Phenotypic and genetic characterization of macrolide resistance in Francisella tularensis subsp. holarctica biovar I

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Objectives: Francisella tularensis subsp. holarctica strains are classified as biovars I and II, which are susceptible and naturally resistant to the macrolide erythromycin, respectively. The present study was aimed at both selecting biovar I strains with increased levels of erythromycin resistance and characterizing the underlying genetic mechanisms.

Methods: Serial cultures in the presence of increasingly high erythromycin concentrations were performed to select independent high- and intermediate-level erythromycin-resistant mutants from each of three different biovar I strains. The mutants were characterized for cross-resistance to several antibiotics, presence of mutations in the genes encoding the 23S rRNA and the L4 and L22 ribosomal proteins, and overexpression of efflux pumps.

Results: Mutants displayed cross-resistance to all macrolide compounds tested but not to other classes of antibiotics. We found mutations in domain V of the 23S rRNA gene (G2057A, A2058G, A2058T and C2611T) and in the gene encoding L22, leading to either the G91D substitution or the M82K83R84 deletion. Analysis of mutants with intermediate resistance levels obtained over the course of the selection process revealed both a positive correlation between the number of mutated ribosomal operons and the resistance level, and an additional resistance mechanism in the early steps of selection.

Conclusions: We showed that high-level resistance to macrolides can be easily obtained in vitro in F. tularensis subsp. holarctica biovar I strains, thereby suggesting that in vivo selection for resistance may explain reported failures of antibiotic treatment. Ketolides were the most effective macrolides tested, which may limit the risk of selection for resistance.

Keywords: tularemia, treatment, resistance mechanisms, mutations, evolution

Introduction

Francisella tularensis, a Gram-negative facultative intracellular bacterium, is the aetiologcal agent of tularemia and a class A biological weapons agent according to the US CDC.1 Human infections are mainly caused by F. tularensis subsp. holarctica (type B) strains in the northern hemisphere, but also by F. tularensis subsp. tularensis (type A) strains in North America.2,3 Contamination may occur from direct contacts with infected animals (especially lagomorphs), via arthropod bites (primarily ticks of the family Ixodidae) or by exposure to contaminated environments (water, soils). Tularemia is transmissible to humans at infectious doses as low as 10 bacteria by aerosol inhalation or skin inoculation. The clinical manifestations of tularemia vary depending on the particular mode of infection and six major forms are recognized: ulceroglandular and glandular (skin inoculation), oculoglandular (conjunctival inoculation), oropharyngeal (digestive route), pneumatic (respiratory tract or haematogenous) and typhoidal (severe sepsis often with undetermined portal of entry of bacteria).2,3 Tularemia is usually much more severe when associated with type A strains, although recent data suggest that their virulence traits vary according to genotype.3,4 Aminoglycosides (streptomycin and gentamicin), fluoroquinolones and tetracyclines are considered the mainstays of the antibiotic treatment of tularemia, whereas β-lactams are ineffective.5–7 Although anecdotal reports indicate that clinical successes can be obtained with macrolide compounds,8–10 these antibiotics are not considered to be reliable in tularemia treatment.
patients, partly because they display only a bacteriostatic activity against *F. tularensis* in both axenic medium and cell systems.\(^{11}\) In addition, type B strains from Central and Eastern Europe and Asia (i.e. biovar II) are naturally resistant to erythromycin and other macrolides,\(^{12}\) including the virulence-attenuated live vaccine strain (LVS), which was obtained from a type B biovar II strain,\(^{13}\) although the underlying genetic mechanism has not been experimentally determined. However, another possibility to explain treatment failures could be related to acquired resistance to macrolides, although it has not yet been reported in *F. tularensis*. On the other hand, since the newly available ketolide compounds (telithromycin and cethromycin) display improved *in vitro* activity against *F. tularensis* compared with other macrolides,\(^{11,14}\) it would be useful to determine this bacterium's ability to become resistant to these compounds as well. Here we developed an *in vitro* selection procedure to select for erythromycin resistance as a model study in *F. tularensis* subsp. *holarctica* biovar I strains and determined the underlying resistance mechanisms compared with that naturally found in biovar II strains.

### Materials and methods

#### Bacterial strains and culture media

Seven French biovar I strains of *F. tularensis* subsp. *holarctica* were used in the present study, including four strains isolated from hares (Ft1, Ft2, Ft3, Ft4) and three human strains isolated from either blood samples (Ft5, Ft6) or a conjunctival swab (Ft7). We also used the reference LVS strain (NCTC 10857) kindly provided by the Emile Pardé Research Centre (CRSSA, Grenoble, France). The animal and human strains were identified at the species and subspecies levels by PCR amplification and sequencing of the 16S rRNA and 23S RNA-encoding genes, using previously described primers.\(^{15}\)

All strains were grown on chocolate agar plates supplemented with PolyVitex (CPV, Biomérieux, Lyon, France) at 37°C in a 5% CO\(_2\)-enriched atmosphere. Liquid cultures for the selection of increased erythromycin resistance were grown in Mueller-Hinton broth (AES Laboratory, Combourg, France) supplemented with 2% PolyVitex (MHPV). All cultures were performed in a biosafety level 3 laboratory using appropriate safety precautions. The strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as controls for MIC determination.

#### Antibiotics

The following antibiotics were tested: amoxicillin (GlaxoSmithKline, Mary-le-Roi, France), ceftriaxone (Sandoz, Levallois-Perret, France), streptomycin (Panpharma, Fougères, France), gentamicin (Dokata Pharm, Le Plessis Robinson, France), doxycycline (Sigma Aldrich, Saint-Quentin-Fallavier, France), rifampicin (Sanofi-Aventis, Paris, France), co-trimoxazole (trimethoprim and sulfamethoxazole; Roche, Neuilly-sur-Seine, France), ciprofloxacin (Bayer, Puteaux, France), and the macrolide compounds erythromycin (Sigma-Aldrich), dirithromycin (Sigma Aldrich), roxithromycin (Sigma Aldrich), azithromycin (Pfizer, Paris, France), josamycin (Sigma Aldrich), telithromycin (Sanofi-Aventis), spiramycin (Sigma Aldrich), lincomycin (Sigma-Aldrich), pristinamycin (Sanofi-Aventis) and quinupristin/dalfopristin (Sanofi-Aventis). Stock solutions were dissolved as recommended by the manufacturers and stored at −80°C until use.

#### Selection of erythromycin-resistant mutants in *F. tularensis* subsp. *holarctica*

Two independent lineages were founded from each of the three human strains of *F. tularensis* subsp. *holarctica* (L1 and L2 from Ft5, L3 and L4 from Ft6, L5 and L6 from Ft7) and propagated by serial passages in culture medium containing increasing concentrations of erythromycin. Each strain was first grown on CPV plates and incubated in a 5% CO\(_2\) atmosphere for 2 days at 37°C. A few colonies were harvested to prepare a bacterial suspension of \(\sim 10^9\) cfu/mL in MHPV broth, which was dispensed in 24-well microtitre plates at 1.8 mL per well. Erythromycin (0.2 mL per well) was added to obtain final 2-fold serial concentrations ranging from 0.5- to 16-fold the MIC determined for the parental strains (1-2 mg/L). After 4—5 days of incubation at 37°C, the minimum concentration of erythromycin inhibiting bacterial growth was recorded. Bacterial cells in the wells with the highest erythromycin concentration allowing growth were serially transferred, after a 1:20 dilution, into new 24-well microtitre plates containing fresh medium, again with 2-fold serial concentrations of erythromycin ranging from 0.5- to 16-fold the erythromycin MIC of the previous selection step. Ten such selection passages were performed for the six independent lineages, for a total of 43 bacterial generations (assuming that at each cycle the 20-fold dilution and regrowth allowed log\(_2\), \(20 = 4.3\) bacterial generations). To avoid any contamination, replicate lineages for each of the three strains were propagated in separate microtitre plates. At each passage, mixed samples of each of the six lineages were frozen at −80°C as glycerol suspensions. From each sample, individual clones could be isolated after streaking on CPV plates. We isolated one high-level erythromycin-resistant mutant from each of the six independent lineages after 10 passages. Erythromycin resistance was found to be stable in all six high-level resistant mutants even after 12 subsequent subcultures in antibiotic-free medium.

#### MIC determination for reference Francisella sp. strains and erythromycin-resistant mutants

MICs were determined using a microdilution technique in MHPV broth with \(\sim 10^8\) cfu/mL of bacterial suspension, as recommended by the CLSI.\(^{16}\) Each bacterial suspension was dispensed in the wells of 96-well microtitre plates (180 \(\mu\)L per well) at 10 times the desired final concentrations. Plates were incubated at 37°C in a 5% CO\(_2\) atmosphere and MICs were read after 48 h of incubation, defined as the lowest antibiotic concentration that completely inhibited growth. The MICs were determined for three sets of samples: the mixed populations isolated after each of 10 passages from each of the six independent lineages, the seven reference strains of *F. tularensis* subsp. *holarctica* biovar I, including the three human strains that were used to propagate the six lineages, and individual erythromycin-resistant mutant clones that were isolated from the six lineages. Experiments were repeated twice to confirm the results. For *E. coli* ATCC 25922 and *S. aureus* ATCC 25923, MICs were determined using the same procedure except for the incubation time (18 h). They were within the expected range.

#### PCR amplification and sequencing of the genes encoding the 23S rRNA and the L4 and L22 ribosomal proteins

Bacterial suspensions were prepared by harvesting a single colony from each mutant strain in 1 mL of sterile distilled water. Bacterial cells were disrupted by heating at 90°C for 1 h. Genomic DNA was extracted using the QIamp DNA mini kit (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions. The *rlf, rplD* and *rplV* genes encoding the 23S *F. tularensis* RNA and the L4 and L22 ribosomal proteins, respectively, were PCR-amplified from the genomic DNA of all three reference *F. tularensis* human strains and the derived erythromycin-resistant mutants using the primers (MWG Biotech, Roissy, France) described in Table 1. The same nine primer pairs were used to amplify the entire *rlf* gene in each of the three RNA operons of the *F. tularensis* genome. Two primer pairs were used for *rplD* and one for *rplV*. Since we found mutations in
the 3′ region of rrl, we also designed three additional primer pairs for specific amplification and sequencing of that region for each of the three 23S rRNA-encoding genes, rrl1, rrl2 and rrl3 (Table 1), in order to determine the number of mutant alleles in erythromycin-resistant mutants. All primers were designed in conserved regions by aligning the sequences of the rrl, rplD and rplV genes retrieved from the available genome sequences of the four strains F. tularensis subsp. holarctica OSU18, LVS, F. tularensis subsp. novicida U112 and F. tularensis subsp. tularensis Schu4.17,18 The specificity of all primers was checked using the BLAST alignment software (National Center for Biotechnology Information, Bethesda, MD, USA).

All PCRs were performed using the Pwo Master Kit (Roche Diagnostics, Meylan, France) according to the manufacturer’s instructions. Amplification reactions were carried out in 50 μL volumes containing 0.4 μM of each of the reverse and forward primers and 5 μL of genomic DNA. The PCRs were initiated by denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, before a final elongation step at 72 °C for 5 min. The PCR products were verified by agarose gel electrophoresis followed by ethidium bromide staining and were then purified using Sephadex-containing Millipore MAH N45 plates (Sigma Aldrich, Saint-Quentin-Fallavier, France). DNA sequencing was performed on a CEQ2000XL apparatus using the GenomeLab DTCs-Quick Start Kit with a CEQ2000 Dye Terminating cycle sequencing protocol (Beckman Coulter, Roissy, France). Sequence analyses were performed using the CEQ2000XL DNA analysis system (Beckman Coulter).

**Evaluation of efflux pump overexpression in erythromycin-resistant mutants**

Erythromycin MICs were determined for resistant mutants in the presence or absence of efflux pump inhibitors (Sigma Aldrich), including verapamil (25 μM), carbonyl cyanide 3-chlorophenylhydrazone (CCCP1, 100 μM; CCCP2, 50 μM), sodium orthovanadate (50 μM), Phe-Arg-β-naphthylamide dihydrochloride (PAAβN1, 38.5 μM; PAAβN2, 77 μM) and 1-naphthylmethyl-piperazine (1-NMP, 88 μM). Triplicate assays were performed for all experiments.

### Results

**Selection of erythromycin resistance in F. tularensis subsp. holarctica**

Two independent lineages from each of the three F. tularensis subsp. holarctica biovar I human strains Ft5, Ft6 and Ft7 were propagated by serial passages in liquid medium with increasing concentrations of erythromycin. For each passage, up to 10, we measured the lowest erythromycin concentration inhibiting growth for the mixed population (therefore equivalent to the MIC) in each of the six lineages [Table S1, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)]. We detected a progressive increase in erythromycin MICs, up to 2048–4096 mg/L, suggesting the appearance and substitution within the lineages of successive erythromycin resistance mutations. Small differences between the two replicate lineages founded from the same ancestral strain probably denote either that erythromycin growth-inhibiting concentrations were measured on mixed populations or that distinct evolutionary paths towards an increased resistance level may exist. However, the two replicate lineages propagated from each individual ancestral strain reached the same high level of resistance. After 10 passages, we sampled one high-level erythromycin-resistant mutant from each lineage, corresponding to a total of six independent mutants, called RCL1 to RCL6, for resistant clone lineages 1 to 6.

**MIC determination for reference strains and erythromycin-resistant mutants**

For the hare and human reference strains (Table 2), we determined the MICs of several macrolides (erythromycin, dirithromycin, roxithromycin, azithromycin, josamycin, telithromycin, spiramycin, lincomycin, pristinamycin and quinupristin/dalfopristin) and other antibiotics from different families:

### Table 1. Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Targeted gene</th>
<th>Forward primer (5′–3″)</th>
<th>Reverse primer (5′–3″)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rrl (3 copies)</td>
<td>(1) TCGTGTAGTTCAAGTCTACTAG (1) CGATTTCAAGTCTACGACCA</td>
<td>(1) CCATATTCAAGACAGTCTCCAC (1) CAGTATTGGTGTCGTCCTC</td>
</tr>
<tr>
<td></td>
<td>(2) GTGAGAAGGCGGCTGGTAAGA (2) CGGTTAGGGAGTACGAAGGTG</td>
<td>(2) CCATATTCAGACAAGGTTCCAC (2) ACCCCAGGCTATCAACGGTC</td>
</tr>
<tr>
<td></td>
<td>(3) TGATACAACAGTAGGAGCA (3) GTGAAATTGAAATCGCTGTG</td>
<td>(3) CCATATTCAGACAAGGTTCCAC (3) ACCCCAGGCTATCAACGGTC</td>
</tr>
<tr>
<td></td>
<td>(4) CTGGTGAGTGGAGTAGAAGG (4) CGGTTAGGGAGTACGAAGGTG</td>
<td>(4) CCATATTCAGACAAGGTTCCAC (4) ACCCCAGGCTATCAACGGTC</td>
</tr>
<tr>
<td></td>
<td>(5) CGCAAGTGTGATCACAGTAGGA (5) GTGAAATTGAAATCGCTGTG</td>
<td>(5) CCATATTCAGACAAGGTTCCAC (5) ACCCCAGGCTATCAACGGTC</td>
</tr>
<tr>
<td></td>
<td>(6) CCGCCGGTGCTGGAAAGATTA (6) CGGTTAGGGAGTACGAAGGTG</td>
<td>(6) CCATATTCAGACAAGGTTCCAC (6) ACCCCAGGCTATCAACGGTC</td>
</tr>
<tr>
<td></td>
<td>(7) GTGAAATTGAAATCGCTGTG (7) GTGAAATTGAAATCGCTGTG</td>
<td>(7) CCATATTCAGACAAGGTTCCAC (7) ACCCCAGGCTATCAACGGTC</td>
</tr>
<tr>
<td></td>
<td>(8) AACGGAGAGTGAGGAGTAGG (8) CCAGTCAAACTACCCACCAT</td>
<td>(8) CCATATTCAGACAAGGTTCCAC (8) ACCCCAGGCTATCAACGGTC</td>
</tr>
<tr>
<td></td>
<td>(9) GCCGTAGAGATTTGAGAGAGA (9) GCCCAATTCACTTTCCCT</td>
<td>(9) GCCCAATTCACTTTCCCT</td>
</tr>
</tbody>
</table>

* Nine primer pairs (numbered 1 to 9) were used for PCR amplification of the three copies of rrl, one for a specific region overlapping each of rrl1, rrl2 and rrl3, two for rplD (numbered 1 and 2) and one for rplV.
tetracyclines (doxycycline), rifamycins (rifampicin), fluoroquinolones (ciprofloxacin), aminoglycosides (gentamicin and streptomycin), sulphonamides (trimethoprim and sulfamethoxazole) and β-lactams (amoxicillin and ceftriaxone).

Reference strains, of either hare or human origin, revealed similar MICs of all non-macrolide antibiotics (Table 2), including full resistance to amoxicillin, with between-strain variation ranging from only 1- to 4-fold (except for an 8-fold difference for streptomycin and rifampicin). The same trend was observed for the macrolide MICs, with high-level resistance to lincomycin, whereas telithromycin was the most active compound. As expected, the LVS strain, used as control, displayed high-level resistance to all macrolides tested, with no difference, however, for the other antibiotics compared with the seven reference strains (Table 2). The three reference human strains, Ft5, Ft6 and Ft7, from which erythromycin resistance mutants were selected had erythromycin MICs of 2, 4 and 2 mg/L, respectively (Table 2). Their six derived erythromycin-resistant selected clones, RCL1 to RCL6, displayed high-level MICs of erythromycin with 512- to 2048-fold increases compared with their respective parental strains (Table S2, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)). All MICs for these individual clones fitted very well with those determined for the mixed populations (Table S1). The small 2-fold differences between the selected resistance mutants (RCL3, RCL5 and RCL6) and their respective lineages (L3, L5 and L6) likely reflect the fact that the MICs were measured for individual clones (Table S2) and mixed populations (Table S1). Parallel phenotypic resistance changes were observed for the two replicate lineages that were propagated from each of the three reference human strains, with the erythromycin MIC being similar for the two replicate lineages. Moreover, cross-resistance to all macrolide compounds tested was detected, with again similar related changes in each set of two replicate lineages, except for the resistance level to both telithromycin and quinupristin/dalfopristin in the two RCL1 and RCL2 mutants (Table S2). These differences may indicate that the mutational changes selected on erythromycin-containing medium in the two Ft5-derived lineages have differential effects on resistance to telithromycin and quinupristin/dalfopristin. The six erythromycin-selected mutants showed no cross-resistance to other antibiotic classes, including β-lactams, aminoglycosides, tetracyclines, fluoroquinolones, and...
rifamycins and sulphonamides (Table S2), suggesting genetic mechanisms specific to macrolide resistance. We therefore focused on characterizing the \textit{rrl}, \textit{rplD} and \textit{rplV} genes, encoding the 23S rRNA and L4 and L22 ribosomal proteins, respectively, in the RCL1 to RCL6 mutants compared with their respective parental strains. These genes have been shown to be targets of macrolides.\textsuperscript{19–21}

**PCR amplification and sequencing of the \textit{rrl}, \textit{rplD} and \textit{rplV} genes**

We determined the DNA sequence of \textit{rrl}, \textit{rplD} and \textit{rplV} in the seven natural strains of \textit{F. tularensis} subsp. \textit{holarctica} and in the high-level resistant mutants sampled after 10 passages from the six independent lineages founded from the Ft5, Ft6 or Ft7 human strains. For comparison purposes, all mutation positions will be given according to the \textit{E. coli} corresponding coordinates.

All seven hare and human strains revealed identical DNA sequences for the three genes similar to the \textit{F. tularensis} subsp. \textit{holarctica} OSU18 strain sequences.\textsuperscript{18} The LVS strain also revealed the same sequences, except for the A2059C mutation in \textit{rrl}.

In five mutants (RCL1 to RCL3, RCL5 and RCL6), we found a single mutation in domain V of \textit{rrl} (Table 3), whereas no mutation was found in domain II. Four mutant alleles were found: G2057A in RCL3, A2058T in RCL1, A2058G in RCL6 and C2611T in RCL2 and RCL5. By using three sets of primer pairs specific to each of the three \textit{Francisella} ribosomal operons (Table 1), we further showed that each mutation affected one (in RCL1), two (in RCL6) or all ribosomal operons (in RCL2, RCL3 and RCL5). In addition, we found a point mutation and a small in-frame deletion of nine bp (ATGAAGCGT) in \textit{rplV} in the RCL1 and RCL4 clones, respectively, leading to the amino-acid G91D substitution in the first case and to the deletion of M82K83R84 in the second case in L22. No mutation was found in \textit{rplD}.

To both determine the dynamics of substitutions of all detected mutations in the six propagated lineages and tentatively correlate these mutations with the MIC levels, we sampled one intermediate-level resistant mutant from each lineage at each of two earlier passages where significant increases of the erythromycin MICs had been observed. We determined the erythromycin MICs for all these intermediate clones (Table 3). Compared with the values obtained for the mixed populations (Table S1), differences were observed but were never larger than 2-fold. Of the seven mutations found in

### Table 3. Mutations detected in erythromycin-resistant mutants sampled at different passages during propagation with increasing erythromycin concentrations of the three human strains of \textit{F. tularensis} subsp. \textit{holarctica}

<table>
<thead>
<tr>
<th>Lineage</th>
<th>Passage</th>
<th>Erythromycin MIC (mg/L)\textsuperscript{b}</th>
<th>Mutational change in \textit{rrl} (number of mutated operons)</th>
<th>Mutational change in \textit{rplV}c</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>P10</td>
<td>4096</td>
<td>A2058T (1)</td>
<td>G91D</td>
</tr>
<tr>
<td></td>
<td>P7</td>
<td>256</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>16</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>L2</td>
<td>P10</td>
<td>4096</td>
<td>C2611T (3)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>P5</td>
<td>128</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>L3</td>
<td>P10</td>
<td>4096</td>
<td>G2057A (3)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>P8</td>
<td>512</td>
<td>G2057A (1)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>P5</td>
<td>32</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>L4</td>
<td>P10</td>
<td>2048</td>
<td>—</td>
<td>M82K83R84</td>
</tr>
<tr>
<td></td>
<td>P8</td>
<td>64</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>P4</td>
<td>8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>L5</td>
<td>P10</td>
<td>2048</td>
<td>C2611T (3)</td>
<td>—</td>
</tr>
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<td></td>
<td>P8</td>
<td>64</td>
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<td>—</td>
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<tr>
<td></td>
<td>P5</td>
<td>8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>L6</td>
<td>P10</td>
<td>2048</td>
<td>A2058G (2)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>P5</td>
<td>64</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>8</td>
<td>—</td>
<td>—</td>
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</tbody>
</table>

\textsuperscript{a}For passage P10, all MICs correspond to the high-level resistant clones RCL1 to RCL6.

\textsuperscript{b}The erythromycin MICs were determined here for individual resistant clones sampled from the six independent lineages at each of three different passages, compared with minimal erythromycin concentrations that inhibited growth of the mixed populations (Table S1).

\textsuperscript{c}The change in the protein sequence is given.
the six resistant clones sampled after 10 passages, only two were detected in earlier samples; the mutation leading to the G91D change in L22 in lineage L1 after 7 passages and the G2057A mutation in rrl in L3 after 8 passages, although in only one ribosomal operon instead of three after 10 passages. Three conclusions can be derived from these results: (i) the rplV mutation in L1 and the mutation in one copy of rrl in L3, either alone or in combination with other yet unknown mutations, are involved in the increases of the MIC to 256 mg/L after seven passages for lineage L1 and to 512 mg/L after eight passages for L3, respectively; (ii) the additional A2058T mutation in lineage L1 is responsible, potentially with other unknown mutations, for the MIC increase from 256 to 4096 mg/L, while the G2057A mutations in each of the two additional rrl copies in lineage L3 were responsible for the MIC increase from 512 to 4096 mg/L; and (iii) since no mutation was detected whatsoever during the early passages, corresponding to MICs less than 256 mg/L, the three rrl, rplD and rplV genes were not the first targets of selection for low levels of erythromycin resistance. Therefore, other mutational targets must exist, for example genes encoding efflux pumps.

**Evaluation of efflux pump overexpression in erythromycin-resistant mutants**

Five different types of efflux pump inhibitors (EPIs) were used to evaluate efflux pump overexpression in all the high- and intermediate-level erythromycin-resistant mutants that we selected during this study. Although the mechanism of action of most EPIs is not fully characterized, two main mechanisms may be distinguished: (i) blocking of the efflux of antibiotics in a competitive or non-competitive manner by direct action within the pump’s cavities (e.g. PAβN); and (ii) alteration of efflux pump function by depletion of energy, either by inhibiting ATP-binding or by disturbing the proton-motive force (e.g. CCCP, verapamil).22 The tested EPIs displayed no bacteriostatic effect against both the reference and mutant strains of *F. tularensis* subsp. *holarctica* at the concentrations used. None of the EPIs significantly reduced the erythromycin MICs for the resistant mutants (data not shown), thereby potentially eliminating the corresponding efflux pumps as targets of selection for increased erythromycin resistance under the conditions used.

**Discussion**

As suggested by Kudelina and Olsufiev,12 *F. tularensis* subsp. *holarctica* was subdivided into biovar I and II strains, which are, respectively, naturally susceptible and resistant to erythromycin. This dichotomy was later confirmed, with reports of either susceptibility or resistance to macrolides in this subspecies,23–26 sometimes in a single area.12,27 We further characterized the activity of a large panel of macrolide compounds, together with antibiotics from other families, against seven *F. tularensis* subsp. *holarctica* biovar I strains collected from hares and humans in France during the last 4 years. The seven human and hare strains revealed low MICs of most macrolides tested, except for lincomycin, the 16-membered ring spiramycin and pristinamycin, for which intermediate levels of resistance were detected. The ketolide telithromycin was the most effective macrolide. The MICs of the macrolides were close to maximum serum levels achievable in human sera, which may limit their clinical usefulness. However, it should be noted that *F. tularensis* is a facultative intracellular bacterium that multiplies within macrophages, and the macrolides can concentrate within these eukaryotic cells.28 In *in vitro* studies, we have shown that the macrolides are active against *F. tularensis* grown in macrophages.11,29 As a positive control, we confirmed that the LVS strain, a biovar II strain, displayed high-level MICs of macrolides that have been suggested to be related to the A2059C change in rrl,13 as reported for other bacterial species,19 although this has not been confirmed experimentally. The seven strains were all susceptible to aminoglycosides (streptomycin and gentamicin), doxycycline, ciprofloxacin and rifampicin, with MIC values close to those reported in previous studies,11,13,23–26,30 but were resistant to amoxicillin and displayed intermediate-level MICs of the third-generation cephalosporin ceftriaxone. Previous studies have reported variability in the activity of third-generation cephalosporins, including among strains collected in the same area.26

To select for erythromycin-resistant mutants, we propagated in *in vitro* independent lineages of the three human strains with increasing concentrations of erythromycin. (Natural *F. tularensis* biovar I strains with acquired macrolide resistance are not available.) High-level erythromycin-resistant mutants (MICs of 2048–4096 mg/L, i.e. 512- to 2048-fold the MIC for the parental strains) were easily obtained after only 10 growth passages. Cross-resistance was observed for all other macrolide compounds tested but not for other antibiotic classes. Acquired resistance to macrolides has been characterized in many bacterial species.19–21 In most cases, it involves modifications of the cellular targets, including the peptidyltransferase region of the bacterial ribosome and the ribosomal proteins L4 and L22, which are involved in reconstitution of the peptidyltransferase activity of the 50S ribosomal subunit. Mutations in *rlf* genes encoding the 23S rRNA (in particular in domains II and V at nucleotide positions 2058 and 2059 and to a lesser extent 754, 2032, 2057, 2611 and 2452) and post-transcriptional methylation of the 23S rRNA at nucleotide positions 2058 and 2059 disrupt the interaction site between macrolides and ribosomes, thereby preventing inhibition of protein synthesis. Only mutations or methylation at positions 2058 and 2059 are associated with high-level resistance to macrolides. Mutations in *rrl* usually predominate in bacterial species with few ribosomal operons (one to four), whereas 23S rRNA methylation is more common in species with more operons.19 Other resistance mechanisms include efflux pump systems and, to a lesser extent, enzymatic inactivation of the drugs.20,31

Strong phenotypic and genetic parallel evolution towards increased erythromycin resistance was observed in all six independent high-level erythromycin-resistant mutants selected after 10 passages, with similar high levels of achieved resistance and mutations in either *rlf* or *rplV*. However, strong allelic divergence was detected with no unique mutant allele present in more than one independent lineage, suggesting different evolutionary paths even for the two lineages propagated from each of the three parental strains. This therefore reflects the many ways bacterial cells possess to increase their resistance levels. A single mutation in *rlf* was found in five mutants, including G2057A, A2058G, A2058T and C2611T changes. A G2057A
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mutation has previously been described in *E. coli*, *Mycoplasma hominis* and *Propionibacterium* sp. It is usually responsible for low-level resistance to macrolide compounds with a 14-membered lactone ring such as erythromycin, whereas 16-membered lactone ring macrolides (spiramycin, josamycin) and synergistins remain active. In the RCL3 mutant bearing this mutation, however, we observed high-level resistance to both erythromycin and the quinupristin/dalfopristin combination. A C2611T mutation has been described previously in *E. coli* and *Streptococcus pneumoniae*. The C2611T mutation found in RCL2 and RCL5 was associated with the same phenotypic resistance to macrolide compounds as the G2057A mutation in RCL3 and also affected the three rrl genes. The A2058G and A2058T mutations have been described in many pathogenic bacteria, including *E. coli*, *Helicobacter pylori*, *Campylobacter* sp., *Mycobacterium* sp. and *S. pneumoniae*. The adenine at position 2058 is considered to be the key nucleotide involved in the interaction between the 23S rRNA and macrolides, and mutations at that position usually confer high-level resistance to all macrolides, lincosamides and synergistin B (MLSB phenotype). Interestingly, we did not identify mutations at position 2059, especially the position usually confer high-level resistance to all macrolides, lincosamides and synergistin B. The A2059C mutation, which is naturally present in biovar II strains, was not identified. Between the 23S rRNA and macrolides, and mutations at that position usually confer high-level resistance to all macrolides, lincosamides and synergistin B (MLSB phenotype). A multidrug efflux pump of the resistance nodulation division family has been described in the *F. tularensis* LVS strain, analogous to the AcrAB-ToIC system involved in erythromycin resistance in *E. coli*. However, acrB, tolC and rfcL VLS deletion mutants resulted in increased susceptibility to β-lactams, rifampicin and tetracyclines, but not to aminoglycosides, fluoroquinolones and erythromycin. Moreover, the genome of *F. tularensis* subsp. *tularensis* strain Schu4 encodes 15 functional ATP-binding cassette transporter systems, of which two may be involved in drug resistance. However, based on two lines of evidence, we most likely ruled out the involvement of mutations overexpressing efflux pumps in the resistant mutants that we selected; we detected no cross-resistance to antibiotic families other than macrolides and no reduction of the erythromycin MICs after addition of different EPIs for both high- and intermediate-level resistant mutants. As an alternative possibility, changes in post-transcriptional 23S rRNA methylation may be involved in increased erythromycin resistance. Methylenes encoded by the erm genes in *Staphylococcus* and *Streptococcus* species are the best-known enzymes responsible for erythromycin resistance, but no erm-like genes are currently described in the available *F. tularensis* genomes.

Macrolides are currently not recommended for the treatment of tularemia because of frequent failures and relapses upon antibiotic withdrawal. This may be partly related to natural resistance in *F. tularensis* subsp. *holarctica* biovar II strains. Moreover, we showed here that selection of high-level erythromycin resistance is very easy to obtain in naturally susceptible biovar I strains. Telithromycin was the most effective macrolide compound against not only the natural biovar I strains, but also the erythromycin-resistant mutants. Previous studies have shown that the ketolide compounds telithromycin and cethromycin display higher activity than erythromycin against *F. tularensis* in both axenic medium and eukaryotic cells. compared with other macrolides, these compounds have a number of advantages, including a higher affinity for the 50S ribosomal subunit, a lower risk of selection for macrolide resistance and better stability at acidic pH, which may partly explain the better activity in the intracellular environment of phagocytic cells where *F. tularensis* multiplies. Thus, ketolides potentially represent a new oral therapeutic option in areas where macrolide-resistant strains are not found, such as in Western Europe and the USA, at least in tularemia patients with mild to moderate disease. This may be particularly true when fluoroquinolones and tetracyclines are contraindicated, such as for children less than 8 years old and pregnant women, although the use of telithromycin is currently limited due to potential side effects such as hepatotoxicity. Finally, ketolides are also effective against common pathogens responsible for community-acquired pneumonia as well as the other biowarfare agents *Bacillus anthracis* and *Yersinia pestis*.

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Transparency declarations
None to declare.

Supplementary data
Tables S1 and S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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