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Differences in Antibiotic-Induced Oxidative Stress Responses between Laboratory and Clinical Isolates of *Streptococcus pneumoniae*

Bédis Dridi, Andréanne Lupien, Michel G. Bergeron, Philippe Leprohon, Marc Ouellette

Centre de Recherche en Infectiologie du Centre de Recherche du CHU de Québec and Département de Microbiologie, Infectiologie et Immunologie, Faculté de Médecine, Université Laval, Québec, Canada

Oxidants were shown to contribute to the lethality of bactericidal antibiotics in different bacterial species, including the laboratory strain *Streptococcus pneumoniae* R6. Resistance to penicillin among *S. pneumoniae* R6 mutants was further shown to protect against the induction of oxidants upon exposure to unrelated bactericidal compounds. In the work described here, we expanded on these results by studying the accumulation of reactive oxygen species in the context of antibiotic sensitivity and resistance by including *S. pneumoniae* clinical isolates. In *S. pneumoniae* R6, penicillin, ciprofloxacin, and kanamycin but not the bacteriostatic linezolid, erythromycin, or tetracycline induced the accumulation of reactive oxygen species. For the three bactericidal compounds, resistance to a single molecule prevented the accumulation of oxidants upon exposure to unrelated bactericidal antibiotics, and this was accompanied by a reduced lethality. This phenomenon does not involve target site mutations but most likely implicates additional mutations occurring early during the selection of resistance to increase survival while more efficient resistance mechanisms are being selected or acquired. Bactericidal antibiotics also induced oxidants in sensitive *S. pneumoniae* clinical isolates. The importance of oxidants in the lethality of bactericidal antibiotics was less clear than for *S. pneumoniae* R6, however, since ciprofloxacin induced oxidants even in ciprofloxacin-resistant *S. pneumoniae* clinical isolates. Our results provide a clear example of the complex nature of the mode of action of antibiotics. The adaptive approach to oxidative stress of *S. pneumoniae* is peculiar, and a better understanding of the mechanism implicated in response to oxidative injury should also help clarify the role of oxidants induced by antibiotics.

Streptococcus pneumoniae is an opportunistic colonizer of the nasopharynx and the causative agent of many serious diseases, such as pneumonia, sepsis, meningitis, and otitis media (1, 2). Antimicrobial therapy based on β-lactam antibiotics is the recommended treatment regimen against pneumococcal diseases (3–5). However, resistance is now common in many countries, resulting into a shift toward the use of other molecules, including respiratory fluoroquinolones and third-generation cephalosporins (6–11). While the rates of resistance to these alternative agents remain globally low, some countries are nonetheless experiencing decreased susceptibilities (6, 8, 12–16), and a precise understanding of the mode of action (MOA) of antibiotics and of the cellular response that they induce should prove useful for the prevention of further resistance.

Bactericidal antibiotics have been proposed to contribute to bacterial death through a common mechanism involving reactive oxygen species (ROS) as a common effector (17–24). While generating great enthusiasm regarding novel therapeutic strategies (25, 26), this unified model remains a matter of debate given recent contradictory findings about the role of oxidants in the MOA of bactericidal antibiotics (27–29). While ROS are not the sole arbiters, recent additional work convincingly showed that they contribute to the lethality of antibiotics (30, 31). In the case of *S. pneumoniae*, the proposed model is difficult to reconcile with the fact that, while being vulnerable to killing by bactericidal antibiotics, *S. pneumoniae* lacks genes encoding a complete electron transport chain or the tricarboxylic acid cycle (32), which are both central to the production of antibiotic-induced ROS (17, 18, 22, 33). *S. pneumoniae* is also apparently tolerant to the adverse effects of the Fenton reaction (34) owing to the sequestration of the majority of the Fe^{2+} (and thus of the reactive $OH^{\bullet}$) away from DNA (35) and to the scarcity of pneumococcal proteins containing iron-sulfur clusters (20, 35). *S. pneumoniae* produces substantial levels of H_2O_2 through the activity of its pyruvate oxidase SpxB (36), and it is possible that deregulation in iron homeostasis following exposure to bactericidal antibiotics could feed the Fenton reaction for the production of $OH^{\bullet}$ to a point no more sustainable by the cell. Indeed, bactericidal antibiotics but not their bacteriostatic counterparts were previously shown to induce oxidative stress in the laboratory strain *S. pneumoniae* R6 (37), and in the case of the fluoroquinolone levofloxacin, this was shown to result from an increased expression of iron import genes that contributed to cell death (38). Interestingly, the selection for a nonsense mutation in a putative iron importer in penicillin (PEN)-resistant *S. pneumoniae* R6 was shown to protect against the triggering of ROS not only by PEN but also upon exposure to unrelated bactericidal molecules, including fluroroquinolones (37). Such cross-tolerance to antibiotic-induced ROS did not translate into substantial cross-resistance, however, suggesting a role for additional mechanisms in antibiotic-induced lethality in *S. pneumoniae* (37). ROS could also be formed when molecular oxygen collides with

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Address correspondence to Marc Ouellette, Marc.Ouellette@crchul.ulaval.ca.

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TABLE 1 List of strains and transformants used in this study

Strain	Description	MIC (μg/ml)[a]					
		PEN	CIP	KAN	LZD	ERY	TET
R6	Wild type	0.03	0.5	32	0.5	0.03	0.125
R6M2B-CIP	R6 clone selected for CIP resistance	0.03	128	32	0.5	NA	NA
R6M2-PEN	R6 clone selected for PEN resistance	2	0.5	32	0.5	0.03	0.125
R6M2-KAN	R6 clone selected for KAN resistance	0.03	0.5	1,024	0.5	0.03	0.06
$R6^{parCgyrA\text{-}R6M2BCIP}$	R6 transformed with *gyrA* and *parC* derived from R6M2B-CIP (*gyrA* G253A; *parC* C245T)	0.03	32	32	NA	NA	NA
$R6^{parC\text{-}R6M2BCIP}$	R6 transformed with *parC* derived from R6M2B-CIP (*parC* C245T)	0.03	2	32	NA	NA	NA
CCRI-14635	Susceptible *S. pneumoniae* clinical isolate	0.03	0.5	32	0.5	NA	NA
CCRI-14703	Susceptible *S. pneumoniae* clinical isolate	0.06	0.5	35	0.5	0.125	0.125
CCRI-14598	Susceptible *S. pneumoniae* clinical isolate	0.12	0.5	32	0.5	0.125	0.125
CCRI-14635 M2-CIP	CCRI-14635 selected for resistance to CIP (*gyrA* G253A; *parC* C245T)	0.03	128	32	0.5	0.125	0.125
CCRI-14598 M2-CIP	CCRI-14598 selected for resistance to CIP (*gyrA* G253A; *parC* C245T)	0.12	128	32	0.5	0.125	0.125
CCRI-14703 M2-CIP	CCRI-14703 selected for resistance to CIP (*parC* C245T)	0.06	4	32	0.5	0.125	0.125
CCRI-45693	CIP-resistant *S. pneumoniae* clinical isolate (*gyrA* G253A; *parC* C245T)	0.06	128	32	NA	NA	NA
CCRI-50154	CIP-resistant *S. pneumoniae* clinical isolate (*parC* C245T)	0.03	4	32	NA	NA	NA

[a] MICs were determined from three independent biological replicates. NA, not available.

flavoenzymes and steals their electrons (reviewed in reference 20). The autoxidation rates of flavoenzymes vary widely, however, depending on the reduction potential of the flavin and the degree to which it is solvent exposed. Flavoenzymes have been described for *S. pneumoniae* (39, 40), but whether they represent a significant source of oxidant remains unknown.

Due to the peculiarity of *S. pneumoniae* regarding oxidative stress (34, 35, 41), we sought in this study to determine whether a similar connection between bactericidal antibiotics, ROS, and resistance also occur in resistant clinical isolates, more specifically, if resistance to a single bactericidal antibiotic confers tolerance to oxidative stress induced by unrelated bactericidal molecules. Our results confirmed previous observations obtained with the laboratory strain *S. pneumoniae* R6 but revealed a more complex situation for clinical isolates, whereby bactericidal antibiotics are potent inducers of ROS in a context of antibiotic sensitivity but also often in a context of antibiotic resistance.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in this study are listed and described in Table 1. Pneumococci were grown on blood agar containing 5% defibrinated sheep's blood or in brain heart infusion broth (BHI; Difco). Cultures were incubated for 16 to 24 h at 35°C in a 5% CO_2 atmosphere. All strains were conserved frozen at −80°C in BHI containing 15% glycerol. The selection of resistant mutants was conducted from independent clones of *S. pneumoniae* R6 (wild type [WT]), CCRI-14635, CCRI-14703, or CCRI-14598. The selection of resistance was performed on Szybalski plates containing antibiotic concentration gradients. For subculturing, colonies were picked from the area of highest antibiotic concentrations and streaked onto agar plates containing either the same concentration of antibiotic or a gradient of increased antibiotic concentrations. The MIC of the resistant cells isolated from the plates with the highest concentrations of antibiotic was determined to confirm the resistance phenotype.

Antibiotic susceptibility determination. The MICs of all drugs used in this study were determined by microdilution according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The MICs were recorded as the lowest dilution showing no growth. When it was possible, we also used Etest strips (AB bioMérieux, Stockholm, Sweden) on Mueller-Hinton agar plates supplemented with 5% sheep blood according to the manufacturer's instructions. MIC measurements were done at least in triplicate.

Detection of ROS. Intracellular ROS measurements relied on dichlorodihydrofluorescein diacetate (DCF-DA; Invitrogen, Grand Island, NY), whose fluorescence intensity is proportional to the level of ROS. In a typical experiment, cells were grown to the onset of exponential phase (optical density at 600 nm [OD_{600}], 0.12) before addition of the antibiotic. PEN and ciprofloxacin (CIP) were added at a final concentration corresponding to twice the MIC, and kanamycin (KAN) was added at 13 times the MIC. The bacteriostatic antibiotics linezolid (LZD), erythromycin (ERY), and tetracycline (TET) were added at a final concentration corresponding to 20 times the MIC. One-milliliter culture aliquots were collected at baseline (prior to the addition of antibiotic) and at 1, 2, and 3 h following the addition of antibiotic. The aliquots were washed once with 1× phosphate-buffered saline (PBS; pH 7.2), resuspended in 500 μl of 1× PBS (pH 7.2) containing 5 μM DCF-DA, and incubated at 37°C in the dark for 30 min. The labeled cells were washed once with 1× PBS (pH 7.2) and resuspended in 500 μl of 1× PBS. The fluorescence signal of a 200-μl aliquot was analyzed using a Victor fluorometer (Perkin-Elmer, Waltham, MA) at 485-nm excitation and 535-nm emission wavelengths. Results were normalized according to viable cell counts and are expressed as rates of ROS production in the presence of antibiotics compared to that in a no-drug control. Rates were calculated after 3 h of exposure to drugs. Each experiment was performed with three technical and three biological replicates.

Genetic transformation. DNA transformation was done as previously described (37, 42, 43). For reconstructing CIP resistance, the CIP-susceptible *S. pneumoniae* R6 was transformed with PCR fragments covering the quinolone-resistance-determining regions (QRDR) of *parC* and *gyrA* derived from the CIP-resistant mutant *S. pneumoniae* R6M2B (44). When needed, an $rpsL^{+}$ fragment conferring resistance to streptomycin (Lys57Thr) was cotransformed as a surrogate selection marker along with the DNA fragment of interest (37).

RESULTS

Bactericidal antibiotics induce ROS in *S. pneumoniae* R6. WT *S. pneumoniae* R6 subjected to a 3-h exposure to PEN, CIP, and KAN at concentrations equivalent to two times the respective MICs exhibited a 100- to 450-fold increase in ROS levels compared to that of the untreated control (Fig. 1). The accumulation of ROS was measured using the dichlorofluorescein diacetate dye, whose

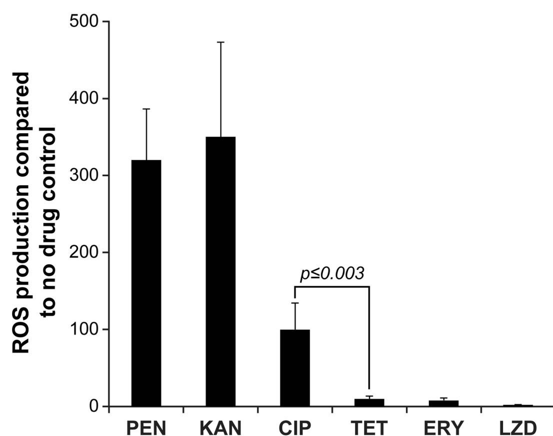

FIG 1 ROS production in WT *S. pneumoniae* R6 after 3 h of exposure to bactericidal and bacteriostatic antibiotics. Results were normalized according to viable-cell counts and are expressed as the rate of ROS production compared to that in the no-drug control. Results are averages from three independent experiments. The significance of differences in ROS production was confirmed by Student's *t* test. PEN, penicillin G; KAN, kanamycin; CIP, ciprofloxacin; TET, tetracycline; ERY, erythromycin; LZD, linezolid.

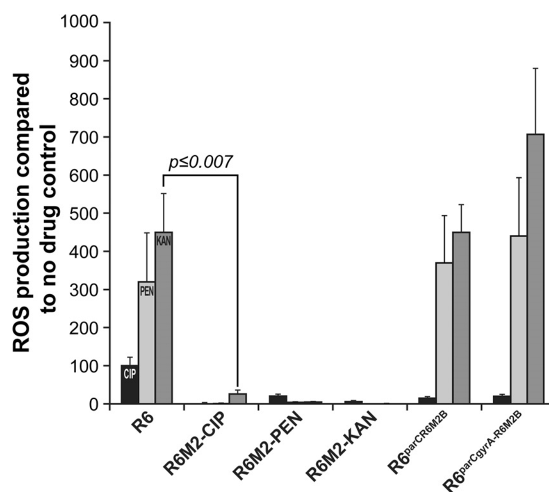

FIG 2 ROS production in WT *S. pneumoniae* R6 and laboratory-derived resistant mutants and resistant transformants after 3 h of exposure to bactericidal antibiotics. Results were normalized according to viable cell counts and are expressed as the rate of ROS production compared to that in the no-drug control. Results are averages from three independent experiments. The significance of differences in ROS production was confirmed by Student's *t* test. The last two strains were WT *S. pneumoniae* R6 transformed with the *parC* or the *parC* and *gyrA* genes derived from R6M2B-CIP.

fluorescence intensity is indicative of the levels of intracellular ROS (37, 42). In contrast, the bacteriostatic antibiotics ERY, LZD, and TET only minimally increased ROS levels, even when they were added at concentrations equivalent to 20 times their MICs ($P \leq 0.003$) (Fig. 1).

Antibiotic resistance in *S. pneumoniae* R6 protects against the induction of ROS by unrelated antibiotics. Resistance to PEN and CIP among *S. pneumoniae* R6 laboratory-derived mutants was previously shown to prevent the accumulation of ROS that these antibiotics usually induce (37, 44). This was confirmed and extended here, whereby the highly resistant mutants *S. pneumoniae* R6M2-PEN, R6M2B-CIP, and R6M2-KAN (Table 1) failed to produce ROS not only when exposed to the antibiotic defining their resistance but also when challenged with any of the three ROS-inducing antibiotics (Fig. 2). We further studied the consequences of this inability of R6 mutants to produce ROS in the presence of bactericidal antibiotics. Bacterial cells resistant to one bactericidal antibiotic were not cross resistant to the other bactericidal antibiotics (Table 1). While PEN and CIP inhibit the growth of WT *S. pneumoniae* R6 by killing it (Fig. 3A), it would appear that PEN inhibits the CIP-resistant mutant *S. pneumoniae* R6M2B-CIP by arresting growth (Fig. 3B). This reduced lethality of PEN for *S. pneumoniae* R6M2B-CIP was further confirmed by determining the MBC/MIC ratios of PEN, CIP, and LZD as previously described (45–47) (Table 2).

In the case of *S. pneumoniae* R6M2-PEN, the cross-tolerance to ROS accumulation was previously shown to be unrelated to the primary determinants of resistance (i.e., penicillin-binding proteins with a decreased affinity for β-lactams) but instead to result from the selection of a mutation in a putative iron permease (37). We show here that cross-tolerance to ROS accumulation in *S. pneumoniae* R6M2B-CIP was also independent of target mutations. The CIP resistance of R6M2B-CIP was previously shown to result mostly from the acquisition of mutations in the genes *parC* and *gyrA*, coding for DNA topoisomerase IV and DNA gyrase, respectively (44). As expected, the introduction of a *parC* allele derived from R6M2B-CIP alone and in combination with a mutated *gyrA* gene into WT *S. pneumoniae* R6 increased the CIP MIC for the resulting *S. pneumoniae* transformants 4-fold and 64-fold, respectively (Table 1). However, while the mutations in *parC* and *gyrA* were sufficient for preventing CIP from inducing ROS (Fig. 2), these could not prevent the accumulation of ROS triggered by PEN and KAN (Fig. 2). The extended protection against antibiotic-induced ROS should thus also require additional events besides target site mutations in *S. pneumoniae*.

Bactericidal antibiotics also induce ROS in *S. pneumoniae* sensitive clinical isolates. The antibiotic-sensitive clinical isolates CCRI-14635, CCRI-14703, and CCRI-14598 (Table 1) exhibited a 50- to 200-fold increase in ROS levels when exposed for 3 h to PEN and CIP at concentrations equivalent to two times their MICs (Fig. 4). Variations in ROS levels were noted between the isolates, however, with PEN, producing a 10-fold difference in ROS between clinical isolates (Fig. 4). Similar to the case with WT *S. pneumoniae* R6, the bacteriostatic antibiotic LZD induced only a minimal increase in ROS levels in the three antibiotic-susceptible clinical isolates ($P \leq 0.04$), even when added at a concentration equivalent to 20 times the MIC (Fig. 4).

Complex association between ROS-inducing antibiotics and resistance in clinical isolates of *S. pneumoniae*. CIP induced ROS in the sensitive isolates *S. pneumoniae* CCRI-14635 and CCRI-14598 (Fig. 4). These two clinical isolates were then selected *in vitro* for resistance to CIP until they reached a 250-fold increase in resistance (CCRI-14635 M2-CIP and CCRI-14598 M2-CIP in Table 1) and both mutants had mutations in *parC* and *gyrA* (Table 1). Resistance to CIP was also selected in CCRI-14703, but we could not achieve high-level resistance in this strain (CCRI-14703 M2-CIP in Table 1). The CCRI-14635 M2-CIP and CCRI-14598 M2-CIP resistant mutants exhibited a significantly reduced ROS production ($P \leq 0.01$) upon exposure to CIP in comparison to that of their sensitive parents (Fig. 5A). Similar to the case with R6, we did observe a cross-protection against the induction of ROS by

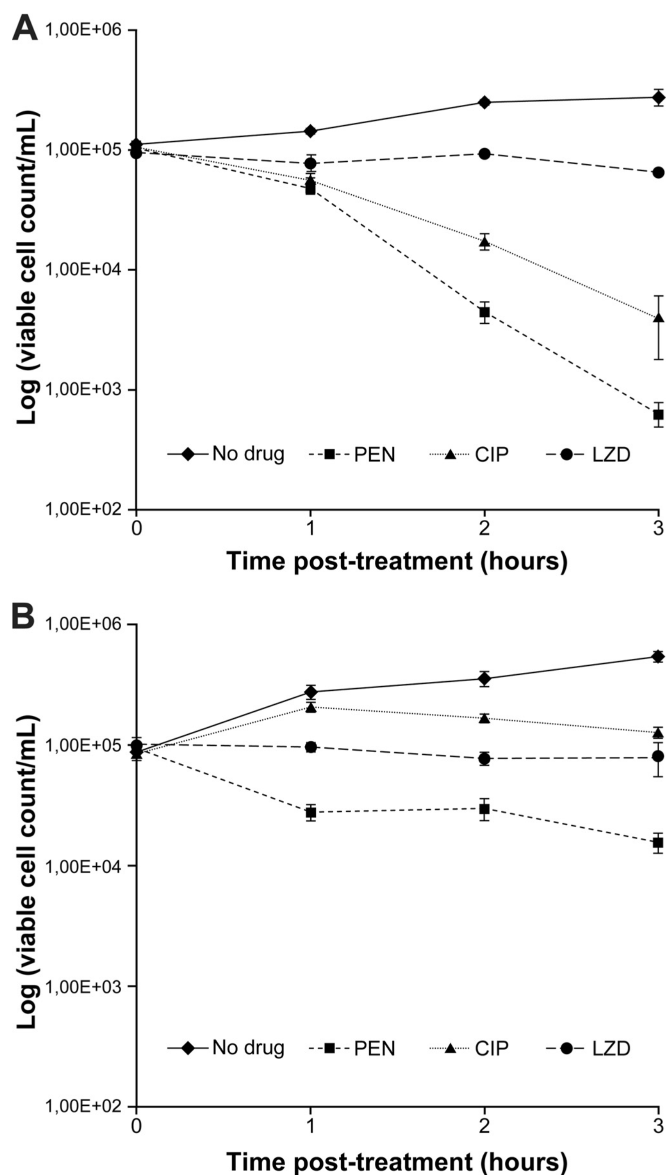

FIG 3 Killing efficiency of bactericidal antibiotics in ROS-proficient and ROS-deficient *S. pneumoniae* backgrounds. The viable cell counts of WT *S. pneumoniae* R6 (A) and *S. pneumoniae* R6M2B-CIP (B) were monitored after 3 h of exposure to the bactericidal antibiotics CIP and PEN (at twice the MIC for the strain) and to the bacteriostatic antibiotic LZD (at 20 times the MIC for the strain) by plating in the absence of drugs. Results represent means from three independent experiments.

PEN for CCRI-14598 M2-CIP ($P \leq 0.05$) (Fig. 5A). However, ROS levels monitored in the CCRI-14635M2-CIP mutant and its CCRI-14635 parent after a 3-h exposure to PEN were almost identical (Fig. 5A). For mutant CCRI-14703 M2-CIP, differences in ROS levels were not significant with either PEN or CIP compared to its CCRI-14703 parent (Fig. 5A), a phenomenon that might be explained by the low level of resistance of this mutant (Table 1).

We tested ROS production in clinical isolates for which resistance was induced *in vitro*. We also tested for antibiotic-induced ROS production in clinical CIP-resistant isolates. The resistant isolates CCRI-50154 and CCRI-45693 display low- and high-level resistance to CIP, respectively, owing to mutations in *parC* alone

TABLE 2 MBC/MIC ratios of PEN, CIP, and LZD for *S. pneumoniae* R6 and R6M2B-CIP

	Value for[a]:					
	S. pneumoniae R6			*S. pneumoniae* R6M2B-CIP		
Drug	MBC	MIC	MBC/MIC	MBC	MIC	MBC/MIC
PEN	0.03	0.03	1	0.12	0.03	4
CIP	0.5	0.5	1	2,048	128	16
LZD	16	0.5	32	16	0.5	32

[a] MBCs and MICs are expressed as micrograms per milliliter and were determined from three independent biological replicates.

(CCRI-50154) or in combination with mutations in *gyrA* (CCRI-45693) (Table 1). Despite their resistance, these isolates did not exhibit ROS protection against either CIP (i.e., the drug defining their resistance) or PEN (Fig. 5B). The ROS levels monitored in CCRI-50154 after exposure to CIP were indeed not significantly different from those induced in the CIP-sensitive clinical isolates CCRI-14703 and CCRI-14598 (Fig. 5B). The ROS levels induced in the highly CIP-resistant clinical isolate CCRI-45693 were higher than in any other strain included in this study (Fig. 5B).

DISCUSSION

The bactericidal antibiotics PEN and CIP, but not the bacteriostatic TET, chloramphenicol, or LZD, had previously been shown to induce oxidative stress in the laboratory strain *S. pneumoniae* R6 (37, 44). Resistance to either PEN or CIP also protected *S. pneumoniae* R6 mutants against the accumulation of ROS upon exposure to the antibiotic defining their resistance (37, 44). In the case of *S. pneumoniae* R6 PEN-resistant mutants, such protection against antibiotic-induced ROS was further shown to extend to unrelated bactericidal antibiotics (37). In this study, we confirmed that this phenomenon most likely generalizes to any bactericidal antibiotics in the case of *S. pneumoniae* R6 mutants, with cross-tolerance to ROS production also occurring among mutants resistant to either of the unrelated bactericidal antibiotics CIP and

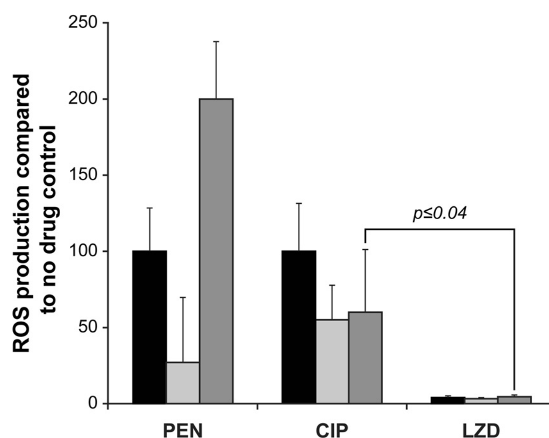

FIG 4 ROS production in antibiotic-susceptible *S. pneumoniae* clinical isolates after 3 h of exposure to bactericidal and bacteriostatic antibiotics. Results were normalized according to viable cell counts and are expressed as the rate of ROS production compared to that of the no-drug control. Results are averages from three independent experiments. The significance of differences in ROS production was confirmed by Student's *t* test. Black bars, CCRI-14635; light gray bars, CCRI-14703; dark gray bars, CCRI-14598.

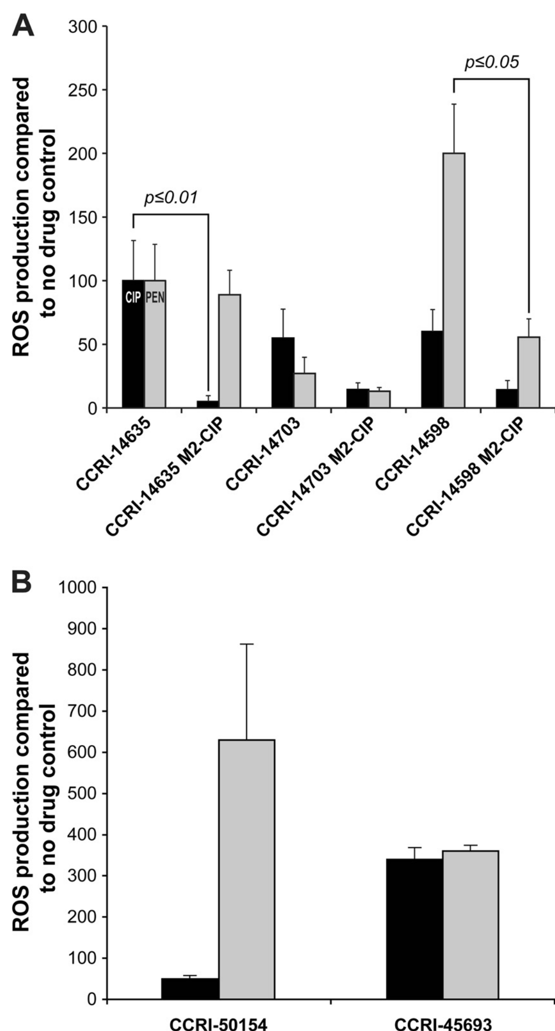

FIG 5 ROS production in antibiotic-resistant *S. pneumoniae* clinical isolates after 3 h of exposure to bactericidal antibiotics. *S. pneumoniae* clinical isolates either were selected for resistance to CIP *in vitro* (A) or were CIP-resistant clinical isolates (B). Results were normalized according to viable cell counts and expressed as the rate of ROS production compared to no drug control. Results are the average of three independent experiments. The significance of differences in ROS production was confirmed by Student's *t* test.

KAN. These cross-protections do not translate into cross-resistance (Table 1), but we observed a switch from a clean bactericidal MOA to a phenomenon reminiscent of the bacteriostatic MOA, as indicated by the survival growth curves (Fig. 3) and the higher MBC/MIC ratio (Table 2). This is in line with the connection between ROS production and antibiotic-induced lethality extensively discussed in the literature (17, 18, 22, 23, 26, 30, 48). The acquisition of mutations in a given antibiotic's target is not enough for conferring protection against ROS induced by unrelated bactericidal counterparts, as indicated for PEN previously (37) or with the *parC* and *gyrA* genes for CIP in this study (Fig. 2). Mutations preventing ROS accumulation are likely to be selected early during the acquisition of resistance (37, 44). Combined with the altered lethality of bactericidal antibiotics in the absence of ROS, this suggests an early survival benefit to endure antibiotic pressure while more efficient mechanisms of resistance are being selected or acquired.

Our analysis of further linkages between bactericidal antibiotics and ROS production in the context of antibiotic sensitivity and resistance indicated that our findings for the lab strain R6 are not as easily extrapolated to clinical isolates. ROS-producing antibiotics were also competent in producing ROS in clinical isolates (Fig. 4 and 5). Interestingly, we found that resistance to one bactericidal antibiotic in R6 led to cross-protection to antibiotic-induced ROS (Fig. 2). A similar ROS cross-protection was observed with the drug-resistant protozoan parasite *Leishmania* (49). This phenomenon of resistance-mediated absence of ROS production was observed with one clinical isolate selected for CIP resistance *in vitro* (CCRI-14598M2-CIP) but not in CCRI-14635 (Fig. 5A). In the *S. pneumoniae* R6 resistant mutants, cross-tolerance to ROS induction implicated mutations other than target site mutations (see the resistant transformants in Fig. 2A), and it is possible that such mutations have not been selected in the resistant mutants derived from CCRI-14635. Neither R6 (Fig. 2) nor clinical isolates (Fig. 5A) selected *in vitro* for CIP resistance produced ROS in the presence of CIP. CIP resistance generated by DNA transformation in R6 will also lead to the absence of ROS production in the presence of CIP (Fig. 2). However, this is not observed with clinical CIP-resistant isolates for which ROS is produced when these cells are incubated with CIP (Fig. 5B). Thus, resistance *per se* is not sufficient to decrease antibiotic-induced ROS.

Recent work highlighted the complex nature of antibiotics action and reinforced the notion that ROS contribute causatively to drug lethality in addition to the lethal cellular damage induced by the inhibition of target-specific processes (30). *S. pneumoniae* provides a clear example of such complexity, with the role of ROS differing between laboratory-derived and naturally selected antibiotic-resistant mutants. *S. pneumoniae* is adapted to survive in the presence of high concentrations of endogenously generated oxidants (H_2O_2), but it is not entirely immune to their adverse effects (50). It is also peculiar in lacking canonical enzymes to detoxify oxygen radicals or homologues of oxidative stress response regulators (reviewed in reference 34). Still, pneumococcal enzymes implicated in the removal of ROS have been described, which include NADH oxidase, superoxide dismutase, thiol peroxidase, and alkyl hydroperoxidase (reviewed in reference 34), and differences in expression of such detoxifying enzymes or pathways could provide protection against antibiotic-induced oxidants, as recently reported (30). For example, the pneumococcal population was shown to display high genetic diversity, and differences in the ability to survive oxidative stress have been noted between strains (51). We also have previously shown that polymorphism in a putative NAD(P)H-dependent glycerol-3-phosphate dehydrogenase can protect *S. pneumoniae* against ciprofloxacin-mediated ROS (44). It is therefore possible that depending on their genetic background relative to oxidative stress defense, some clinical isolates do not require additional mutations to thrive in the presence of oxidants induced by antibiotics.

In conclusion, our results suggest that tolerance to antibiotic-induced oxidative stress in *S. pneumoniae* is not universal and varies according to the genetic background of the strains. Monitoring for new mutations providing ROS protection and the chronology of their appearance under various bactericidal conditions could increase our understanding of the response of *S. pneumoniae* against bactericidal antibiotics.

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The authors have no conflict of interest to declare.

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