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Reactive Oxygen Species Production is a Major Factor Directing the Post-antibiotic Effect of Fluoroquinolones in *Streptococcus pneumoniae*

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Running title*:* Role of ROS in PAE of fluoroquinolones

ABSTRACT

We studied the molecular mechanisms involved in the post-antibiotic effect of the fluoroquinolones levofloxacin and moxifloxacin in *Streptococcus pneumoniae*. Wild-type strain R6 had post-antibiotic effects of 2.05±0.10 h (mean±SD) and 3.23±0.45 h at 2.5× and 10× MIC of levofloxacin, respectively. Moxifloxacin exhibited a lower effect, of 0.87±0.1 and 2.41±0.29 h at 2.5× and 10× MIC, respectively. Fluoroquinolone-induced chromosome fragmentation was measured at equivalent post-antibiotic effects for levofloxacin (2.5 × MIC) and moxifloxacin (10 × MIC). After 2 h of drug removal, reductions were of about 7-fold for levofloxacin and 3-fold for moxifloxacin, without further decrease at later times. Variations in reactive oxygen species production were detected afterwards, after 4-6 h of drug withdrawals, with decreases ≥400-fold for levofloxacin and ≥800-fold for moxifloxacin at 6 h. In accordance, after 4-6 h of drug withdrawal, the levofloxacin-induced up-regulation of the *fatCDEB* operon, introducing iron in the bacteria, decreased up to 2-to-3 fold, and the moxifloxacin-induced up-regulation of several genes involved in the production of pyruvate was reduced 3-to-7 fold. In accordance, lower post-antibiotic effects (up to 1h) were observed in strain R6Δ*spxB*, lacking the main enzyme involved in oxygen peroxide production, than in R6. Although no change in the recovery of chromosome fragmentation was observed between R6 and R6Δ*spxB*, a 3.5 × 10^3-fold lower reactive oxygen species production was observed in R6Δ*spxB*, without changes after drug removal. These results show that reactive oxygen species are the main factors directing the post-antibiotic effect of levofloxacin and moxifloxacin in *S. pneumoniae*.

Streptococcus pneumoniae, a main human pathogen, is the primary cause of community-acquired pneumonia, meningitis, bacteremia, and otitis media in children. Worldwide, one million children ≤5 years old die annually of pneumococcal infections (1). Given the spread of pneumococcal isolates resistant to beta-lactams and macrolides (2), the fluoroquinolones (FQs) levofloxacin (LVX) and moxifloxacin (MOX) are currently recommended for the treatment of adult patients with pneumonia (3). FQ-resistance in *S. pneumoniae* has a low prevalence (< 3%) in Europe (4, 5), although is higher in Canada (7.3%) (6) and in some locations of Asia (10.5%) (7). However, an increase in resistance may occur if the use of FQs is increased (8). An important pharmaco-dynamic parameter of antibiotics, which has clinical impact on the antibiotic dosing regimens, is the post-antibiotic effect (PAE). PAE is defined as the delayed regrowth of bacteria following short exposure to supra inhibitory concentrations of an antibiotic (9). PAEs of FQS in *S. pneumoniae* have been previously determined (10-14). However, the mechanisms involved in this FQs-induced PAE have not been studied. FQs target DNA gyrase and DNA topoisomerase IV, essential enzymes for the maintenance of DNA topology, and, subsequently, of replication and transcription (15). They interact with these topoisomerases and stabilize an intermediate of their reaction, DNA-FQ-topoisomerase complexes, which lead to the generation of harmful double-stranded DNA breaks (16). In addition, the inhibition of gyrase triggers the generation of hydroxyl radical via the Fenton reaction, contributing to the lethality of FQs (17-19). We have previously detected the production of reactive oxygen species (ROS, chemical species formed upon incomplete reduction of oxygen, including superoxide anion, hydrogen peroxide, and hydroxyl radical) in *S. pneumoniae* after FQ treatment. The inhibition of topoisomerase IV by LVX (20) or of both topoisomerase IV and gyrase by MXF (21) triggered global changes in the transcriptome. We proposed these changes to

be mediated by local changes in supercoiling. The differences in the transcriptome triggered by either drug would be due to differences in sequence recognition by the topoisomerases, a process which is itself affected by DNA supercoiling. LVX treatment up-regulates the *fatDCEB* operon, which is responsible for iron intake. This up-regulation leads to an increase of intracellular iron, and in turn, to the shift in the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$) toward the production of hydroxyl radicals. Treatment with MXF up-regulates pathways affecting pyruvate levels, which lead to a parallel increase in intracellular H_2O_2 mediated by the pyruvate oxidase enzyme SpxB (EC 1.2.3.3). Then, both LVX and MXF stimulate the Fenton reaction by causing an increase in the concentration of either Fe^{2+} or H_2O_2, respectively. In consequence, an increase in the amount of hydroxyl radicals occurs, contributing to the lethality of these FQs

We present here a study whose aim was to understand the mechanism involved in the PAEs of LVX and MXF in *S. pneumoniae*. Chromosomal DNA fragmentation, ROS production, and transcriptional alterations were monitored in the recovery phase.

RESULTS

The length of PAE depends on the presence of an active SpxB enzyme. In strain R6, PAE of LVX was dependent on its concentration, being of 1.6-fold higher at 10× MIC than at 2.5× MIC ($P = 0.01$). Likewise, PAE of MXF was 2.8-fold higher at 10× MIC than at 2.5× MIC ($P = 0.001$). These proportions were maintained in the R6Δ*spxB* strain, LVX PAE was 1.8-fold higher at 10× MIC than at 2.5× MIC ($P = 0.001$), and 2.2-fold higher at 10× MIC than at 2.5× MIC ($P = 0.0007$) in the case of MXF. However, R6Δ*spxB* had shorter PAEs than R6 at 2.5× MIC of LVX (2.05±0.10 h versus 1.18±0.13 h, mean ± SD) and 10× MIC of MXF (2.41±0.29 h versus 1.53±0.12

h) (Fig. 1, Table 1). Strain R6Δ*spxB* is isogenic with R6, except for a deletion in the *spxB* gene, which codes for the pyruvate oxidase enzyme, responsible for the production of oxygen peroxide (22, 23). Then, it can be concluded that duration of FQs PAEs is related to the presence of a pyruvate oxidase enzyme encoded by *spxB*.

Fragmentation of chromosomal DNA reverted soon after drug removal. The effects of LVX and MXF on R6 were tested at concentrations in which the bacteria exhibited similar PAEs, i. e., 2.5× MIC of LVX (2.05±0.10 h) and 10× MIC of MXF (2.41±0.29 h). For R6Δ*spxB*, 10× MIC of both FQs were used, given they conferred the highest PAEs to the strain (2.17±0.15 h for LVX and 1.53±0.12 h for MXF). In R6, the reduction in DNA fragmentation was observed at 2 h, with no further decrease. This was detected in the LVX treatment both in the decrease of the fragmented DNA (CZ, compression zone, the band in which the nicked fragments of chromosomal DNAs that are unresolved in the gel migrate) and in the increase of non-fragmented DNA in the well. However, in the MXF treatment, the decrease was only observed in the fragmented DNA (Fig. 2). This difficult in reproducibly measuring non-fragmented DNA trapped in the well, and the lack of correlation between the reduction in the CZ band and the recovery of non-fragmented DNA suggests that, in the case of MXF treatment, this approach is semi-quantitative.

For LVX, reductions of the CZ signal were of up to 7-fold at 2 and 4 h and the increase in the non-fragmented DNA was of 2-fold (2 h) and up to 3-fold (4 h). For MXF, a reduction in the CZ signal of up to 3-fold was detected at 2 and 4 h. In R6Δ*spxB* reductions in the CZ signal were of up to 20-fold (LVX) and up to 3-fold (MXF) after 2 and 4 h. The increase in the non-fragmented DNA was of up to 1.5-fold after 2 and 4 h of MXF treatment. Although the recovery in the R6Δ*spxB* after LVX

112 treatment was higher, no differences were observed between R6 and R6Δ*spxB* after
113 MXF treatment. In both strains, recovery after 2 h and 4 h treatment were equivalent.

114 **ROS production reverted at 4 and 6 h in the PAE phase accordingly to the**
115 **transcriptional alterations induced by LVX and MXF.** The effects of LVX and MXF
116 on R6 were tested at the same concentrations used in the fragmentation assays, to
117 maintain similar PAEs for both drugs. The amount of ROS production after 1 h
118 treatment (time 0 min in Fig. 3) was similar with both drugs. While no changes were
119 observed 2 h after drug withdrawal, decreases in ROS production were detected after 4-
120 6 h of drug withdrawals. Similar decreases were observed for both FQs. After 6 h of
121 drug withdrawal, decreases at 6 h of ≥400-fold for LVX and ≥800-fold for MXF were
122 detected (Fig. 3A). In R6Δ*spxB*, the intrinsic production of ROS was 3.5×10^3 fold
123 lower that of R6 and not significant changes were observed under FQ treatment (Fig.
124 3A).

125 In accordance, after 4-6 h of LVX withdrawal, the induced up-regulation of the
126 transcription of the *fatCDEB* operon, involved in the introduction of iron in the bacteria,
127 was reduced up to 2-to-3 fold (Fig. 4A), and the MXF-induced up-regulation of several
128 genes encoding enzymes involved in the production of pyruvate was reduced 3-to-7 fold
129 in the PAE phase (Fig. 4B). In the MXF treatment, 6 genes were chosen to test
130 transcription by qRT-PCR. These genes have been previously shown to be up-regulated
131 by MXF (21). These included *tktA* and *pmi*, whose products convert manose-6P to
132 fructose-6P and ribulose-5P to glucose-6P, respectively. Glucose-6P is further
133 converted to fructose-6P, the first substrate of the glycolysis. After an initial increase in
134 the transcription of these genes, a reversion was observed in the PAE phase (Fig. 4B).
135 There were also three genes coding glycolytic enzymes (*fba*, *gpdA*, and *gpmA*), only for

gpdA a significant increase, and subsequent recovery was observed. Although no significant variations were observed for *fba*, this gene showed a behavior consistent with up-regulation and recovery. The same behaviour was observed for *pfl*, which encodes formate acetyltransferase, which converts acetyl-coA in pyruvate. The differences observed in the transcription of the chosen genes and that previously reported (21) could be due to the different MXF concentrations and times used. Anyway, 4 out of the 6 genes analyzed showed an up-regulation after 1 h treatment and further recovery during the PAE phases, the recovery was mostly observed at 4-6 h after FQ withdrawal.

DISCUSSION

Postantibiotic effect induced by FQs is fundamental to determine optimal dosage regimens that achieve therapeutic success and avoid the emergence of resistance. In addition, knowledge of the postantibiotic recovery period is critical to understand persistence phenotypes. However, there are few studies on its mechanism and the factors on which it depends. This study related the recovery of DNA cleavage and ROS levels with its duration.

By measuring the PAE of LVX and MXF in *S. pneumoniae* R6 strain and its isogenic R6Δ*spxB* derivative, we showed PAEs of up to 1 h longer, in R6. In both strains the resealing of DNA breaks after drug withdrawals was equivalent in time, occurring after 2 h without further decrease at latter times. At equivalent LVX PAEs, 2.05±0.10 h and 2.17±0.15 for R6 and R6Δ*spxB*, respectively, reductions in fragmented DNA, estimated as the amount of CZ species, were higher for R6Δ*spxB* than for R6 (20-fold versus 7-fold). In the case of MXF, PAEs of 3.23±0.45 h for R6 and 1.53±0.12 h for R6Δ*spxB*

159 did not produce differences in the reductions of fragmented DNA (3-fold in both
160 strains). These results indicate that the main factor determining the duration of PAE was
161 not the recovery from chromosome fragmentation mediated by the FQs. However, big
162 differences were observed in ROS production. The decrease in ROS production was
163 detected only in R6, and after 4 and 6 h of drug withdrawal. No changes in ROS
164 production after FQ removal were detected in R6Δ*spxB*. The delayed decrease of ROS,
165 with respect of the reduction in DNA fragmentation, could be related to the stability of
166 this species. Production of ROS has been also recently suggested as the mechanism of
167 antibacterial and antibiofilm activity of other drugs, such as thymoquinone (24),
168 ciprofloxacin and meropenem (25). Previous studies also related ROS increases with
169 longer PAEs induced by aminoglycosides in *P. aeruginosa* under hyperoxia conditions
170 (26). The photolysis of hydrogen peroxide induced significantly longer PAE in
171 *Staphylococcus aureus* and *Streptococcus salivarius* due to the generation of hydroxyl
172 radicals (27). Another factor to consider is the slower growth rate of R6Δ*spxB*, which
173 might also confer some protection against FQs and thereby reduce the PAE. The role of
174 ROS in the lethality of antibiotics in anaerobic conditions has been a matter of
175 controversy (28). Some studies denied this role (29, 30), although others clearly show a
176 connection, especially for FQs (17, 19). The model was proposed in *Escherichia coli*, an
177 aerobic bacterium. It was shown that the primary drug-target interactions stimulate the
178 tricarboxylic acid cycle, this in turn lead to the hyper-activation of the electron transport
179 chain that stimulates H_2O_2 formation. Superoxide damages Fe-S clusters of enzymes,
180 making Fe^{2+} available for oxidation by the Fenton reaction. There are several
181 differences between this model and the one we propose for *S. pneumoniae* (31), a
182 facultative anaerobe that obtains energy from carbohydrate fermentation via glycolysis.
183 The genome of this bacterium does not code for enzymes of the tricarboxylic acid cycle,

nor for those coding the cytochromes and heme-containing proteins involved in aerobic respiration. In addition, its F_0F_1-ATPase does not synthesizes ATP, it uses instead ATP to pump out protons out of the cell, being the main regulator of the intracellular pH (32). In our model, both LVX and MXF, by means of transcriptional alterations, stimulate the Fenton reaction by increasing the concentration of either Fe^{2+} or H_2O_2, respectively. We had shown that these effects have a low contribution to the lethality of the FQs (20, 21). In this study we show that ROS production is the main factor determining the length of PAE. The efficacy of FQs could be increased either by elevating the levels of intracellular ferrous iron or by increasing the accumulation of ROS. This could also prolong PAE, determining longer dosing intervals, reducing adverse effects and lower costs while formulating a daily administration dosage.

MATERIALS AND METHODS

Post-antibiotic effect (PAE). *S. pneumoniae* was grown in a casein hydrolysate based medium (AGCH) with 0.2% yeast extract and 0.3% sucrose (33). MICs for R6 and R6Δ*spxB* strains were 0.25 μg/ml for LVX and 0.06 μg/ml for MXF. PAE was calculated measuring bacteria re-growth after antibiotic treatment by the viable plate count method (34). Cultures were grown in AGCH broth to about 10^8 CFU/ml, treated for 1 h with diverse drug concentrations in 24-well (flat bottom) polystyrene microtiter dishes. Growth controls without antibiotic were included with each experiment. After 1/1000 dilution with fresh media cultures were incubated for 6 h and viable bacteria were counted every 2 h by plating on Mueller-Hinton blood agar plates. PAE was quantified with the formula PAE = T- C. It measures the time required for the viable bacteria counts to increase by 1 $\log_{10}$ above the counts observed immediately after

washing in exposed cultures (T) compared to culture not exposed to antibiotic (C). PAE $\geq$ 0.5 h was considered significant (35).

Analysis of chromosomal fragmentation. Pulse-field gel electrophoresis (PFGE) was used to detect chromosomal fragmentation as described previously (36). About 3×10^6 cells were lysed in solid agarose inserts in a buffer containing 10 mM Tris HCl pH 8, 1 M NaCl, 0.1 M EDTA, 0.5% Brij58, 0.2% deoxycholate, 0.5% sarkosyl, 20 μg/ ml RNaseE, and 100 μg/ ml lysozyme. Inserts were treated with 1 mg/ ml proteinase K and washed before to place them into a 1% low gelling agarose (Pronadisa) gel in 0.5% × TBE buffer (45 mM Tris-borate pH 8, 1 mM EDTA). Gels were electrophoresed in a Cheff-DR III System (BioRad), during 20 hours at 5.8 V/ cm with a 0.1 to 40 seconds switch-time ramp at 14°C. Gels were stained with 0.5 μg/ml of ethidium bromide and bands visualized under ultraviolet light.

Detection of ROS. The intracellular oxidation levels were measured using dihydrorhodamine 123 dye (Sigma-Aldrich). This compound is non-fluorescent, but oxidation converts it to the fluorescent product rhodamine 123, therefore, measured fluorescence is proportional to the level of oxidation (37). Cells were grown exponentially to an OD_{620} = 0.4 before fluoroquinolones were added. Samples (1 ml) were collected and processed as previously described (20). Briefly, cells were washed with phosphate-buffered saline (PBS) (pH 7.2). They were incubated in PBS with 2.5 μg/ml of dihydrorhodamine 123, for 30 min at 37ºC in the dark. Cells were then washed and fluorescence was measured using a Tecan Infinite 2000 device and a filter with excitation/emission wavelengths of 485 nm/535 nm. Results expressed as relative fluorescence units (RFU), were normalized according to the number of live cells at each time point

RNA extraction and qRT-PCR experiments. Total RNA was extracted with an RNeasy midi kit (Quiagen), according to the manufacturer's instructions. Three DNase treatments were performed to avoid any DNA contamination. Synthesis of cDNA was carried out in 20-µl reaction mixtures containing 3 µg of RNA, 0.5 mM of each deoxynucleotide triphosphate, 2 pmol of random hexanucleotides, 40 U of RNaseOUT RNase inhibitor (Invitrogen) and 200 U of SuperScript IV reverse transcriptase (Invitrogen), and 1U of RNAse H (Invitrogen) in the buffer recommended by the manufacturer. These cDNAs were subjected to qRT-PR (chromo 4, Bio-Rad) in 20-µl reaction mixtures containing 2 µl of cDNA, 0.4 µM each specific primer, and 10 µl of LigthCycler FastSatrt Universal A SYBR green Master (Roche). Amplification was achieved with 42 cycles of a three-segment program: denaturation (30 s at 94ºC), annealing (30 s at 45 to 56 ºC), and elongation (30 s at 68ºC). To normalize the three independent cDNA replicates, values were divided by those obtained from the amplification of internal fragment of 16S rDNA. Oligonucleotide pairs used were: : 5'-CCATCGGCTAGTCTGACCCAAAA-3' and 5'-ATCCCAATCAGAGGCAACATCCAC-3' (*fatD*); 5'-TTGGCTAAAGAAGTTGTTGAAAAA-3' and 5'-AAGGGCCGTGGATGTTACC-3' (*fba)*; 5'-GAAAAACAAACCGTCGCCGTCTT-3' and 5'-ACAACAAACAAAATCGCATCCACA-3' (*gpdA*); 5'-AGGGAACAGATCTTGCTACTTTG-3' and 5'-CTTCTTTTGACTTGGCATTGTGAC-3' (*pmi*), 5'-GAAACGTGCTATCAAAACAACTAA-3' and 5' TGCTCATCATCACGGTCCATA 3'(*gpmA)*, 5'-GGGCAGAGGCTCCGAAGGTA-3' and 5'-TGGCTAGTCAAGGCGAAAAA-3' (tktA), 5'-ACAGAGCGTTCACTTCACATCA-3' and 5'-AGCTGGGTCTGGTTCGTATCC-3' *(pfl).*

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FIGURE LEGENDS

FIG 1 PAE in *S. pneumoniae* R6 (A) and R6Δ*spxB (*B). Cultures containing about 10^8 CFU/ ml were subjected to 1 h treatment with either LVX or MXF at the indicated concentrations. Cultures were diluted 1000-fold in media without drug and growth was followed during 6 h. Viable cells were determined by plating on blood agar plates. Log_{10} of CFU/ml (mean ± SD) of three independent replicates are represented.

FIG 2 PAE and chromosome fragmentation. Cultures of *S. pneumoniae* R6 (A) or R6Δ*spxB* (B) containing 10^8 CFU/ml were treated with antibiotics as described in FIG 1. DNAs from cells taken at different times in the PAE phase were subjected to pulse-field gel electrophoresis. CZ: compression zone, the band in which the nicked fragments of chromosomal DNAs that are unresolved in the gel migrate. Quantification of the fragmented chromosome (signal in the CZ relative to the combined signal of the lane plus well) and of the no-fragmented chromosome (signal in the well relative to the combined signal of the lane plus well) relative to time 0 is shown. Fold variations (mean ± SD) of three independent replicates are represented. Student T-test significances with respect time 0 h: *P* value *<0.05, ***<0.0001, ****<0.00001.

FIG 3 ROS production in PAE phases. Cultures of *S. pneumoniae* R6 (A) or R6ΔspxB (B) containing 10^8 CFU/ml were treated for 1 h with either LVX or MXF at the indicated concentrations, diluted 1000-fold and incubated in media without drug. Samples were taken at the indicated times, and the total ROS content of the bacteria was measured as described in Material and Methods. FU, fluorescence units; values were divided by the number of viable cells (CFU). Results (mean ± SD) of three independent replicates are represented. Student T-test significances with respect time 0 h: *P* value *<0.05, **<0.001, ***<0.0001.

FIG 4 Transcriptional alterations in the PAE phases. Cultures of *S. pneumoniae* R6 containing 10^8 CFU/ml were subjected to 1 h treatment with either LVX (A) or MXF (B) at the indicated concentrations. Cultures were diluted as described in FIG 1. Samples were taken, total RNA was extracted, cDNAs were synthetized and subjected to qRT-PCR determinations. Values were made relative to 16SrDNA. Fold changes variations (mean ± SD) with respect the non-treated cultures of three independent replicates are represented. Student T-test significances: *P* value *<0.05, **<0.01, ***<0.0001.

435 **TABLE 1. PAE in *S. pneumoniae* R6 and R6Δ*spxB*.** Bacteria grew to 10^8 CFU/ml
436 were exposed to the drugs for 1 hour. Treatment finished by 1/1000 dilution with fresh
437 media. PAE was measured by viable count method. Results are the average of three
438 independent replicates.

Strain[a]	PAE in hours: mean ± SD[b]			
	LVX (× MIC)[c]		MXF (× MIC)	
	2.5	10	2.5	10
R6	2.05 ± 0.10	3.23 ± 0.45	0.87 ± 0.1	2.41 ± 0.29
R6Δ*spxB*	1.18 ± 0.13 (0.0008)	1.53 ± 0.06 (0.003)	0.43 ± 0.15 (0.015)	1.53 ± 0.12 (0.008)

439
440 [a] R6Δ*spxB* has a deletion in *spxB* (20).

441 [b] *P* values of the differences between R6 and R6Δ*spxB* are in parentheses.

442 [c] The MICs of LVX and MXF for both strains were 1 μg/ml and 0.06 μg/ml,
443 respectively.

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