4, 14) that described a role for TCA cycle-dependent oxidative stress in antibiotic-mediated cell death. Indeed, we were also able to confirm that sub-MIC oxacillin (32 ng/μliter)-challenged *S. epidermidis* cultured with thiourea (antioxidant) or dipyridyl (iron scavenger that suppresses Fenton chemistry) partially rescued oxacillin-dependent lysis of the WT (see Fig. S2B). However, somewhat surprisingly, we also noticed that the ΔTCA mutant itself underwent relatively high oxidative stress (Fig. 2C), independent of oxacillin treatment, and yet did not exhibit a lysis phenotype in the presence of oxacillin (Fig. 2A). The increased oxidative stress may account for the accumulation of dead cells in the ΔTCA mutant during growth, as estimated from a reduction of viable cell counts recovered per unit of OD_{600} (see Fig. S2C). We reasoned that the observed cell death in the ΔTCA mutant may have enriched for a population that is oxidative stress tolerant and hence not susceptible to the levels of reactive oxygen species (ROS) observed in this mutant or to the lytic effects of oxacillin. To test this argument, we challenged both the WT and the ΔTCA mutant with various doses of hydrogen peroxide (0 to 10 mM). Indeed, the ΔTCA mutant was more resistant to hydrogen peroxide-mediated oxidative stress than the WT (Fig. 2D). Interestingly, such a phenotype is not restricted to staphylococci but was also recently reported in *Salmonella*, wherein decreased flux through the TCA cycle resulted in increased resistance to oxidative stress (15). Collectively, these results raise two intriguing possibilities whereby oxacillin may drive ROS production: via inhibition or activation of the TCA cycle. The former possibility, however, appears unlikely given that the WT challenged with sub-MIC levels of oxacillin did not exhibit the characteristic growth defect of the ΔTCA mutant (Fig. 2A). It is not clear as to what constitutes the source of the observed oxidative stress in the ΔTCA mutant. But one possibility lies in the depletion of a powerful antioxidant (α-ketoglutarate) in this mutant (16). Alternately, a potential deficit of intracellular malate and reducing equivalents in the ΔTCA mutant may affect functional pools of the low-molecular-weight thiol, bacillithiol, that is crucial for maintaining the reducing environment in the cytoplasm (17). Indeed, *Bacillus subtilis* mutants incapable of synthesizing bacillithiol exhibit increased sensitivity to penicillin (18). Taken together, our results not only suggest that TCA cycle-dependent oxidative stress may be a trigger for oxacillin-mediated cell death, but they also point to additional synergistic determinants that limit lysis of the ΔTCA mutant in the presence of oxacillin.

Since oxacillin-mediated lysis is dependent on the activity of autolysins (19), we performed zymography to detect autolytic profiles of both the WT and the ΔTCA mutant in the presence or absence of sub-MIC oxacillin (32 ng/ml). Interestingly, the presence of oxacillin itself did not significantly alter the cell surface autolytic profile of cells. However, we observed significant autolysin pattern alterations and decreased autolytic activities of the ΔTCA mutant relative to those of the WT (Fig. 2E, see cell wall fraction), particularly in the range of 40 to 100 kDa. It is possible that this reduced autolysin activity in the ΔTCA mutant resulted from an inability of the secreted autolysins to bind the ΔTCA mutant surface, resulting in their enhanced proteolytic degradation (<25 kDa) within the culture supernatant (Fig. 2E, see cell supernatant fraction). Consistent with this, we observed that the ΔTCA mutant had a more positively charged cell surface relative to that of the WT using the cationic cytochrome *c* binding assay (Fig. 2F). Although the mechanism by which the ΔTCA mutant maintains a positively charged surface is not known, one possibility involves the diversion of carbon into the production of PIA in this mutant (9), where the deacetylation of Polysaccharide Intercellular Adhesin (PIA) gives rise to an increased positive charge (20). Irrespective of the mechanism, a positive cell surface charge may hinder binding of the major *S. epidermidis* autolysins to the surface. In addition, the repeat domains that target autolysins to the cell surface are also positively charged (21, 22), effectively encouraging their electrostatic repulsion from the surface. Collectively, these results suggest that the nature of the fitness cost of an active TCA cycle in the presence of β-lactam antibiotics may be multifactorial, involving adventitious production of free radicals, increased susceptibility to oxidative stress, and changes in cell surface charge that may make it vulnerable to autolysis.

Finally, we hypothesized that under selective pressure, a fitness cost would be evolutionarily selected against. Hence, despite the crucial metabolic role of the TCA cycle, we predicted the existence of clinical *S. epidermidis* isolates with TCA cycle dysfunctions, especially due to the widespread use of β-lactam antibiotics. To test this hypothesis, we took advantage of the well-known ability of *S. epidermidis* to oxidize metabolically excreted acetate via the TCA cycle under aerobic conditions (1). A library of 126 clinical *S. epidermidis* isolates were grown in TSB (0.25% glucose) under aerobic conditions, and the acetate concentrations were measured from the culture supernatants after 24 h of growth. Under these conditions and this time frame, *S. epidermidis* isolates with a functional TCA cycle completely oxidized the acetate (usually in excess of 20 mM) generated from the oxidation of glucose in the media. We used both the WT and the ΔTCA mutant as qualitative controls in this screen and, based on the concentrations of acetate remaining in the supernatant, classified strains as strong (less than

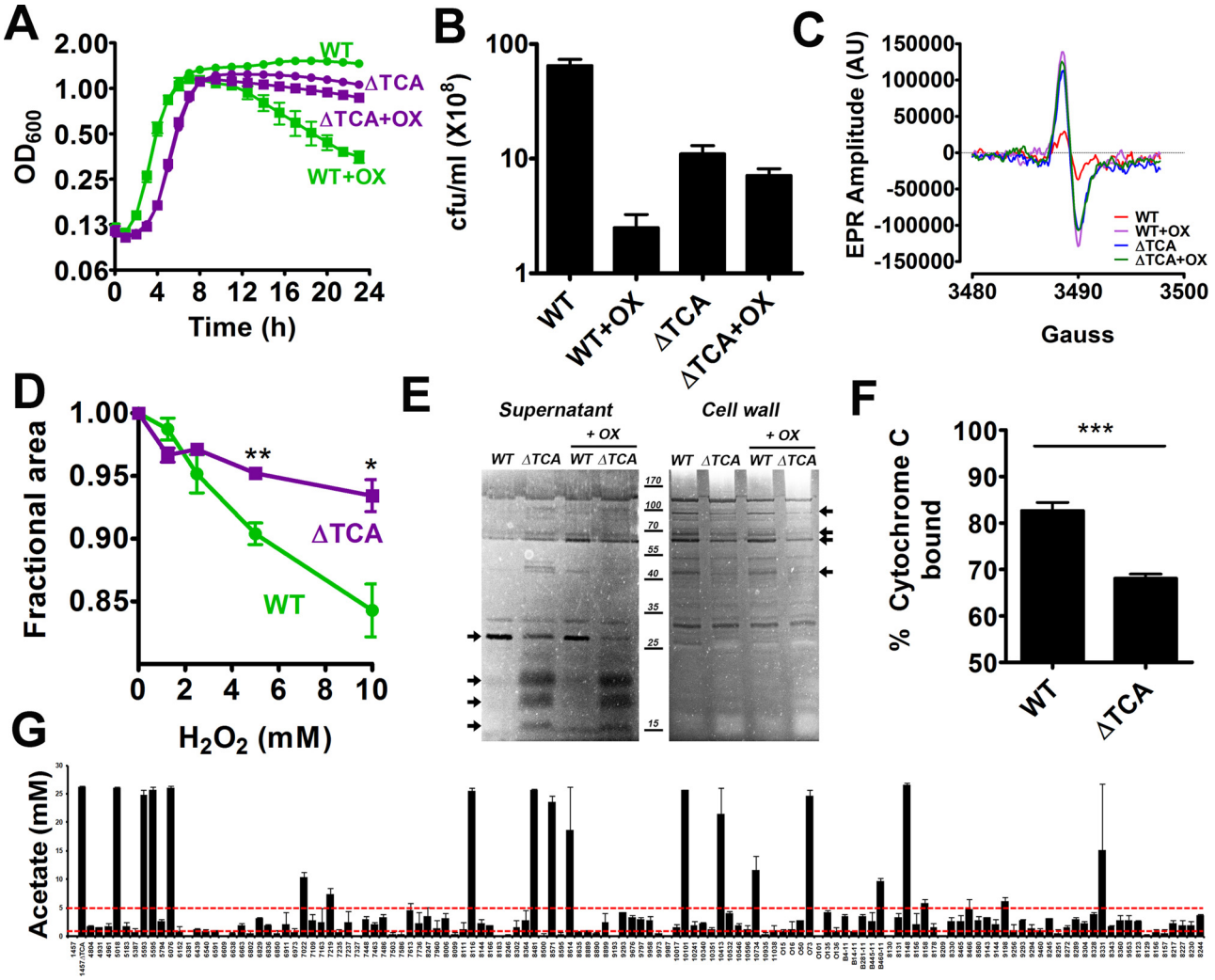

FIG 2 Nature of the fitness cost imposed by an active TCA cycle and its clinical implications. (A) *S. epidermidis* growth and lysis in the presence or absence of sub-MIC concentrations of oxacillin (OX; 32 ng/ml) was determined by monitoring OD_{600}. (B) Cell viabilities of the WT and ΔTCA mutant following growth in 32 ng/μliter oxacillin were determined 24 h postinoculation by plating on solid media ($n = 3$). (C) Generation of oxygen radicals following antibiotic challenge was determined by whole-cell EPR analysis for all isolates (as described in text) after normalizing to an OD_{600} of 10 ($n = 3$; AU, arbitrary units). (D) Sensitivity of strains to hydrogen peroxide was carried out in TSB (no glucose) using the Lambert and Pearson method (10) ($n = 3$; Student's *t* test; *, $P < 0.05$; **, $P < 0.005$). (E) Zymogram analysis was performed using *S. epidermidis* native peptidoglycan as the substrate for visualization of autolysin activity. Equal concentrations of cell supernatant and whole-cell proteins (12 ng) were loaded in each well. Black arrows indicate autolytic activities of interest described in text. (F) Cytochrome *c* binding assays (measure of cell surface charge) were performed as previously described (23) (unpaired Student's *t* test; $n = 6$; ***, $P < 0.0005$). (G) Acetate concentrations in culture supernatants of clinical *S. epidermidis* isolates were measured 24 h postinoculation using a commercially available kit (R-Biopharm, Germany). Red (dashed) lines indicate boundaries used for classification of TCA activity in clinical isolates based on acetate levels in culture supernatants.

1 mM acetate), moderate (1 to 5 mM acetate), and low (more than 5 mM acetate) oxidizers of acetate. Our results suggest that at least 14.2% of the isolates had strong TCA cycle dysfunctions, while a majority of the remaining isolates (57.9%) had adaptations that led to a slower metabolic flux through the TCA cycle in comparison to that of the WT (Fig. 2G).

In conclusion, although our studies do not entirely support a common, ROS-dependent mechanism of action for all bactericidal antibiotics (as proposed by Kohanski et al. [4]), several distinct lines of evidence suggest a partial dependence of the bactericidal action of β-lactam antibiotics on the generation of TCA cycle-dependent ROS. First, while the addition of sub-MIC oxacillin to the WT resulted in a significant increase in the production of ROS, no such increase was observed for the ΔTCA mutant upon antibiotic challenge, suggesting that oxacillin indeed mediated TCA cycle-dependent ROS production. Further, the lack of cell lysis in the ΔTCA mutant upon oxacillin challenge correlates with the absence of oxacillin-dependent ROS production. Second, alleviation of antibiotic-induced ROS by thiourea or dipyridyl partially rescued the β-lactam-mediated lysis of *S. epidermidis* 1457. A similar decrease in antibiotic-dependent lethality was also recently confirmed in *Staphylococcus aureus* by an independent study (5). Finally, mutations in the TCA cycle not only passively alleviated β-lactam-induced ROS production but also actively increased oxidative stress tolerance and altered cell surface properties that countered the bactericidal action of β-lactam antibiotics. It is

likely for this very same reason (altered cell surface properties) that other classes of antibiotics that do not target the cell wall or whose action is not dependent on autolysins have failed to show a TCA cycle-dependent phenotype. Unsurprisingly, several *S. epidermidis* clinical isolates exhibit TCA cycle dysfunctions. Such metabolic anomalies may be advantageous in antibiotic-selective environments, such as hospitals, as they promote protection against antibiotics. In addition, reduced TCA cycle activity is also known to promote biofilm development in a PIA-dependent manner (2). Thus, modulation of TCA cycle activity may represent a common priming mechanism against various stresses bacteria encounter in the clinical environment.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00437-13/-/DCSupplemental.

Figure S1 , TIF file, 2.8 MB.

Figure S2 , TIF file, 2.6 MB.

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