Objectives: Fluoroquinolone resistance has been poorly studied in Legionella pneumophila, an intracellular pathogen responsible for legionellosis. Our goal was to further characterize molecular mechanisms involved in fluoroquinolone resistance in this species.

Methods: Eight independent lineages were founded from a common fluoroquinolone-susceptible L. pneumophila ancestor and propagated by serial passages in moxifloxacin-containing culture medium. We identified the substituted mutations that affected the DNA topoisomerase II-encoding genes, determined the order of substitution of the mutations leading to the stepwise MIC increases of moxifloxacin over evolutionary time and demonstrated their direct involvement in the resistance process.

Results: Adaptation occurred through parallel stepwise increases in the moxifloxacin MICs up to 512-fold the MIC for the parental strain. Mutations affected the topoisomerase II-encoding genes gyrA, parC and gyrB, reflecting a high degree of genetic parallelism across the independent lineages. During evolution, the T83I change in GyrA occurred first, followed by G78D or S80R in ParC and D87N in GyrA, or S464Y or D426N in GyrB. By constructing isogenic strains, we showed that the progressive increase in resistance was linked to a precise order of mutation substitution, but also to the co-existence of several subpopulations of bacteria bearing different mutations.

Conclusions: Specific mutational trajectories were identified, strongly suggesting that intermolecular epistatic interactions between DNA topoisomerases underlie the mechanism of fluoroquinolone resistance in L. pneumophila. Our results suggest that L. pneumophila has strong potential to become resistant to fluoroquinolone compounds and warrant further investigation of resistance in clinical and environmental strains of this pathogen.

Keywords: Legionella pneumophila, resistance mechanisms, mutations, evolution

Introduction

Antibiotics have allowed tremendous progress in the medical management of infectious diseases since the middle of the 20th century, yet these successes are now being eroded due to rapid emergence and dissemination of antibiotic resistance. Among widely prescribed antibiotics, the fluoroquinolone family represents about one-fifth of the antibacterial market. Fluoroquinolones are used to treat a wide range of bacterial infections because of their efficient pharmacokinetic and pharmacodynamic properties, including a large distribution in the body, a wide antibacterial spectrum and bactericidal activity. However, after their introduction >40 years ago, the increased use of fluoroquinolones in clinical medicine has led to selection of resistance towards these drugs in many Gram-positive and Gram-negative bacterial species. Three major resistance mechanisms have been described, including reduced drug accumulation by efflux systems and protection of the targets of fluoroquinolones.
Mutational paths towards fluoroquinolone resistance

fluoroquinolones by Qnr, a plasmid-encoded protein. However, in most cases, mutations are selected first in the genes encoding type II topoisomerases, i.e. DNA gyrase (gyrA and gyrB) and topoisomerase IV (parC and parE), which are the targets of fluoroquinolones. Resistance-conferring mutations occur within specific regions of these genes, called the quinolone resistance-determining regions (QRDRs), frequently affecting one or several of the positions corresponding to Escherichia coli codons 83 and 87 of gyrA and 80 and 84 of parC.

Fluoroquinolone resistance mechanisms have been extensively studied in many bacterial species, except in intracellular pathogens. Rickettsioses, Q fever, chlamydioidoses, legionellosis, tularemia, bartonelloses and brucelloses are examples of infectious diseases due to intracellular bacteria, for which fluoroquinolones represent a possible therapeutic alternative. Relapses and failures despite administration of fluoroquinolone therapy have been occasionally reported in patients suffering from these diseases, for example murine typhus, Q fever, Chlamydia trachomatis urogenital infection, legionellosis, bartonelloses and brucelloses. Yet, bacteria responsible for these diseases are usually considered not able to develop resistance to these antibiotics. Hence, few clinical strains of these species harbouring acquired fluoroquinolone resistance mechanisms have been isolated so far, except for C. trachomatis. It should be mentioned, however, that for most of these pathogens fluoroquinolone-resistant mutants have been obtained in vitro, e.g. Coxiella burnetii, C. trachomatis, Legionella pneumophila, Bartonella bacilliformis and Brucella melitensis.

L. pneumophila, a Gram-negative bacterium, is responsible for most legionellosis cases. Human infections occur via inhalation of infected aerosols, mainly from man-made aquatic systems. Following the first large outbreak of legionellosis during an American Legion convention in Philadelphia in 1976, erythromycin was proposed as the drug of choice to treat legionellosis patients. More recently, fluoroquinolone compounds have been proposed as first-line drugs in legionellosis patients. However, mortality rates of 10%–15% are usually reported in legionellosis patients and death may occur despite fluoroquinolone therapy. On the other hand, Legionella species are present in most aquatic environments, where they interact with protozoa and where they may be exposed to residual concentrations of these antibiotics.

In L. pneumophila, in vitro selection of fluoroquinolone resistance has been reported. Resistance has been related to mutations affecting codons 83 and 87 of gyrA (E. coli numbering), although the last mutation resulted in resistance to only nalidixic acid, whereas MICs of fluoroquinolones remained unchanged. However, no precise genetic reconstructions were performed to demonstrate their direct involvement in the resistance levels. In the present study, we further characterized fluoroquinolone resistance mechanisms in L. pneumophila by designing evolution experiments in which cells of L. pneumophila were propagated under increasing concentrations of moxifloxacin. We identified new fluoroquinolone resistance mutations in the QRDRs of the topoisomerase-encoding genes compared with both previous studies and other bacteria. We rigorously reconstructed these mutations by genetic manipulation, either alone or in various combinations, to specifically investigate the relationship between their substitution order and the different levels of resistance. We found that mutational diversity and trajectories dictate Darwinian evolution towards increased levels of resistance and this has only been previously demonstrated in E. coli and Haemophilus influenzae. We consider that these in vitro data strengthen the hypothesis that resistance to fluoroquinolone compounds may arise in clinical and/or environmental strains of L. pneumophila and our data will allow us to design specific molecular biological tools to further investigate this possibility.

Materials and methods

Bacterial strains and media

Bacterial strains used in the present study are listed in Table 1. L. pneumophila serogroup 1 strain Paris (CIP107629T) was used as the ancestral strain since this strain is endemic in France and other European countries, widely distributed in the environment and responsible for sporadic cases of legionellosis in humans. It was kindly provided by Professor J. Etienne (French National Reference Center for Legionella, Lyon, France). Bacteria were grown in N-(2-acetamido)-2-aminooethanesulfonic acid (ACES)-buffered yeast extract broth (BYE-α) at 37°C with agitation or on solid ACES-buffered charcoal–yeast extract (BCYE-α) agar medium.

Antibiotics

Levofloxacin and rifampicin (Aventis, France), ciprofloxacin and moxifloxacin (Bayer Pharma, France), erythromycin (Abbott, France) and doxycycline (Sigma, France) were prepared as 2 mg/mL stock solutions and stored at −80°C. Moxifloxacin was chosen for the evolution experiment because it is a recently developed fluoroquinolone.

MIC determination

A bacterial suspension of ~10⁵ cfu/mL was prepared in BYE-α medium and 180 μL was dispensed into each well of a 96-well microtiter plate. Twenty microlitres of 10-times concentrated antibiotic suspensions were added to obtain the desired final concentrations. MICs corresponded to the lowest antibiotic concentrations for which no visible growth was detected after 48 h incubation at 37°C. Antibiotic-free wells containing 200 μL of bacterial suspension were used as growth standards. MICs were also determined in Mueller–Hinton broth for Staphylococcus aureus ATCC 25923 and E. coli ATCC 25922, as controls. All experiments were duplicated and checked for reproducibility.

Selection of resistant mutants by serial passages

Eight independent lineages (L1–L8) were founded from L. pneumophila strain Paris and propagated by serial passages with increasing concentrations of moxifloxacin. A suspension of L. pneumophila strain Paris was prepared and dispensed in each well (1.8 mL/well) of 24-well microtiter plates to obtain a final bacterial inoculum of ~10⁶ cfu/mL. To avoid contamination across the different cultures, each culture was surrounded by empty wells. Moxifloxacin (0.2 mL/well) was added to obtain final 2-fold serial concentrations ranging from 0.5 to 16 times the MIC determined for the parental strain (0.0625 mg/L). After 4 days of incubation at 37°C, the minimum concentration of moxifloxacin inhibiting bacterial growth was recorded. It is important to note that the recorded MIC corresponded to that of the entire population present at each timepoint. Bacterial cells in the wells...
with the highest moxifloxacin concentration allowing growth were serially transferred, using a 1:40 dilution, into new 24-well microtitre plates containing fresh medium with, again, 2-fold serial concentrations of moxifloxacin ranging from 0.5 to 16 times the moxifloxacin concentration inhibiting growth of the previous selection cycle. This procedure was repeated until growth was obtained at a moxifloxacin concentration of 16 mg/L, i.e. the moxifloxacin concentration inhibiting growth was 32 mg/L, which corresponded to a 512-fold increase in moxifloxacin MIC for the ancestral strain. Genetically heterogeneous mixtures were sampled and frozen from each of the eight lineages and at each 4 day cycle to determine the dynamics of substitution of the mutations conferring fluoroquinolone resistance (see below).

Sequencing the QRDRs of the topoisomerase II-encoding genes

The bacterial frozen mixtures from each of the eight lineages, sampled at a moxifloxacin concentration of 16 mg/L, were plated onto BCYE-a Petri dishes. One evolved clone was picked from each lineage, grown in BYE-a and subsequently preserved as glycerol suspensions (Table 1). Genomic DNA was extracted from the L. pneumophila CIP107629T ancestral and antibiotic-resistant selected clones, using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany), according to the manufacturer’s recommendations. Fragments including the QRDRs of the genes encoding DNA gyrase (gyrA and gyrB) and topoisomerase IV (parC and parE) were PCR-amplified using the respective primer pairs PLPF7–PLPR8, PLPF9–PLPR10, PLPF11–PLPR12 and LpParEF–LpParER (Table 2) and subsequently sequenced using the same primers.

Dynamics of substitution of fluoroquinolone resistance mutations

Frozen bacterial suspensions collected at various timepoints during propagation of the eight lineages were plated onto BCYE-α plates and individual colonies were isolated and grown in BYE-α liquid medium. The mutations identified in the previous section were

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**Table 1.** Strains used and constructed in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIP107629T</td>
<td>L. pneumophila -1 strain Paris</td>
<td>CNRL</td>
</tr>
<tr>
<td>LP1</td>
<td>resistant clone isolated from lineage L1</td>
<td>this work</td>
</tr>
<tr>
<td>LP2</td>
<td>resistant clone isolated from lineage L2</td>
<td>this work</td>
</tr>
<tr>
<td>LP3</td>
<td>resistant clone isolated from lineage L3</td>
<td>this work</td>
</tr>
<tr>
<td>LP4</td>
<td>resistant clone isolated from lineage L4</td>
<td>this work</td>
</tr>
<tr>
<td>LP5</td>
<td>resistant clone isolated from lineage L5</td>
<td>this work</td>
</tr>
<tr>
<td>LP6</td>
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<td>this work</td>
</tr>
<tr>
<td>LP7</td>
<td>resistant clone isolated from lineage L7</td>
<td>this work</td>
</tr>
<tr>
<td>LP8</td>
<td>resistant clone isolated from lineage L8</td>
<td>this work</td>
</tr>
<tr>
<td>LPP11</td>
<td>CIP107629T + gyrA83 (T83I)</td>
<td>this work</td>
</tr>
<tr>
<td>LPP14</td>
<td>CIP107629T + gyrA83 (T83I) + gyrA87 (D87N)</td>
<td>this work</td>
</tr>
<tr>
<td>LPP15</td>
<td>CIP107629T + gyrA83 (T83I) + gyrA87* (D87H)</td>
<td>this work</td>
</tr>
<tr>
<td>LPP18</td>
<td>LPP11 + parC78 (G78D)</td>
<td>this work</td>
</tr>
<tr>
<td>LPP19</td>
<td>LPP11 + parC80 (S80R)</td>
<td>this work</td>
</tr>
<tr>
<td>LPP10</td>
<td>LPP14 + parC78 (G78D)</td>
<td>this work</td>
</tr>
<tr>
<td>LPP111</td>
<td>LPP15 + parC78 (G78D)</td>
<td>this work</td>
</tr>
<tr>
<td>LPP114</td>
<td>LPP18 + gyrB464 (S464Y)</td>
<td>this work</td>
</tr>
<tr>
<td>LPP117</td>
<td>LPP14 + parC80 (S80R)</td>
<td>this work</td>
</tr>
</tbody>
</table>

*All resistant clones have been isolated at the last evolutionary passage allowing growth (16 mg/L moxifloxacin), i.e. just before the passage corresponding to a 512-fold increase in the moxifloxacin concentration.

The mutations introduced by homologous recombination into the chromosome of the moxifloxacin-susceptible parental strain are indicated by the changes they produce in the amino acid sequence of the corresponding protein.

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**Table 2.** Primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>gyrA</td>
<td>LpGyrAF</td>
<td>5'-AGG CGT GTT CTT TTT GCG AT-3'</td>
</tr>
<tr>
<td>gyrB</td>
<td>LpGyrBR</td>
<td>5'-ATT GCT GGT TTA CCA GGT AAA TT-3'</td>
</tr>
<tr>
<td>parC</td>
<td>LpParCF</td>
<td>5'-GCG TCC GTA TGG AGA GTA ATC AG-3'</td>
</tr>
<tr>
<td>parE</td>
<td>LpParEF</td>
<td>5'-ATA CTT AAT TCA TGG GAA GTA GAT-3'</td>
</tr>
</tbody>
</table>

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Downloaded from https://academic.oup.com/jac/article-abstract/64/2/284/713858 by guest on 03 January 2019
investigated in these additional clones by PCR amplification of the QRDRs and sequencing of the PCR products. In these cases, we used the following pairs of primers: LpGyrAF–LpGyrAR, LpGyrBF–LpGyrBR and LpParCF–LpParCR (Table 2). We were therefore able to reconstitute the order of substitution of the identified mutations in each of the eight lineages. The QRDR of parE was also resequenced.

Reconstruction of resistance alleles in the ancestral strain by gene allele exchange

The direct involvement of topoisomerase mutations in fluoroquinolone resistance was verified by gene allele exchange. The different resistance mutations were introduced, alone and in combination, into the fluoroquinolone-susceptible ancestral genetic background by homologous recombination using a natural transformation procedure. Briefly, a PCR product containing ~500 bp (for gyrA or parC) or ~1100 bp (for gyrB) of adjacent DNA sequences on each side of the mutation was cloned into the pCRII-Topo plasmid using the Topo-TA cloning kit (Invitrogen) and subsequently sequenced to verify the absence of undesired mutations. Mutations within both gyrA 83 and 87 codons were simultaneously PCR-amplified from clones bearing both mutations. All inserts were subsequently recovered from the plasmid backbone by restriction enzyme digestions. The resulting linear fragments were purified and used for natural transformation of the ancestral strain L. pneumophila Paris CIP107629T by serial passages in liquid medium with increasing concentrations of moxifloxacin, until we obtained a moxifloxacin concentration inhibiting growth of 32 mg/L. As shown in Table 3, this was readily obtained for all lineages, after 8 passages for L8, 9 passages for L1, L2, L3 and L6, 10 passages for L5 and L7, and 12 passages for L4. It is important to note here that, at each timepoint of the evolution experiment, the moxifloxacin concentration inhibiting growth we measured was that of the population mixture. The progressive increase in moxifloxacin resistance level is indicative of successive substitutions of resistance mutations. One evolved resistant clone was sampled from each lineage (L1–L8) at the last evolutionary timepoint (16 mg/L moxifloxacin). These clones were called LP1–LP8, respectively.

Characterization of the resistance mechanisms

We sequenced the QRDRs of gyrA, gyrB, parC and parE in each of the LP1–LP8 evolved resistant clones. Three different mutations were found in each clone, except for LP5 where four mutations were present (Table 4). We adopted E. coli numbering to characterize the amino acid position of the relevant protein affected by the gene mutations. A very high level of genetic parallelism was evident in all lineages, leading to the consistent increase in the moxifloxacin MIC. First, in all evolved resistant clones, two mutations were consistently observed: one affecting the second position of codon 83 of gyrA (gyrA83), leading to the replacement of the threonine residue 83 by an isoleucine (T83I), and one affecting the second position of codon 78 of parC (parC78), leading to the replacement of the glycine residue 78 by an aspartic acid (G78D). Second, in evolved resistant clones isolated from four lineages (LP5–LP8), a second mutation in gyrA was also present, except for LP5 where four mutations were found (mutation gyrA83). Third, among the eight evolved resistant clones, five also revealed mutations in gyrB, leading to the amino acid changes S464Y in LP1–LP4 (mutation gyrB464Y) and

Table 3. Lowest concentrations (mg/L) of moxifloxacin inhibiting growth in each evolution lineage at the different evolutionary passages

<table>
<thead>
<tr>
<th>Lineage</th>
<th>Passage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P1</td>
</tr>
<tr>
<td>L1</td>
<td>0.0625</td>
</tr>
<tr>
<td>L2</td>
<td>0.0625</td>
</tr>
<tr>
<td>L3</td>
<td>0.0625</td>
</tr>
<tr>
<td>L4</td>
<td>0.0625</td>
</tr>
<tr>
<td>L5</td>
<td>0.0625</td>
</tr>
<tr>
<td>L6</td>
<td>0.0625</td>
</tr>
<tr>
<td>L7</td>
<td>0.0625</td>
</tr>
<tr>
<td>L8</td>
<td>0.0625</td>
</tr>
</tbody>
</table>

Results

Experimental evolution strategy for selection of fluoroquinolone resistance mutants

Eight independent lineages were propagated from L. pneumophila Paris CIP107629T by serial passages in liquid medium with increasing concentrations of moxifloxacin, until we obtained a moxifloxacin concentration inhibiting growth of 32 mg/L. As shown in Table 3, this was readily obtained for all lineages, after 8 passages for L8, 9 passages for L1, L2, L3 and L6, 10 passages for L5 and L7, and 12 passages for L4. It is important to note here that, at each timepoint of the evolution experiment, the moxifloxacin concentration inhibiting growth we measured was that of the population mixture. The progressive increase in moxifloxacin resistance level is indicative of successive substitutions of resistance mutations. One evolved resistant clone was sampled from each lineage (L1–L8) at the last evolutionary timepoint (16 mg/L moxifloxacin). These clones were called LP1–LP8, respectively.
D426N in LP5 (mutation gyrB<sup>426</sup>). No mutational change was detected in the QDRD of parE.

The eight clones LP1–LP8 were further characterized by measuring their MICs of the fluoroquinolones moxifloxacin, levofloxacin and ciprofloxacin and of other antibiotic classes used for the treatment of Legionnaires’ disease. The MICs of all three fluoroquinolones tested were twice as low for LP1–LP3 (16 mg/L) as compared with clones LP4–LP8 (32 mg/L). While cross-resistance between the fluoroquinolone compounds was clearly evident, no resistance was detected to erythromycin, rifampicin and doxycycline (data not shown). We next characterized the order of substitution of the identified mutations in the QDRDs of gyrA, gyrB and parC in the eight evolving lineages.

**Dynamics of substitution of fluoroquinolone resistance mutations**

Frozen bacterial suspensions, sampled from each of the eight lineages at different passages of the evolution experiment, were plated onto BCYE-α medium and 5–10 resistant clones were isolated from each sample. For all selected clones, the QDRDs of the four genes gyrA, gyrB, parC and parE were PCR-amplified and sequenced to check for the presence of the mutations detected in clones LP1–LP8 and their moxifloxacin MIC was determined (Figure 1). The first mutation substituted (i.e. present in all tested evolved clones from a single timepoint) was the same in all lineages and led to the T83I change in GyrA (gyrA<sup>83</sup> mutation). This amino acid substitution was present in all evolved clones and was detected as early as the fourth passage in all eight lineages. It was always associated with a moxifloxacin MIC of 0.5 mg/L (Table 3), i.e. an 8-fold increase compared with the ancestral strain.

The second mutation affected parC in all lineages and all tested evolved clones. From our analyses of clones LP1–LP8, the mutation was expected to affect codon 78 (parC<sup>78</sup> mutation) and this was indeed the case for six lineages. This mutational event was detected in all tested evolved clones at passages P6 for L1 and L8 and P7 for L3, L5, L6 and L7. In all six cases, the additional mutation was associated with a moxifloxacin MIC of 4 mg/L, fitting very well with the timecourse of increased resistance measured in these six different lineages (Figure 1 and Table 3). Two exceptions were observed, however, for lineages L2 and L4, where a mutation was found in all tested evolved clones in codon 80 of parC (parC<sup>80</sup>), leading to the amino acid change S80R in ParC) at passages P6 for L2 and P8 for L4. They were associated with a moxifloxacin MIC of 2 mg/L, again corresponding well with the timecourse of resistance increase in these two lineages (Figure 1 and Table 3). However, even in those two lineages we found that parC<sup>35</sup> was the substituted mutation at the end of the evolution experiment (see previous section). This may be explained by the higher level of resistance conferred by this last mutation, but it may also imply some transient polymorphism in the L2 and L4 lineages, with different evolved clones from the same evolutionary timepoint bearing different mutations in parC (at codons 78 and 80). We indeed detected this situation (see next section below).

The third substitution was the gyrA<sup>87</sup> mutation for lineages L6–L8 at passages P9, P10 and P8, respectively, leading to the expected final moxifloxacin MIC of 32 mg/L (Figure 1 and Table 3). The five other lineages (L1–L5) experienced substitution of either the gyrB<sup>464</sup> or gyrB<sup>426</sup> mutations at passages P9 for L1, L2, L3 and L5 and P10 for L4 (Figure 1). In all five lineages, the three mutations gyrA<sup>83</sup> + parC<sup>78</sup> + gyrB<sup>464</sup> or gyrB<sup>426</sup> were associated with a moxifloxacin MIC of 16 mg/L (Figure 1), which is consistent only for L4 and L5 with the timecourse of resistance increase (Table 3). The L5 lineage subsequently experienced the additional mutation gyrA<sup>81</sup> at passage P10, leading to the expected final MIC of 32 mg/L (Figure 1 and Table 3). No additional mutation was found in the QDRDs for lineages L1–L4, suggesting that one or several mutations outside the topoisomerase-encoding genes were missed. This is particularly evident in lineage L4 where the moxifloxacin MIC increased from 16 mg/L in 5/5 clones at passage P10 to 32 mg/L.

**Table 4. Mutations in the QDRRs of the gyrA, gyrB and parC genes in the evolved resistant clones LP1–LP8**

<table>
<thead>
<tr>
<th>Strain</th>
<th>gyrA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>gyrB&lt;sup&gt;a&lt;/sup&gt;</th>
<th>parC&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP1</td>
<td>(ACA → ATA) T83I</td>
<td>(TCT → TAT) S464Y</td>
<td>(GAT → GAT) G78D</td>
</tr>
<tr>
<td>LP2</td>
<td>(ACA → ATA) T83I</td>
<td>(TCT → TAT) S464Y</td>
<td>(GAT → GAT) G78D</td>
</tr>
<tr>
<td>LP3</td>
<td>(ACA → ATA) T83I</td>
<td>(TCT → TAT) S464Y</td>
<td>(GAT → GAT) G78D</td>
</tr>
<tr>
<td>LP4</td>
<td>(ACA → ATA) T83I</td>
<td>(TCT → TAT) S464Y</td>
<td>(GAT → GAT) G78D</td>
</tr>
<tr>
<td>LP5</td>
<td>(ACA → ATA) T83I</td>
<td>(GAC → AAC) D426N</td>
<td>(GAT → GAT) G78D</td>
</tr>
<tr>
<td>LP6</td>
<td>(GAC → AAC) D87N</td>
<td>none</td>
<td>(GAT → GAT) G78D</td>
</tr>
<tr>
<td>LP7</td>
<td>(ACA → ATA) T83I</td>
<td>none</td>
<td>(GAT → GAT) G78D</td>
</tr>
<tr>
<td>LP8</td>
<td>(ACA → ATA) T83I</td>
<td>none</td>
<td>(GAT → GAT) G78D</td>
</tr>
</tbody>
</table>

<sup>a</sup>The mutational event is given for each codon and the subsequent amino acid substitution is indicated using *E. coli* coordinates.
Figure 1. Order of substitution of the different mutations in the eight lineages L1–L8 across evolutionary timepoints. Several clones were sampled at different passages (indicated above the arrows). The number of clones with the different mutations out of the number of tested clones at each passage is indicated in parentheses. The moxifloxacin MIC that is shown corresponds to the value measured at each timepoint and for each evolved clone, whereas in Table 3 the moxifloxacin concentration inhibiting growth is given for the population mixture at each timepoint. When a mixture was detected at a single passage (lineages L4, L6 and L7), the subpopulation that will be predominant is shown in bold.
in 5/5 clones at passage P12, despite the fact that no additional mutation was detected in the QRDRs.

**Transient polymorphism during adaptation to increasingly higher moxifloxacin concentrations**

The dynamics of substitution that we observed in lineages L2 and L4 for the parC mutations (see above) suggested that a transient polymorphism may exist at some evolutionary timepoints. Hence, sequencing the QRDRs across evolutionary time revealed that different evolved clones isolated at defined passages carry different resistance mutations, including different changes in the same gene. For example, one-fifth of clones sampled at passage P9 of lineage L4 carried the mutation parC^{80} and co-existed with clones (4/5) carrying the mutation parC^{78}, leading to the transient co-existence of both subpopulations (Figure 1). We confirmed the previously measured moxifloxacin MIC of 2 and 4 mg/L for the two subpopulations carrying parC^{80} and parC^{78}, respectively. The higher MIC conferred by parC^{78} probably explains why at passage P9 the subpopulation carrying parC^{78} was already predominant. This also fits with the concentration of moxifloxacin inhibiting growth measured for the mixed population in lineage L4 at this passage P9, i.e. 4 mg/L (Table 3). The subsequent substitution of the mutation gyrB^{464} probably occurred in the subpopulation bearing parC^{78}, increasing further the resistance level of those clones to 16 mg/L and leading to the complete disappearance of the parC^{80}-carrying clones. Such a transient polymorphism must have been present in lineage L2 but was not detected, probably because we did not test a high enough number of evolved clones at passage P8.

Analyses of evolved resistant clones sampled from the other lineages revealed that a transient polymorphism was common during this evolution towards higher resistance levels. Hence, a polymorphic state was detected in lineage L6 where two types of clones co-existed at passage P8, one (2/5 clones) with mutation gyrA^{87} and one (3/5 clones) with a mutation at the same codon of gyrA but leading to the D87H amino acid change of GyrA (mutation gyrA^{87*}). The first type of clones had a moxifloxacin MIC of 32 mg/L and was subsequently predominant in the lineage, whereas the MIC of the second type of clones was 16 mg/L (Figure 1). This is consistent with the moxifloxacin concentration inhibiting growth measured for the mixed population in lineage L6 from passages P7 to P9 (Table 3). In lineage L7 a mixture of two subpopulations co-existed at passage P8 with either the gyrB^{464} (3/6 clones) or the gyrB^{426} (3/6 clones) mutations that conferred, together with the gyrA^{83} and parC^{78} mutations that were already present, moxifloxacin MICs of 16 and 8 mg/L, respectively (Figure 1). At passage P9 in lineage L7, a third subpopulation of cells carrying the mutations gyrA^{83}, parC^{78} and the additional gyrA^{87*} was detected, which will be the predominant evolved resistant clones at subsequent passages due to their high level of resistance (moxifloxacin MIC of 32 mg/L). Each of these three subpopulations represented one-third of the entire population. This polymorphism in lineage L7 explains the resistance level measured for the mixed population between passages P7 and P10 (Table 3). Therefore, the progressive increase in the moxifloxacin concentration inhibiting growth measured for the mixed populations in the different lineages across evolutionary time is explained by both the successive substitution of resistance-conferring mutations and the transient co-existence of clones bearing different types of alleles.

**Reconstruction of resistance alleles in the moxifloxacin-susceptible ancestral strain by gene allele exchange**

To analyse precisely the level of resistance conferred by each QRDR mutation selected during experimental evolution, we constructed a set of isogenic mutant strains by allelic replacements. We moved mutations, alone and in combination, into the ancestral chromosome, replacing the corresponding moxifloxacin-susceptible alleles. These isogenic strains (Table 1) were then used to examine the effects of the evolved alleles on the moxifloxacin MIC (Figure 2a). Several trends emerged from these data. First, the MIC of the strain bearing the mutation gyrA^{83} was 0.5 mg/L compared with 0.0625 mg/L for the ancestral strain. The combination of the two first substituted mutations (gyrA^{83} + parC^{78}) further increased the MIC to 4 mg/L. The additional introduction of the third mutation that was substituted in lineages L1–L4 (gyrB^{464}) and in lineages L6–L8 (gyrA^{87*}) further increased the MIC to 16 and 32 mg/L, respectively. All these data are consistent with both the order of substitution of the mutations and the associated increase in moxifloxacin resistance levels of the evolving lineages (Figure 1 and Table 3). Thus, the observed mutations in the QRDRs of the topoisomerase-encoding genes account for the increased resistance to moxifloxacin of evolved clones.

Second, whereas the strain bearing the combination gyrA^{83} + parC^{78} increased the MIC to 4 mg/L, the one bearing gyrA^{83} + parC^{80} increased the MIC to only 2 mg/L. Similarly, the isogenic strain containing the three mutations gyrA^{83} + parC^{78} + gyrA^{87} increased the MIC to 32 mg/L, while both isogenic strains containing gyrA^{83} + parC^{78} + gyrA^{87*} or gyrA^{83} + parC^{80} + gyrA^{87} increased the MIC to only 16 mg/L. This is consistent with the final substitution of the parC^{78} and gyrA^{87*} mutations in the different lineages where we detected...
transient polymorphisms, i.e. in L4 at passage P9, L6 at passage P8 and L7 at passage P9.

Third, none of the mutations gyrA<sup>87</sup> and gyrA<sup>87*</sup> further increased the MIC when introduced together with the gyrA<sup>83</sup> mutation in the ancestral genetic background, whereas each one increased it by 8- and 4-fold, respectively, in the ancestral genetic background already containing the combination of gyrA<sup>83</sup> + parC<sup>78</sup> mutations. This strongly suggests that a particular order of mutation substitution is required to reach high levels of fluoroquinolone resistance during adaptation to increasingly higher concentrations of antibiotics. A summary of the order of mutation substitution, the transient polymorphisms and the associated moxifloxacin MIC is given in Figure 2(b).

**Discussion**

By using a strategy of experimental evolution where eight lineages were propagated from a common fluoroquinolone-susceptible ancestral strain of *L. pneumophila* in the presence of increasing concentrations of moxifloxacin, we obtained, very quickly and easily, independent evolved clones with low to high levels of resistance. These clones revealed cross-resistance to other fluoroquinolones (levofloxacin and ciprofloxacin), but not to other antibiotic families (doxycycline, rifampicin and erythromycin). This suggests first that the bacterial response to the moxifloxacin challenge involves resistance mechanisms specific to the fluoroquinolone compounds and second that no efflux mechanism has been selected for. Hence, we identified mutations in the QRDRs of gyrA, gyrB and parC, revealing a high level of genetic parallelism across the independent lineages. By isolating and analysing evolved clones from samples of the independent lineages frozen across evolutionary time, we showed that the increased levels of moxifloxacin resistance involved mutations that were successively substituted within the independent lineages. Moreover, transient polymorphisms were detected within different lineages, with a mixture of clones bearing different mutations. We then moved the mutations, either alone or in combination, into the chromosome of the susceptible ancestral strain. Determination of the moxifloxacin MIC of this set of isogenic strains except for the selected mutations were selectively neutral with respect to moxifloxacin resistance in a genotype with only one mutation substituted.

In vitro selection of *L. pneumophila* resistance to fluoroquinolones has previously been related only to mutations affecting gyrA and leading to the amino acid changes T83K or T83I in GyrA<sup>26,27</sup>. A substitution leading to D87Y has also been reported<sup>27</sup>, but resulted in resistance to only nalidixic acid, whereas fluoroquinolone MICs remained unchanged. This may now be explained in the light of our results showing that changes at codon position 87 enhanced moxifloxacin MIC only if previous mutations in gyrA (codon 83) and parC occurred. We extended these data by detecting mutations in gyrA, gyrB and parC and by showing directly their involvement in the resistance mechanism by construction of isogenic strains. Many of the mutations we describe are common to other bacteria but some affect more specific residues.

The first substituted mutation always affected codon 83 of gyrA. Gyrase has been shown to be the primary target of quinolones in most Gram-negative bacteria<sup>45</sup> and the mutation at codon 83 of gyrA is the most frequent mutation observed in enterobacteria.<sup>5</sup> The second most frequent gyrA mutation in clinical isolates affects codon 87 and we found such a mutation in *Legionella*. Gram-positive bacteria also present mutations in both gyrA codons.<sup>3</sup> We detected a third type of mutation in gyrA, leading to the amino acid change G81A. This position is rarely affected by resistance mutations and has been described only in two cases in *E. coli*, including an in vitro mutant (G81C) and a clinical isolate (G81D).<sup>36</sup>

The second substituted mutation affected either of codons 78 (G78D) or 80 (S80R) of parC. In *E. coli*, the most common substitutions in parC affect codons 80 and 84. However, the substitution leading to G78D has also been found either in clinical isolates or in laboratory experiments.<sup>37,38</sup> We did not isolate *Legionella* resistant clones bearing mutations at codon 84 and we also showed that the mutation at codon 78 conferred higher resistance compared with the one at codon 80.

Further increase in moxifloxacin resistance levels in *L. pneumophila* involved gyrB mutations at codons 426 (D426N) or 464 (S464Y). In *E. coli*, mutations have been described in gyrB, leading to the amino acid changes D426N and K447E in GyrB<sup>39</sup> whereas the S463Y change has been described in *Salmonella typhimurium*.<sup>40</sup> Many other mutations have been detected in gyrA and gyrB.<sup>29-31,41</sup> Equivalent positions are also affected in Gram-positive resistant bacteria.<sup>42</sup> Therefore, we detected some specificity in the resistance profiles of *Legionella*.

At one extreme, one could envisage the paths towards high-level resistance as totally random with any combinations of mutations leading to the maximum level. At the other extreme, high resistance may involve only a small number of specific paths with a particular order of mutations. By our experimental evolution strategy, we showed that mutations were successively substituted first in gyrA, second in parC and third into either gyrA or gyrB. We also showed that the gyrA<sup>87</sup> or gyrA<sup>87*</sup> mutations further increased the resistance level only if they occurred in a genotype already present in both the gyrA<sup>83</sup> and parC mutated alleles, whereas these mutations were selectively neutral with respect to moxifloxacin resistance in a genotype with only gyrA<sup>83</sup>. Therefore, these mutations only conditionally increase the resistance level, a phenomenon known as sign epistasis.<sup>43</sup> Therefore, Darwinian selection acts to increase fluoroquinolone resistance through only a fraction of all possible mutational pathways, owing to intermolecular interactions between DNA gyrase and topoisomerase IV. Intramolecular interactions in a β-lactamase-encoding gene leading to specific mutational trajectories to increase the resistance towards cefotaxime have similarly been detected.<sup>44</sup> Stepwise increases in fluoroquinolone resistance have also been shown to occur by successive mutations in the QRDRs of gyrA and parC in *E. coli* and in *H. influenzae*.<sup>32,33</sup> However, no isogenic strains have been constructed in these two studies and it cannot therefore be completely excluded that genetic background differences between strains may affect MICs in addition to the resistance mutations. A recent study used genetic allele exchange to reconstruct isogenic *E. coli* strains bearing different alleles of gyrA and parC either alone or in combination.<sup>28</sup> Some mutations have been shown to be contingent on other alleles to increase the resistance level to fluoroquinolones. However, no precise mutation order has been experimentally demonstrated as in our evolution strategy.

Current treatment recommendations for legionellosis are mainly based on clinical experience.<sup>45</sup> with macrolides and fluoroquinolones being first-line drugs. However, treatment failures have been
reported in treated patients. Also, a treatment duration of 2–3 weeks is advocated to prevent relapses. These data may indicate a potential antibiotic resistance opportunity in vivo. Legionella spp. are susceptible to macrolide and fluoroquinolone compounds either in axenic medium, in cell systems or in animal models. Acquired resistance in clinical strains of L. pneumophila has not yet been described. However, it should be emphasized that the isolation of L. pneumophila remains difficult and the cultures may be negative in patients who have received previous antibiotic therapy despite persistence of the infection. We demonstrated that in vitro selection of fluoroquinolone-resistant mutants occurred rapidly, with an 8-fold increase in moxifloxacin MIC after only three passages (Table 3). A 512-fold increase in moxifloxacin MIC could be obtained. A mutation rate to fluoroquinolone resistance of $10^{-8}$ has been reported in L. pneumophila and bacterial loads $>10^6$ cfu/mL of sputum sample occur in infected patients. Thus, the possibility of in vivo selection of resistant mutants in legionellosis patients receiving a fluoroquinolone compound should be considered. On the other hand, Legionella spp. are able to colonize environmental water systems. In these environments they may be exposed to antibiotics, especially those that are naturally secreted by other microorganisms or residual concentrations of antibiotics used in medical or veterinary practice. It has been shown that the presence of antibiotics in the environment may promote the evolution of resistance mechanisms. In another independent study, bacteria sampled from soil revealed mutations in the QRDR of gyrA even without apparent selection. Thus, environmental exposure to fluoroquinolones is another potential mode of selection of resistance in Legionella species.

In conclusion, the population dynamics that we detected in experimentally evolving lineages of L. pneumophila demonstrates that fluoroquinolone resistance in this pathogen involves mutational paths in type II topoisomerase-encoding genes. GyrA is the primary fluoroquinolone target in L. pneumophila. Once gyrA mutation reduces the susceptibility of DNA gyrase towards fluoroquinolones, topoisomerase IV and, especially, ParC start to represent a target for the antibiotics. Following decreased susceptibility of topoisomerase IV to fluoroquinolone because of parC mutation, DNA gyrase is again a suitable target with mutations either in gyrA or gyrB. Substitutions in L. pneumophila topoisomerase-encoding genes were very easily obtained in vitro, leading to high-level resistance to fluoroquinolones. Our data warrant further investigation of the presence of acquired fluoroquinolone resistance in clinical or environmental strains of L. pneumophila.

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Transparency declarations

None to declare.

References

Mutational paths towards fluoroquinolone resistance


