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RESEARCH ARTICLE

Anaerobic *Mycobacterium tuberculosis* Cell Death Stems from Intracellular Acidification Mitigated by the DosR Regulon

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ABSTRACT *Mycobacterium tuberculosis* is a strict aerobe capable of prolonged survival in the absence of oxygen. We investigated the ability of anaerobic *M. tuberculosis* to counter challenges to internal pH homeostasis in the absence of aerobic respiration, the primary mechanism of proton efflux for aerobic bacilli. Anaerobic *M. tuberculosis* populations were markedly impaired for survival under a mildly acidic pH relative to standard culture conditions. An acidic environmental pH greatly increased the susceptibilities of anaerobic bacilli to the collapse of the proton motive force by protonophores, to antimicrobial compounds that target entry into the electron transport system, and to small organic acids with uncoupling activity. However, anaerobic bacilli exhibited high tolerance against these challenges at a near-neutral pH. At a slightly alkaline pH, which was near the optimum intracellular pH, the addition of protonophores even improved the long-term survival of bacilli. Although anaerobic *M. tuberculosis* bacilli under acidic conditions maintained 40% lower ATP levels than those of bacilli under standard culture conditions, ATP loss alone could not explain the drop in viability. Protonophores decreased ATP levels by more than 90% regardless of the extracellular pH but were bactericidal only under acidic conditions, indicating that anaerobic bacilli could survive an extreme ATP loss provided that the external pH was within viable intracellular parameters. Acidic conditions drastically decreased the anaerobic survival of a DosR mutant, while an alkaline environment improved the survival of the DosR mutant. Together, these findings indicate that intracellular acidification is a primary challenge for the survival of anaerobic *M. tuberculosis* and that the DosR regulon plays a critical role in sustaining internal pH homeostasis.

IMPORTANCE During infection, *M. tuberculosis* bacilli are prevalent in environments largely devoid of oxygen, yet the factors that influence the survival of these severely growth-limited and metabolically limited bacilli remain poorly understood. We determined how anaerobic bacilli respond to fluctuations in environmental pH and observed that these bacilli were highly susceptible to stresses that promoted internal acidic stress, whereas conditions that promoted an alkaline internal pH promoted long-term survival even during severe ATP depletion. The DosR regulon, a major regulator of general hypoxic stress, played an important role in maintaining internal pH homeostasis under anaerobic conditions. Together, these findings indicate that in the absence of aerobic respiration, protection from internal acidification is crucial for long-term *M. tuberculosis* survival.

KEYWORDS DosR, *Mycobacterium tuberculosis*, anaerobic, dormancy, hypoxia, pH homeostasis

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Tuberculosis (TB) remains humanity's deadliest bacterial disease, with nearly 10 million new cases and 1.5 million deaths per year (1). A substantial proportion of new active cases arises from the spontaneous reactivation of latent disease originating from the vast reservoir of the world's nearly 2 billion latently infected individuals. The reservoir of latently infected individuals and the diversity of infection environments necessitate an understanding of *M. tuberculosis* physiology in different host microenvironments when considering new approaches for TB treatment.

As a strict aerobe, the growth of *Mycobacterium tuberculosis* is dependent on O_2, but during infection, *M. tuberculosis* experiences a wide range of O_2 concentrations ranging from high atmospheric O_2 concentrations during aerosolization, transmission, and initial infection to prolonged exposure to hypoxic and anaerobic microenvironments within necrotic granulomas. Hypoxia exposure is often used as a proxy for latency, as bacilli in latent infection appear to reside in environments largely devoid of O_2. Antigens encoded by genes in the DosR regulon, the primary hypoxia-induced regulon (2, 3), are highly recognized by T cells from latently infected individuals (4). A *dosR* mutant exhibited disease progression comparable to that of wild-type *M. tuberculosis* in immunocompetent BALB/c mice that do not form necrotic hypoxic granulomas (5). However, a study performed in macaques, which form hypoxic caseous necrotic granulomas, demonstrated that the DosR regulon is required for persistence and active disease (6). Under *in vitro* anaerobic conditions, *M. tuberculosis* bacilli cease growth, dramatically alter gene expression, and adopt a nonreplicating, quasidormant physiological state. Additionally, nonreplicating anaerobic bacilli exhibit decreased susceptibility to numerous antimycobacterial compounds (e.g., isoniazid, rifampin, and ciprofloxacin), similar to the difficult-to-eradicate persister populations characteristic of mycobacterial infections (7–10).

The high-level expression of the DosR regulon under conditions of limited aerobic respiration (e.g., exposure to hypoxia or NO or CO gasses) has been well described for *M. tuberculosis* and other related mycobacteria (11). DosR-controlled proteins mediate the adaptation to hypoxia and anaerobiosis in part by slowing O_2 consumption; inducing nitrate uptake for respiration, if available (12, 13); and synthesizing triacylglycerides as a potential electron sink and food source following reaeration (8, 14). These physiological adaptations protect bacilli during long-term exposure to hypoxia, enable the maintenance of ATP levels and redox balance, and assist in recovery from anaerobic dormancy upon reaeration (2, 15).

While our understanding of anaerobic metabolism in *M. tuberculosis* is incomplete, multiple investigations have observed that succinate accumulates to high levels in the extracellular medium upon entry into a hypoxic or anaerobic state (16, 17). It was proposed that succinate secretion is essential for maintaining the proton motive force (PMF) in hypoxic and anaerobic bacilli. Secreted succinate likely arises from one of two routes: through the reduction of fumarate to succinate in a truncated reductive tricarboxylic acid (rTCA) cycle (16) or through the action of isocitrate lyase via the glyoxylate shunt (17). Both proposed routes of succinate production have been suggested to assist in the maintenance of redox potential either by consuming excess reducing equivalents via the rTCA cycle (16) or by slowing NADH generation through the use of isocitrate lyase and the glyoxylate shunt (17).

In addition to hypoxia, pH is another important environmental factor that varies widely for different subpopulations of *M. tuberculosis* during infection. Within the phagosome, the pH fluctuates from roughly 6.2 in the phagosome of immature macrophages to as low as 4.5 in the phagolysosome following gamma interferon (IFN-γ) activation (18). However, the environmental pH can be considerably higher for extracellular bacilli such as those within the necrotic core of a granuloma, in which the pH is considerably more alkaline and approaches the blood pH of 7.4 (19). Phagolysosome acidification is important for the immune control of *M. tuberculosis* and has prompted numerous investigations into the response of *M. tuberculosis* to acid stress (18, 20–26). While the majority of those investigations looked at aerobic bacilli, it is also important to consider the importance of acidic stress during hypoxia and anaerobiosis.

During exposure to hypoxia, respiratory proton efflux slows dramatically. As a consequence, the capacity of bacilli to maintain cytosolic pH homeostasis and protect against acidification is compromised. Furthermore, it has been shown that under hypoxic conditions, *M. tuberculosis* bacilli cultured at pH 5.5 exhibit impaired survival compared to bacilli incubated at pH 6.6. Bacillus survival was restored by the addition of nitrate, indicating that respiration could play an important role in anaerobic pH homeostasis (27).

In this study, we investigated the importance of pH homeostasis for the survival of anaerobic *M. tuberculosis* populations. We found that moderate acid stress significantly impaired the long-term survival of anaerobic *M. tuberculosis* bacilli. Additionally, acid stress rendered the bacilli highly sensitive to the protonophore-mediated collapse in the PMF, the inhibition of the electron transport system, and the uncoupling activity of small organic acids. Conversely, an environmental pH near the normal intracellular pH allowed *M. tuberculosis* to be highly tolerant to these challenges against the energized membrane. Thus, we conclude that these challenges were lethal primarily because they promoted intracellular acidification, and they were nonlethal if the extracellular pH was close to the intracellular pH. Furthermore, we discovered a new role for DosR regulon proteins, from the finding that under anaerobic conditions, a *dosR* mutant was highly susceptible to disruptions in intracellular pH homeostasis. This finding indicated that the DosR regulon plays a protective role against acidic stress in anaerobic *M. tuberculosis* populations in addition to its role in general protection from hypoxic stress.

RESULTS

Acidic extracellular pH decreases the viability of anaerobic *M. tuberculosis* bacilli. To test the impact of pH fluctuation on anaerobic bacilli, *M. tuberculosis* cultures were subjected to a shift from aerobic growth to anaerobic stasis by using the rapid anaerobic dormancy (RAD) model (8) followed by adjustment of the culture pH. Within 48 h of complete O_2 depletion, RAD model-cultured bacilli achieve homeostatic ATP levels and NAD/NADH ratios (7, 8, 16). Both parameters remained relatively constant until at least day 20 in the model and possibly longer, as bacilli do not die until after 40 days in this closed system (8). Likewise, the culture pH was maintained at 6.6 $\pm$ 0.1 (see Fig. S1 in the supplemental material). While a pH of 6.6 is typical for the macrophage intraphagosomal environment (18, 19, 28), the slightly acidic pH of typical *M. tuberculosis* media does not reflect the full pH range experienced by hypoxic/anoxic *M. tuberculosis* bacilli within different tissue microenvironments or at different stages of granuloma development (28–30). Thus, at day 12, the pH of RAD model cultures was adjusted to either pH 5.6, similar to the pH of the phagosomal environment (28–30), or pH 7.4, which is reflective of late-stage granulomas (19). Viability was assessed periodically for 29 days following pH adjustment. While long-term viabilities were comparable for cultures at pHs 6.6 and 7.4, viability was dramatically reduced in cultures adjusted to pH 5.6 (Fig. 1), indicating that anaerobic bacilli require an approximately neutral extracellular pH for optimal long-term survival.

Cellular ATP levels and membrane potential change as a function of extracellular pH. To determine the cause of cell death during low pH, we investigated two factors likely impacted by a change in the extracellular pH: ATP, a measure of the cellular energetic state, and PMF. Both parameters were measured 24 h following adjustment of the extracellular pH as described previously (7, 8, 31) (Table 1). ATP levels and membrane potential ($\Delta\Psi$) values changed as a function of the extracellular pH, with cells at pH 5.6 exhibiting approximately 2-fold less ATP than cells at pH 7.4 (Table 1). Likewise, when the culture pH was reduced to 5.6, we observed a decrease in the $\Delta\Psi$ relative to unchallenged controls and an increase in the $\Delta\Psi$ following adjustment up to pH 7.4 (Table 1).

Anaerobic *M. tuberculosis* bacilli in acidic but not neutral extracellular pH are susceptible to PMF collapse. To amplify internal pH stress, anaerobic *M. tuberculosis* cultures were challenged by the addition of two different protonophores, 2,4-dinitrophenol (DNP) and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). Proto-

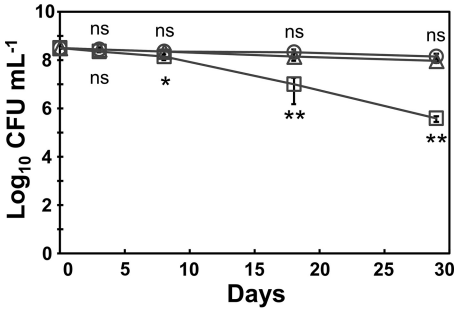

FIG 1 Anaerobic survival of *M. tuberculosis* is impaired following mild acidic challenge. *M. tuberculosis* H37Rv bacilli cultured for 12 days to anaerobiosis in the RAD model were pH stressed by shifting the culture pH to pH 5.6 or pH 7.4. Circles represent population survival for unadjusted cultures at pH 6.6, squares represent cultures adjusted to pH 5.6, and triangles represent cultures adjusted to pH 7.4. All symbols represent averages and standard deviations of results from triplicate biological replicates. Asterisks represent statistical significance comparing the survival of cultures at pHs 5.6 and 7.4 to that of cultures at pH 6.6 (*, $P \leq 0.05$; **, $P \leq 0.01$; ns, not significant).

nophores challenge intracellular pH homeostasis as they drive the internal pH toward the external pH (32–34). At pH 5.6, both protonophores were bactericidal against anaerobic bacilli. The addition of 0.1 mM CCCP led to a decrease in viability versus that of control cultures (Fig. 2). A similar decrease in viability was observed following the addition of 1.0 mM DNP at pH 5.6, and doubling the concentration of DNP greatly enhanced its bactericidal activity (Fig. 2). Conversely, neither protonophore exhibited appreciable bactericidal activity at pH 6.6 or 7.4, even after 8 days of exposure (Fig. 2). Furthermore, increasing the DNP concentration from 1.0 to 2.0 mM did not change the bactericidal activity at pH 6.6 or 7.4 (Fig. 2). Impaired long-term survival at low pH, together with the inability of anaerobic bacilli to combat protonophore-induced stress, strongly suggested that a failure to maintain internal pH homeostasis was a major cause of anaerobic death. We observed a similar trend in protonophore-induced bactericidal activity against aerobic bacilli under nutrient-deprived conditions. Bacilli in this severely limited growth and respiratory state exhibited substantially greater susceptibility to protonophore activity at acidic pH than did bacilli at neutral or alkaline pH, further supporting the concept that under conditions that reduce respiration, bacilli are highly susceptible to acidic stress (see Fig. S2 in the supplemental material).

Protonophore activity decreased both ATP levels and membrane potential at every extracellular pH (Tables 1 and 2). This was expected given that protonophores partially uncouple ATP synthase from the electron transport system by reducing the PMF. ATP depletion likely exacerbated problems associated with acidic pH; however, decreased ATP levels alone were not sufficient to kill anaerobic bacilli provided that the external

TABLE 1 ATP and membrane potential[a]

Strain	pH	Avg ATP level (RLU) ± SD	Avg ΔΨ (mV) ± SD
H37Rv	5.6	$60.8 \pm 8.8^{*}$	$52.1 \pm 4.3^{***}$
	6.6	87.4 ± 11.2	73.0 ± 2.2
	7.4	$110 \pm 4^{*}$	83.4 ± 6.7^{NS}
Δ3132c-4c	5.6	$9.80 \pm 3.51_{**}^{NS}$	$39.4 \pm 9.2_{*}^{*}$
	6.6	$20.3 \pm 4.7_{***}$	$59.3 \pm 4.1_{**}$
	7.4	$41.2 \pm 5.7_{***}^{*}$	$59.8 \pm 11.1_{**}^{NS}$
Δ3132c-4c complement	5.6	$54.3 \pm 10.7_{NS}^{*}$	$60.9 \pm 2.9_{NS}^{NS}$
	6.6	$106 \pm 0_{NS}$	$\mathit{72.9 \pm 4.9}_{NS}^{NS}$
	7.4	$125 \pm 5_{NS}^{*}$	$85.0 \pm 11.2_{NS}^{NS}$

[a]Superscript symbols represent statistical significance comparing either ATP or ΔΨ values for cultures at pH 5.6 or 7.4 to those of cultures at pH 6.6 for the same strain, and subscript symbols represent statistical significance comparing H37Rv and Δ3132-4c or Δ3132-4c complement at the same pH (single asterisk, $P \leq 0.05$; double asterisks, $P \leq 0.01$; triple asterisks, $P \leq 0.001$; NS, not significant). RLU, relative light units.

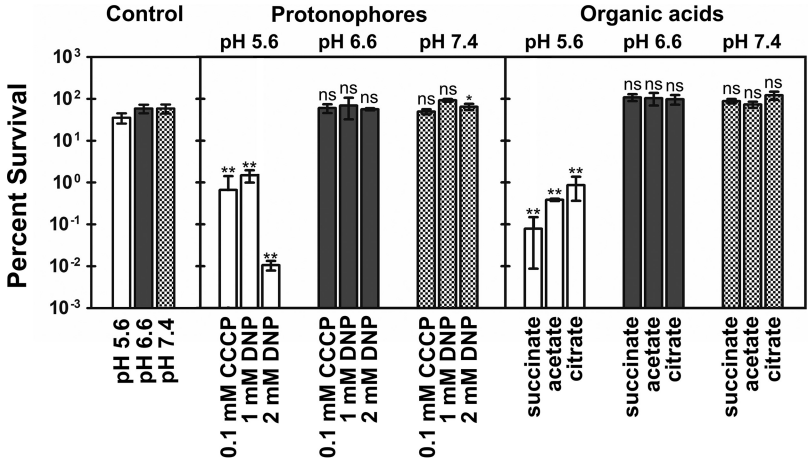

FIG 2 Anaerobic *M. tuberculosis* populations are susceptible to collapse of the PMF only under acidic conditions. *M. tuberculosis* H37Rv bacilli cultured to day 12 in the RAD model were treated for 8 days with 0.1 mM CCCP; 1 or 2 mM DNP; or 20 mM succinate, acetate, or citrate following pH adjustment of cultures to pH 5.6, 6.6, or 7.4. The left panel depicts the percent survival of control cultures at pHs 5.6, 6.6, and 7.4; the middle panel (protonophores) depicts the percent survival of CCCP- and DNP-treated cultures at pHs 5.6, 6.6, and 7.4; and the right panel (organic acids) depicts the survival of succinate-, acetate-, and citrate-treated cultures at pHs 5.6, 6.6, and 7.4. White bars represent cultures at pH 5.6, black bars represent cultures at pH 6.6, and checkered bars represent cultures at pH 7.4. Data represent averages and standard deviations of results from triplicate biological cultures. Asterisks represent statistical significance, where indicated, comparing protonophore- or organic acid-treated cultures to control cultures at the same pH (*, $P \leq 0.05$; **, $P \leq 0.01$; ns, not significant).

pH was near the optimal internal pH, as the addition of protonophores at pHs 6.6 and 7.4 was not lethal, even though ATP levels were very low.

Like protonophores, small organic acids can function as uncoupling agents under conditions of acidic pH (35, 36). Due to low pK_a values of generally <5, most small organic acids exist primarily in the unprotonated anionic state at physiological pH. Under acidic conditions, increasing proportions of the uncharged protonated forms exist, which can freely transverse the cell membrane and then dissociate, becoming sequestered inside the more alkaline cytosolic environment. This net movement of extracellular protons into the cell decreases the internal pH and compromises the pH gradient (35, 36). We tested the impact of three common organic acids, acetate, succinate, and citrate, on the survival of *M. tuberculosis* under strict anaerobic conditions. These compounds are all common metabolic intermediates in central metabolism and are representative of mono-, di-, and tricarboxylic acids. A concentration of 20 mM each acid was added to anaerobic cultures following pH adjustment. As was observed with protonophores, all three organic acids led to a dramatic drop in cell viability at pH 5.6 and no drop in viability at pH 6.6 or 7.4 (Fig. 2). Likewise, ATP levels and $\Delta\Psi$ values were substantially lower for bacilli treated with succinate than for nontreated bacilli at pH 5.6. However, the addition of succinate had no negative impact on the ATP level or the $\Delta\Psi$ for bacilli at pH 6.6 or 7.4, indicating that the uncoupling activity of succinate required acidic conditions (Table 2).

TABLE 2 ATP and membrane potential after 24 h of treatment with protonophores, succinate, or chlorpromazine[a]

	Avg ATP level (RLU) ± SD at pH:			Avg $\Delta\Psi$ (mV) ± SD at pH:		
Condition	**5.6**	**6.6**	**7.4**	**5.6**	**6.6**	**7.4**
DNP (1 mM)	$2.08 \pm 0.86^{***}$	$20.9 \pm 2.1^{**}$	$26.4 \pm 1.9^{***}$	$38.9 \pm 2.1^{*}$	$45.1 \pm 1.2^{**}$	70.2 ± 4.0^{NS}
CCCP (0.1 mM)	$20.4 \pm 2.5^{**}$	$15.7 \pm 0.6^{**}$	$27.0 \pm 3.3^{***}$	$35.4 \pm 2.1^{*}$	$45.1 \pm 1.2^{**}$	$54.2 \pm 4.5^{*}$
Succinate (20 mM)	$0.058 \pm 0.012^{***}$	97.8 ± 5.1^{NS}	$82.7 \pm 6.0^{*}$	$20.0 \pm 3.0^{***}$	75.2 ± 3.7^{NS}	96.7 ± 3.6^{NS}
CPZ (50 μg/ml)	$0.52 \pm 0.35^{***}$	$7.95 \pm 3.02^{**}$	$55.0 \pm 15.5^{*}$	$28.8 \pm 2.1^{**}$	$43.2 \pm 6.2^{**}$	$64.5 \pm 4.3^{*}$

[a]Asterisks represent statistical significance comparing either ATP or $\Delta\Psi$ values under the indicated conditions to those of untreated cultures of H37Rv (Table 1) at the same pH (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; NS, not significant).

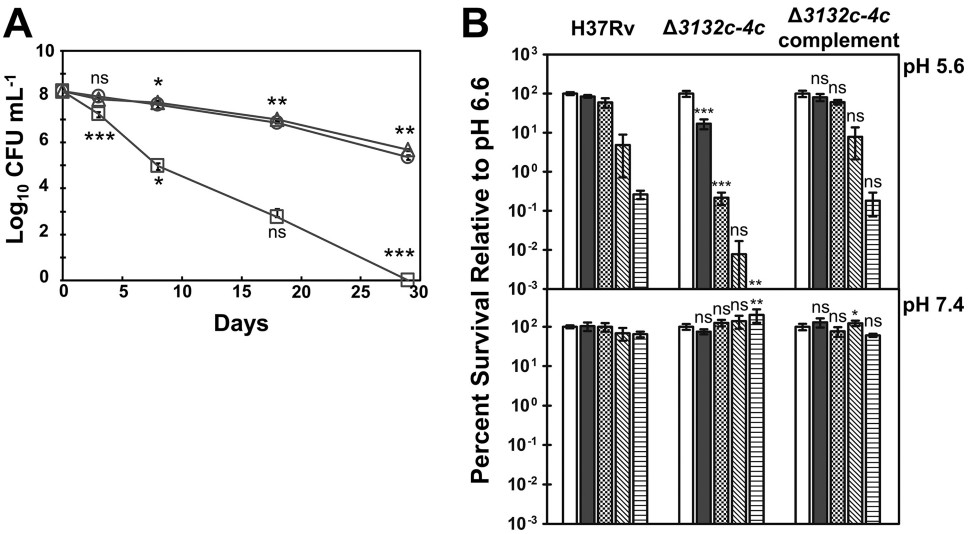

FIG 3 A DosR mutant exhibits enhanced susceptibility to acidic stress under anaerobic conditions. Cultures of *M. tuberculosis* H37Rv, Δ3132c-4c, and Δ3132c-4c complement were cultured to day 12 in the RAD model and pH stressed by shifting the culture pH to 5.6 or 7.4. (A) Survival of Δ3132c-4c cultures following pH adjustment. Circles represent population survival for unchallenged cultures at pH 6.6, squares represent cultures adjusted to pH 5.6, and triangles represent cultures adjusted to pH 7.4. Data represent averages and standard deviations of results from triplicate biological replicates. (B) Relative survival of H37Rv, Δ3132c-4c, and Δ3132c-4c complement adjusted to pH 5.6 (top) or pH 7.4 (bottom) versus unchallenged controls at pH 6.6. White bars represent day 0 following the pH shift, black bars represent day 3, checkered bars represent day 8, diagonally striped bars represent day 18, and horizontally striped bars represent day 29. Statistics in panel A compare the survival of Δ3132c-4c cultures at pH 6.6 to that at pH 5.6 or 7.4. Statistics in panel B compare the relative survival of H37Rv to the survival of Δ3132c-4c and Δ3132c-4c complement at each indicated pH and time point (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns, not significant).

The DosR regulon confers anaerobic acidic stress tolerance. As the induction of the DosR regulon is critical for numerous aspects of adaptation to hypoxic survival and metabolism (8), we tested whether DosR expression contributed to the protection of anaerobic bacilli from acidic stress. The DosR regulon mutant strain (H37Rv ΔRv3132c-4c::*hyg*) deleted for both the response regulator DosR and the sensor kinase DosS in the DosR regulon and the complemented strain (Δ3132c-4c complement) (8) were grown under anaerobic conditions in the RAD model and subjected to pH adjustments as described above for H37Rv. The medium pHs for both Δ3132c-4c (pH 6.59 ± 0.01) and Δ3132c-4c complement (pH 6.61 ± 0.01) were nearly indistinguishable from that for H37Rv (see Fig. S1 in the supplemental material) prior to pH adjustment. Survival defects observed at pH 5.6 for H37Rv were magnified for Δ3132c-4c (Fig. 3A and B), suggesting that in addition to providing protection against general hypoxic stress, the DosR regulon protects anaerobic *M. tuberculosis* from acidic stress. Interestingly, Δ*3132c-4c* survival was improved at pH 7.4 relative to pH 6.6 (Fig. 3B). The severe defect in Δ3132c-4c survival at acidic pH relative to the wild-type and complemented strains and the finding that increased extracellular pH improved the survival of Δ3132c-4c suggest that a failure to prevent intracellular acidification is a major cause of cell death in the DosR regulon mutant at both pH 5.6 and pH 6.6. Additionally, as with H37Rv, the ATP level decreased as a function of extracellular pH in Δ3132c-4c (Table 1). However, as was previously observed (8), ATP levels were far lower in Δ3132c-4c than in H37Rv at all extracellular pH values. Furthermore, the drop in the ATP level at pH 5.6 was more severe in Δ3132c-4c than in H37Rv or the complemented strain. Thus, the combination of internal acid stress and extreme ATP depletion likely further exacerbated the killing of the DosR regulon mutant at pH 5.6.

The survival of Δ3132c-4c following protonophore-induced stress was also tested. DNP was highly bactericidal under acidic conditions. However, the rate and magnitude of the DNP bactericidal activity were far higher in Δ3132c-4c than in H37Rv (Fig. 4 and Fig. S3). Viable Δ3132c-4c bacilli were undetectable after 18 days of DNP exposure,

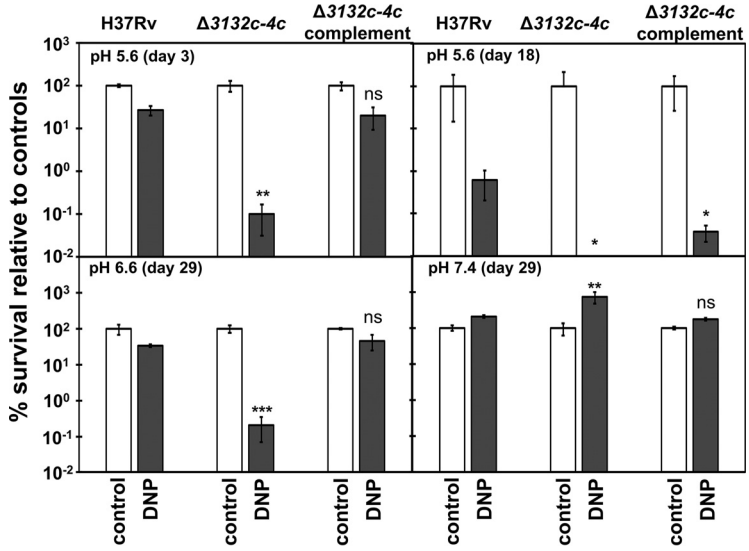

FIG 4 Protonophore treatment is highly bactericidal to a DosR mutant at pH 5.6 but improves long-term survival at pH 7.4. Wild-type strain H37Rv, Δ3132c-4c, and Δ3132c-4c complement were cultured to day 12 in the RAD model and challenged with 1 mM DNP immediately following culture pH adjustment. Survival of untreated control cultures at pHs 5.6, 6.6, and 7.4 was normalized to 100% at the indicated time points. Relative survival of DNP-treated versus untreated controls at the same pH is shown for days 3 and 18 postchallenge. Day 29 is not shown for pH 5.6 due to the CFU being below the limit of detection for both control and DNP-treated samples of H37Rv ΔRv3132c-4c::*hyg*. White bars depict control cultures at the indicated pH, and gray bars depict DNP-treated cultures at the indicated pH. Asterisks represent statistical significance, where indicated, comparing the relative survival of DNP-treated Δ3132c-4c cultures to that of DNP-treated H37Rv cultures at the same time point (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns, not significant).

whereas viability assays showed that H37Rv and Δ3132c-4c complement bacilli did not fall below the limit of detection until after 29 days (Fig. 4 and Fig. S3). Protonophore bactericidal activity was also much higher against Δ3132c-4c than against the wild type at pH 6.6 (Fig. 4). Treatment of Δ3132c-4c with 1 mM DNP at pH 6.6 led to a progressive drop in culture viability. The same treatment had virtually no bactericidal activity against H37Rv after 18 days and decreased viability only slightly by day 29 (Fig. 4 and Fig. S3). Survival dynamics in Δ3132c-4c complement cultures were similar to those in H37Rv cultures (Fig. 4 and Fig. S3). Interestingly, long-term DNP treatment at pH 7.4 improved the survival of anaerobic bacilli relative to mock treatment (Fig. 4). The observation that protonophore activity increased survival when the extracellular pH was near the homeostatic intracellular pH further supports the model that a failure to maintain intracellular pH and not primarily the loss of PMF is a primary contributor to cell death for anaerobic bacilli.

Loss of DosR expression and acidic pH enhance susceptibility of anaerobic bacilli to the Ndh2 inhibitor chlorpromazine. PMF is generated and maintained primarily by the electron transport system (37). Therefore, decreases in $\Delta\Psi$ values and ATP levels combined with increased susceptibility to protonophores suggested the involvement of an electron transport system in the protection of anaerobic *M. tuberculosis* from acidification. Furthermore, the greater susceptibility of the DosR regulon mutant to mild acidity and protonophore activity implicates the involvement of the DosR regulon in this protection. We examined the role of the electron transport system using the phenothiazine chlorpromazine (CPZ), a proposed inhibitor of the membrane-bound type 2 NADH dehydrogenase (Ndh2) (38, 39). Ndh2 is an essential component of both the aerobic and anaerobic electron transport systems in *M. tuberculosis* and the primary entry point to the electron transport system for electrons coming from NADH (31). Immediately following culture pH adjustment, chlorpromazine was administered at day 12 in the RAD model to H37Rv, Δ3132c-4c, and Δ3132c-4c complement cultures at pH 6.6 and to H37Rv RAD model cultures at pHs 5.6, 6.6, and 7.4. Consistent with the

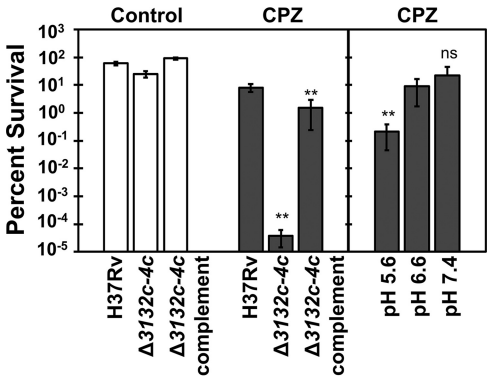

FIG 5 The DosR regulon and neutral to alkaline pH are required for protection against chlorpromazine treatment. Cultures were treated for 8 days with 50 μg ml^{-1} CPZ administered at day 12 in the RAD model. (Left) Percent survival for CPZ-treated cultures versus untreated control cultures of H37Rv, Δ3132c-4c, and Δ3132c-4c complement. (Right) Percent survival for CPZ-treated cultures of H37Rv at pHs 5.6, 6.6, and 7.4. Data represent averages and standard deviations of results from three biological replicate cultures. Statistics compare the survival of CPZ-treated H37Rv bacilli at pH 6.6 to that under all other conditions (*, $P \leq 0.05$; **, $P \leq 0.01$; ns, not significant).

induction of the DosR regulon playing a critical role in the protection of anaerobic bacilli against Ndh2 inhibition, Δ3132c-4c was vastly more susceptible to chlorpromazine than was H37Rv (Fig. 5). As was the case for the protonophores and organic acids, chlorpromazine-mediated bactericidal activity against H37Rv was enhanced at pH 5.6 relative to pHs 6.6 and 7.4 (Fig. 5). Furthermore, ATP levels and $\Delta\Psi$ values following chlorpromazine treatment were substantially lower at pH 5.6 than at either pH 6.6 or 7.4 (Table 2).

DISCUSSION

We investigated how anaerobic *M. tuberculosis* copes with challenges to internal pH homeostasis. Our findings indicate that the prevention of internal acidification is critical for long-term survival in the absence of respiration and growth. A failure to maintain cytosolic pH homeostasis appears to be the basis for the death of bacilli in the absence of oxygen. Furthermore, the DosR regulon plays a key role in internal pH homeostasis for anaerobic *M. tuberculosis*, although the entirety of the mechanisms used by anaerobic bacilli to maintain internal pH homeostasis remains unclear. These findings may provide novel strategies for targeting difficult-to-eradicate hypoxic bacilli that take into consideration pH homeostasis.

One of the primary challenges faced by *M. tuberculosis* during exposure to extreme oxygen limitation is the generation of a proton gradient necessary for ATP production, transport functions, and the maintenance of intracellular pH homeostasis. The addition of protonophores to anaerobic *M. tuberculosis* cultures induced pronounced drops in both ATP levels and $\Delta\Psi$ values. However, protonophores proved lethal only at pH 5.6, which suggested that anaerobic bacilli could tolerate severe drops in either ATP levels or membrane potential provided that the pH of the external environment was within the intracellular pH homeostatic range, which is close to pH 7.6 (33, 40, 41). A DosR mutant strain, which had impaired anaerobic survival, ATP, and $\Delta\Psi$, was hypersensitive to the addition of protonophores relative to wild-type H37Rv at both pHs 5.6 and 6.6, suggesting that the combined stresses of acidic pH and low ATP levels exacerbated cell death. However, the fact that survival of the DosR mutant was improved by the addition of protonophores at pH 7.4 strongly suggests that the failure to maintain internal pH is a primary cause of cell death in anaerobic cells not expressing the DosR regulon.

M. tuberculosis populations experiencing acidic stress are susceptible to agents known to collapse the PMF (33, 40). However, the present findings with respect to protonophores appear to be at odds with data from a study that found that anaerobic *M. tuberculosis* bacilli were exquisitely sensitive to the ionophores valinomycin and

nigericin, which collapse $\Delta\Psi$ and the membrane proton gradient (ΔpH), respectively (31). It is possible that valinomycin and nigericin, which are K^+ and K^+/H^+ ionophores, induce particular bactericidal effects not exhibited by the specific protonophores used in this study. The use of related but different *in vitro* models of hypoxia (i.e., the Wayne model versus the RAD model) may also explain this discrepancy (7, 8, 42). Although these models are conceptually similar, the long-term survival of bacilli is substantially improved in the RAD model versus the Wayne model. Wayne model-cultivated bacilli exhibit a half-life of 11 days from the onset of anaerobiosis, whereas RAD model-cultured bacilli exhibit near-complete survival for over 30 days after the onset of anaerobiosis (7, 8). Thus, it is possible that bacilli cultivated by using the RAD model are experiencing less stress overall than those cultivated by using the Wayne model and are more poised to deal with challenges to internal pH homeostasis.

Tan et al. found that under nonreplicating hypoxic conditions, *M. tuberculosis* bacilli cultured at pH 5.5 survive poorly compared to bacilli incubated at pH 6.6 (27). Those authors attributed this impaired survival to decreases in the ATP level and membrane potential induced by acidity. Interestingly, both ATP and membrane potential could be restored by the addition of nitrate as a terminal electron acceptor for anaerobic respiration, indicating that respiration plays a critical role in cellular pH homeostasis (27). Although we did not revisit the use of nitrate respiration in the present study, and we did not include nitrate in our media, the use of nitrate for protection against acidic stress implicates the DosR regulon in other mechanisms for acidic stress tolerance. The terminal nitrate reductase complex NarGHIJ is not DosR controlled (13). However, hypoxic and anaerobic nitrate respiration require the expression of the DosR regulon protein NarK2, which is an antiporter required for nitrate uptake and nitrite export (12, 13), and DosR mutants are highly attenuated for nitrate respiration (13). Thus, wild-type levels of the DosR regulon are necessary for protection against acidic stress under both anoxic nitrate-respiring and nonrespiring conditions.

The differential usage of terminal cytochrome oxidases could also partially explain the heightened sensitivity to acidic stress during hypoxia. During growth under oxygen-replete conditions, *M. tuberculosis* and other mycobacterial species preferentially utilize the low-affinity, higher-energy-conserving cytochrome bc_1 reductase and cytochrome aa_3 oxidase, which translocates 3 protons for every electron transferred to oxygen. Conversely, the high-affinity, lower-energy-conserving cytochrome *bd* menaquinol oxidase, which translocates only one proton per electron transferred to oxygen, is preferentially utilized under conditions of severe hypoxia. Thus, in addition to having less free oxygen to utilize as a terminal oxygen acceptor, hypoxic *M. tuberculosis* bacilli likely also have a reduced capacity to combat challenges to internal acidification due to the use of a lower-proton-translocating terminal cytochrome oxidase (43–45).

Our findings with respect to chlorpromazine further highlight the importance of respiration in maintaining pH homeostasis. We previously observed that under aerobic conditions, the bactericidal activity of chlorpromazine against both *M. tuberculosis* and *Mycobacterium smegmatis* is increased at an alkaline pH of 7.6 relative to pH 5.6 (41). We speculated that this pattern of killing by chlorpromazine under aerobic conditions results from the alkalization of the cytosol due to excessive respiratory proton efflux without a concomitant increase in ATP synthase activity. In support of this model, chlorpromazine treatment against *M. smegmatis* increases both the mean intracellular pH and the proportion of the bacillus population experiencing an extremely high intracellular pH ($\geq$8.6). Furthermore, the addition of protonophores in conjunction with chlorpromazine inhibits the bactericidal, but not the bacteriostatic, activity of the drug and prevents a large-scale increase in the population intracellular pH. Interestingly, the findings in the present study indicate that the situation is reversed under anaerobic conditions, with chlorpromazine exhibiting maximum bactericidal activity at pH 5.6. Thus, it may be that when terminal electron acceptors such as oxygen and nitrate are abundant, the inhibition of Ndh2 by chlorpromazine leads to a rerouting of electrons through higher-proton-translocating alternatives to Ndh2. Such a rerouting could

ultimately lead to a dangerous increase in the intracellular pH and cell death. Conversely, when terminal electron acceptors are limited, the use of chlorpromazine presumably hinders the ability of bacilli to pump protons out of the cell, resulting in the acidification of the cytosol.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains used in this study include *M. tuberculosis* H37Rv (ATCC 27294), H37Rv ΔRv3132c-4c::*hyg* (DosR regulon mutant) (Δ3132c-4c) (5), and H37Rv ΔRv3132c-4c::*hyg*/*attB*::Rv3132c-4c (Δ3132c-4c complement), which contains the intact native promoter sequence for this regulon (5). Cultures used in RAD models were first grown with rapid stirring at 37°C in 125-ml sterile Erlenmeyer flasks with DTA medium, composed of Dubos broth base (Difco/Becton Dickinson, East Rutherford, NJ) supplemented with Tween 80 (0.05%); NaCl (0.17%); bovine serum albumin (BSA) (0.5%); glucose (0.75%); and a mineral supplement consisting of EDTA (12 μM), $MnCl_2$ (309 nM), $CoCl_2$ (84 nM), $CuSO_4$ (32 nM), Na_2MoMO_4 (62 nM), $ZnCl_2$ (73.5 nM), $LiCl_2$ (59 nM), $SnCl_2$ (11 nM), H_3BO_3 (81 nM), KBr (84 nM), KI (60 nM), $BaCl_2$ (12 nM), Na_2SeO_4 (1 μM), $NiCl_2$ (2 μM), and Na_2WO_4 (1 μM). The RAD model experiments were performed as described previously (8). Sterile glass tubes (20 by 125 mm) containing stir bars (12 mm by 4.5 mm) and 16 ml DTA medium at a culture-to-headspace ratio of 0.65 were inoculated with 360 μl of the culture at an optical density at 600 nm (OD_{600}) of 0.2. Tubes were sealed with Teflon-lined phenolic caps, and vacuum grease was applied to the threads to create an airtight seal. Cultures were stirred rapidly (200 rpm) by using a rotary magnetic tumble stirrer obtained from V&P Scientific (San Diego, CA). At the indicated time points during the RAD model experiments, cultures were opened inside an anaerobic chamber for pH adjustment, drug additions, ATP and membrane potential assays, and CFU enumerations. All CFU determinations were carried out on DTA agar plates containing 0.2% (wt/vol) activated charcoal (Sigma, St. Louis, MO).

pH adjustments. Cultures grown for 12 days in the RAD model were transferred to an anaerobic environmental chamber (Shell Lab, Cornelius, OR). The culture pH was adjusted by using sterile solutions of 5 N HCl or NaOH. Volumes of HCl and NaOH required to reach the desired pH were determined experimentally by using test cultures of H37Rv grown by using the RAD model to day 12. A volume of 38 μl of 5 N HCl was used to achieve pH 5.6, and 60 μl of 5 N NaOH was used to achieve pH 7.4. In the presence of 20 mM succinate, acetate, and citrate, volumes of 64, 38, and 64 μl of 5 N HCl, respectively, were used to achieve pH 5.6, and 64, 64, and 52 μl of 5 N NaOH, respectively, were used to achieve pH 7.4.

ATP measurements. ATP measurements were performed as described previously (7, 8). On the indicated days, sealed cultures were opened inside an anaerobic chamber, and 1 ml of each culture was pelleted for 1 min at 13,000 rpm, snap-frozen on dry ice, and stored at −80°C until processing. Frozen cell pellets were suspended in 100 μl 25 mM HEPES (pH 7.75) with 0.02% Tween 80, added to glass tubes (12 by 75 mm) containing 40 μl chloroform, and heated at 80°C for 20 min. Samples were then diluted with 4.9 ml 25 mM HEPES (pH 7.75), and diluted samples were frozen at −80°C until use. ATP assays were performed according to the manufacturer's guidelines for the Enliten ATP assay system (Promega, Madison, WI). Ten-microliter samples were added to a 96-well plate (Costar solid white), which was loaded into an L-Max microplate luminometer (Molecular Devices, Sunnyvale, CA). Enliten luciferase-luciferin reagent dissolved in sample buffer was added immediately before the results were read. Sample luminescence was determined every 0.1 s for a total of 100 s. Data are the averages of results from three independent experiments.

Membrane potential measurements. The $\Delta\Psi$ was measured as described previously (31). Following 24 h of pH challenge and/or drug addition, cultures were transferred to an anaerobic chamber, and [^{3}H]TTP (tetraphenylphosphonium bromide) or [^{3}H]water and [^{14}C]sucrose were used to measure the $\Delta\Psi$ and the internal cell volume, respectively. Culture aliquots of 1 ml were added to 2-ml screw-cap microcentrifuge tubes containing either 1 μCi [^{3}H]TTP (40 μCi mM^{-1}) or 5 μCi [^{3}H]water and 0.2 μCi [^{14}C]sucrose. For $\Delta\Psi$ determinations, samples were incubated for 30 min at 37°C, while samples for cell volume determinations were incubated for 10 min at room temperature. Samples were then pelleted for 5 min at 13,000 rpm. Twenty-microliter supernatant aliquots were diluted into 180 μl 20% (wt/vol) perchloric acid, and the pellets were suspended in 100 μl 20% (wt/vol) perchloric acid. Aliquots of 50 μl of the pellet and supernatant samples were added to 5 ml EconoSafe scintillation fluid (RPI, Mt. Prospect, IL). The radioactivity retained in each fraction was measured by scintillation counting performed in triplicate. The internal cell volume was calculated by using the following equation: internal volume = $\text{volume}_{\text{supernatant}}\{([^3\text{H}]\text{water}_{\text{pellet}}/[^3\text{H}]\text{water}_{\text{supernatant}}) - ([^{14}\text{C}]\text{sucrose}_{\text{pellet}}/[^{14}\text{C}]\text{sucrose}_{\text{supernatant}})\}$. The $\Delta\Psi$ was calculated by using the following equation: $\Delta\Psi = -2.3RT/F \log([^3\text{H}]\text{TTP}_{\text{pellet}}/[^3\text{H}]\text{TTP}_{\text{supernatant}})$, where R is the molar universal constant 8.314 J mol^{-1} K^{-1}, T is temperature in kelvins, and F is Faraday's constant (96,485 J mol^{-1}). All values represent the averages and standard deviations of results from three biological replicates, each assayed in triplicate.

Statistics. For all statistical comparisons, a paired, one-tailed t test was performed. All statistical comparisons made are described in the figure legends.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00320-17.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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