Reactive Oxygen Species Contribute to the Bactericidal Effects of the Fluoroquinolone Moxifloxacin in *Streptococcus pneumoniae*

M. J. Ferrándiz,^a A. J. Martín-Galiano,^a C. Aranz,^a T. Zimmerman,^a A. G. de la Campa^{a,b}

Unidad de Genética Bacteriana, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain^a; Presidencia. Consejo Superior de Investigaciones Científicas, Madrid, Spain^b

We studied the transcriptomic response of *Streptococcus pneumoniae* to the fluoroquinolone moxifloxacin at a concentration that inhibits DNA gyrase. Treatment of the wild-type strain R6, at a concentration of 10× the MIC, triggered a response involving 132 genes after 30 min of treatment. Genes from several metabolic pathways involved in the production of pyruvate were upregulated. These included 3 glycolytic enzymes, which ultimately convert fructose 6-phosphate to pyruvate, and 2 enzymes that funnel phosphate sugars into the glycolytic pathway. In addition, acetyl coenzyme A (acetyl-CoA) carboxylase was down-regulated, likely leading to an increase in acetyl-CoA. When coupled with an upregulation in formate acetyltransferase, an increase in acetyl-CoA would raise the production of pyruvate. Since pyruvate is converted by pyruvate oxidase (SpxB) into hydrogen peroxide (H₂O₂), an increase in pyruvate would augment intracellular H₂O₂. Here, we confirm a 21-fold increase in the production of H₂O₂ and a 55-fold increase in the amount of hydroxyl radical in cultures treated during 4 h with moxifloxacin. This increase in hydroxyl radical through the Fenton reaction would damage DNA, lipids, and proteins. These reactive oxygen species contributed to the lethality of the drug, a conclusion supported by the observed protective effects of an SpxB deletion. These results support the model whereby fluoroquinolones cause redox alterations. The transcriptional response of *S. pneumoniae* to moxifloxacin is compared with the response to levofloxacin, an inhibitor of topoisomerase IV. Levofloxacin triggers the transcriptional activation of iron transport genes and also enhances the Fenton reaction.

Streptococcus pneumoniae (the pneumococcus) is an important human pathogen. It is the primary cause of community-acquired pneumonia, meningitis, bacteremia, and otitis media in children. Worldwide, one million children 5 years of age and younger die annually of pneumococcal infections (1). Isolates of *S. pneumoniae* resistant to beta-lactams, which act on the cell wall, and macrolides, which inhibit protein synthesis, have proliferated in the past 3 decades (2). The fluoroquinolones (FQs) levofloxacin (LVX) and moxifloxacin (MOX) are currently used for the treatment of adult patients with pneumonia. FQ resistance in *S. pneumoniae* is minimally prevalent (<3%) in Europe (3, 4), although higher rates have been detected in Asia (10.5%) (5) and in Canada (7.3%) (6). This low prevalence allows successful treatment of pneumococcal pneumonia with FQs. However, an increase in resistance may occur in tandem with the increased use of FQs (7). FQs target the type II DNA topoisomerases: DNA gyrase (gyrase) and DNA topoisomerase IV (topo IV). These enzymes manage DNA topology and solve topological problems associated with DNA replication, transcription, and recombination (8). Gyrase introduces negative supercoils into DNA (9), whereas topo IV relaxes DNA and participates in chromosome partitioning (10). In *S. pneumoniae*, the primary target for most FQs, including LVX, is topo IV (11, 12), while gyrase has been described as the primary target for MOX (13).

The mechanism of action of FQs is the formation of DNA-FQ-topoisomerase complexes and the subsequent generation of detrimental double-stranded DNA breaks (14). In *Escherichia coli*, this appears to occur through two pathways, one dependent and the other independent of protein synthesis. Reactive oxygen species (ROS), such as superoxide, hydrogen peroxide (H₂O₂), and hydroxyl radical, contribute to FQ-mediated cell death via the protein synthesis-dependent pathway (15). This observation is consistent with a model proposed recently suggesting that, as a

consequence of gyrase inhibition, hydroxyl radicals are generated via the Fenton reaction (16), adding to the lethality of FQs (17–20). ROS production is observed with antibiotics from a variety of bactericidal antibiotic families in addition to FQs, each with a different intracellular target. However, the intervening pathways lying between the initial antibiotic-target interaction and ROS formation are not fully characterized, although some are beginning to be described (21–23). We have previously investigated these pathways for LVX in *S. pneumoniae*. We observed a global transcriptional response at concentrations of LVX required to inhibit the topo IV enzyme but not gyrase. This response included the upregulation of *fatDCEB*, an operon responsible for iron uptake (Fe²⁺ and Fe³⁺). These were the only genes upregulated at every concentration (0.5× MIC and 10× MIC) and time point (5, 15, and 30 min) studied. In addition, they were the only genes various at 0.5× MIC and 5 min of LVX treatment. LVX-induced upregulation of *fatDCEB* leads to an increase of intracellular iron and, in turn, to the shift in the Fenton reaction favoring the production of hydroxyl radical. We established a relationship between an increase in ROS and LVX lethality and demonstrated that local topological changes were involved in the upregulation of *fatDCEB*. LVX did not change global supercoiling. However, increases in

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Address correspondence to A. G. de la Campa, agcampa@isciii.es.

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negative supercoiling and *fatDECB* transcription after topoisomerase I inhibition were observed, while the opposite occurred after gyrase inhibition with novobiocin. In accordance, the *fatDCEB* operon is located in the chromosome in a topological domain downregulated by DNA relaxation (24). We constructed a strain in which a P_{fatcat} fusion was positioned 106 kb away from the P_{fat} *fatDCEB* native position. In this strain, upregulation of the operon in the presence of LVX was observed, while no change was observed in P_{fatcat} . Results indicated that *fatDCEB* transcription depends on its location in a topological domain. These results illustrated a link between the initial LVX interaction with its topo IV target and the production of ROS (25).

We present here a complementary study in which the aim was to understand the transcriptional response of *S. pneumoniae* to MOX at concentrations that inhibited gyrase. Chromosomal DNA fragmentation and changes in DNA topology were monitored. The global transcriptional response was analyzed by microarray technology. The results sketch a pathway from gyrase inhibition to ROS production and suggest that *S. pneumoniae* accumulates H_2O_2 as part of the death process associated with MOX treatment.

MATERIALS AND METHODS

Bacterial strains and conditions for growth and transformation. *S. pneumoniae* was grown in a casein hydrolysate-based medium (AGCH) with 0.3% sucrose (26). The MICs of MOX and LVX (Sigma-Aldrich) for the R6 strain were 0.125 μ g/ml and 0.250 μ g/ml, respectively. Two R6-derived strains, T1 and T2 (27), were also used. T1 carried the ParC S79F change and had MICs of 2 μ g/ml (LVX) and 0.125 μ g/ml (MOX). T2 carried both ParC S79F and GyrA S81F changes and had MICs of 16 μ g/ml (LVX) and 4 μ g/ml (MOX). *S. pneumoniae* R6 carrying the plasmid pLS1 was cultured in AGCH medium with 1 μ g/ml of tetracycline.

Analysis of chromosomal fragmentation and of the topology of covalently closed circles. Chromosomal fragmentation was detected by pulsed-field gel electrophoresis (PFGE). 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% Brij 58, 0.2% deoxycholate, 0.5% Sarkosyl, 20 μ g/ml RNase E, and 100 μ g/ml lysozyme. Agarose inserts were treated with 1 mg/ml proteinase K and washed before placing them into a 1% low-gelling agarose (Pronadisa) gel in $0.5 \times$ Tris-borate-EDTA (TBE) buffer (45 mM Tris-borate [pH 8], 1 mM EDTA). Gels were electrophoresed in a Cheff-DR III system (Bio-Rad), during 20 h at 5.8 V/cm with a 0.1- to 40-s switch-time ramp at 14°C. Plasmid DNA isolation from *S. pneumoniae* (pLS1) was performed using the neutral method already described (28). Circular DNA molecules were analyzed in neutral/neutral two-dimensional agarose gels. The first dimension was run at 1.5 V/cm in a 0.4% agarose (Seakem; FMC Bioproducts) gel in $1 \times$ TBE buffer for 17 to 19 h at room temperature. The second dimension was run at 7.5 V/cm in a 1% agarose gel in $1 \times$ TBE buffer for 7 to 9 h at 4°C. Chloroquine (Sigma-Aldrich) was added to the TBE buffer before use. After electrophoresis, gels were subjected to Southern hybridization with a 240-bp probe specific for pLS1, as described previously (24). The DNA linking number (Lk) was calculated by quantifying every topoisomer. The DNA supercoiling density (σ) was calculated using the equation $\sigma = \Delta Lk/Lk_0$. The change in linking number (ΔLk) was determined using the equation $Lk = Lk - Lk_0$, where $Lk_0 = N/10.5$, N is the size of the molecule (in bp), and 10.5 is the number of bp per one complete turn of B-DNA.

Normalization and analysis of microarray data. High-density expression arrays from Agilent were processed at the Functional Genomics Core Facility, Institut de Recerca Biomèdica, Barcelona (Spain). Arrays were designed as described previously (29). cDNAs were obtained from 25 ng of total RNA using the Transplex WTA2 (whole transcriptome amplification 2) kit (Sigma). These cDNAs were labeled with Cy3 using the

Agilent genomic DNA ULS (universal linkage system) kit following the manufacturer's instructions. A total of 250 ng of labeled cDNA was used for hybridization (40 h at 65°C), and scanning was performed with a Roche MS200 scanner. Raw data were extracted and normalized using Agilent Feature extraction software v11.5.1.1. The median expression of every probe set was obtained, scaled by log 2, and normalized by quantile using the Bioconductor software (www.bioconductor.org/). A principal component analysis was then carried out using the Partek genomics suite 6.4, and the significance of differential gene expression was tested using analysis of variance. Each microarray experiment was carried out in duplicate with cDNA prepared from two independent cultures. All microarray data are available at the GEO (NCBI, USA) database via accession number GSE68947.

Microarrays results were validated by quantitative real-time reverse transcription (RT)-PCR (Chromo 4, Bio-Rad), as previously described (25). Genes selected to be amplified were the following: *fatC*, *fatD*, *gyrA*, *gyrB*, *glnA*, *mutM*, *lmb*, *parE*, *psaB*, *pyrB*, *spr1680*, and *topA*. For most genes, previously described oligonucleotides were used (24, 25). For the rest of genes, oligonucleotides (forward/reverse) were as follows: *mutM* (5'-AGATGGTGGCAGCGTTGTTA-3'/5'-GTGTATCCATAGCACC GAGTCC-3'), *lmb* (5'-AAGGGGATGAAAATTGTGACCAG-3'/5'-GCCAAGATTTCGAGTGTATGAGA-3') *pyrB* (5'-AACTCTTTTTCGCTG GACCTG-3'/5'-ACTATCATGGCGTTCGTGTTG-3'), *psaB* (5'-ATAC GTATCGAAAACCTCAGTGTC-3'/5'-CCCAGCATACCTTTTAATAG TGTT-3'). Annealing temperatures for the oligonucleotides used were between 51°C and 56°C. PCR products had sizes that ranged from 128 to 205 bp.

Detection of ROS. The intracellular oxidation levels were measured using dihydrorhodamine 123 dye (Sigma-Aldrich), a nonfluorescent compound which diffuses across membranes. Oxidation converts it to the fluorescent product rhodamine 123. Measured fluorescence, therefore, is proportional to the level of oxidation (30). Cells were grown to an optical density at 620 nm (OD_{620}) of 0.4 before MOX was added. Samples were collected and processed as previously described (25). Fluorescence was measured using a Tecan Infinite 2000 device and a filter with excitation and emission wavelengths of 485 nm and 535 nm, respectively. Results were normalized according to the number of live cells at every given time point and expressed as relative fluorescence units (RFUs) (31). Hydrogen peroxide was detected with the Amplex Red hydrogen peroxide/peroxidase assay kit (Invitrogen). Horseradish peroxidase catalyzes a reaction with the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) and H_2O_2 to produce resorufin. Resorufin was detected by absorbance at 540 nm. In order to check the effects of MOX or LVX on the levels of H_2O_2 , cells were grown to an OD_{620} of 0.4 and then diluted 1:100 in growth media before MOX and LVX were added. Cells were then incubated at 37°C for 4 h. Samples were taken at 0, 3, and 4 h, washed 3 times in phosphate-buffered saline, resuspended in a buffer containing 100 mM phosphate (pH 8) and 1% deoxycholate, and incubated for 30 min at room temperature. The samples were centrifuged for 30 min at $10,000 \times g$, and the supernatants were analyzed with Amplex Red. The concentration of H_2O_2 in samples was calculated using a standard curve derived from assays of serial dilutions of a defined concentration of H_2O_2 . Final values were normalized according to the number of viable cells.

RESULTS

MOX caused fragmentation of chromosomal DNA. MOX was tested at fully inhibitory ($10 \times$ MIC) concentrations against the reference strain R6 carrying plasmid pLS1. Topoisomer distributions of the replicating pLS1 plasmid were analyzed and used as an estimation of changes in chromosomal supercoiling (32). At the chloroquine concentration used, the induced ΔLk was -14 (24). In the autoradiograms, topoisomers were found to be distributed in a bubble-shaped arc. Negative and positive supercoiled molecules were located in the right or left sides, respectively. No significant differences in supercoiling densities (σ) were detected under

FIG 1 Global supercoiling did not vary under treatment of *S. pneumoniae* R6 (pLS1) with MOX, although chromosomal DNA became fragmented. Cultures in AGCH that reached the exponential phase at an OD_{620} of 0.4 were treated with MOX at 10 $\times$ the MIC. Samples were taken before the addition of the drug (zero time), and, at the indicated times, plasmid DNA was isolated and subjected to two-dimensional agarose gel electrophoresis. (A) First-dimension run in the presence of 1 μ g/ml chloroquine, and second-dimension run in the presence of 2 μ g/ml chloroquine. Indicated supercoiling density (σ) values are averages $\pm$ standard deviations (SD) from three independent replicates. An open arrowhead indicates the topoisomer that migrated with $\Delta Lk = 0$ in the second dimension and that has a ΔWr of -14 . A blackened arrowhead points to the more abundant topoisomer. (B) PFGE gel of R6 at the indicated times and MOX concentrations. CZ, compression zone. (C) Quantification of the fragmentation: signal in the CZ divided by the combined signal of the lane plus well. Results are represented as the means $\pm$ SD from three independent replicates.

any condition. These results demonstrated that the inhibition of gyrase by MOX did not have a detectable effect on the overall level of supercoiling (Fig. 1A). However, consistent with the known mechanism of action of FQs (14), an increase in chromosomal fragmentation was observed by PFGE. The compression zone, the band in which the nicked fragments of chromosomal DNA that are unresolved in the gel migrate, was used to estimate fragmentation (33). As shown in Fig. 1B and C, fragmentation increased both with the MOX concentration used and with the time of treatment. These results are in agreement with the decrease in chromosomal DNA size detected by sucrose gradient sedimentation of *E. coli* cells treated with nalidixic acid (34).

MOX triggers a global transcriptional response in *S. pneumoniae*. The global transcriptional response of R6 cells to 10 $\times$ MIC (1.25 μ g/ml) of MOX was measured after 15 and 30 min. Changes in gene expression were normalized with respect to values at time zero. Normalized values are shown in Table 1. In total, the transcription of only 28 genes (1.4% of the genome) varied at 15 min. The response was more pronounced at 30 min, when the transcription of 132 genes (6.5% of the genome) varied. The responses at 15 and 30 min were consistent: 17 of 28 genes for which transcription had varied at 15 min also had varied transcription at 30 min. In addition, the transcriptional response of two R6-derived strains, T1 (ParC S97F; MOX MIC of 0.125 μ g/ml) and T2 (ParC S79F, GyrA S81F; MOX MIC of 4 μ g/ml) was also analyzed

in the presence of the same concentration used for R6, 1.25 μ g/ml, after 30 min of treatment. No significant responses were observed for T1 and T2 in the presence of MOX compared with no treatment. Only 5 genes were differentially expressed in T1, and 3 were in common with the R6 response. For the T2 strain, only 28 genes showed differential expression, and 5 were common to the R6 response. The transcriptome data from microarrays were validated by quantitative RT-PCR (qRT-PCR). The reverse transcription products of 12 genes were measured with samples taken at 10 $\times$ the MIC of MOX. Ratios of microarray and qRT-PCR levels showed a good correlation ($r^2 = 0.75$; $P = 3 \times 10^{-4}$) and a slope of 0.9, indicating similar sensitivities for qRT-PCR and microarray hybridization (Fig. 2A).

The transcriptional response induced by MOX in R6 was clearly different than the one induced by LVX. In fact, they overlapped with only 8 (29%) and 22 (17%) genes at 15 min and 30 min, respectively (Fig. 2B). A stratified categorization of functional classes based on the number of responsive genes at 30 min revealed 4 gene groups (I through IV) (Fig. 2C). Group I represented >15% of the total affected genes, which included those coding for hypothetical proteins and membrane transport systems. These two classes were also predominant in the transcriptional response to LVX (25). They formed the largest group of genes in the R6 genome (35). No bias in the kind of regulation was observed in this group: about half of the genes was upregulated,

TABLE 1 Genes involved in the transcriptomic response of *S. pneumoniae* to a 30-min treatment with MOX

Role or subrole	R6 locus (gene) ^a	Mean relative fold change ^b
Amino acid biosynthesis: Asp	spr1907 (<i>dapD</i>)	2.0
Amino acid biosynthesis: Gln	spr0443 ^c -0444 (<i>glnRA</i>)	2.0
Amino acid biosynthesis: Ser	spr1095 (<i>metY</i>)	3.9
	spr0823 (<i>but</i>) ^d	-2.3
Biosynthesis of cofactors	spr1324 (<i>apbE</i>) ^d	-2.8
Carbohydrate metabolism: glycolysis	spr0530 (<i>fba</i>)	2.7
	spr1902 (<i>gpdA</i>)	2.6
	spr1499 (<i>gpmA</i>)	2.3
	spr0647 (<i>pml</i>)	3.8
Carbohydrate metabolism: pyruvate	spr0415 (<i>pfl</i>)	2.9
Carbohydrate metabolism: pentose-P pathway	spr1438 (<i>entB</i>) ^d	-2.4
Carbohydrate metabolism: other	spr1841 (<i>tktA</i>)	2.8
	spr0064 (<i>agaS</i>) ^d	-2.2
	spr1667 (<i>galT</i>)	-4.4
	spr1833 (<i>bgl2</i>) ^d	-2.6
	spr1842 (<i>ulaG</i>) ^d	2.8
Cell envelope	spr1459 (<i>gtrB</i>)	2.9
	spr0540 ^c -0541 (<i>murMN</i>)	2.1
Cellular processes: detoxification	spr0864 (<i>lguL</i>)	-2.3
Cellular processes: pathogenesis	spr0328 (<i>eng</i>) ^d	-2.3
	spr0906 (<i>lmb</i>)	-3.4
	spr0910 (<i>phtE</i>)	-2.6
	spr1060 (<i>phpA</i>) ^d	-3.6
	spr1492 (<i>psaB</i>)	-2.2
Cellular processes: stress	spr0453 ^c -0456 (<i>hrcA-grpE-dnaK-dnaJ</i>)	2.6
	spr0692 (<i>gor</i>)	2.2
	spr1723 ^c -1722 (<i>groES-groEL</i>)	2.1
Cellular processes: cell division	spr0844 (<i>gidA</i>)	2.2
Cellular processes: toxin production	spr0880 (<i>tehB</i>)	-2.1
Central intermediary metabolism	spr0816 (<i>cad</i>)	-2.4
	spr0284 (<i>xylS</i>)	2.1
DNA metabolism	spr0673 (<i>holA</i>)	2.3
	spr0790 (<i>hsdR</i>)	2.6
	spr0872 (<i>mutM</i>)	-2.0
Fatty acid metabolism	spr0386 ^c -0387 (<i>accDA</i>)	-2.2
Protein export and trans-translation	spr0877 ^c -0879 (<i>secG-rnr-smpB</i>)	-2.7
Purines, pyrimidines	spr0028 (<i>prsA</i>)	2.6
	spr0655 (<i>upp</i>)	2.0
	spr0865 (<i>pyrK</i>) ^d	9.4
	spr1156 ^c -1153 (<i>pyrRB-carAB</i>)	3.5
	spr1165 (<i>pyrP</i>)	3.9
	spr2033 (<i>imdH</i>)	3.7
Regulatory functions: TCS12 (ComCDE)	spr0013 ^c (<i>comX2</i>)	2.1
	spr0654 (<i>comEB</i>)	2.1
	spr1962 ^c	3.7
Regulatory functions: TCS11	spr1814 (<i>rr11</i>)	-2.0
Transcription	spr1336	-2.9
	spr0336 (<i>ritR</i>)	2.0
	spr0679 (<i>ppiA</i>)	2.6

TABLE 1 (Continued)

Role or subrole	R6 locus (gene) ^a	Mean relative fold change ^b
Translation	spr0682 (<i>rpsP</i>) ^d	-2.1
	spr0861 ^c -0864 (<i>infC-rpmI-rplT-lguL</i>)	-2.4
	spr0876 (<i>rpmG</i>)	-2.4
	spr0913 (<i>pepT</i>)	3.1
	spr0920 (<i>trmE</i>)	2.2
	spr1082 (<i>lepA</i>)	-2.0
	spr1204 (<i>ptrB</i>)	3.3
	spr1211 (<i>rplL</i>) ^d	-2.1
	spr1871	-2.0
	spr1943 ^c -1944 (<i>rpmFG</i>) ^d	-2.0
Transport: amino acids, peptides, and amines	spr0327 (<i>aliA</i>) ^d	-3.8
	spr0369 (<i>dagA</i>)	-2.0
	spr0524	-2.3
	spr0535 ^c -0532	2.6
	spr1704 ^c -1703 (<i>amiEF</i>)	-2.0
Transport: anions	spr0648	3.1
Transport: sugars	spr0063	-2.1
Transport: other	spr0875 (<i>pmrA</i>)	-3.7
	spr1203	3.1
	spr1216 ^c -1215	-2.4
	spr1352 (<i>bta</i>)	2.1
	spr1559	-2.0
	spr1817	-2.9
	spr2035	2.5

^a The responsive genes included in this list showed significant fold variations (≥ 2 and $P < 0.01$). All genes showing variations are included, with the exception of 22 encoding hypothetical proteins and 7 encoding transposases. Genes involved in the growth-related response are not included.

^b In operons, values indicated correspond to those of the first gene of the operon.

^c The first gene of the operon.

^d Genes affected in the response to LVX and to MOX.

^e Gene with a ComE box.

while the other half was downregulated. Group II represented 5% to 15% of the total of affected genes. These had a role in 1 of 5 different processes: translation, purine and pyrimidine metabolism, carbohydrate metabolism, transposon functions, or stress response. Biases in regulation were linked to the role the genes played. Genes involved in translation, which included translation factors and genes of ribosomal proteins, were mostly downregulated (11 of 14). However, affected genes involved in purine and pyrimidine metabolism were upregulated (Fig. 3A). Most responsive genes involved in carbohydrate metabolism, in particular the genes of several pathways involved in the production of pyruvate, were also upregulated. These included 2 genes, *tktA* and *pml*, whose products convert 6-phosphate (6P) sugars to fructose 6-phosphate (fructose-6P). Among these were 3 of 7 glycolysis genes (*fba*, *apdA*, and *gpmA*). Glycolysis is the conversion of glucose to pyruvate through a fructose-6P intermediate. Meanwhile, the genes coding for acetyl coenzyme A (acetyl-CoA) carboxylase (*accDA*) were downregulated, which would be expected to lead to an increase in the concentration of acetyl-CoA. This would, in turn, lead to an increase in the amount of pyruvate through the action of formate acetyltransferase (*pfl*), whose gene was upregulated (Fig. 3B). Meanwhile, 8 of the 9 genes involved in transposon functions were downregulated. All responsive genes involved in stress responses were also upregulated. These included the *hrcA*-

FIG 2 Gene expression analysis under the two conditions assayed. (A) Correlation of the changes in RNA transcript levels using data from qRT-PCR and microarray hybridizations. (B) Localization of responsive genes in the chromosome of *S. pneumoniae*. The relative fold variation of each gene is plotted against the location of the 3' end of each open reading frame in the *S. pneumoniae* R6 chromosome (bases 1 to 2,038,615). (C) Classification of responsive genes by functional classes: AaB, amino acid biosynthesis; CaM, carbohydrate metabolism; CE, cell envelope; DNA, DNA metabolism; Pat, pathogenesis; Str, stress; FaM, fatty acid metabolism; PPy, purines and pyrimidines metabolism; TCS, two-component systems; Tr, translation; TBP, transport and binding proteins; Hy, hypothetical proteins; Tns, transposon functions; Oth, other (classes with a representation of <2.5%). Column colors: black, upregulated responsive genes; white, downregulated responsive genes.

grpE-dnaK-dnaJ and *groES-groEL* operons, which encode chaperones that modulate protein folding and promote refolding and proper assembly (36). Group III represented 2.5% to 5% of the affected genes. These included genes involved in pathogenesis (all 7 downregulated); amino acid biosynthesis (4 upregulated, 1 downregulated); genes of two-component systems (TCSs) (3 of TCS12 upregulated, 1 of TCS11 downregulated); DNA metabolism; fatty acid metabolism; cell envelope; and the *secG-rnr-smgB* (37) operon that is involved in protein secretion (SecG), RNA degradation (RNase R), and *trans*-translation (SmpB). Group IV represented <2.5% of the affected genes and included diverse functions, with no clear pattern in responses.

ROS contribute to the lethality of MOX. The transcriptomic response described above implied an increase in the intracellular concentration of pyruvate. This would, in turn, lead to increases in

FIG 3 The regulation of two metabolic pathways by MOX treatment would yield increases in UMP (A) or pyruvate (B). The increase in pyruvate would produce a consequent increase in hydrogen peroxide and, in turn, an increase in hydroxyl radicals via the Fenton reaction.

H₂O₂, through the activity of pyruvate oxidase (SpxB), and in hydroxyl radical, through the Fenton reaction (Fig. 3B). To assess the relevance of H₂O₂ production in MOX lethality, a previously constructed *spxB*-deletion mutant was assayed (25). This mutant produces 80% less hydrogen peroxide than the wild-type strain (38). Consistent with this observation, the R6Δ*spxB* strain was less susceptible to MOX (Fig. 4A), while deletion of *spxB* in strains T1

FIG 4 MOX lethality is linked to the level of intracellular hydrogen peroxide. (A) Viability of *S. pneumoniae* strains either in AGCH or in AGCH plus MOX at 0.125 μg/ml (1× the MIC for R6 and T1). Values were normalized to the zero time value. The mean ± standard error of the mean from three independent replicates is shown. (B) Accumulation of reactive oxygen species, measured using dihydrorhodamine 123 dye, a nonfluorescent compound which is converted by oxidation to fluorescent rhodamine 123. Strains R6 and R6ΔspxB were grown to an OD₆₂₀ of 0.4 before MOX at 1× the MIC was added, samples were processed, and fluorescence was measured, as described in Materials and Methods. RFU, relative fluorescence units; values were divided by the number of viable cells. (C) Accumulation of hydrogen peroxide in the indicated conditions, detected with the Amplex Red hydrogen peroxide/peroxidase assay kit, as described in the Materials and Methods. Cells were grown to an OD₆₂₀ of 0.4 and then diluted 1:100 in growth media before MOX and LVX were added, both at 0.5× the MIC for R6. Cells were then incubated at 37°C for 4 h. Samples were taken at 0, 3, and 4 h and processed as described in Materials and Methods. Values were calculated as the concentration of hydrogen peroxide in μM normalized against the number of viable cells and calculated as the fold difference with respect to the initial zero time value.

and T2 did not change this susceptibility. No appreciable differences in susceptibility were observed in the presence of the iron chelator 1,10-phenanthroline. This result indicated that the absence or presence of iron did not affect cell survival, despite iron

being a component of the Fenton reaction. Meanwhile, accumulation of ROS was observed in R6 cultures treated with MOX (Fig. 4B). Sharp increases with respect to time zero of 22.1-fold, 54.9-fold, and 80.8-fold were observed at 3, 4, and 5 h of treatment,

respectively. However, there was a less than 5-fold increase for the ΔspxB strain (Fig. 4B). Only a 15-fold increase in ROS was observed at 3 to 5 h in the presence of 1,10-phenantroline (Fig. 4B). In addition, we observed a comparable increase of about 21-fold in the production of H_2O_2 in R6 cultures after 3 and 4 h with $0.5\times$ the MIC of MOX (Fig. 4C). However, no increase was detected in the presence of LVX. A plausible interpretation is that the 15-fold increase of signal in the presence of 1,10-phenantroline happens independent of the Fenton reaction and is really a consequence of an increase in H_2O_2 , which was also detected by dihydrorhodamine 123. These results suggest a link between MOX lethality and the Fenton reaction via an increase of H_2O_2 . In summary, ROS contributes to the lethality of MOX in two ways: directly by an increase in endogenous H_2O_2 and indirectly via the Fenton reaction. No increases in the production of H_2O_2 in the T1 and T2 strains with respect to the time zero levels were observed in the presence of MOX. This is consistent with the fact that the susceptibility of MOX for strain T1 ΔspxB did not change.

DISCUSSION

In an effort to understand the transcriptional alterations mediated by inhibitors of DNA topoisomerases, we analyzed the global transcriptomic response of *S. pneumoniae* to MOX. As far as we are aware, this is the first study of the effects of this drug on transcription in pathogenic bacteria. In order to selectively inhibit gyrase, we used $10\times$ the MIC of MOX. At this concentration, gyrase was expected to be fully inhibited. However, the possibility could not be discarded that topo IV was also partially inhibited (13, 39, 40). To test this possibility, the transcriptomic responses of two isogenic strains, T1 (as R6, ParC S79F) and T2 (as R6, ParC S79F, GyrA S81F), were analyzed. No significant transcriptional response was detected in any strain, suggesting that topo IV is also inhibited by MOX at this concentration. In accordance, treatment of T1 with MOX caused the inhibition of growth, although the decrease in viability detected in R6 was not observed (Fig. 4A). Likewise, no inhibition of growth was detected in T2.

A global transcriptional response induced by MOX in R6 was observed. A similar number of genes was affected with MOX as with LVX at $10\times$ the MIC; however, these had different patterns (25). Those genes for which transcription varied in the control (nontreated) culture were considered growth-related genes and were filtered out in the analysis of the treated cultures (25). When excluding these genes, we found that there were only 2 genes similarly affected by LVX and MOX at 15 min. At 30 min of treatment, 15 genes were similarly affected. From these results, we can conclude that the transcriptomic responses to treatment with LVX or MOX are different, given that a low proportion of the responsive genes were similarly affected. These differences revealed the different modes of action of the two drugs: LVX inhibited topo IV (12, 13, 40), and MOX inhibited both topo IV and gyrase.

However, LVX and MOX affected the activation of competence in a similar fashion. We previously observed that, over time, a control culture had a general downregulation of the 53 genes of the competence regulon (25). Meanwhile, treatment with LVX led to a decrease in this downregulation (25). The effect was even stronger with MOX: downregulation was reversed and upregulation was even observed in the case of 3 competence genes (Table 1), including the gene coding for the alternative sigma factor ComX, which activates transcription of late competence genes (41, 42). Thus, activation of competence in

S. pneumoniae is a common stress response to FQs in a bacterium that lacks an SOS-like system (43).

We have not detected changes in general supercoiling with LVX (25) or with MOX treatments. However, we observed a different pattern of gene expression alteration with these drugs. A possibility is that the transcriptional alterations induced by MOX and LVX are due to local changes in supercoiling, which are dependent on whether topo IV (LVX) or gyrase (MOX) is inhibited. We have proved that this is the case for the *fatCDE* operon (25). Since both topo IV and gyrase produce double-stranded breaks in the DNA when inhibited by FQs, the differential transcriptional alterations caused by these drugs could also be related to differences in sequence recognition (44), which is affected by DNA supercoiling and bendability (45). Sequence recognition mediated by local supercoiling levels might explain the unique distribution of the genes affected by LVX or MOX (Fig. 2C). In addition, the location of the FQ-topoisomerase complexes relative to the replication forks, which is different for gyrase and topo IV (46), could also have a role in the different transcription alterations.

Several genes related to purine and pyrimidine metabolism were upregulated with MOX but not with LVX. Among them was *prsA*, coding for an enzyme that catalyzes the synthesis of 5-phosphoribosyl- α -1-pyrophosphate (PRP). PRP is an intermediary in both purine and pyrimidine metabolism (47). The product of *pyrP* is a dedicated transporter that facilitates the entrance of uracil into the cell. Uracil is converted to UMP by the product of *upp*. The intracellular concentration of UMP would be expected to increase through the activation of the CO_2 biosynthesis pathway; 6 of 8 of these genes were upregulated (Table 1, Fig. 3A). These genes are regulated by an attenuator mechanism and would form part of the PyrR regulon. PyrR senses UMP levels and, when the concentration is high, binds to both UMP and the antiterminator, impeding the transcription of downstream genes. As in other lactic acid bacteria (47), the *pyrFE*, *pyrK*, and *pyrR-pyrB-carA-carB* genes would be modulated by this system.

The pathways affecting pyruvate levels were upregulated by MOX but not by LVX. These pathways include the *tktA* and *pmi* genes, which encode enzymes that convert ribulose-5P to fructose-6P and manose-6P to fructose-6P, respectively. They also include 3 of 7 genes of the glycolysis pathway (Fig. 3B). Likewise, acetyl-CoA carboxylase was downregulated in a MOX-dependent manner, likely leading to an increase in acetyl-CoA levels. This increase would lead to increased production of pyruvate by formate acetyltransferase (*pfl*), also upregulated by MOX. An increase in pyruvate would lead to a parallel increase, mediated by SpxB, in intracellular H_2O_2 . This would, in turn, increase the amount of hydroxyl radical produced through the Fenton reaction. We observed comparable increases in the production of H_2O_2 and hydroxyl radical as a result of MOX treatment (Fig. 4).

We have demonstrated that ROS have a role in the lethality of MOX in *S. pneumoniae* R6. Consistent with this model, the R6 ΔspxB and T1 strains were less susceptible to this drug (Fig. 4). These results support a model whereby the accelerated production of redox alterations by FQs contributes to their lethality (18, 20). Also consistent is the fact that the glutathione reductase (*gor*) gene was upregulated, presumably in an attempt to counteract the lethal level of oxidation. Glutathione reductase reduces glutathione (γ -L-glutamyl-L-cysteinyl-glycine), which lowers the level of oxidation. This provides protection from H_2O_2 accumulation (48).

Stress-related genes coding for chaperones were also upregulated by MOX. These were the *hrcA-grpE-dnaK-dnaJ* and *groES-groEL* operons. These operons are induced in bacteria by a wide variety of stresses, including starvation, exposure to free radicals, and heat shock. In *S. pneumoniae*, the *hrcA-grpE-dnaK-dnaJ* operon is also known to be regulated by heat shock (49). In addition, the *secG-rnr-smpB* (37) operon was downregulated in the presence of MOX. This operon codes for the SecG integral membrane protein that forms part of the SecYEG complex, which is involved in protein secretion. The operon also codes for RNase R, involved in RNA degradation, and SmpB, which mediates trans-translation. This operon is also regulated by stress and is upregulated by cold shock (37). In addition, *lepA*, the gene coding EF-4, which is involved in trans-translation, is also downregulated (Table 1). In *E. coli*, LepA contributed to ROS lethality (50). Then, downregulation would be a protective effect. In this light, a reduction in the repair systems increases MOX lethality in *Mycobacterium smegmatis* (51). Whether or not these operons are regulated by ROS in *S. pneumoniae* remains to be determined.

The results presented here complement our previous findings that LVX, an inhibitor of topo IV, triggers the transcriptional activation of iron transport genes (25), a result not observed with MOX. LVX and MOX stimulate the Fenton reaction by causing an increase in the concentration of iron and H₂O₂, respectively. In addition to causing hydroxyl radical to accumulate, MOX increases endogenous H₂O₂, which has been shown to alter membrane composition (52) and survival (53) in *S. pneumoniae*. This study suggests that a strategy for improving the efficacy of FQs may be to potentiate their ability to provoke the accumulation of intracellular hydroxyl radical and H₂O₂.

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