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The Fluoroquinolone Levofloxacin Triggers the Transcriptional Activation of Iron Transport Genes That Contribute to Cell Death in Streptococcus pneumoniae

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6 # *Streptococcus pneumoniae*

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18 **Running title**: Levofloxacin-mediated iron transport

19 **ABSTRACT**

20 **We studied the transcriptomic response of *Streptococcus pneumoniae* to levofloxacin,**
21 **under conditions inhibiting topoisomerase IV, but not gyrase. Although a complex**
22 **transcriptomic response was observed, the most outstanding result was the up-**
23 **regulation of the genes of *fatDCEB* operon, involved in iron (Fe^{2+} and Fe^{3+}) uptake,**
24 **which were the only genes varying at every condition tested. Although the inhibition**
25 **of topoisomerase IV by levofloxacin did not have a detectable effect in the level of**
26 **global supercoiling, increases in general supercoiling and *fatD* transcription were**
27 **observed after topoisomerase I inhibition, while the opposite was observed after**
28 **gyrase inhibition with novobiocin. Since *fatDCEB* is located in a topological**
29 **chromosomal domain down-regulated by DNA relaxation, we studied the**
30 **transcription of a copy of the 422-bp (including the P_{fat} promoter) region located**
31 **upstream of *fatDCEB* fused to the *cat* reporter inserted into the chromosome 106-kb**
32 **away from its native position: P_{fat}*fatD* was up-regulated in the presence of LVX in its**
33 **native location, whereas no change was observed in the P_{fat}*cat* construction. Results**
34 **suggest that topological changes are indeed involved in P_{fat}*fatDCE* transcription. Up-**
35 **regulation of *fatDCEB* would lead to an increase of intracellular iron, and in turn, to**
36 **the activation of the Fenton reaction and the increase of reactive oxygen species. In**
37 **accordance, we observed an attenuation of levofloxacin lethality in iron-deficient**
38 **media and in a strain lacking the gene coding for SpxB, the main source of hydrogen**
39 **peroxide. In addition, we observed an increase of reactive oxygen species that**
40 **contributed to levofloxacin lethality.**

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42 *Streptococcus pneumoniae* (the pneumococcus) acts as an opportunistic pathogen. It forms
43 part of the commensal microbiota of the human nasopharynx. Under specific
44 circumstances, it migrates to other niches (ear, lung, bloodstream, cerebrospinal fluid)
45 causing diverse pathologies. One million children aged <5 years die annually of
46 pneumococcal infections worldwide (1). After the usage of the pneumococcal 7-valent
47 conjugate vaccine, which includes the serotypes more often associated with resistance to
48 antibiotics, the incidence of invasive pneumococcal disease declined (2, 3) coincidentally
49 with a decrease of penicillin resistance rates in many countries (3-5). However, emergence
50 of serotypes not included in the vaccine has been observed (6, 7). Therefore, knowledge of
51 the molecular bases of antimicrobial action, including the mechanisms of killing, is
52 essential for developing improved therapeutics.

53 Resistance in *S. pneumonia*e to antibiotics acting either in cell wall (beta-lactams) or
54 protein synthesis (macrolides) has spread worldwide in the last three decades (8). The
55 fluoroquinolones (FQs) levofloxacin (LVX) and moxifloxacin are used nowadays for
56 treatment of adult patients with pneumonia. FQ-resistance in *S. pneumoniae* is maintained
57 at low prevalence (< 3%) in Europe (9, 10), although higher rates have been detected in
58 Asia (11) and in Canada (12). However, an increase in resistance in this bacterium may
59 occur if FQ use is increased (13). FQs target the type II DNA topoisomerases. Despite the
60 functional similarities between topoisomerase (topo) IV and gyrase, their susceptibility to
61 FQs varies across bacterial species (14). In *S. pneumoniae*, the primary target for LVX is
62 topo IV (15-18), while gyrase is the primary target for moxifloxacin (19). Type II
63 topoisomerases maintain DNA topology and solve the topological problems associated with
64 DNA replication, transcription, and recombination (20). Gyrase introduces negative
65 supercoils into DNA (21) whereas topo IV relaxes DNA and participates in chromosome

66 partitioning (22). Chromosomal topology in *Escherichia coli* is maintained homeostatically
67 by the opposing activities of topoisomerases which relax DNA (topo I and topo IV), and by
68 gyrase. In these bacterium, transcription of the *topA* gene encoding topo I increases when
69 negative supercoiling increases (23), while that of *gyrA* and *gyrB* increases after DNA
70 relaxation (24-26). Changes in DNA supercoiling also have a global effect on genome
71 transcription in *E. coli* (27, 28) and *Haemophilus influenzae* (29). We have also shown that
72 relaxation of the *S. pneumoniae* chromosome with novobiocin (NOV, a GyrB inhibitor)
73 causes up-regulation of gyrase genes and down-regulation of topo I and IV genes, and
74 triggers a global transcriptional response affecting *ca*. 14% of the genome (30). Most
75 (>68%) responsive genes are closely positioned forming 15 gene clusters (up- and down-
76 regulated topological domains), which showed a coordinated response (30).
77 The killing effect of FQs has been related to the resolution of reaction intermediates of
78 DNA-FQ-topoisomerase complexes, which generates irreparable double-stranded DNA
79 breaks (31). This could occur in *E. coli* by two pathways, one dependent on protein
80 synthesis and the other independent. It has been shown that hydroxyl radical action
81 contributes to FQ-mediated cell death occurring via a protein-dependent pathway (32). This
82 result agrees with a recently proposal suggesting that, following gyrase poisoning, hydroxyl
83 radical formation utilizing internal iron and the Fenton reaction (33) are generated and
84 contributes to cell killing by FQs (34) as well as by other bactericidal antibiotics (35, 36).
85 In this mechanism, proposed for Enterobacteriaceae (35, 37), the primary drug-interactions
86 stimulate oxidation of NADH via the electron transport chain that is dependent of the
87 tricarboxylic acid cycle. Hyperactivation of the electron transport chain stimulates
88 superoxide formation. Superoxide destabilizes the iron-sulfur clusters of enzymes, making
89 Fe^{2+} available for oxidation by the Fenton reaction. The Fenton reaction leads to the

90 formation of hydroxyl radicals that would damage DNA, proteins and lipids (38), which
91 results in cell death. Instead a generalized oxidative damage, a recent study supports that
92 the main action of hydroxyl radicals is the oxidation of guanine (to 8-oxo-guanine) of
93 nucleotide pool. The incomplete repair of closely spaced 8-oxo-deoxyguanosine lesions
94 caused lethal double-strand DNA breaks, which would underlie much of the cell death
95 caused by beta-lactams and FQs (39). However, recent investigations have questioned the
96 role of hydroxyl radicals and intracellular iron levels in antibiotic-mediated lethality using
97 either similar antibiotic concentrations (40) or higher concentrations (41) than used
98 previously. The disparate results obtained using diverse antibiotic concentrations and times
99 of treatment emphasize the complexity of the lethal stress response (42).

100 Given that different antibiotic families have different intracellular targets, it is essential
101 to know the pathway between the initial antibiotic-target interaction and the promotion of
102 hydroxyl radical formation. These pathways are mostly unknown. A model has been
103 proposed for aminoglycosides in *E. coli* in which, the interference of these drugs with
104 ribosome progression would release incomplete polypeptides, which are translocated to the
105 cell membranes where they may trigger envelope stress. The Arc regulatory system is
106 perturbed, accelerating respiration and thereby increasing the flux of superoxide and
107 hydrogen peroxide into the cell (43). However, for FQs, the specific pathway has not been
108 established, although a general scheme for stress-response regulation in *E. coli*, which
109 involves the hydroxyl radical cascade, has been proposed (42). The present study was
110 aimed to understand the transcriptional response to levofloxacin in *S. pneumoniae* at
111 concentrations that inhibited its primary target, topo IV, without inhibiting gyrase, to avoid
112 the opposite effects of these two enzymes on DNA topology. Changes in DNA topology
113 were tested by analyzing the distribution of topoisomers of a replicating plasmid. Global

114 transcription response was analyzed using microarrays technology after cells' exposure to
115 two LVX concentrations. Microarray data were validated by quantitative real-time PCR
116 (qRT-PCR). In addition, transcriptional regulation of the *fatDCEB* operon, coding for an
117 iron transporter was analyzed. The relation between iron transport and lethality was also
118 tested. Results provide a pathway between topo IV inhibition and hydroxyl radical
119 production and suggest that *S. pneumoniae* uses iron accumulation as part of the death
120 process associated with LVX treatment.

121 MATERIALS AND METHODS

122 **Bacterial strains, growth and transformation of bacteria**. *S. pneumoniae* was grown in
123 AGCH medium with 0.3% sucrose and transformed as described previously (44). MICs of
124 LVX (Sigma) and chloramphenicol (CHL) for R6 strain were 0.25 μg/ml and 1.25 μg/ml,
125 respectively. To construct the Δ*spxB* strain, two fragments of 1481 bp and 1374 bp flanking
126 *spxB* were amplified with oligonucleotide pairs SpxBUPF1/SpxBUPR1 and
127 SpxBDOWNF1/SpxBDOWNR1 (Table S1), digested with SphI and XbaI and ligated to the
128 CHL-acetyl transferase gene (*cat*) of plasmid pJS3 digested with the same enzymes. R6
129 was transformed; recombinant colonies were selected in medium containing 2.5 μg/ml
130 CHL and checked by PCR amplification with external oligonucleotides
131 SpxBUPF2/SpxBDOWNR2 (Table S1). Those with the appropriate size (4574 bp versus
132 5352 bp of R6) were sequenced using oligonucleotide CATMED. To construct the R6-
133 P_{fat}*cat* strain, five PCR products were obtained. Two from genes *spr1793* (1061 bp) and
134 *spr1794* (1036 bp), flanking the site of insertion, by amplifications with primers
135 1793F1(XbaI)/spr1793R1 and spr1794F1/spr1794R1(SphI). The third fragment (144 bp),
136 was amplified with UptrcatXba/UptrcatEco, and contains the transcriptional terminator that

137 precedes the *cat* cassette in plasmid pJS3. The fourth fragment (422 bp) containing the 5´-
138 upstream region of the iron transport operon *fatDCEB* was amplified with
139 UpfatDF1(EcoRI) and phosphorylated UpFatDR1. The fifth fragment (758 bp) contains *cat*
140 and was amplified with Cat1 phosphorylated and CatDownSph(SphI). Each fragment was
141 digested with the appropriate enzyme, and all fragments ligated together. The ligation mix
142 was used to transform strain R6, and transformants selected in medium containing 2.5
143 μg/ml CHL. This rendered strain R6-P_{fat}*cat* (Fig. 3A), whose genetic structure was checked
144 by PCR with primers Spr1793R2 and Spr1794F2, and by sequencing with CATMED,
145 CAT191, and spr1793R3.

146 **Analysis of the topology of covalently closed circles.** Plasmid DNA isolation from *S.*
147 *pneumoniae* cultures grown on AGCH medium with 1 μ/g/ml tetracycline (for
148 pLS1selection) was performed as described before (30). Circular DNA molecules were
149 analyzed in neutral/neutral two-dimensional agarose gels, which were subjected to
150 Southern hybridization with a 240-bp specific pLS1 probe as described previously (30).
151 DNA linking number (Lk) was calculated by quantifying the amount of every topoisomer.
152 DNA supercoiling density (σ) was calculated from the equation $\sigma = \Delta Lk / Lk_0$. Linking
153 number differences (ΔLk) were determined using the equation $Lk = Lk - Lk_0$, in which Lk_0
154 $= N/10.5$, where N is the size of the molecule (in bp) and 10.5 the number of bp per one
155 complete turn in B-DNA.

156 **RNA extraction and real time RT-PCR experiments.** Synthesis of cDNAs from 5 μg
157 of total RNA was performed as previously described (52). These cDNAs were subjected to
158 quantitative qRT-PCR (Chromo 4, BioRad) in 20 μl reactions containing 2 μl of cDNA, 0.3
159 μM of each specific primer, and 10 μl of LightCycler FastStart Universal A SYBR Green

160 Master (Roche). Amplification was achieved with 42 cycles of a tree-segment program:
161 denaturation (30 s at 94°C), annealing (30 s at 45–56°C), and elongation (30 s at 68°C). To
162 normalize the three independent cDNA replicate samples, values were divided by those
163 obtained of the amplification of internal fragments of *rpoB* (52) and 16S rDNA. The
164 oligonucleotides used are shown in Table S1.

165 **Microarray data normalization and analysis.** High density arrays A6701-00-01 from
166 Roche NimbleGen were used. Double-stranded cDNAs were obtained from total RNA with
167 the SuperScript^{TM} Double-Stranded cDNA Synthesis Kit (Invitrogen). Labeling of cDNAs
168 with Cy3 and hybridization were performed at the Institut de Recerca Biomèdica,
169 Barcelona (Spain). A GenePix 4000B scanner at 5 µm resolution was used and raw data
170 were extracted and RMA normalized using NimbleScan v2.4. After this normalization,
171 Partek Genomics Suite 6.4 was used to do a principal component analysis and test for
172 significance for differential gene expression using ANOVA. Each microarray experiment
173 was carried out in duplicate with cDNA prepared from two independent cultures. All
174 microarray data are available at the Array Express (EBI, UK) database via accession
175 number E-MEXP-3809.

176 **Detection of reactive oxygen species.** The intracellular oxidation levels were measured
177 using dihydrorhodamine 123 dye (Sigma-Aldrich), a non-fluorescent compound which
178 diffuses passively across membranes. Oxidation converts it to the fluorescent product
179 rhodamine 123, and this fluorescence is proportional to the level of oxidation (37). In a
180 typical experiment, cells were grown exponentially to an optical density at 620 nm (OD_{620})
181 = 0.4 before LVX was added. One ml samples were collected, cells were washed once in
182 500 µl of 1× PBS (pH 7.2) and suspended in 250 µl of 1× PBS containing 2.5 µg/ml of

183 dihydrorhodamine 123 and incubated at 37°C in the dark for 30 min. Cells were washed
184 once in 500 μl of 1× PBS and suspended in 250 μl of 1× PBS. A volume of 200 μl was
185 used to measure fluorescence. The fluorescence signal was analyzed using a Tecan Infinite
186 2000 with excitation λ/emission λ of 485 nm/535 nm. Results were expressed as relative
187 fluorescence units (RFU) and were normalized according to the number of live cells at each
188 time point (45).

189 RESULTS

190 **DNA topoisomer distribution did not vary under treatment with LVX.** The effect of
191 LVX was tested in the reference strain R6 carrying plasmid pLS1 at subinhibitory (0.5×
192 MIC) and fully inhibitory (10× MIC) concentrations. The change in OD_{260} along the 60 min
193 of the experiment was from 0.4 to 0.8. Cell division was inhibited only when the culture
194 was treated with LVX at 10× MIC, with decreases in cell viability to about 70% and 97% at
195 30 and 60 min, respectively (Fig. 1A). To measure supercoiling alterations, topoisomer
196 distributions of the replicating pLS1 plasmid were analyzed. Under the chloroquine
197 concentration used, the induced ΔLk is −14 (40). Topoisomers appeared distributed in the
198 autoradiograms in a bubble-shaped arc, where negative and positive supercoiled molecules
199 are located to the right- or to the left-side, respectively (Fig. 1B). Although we have not
200 measured the supercoiling level of the bacterial chromosome, the values obtained on small
201 plasmids provide a good estimation of chromosomal supercoiling (46). No significant
202 differences in supercoiling densities (σ) were detected in any condition, showing that the
203 inhibition of topo IV by LVX did not have a detectable effect in the level of global
204 supercoiling and that gyrase was not inhibited at the LVX concentrations used.

Two kinds of transcriptional responses in LVX-treated cultures: growth-related, LVX-related. The transcriptional response was measured in cultures of strain R6 at three time points (5, 15, and 30 min) after treatment with LVX concentrations of 0.5× MIC and 10× MIC. In addition, samples taken at 15 and 30 min of an untreated culture (No-LVX) were also analyzed and used to distinguish those genes varying along the growth curve. Only gene expression variations ≥ 2 (P values <0.01) with respect to time 0 min were considered. The whole transcriptomic response is shown in Table 1. Based on the results obtained, responsive genes were classified into two categories: growth-related and LVX-related (Fig. 2, Table 1). Growth-related genes included 108 genes showing transcription variations in the No-LVX culture. Additionally, 10 genes forming part of operons with these genes were also considered to be growth-related. In total, 118 genes (5.8% of the genome), showed variations associated with growth (Fig. 2A).

Genes controlled by two-component systems (TCS)-12 and -13 represented the greatest proportion (50.8%) of responsive genes (Table 1, Fig. 2C). TCS-12 regulates competence for genetic transformation (47). The regulatory cascade begins with the secretion and processing of ComC by the dedicated ABC transporter ComAB. Processed ComC activates TCS-12 ComDE: ComD is the histidine kinase (that senses the stimulus), ComE is the response regulator (the transcriptional modulator of the responsive genes). Phosphorylated ComE activates the transcription of early genes, including the alternative sigma factor ComX (48), which activates transcription of late competence genes (49, 50). Among competence genes, 53 (11 early and 42 late genes) showed down-regulation. These included most (10 out of 18) genes of the early competence response (49), which are transcribed from 7 out of the 10 promoters (Table 1) containing the binding site of the

228 transcriptional activator ComE (51). Early down-regulated genes included those coding for
229 the two transcriptional regulators of competence, *comE* and two genes coding for the
230 alternative sigma factor ComX (52), required for induction of many late genes. In
231 accordance, 42 out of the 81 late-competence genes (49) were down-regulated. These 42
232 genes were transcribed from 13 out of 19 promoters containing a ComX box. Concerning
233 TCS-13 (SpiRH), 13 genes containing regulatory sequences for its response regulator SipR
234 (53, 54) were up-regulated, including *spiP* encoding the bacteriocin with a Gly-Gly motif
235 and the dedicated ABC transporter (*spiABCD*).

236 The LVX-related response involved 108 out of 174 genes that did not show
237 variations in the No-LVX culture (Fig. 2B). Of them, 4 vary only at 5 min after 10× MIC
238 treatment. Among LVX-responsive genes, 24.1% code for hypothetical proteins, and the
239 same proportion for transport proteins (Fig. 2C). Interestingly, the only genes up-regulated
240 at 5 min in 0.5× MIC were the four genes of the *fatDCEB* operon (55). These genes were
241 up-regulated at every time and LVX concentration used. We have previously shown that
242 the *fatDCEB* operon is located in a topological domain (D14, Fig. 3A) showing down-
243 regulation under NOV treatment, as tested by microarray experiments (30). We validated
244 these results by qRT-PCR, showing that treatment with NOV caused a decrease in *fatD*
245 transcription at any time tested at 10× MIC, and at 5 min at 0.5× MIC. At 0.5× MIC, a
246 recovery in *fatDCE* transcription was observed (Fig. 3B), as expected from the general
247 supercoiling recovery (30). On the contrary, qRT-PCR confirmed the up-regulation of
248 *fatDCEB* at all times regardless of LVX concentration (Fig. 3C), with similar fold-variation
249 values that in microarrays. To test the role of topo IV inhibition in the up-regulation of
250 *fatD,* qRT-PCR determinations in a LVX-resistant R6 mutant containing a ParCS79F

251 change in topo IV (56), treated with the same LVX concentrations that R6, were performed.
252 No increase of *fatD* transcription in this strain was observed.

253 ***fatDCEB* transcription is affected by supercoiling levels.** Even when no changes in the
254 general supercoiling levels were detected in the presence of LVX (Fig. 1C), we assumed
255 that local changes in supercoiling could be involved in the regulation of the *fatDCEB*
256 operon. To check this possibility, a strain (R6-P_{fat}*cat* in Fig. 3A), which contains a 422-bp
257 region located upstream of *fatDCEB* that includes the promoter of the operon (P_{fat}), fused to
258 the *cat* reporter gene, was inserted into the chromosome 106 kb away from *fatDCEB* (Fig
259 3A). The levels of transcription of *fatD* and *cat* were tested by qRT-PCR in cultures treated
260 with two LVX concentrations (Fig. 3C). While *fatD* showed up-regulation in the presence
261 of LVX, almost no change was observed in *cat* transcription. Thus, supercoiling alteration
262 induced by LVX is acting as a regulator of P_{fat}, given that its transcriptional up-regulation is
263 dependent on its location in a topological chromosomal domain.

264 In addition, the level of transcription of *fatDCEB* was tested by qRT-PCR in
265 cultures treated either with NOV, an inhibitor of GyrB (21, 57) (Fig. 3B) or with *N*-methly-
266 seconeolitsine (a topo I inhibitor) (58). Treatment with *N*-methly-seconeolitsine caused, as
267 expected (58), a general increase in supercoiling (Fig. 4A). This increase was accompanied
268 by a rise in the transcription of *fatD* and *fatC* at every concentration tested (Fig. 4B).

269 **Transcriptional activation of iron transport induced by LVX is involved in cell**
270 **death.** The increase in transcription of the *fatDCEB* operon would lead to the accumulation
271 of toxic concentrations of iron within the cell. This toxicity would be related to the
272 activation of the Fenton reaction, which utilizes unincorporated intracellular iron and
273 transfers an electron to hydrogen peroxide (Fig. 5A). To test if intracellular iron is an
274 important component of the LVX-mediated killing, R6 was grown in the presence of LVX

275 in three different media: AGCH (containing 1.58 μM of SO_4Fe); AGCH plus the iron
276 chelator *o*-phenantroline, or AGCH deficient in SO_4Fe (AGCH*). Attenuation of the
277 bactericidal effect of LVX, both in AGCH + *o*-phenantroline and in AGCH*, was
278 observed, suggesting a role for intracellular iron in LVX lethality (Fig. 5B).

279 The main source of endogenous hydrogen peroxide in *S. pneumoniae* is SpxB (59), the
280 pyruvate oxidase enzyme (EC 1.2.3.3) which decarboxylates pyruvate to acetyl phosphate
281 plus H_2O_2 and CO_2 (Fig. 5A). To assess that LVX lethality was related to the production of
282 hydroxyl radicals via the Fenton reaction, a SpxB-deletion mutant was constructed as
283 detailed in Material and Methods. The Δ*spxB* strain was less susceptible to the killing by
284 LVX, the attenuation being similar to that exhibited by R6 grown either in the presence of
285 *o*-phenantroline or in AGCH* (Fig. 5B). These results provided a relation between LVX
286 lethality and the Fenton reaction via the increase of intracellular iron. In addition,
287 accumulation of reactive oxygen species was measured by the oxidation of
288 dihydrorhodamine 123. Accumulation was observed in R6 cultures treated with LVX (Fig.
289 5C), with increases with respect to time 0 min higher than 35-fold at 3, 4, and 5 h of
290 treatment. Similar increases in reactive oxygen species had been observed in ciprofloxacin-
291 treated *S. pneumoniae* with a different dye probe (45), and also in norfloxacin-treated *E.*
292 *coli* (35). This accumulation was reverted about 10-fold by *o*-phenantroline. A similar
293 reversion was observed in the Δ*spxB* strain.

294 **DISCUSSION**

295 The complex transcriptional response observed in our microarray experiments lead
296 us to differentiate among those genes whose transcription was altered as a consequence of
297 growth and those that were LVX-dependent. We detected that 5.8% of the genome varied

298 as a consequence of growth, involving mainly genes of two of the 13 pneumococcal two-
299 component-systems, TCS-12 and -13. The major response corresponded to genes dependent
300 of TCS-12 (ComDE), involved in the regulation of competence for genetic transformation.
301 However, in the presence of LVX, competence genes showed decreases in transcription
302 lower (2.7 ± 0.8, average ± SD) than those observed at 30 min in the untreated culture.
303 These results suggest that two opposed regulation mechanisms are acting over competence
304 development in the LVX-treated cultures: the growth-phase caused down-regulation of
305 competence genes, while LVX counteracted this down-regulation. These results are in
306 agreement with the described transcriptional activation of *ssbB*, a late competence gene,
307 after 2.5 hours of FQ treatment, with the subsequent induction of transformability (60).
308 Thus, in response to FQs, *S. pneumoniae*, a bacterium lacking an SOS-like system,
309 activates the competence regulon, supporting the hypothesis that competence is a general
310 stress response of *S. pneumoniae* (60). Conversely, the up-regulation of the genes
311 controlled by TCS-13 (SpiRH) was not affected by LVX treatment.
312 The LVX-related response included 108 genes (5.2% of the genome). The most striking
313 result in the LVX response was the up-regulation of the *fatDCEB* operon at the earliest time
314 analyzed (5 min) and at the subinhibitory (0.5× MIC) concentration, being the only genes
315 varying in this condition. We tested *fatD* transcription in a LVX-resistant R6 mutant and no
316 up-regulation was observed in the presence of the antibiotic (Fig. 3). These results indicate
317 that the LVX transcriptional effects were indeed due to the inhibition of topo IV. However,
318 changes in the general supercoiling levels in the presence of LVX were not found (Fig. 1C).
319 Likewise, no changes in general chromosomal supercoiling were observed in *E. coli* cells
320 treated with oxolinic acid, an inhibitor of gyrase (61).We assumed that local changes in

321 supercoiling could be involved in the regulation of *fatDCEB*. To test the role of
322 supercoiling in *fatDC* transcription, we altered global supercoiling in both directions. On
323 one side, we increased global supercoiling by using the DNA topo I inhinitor *N*-methly-
324 seconeolitsine (58). On the other, we decreased supercoiling by using the gyrase B inhibitor
325 NOV (57). We observed both an increase in supercoiling (Fig. 4A) and of *fatDC*
326 transcription with the topo I inhibitor (Fig. 4B). On the contrary, treatment with NOV
327 caused a decrease in *fatDC* transcription, as detected by qRT-PCR (Fig. 4C), in accordance
328 with a general supercoiling decrease and down-regulation of *fatDCE* transcription in
329 microarrays (30). Microarrays results have shown that *fatDCEB* is located in topological
330 cluster D14, which contains genes down-regulated when DNA supercoiling decreases (30).
331 We constructed a strain with a copy of the 422-bp *fatDCEB*-upstream region fused to *cat*
332 inserted 106-kb away from its native position. Transcription from P_{fat} in the presence of
333 LVX varied depending on its chromosomal location. It was up-regulated in its appropriate
334 chromosomal location in down-regulated cluster D14, but was almost not regulated when
335 located 106 kb away (Fig. 3), in a non-regulated domain.

336 Besides this supercoiling regulation, the *fatDCE* operon has been shown to be regulated
337 in several ways, as expected for an operon essential for iron homeostasis. Among them,
338 environmental factors, such as high levels of extracellular Mn^{2+} (62) and low pH (63)
339 caused its transcriptional up-regulation, whereas aerobiosis caused its down-regulation
340 (64). Other regulators of the operon are the RitR repressor and (55) the CodY repressor,
341 whose DNA binding capacity is modulated by branched chain amino acids (65, 66). In
342 accordance, we observed *codY* down-regulation in the LVX response (Table 1), probably
343 contributing to the up-regulation of *fatDCEB*.

344 The genome of *S. pneumoniae* R6 encodes three operons for iron transport systems
345 (spr0224–spr0220, spr0934–spr0936, and *fatDCEB*). Out of them, only *fatDCEB* is
346 involved in iron (Fe^{2+} and Fe^{3+}) uptake (67). In this way, the up-regulation of the operon
347 would cause an increased uptake of iron and its intracellular accumulation, which in turn
348 would activate the Fenton reaction (Fig. 5A). We have observed attenuation of the LVX
349 bactericidal effect in media defective in iron (Fig. 5B), confirming that intracellular iron is
350 a component of LVX-mediated killing. In addition, the accumulation of reactive oxygen
351 species (Fig. 5C) was in accordance with this interpretation. These results agree with the
352 proposed mechanism of killing by bactericidal antibiotics, including FQs (35). The
353 stimulation of the Fenton reaction is the final common step. However, there are several
354 differences between this model and the one we propose in this study (Fig. 6). *S.*
355 *pneumoniae* is a lactic acid bacterium that obtains its metabolic energy exclusively from the
356 fermentation of carbohydrates via glycolysis. Its genome does not contain genes for the
357 tricarboxylic acid cycle, and lacks the cytochromes and heme-containing proteins involved
358 in aerobic respiration. In addition, although genes coding the F_0F_1-ATPase, are present, this
359 proton pump does not synthesize ATP; conversely, it works at the expense of ATP, and
360 serves as the major regulator of intracellular pH (68). Consequently, the only enzymes
361 annotated as iron-sulfur-dependent in the *S. pneumoniae* R6 genome are the two subunits of
362 the L-Ser dehydratase (Spr0094 and Spr0095). The main reason for the increase of
363 intracellular Fe^{2+} in the presence of LVX should be transcriptional activation of the
364 *fatDCEB* transporter. The importance of iron in the susceptibility to antibiotics has been
365 recently reinforced by the demonstration that overexpression of an iron efflux system in
366 *Salmonella typhimurium* protects cells against ampicillin and ciprofloxacin (69). With
367 respect to the other component of the Fenton reaction —hydrogen peroxide— it is mainly

368 produced in *S. pneumoniae* by the SpxB enzyme (59, 70). We deleted the gene encoding
369 this enzyme (*spxB*) and observed that this strain was more resistant to the killing by LVX
370 than its *spxB*$^+$ parental strain, the attenuation being similar to that exhibited by R6 grown in
371 iron-deficient media (Fig. 5B). The difference in lethality between the wild-type R6 strain
372 and the R6-Δ*spxB* mutant in the presence of 2.5 × MIC LVX was in the same range to that
373 observed between *E. coli* wild-type and mutant strains lacking either superoxide dismutase
374 activities (36) or both catalase and peroxidase activities (36, 40), which accumulate H_2O_2,
375 in the presence of norfloxacin at 4- to 10 × MIC. We have observed protection to FQ
376 lethality using low LVX concentrations (2.5 × MIC), in agreement with results of *E. coli*
377 treatment with norfloxacin at 2 × to 4 × MIC (41). In conclusion, we have shown for the
378 first time that *fatDCEB* transcription is regulated by supercoiling level. The primary effect
379 of the interaction of LVX-topo IV is the up-regulation of the operon by local increase in
380 DNA supercoiling. This up-regulation would increase the intracellular level of iron, which
381 activates the Fenton reaction, increasing the concentration of hydroxyl radicals. These
382 effects were observed before the inhibition of protein synthesis mediated by LVX. All these
383 effects, together with the DNA damage caused by the inhibition of topo IV, would account
384 for LVX lethality. The possibility to increase FQs efficacy by elevating the levels of
385 intracellular ferrous iron remains open.

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614 **FIGURE LEGENDS**

615 **FIG 1.** Global supercoiling did not vary under treatment of *S. pneumoniae* R6 (pLS1) with
616 levofloxacin. (A) Viability. (B) Topoisomer distribution of pLS1**.** Exponentially growing
617 cultures in AGCH at OD_{620} = 0.4 were treated with the indicated LVX concentrations.
618 Values of a typical experiment are indicated. Samples were taken before the addition of the
619 drug (time 0 min), and, at the indicated times, plasmid DNA was isolated and subjected to
620 two-dimensional agarose gel electrophoresis run in the presence of 1 and 2 μg/ml
621 chloroquine in the first and second dimensions, respectively. Supercoiling density (σ)
622 values are indicated. A blackened arrowhead indicates the topoisomer that migrated with
623 $\Delta Lk = 0$ in the second dimension that has a $\Delta Wr = -14$. An open arrowhead points to the
624 more abundant topoisomer.

625 **FIG 2.** Gene expression analysis in the three conditions assayed. (A and B) Responsive
626 genes represented in Venn diagrams with 3 circles, each one corresponding to one time
627 interval in each condition, showing the differentially expressed genes in microarrays. (B)
628 All genes (left diagram), or only those genes that differed from those present in the No-
629 LVX sample (right diagram), are indicated. A complete list of these genes can be found in
630 Table 1. (C) Classification of responsive genes by functional classes: CiM, central
631 intermediary metabolism; EnM, energy metabolism; Hy, hypothetical proteins; PPy,
632 purines, pyrimidines, nucleosides, and nucleotides; Pat, pathogenesis; PrS, protein
633 synthesis; R-M, restriction-modification; TBP, transport and binding proteins; TCS, two-
634 component systems; Tns, transposon functions; Tr, transcription; U, unclassified; Oth,
635 other (classes with a representation < 2%).

636 **FIG 3**. Transcription of *fatD* depended on the inhibition of topoisomerase IV by LVX. (A)
637 Genetic structure of strain R6-P_{fat}*cat* showing the chromosomal location of P_{fat}*fatDCEB*
638 and P_{fat}*cat*. Topology-reacting gene clusters detected after DNA relaxation with NOV are
639 indicated: U1–15, up-regulated domains; D1–14, down-regulated domains. (B)
640 Transcriptional response after NOV treatment measured by qRT−PCR on exponentially
641 growing cultures of strain R6. (C) Transcriptional response of R6, of a LVX-resistant
642 derivative (R6-ParCS79F), and of the R6-P_{fat}*cat* strain. Cultures were growth in AGCH to
643 OD_{620} = 0.4, treated with LVX at 0.125 μg/ml LVX (0.5× MIC of R6 and R6-P_{fat}*cat;* 0.05×
644 MIC of R6-ParCS79F) and at 2.5 μg/ml LVX (10× MIC of R6 and R6-P_{fat}*cat*; 0.5× MIC of
645 R6-ParCS79F). Total RNA was isolated; cDNA was synthesized and subjected to
646 qRT−PCR. Data were normalized to time 0 min. Transcription represented the mean of
647 qRT-PCR values of three independent replicates ± SEM.
648 **FIG 4**. Transcription of *fatD* depended on the general supercoiling level. Cultures were
649 grown as in Fig. 3 and treated with *N*-methyl-seconeolitsine at the indicated concentrations.
650 (A) Plasmid DNA was isolated at the indicated times and subjected to two-dimensional
651 agarose gel electrophoresis in the presence of 5 and 15 μg/ml chloroquine in the first and
652 second dimensions, respectively. Supercoiling density (σ) values are indicated. A black
653 arrowhead indicates the topoisomer that migrated with ΔLk = 0 in the second dimension
654 that has a ΔWr = −30 (53). An open arrowhead indicates the more abundant topoisomer.
655 (B) Total RNA was isolated; cDNA was synthesized and subjected to qRT−PCR, *fatD*
656 (black bars) and *fatC* (white bars) values were normalized to time 0 min. Transcription
657 represented the mean of qRT-PCR values of three independent replicates ± SEM.

658 **FIG 5.** LVX lethality is related to the level of intracellular iron. (A) Enzymatic reaction of
659 SpxB that renders H_2O_2, a substrate of the Fenton reaction. P, phosphate. (B) Viability of *S.*
660 *pneumoniae* R6 (black symbols) or R6Δ*spxB* (red symbols) either in AGCH, in AGCH plus
661 the iron chelator *o*-phenantroline (AGCH+Ph), in AGCH deficient in SO_4Fe (AGCH*).
662 Cultures grown as indicated in Fig. 3 in the diverse media were treated, when indicated,
663 with LVX at concentrations equivalent to 2.5× MIC. (C) Accumulation of reactive oxygen
664 species. Results are the mean ± SEM of three independent replicates. RFU, relative
665 fluorescence units, values were made relative to 0 min and divided by the number of viable
666 cells.
667 **FIG 6.** Oxidative damage cell death pathway. The inhibition of topo IV by levofloxacin
668 (LVX) or of topoisomerase I by *N*-methyl-seconeolitsine (SCN) would cause a local
669 increase in supercoiling resulting in the up-regulation of the *fatDCEB* operon. The
670 consequent increase in this iron transporter causes an increase of intracellular ferrous iron
671 (Fe^{2+}). This compound and hydrogen peroxide (produced by the activity of the SpxB
672 pyruvate oxidase) are the substrates of the Fenton reaction. The Fenton reaction renders
673 hydroxyl radicals, which oxidatively damage DNA, proteins and lipids.
674

675 TABLE 1. Genes involved in the transcriptomic response of *S. pneumoniae* R6 to
676 levofloxacin (LVX)
677

Role or subrole	R6 locus (gene)[a]	Mean relative fold change[b]							
		NO LVX		LVX 0.5 × MIC			LVX 10 × MIC		
		15’	30’	5’	15’	30’	5’	15’	30’
Amino acid biosynthesis	spr0515 (*metF*)	—	–2.3	—	—	–2.1	—	—	—
Biosynthesis of cofactors	spr0636	—	—	—	—	—	**2.0**	—	—
	spr1438 (*entB*)	—	—	—	—	—	—	—	–2.2
Cell envelope	spr0867 (*lytB*)	**2.9**	**2.5**	—	**3.6**	**2.7**	—	**3.5**	**3.2**
	spr1324 (*apbE*)	—	—	—	—	—	—	—	–2.1
TCS12/ComCDE (°ComE box)	spr0013° (*comX1*)	—	–7.8	—	—	—	—	—	—
	spr0020° (*comW*)	—	–2.5	—	—	—	—	—	—
	spr0043*°–0044 (*comAB*)	—	–3.5	—	—	—	—	—	—
	spr1017° (*mreA*)	—	–3.0	—	—	—	—	—	—
	spr1819° (*comX2*)	—	–7.8	—	—	—	—	—	—
	spr1762°	—	–6.0	—	—	–2.0	—	—	—
	spr2043*°–2041 (*comC*)	—	–8.8	—	—	–2.4	—	—	—
(°°ComX box)	spr0027°°	—	–2.8	—	—	—	—	—	—
	spr0031*°°–0030	—	–4.8	—	—	–3.0	—	—	—
	spr0128*°°–0127 (*cibA*)	—	–2.7	—	—	–2.2	—	—	—
	spr0181°° (*orf47*)	—	–3.6	—	—	–	—	—	—
	spr0856*°°–0860 (*comEA/EC*)	—	–6.9	—	—	–2.2	—	—	—
	spr0881°° (*coiA*)	—	–8.3	—	—	–2.7	—	—	—
	spr0996°° (*radC*)	—	–6.6	—	—	–2.3	—	—	—
	spr0997	—	–3.9	—	—	—	—	—	—
	spr1144 (*dprA*)	—	–5.3	—	—	–2.1	—	—	—
	spr1628*°°–1631 (*cclA*)	—	–4.5	—	—	–2.6	—	—	—
	spr1758*°°–1756 (*cinA/recA/dinF*)	—	–3.1	—	—	—	—	—	—
	spr1831*°°–1826	—	–9.2	—	—	–3.0	—	—	–2.1
	spr1864*°°–1855 (*cglABCDFG*)	—	–2.5	—	—	—	—	—	—
	spr2006°° (*cbpD*)	—	-6.2	—	—	–2.4	—	—	—
	spr2013*°°–2012 (*comF/FC*)	—	-11.9	—	—	–3.0	—	—	—
TCS13/ SpiRH (•SpiR box)	spr0040• (*pncE*)	—	**2.7**	—	—	**2.2**	—	—	**2.0**
	spr0461•	—	**2.1**	—	—	**2.9**	—	—	**2.7**
	spr0469*•–0465 (*spiABCDP*)	—	**2.4**	—	**2.2**	**3.3**	—	—	**2.5**
	spr0470*•–0475 (*pncW*)	—	**3.1**	—	**2.2**	**4.1**	—	—	**3.2**
Pathogenesis	spr0121 (*pspA*)	—	—	—	—	—	—	—	–2.6
	spr0328 (*eng*)	—	—	—	—	—	—	—	–2.1
	spr0565 (*bgaA*)	—	—	—	—	—	—	—	–2.6
	spr1652*–1649 (*pfbA*)	—	—	—	—	—	—	—	–2.4
Central metabolism	spr1867 (*nagA*)	—	—	—	—	—	—	—	–2.5
	spr1833 (*bgl2*)	—	—	—	—	—	—	—	–2.2
	spr1285*–1287	—	—	—	—	—	—	—	–2.3

	spr1666 (*dpnD*)	—	—	—	**2.0**	—	—	**2.1**	—
Energy metabolism	spr1029 (*glgB*)	—	—	—	—	**2.1**	—	—	**2.2**
	spr0226 (*pflE*)	—	—	—	—	—	—	—	−2.2
	spr1837 (*adhE*)	—	—	—	—	—	−2.2	—	—
	spr0064 (*agaS*)	—	—	—	—	—	—	—	−2.5
	spr1028 (*gapN*)	—	—	—	—	—	−2.5	—	—
	spr0065 (*galM*)	—	—	—	—	—	—	—	−2.2
	spr0276	—	—	—	—	**3.8**	—	—	**3.7**
	spr1647*–1648 (*galET*)	—	—	—	—	—	—	−2.0	−3.2
	spr1668*–1667 (*galK*)	—	**2.7**	—	—	—	—	—	—
	spr1974 (*fcsR*)	—	—	—	—	—	—	−2.0	−4.1
Protein fate	spr1204 (*ptrB*)	—	—	—	—	**2.0**	—	—	—
Ribosomal proteins synthesis	spr0078 (*rpsD*)	—	—	—	—	—	—	—	−2.1
	spr1211 (*rplL*)	—	—	—	—	—	—	—	−2.4
	spr0682 (*rpsP*)	—	—	—	—	—	—	—	−2.4
	spr1271 (*rpsU*)	—	—	—	—	—	—	—	−2.1
	spr1943*–1944 (*rpmFG*)	—	—	—	—	—	—	—	−2.0
Purines, pyrimidines, nucleosides & nucleotides	spr0045*–0055 (*pur,van, pyr*)	**8.0**	**8.1**	—	**16.9**	**15.1**	—	**16.2**	**11.2**
	spr0613*-0614	**2.6**	—	—	**3.8**	**2.4**	—	**3.6**	**2.8**
	spr0865*–0866 (*pyrDIID*)	—	—	—	**2.5**	**2.0**	—	**2.6**	**2.4**
	spr1153 (*carB*)	—	—	—	**2.1**	**2.1**	—	**2.0**	—
	spr1662*–1663 (*xpt, pbuX*)	—	—	—	**2.8**	**3.8**	—	**3.1**	**2.8**
	spr1709 (*gtfA*)	—	−2.9	—	—	—	—	—	—
Transcription	spr0634	—	—	—	—	—	**2.0**	—	—
	spr0227 (*deoR*)	—	—	—	—	—	—	—	−2.4
	spr0279 (*bglG*)	—	—	—	**3.2**	**4.1**	—	—	**3.8**
	spr1067 (*lacR*)	—	—	—	—	—	—	—	−2.1
	spr1439 (*codY*)	—	—	—	—	—	—	—	−2.0
	spr1889	—	—	—	—	—	—	—	−2.8
	spr1899 (*phoU*)	−2.3	−2.3	—	—	—	—	—	—
	spr1933 (*rgg*)	—	−3.2	—	—	—	—	—	—
Transport and binding	spr0551 (*brnQ*)	—	—	—	—	—	**2.3**	—	**2.1**
	spr0624*–0622 (*glnQ*)	—	—	—	—	—	—	**2.1**	**2.1**
	spr1895*–1898	−2.4	−2.2	—	—	—	—	—	—
	spr1641 (*ctpA*)	—	—	—	—	—	—	−2.4	−2.9
	spr1684*–1687 (*fatDCEB*)	—	—	**3.7**	**4.7**	**3.3**	**3.8**	**6.2**	**5.6**
	spr0264*–0265	—	—	—	**3.0**	**2.9**	—	**3.0**	**2.0**
	spr0278	—	—	—	—	**5.2**	—	—	**4.7**
	spr0280	—	—	—	**2.4**	**2.6**	—	—	**2.6**
	spr1710	—	−2.0	—	—	—	—	—	—
	spr1834*–1836 (*ptcAB*)	—	—	—	—	—	—	—	−2.2
	spr1836	—	—	—	—	−2.7	—	—	−3.0
	spr0081	—	—	—	**2.0**	—	—	—	—
	spr0619	**2.5**	**2.9**	—	**2.2**	**2.2**	—	**2.3**	**2.0**
	spr0621*–0620	—	—	—	**2.0**	**2.0**	—	**2.3**	**2.2**
	spr1097 (*nirC*)	**2.2**	**2.1**	—	—	—	—	—	—

	spr1202	—	**2.1**	—	—	—	—	—	—
	spr1203	—	**2.1**	—	—	**2.4**	—	—	—
	spr1381*–1378	**2.1**	—	—	**2.2**	—	—	—	—
	spr1441 (*oxlT*)	—	—	—	**3.0**	**4.3**	**2.2**	**3.5**	**4.0**
	spr1546	—	**3.7**	—	—	—	—	—	—
	spr1646*–1643	—	—	—	—	—	—	—	-2.1
	spr1801	—	—	—	**3.0**	**2.8**	–	**3.4**	**2.4**
	spr1817	—	—	—	-2.0	—	—	—	—
Unclassified	spr0907*–0908 (*phtDE*)	—	—	—	—	—	—	—	–2.8
	spr1060 (*phpA*)	—	—	—	—	—	—	—	–2.2
Transposon functions	spr0018	—	—	—	–2.2	–2.1	—	—	—
	spr0019	—	—	—	—	**3.1**	—	—	**2.7**
	spr0041	—	—	—	—	—	—	**2.5**	—
	spr0612	—	—	—	—	—	—	**2.5**	—
	spr0273	—	–2.6	—	—	—	—	—	—
	spr0523	—	—	—	—	—	—	**2.5**	—
	spr1046	—	—	—	–2.2	—	—	—	—
	spr1349*–1347	–2.4	–2.0	—	–2.0	—	—	—	—
	spr1367	–2.4	–2.3	—	—	—	—	—	—
	spr1563	—	—	—	—	–2.0	—	—	—

678

679 [a] The responsive genes included showed significant fold variations (≥2 and P <0.01). All genes showing
680 variations, with the exception of 47 encoding hypothetical proteins are included. Genes considered to be
681 involved in the LVX-mediated transcriptomic response (*i.e.*, that did not showed variations in the no-LVX
682 culture) are shadowed in grey. No shadowed genes are considered to be involved in the growth-related
683 response. * indicates the first gene of the putative operon: °gene with a ComE box; °°gene with a ComX box;
684 • gene with a SpiR box.

685 [b] In operons, values indicated correspond to those of the first gene of the operon, except in spr0045–0055,
686 spr0613–0614, spr1859–1898, spr1864–1855, in which variations corresponded to the second gene. Values
687 above 2 are shown in boldface; —, no change.