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

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

## ABSTRACT

We studied the transcriptomic response of *Streptococcus pneumoniae* to the fluoroquinolone moxifloxacin at a concentration that inhibits DNA gyrase. Treatment of the wild-type strain R6, at a concentration of 10× MIC, triggered a response involving 132 genes after 30 minutes of treatment. Genes from several metabolic pathways involved in the production of pyruvate were up-regulated. These included 3 glycolytic enzymes, which ultimately convert fructose-6P to pyruvate, and 2 enzymes that funnel P-sugars into the glycolytic pathway. In addition, acetyl-CoA carboxylase was down-regulated, likely leading to an increase in acetyl-CoA. When coupled with an up-regulation in formate acetyltransferase, an increase in acetyl-CoA would raise the production of pyruvate. Since pyruvate is converted by pyruvate oxidase (SpxB) into hydrogen peroxide ($H_2O_2$), an increase in pyruvate would augment intracellular $H_2O_2$. Here, we confirm a 21-fold increase in the production of $H_2O_2$ and a 55-fold increase in the amount of hydroxyl radical in cultures treated during 4 h with moxifloxacin. This increase in hydroxyl radical through the Fenton reaction, would damage DNA, lipids, and proteins. These reactive oxygen species contributed to the lethality of the drug, a conclusion supported by the observed protective effects of a SpxB deletion. These results support the model whereby fluoroquinolones cause redox alterations. The transcriptional response of *S. pneumoniae* to moxifloxacin is compared with the response to levofloxacin, an inhibitor of topoisomerase IV. Levofloxacin triggers the transcriptional activation of iron transport genes and also enhances the Fenton reaction.

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

The mechanism of action of FQs is the formation of DNA-FQ-topoisomerase complexes and the subsequent generation of detrimental double-stranded DNA breaks (14). In *Escherichia coli*, this appears to occur through two pathways, one dependent and the other independent of protein synthesis. Reactive oxygen species (ROS), such as superoxide, hydrogen peroxide ($H_2O_2$), and hydroxyl radical, contribute to FQ-mediated cell death via the protein synthesis-dependent pathway (15). This observation is consistent with a model proposed recently suggesting that, as a consequence of gyrase

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

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

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

## MATERIALS AND METHODS

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

**Analysis of chromosomal fragmentation and of the topology of covalently closed circles.** Chromosomal fragmentation was detected by pulse-field gel electrophoresis (PFGE). About $3\times 10^6$ cells were lysed in solid agarose inserts in a buffer containing 10 mM Tris HCl pH 8, 1 M NaCl, 0.1 M EDTA, 0.5% Brij58, 0.2% deoxycholate, 0.5% sarkosyl, 20 μg/ml RNaseE and 100 μg/ ml lysozyme. Agarose inserts were treated with

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

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

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

Microarrays results were validated by quantitative real-time RT-PCR (Chromo 4, BioRad) as previously described (25). Genes selected to be amplified were the following: *fatC, fatD, gyrA*, *gyrB*, *glnA*, *mutM*, *lmb*, *parE*, *psaB*, *pyrB spr1680*, and *topA*. For most genes, previously described oligonucleotides were used (24, 25). For the rest of genes, oligonucleotides (forward/ reverse) were: m*utM* (5'-AGATGGTGGCACGCTTGTTTA-3'/ 5'-GTGTATCCATAGCACCGAGTCC-3'), *lmB* (5'-AAGGGGATGAAAATTGTGACCAG-3'/ 5'-GCCCAAGATTCGAGTGTATGAGA-3') *pyrB* (5'-AACTCTTTTTCGCTGGACCTG-3'/ 5'-ACTATCATGGCGTTCGTGTTG-3'), *psaB* (5'-ATACGTATCGAAAACCTCAGTGTC-3'/ 5'-CCCAGCATACCTTTTAATAGTGTT-3'). Annealing temperatures for the oligonucleotides used were comprised between 51 and 56 ºC. PCR products had sizes varying from 128 to 205 bp,

**Detection of ROS.** The intracellular oxidation levels were measured using dihydrorhodamine 123 dye (Sigma-Aldrich), a non-fluorescent compound which diffuses across membranes. Oxidation converts it to the fluorescent product rhodamine 123. Measured fluorescence is therefore proportional to the level of oxidation (30). Cells were grown to an optical density at 620 nm ($OD_{620}$) = 0.4 before MOX was added. 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 $H_2O_2$ 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 $H_2O_2$, cells were grown to an optical
167 density $OD_{620}$ of 0.4 and then diluted 1:100 in growth media before MOX and LVX
168 were added. Cells were then incubated at 37ºC 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 $H_2O_2$ in samples was calculated using a
173 standard curve derived from assays of serial dilutions of a defined concentration of
174 $H_2O_2$. 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 $\Delta Lk$ 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 μg/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 μg/ml) and T2 (ParCS79F,
203 GyrAS81F; MOX MIC=4 μg/ml) was also analysed in the presence of the same
204 concentration used for R6, 1.25 μg/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= $3\times10^{-4}$) 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 $H_2O_2$, through the activity of pyruvate oxidase (SpxB),
252 and in hydroxyl radical, through the Fenton reaction (Fig. 3B). To assess the relevance
253 of $H_2O_2$ 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

presence of the iron chelator *0*-phenantroline. This result indicated that absence or presence of iron did not affect cell survival, despite iron being a component of the Fenton reaction. Meanwhile, accumulation of ROS was observed in R6 cultures treated with MOX (Fig. 4B). Sharp increases with respect to time 0 of 22.1-fold, 54.9-fold and 80.8-fold were observed at 3, 4, and 5 hours of treatment, respectively. However, there was less than 5-fold increase for the Δ*spxB* strain (Fig. 4B). Only a 15-fold increase in ROS was observed at 3 to 5 hours in the presence of *0*-phenantroline (Fig. 4B). In addition, we observed a comparable increase of about 21-fold in the production of $H_2O_2$ in R6 cultures after 3 and 4 hours with 0.5× MIC of MOX (Fig. 4C). However, no increase was detected in the presence of LVX. A plausible interpretation is that the 15-fold increase of signal in the presence of *0*-phenantroline, happens independently of the Fenton reaction and is really a consequence of an increase in $H_2O_2$, which was also detected by dihydrorhodamine 123. These results suggest a link between MOX lethality and the Fenton reaction via an increase of $H_2O_2$. In summary, ROS contributes to the lethality of MOX in two ways: directly by an increase in endogenous $H_2O_2$ and indirectly via the Fenton reaction. No increases in the production of $H_2O_2$ in the T1 and T2 strains with respect to the time 0 min were observed in the presence of MOX. This is consistent with the fact that the susceptibility of MOX for strain T1Δ*spxB* did not change.

## DISCUSSION

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

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

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

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

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

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

Several genes related to purine and pyrimidine metabolism were up-regulated with MOX but not with LVX. Among them was *prsA*, coding for an enzyme that catalyzes the synthesis of 5-phospho ribosyl-α-1-pyrophosphate (PRP). PRP is an intermediary in both purine and pyrimidine metabolism (47). The product of *pyrP* is a dedicated transporter that facilitates the entrance of uracil into the cell. Uracil is converted to UMP by the product of *upp*. The intracellular concentration of UMP would be expected to increase through the activation of the $CO_2$ biosynthesis pathway: 6 out of

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

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

We have demonstrated that ROS have a role in the lethality of MOX in *S. pneumoniae* R6. Consistent with this model, the R6Δ*spx*B and T1 strains were less susceptible to this drug (Fig. 4). These results support a model whereby the accelerated production of redox alterations by FQs contributes to their lethality (18, 20). Also consistent is the fact that glutathione reductase (*gor*) gene was up-regulated, presumably in an attempt to counteract the lethal level of oxidation. Glutathione reductase reduces glutathionine (γ-L-glutamyl-L-cysteinyl-glycine) which lowers the level of oxidation. This provides protection from $H_2O_2$ accumulation (48).

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

The results presented here complement our previous findings that LVX, an inhibitor of topo IV, triggers the transcriptional activation of iron transport genes (25), a result not observed with MOX. Both LVX and MOX stimulate the Fenton reaction by causing an increase in the concentration of either iron or $H_2O_2$, respectively. In addition to causing hydroxyl radical to accumulate, MOX increases endogenous $H_2O_2$, which has been shown to alter membrane composition (52) and survival (53) in *S. pneumoniae*. This study suggests that a strategy for improving the efficacy of FQs may be to potentiate their ability to provoke the accumulation of intracellular hydroxyl radical and $H_2O_2$.

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## REFERENCES

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

penicillin-susceptible and-nonsusceptible *Streptococcus pneumoniae* isolates in Hong Kong, 2000 to 2005. Antimicrob. Agents Chemother. **51:**2192-2194.

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

14. **Drlica K, Malik M, Kerns RJ, Zhao X.** 2008. Quinolone-mediated bacterial death. Antimicrob. Agents Chemother. **52:**385-392.

15. **Wang X, Zhao X, Malik M, Drlica K.** 2010. Contribution of reactive oxygen species to pathways of quinolone-mediated bacterial cell death. J. Antimicrob. Chemother. **65:**520-524.

16. **Imlay JA, Chin SM, Linn S.** 1988. Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. Science **240:**640-642.

17. **Dwyer DJ, Kohanski MA, Hayete B, Collins JJ.** 2007. Gyrase inhibitors induce an oxidative damage cellular death pathway in *Escherichia coli*. Mol. Syst. Biol. **3:**91.

18. **Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ.** 2007. A common mechanism of cellular death induced by bactericidal antibiotics. Cell **130:**797-810.

19. **Wang X, Zhao X.** 2009. Contribution of oxidative damage to antimicrobial lethality. Antimicrob. Agents Chemother. **53:**1395-1402.

20. **Dwyer DJ, Belenky PA, Yang JH, MacDonald IC, Martell JD, Takahashi N, Chan CT, Lobritz MA, Braff D, Schwarz EG, Ye JD, Pati M, Vercruysse M, Ralifo PS, Allison KR, Khalil AS, Ting AY, Walker GC, Collins JJ.** 2014. Antibiotics induce redox-related physiological alterations as part of their lethality. Antibiotics induce redox-related physiological alterations as part of their lethality. Proc. Natl Acad. Sci. USA. **111:**E2100-2109.

21. **Zhao X, Hong Y, Drlica K.** 2015. Moving forward with reactive oxygen species involvement in antimicrobial lethality. J. Antimicrob. Chemother. **70:**639-642.

22. **Dwyer DJ, Collins JJ, Walker GC.** 2015. Unraveling the physiological complexities of antibiotic lethality. Ann. Rev. Pharmacol. Toxicol. **55:**313-332.

23. **Zhao X, Drlica K.** 2014. Reactive oxygen species and the bacterial response to lethal stress. Curr. Op. Microbiol. **21:**1-6.

24. **Ferrándiz MJ, Martín-Galiano AJ, Schvartzman JB, de la Campa AG.** 2010. The genome of Streptococcus pneumoniae is organized in topology-reacting gene clusters. Nucl. Acids Res. **38:**3570-3581.

25. **Ferrandiz MJ, de la Campa AG.** 2014. The fluoroquinolone levofloxacin triggers the transcriptional activation of iron transport genes that contribute to cell death in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **58:**247-257.

26. **Lacks SA, Lopez P, Greenberg B, Espinosa M.** 1986. Identification and analysis of genes for tetracycline resistance and replication functions in the broad-host-range plasmid pLS1. J. Mol. Biol. **192:**753-765.

27. **Balsalobre L, de la Campa AG.** 2008. Fitness of *Streptococcus pneumoniae* fluoroquinolone-resistant strains with topoisomerase IV recombinant genes. Antimicrob. Agents Chemother. **52:**822-830.

28. **Martín-Parras L, Lucas I, Martínez-Robles ML, Hernández P, Krimer DB, Hyrien O, Schvartzman JB.** 1998. Topological complexity of different populations of pBR322 as visualized by two-dimensional agarose gel electrophoresis. Nucl. Acids Res. **26:**3424-3432.

29. **Ferrandiz MJ, Arnanz C, Martin-Galiano AJ, Rodriguez-Martin C, de la Campa AG.** 2014. Role of global and local topology in the regulation of gene expression in *Streptococcus pneumoniae*. PloS one **9:e101574**.

30. **Yeom J, Imlay JA, Park W.** 2010. Iron homeostasis affects antibiotic-mediated cell death in Pseudomonas species. J. Biol. Chem. **285:**22689-22695

31. **Fani F, Leprohon P, Legare D, Ouellette M.** 2011. Whole genome sequencing of penicillin-resistant *Streptococcus pneumoniae* reveals mutations in penicillin-binding proteins and in a putative iron permease. Genome Biol **12:**R115.

32. **Pruss GJ, Manes SH, Drlica K.** 1982. *Escherichia coli* DNA topoisomerase I mutants: increased supercoiling is corrected by mutations near gyrase genes. Cell **31:**35-42.

33. **Khan SR, Kuzminov A.** 2013. Trapping and breaking of in vivo nicked DNA during pulsed field gel electrophoresis. Anal. Biochem. **443:**269-281.

34. **Malik M, Zhao X, Drlica K.** 2006. Lethal fragmentation of bacterial chromosomes mediated by DNA gyrase and quinolones. Mol. Microbiol. **61:**810-825.

35. **Hoskins J, Alborn WE, Arnold J, Blaszczak LC, Burgett S, DeHoff BS, Estrem ST, Fritz L, Fu DJ, Fuller W, Geringer C, Gilmour R, Glass JS, Khoja H, Kraft AR, Lagace RE, LeBlanc DJ, Lee LN, Lefkowitz EJ, Lu J, Matsushima P, McAhren SM, McHenney M, McLeaster K, Mundy CW, Nicas TI, Norris FH, O'Gara M, Peery RB, Robertson GT, Rockey P, Sun PM, Winkler ME, Yang Y, Young-Bellido M, Zhao G, Zook CA, Baltz RH, Jaskunas SR, Rosteck PR, Skatrud PL, Glass JI.** 2001. Genome of the bacterium *Streptococcus pneumoniae* strain R6. J. Bacteriol. **183:**5709-5717

36. **Gottesman S, Wickner S, Maurizi MR.** 1997. Protein quality control: triage by chaperones and proteases. Genes Develop. **11:**815-823.

37. **Moreira RN, Domingues S, Viegas SC, Amblar M, Arraiano CM.** 2012. Synergies between RNA degradation and trans-translation in *Streptococcus*

*pneumoniae*: cross regulation and co-transcription of RNase R and SmpB. BMC Microbiol. **12:**268.

38. **Spellerberg B, Cundell, D.R., Sandros, J., Pearce, B.J., Idanpaan-Heikkila, I., Rosenow, C., Masure, H.R.** 1996. Pyruvate oxidase, as a determinant of virulence in *Streptococcus pneumoniae*. Mol. Microbiol. **19:**803-813.

39. **Yague G, Morris JE, Pan XS, Gould KA, Fisher LM.** 2002. Cleavable-complex formation by wild-type and quinolone-resistant *Streptococcus pneumoniae* type II topoisomerases mediated by gemifloxacin and other fluoroquinolones. Antimicrob. Agents Chemother. **46:**413-419.

40. **Kishii R, Takei M, Fukuda H, Hayashi K, Hosaka M.** 2003. Contribution of the 8-methoxy group to the activity of gatifloxacin against type II topoisomerases of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **47:**77-81.

41. **Peterson SN, Sung CK, Cline R, Desai BV, Snesrud EC, Luo P, Walling J, Li H, Mintz M, Tsegaye G, Burr PC, Do Y, Ahn S, Gilbert J, Fleischmann RD, Morrison DA.** 2004. Identification of competence pheromone responsive genes in *Streptococcus pneumoniae* by use of DNA microarrays. Mol. Microbiol. **51:**1051-1070.

42. **Dagkessamanskaia A, Moscoso M, Henard V, Guiral S, Overweg K, Reuter M, Martin B, Wells J, Claverys JP.** 2004. Interconnection of competence, stress and CiaR regulons in *Streptococcus pneumoniae*: competence triggers stationary phase autolysis of *ciaR* mutant cells. Mol. Microbiol. **51:**1071-1086.

43. **Prudhomme M, Attaiech L, Sanchez G, Martin B, Claverys JP.** 2006. Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*. Science **313:**89-92.

44. **Leo E, Gould KA, Pan XS, Capranico G, Sanderson MR, Palumbo M, Fisher LM.** 2005. Novel symmetric and asymmetric DNA scission determinants for *Streptococcus pneumoniae* topoisomerase IV and gyrase are clustered at the DNA breakage site. J. Biol. Chem. **280:**14252-14263.

45. **Arnoldi E, Pan XS, Fisher LM.** 2013. Functional determinants of gate-DNA selection and cleavage by bacterial type II topoisomerases. Nucl. Acids Res **41:**9411-9423.

46. **Postow L, Crisona NJ, Peter BJ, Hardy CD, Cozzarelli NR.** 2001. Topological challenges to DNA replication: conformations at the fork. Proc. Natl. Acad. Sci. USA **98:**8219-8226.

47. **Kilstrup M, Hammer K, Ruhdal Jensen P, Martinussen J.** 2005. Nucleotide metabolism and its control in lactic acid bacteria. FEMS Microbiol. Rev. **29:**555-590.

48. **Potter AJ, Trappetti C, Paton JC.** 2012. *Streptococcus pneumoniae* uses glutathione to defend against oxidative stress and metal ion toxicity. J. Bacteriol. **194:**6248-6254.

49. **Kim SW, Bae YG, Pyo SN, Rhee DK.** 2007. Differential regulation of the genes of the *Streptococcus pneumoniae dnaK* operon by $Ca^{++}$. Mol. Cel. **23:**239-245.

50. **Li L, Hong Y, Luan G, Mosel M, Malik M, Drlica K, Zhao X.** 2014. Ribosomal elongation factor 4 promotes cell death associated with lethal stress. mBio **5:**e01708.

51. **Long Q, Du Q, Fu T, Drlica K, Zhao X, Xie J.** 2015. Involvement of Holliday junction resolvase in fluoroquinolone-mediated killing of *Mycobacterium smegmatis*. Antimicrob Agents Chemother. **59**:1782-1785.

52. **Pesakhov S, Benisty R, Sikron N, Cohen Z, Gomelsky P, Khozin-Goldberg I, Dagan R, Porat N.** 2007. Effect of hydrogen peroxide production and the Fenton reaction on membrane composition of *Streptococcus pneumoniae*. Bioch. Bioph. Acta **1768:**590-597.

53. **Regev-Yochay G, Trzcinski K, Thompson CM, Lipsitch M, Malley R.** 2007. SpxB is a suicide gene of *Streptococcus pneumoniae* and confers a selective advantage in an in vivo competitive colonization model. J. Bacteriol. **189:**6532-6539.

## FIGURE LEGENDS

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

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

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 μg/ml (1× MIC for R6 and T1). Values normalized to the 0 time value. The mean ±
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 R6Δ*spxB* were
597 grown to an optical density at 620 nm ($OD_{620}$) = 0.4 before MOX at 1× 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 $OD_{620}$=0.4 and then diluted 1:100 in growth media before MOX and
603 LVX were added, both at 0.06 μg/ml (0.5× MIC for R6). Cells were then incubated at
604 37ºC 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 min

610 treatment with MOX[a]

| Role or subrole | R6 locus (gene)[a] | Mean relative fold change[b] |
|---|---|---|
| **Amino acid biosynthesis:** Asp | spr1907 (*dapD*) | **2.0** |
| **Amino acid biosynthesis:** Gln | spr0443*-0444 (*glnRA*) | **2.0** |
| **Amino acid biosynthesis**: Ser | spr1095 (*metY*) | **3.9** |
| | **spr0823 (*but*)** | -2.3 |
| **Biosynthesis of cofactors** | **spr1324 (*apbE*)** | -2.8 |
| **Carbohydrate metabolism:** Glycolisis | spr0530 (*fba*) | **2.7** |
| | spr1902 (*gpdA*) | **2.6** |
| | spr1499 (*gpmA*) | **2.3** |
| | spr0647 (*pmi*) | **3.8** |
| **Carbohydrate metabolism:** Pyruvate | spr0415 (*pfl*) | **2.9** |
| | **spr1438 (*entB*)** | -2.4 |

| | | |
|---|---|---|
| **Carbohydrate metabolism:** Pentose-P pathway | spr1841 (*tktA*) | **2.8** |
| **Carbohydrate metabolism:** Other | **spr0064 (*agaS*)** | -2.2 |
| | spr0778*-0779 (*fruRB*) | **—** |
| | spr1667 (*galT*) | -4.4 |
| | **spr1833 (*bgl2*)** | -2.6 |
| | **spr1842 (*ulaG*)** | **2.8** |
| **Cell envelope** | spr1459 (*gtrB*) | **2.9** |
| | spr0540*-0541 (*murMN*) | **2.1** |
| **Cellular processes:** Detoxification | spr0864 (*lguL*) | -2.3 |
| **Cellular processes:** Pathogenesis | **spr0328 (*eng*)** | -2.3 |
| | spr0906 (*lmb*) | -3.4 |
| | spr0910 (*phtE*) | -2.6 |
| | **spr1060 (*phpA*)** | -3.6 |
| | spr1492 (*psaB*) | -2.2 |
| **Cellular processes:** Stress | spr0453*-0456 (*hrcA-grpE-dnaK-dnaJ*) | **2.6** |
| | spr0692 (*gor*) | **2.2** |
| | spr1723*-1722 (*groES-groEL*) | **2.1** |

| | | |
|---|---|---|
| **Cellular processes:** Cell division | spr0844 (*gidA*) | **2.2** |
| **Cellular processes:** Toxin production | spr0880 (*tehB*) | -2.1 |
| **Central intermediary metabolism** | spr0816 (*cad*) | -2.4 |
| | spr0284 (*xylS*) | **2.1** |
| **DNA metabolism** | spr0673 (*holA*) | **2.3** |
| | spr0790 (*hsdR*) | **2.6** |
| | spr0872 (*mutM*) | -2.0 |
| **Fatty acid metabolism** | spr0386*-0387 (*accDA*) | -2.2 |
| **Protein Export and Trans-translation** | spr0877*-0879 (*secG-rnr-smpB*) | -2.7 |
| **Purines, pyrimidines** | spr0028 (*prsA*) | **2.6** |
| | spr0655 (*upp*) | **2.0** |
| | **spr0865 (*pyrK*)** | **9.4** |
| | spr1156*-1153 (*pyrRB-carAB*) | **3.5** |
| | spr1165 (*pyrP*) | **3.9** |
| | spr2033 (*imdH*) | **3.7** |
| **Regulatory functions:**TCS12 (ComCDE) | spr0013° (*comX2*) | **2.1** |
| | spr0654 (*comEB*) | **2.1** |
| | spr1962° | **3.7** |

| | | |
|---|---|---|
| **Regulatory functions:**TCS11 | spr1814 (*rr11*) | -2.0 |
| **Transcription** | spr1336 | -2.9 |
| | spr0336 (*ritR*) | **2.0** |
| | spr1569 (*scrR*) | **—** |
| | spr0679 (*ppiA*) | **2.6** |
| **Translation** | **spr0682 (*rpsP*)** | -2.1 |
| | spr0861*-0864 (*infC- rpmI- rplT- lguL*) | -2.4 |
| | spr0876 (*rpmG*) | -2.4 |
| | spr0913 (*pepT*) | **3.1** |
| | spr0920 (*trmE*) | **2.2** |
| | spr1082 (*lepA*) | -2.0 |
| | spr1123 (*ftsY*) | **—** |
| | spr1204 (*ptrB*) | **3.3** |
| | **spr1211 (*rplL*)** | -2.1 |
| | spr1871 | -2.0 |
| | **spr1943*-1944 (*rpmFG*)** | -2.0 |
| **Transport:** Amino acids, peptides and amines | **spr0327 (*aliA*)** | -3.8 |

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

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

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

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