

This is the peer reviewed version of the following article:

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

M. J. Ferrándiz, A. J. Martín-Galiano, C. Arnanz, T. Zimmerman, and A. G. de la Campa corresponding author.

Antimicrob Agents Chemother. 2015 Nov 2;60(1):409-17.

which has been published in final form at <https://doi.org/10.1128/AAC.02299-15>

1

2

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

5 **MJ Ferrándiz ^a, AJ Martín-Galiano ^a, C Arnanz ^a, T Zimmerman ^a, and AG de la**
6 **Campa ^{a, b}**

7 ^aUnidad de Genética Bacteriana, Centro Nacional de Microbiología, Instituto de Salud
8 Carlos III, 28220 Majadahonda, Madrid, Spain. ^bPresidencia. Consejo Superior de
9 Investigaciones Científicas, Madrid, Spain.

10

11 Corresponding author. Mailing address: Unidad de Genética Bacteriana, Centro
12 Nacional de Microbiología. Instituto de Salud Carlos III, 28220 Majadahonda, Madrid,
13 Spain. Phone: (34) 91 822 3944. Fax: (34) 91 509 7966. E-mail: E-mail:
14 agcampa@isciii.es

15 **Running title:** Increase of reactive oxygen species with moxifloxacin

16 **ABSTRACT**

17 We studied the transcriptomic response of *Streptococcus pneumoniae* to the
18 fluoroquinolone moxifloxacin at a concentration that inhibits DNA gyrase. Treatment of
19 the wild-type strain R6, at a concentration of 10× MIC, triggered a response involving
20 132 genes after 30 minutes of treatment. Genes from several metabolic pathways
21 involved in the production of pyruvate were up-regulated. These included 3 glycolytic
22 enzymes, which ultimately convert fructose-6P to pyruvate, and 2 enzymes that funnel
23 P-sugars into the glycolytic pathway. In addition, acetyl-CoA carboxylase was down-
24 regulated, likely leading to an increase in acetyl-CoA. When coupled with an up-
25 regulation in formate acetyltransferase, an increase in acetyl-CoA would raise the
26 production of pyruvate. Since pyruvate is converted by pyruvate oxidase (SpxB) into
27 hydrogen peroxide (H₂O₂), an increase in pyruvate would augment intracellular H₂O₂.
28 Here, we confirm a 21-fold increase in the production of H₂O₂ and a 55-fold increase in
29 the amount of hydroxyl radical in cultures treated during 4 h with moxifloxacin. This
30 increase in hydroxyl radical through the Fenton reaction, would damage DNA, lipids,
31 and proteins. These reactive oxygen species contributed to the lethality of the drug, a
32 conclusion supported by the observed protective effects of a SpxB deletion. These
33 results support the model whereby fluoroquinolones cause redox alterations. The
34 transcriptional response of *S. pneumoniae* to moxifloxacin is compared with the
35 response to levofloxacin, an inhibitor of topoisomerase IV. Levofloxacin triggers the
36 transcriptional activation of iron transport genes and also enhances the Fenton reaction.

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

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

62 inhibition, hydroxyl radical are generated via the Fenton reaction (16) adding to the
63 lethality of FQs (17-20). ROS production is observed with antibiotics from a variety of
64 bactericidal antibiotic families in addition to FQs, each with a different intracellular
65 target. However, the intervening pathways lying between the initial antibiotic-target
66 interaction and ROS formation are not fully characterized, although some are beginning
67 to be described (21-23). We have previously investigated these pathways for LVX in *S.*
68 *pneumoniae*. We observed a global transcriptional response at concentrations of LVX
69 required to inhibit the topo IV enzyme but not gyrase. This response included the up-
70 regulation of *fatDCEB*, an operon responsible for iron uptake (Fe^{2+} and Fe^{3+}). These
71 were the only genes up-regulated at every concentration (0.5× MIC and 10× MIC) and
72 time point studied (5, 15 and 30 min). In addition, they were the only genes varying at
73 0.5× MIC and 5 min of LVX treatment. LVX-induced up-regulation of *fatDCEB* leads
74 to an increase of intracellular iron, and in turn, to the shift in the Fenton reaction
75 favoring the production of hydroxyl radical. We established a relationship between an
76 increase in ROS and LVX lethality and demonstrated that local topological changes
77 were involved in the up-regulation of *fatDCEB*. LVX did not changed global
78 supercoiling. However, increases in negative supercoiling and *fatDECB* transcription
79 after topoisomerase I inhibition were observed, while the opposite occurred after gyrase
80 inhibition with novobiocin. In accordance, the *fatDCEB* operon is located in the
81 chromosome into a topological domain down-regulated by DNA relaxation (24). We
82 constructed a strain in which a P_{fatcat} fusion was positioned 106-kb away from the
83 $P_{fatfatDCEB}$ native position. In this strain, up-regulation of the operon in the presence of
84 LVX were observed, while no change was observed in P_{fatcat} . Results indicated that
85 *fatDCEB* transcription depends on its location in a topological domain. These results

86 illustrated a link between the initial LVX interaction with its topo IV target and the
87 production of ROS (25).

88 We present here a complementary study whose aim was to understand the
89 transcriptional response of *S. pneumoniae* to MOX at concentrations that inhibited
90 gyrase. Chromosomal DNA fragmentation and changes in DNA topology were
91 monitored. The global transcriptional response was analyzed by microarray technology.
92 The results sketch a pathway from gyrase inhibition to ROS production and suggest that
93 *S. pneumoniae* accumulates H₂O₂ as part of the death process associated with MOX
94 treatment.

95 **MATERIALS AND METHODS**

96 **Bacterial strains and conditions for growth and transformation.** *S. pneumoniae* was
97 grown in a casein hydrolysate based medium (AGCH) with 0.3% sucrose (26). The
98 MICs of MOX and LVX (Sigma-Aldrich) for the R6 strain were 0.125 µg/ml and 0.250
99 µg/ml, respectively. Two R6-derived strains, T1 and T2 (27) were also used. T1 carried
100 the ParCS79F change and had MICs of 2 µg/ml (LVX) and 0.125 µg/ml (MOX). T2
101 carried both ParCS79F and GyrAS81F changes and had MICs of 16 µg/ml (LVX) and 4
102 µg/ml (MOX). *S. pneumoniae* R6 carrying the plasmid pLS1 was cultured in AGCH
103 medium with 1 µg/ml tetracycline.

104 **Analysis of chromosomal fragmentation and of the topology of covalently closed**
105 **circles.** Chromosomal fragmentation was detected by pulse-field gel electrophoresis
106 (PFGE). About 3× 10⁶ cells were lysed in solid agarose inserts in a buffer containing 10
107 mM Tris HCl pH 8, 1 M NaCl, 0.1 M EDTA, 0.5% Brij58, 0.2% deoxycholate, 0.5%
108 sarkosyl, 20 µg/ml RNaseE and 100 µg/ml lysozyme. Agarose inserts were treated with

109 1 mg/ ml proteinase K and washed before to place them into a 1% low gelling agarose
110 (Pronadisa) gel in 0.5% × TBE buffer (45 mM Tris-borate pH 8, 1 mM EDTA). Gels
111 were electrophoresed in a Cheff-DR III System (BioRad), during 20 hours at 5.8 V/cm
112 with a 0.1 to 40 seconds switch-time ramp at 14°C. Plasmid DNA isolation from *S.*
113 *pneumoniae* (pLS1) was performed using the neutral method already described (28).
114 Circular DNA molecules were analyzed in neutral/neutral two-dimensional agarose
115 gels. The first dimension was run at 1.5 V/cm in a 0.4% agarose (Seakem; FMC
116 Bioproducts) gel in 1 × TBE buffer for 17–19 h at room temperature. The second
117 dimension was run at 7.5 V/cm in a 1% agarose gel in 1 × TBE buffer for 7–9 h at 4°C.
118 Chloroquine (Sigma-Aldrich) was added to the TBE buffer before use. After
119 electrophoresis, gels were subjected to Southern hybridization with a 240-bp probe
120 specific for pLS1, as described previously (24). The DNA linking number (Lk) was
121 calculated by quantifying every topoisomer. The DNA supercoiling density (σ) was
122 calculated using the equation $\sigma = \Delta Lk / Lk_0$. The change in linking number (ΔLk) was
123 determined using the equation $Lk = Lk - Lk_0$, where $Lk_0 = N/10.5$, N is the size of the
124 molecule (in bp) and 10.5 the number of bp per one complete turn of B-DNA.

125 **Normalization and analysis of microarray data.** High density expression arrays
126 from Agilent were processed at the Functional Genomics Core Facility, Institut de
127 Recerca Biomèdica, Barcelona (Spain). Arrays were designed as described (29). cDNAs
128 were obtained from 25 ng of total RNA using the Trans-Plex WTA2 kit (Sigma). These
129 cDNAs were labelled with Cy3 using the Agilent Genomic DNA ULS™ (Universal
130 Linkage System) kit following manufacturer's instructions. A total of 250 ng of labelled
131 cDNA were used for hybridization (40 hours at 65°C) and scanning was performed with
132 a Roche MS200 scanner. Raw data were extracted and normalized using Agilent
133 Feature extraction software v11.5.1.1. The median expression of every probeset was

134 obtained, scaled by log₂ and normalized by quantile using the Bioconductor software
 135 (www.bioconductor.org/). A principal component analysis was then carried out using
 136 the Partek Genomics Suite 6.4 and the significance of differential gene expression was
 137 tested using ANOVA. Each microarray experiment was carried out in duplicate with
 138 cDNA prepared from two independent cultures. All microarray data are available at the
 139 GEO (NCBI, USA) database via accession number GSE68947.

140 Microarrays results were validated by quantitative real-time RT-PCR (Chromo 4,
 141 BioRad) as previously described (25). Genes selected to be amplified were the
 142 following: *fatC*, *fatD*, *gyrA*, *gyrB*, *glnA*, *mutM*, *lmb*, *parE*, *psaB*, *pyrB* *spr1680*, and
 143 *topA*. For most genes, previously described oligonucleotides were used (24, 25). For the
 144 rest of genes, oligonucleotides (forward/ reverse) were: *mutM* (5'-
 145 AGATGGTGGCACGCTTGTTTA-3'/ 5'-GTGTATCCATAGCACCGAGTCC-3'),
 146 *lmb* (5'-AAGGGGATGAAAATTGTGACCAG-3'/ 5'-
 147 GCCCAAGATTCGAGTGTATGAGA-3') *pyrB* (5'-
 148 AACTCTTTTTTCGCTGGACCTG-3'/ 5'-ACTATCATGGCGTTCGTGTTG-3'), *psaB*
 149 (5'-ATACGTATCGAAAACCTCAGTGTC-3'/ 5'-
 150 CCCAGCATACTTTTAATAGTGTT-3'). Annealing temperatures for the
 151 oligonucleotides used were comprised between 51 and 56 °C. PCR products had sizes
 152 varying from 128 to 205 bp,

153 **Detection of ROS.** The intracellular oxidation levels were measured using
 154 dihydrorhodamine 123 dye (Sigma-Aldrich), a non-fluorescent compound which
 155 diffuses across membranes. Oxidation converts it to the fluorescent product rhodamine
 156 123. Measured fluorescence is therefore proportional to the level of oxidation (30).
 157 Cells were grown to an optical density at 620 nm (OD₆₂₀) = 0.4 before MOX was added.
 158 Samples were collected and processed as previously described (25). Fluorescence was

159 measured using a Tecan Infinite 2000 device and a filter with excitation/emission
160 wavelengths of 485 nm/535 nm. Results were normalized according to the number of
161 live cells at every given time point and expressed as relative fluorescence units
162 (RFU)(31). Hydrogen peroxide was detected with the Amplex® Red Hydrogen
163 Peroxide/Peroxidase Assay Kit (Invitrogen). Horseradish peroxidase catalyzes a
164 reaction with the Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) and
165 H₂O₂ to produce resorufin. Resorufin was detected by absorbance at 540 nm. In order to
166 check the effects of MOX or LVX on the levels of H₂O₂, cells were grown to an optical
167 density OD₆₂₀ of 0.4 and then diluted 1:100 in growth media before MOX and LVX
168 were added. Cells were then incubated at 37°C for 4 hours. Samples were taken at 0, 3,
169 and 4 hours, washed 3 times in PBS, resuspended in a buffer containing 100 mM
170 phosphate, pH 8 and 1% deoxycholate, and incubated for 30 min at room temperature.
171 The samples were centrifuged for 30 min at 10000 g and the supernatants were analyzed
172 with Amplex Red. The concentration of H₂O₂ in samples was calculated using a
173 standard curve derived from assays of serial dilutions of a defined concentration of
174 H₂O₂. Final values were normalized according to number of viable cells.

175

176 **RESULTS**

177 **MOX caused fragmentation of chromosomal DNA.** MOX was tested at fully
178 inhibitory (10× MIC) concentrations against the reference strain R6 carrying plasmid
179 pLS1. Topoisomer distributions of the replicating pLS1 plasmid were analyzed and
180 used as an estimation of changes in chromosomal supercoiling (32). At the chloroquine
181 concentration used, the induced ΔLk is -14 (24). In the autoradiograms, topoisomers
182 were found to be distributed in a bubble-shaped arc. Negative and positive supercoiled

183 molecules are located in the right or left sides, respectively. No significant differences
184 in supercoiling densities (σ) were detected under any condition. These results
185 demonstrated that the inhibition of gyrase by MOX did not have a detectable effect on
186 the overall level of supercoiling (Fig. 1A). However, consistent with the known
187 mechanism of action of FQs (14), an increase in chromosomal fragmentation was
188 observed by PFGE. The compression zone (CZ), the band in which the nicked
189 fragments of chromosomal DNA that are unresolved in the gel migrate, was used to
190 estimate fragmentation (33). As shown in Fig. 1B and 1C, fragmentation increased both
191 with the MOX concentration used and with the time of treatment. These results are in
192 agreement with the decrease in chromosomal DNA size detected by sucrose gradient
193 sedimentation of *E. coli* cells treated with nalidixic acid (34).

194 **MOX triggers a global transcriptional response in *S. pneumoniae*.** The global
195 transcriptional response of R6 cells to 10× MIC (1.25 $\mu\text{g/ml}$) of MOX was measured
196 after 15 and 30 min. Changes in gene expression were normalized with respect to values
197 at time 0. Normalized values are shown in Table 1. In total, the transcription of only 28
198 genes (1.4% of the genome) varied at 15 min. The response was more pronounced at 30
199 min, when the transcription of 132 genes (6.5% of the genome) varied. The responses at
200 15 and 30 min were consistent: 17 out of 28 genes whose transcription had varied at 15
201 min had also varied at 30 min. In addition, the transcriptional response of two R6-
202 derived strains, T1 (ParCS97F; MOX MIC=0.125 $\mu\text{g/ml}$) and T2 (ParCS79F,
203 GyrAS81F; MOX MIC=4 $\mu\text{g/ml}$) was also analysed in the presence of the same
204 concentration used for R6, 1.25 $\mu\text{g/ml}$, after 30 min of treatment. No significant
205 responses were observed for T1 and T2 in the presence of MOX compared with not
206 treatment. Only 5 genes were differentially expressed in T1, 3 being in common with
207 the R6 response. For the T2 strain, only 28 genes showed differential expression, 5

208 being common to the R6 response. The transcriptome data from microarrays were
209 validated by qRT-PCR. The reverse transcription products of 12 genes were measured
210 with samples taken at 10× MIC of MOX. Ratios of microarray and qRT-PCR showed a
211 good correlation ($r^2=0.75$, p value= 3×10^{-4}) and a slope of 0.9, indicating similar
212 sensitivities for qRT-PCR and microarray hybridization (Fig. 2A).

213 The transcriptional response induced by MOX in R6 was clearly different to the one
214 induced by LVX. In fact, they overlapped with only 8 (29%) and 22 (17%) genes at 15
215 min and 30 min, respectively (Fig. 2B). A stratified categorization of functional classes
216 based on the number of responsive genes at 30 min revealed 4 gene groups (Fig. 2C).
217 Group I represented >15% of the total affected genes, which included those coding for
218 hypothetical proteins and membrane transport systems. These two classes were also
219 predominant in the transcriptomic response to LVX (25). They form the largest group of
220 genes in the R6 genome (35). No bias in the kind of regulation was observed in this
221 group: about half of the genes was up-regulated while the other half was down-
222 regulated. Group II represented 5-15% of the total of affected genes. These had a role in
223 one of five different processes: translation, purine and pyrimidine metabolism,
224 carbohydrate metabolism, transposon functions, and stress response. Biases in
225 regulation were linked to the role the genes played. Genes involved in translation, which
226 included translation factors and genes of ribosomal proteins, were mostly down-
227 regulated (11 out of 14). However, affected genes involved in purine and pyrimidine
228 metabolism were up-regulated (Fig. 3A). Most responsive genes involved in
229 carbohydrate metabolism were also up-regulated, in particular, the genes of several
230 pathways involved in the production of pyruvate. These included 2 genes, *tktA* and *pmi*,
231 whose products convert 6P-sugars to fructose 6P. Among these were 3 out of 7
232 glycolysis genes (*fba*, *apdA*, *gpmA*). Glycolysis is the conversion of glucose to pyruvate

233 through a fructose-6P intermediate. Meanwhile, the genes coding for acetyl-CoA
234 carboxylase (*accDA*) were down-regulated, which would be expected to lead to an
235 increase in the concentration of acetyl-CoA. This would, in turn, lead to an increase in
236 the amount of pyruvate through the action of formate acetyltransferase (*pfl*), whose gene
237 was up-regulated (Fig. 3B). Meanwhile, 8 out of the 9 genes involved in transposon
238 functions were down-regulated. All responsive genes involved in stress responses were
239 also up-regulated. These included the *hrcA-grpE-dnaK-dnaJ* and *groES-groEL* operons,
240 which encode chaperones that modulate protein folding, promote refolding and proper
241 assembly (36). Group III represented 2.5-5% of the affected genes. These included
242 genes involved in pathogenesis (all 7 down-regulated); amino acid biosynthesis (4 up-
243 regulated, 1 down-regulated); genes of two-component systems (3 of TCS12 up-
244 regulated, 1 of TCS11 down-regulated); DNA metabolism; fatty acid metabolism; cell
245 envelope; and the *secG-rnr-smpB* (37) operon that is involved in protein secretion
246 (SecG), RNA degradation (RNaseR) and trans-translation (SmpB). Group IV
247 represented <2.5% of the affected genes, and included diverse functions with no clear
248 pattern in responses.

249 **ROS contribute to the lethality of MOX.** The transcriptomic response described
250 above implied an increase in the intracellular concentration of pyruvate. This would, in
251 turn, lead to increases in both H₂O₂, through the activity of pyruvate oxidase (SpxB),
252 and in hydroxyl radical, through the Fenton reaction (Fig. 3B). To assess the relevance
253 of H₂O₂ production in MOX lethality, a previously constructed *spxB*-deletion mutant
254 was assayed (25). This mutant produces 80% less hydrogen peroxide than the wild-type
255 strain (38). Consistent with this observation, the R6Δ*spxB* strain was less susceptible to
256 MOX (Fig. 4A), while deletion of *spxB* in strains T1 and T2 did not change this
257 susceptibility. No appreciable differences in susceptibility were observed in the

258 presence of the iron chelator *o*-phenantroline. This result indicated that absence or
259 presence of iron did not affect cell survival, despite iron being a component of the
260 Fenton reaction. Meanwhile, accumulation of ROS was observed in R6 cultures treated
261 with MOX (Fig. 4B). Sharp increases with respect to time 0 of 22.1-fold, 54.9-fold and
262 80.8-fold were observed at 3, 4, and 5 hours of treatment, respectively. However, there
263 was less than 5-fold increase for the $\Delta spxB$ strain (Fig. 4B). Only a 15-fold increase in
264 ROS was observed at 3 to 5 hours in the presence of *o*-phenantroline (Fig. 4B). In
265 addition, we observed a comparable increase of about 21-fold in the production of H₂O₂
266 in R6 cultures after 3 and 4 hours with 0.5× MIC of MOX (Fig. 4C). However, no
267 increase was detected in the presence of LVX. A plausible interpretation is that the 15-
268 fold increase of signal in the presence of *o*-phenantroline, happens independently of the
269 Fenton reaction and is really a consequence of an increase in H₂O₂, which was also
270 detected by dihydrorhodamine 123. These results suggest a link between MOX lethality
271 and the Fenton reaction via an increase of H₂O₂. In summary, ROS contributes to the
272 lethality of MOX in two ways: directly by an increase in endogenous H₂O₂ and
273 indirectly via the Fenton reaction. No increases in the production of H₂O₂ in the T1 and
274 T2 strains with respect to the time 0 min were observed in the presence of MOX. This is
275 consistent with the fact that the susceptibility of MOX for strain T1 $\Delta spxB$ did not
276 change.

277

278 **DISCUSSION**

279 In an effort to understand the transcriptional alterations mediated by inhibitors of DNA
280 topoisomerases we analyzed the global transcriptomic response of *S. pneumoniae* to
281 MOX. As far as we are aware, this is the first study of the effects of this drug on

282 transcription in pathogenic bacteria. In order to selectively inhibit gyrase, we used 10×
283 MIC of MOX. At this concentration gyrase was expected to be fully inhibited.
284 However, the possibility could not be discarded that topo IV was also partially inhibited
285 (13, 39, 40). To test this possibility, the transcriptomic responses of two isogenic
286 strains, T1 (as R6, ParCS79F) and T2 (as R6, ParCS79F, GyrAS81F), were analyzed.
287 No significant transcriptional response was detected in any strain, suggesting that topo
288 IV is also inhibited by MOX at this concentration. In accordance, treatment of T1 with
289 MOX caused the inhibition of growth, although the decrease in viability detected in R6
290 was not observed (Fig. 4A). Likewise, no inhibition of growth was detected in T2.

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

302 However, LVX and MOX affected the activation of competence in a similar
303 fashion. We previously observed that, over time, a control culture had a general down-
304 regulation of the 53 genes of the competence regulon (25). Meanwhile, treatment with
305 LVX led to a decrease in this down-regulation (25). The effect was even stronger with
306 MOX: down-regulation was reversed and even up-regulation was observed in the case

307 of three competence genes (Table 1), including the gene coding for the alternative sigma
308 factor ComX, which activates transcription of late competence genes (41, 42). Thus
309 activation of competence in *S. pneumoniae* is a common stress response to FQs in a
310 bacterium that lacks an SOS-like system (43).

311 We have not detected changes in general supercoiling neither with LVX (25) or
312 MOX treatments. However, we observed a different pattern of gene expression
313 alteration with these drugs. A possibility is that the transcriptional alterations induced
314 by MOX and LVX are due to local changes in supercoiling, which are dependent on
315 whether topo IV (LVX) or gyrase (MOX) are inhibited. We have proved that this is the
316 case for the *fatCDE* operon (25). Since both topo IV and gyrase produce double-
317 stranded breaks in the DNA when inhibited by FQs, the differential transcriptional
318 alterations caused by these drugs could also be related to differences in sequence
319 recognition (44), which is affected by DNA supercoiling and bendability (45). Sequence
320 recognition mediated by local supercoiling levels might explain the unique distribution
321 of the genes affected by LVX or MOX (Fig. 2C). In addition, the location of the FQ-
322 topoisomerase complexes relative to the replication forks, which is different for gyrase
323 and topo IV (46), could also have a role in the different transcription alterations.

324 Several genes related to purine and pyrimidine metabolism were up-regulated
325 with MOX but not with LVX. Among them was *prsA*, coding for an enzyme that
326 catalyzes the synthesis of 5-phospho ribosyl- α -1-pyrophosphate (PRP). PRP is an
327 intermediary in both purine and pyrimidine metabolism (47). The product of *pyrP* is a
328 dedicated transporter that facilitates the entrance of uracil into the cell. Uracil is
329 converted to UMP by the product of *upp*. The intracellular concentration of UMP would
330 be expected to increase through the activation of the CO₂ biosynthesis pathway: 6 out of

331 the 8 these genes were up-regulated (Table 1, Fig. 3A). These genes are regulated by an
332 attenuator mechanism and would form part of the PyrR regulon. PyrR senses UMP
333 levels and when the concentration is high, it binds to both UMP, and the antiterminator,
334 impeding the transcription of downstream genes. As in other lactic acid bacteria (47),
335 the *pyrFE*, *pyrK*, and *pyrR-pyrB-carA-carB* genes would be modulated by this system.

336 The pathways affecting pyruvate levels were up-regulated by MOX but not by
337 LVX. These pathways include the *tktA* and *pmi* genes, which encode enzymes that
338 convert ribulose-5P to fructose-6P and manose-6P to fructose 6P, respectively. They
339 also include 3 out of 7 genes of the glycolysis pathway (Fig. 3B). Likewise, acetyl-CoA
340 carboxylase was down-regulated in a MOX-dependent manner, likely leading to an
341 increase in acetyl-CoA levels. This increase would lead to increased production of
342 pyruvate by formate acetyltransferase (*pfl*), also up-regulated by MOX. An increase in
343 pyruvate would lead to a parallel increase, mediated by SpxB, in intracellular H₂O₂.
344 This would, in turn, increase the amount of hydroxyl radical produced though the
345 Fenton reaction. We observed comparable increases in the production of H₂O₂ and
346 hydroxyl radical as a result of MOX treatment (Fig. 4).

347 We have demonstrated that ROS have a role in the lethality of MOX in *S.*
348 *pneumoniae* R6. Consistent with this model, the R6 Δ *spxB* and T1 strains were less
349 susceptible to this drug (Fig. 4). These results support a model whereby the accelerated
350 production of redox alterations by FQs contributes to their lethality (18, 20). Also
351 consistent is the fact that glutathione reductase (*gor*) gene was up-regulated, presumably
352 in an attempt to counteract the lethal level of oxidation. Glutathione reductase reduces
353 glutathionine (γ -L-glutamyl-L-cysteinyl-glycine) which lowers the level of oxidation.
354 This provides protection from H₂O₂ accumulation (48).

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

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

379 **ACKNOWLEDGMENTS**

380 This study was supported by grants BIO2011-25343 and BIO2014-55462-R from
381 Plan Nacional de I+D+I of the Ministry of Economy and Competitiveness. AJMG is the
382 recipient of a Miguel Servet contract from the Spanish Ministry of Economy and
383 Competitiveness.

384

385 **REFERENCES**

- 386 1. **World Health Organization.** 2007. Pneumococcal conjugate vaccine for childhood
387 immunization-WHO position paper. Wkly. Epidemiol. Rec. **82**:93-104.
- 388 2. **Jacobs MR, Felmingham D, Appelbaum PC, Grüneberg RN, the Alexander**
389 **Project Group.** 2003. The Alexander project 1998-200: susceptibility of pathogens
390 isolated from community-acquired respiratory tract infection to commonly used
391 antimicrobial agents. J. Antimicrob. Chemother. **52**:229-246.
- 392 3. **Riedel S, Beekmann SE, Heilmann KP, Richter SS, García de Lomas J, Ferech**
393 **M, Goosens H, Doern GV.** 2007. Antimicrobial use in Europe and antimicrobial
394 resistance in *Streptococcus pneumoniae*. Eur. J. Clin. Microb. Infect. Dis. **26**:485-
395 490.
- 396 4. **Domenech A, Tirado-Vélez JM, Fenoll A, Ardanuy C, Yuste J, Liñares J, de la**
397 **Campa AG.** 2014. Fluoroquinolone-resistant pneumococci: dynamics of serotypes
398 and clones in Spain in 2012 compared with those from 2002 and 2006. Antimicrob.
399 Agents Chemother. **58**:2393-2399.
- 400 5. **Ip M, Chau SS, Chi F, Cheuk ES, Ma H, Lai RW, Chan PK.** 2007.
401 Longitudinally tracking of fluoroquinolone resistance and its determinants in

- 402 penicillin-susceptible and-nonsusceptible *Streptococcus pneumoniae* isolates in
403 Hong Kong, 2000 to 2005. Antimicrob. Agents Chemother. **51**:2192-2194.
- 404 6. **Adam HJ, Hoban DJ, Gin AS, Zhanel GG.** 2009. Association between
405 fluoroquinolone usage and a dramatic rise in ciprofloxacin-resistant *Streptococcus*
406 *pneumoniae* in Canada, 1997-2006. Int. J. Antimicrob. Agents **34**:82-85.
- 407 7. **Chen DK, McGeer A, de Azavedo JC, Low DE.** 1999. Decreased susceptibility of
408 *Streptococcus pneumoniae* to fluoroquinolones in Canada. N. Engl. J. Med.
409 **341**:233-239.
- 410 8. **Champoux JJ.** 2001. DNA topoisomerases: structure, function, and mechanism.
411 Ann. Rev. Biochem. **70**:369-413.
- 412 9. **Gellert M, Mizuuchi K, ODea H, Nash HA.** 1976. DNA gyrase: an enzyme that
413 introduces superhelical turns into DNA. Proc. Natl. Acad. Sci. USA **73**:3872-3876.
- 414 10. **Kato J, Nishimura Y, Imamura R, Niki H, Hiraga S, Suzuki H.** 1990. New
415 topoisomerase essential for chromosome segregation in *E. coli*. Cell **63**:393-404.
- 416 11. **Muñoz R, de la Campa AG.** 1996. ParC subunit of DNA topoisomerase IV of
417 *Streptococcus pneumoniae* is a primary target of fluoroquinolones and cooperates
418 with DNA gyrase A subunit in forming resistance phenotype. Antimicrob. Agents
419 Chemother. **40**:2252-2257.
- 420 12. **Morrissey I, George J.** 1999. Activities of fluoroquinolones against *Streptococcus*
421 *pneumoniae* type II topoisomerases purified as recombinant proteins. Antimicrob.
422 Agents Chemother. **43**:2579-2585.
- 423 13. **Houssaye S, Gutmann L, Varon E.** 2002. Topoisomerase mutations associated
424 with in vitro selection of resistance to moxifloxacin in *Streptococcus pneumoniae*.
425 Antimicrob. Agents Chemother. **46**:2712-2715.

- 426 14. **Drlica K, Malik M, Kerns RJ, Zhao X.** 2008. Quinolone-mediated bacterial
427 death. *Antimicrob. Agents Chemother.* **52**:385-392.
- 428 15. **Wang X, Zhao X, Malik M, Drlica K.** 2010. Contribution of reactive oxygen
429 species to pathways of quinolone-mediated bacterial cell death. *J. Antimicrob.*
430 *Chemother.* **65**:520-524.
- 431 16. **Imlay JA, Chin SM, Linn S.** 1988. Toxic DNA damage by hydrogen peroxide
432 through the Fenton reaction in vivo and in vitro. *Science* **240**:640-642.
- 433 17. **Dwyer DJ, Kohanski MA, Hayete B, Collins JJ.** 2007. Gyrase inhibitors
434 induce an oxidative damage cellular death pathway in *Escherichia coli*. *Mol.*
435 *Syst. Biol.* **3**:91.
- 436 18. **Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ.** 2007. A
437 common mechanism of cellular death induced by bactericidal antibiotics. *Cell*
438 **130**:797-810.
- 439 19. **Wang X, Zhao X.** 2009. Contribution of oxidative damage to antimicrobial
440 lethality. *Antimicrob. Agents Chemother.* **53**:1395-1402.
- 441 20. **Dwyer DJ, Belenky PA, Yang JH, MacDonald IC, Martell JD, Takahashi N,**
442 **Chan CT, Lobritz MA, Braff D, Schwarz EG, Ye JD, Pati M, Vercruyse**
443 **M, Ralifo PS, Allison KR, Khalil AS, Ting AY, Walker GC, Collins JJ.**
444 2014. Antibiotics induce redox-related physiological alterations as part of their
445 lethality. Antibiotics induce redox-related physiological alterations as part of
446 their lethality. *Proc. Natl Acad. Sci. USA.* **111**:E2100-2109.
- 447 21. **Zhao X, Hong Y, Drlica K.** 2015. Moving forward with reactive oxygen
448 species involvement in antimicrobial lethality. *J. Antimicrob. Chemother.*
449 **70**:639-642.

- 450 22. **Dwyer DJ, Collins JJ, Walker GC.** 2015. Unraveling the physiological
451 complexities of antibiotic lethality. *Ann. Rev. Pharmacol. Toxicol.* **55**:313-332.
- 452 23. **Zhao X, Drlica K.** 2014. Reactive oxygen species and the bacterial response to
453 lethal stress. *Curr. Op. Microbiol.* **21**:1-6.
- 454 24. **Ferrándiz MJ, Martín-Galiano AJ, Schwartzman JB, de la Campa AG.**
455 2010. The genome of *Streptococcus pneumoniae* is organized in topology-
456 reacting gene clusters. *Nucl. Acids Res.* **38**:3570-3581.
- 457 25. **Ferrandiz MJ, de la Campa AG.** 2014. The fluoroquinolone levofloxacin
458 triggers the transcriptional activation of iron transport genes that contribute to
459 cell death in *Streptococcus pneumoniae*. *Antimicrob. Agents Chemother.*
460 **58**:247-257.
- 461 26. **Lacks SA, Lopez P, Greenberg B, Espinosa M.** 1986. Identification and
462 analysis of genes for tetracycline resistance and replication functions in the
463 broad-host-range plasmid pLS1. *J. Mol. Biol.* **192**:753-765.
- 464 27. **Balsalobre L, de la Campa AG.** 2008. Fitness of *Streptococcus pneumoniae*
465 fluoroquinolone-resistant strains with topoisomerase IV recombinant genes.
466 *Antimicrob. Agents Chemother.* **52**:822-830.
- 467 28. **Martín-Parras L, Lucas I, Martínez-Robles ML, Hernández P, Krimer DB,**
468 **Hyrien O, Schwartzman JB.** 1998. Topological complexity of different
469 populations of pBR322 as visualized by two-dimensional agarose gel
470 electrophoresis. *Nucl. Acids Res.* **26**:3424-3432.
- 471 29. **Ferrandiz MJ, Arnanz C, Martín-Galiano AJ, Rodríguez-Martin C, de la**
472 **Campa AG.** 2014. Role of global and local topology in the regulation of gene
473 expression in *Streptococcus pneumoniae*. *PloS one* **9**:e101574.

- 474 30. **Yeom J, Imlay JA, Park W.** 2010. Iron homeostasis affects antibiotic-mediated
475 cell death in *Pseudomonas* species. *J. Biol. Chem.* **285**:22689-22695
- 476 31. **Fani F, Leprohon P, Legare D, Ouellette M.** 2011. Whole genome sequencing
477 of penicillin-resistant *Streptococcus pneumoniae* reveals mutations in penicillin-
478 binding proteins and in a putative iron permease. *Genome Biol* **12**:R115.
- 479 32. **Pruss GJ, Manes SH, Drlica K.** 1982. *Escherichia coli* DNA topoisomerase I
480 mutants: increased supercoiling is corrected by mutations near gyrase genes.
481 *Cell* **31**:35-42.
- 482 33. **Khan SR, Kuzminov A.** 2013. Trapping and breaking of in vivo nicked DNA
483 during pulsed field gel electrophoresis. *Anal. Biochem.* **443**:269-281.
- 484 34. **Malik M, Zhao X, Drlica K.** 2006. Lethal fragmentation of bacterial
485 chromosomes mediated by DNA gyrase and quinolones. *Mol. Microbiol.*
486 **61**:810-825.
- 487 35. **Hoskins J, Alborn WE, Arnold J, Blaszcak LC, Burgett S, DeHoff BS,**
488 **Estrem ST, Fritz L, Fu DJ, Fuller W, Geringer C, Gilmour R, Glass JS,**
489 **Khoja H, Kraft AR, Lagace RE, LeBlanc DJ, Lee LN, Lefkowitz EJ, Lu J,**
490 **Matsushima P, McAhren SM, McHenney M, McLeaster K, Mundy CW,**
491 **Nicas TI, Norris FH, O’Gara M, Peery RB, Robertson GT, Rockey P, Sun**
492 **PM, Winkler ME, Yang Y, Young-Bellido M, Zhao G, Zook CA, Baltz RH,**
493 **Jaskunas SR, Rosteck PR, Skatrud PL, Glass JI.** 2001. Genome of the
494 bacterium *Streptococcus pneumoniae* strain R6. *J. Bacteriol.* **183**:5709-5717
- 495 36. **Gottesman S, Wickner S, Maurizi MR.** 1997. Protein quality control: triage by
496 chaperones and proteases. *Genes Develop.* **11**:815-823.
- 497 37. **Moreira RN, Domingues S, Viegas SC, Amblar M, Arraiano CM.** 2012.
498 Synergies between RNA degradation and trans-translation in *Streptococcus*

- 499 *pneumoniae*: cross regulation and co-transcription of RNase R and SmpB. BMC
500 Microbiol. **12**:268.
- 501 38. **Spellerberg B, Cundell, D.R., Sandros, J., Pearce, B.J., Idanpaan-Heikkila,**
502 **I., Rosenow, C., Masure, H.R.** 1996. Pyruvate oxidase, as a determinant of
503 virulence in *Streptococcus pneumoniae*. Mol. Microbiol. **19**:803-813.
- 504 39. **Yague G, Morris JE, Pan XS, Gould KA, Fisher LM.** 2002. Cleavable-
505 complex formation by wild-type and quinolone-resistant *Streptococcus*
506 *pneumoniae* type II topoisomerases mediated by gemifloxacin and other
507 fluoroquinolones. Antimicrob. Agents Chemother. **46**:413-419.
- 508 40. **Kishii R, Takei M, Fukuda H, Hayashi K, Hosaka M.** 2003. Contribution of
509 the 8-methoxy group to the activity of gatifloxacin against type II
510 topoisomerases of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother.
511 **47**:77-81.
- 512 41. **Peterson SN, Sung CK, Cline R, Desai BV, Snesrud EC, Luo P, Walling J,**
513 **Li H, Mintz M, Tsegaye G, Burr PC, Do Y, Ahn S, Gilbert J, Fleischmann**
514 **RD, Morrison DA.** 2004. Identification of competence pheromone responsive
515 genes in *Streptococcus pneumoniae* by use of DNA microarrays. Mol.
516 Microbiol. **51**:1051-1070.
- 517 42. **Dagkessamanskaia A, Moscoso M, Henard V, Guiral S, Overweg K, Reuter**
518 **M, Martin B, Wells J, Claverys JP.** 2004. Interconnection of competence,
519 stress and CiaR regulons in *Streptococcus pneumoniae*: competence triggers
520 stationary phase autolysis of *ciaR* mutant cells. Mol. Microbiol. **51**:1071-1086.
- 521 43. **Prudhomme M, Attaiech L, Sanchez G, Martin B, Claverys JP.** 2006.
522 Antibiotic stress induces genetic transformability in the human pathogen
523 *Streptococcus pneumoniae*. Science **313**:89-92.

- 524 44. **Leo E, Gould KA, Pan XS, Capranico G, Sanderson MR, Palumbo M,**
525 **Fisher LM.** 2005. Novel symmetric and asymmetric DNA scission determinants
526 for *Streptococcus pneumoniae* topoisomerase IV and gyrase are clustered at the
527 DNA breakage site. J. Biol. Chem. **280**:14252-14263.
- 528 45. **Arnoldi E, Pan XS, Fisher LM.** 2013. Functional determinants of gate-DNA
529 selection and cleavage by bacterial type II topoisomerases. Nucl. Acids Res
530 **41**:9411-9423.
- 531 46. **Postow L, Crisona NJ, Peter BJ, Hardy CD, Cozzarelli NR.** 2001.
532 Topological challenges to DNA replication: conformations at the fork. Proc.
533 Natl. Acad. Sci. USA **98**:8219-8226.
- 534 47. **Kilstrup M, Hammer K, Ruhdal Jensen P, Martinussen J.** 2005. Nucleotide
535 metabolism and its control in lactic acid bacteria. FEMS Microbiol. Rev.
536 **29**:555-590.
- 537 48. **Potter AJ, Trappetti C, Paton JC.** 2012. *Streptococcus pneumoniae* uses
538 glutathione to defend against oxidative stress and metal ion toxicity. J. Bacteriol.
539 **194**:6248-6254.
- 540 49. **Kim SW, Bae YG, Pyo SN, Rhee DK.** 2007. Differential regulation of the
541 genes of the *Streptococcus pneumoniae* *dnaK* operon by Ca⁺⁺. Mol. Cel. **23**:239-
542 245.
- 543 50. **Li L, Hong Y, Luan G, Mosel M, Malik M, Drlica K, Zhao X.** 2014.
544 Ribosomal elongation factor 4 promotes cell death associated with lethal stress.
545 mBio **5**:e01708.
- 546 51. **Long Q, Du Q, Fu T, Drlica K, Zhao X, Xie J.** 2015. Involvement of Holliday
547 junction resolvase in fluoroquinolone-mediated killing of *Mycobacterium*
548 *smegmatis*. Antimicrob Agents Chemother. **59**:1782-1785.

- 549 52. **Pesakhov S, Benisty R, Sikron N, Cohen Z, Gomelsky P, Khozin-Goldberg**
550 **I, Dagan R, Porat N.** 2007. Effect of hydrogen peroxide production and the
551 Fenton reaction on membrane composition of *Streptococcus pneumoniae*. *Bioch.*
552 *Bioph. Acta* **1768**:590-597.
- 553 53. **Regev-Yochay G, Trzcinski K, Thompson CM, Lipsitch M, Malley R.** 2007.
554 SpxB is a suicide gene of *Streptococcus pneumoniae* and confers a selective
555 advantage in an in vivo competitive colonization model. *J. Bacteriol.* **189**:6532-
556 6539.
- 557
- 558
- 559

560 **FIGURE LEGENDS**

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

575 **FIG 2.** Gene expression analysis in the two conditions assayed. (A) Correlation of the
 576 changes in RNA transcript levels using data from qRT-PCR and microarray
 577 hybridizations. (B) Localization of responsive genes in the chromosome of *S.*
 578 *pneumoniae*. The relative fold variation of each gene is plotted against the location of
 579 the 3' end of each open reading frame in the *S. pneumoniae* R6 chromosome (bases 1 to
 580 2038615). (C) Classification of responsive genes by functional classes: AaB, amino acid
 581 biosynthesis; CaM, carbohydrate metabolism; CE, cell envelope; DNA, DNA
 582 metabolism; Pat, pathogenesis; Str, stress; FaM, fatty acid metabolism; PPy, purines and
 583 pyrimidines metabolism; TCS, two-component systems; Tr, translation; TBP, transport

584 and binding proteins; Hy, hypothetical proteins; Tns, transposon functions; Oth, other
585 (classes with a representation < 2.5%). Column colors: black , up-regulated responsive
586 genes, white, down-regulated responsive genes.

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

591 **FIG 4.** MOX lethality is linked to the level of intracellular hydrogen peroxide. (A)
592 Viability of *S. pneumoniae* strains either in AGCH, or in AGCH plus MOX at 0.125
593 $\mu\text{g/ml}$ ($1\times$ MIC for R6 and T1). Values normalized to the 0 time value. The mean \pm
594 SEM of three independent replicates is shown. (B) Accumulation of reactive oxygen
595 species, measured using dihydrorhodamine 123 dye, a non-fluorescent compound which
596 in converted by oxidation to fluorescent rhodamine 123. Strains R6 and R6 ΔspxB were
597 grown to an optical density at 620 nm (OD_{620}) = 0.4 before MOX at $1\times$ MIC was added,
598 samples were processed and fluorescence was measured as described in methods. RFU,
599 relative fluorescence units; values were divided by the number of viable cells. (C)
600 Accumulation of hydrogen peroxide in the indicated conditions, detected with the
601 Amplex[®] Red Hydrogen Peroxide/Peroxidase Assay Kit as described in methods. Cells
602 were grown to $\text{OD}_{620}=0.4$ and then diluted 1:100 in growth media before MOX and
603 LVX were added, both at 0.06 $\mu\text{g/ml}$ ($0.5\times$ MIC for R6). Cells were then incubated at
604 37°C for 4 hours. Samples were taken at 0, 3, and 4 hours, processed as described in
605 methods. Values are calculated as concentration of hydrogen peroxide in μM
606 normalized against the number of viable cells and calculated as the fold-difference with
607 respect the initial 0 time value.

608

609 TABLE- 1. Genes involved in the transcriptomic response of *S. pneumoniae* to 30 min610 treatment with MOX^a

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*-0444 (<i>glnRA</i>)	2.0
Amino acid biosynthesis: Ser	spr1095 (<i>metY</i>)	3.9
	spr0823 (<i>but</i>)	-2.3
Biosynthesis of cofactors	spr1324 (<i>apbE</i>)	-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>pmi</i>)	3.8
Carbohydrate metabolism: Pyruvate	spr0415 (<i>pfl</i>)	2.9
	spr1438 (<i>entB</i>)	-2.4

Carbohydrate metabolism: Pentose-P

pathway	spr1841 (<i>tktA</i>)	2.8
---------	-------------------------	------------

Carbohydrate metabolism: Other

	spr0064 (<i>agaS</i>)	-2.2
--	--------------------------------	------

	spr0778*-0779 (<i>fruRB</i>)	—
--	--------------------------------	---

	spr1667 (<i>galT</i>)	-4.4
--	-------------------------	------

	spr1833 (<i>bgl2</i>)	-2.6
--	--------------------------------	------

	spr1842 (<i>ulaG</i>)	2.8
--	--------------------------------	------------

Cell envelope

	spr1459 (<i>gtrB</i>)	2.9
--	-------------------------	------------

	spr0540*-0541 (<i>murMN</i>)	2.1
--	--------------------------------	------------

Cellular processes: Detoxification

	spr0864 (<i>lguL</i>)	-2.3
--	-------------------------	------

Cellular processes: Pathogenesis

	spr0328 (<i>eng</i>)	-2.3
--	-------------------------------	------

	spr0906 (<i>lmb</i>)	-3.4
--	------------------------	------

	spr0910 (<i>phtE</i>)	-2.6
--	-------------------------	------

	spr1060 (<i>phpA</i>)	-3.6
--	--------------------------------	------

	spr1492 (<i>psaB</i>)	-2.2
--	-------------------------	------

Cellular processes: Stress

	spr0453*-0456 (<i>hrcA-grpE-dnaK-dnaJ</i>)	2.6
--	--	------------

	spr0692 (<i>gor</i>)	2.2
--	------------------------	------------

	spr1723*-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*-0387 (<i>accDA</i>)	-2.2
Protein Export and Trans-translation	spr0877*-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>)	9.4
	spr1156*-1153 (<i>pyrRB-carAB</i>)	3.5
	spr1165 (<i>pyrP</i>)	3.9
	spr2033 (<i>imdH</i>)	3.7
Regulatory functions: TCS12 (ComCDE)	spr0013° (<i>comX2</i>)	2.1
	spr0654 (<i>comEB</i>)	2.1
	spr1962°	3.7

Regulatory functions:TCS11	spr1814 (<i>rr11</i>)	-2.0
Transcription	spr1336	-2.9
	spr0336 (<i>ritR</i>)	2.0
	spr1569 (<i>scrR</i>)	—
	spr0679 (<i>ppiA</i>)	2.6
Translation	spr0682 (<i>rpsP</i>)	-2.1
	spr0861*-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
	spr1123 (<i>ftsY</i>)	—
	spr1204 (<i>ptrB</i>)	3.3
	spr1211 (<i>rplL</i>)	-2.1
	spr1871	-2.0
	spr1943*-1944 (<i>rpmFG</i>)	-2.0
Transport: Amino acids, peptides and amines	spr0327 (<i>aliA</i>)	-3.8

	spr0369 (<i>dagA</i>)	-2.0
	spr0524	-2.3
	spr0535*-0532	2.6
	spr1704*-1703 (<i>amiEF</i>)	-2.0
Transport: Anions	spr0648	3.1
Transport: Sugars	spr0063	-2.1
	spr1566	
	spr1836	—
Transport: Other	spr0108	—
	spr0875 (<i>pmrA</i>)	-3.7
	spr1203	3.1
	spr1216*-1215	-2.4
	spr1352 (<i>bta</i>)	2.1
	spr1559	-2.0
	spr1817	-2.9
	spr2035	2.5

 611

612 ^a The responsive genes included in this list showed significant fold variations (≥ 2 and P
 613 < 0.01). All genes showing variations are included, with the exception of 22 encoding

614 hypothetical proteins and 7 encoding transposases. Genes involved in the growth-related
615 response are not included. Genes affected in both the response to LVX and to MOX
616 response are shown in boldface. Symbols: * the first gene of the operon; °gene with a
617 ComE box; —, no change.

618 ^b In operons, values indicated correspond to those of the first gene of the operon. Values
619 above 2 are shown in boldface.

620

621

622