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Ribosomal Mutations Conferring Macrolide Resistance in *Legionella pneumophila*

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ABSTRACT Monitoring the emergence of antibiotic resistance is a recent issue in the treatment of Legionnaires' disease. Macrolides are recommended as first-line therapy, but resistance mechanisms have not been studied in *Legionella* species. Our aim was to determine the molecular basis of macrolide resistance in *L. pneumophila*. Twelve independent lineages from a common susceptible *L. pneumophila* ancestral strain were propagated under conditions of erythromycin or azithromycin pressure to produce high-level macrolide resistance. Whole-genome sequencing was performed on 12 selected clones, and we investigated mutations common to all lineages. We reconstructed the dynamics of mutation for each lineage and demonstrated their involvement in decreased susceptibility to macrolides. The resistant mutants were produced in a limited number of passages to obtain a 4,096-fold increase in erythromycin MICs. Mutations affected highly conserved 5-amino-acid regions of L4 and L22 ribosomal proteins and of domain V of 23S rRNA (G2057, A2058, A2059, and C2611 nucleotides). The early mechanisms mainly affected L4 and L22 proteins and induced a 32-fold increase in the MICs of the selector drug. Additional mutations related to 23S rRNA mostly occurred later and were responsible for a major increase of macrolide MICs, depending on the mutated nucleotide, the substitution, and the number of mutated genes among the three *rrl* copies. The major mechanisms of the decreased susceptibility to macrolides in *L. pneumophila* and their dynamics were determined. The results showed that macrolide resistance could be easily selected in *L. pneumophila* and warrant further investigations in both clinical and environmental settings.

KEYWORDS 23S rRNA, *Legionella pneumophila*, macrolide, resistance, ribosomal mutations, ribosomal proteins

Legionella, the causative agent of Legionnaires' disease (LD), is an intracellular Gram-negative bacterium of environmental origin. Among more than 70 species and serogroups, *Legionella pneumophila* serogroup 1 (Lp1) is involved in more than 85% of cases (1). Upon inhalation of contaminated aerosols from man-made or natural water systems, *L. pneumophila* can infect and replicate within lung macrophages. Treatments with antibiotics such as macrolides or fluoroquinolones that accumulate within these cells are effective therapies.

Following the first outbreak of LD that occurred in 1976 among American Legionnaires in Philadelphia, erythromycin was proposed as the drug of choice for the treatment of LD (2). Several years later, azithromycin and clarithromycin were found to

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be superior to erythromycin against intracellular *L. pneumophila in vitro* and in a guinea pig model (3–5).

The current American and European guidelines recommend macrolides as first-line therapy for the treatment of severe and moderate LD, with a preference for azithromycin (6, 7). However, treatment failures have been described and a mortality rate of over 10% is still reported in LD patients despite timely and adequate therapy (8, 9). No clinical or environmental macrolide-resistant strains have been isolated so far among *Legionella* species, and susceptibility testing is almost never performed on *Legionella* strains (10). However, the recent descriptions of emerging fluoroquinolone resistance in clinical settings may change this outlook (11, 12).

In vitro selection of erythromycin resistance in *Legionella* by one- or two-step procedures has ever been reported, but the molecular mechanisms have not been investigated so far (13, 14). We therefore characterized these mechanisms by investigation of independent *L. pneumophila* lineages from the same *L. pneumophila* ancestral strain propagated to high-level resistance by serial passages under conditions of increasing erythromycin or azithromycin concentrations. We identified mutations that occurred during the evolution procedure by whole-genome sequencing (WGS) performed on one clone from each macrolide-resistant evolved lineage and focused on the major determinants of the decreased susceptibility to macrolides, affecting genes encoding 23S rRNA (*rrl*), L4 (*rplD*), and L22 (*rplV*) ribosomal proteins in all lineages.

RESULTS

Selection of macrolide-resistant lineages of *L. pneumophila*. The selection procedures resulted in high macrolide resistance levels whatever the drug used. A 4,096-fold minimal increase in MICs of the selector drug (from 0.25 to 1,024 mg/liter) was obtained in 9 to 11 passages for erythromycin-selected lineages and in 7 to 14 passages for 3 azithromycin-selected lineages; for the remaining 3 azithromycin-selected lineages, the increase in the MICs was limited to 256 mg/liter after 20 passages (Table 1).

WGS data. WGS data from the 12 clones showed that 4 to 14 mutations, involving 40 coding or noncoding sequences, occurred by lineage during the selection procedure. Mutations involving ribosomal *rrl* genes were common to all lineages. Other mutations notably affected genes encoding L4 and L22 ribosomal proteins (Table 1), putative efflux component proteins, a phosphate transporter, major outer membrane protein, conserved proteins of unknown function, and global transcriptional regulators.

Mutations in genes encoding 23S rRNA and L4 and L22 ribosomal proteins. The observed mutations affected four nucleotides of domain V of 23S rRNA in the final clones. Nine mutations were detected: G2057A (*Escherichia coli* numbering), A2058T, A2058C, A2058G, A2059C, A2059G, C2611A, C2611G, and C2611T (Table 2). Specific PCRs performed for each *rrl* copy ($n = 3$) showed that at least two copies were mutated in each independent lineage. With the exception of Azi2 and Azi6, which had no mutation in genes encoding ribosomal proteins, all mutants showed one or several additional mutations in the *rplD* gene resulting in substitutions or deletions between the Q62 and G66 amino acids of the L4 protein. Only three mutants had substitutions in the *rplV* gene, leading to a P87L, K90M, or G91D substitution in the L22 protein. In most cases, the targeted resequencing (next-generation sequencing [NGS]) performed on the final populations revealed that the ribosomal mutations identified in the 12 evolved clones represented the main population of the sample. One exception was observed; in the Ery1 lineage, mutation A2059C observed in the *rrl* gene of the sequenced clone (Table 2) was identified in only 33% of the whole population, whereas a A2058G substitution occurred in 67% of this population.

Two macrolide resistance profiles. Two distinct macrolide resistance profiles were observed. The first (lineages Ery1, Ery4, Ery5, Azi2, Azi5, and Azi6) corresponded to high-level resistance to both erythromycin and azithromycin (MICs, ≥1,024 mg/liter) and high-level cross-resistance to other macrolides (clarithromycin MIC, ≥256 mg/liter; spiramycin MIC, ≥25,000 IU/ml) and lincomycin (MIC, ≥256 mg/liter). The second (lineages Ery2, Ery3, Ery6, Azi1, Azi3, and Azi4) corresponded to high-level resistance to

TABLE 1 Mutations detected in macrolide-resistant *L. pneumophila* strains selected by erythromycin (Ery1 to Ery6) or azithromycin (Azi1 to Azi6) according to the step of the evolution procedure

L. pneumophila lineage	Passage	Erythromycin MIC (mg/liter)	Azithromycin MIC (mg/liter)	Mutation(s)[a]		
				rrl (no. of mutated copies/ total no. of copies) (23S rRNA)	*rplD* (L4 protein)	*rplV* (L22 protein)
Ery1	P2	1		—	G66D	ND
	P6	4		G2057A (1/3)[b]	G66D	ND
	P7	16		G2057A (3/3)	ND	ND
	P9	32		G2057A (3/3) + A2058G (1/3)	ND	ND
	P10	512		G2057A (3/3) + A2059C (3/3)	ND	ND
	P13	1,024		G2057A (3/3) + A2059C (3/3)	G66D	—
Ery2	P1	0.5		ND	Q62K	ND
	P2	1		—	Q62R + G64E	—
	P8	16		C2611A (1/3)[b]	ND	ND
	P9	128		C2611A (3/3)	ND	—
	P10	1,024		C2611G (3/3)	ND	ND
	P12	1,024		C2611G (3/3)	Q62R + G64E	—
Ery3	P1	0.5		ND	G66A	ND
	P7	256		C2611T (2/3)	ND	ND
	P9	512		C2611T (3/3)	ND	—
	P12	1,024		C2611T (3/3)	G66A	—
Ery4	P1	0.5		ND	K65N	—
	P3	2		ND	G66A	K90M
	P7	16		A2058T (2/3)	G66A	ND
	P8	512		A2058T (3/3)	G66A	ND
	P12	1,024		A2058T (3/3)	G66A	K90M
Ery5	P1	0.5		—	K63Q[b]	ND
	P2	2		G2057A (3/3)	—	ND
	P3	8		G2057A (3/3)	G66S	ND
	P5	32		G2057A (3/3)	G66A	ND
	P7	128		G2057A (3/3) + A2059G (2/3)	G66A	ND
	P10	1,024		G2057A (3/3) + A2058C (1/3) + A2059G (2/3)[c]	G66A	ND
	P12	1,024		G2057A (3/3) + A2058C (2/3) + A2059G (1/3)[c]	G66A	—
Ery6	P3	1		ND	G66D	ND
	P4	2		C2611A (2/3)	ND	ND
	P5	8		C2611A (3/3)	ND	ND
	P8	64		C2611A (1/3) + C2611T (2/3)	ND	—
	P10	1,024		C2611T (3/3)	ND	ND
	P15	1,024		C2611T (3/3)	G66D	—
Azi1	P3		1	—	G66R	—
	P4		1	ND	T65K	ND
	P5		8	—	T65K + G66R	ND
	P8		16	C2611T (1/3)[b]	ND	ND
	P9		128	C2611T (2/3)[b]	ND	ND
	P12		256	C2611T (3/3)	ND	G91D
	P20		256	C2611T (3/3)	T65K + G66R	G91D
Azi2	P4		128	A2058G (2/3)[b]	—	—
	P7		1,024	A2058G (3/3)	ND	ND
	P10		2,048	A2058G (3/3)	—	—
Azi3	P3		2	—	T65K	ND
	P7		32	C2611T (3/3)	T65K	ND
	P9		64	C2611T (3/3)	T65K + G66A	ND
	P12		128	C2611T (3/3)	T65K + G66A	—
	P13		128	ND	T65K + G66C	—
	P20		256	C2611T (3/3)	T65K + G66C	—

(Continued on following page)

TABLE 1 (Continued)

L. pneumophila lineage	Passage	Erythromycin MIC (mg/liter)	Azithromycin MIC (mg/liter)	Mutation(s)[a]		
				rrl (no. of mutated copies/ total no. of copies) (23S rRNA)	*rplD* (L4 protein)	*rplV* (L22 protein)
Azi4	P3		1	ND	del $_{63}$KG$_{64}$	ND
	P8		8	C2611G (2/3)	ND	ND
	P9		128	C2611G (3/3)	del $_{63}$KG$_{64}$	ND
	P20		256	C2611G (3/3)	del $_{63}$KG$_{64}$	—
Azi5	P2		2	ND	T65K	—
	P3		8	ND	T65K	G91D
	P11		16	—	ND	P87L[b] + G91D
	P12		32	A2058G (1/3)[b]	ND	ND
	P13		512	A2058G (3/3)	ND	ND
	P16		1,024	A2058G (3/3)	T65K	P87L + G91D
Azi6	P3		8	C2611G (3/3)	ND	ND
	P8		2,048	A2058G (3/3)	—	—
	P12		2,048	A2058G (3/3)	—	—

[a]*E. coli* numbering. —, no mutation; ND, not determined.
[b]An additional subpopulation(s) was mutated.
[c]Distinct *rrl* copies were mutated.

erythromycin (MIC, ≥1,024 mg/liter) but lower-level resistance to other macrolides (azithromycin MIC, 32 to 256 mg/liter; clarithromycin MIC, 64 to 256 mg/liter; spiramycin MIC, 6,250 to 25,000 IU/ml). The lincomycin MIC increased moderately (from 64 to a maximum of 256 mg/liter).

Irrespective of the resistance profile, the susceptibility to pristinamycin was poorly affected (MICs, 0.5 to 8 mg/liter), unlike the susceptibility to telithromycin (MICs, ≥1 mg/liter). No cross-resistance to other antibiotic families was observed, suggesting the presence of specific molecular mechanisms of macrolide resistance (Table 2).

Dynamics of substitution. The dynamics of substitutions were determined by specific PCR and sequencing of *rrl* ($n = 3$), *rplD*, and *rplV* genes from the whole bacterial populations frozen at each passage. Mutations affecting L4 protein occurred at early steps, after one to three passages. They were responsible for decreased susceptibility to erythromycin or azithromycin, with a 2- to 8-fold increase in the MIC (0.5 to 2 mg/liter). Additional mutations in *rplD* or *rplV* genes were associated with another increase in erythromycin or azithromycin MICs (from 1 to 16 mg/liter). Mutations in *rrl* genes mainly occurred in steps that followed and were correlated to another decrease in susceptibility, with MICs increased to 2 to 2,048 mg/liter (Table 1). The Sanger sequencing revealed a genetic heterogeneity of resistant populations, which may explain the transitions in substitutions observed in several lineages.

Mutations affecting 23S rRNA. The MICs varied according to the number of mutated *rrl* copies, the mutated nucleotide, and the nature of the substitution. Decreased susceptibility to erythromycin (MIC, ≤32 mg/liter) was observed when the mutation G2057A (Ery1 and Ery5) occurred. High-level resistance to erythromycin and azithromycin (MICs, ≥1,024 mg/liter) corresponding to the first resistance profile described above was observed when the A2058 substitution occurred (Ery4, Azi2, Azi5, and Azi6). Mutations of A2059 (Ery1 and Ery5) were combined with other *rrl* mutations; the lineages concerned showed a resistance level similar to that observed when mutations of A2058 occurred. Lineages harboring a C2611G or C2611T mutation (Ery2, Ery3, Ery6, Azi1, Azi3, and Azi4) had high-level resistance to erythromycin (MICs, 1,024 mg/liter) and various levels of decreased susceptibility to azithromycin (MICs, 8 to 256 mg/liter), described above as the second resistance profile. Lineages harboring a C2611A transition (Ery2 and Ery6) showed decreased susceptibility to erythromycin at a level that was lower than that observed with the C2611G and C2611T transitions. Except for the Ery5, Azi2, and Azi6 lineages, the *rrl* mutations were combined with *rplD* and/or *rplV* mutations, which prevented us from rigorously demonstrating the relationship between *rrl* mutations and phenotypes of macrolide susceptibility.

TABLE 2 Ribosomal mutations and MICs observed in *L. pneumophila* sequenced clones and reconstructed mutants

L. pneumophila strain	Nucleotidic or amino acid change(s)			MIC[a]											
	23S rRNA[b] (no. of mutated copies/total no. of copies)	L4	L22	ERY	AZI	CLR	SPI	LIN	PRI	TEL	LZ	LVX	CIP	RIF	DOX
Ery1	G2057A (3/3) + A2059C (3/3)	G66D	—	1,024	2,048	256	100,000	512	0.25	>4	16	0.032	0.016	0.0005	2
Ery2	C2611G (3/3)	Q62R + G64E	—	1,024	128	128	6,250	64	0.25	4	16	0.032	0.016	0.0005	2
Ery3	C2611T (3/3)	G66A	—	1,024	32	256	6,250	32	0.25	1	32	0.032	0.016	0.0005	2
Ery4	A2058T (2/3)	G66A	K90M	1,024	1,024	256	100,000	>512	0.5	>4	8	0.032	0.016	0.0005	2
Ery5	G2057A (3/3) + A2058C (2/3) + A2059G (1/3)	G66A	—	1,024	2,048	>256	100,000	512	0.5	>4	8	0.032	0.016	0.0005	2
Ery6	C2611T (3/3)	G66D	—	1,024	64	64	6,250	256	0.125	1	32	0.032	0.016	0.0005	2
Azi1	C2611T (3/3)	T65K + G66R	G91D	1,024	256	256	25,000	64	1	>4	32	0.032	0.016	0.0005	2
Azi2	A2058G (3/3)	—	—	1,024	2,048	512	12,500	256	0.25	4	16	0.032	0.016	0.0005	2
Azi3	C2611T (3/3)	T65K + G66C	—	2,048	256	256	25,000	32	0.25	4	32	0.032	0.016	0.0005	2
Azi4	C2611G (3/3)	del $_{63}$KG$_{64}$	—	2,048	256	512	25,000	64	1	>4	32	0.032	0.016	0.0005	2
Azi5	A2058G (3/3)	T65K	P87L + G91D	1,024	1,024	256	25,000	>512	1	>4	16	0.032	0.016	0.0005	2
Azi6	A2058G (3/3)	—	—	2,048	2,048	512	12,500	>512	0.125	>4	16	0.032	0.016	0.0005	2
Δ*dotA* L1	A2058G (3/3)	—	—	≥1,024	1,024	256	12,500	>512	0.125	>4	16	0.032	0.016	0.0005	2
Δ*dotA* L2	A2058G (2/3)	—	—	≥1,024	1,024	32	6,250	>512	0.06	>4	16	0.032	0.016	0.0005	2
Δ*dotA* L3	G2057T (3/3)	—	—	16	32	4	780	64	0.125	1	32	0.032	0.016	0.0005	2
Δ*dotA* L4	C2611T (2/3)	—	—	256	8	4	1,560	64	0.25	1	8	0.032	0.016	0.0005	2
Δ*dotA* L5	C2611G (3/3)	—	—	128	8	4	1,560	64	0.25	1	16	0.032	0.016	0.0005	2
Δ*dotA* L6	—	G66R	—	8	4	0.063	1,560	64	0.25	0.25	16	0.032	0.016	0.001	2
Δ*dotA* L7	—	G66A	—	4	2	0.063	1,560	64	0.25	0.25	16	0.032	0.016	0.001	2
Paris WT	—	—	—	0.25	0.25	0.063	390	64	0.125	0.125	16	0.032	0.016	0.0005	2
Paris Δ*dotA*	—	—	—	0.25	0.25	0.063	390	64	0.125	0.125	16	0.032	0.016	0.001	2

[a]All MIC data represent milligrams per liter, except the spiramycin data, which represent IU per milliliter. ERY, erythromycin; AZI, azithromycin; CLR, clarithromycin; SPI, spiramycin; LIN, lincomycin; PRI, pristinamycin; TEL, telithromycin; LZ, linezolid; LVX, levofloxacin; CIP, ciprofloxacin; RIF, rifampin; DOX, doxycycline.

[b]*E. coli* numbering. —, no mutation.

Reconstruction of resistance alleles in the macrolide-susceptible ancestral strain. The *rrl* mutations A2058G, C2611T, and C2611G and the substitutions G66A and G66R in protein L4 were successfully introduced in Δ*dotA* mutant lineages by natural transformation (Table 2). We failed in our attempts to introduce G2057A and *rplV* substitutions. However, a spontaneous mutant grew on an agar plate containing erythromycin and revealed G2057T substitutions in the three *rrl* copies in the Δ*dotA* mutant L3 lineage. Decreased susceptibility to both erythromycin (MIC, ≤8 mg/liter) and azithromycin (MIC, ≤4 mg/liter) was observed when mutations involving the *rplD* gene were introduced. The G2057T transition in the *rrl* gene induced higher erythromycin and azithromycin MICs (MICs, ≤16 mg/liter and ≤32 mg/liter, respectively). High-level resistance to erythromycin (MIC, ≥128 mg/liter) but moderately decreased susceptibility to azithromycin (MIC, 8 mg/liter) was observed when C2611G and C2611T mutations were introduced. High-level resistance to both erythromycin and azithromycin (MICs, ≥1,024 mg/liter) and to all macrolides was observed after the A2058G transition.

DISCUSSION

In this study, we propagated 12 independent lineages of *L. pneumophila* from the same susceptible ancestral strain to a high level of macrolide resistance. The resistant mutants were obtained in a limited number of passages under pressure from erythromycin or azithromycin, two macrolides historically and currently recommended for the treatment of LD (2, 6, 7). The lineages were all highly resistant to erythromycin, with a 4,096-fold increase in MIC, and revealed cross-resistance to other macrolides, with two distinct resistance profiles differing by their levels of azithromycin susceptibility, suggesting two pathways toward high-level resistance. No cross-resistance to other families of antibiotics was observed, suggesting the presence of specific resistance mechanisms.

The characterization of the genetic events consisted first in a WGS strategy using a single evolved clone for each lineage that was representative of the whole population. We focused on mutations affecting L4 and L22 ribosomal proteins and 23S rRNA that were identified in all lineages and are involved in macrolide resistance in many other bacterial species (15, 16). The evolutionary pathway was determined for each lineage from frozen bacterial mixtures in which we observed mixed populations, stabilization of the resistance profiles, or, conversely, loss of the previously acquired mutations during the evolution procedure. In all lineages, additional steps led to a final high resistance level, without any prerequisite mutation necessary to achieve high-level resistance, unlike fluoroquinolone resistance in *L. pneumophila* (17). Most increases in macrolide MICs were correlated with additional mutations in the ribosomal targets. We also demonstrated the direct involvement of any mutations in macrolide susceptibility by reconstructing these mutations in the ancestral strain.

Point mutations or insertions/deletions in *rplD* or *rplV* genes occurred mainly in the early steps of the evolution procedure. They affected two highly conserved regions of 5 amino acids in L4 (Q62 to G66) and L22 (P87 to G91) proteins, which are located on loops that converge to form a narrowing structure in the ribosomal exit tunnel near the macrolide-binding site (18, 19). In *E. coli*, such mutations disrupt the conformation of residues in domains II, III, and V of 23S rRNA and result in a conformational change in the ribosome that hinders binding of macrolides to the peptide exit tunnel (20). In our study, these were associated with a moderate decrease in macrolide susceptibility, with a 2- to 64-fold increase in the MIC of the selector drug, and with moderate and inconsistent cross-resistance to other macrolides. They did not have an impact on lincomycin, pristinamycin, and telithromycin MICs.

Similar resistance levels have been described for other respiratory pathogens. Peric et al. showed that L4/L22 substitutions were responsible for a 2- to 32-fold increase in erythromycin and azithromycin MICs in *Haemophilus influenzae* clinical isolates (21). In *Streptococcus pneumoniae*, L4 and L22 alterations were responsible for a higher macrolide resistance level, with inconstant levels of cross-resistance to telithromycin but no

impact on clindamycin MICs (22, 23). However, the susceptibility to the macrolide antibiotic family has been more widely studied using *in vitro*-selected mutants. As reported for *L. pneumophila*, a moderate increase in the macrolide MICs was observed for L4 and L22 mutants in *in vitro*-selected *H. influenzae* or *Mycoplasma pneumoniae*. Depending on the strain and the mutation, unmodified, increasing, or even decreasing clindamycin MICs have been described in *H. influenzae* mutants whereas telithromycin MICs were unaffected (24, 25). The cumulative effect of L4/L22 alterations observed in *L. pneumophila* was also described for these two species (21, 25). Unlike *M. pneumoniae*, they were not prerequisite for *rrl* mutations in *L. pneumophila* (25).

In *L. pneumophila*, as in *M. pneumoniae*, *S. pneumoniae*, *Campylobacter jejuni*, or *Francisella tularensis*, *rrl* mutations have been correlated to a higher resistance level, with selector drug MICs increased from 2 to 2,048 mg/liter (25–28). They were related to 4 nucleotides within the peptidyltransferase region (positions 2057, 2058, 2059, and 2611 in domain V of 23S rRNA), positions 2058 and 2059 being key nucleotides for macrolide binding and base pair 2057 and 2611 closing the stem preceding the single-stranded portion of the peptidyltransferase region (29).

The macrolide MICs of *L. pneumophila* varied first according to the number of mutated *rrl* copies, irrespective of the mutated nucleotide. Similar results have been described for positions 2057 in *F. tularensis* and 2059 in *C. jejuni*/*C. coli*, species that possess 3 copies of a ribosomal operon (28, 30). However, for positions 2058 and 2611, this contrasted with literature data: high-resistance level was reached as soon as one substitution occurred in a sole position 2058 nucleotide in *F. tularensis*, *Ureaplasma parvum* (2 copies), or *S. pneumoniae* (4 copies) and at position 2611 in *S. pneumoniae* (28, 31–33).

The macrolide MICs of the *L. pneumophila* mutants described here also varied widely according to the affected nucleotide. Although not all of the *rrl* mutations could be reconstructed, we confirmed a high- and cross-resistance profile for all macrolides when A2058 was mutated. Lower macrolide MICs, particularly those of 15- and 16-membered macrolides (azithromycin and spiramycin), were observed when mutations of G2057 or C2611 were introduced. Mutations involving G2057, observed in intermediate passages, were responsible for a moderate decrease in susceptibility for all macrolides. Our results did not allow any formal conclusion with respect to A2059 mutations, but the results observed with the Ery5 lineage let us hypothesize a resistance level similar to that seen with A2058 transitions. These observations correlate with literature data for many other bacterial species: mutations involving base 2058 or base 2059 are responsible for a high level of resistance to all macrolides. They usually give a higher advantage to the bacteria than substitutions in the base pair G2057 and C2611 (16, 25, 28, 31, 34–36).

The macrolide susceptibility of the *L. pneumophila* mutants described here also varied according to the nature of the substitution at C2611. C-to-T and -G transitions were responsible for higher MICs than the C-to-A transition, as observed in *M. pneumoniae* and *S. pneumoniae* (23, 32, 37). As also described for *U. parvum* and *S. pneumoniae*, the ribosomal mutations in *L. pneumophila* did not increase or increased only weakly the MICs of streptogramins, while ketolide MICs were affected, although their use is not recommended in clinical settings (26, 31).

A final point of note is that combinations of substitutions, such as mutations at positions 2058 and 2059 within a given *rrl* copy, were not observed, as described in many species, with the exception of *C. jejuni* (38). Furthermore, mutations in both the 2057–2059 region and position 2611 were not observed in *L. pneumophila* although they have been described in *M. pneumoniae*, *C. jejuni*, and *S. pneumoniae*. But this may have been due to the limited number of *L. pneumophila* lineages selected (23, 38, 39).

Resistance to macrolides can reduce the fitness of bacteria in the absence of antibiotics (40). To determine if the ribosomal macrolide resistance-conferring mutations observed in our study may or may not carry a biological cost, the bacterial fitness of the reconstructed resistant clones was studied. It was unaffected by the ribosomal mutations (data not shown). Bacterial fitness of the 12 sequenced clones was not

studied as these strains possess second-site and nonribosomal mutations that might affect the function of conserved proteins and global transcriptional regulators and thus might positively interfere with their fitness.

Our experimental selection procedure did not allow any resistance mechanism involving horizontal gene transfer that might occur in *Legionella*-infected lungs (41). Mutations in *rrl* usually predominate in species with less than 4 operons, whereas 23S rRNA methylation is observed in species with more operons, such as staphylococci or streptococci, suggesting that the ribosomal mutations described here might be the major macrolide resistance mechanism in *Legionella* species *in vivo* (16). Macrolides are the first-line antibiotics for LD therapy (6, 7). Treatment failures have been reported, suggesting the possibility of resistance acquired *in vivo* (8, 9). Yet no macrolide-resistant clinical strain has been isolated so far. *Legionella* are consistently susceptible to macrolide antibiotics in axenic medium and in cell or animal models (3–5, 42, 43). These data contrast with the ease with which strains with high-level resistance were selected *in vitro* in our study.

Antimicrobial susceptibility testing is not routinely performed on *Legionella* strains, even in expert laboratories, except in cases in which the patient is critically ill or relapsing. Isolation of *Legionella* strains remains difficult, and a preceding antibiotic therapy can cause a negative culture result despite persistence of the infection (44, 45). Moreover, *Legionella* is an environmental pathogen for which humans are accidental hosts. With one exception, no human-to-human spread has been reported so far (46). Unlike respiratory pathogens such as *Staphylococcus aureus* or *Mycobacterium tuberculosis*, for which antibiotic resistance is a major issue, *Legionella* strains that would acquire mutations under macrolide therapy would not be infectious for other hosts. Combination therapy is advocated to treat severe LD, but there is no evidence of its superiority over monotherapy (47–50). Moffie and Mouton described a mutation rate of 10^{-7} for erythromycin, while the *Legionella* burden in lower respiratory tract samples ranges from 2 to 8 log10 DNA copies/ml according to LD severity (51, 52). Thus, the use of combination therapy for critically ill patients may also contribute to limiting the emergence of macrolide-resistant *Legionella* strains. Taking the data together, the combination of a low rate of isolation from clinical samples, the limitation of susceptibility testing to severely ill patients, the absence of human-to-human spread, and the use of combined therapy for patients with high bacterial burden probably help to explain why no macrolide-resistant clinical isolate has been reported so far. In this study, we demonstrated that high-level macrolide-resistant mutants can be easily and quickly selected *in vitro*. Such *in vivo* selection should be considered and may constitute an argument warranting systematic antibiotic combinations for severe LD and for immunosuppressed patients.

Alternatively to mutations occurring under therapy, patients could contract a *Legionella*-resistant strain directly from the environment. Macrolides are present in natural environments, as they are produced by *Streptomycetes* species. They are mostly and extensively used in human and veterinary medicines (notably aquaculture) to control and treat infectious diseases. Administered at subtherapeutic doses, they also act as growth promoters in livestock in veterinary medicine (53). Macrolide antibiotics have been detected in treated wastewater effluents (the main source being hospital and aquaculture effluents) and in river waters but also in surface water, groundwater, and drinking water in a range of nanograms per liter to micrograms per liter, which highlights the direct exposure of *Legionella* to macrolide drugs in its natural habitat (54). The diffusion of macrolides in the environment, particularly in natural water systems, contributes to the development of antibiotic resistance that may affect *Legionella* species (55).

In conclusion, we found that *L. pneumophila* mutants with high-level resistance could be easily selected *in vitro* by the use of subinhibitory concentrations of macrolides. Although they were not prerequisite, initial mechanisms mainly involved ribosomal L4/L22 proteins and induced moderated decreased susceptibility to macrolides. Additional mutations in genes encoding 23S rRNA mostly occurred in a second step

and were responsible for a major increase of the resistance level. The data warrant further investigations in both clinical and environmental settings and promote the development of PCR assays targeting the mutated *Legionella rrl* genes and/or deep-sequencing approaches, which might be notably implemented on culture-negative samples.

MATERIALS AND METHODS

Bacterial strains and culture media. The reference strain *L. pneumophila* serogroup 1 (Lp1) Paris (CIP 107629T) was used to select macrolide-resistant strains. Bacteria were grown in N-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered yeast extract (BYE) broth at 37°C in an aerobic atmosphere or on buffered-charcoal-yeast extract (BCYE) agar plates (Oxoid, Dardilly, France) at 35°C in a 2.5% CO_2-enriched atmosphere.

The avirulent strain Lp1 Paris, deleted for *dotA* by a unique insertion of a kanamycin cassette (*dotA*::*kan*), was used to select macrolide-resistant strains by natural transformation (56, 57).

Antibiotics. The following antimicrobial agents were tested: erythromycin, azithromycin, clarithromycin, rifampin, doxycycline (Sigma-Aldrich, Saint-Quentin-Fallavier, France), spiramycin, pristinamycin, telithromycin, levofloxacin (Sanofi, Gentilly, France), lincomycin, linezolid (Pfizer, Amboise, France), and ciprofloxacin (Bayer, Puteaux, France). Antibiotic solutions were prepared at 64 mg/ml and stored at −20°C until use.

Selection of macrolide-resistant mutants of *L. pneumophila*. Selection of six independent erythromycin-resistant lineages (namely, Ery1 to Ery6) and six independent azithromycin-resistant lineages (Azi1 to Azi6) was performed in a biosafety level 3 laboratory by serial passages in BYE medium containing increasing subinhibitory concentrations of erythromycin and azithromycin, respectively. A suspension of the Lp1 Paris strain (final concentration, 4.10^5 CFU/ml) in BYE broth was dispensed in 24-well microtiter plates (BD Falcon; Becton Dickinson, Le Pont-de-Claix, France) containing erythromycin or azithromycin at increasing 2-fold concentrations ranging from a 0.5- to 16-fold change from the MIC determined for the ancestral strain. The plates were incubated for 3 to 4 days in an aerobic atmosphere at 35°C. The MIC was determined as the lowest antibiotic concentration that inhibited visible bacterial growth. The culture well containing the highest concentration of antibiotics with bacterial growth was used to propagate the lineages. After a 1:100 dilution, a subsequent passage was performed into new 24-well microtiter plates containing fresh medium, with increasing 2-fold concentrations ranging from a 0.5- to 16-fold change from the MIC previously determined. This procedure was repeated until growth was obtained at an erythromycin (Ery1 to Ery6) or azithromycin (Azi1 to Azi6) concentration of 1,024 mg/liter, corresponding to a 4,096-fold increase in the antibiotic MIC for the ancestral strain, or after 20 passages. At each evolution step, the contents of each well (genetically heterogeneous mixtures) were frozen at −80°C. Two additional consecutive passages were performed in a 0.5-fold MIC medium to assess the stability of the resistance phenotype before stopping the evolution procedure. The 12 final bacterial mixtures were sampled and frozen. One evolved clone per lineage was randomly selected for WGS on the basis of the assumption that it would be representative of the whole population.

WGS. Genomes of the 12 evolved clones and the ancestral strain were sequenced using single-read 50-bp Illumina technologies at the ProfileXpert facility (Lyon, France). Reads were mapped against the reference genome (NC_006368). Mapping and variant calling were performed using CASAVA 1.8.2 software.

Dynamics of mutation. The proportion of the population carrying the mutations in *rrl*, *rplD*, and *rplV* identified in the sequenced clone was first determined in the 12 final bacterial mixtures by performing specific PCR targeting the mutated regions and sequencing the amplicons by next-generation sequencing (NGS) technology. The amplifications were performed using Phusion High-Fidelity DNA polymerase (New England BioLabs, Evry, France). The amplicon libraries were generated using an Ion Plus Fragment library kit and were sequenced on an Ion Torrent PGM (Life Technologies, Saint Aubin, France) according to the manufacturer's instructions. Reads were mapped against the reference sequences extracted from the genome (NC_006368). Mapping and variant calling were performed using Torrent Suite 5.0.2.

The dynamics of mutation was determined retrospectively from the frozen intermediate bacterial mixtures by specific PCRs for each lineage (Table 3). The same primers were used for both amplification and sequencing, except for the 23S rRNA gene, for which specific primers were used to amplify independently the 3 copies of the gene and other primers were used to sequence the regions in which there were mutations. Briefly, all PCRs were performed using Expand High Fidelity^{PLUS} *Taq* polymerase (Roche Diagnostics, Meylan, France) and were carried out in 50-μl volumes containing a 0.4 μM concentration of each primer, 1 μl of DNA for the *rrl* PCR, and 10 μl of DNA for the *rplD* and *rplV* PCRs. Double-strand DNA sequencing was performed at the Biofidal facility (Vaulx-en-Velin, France).

Reconstruction of ribosomal mutations in the ancestral strain. To confirm the involvement of *rrl*, *rplD*, and *rplV* mutations in the decreased susceptibility to macrolides, mutation reconstruction was performed by inducing competence for natural transformation in Δ*dotA L. pneumophila* strains in a biosafety level-2 laboratory, as previously described (56). PCR assays were performed using Phusion High-Fidelity DNA polymerase and specific primers (Table 3). Following natural transformation, the bacterial mixtures were plated on BCYE agar plates containing 50 mg/liter erythromycin (approximate final concentration after charcoal absorption, 5 mg/liter [58]) incubated in an aerobic atmosphere at 35°C and examined over 14 days. The presence of mutations in growing colonies was investigated by PCR amplification and sequencing.

TABLE 3 Primers and PCR conditions used in this study

Gene	Primer name	Primer sequence	Product size (bp)	PCR conditions	
				$MgCl_2$ concn (mM)	Amplification conditions
rrl[a] (copies no. 1, 2, and 3)	F2	5′-AAGGCATAGACAGCCAGGAG-3′			
rrl[a] (copy no. 1)	R1.3	5′-GCTTGCTAACTCACACCAAC-3′	2,573	1.5	1 cycle of 5 min at 95°C; 35 cycles of 15 s at 94°C, 30 s at 62°C, and 150 s at 72°C; 1 cycle of 5 min at 72°C
rrl[a] (copy no. 2)	R2″.2	5′-CAAGGAATCACGGTAGG-3′	2,566	1.5	1 cycle of 5 min at 95°C; 35 cycles of 15 s at 94°C, 30 s at 55°C, and 150 s at 72°C; 1 cycle of 5 min at 72°C
rrl[a] (copy no. 3)	R3.2	5′-GAACCAACAAGCATTCTC-3′	2,274	1.5	1 cycle of 5 min at 95°C; 35 cycles of 15 s at 94°C, 30 s at 55°C, and 150 s at 72°C; 1 cycle of 5 min at 72°C
rrl[b] (copies no. 1, 2, and 3)	23S F 23S R	5′-AAGTTCCGACCTGCACGAAT-3′ 5′-GTAGTCTTCAACGGGCTTCA-3′	859		
rplD[a,b]	L4_F L4_R	5′-AAAGGTGCAATTCCTGGTG-3′ 5′-CTGGTTTGTTTGAAGCGTTTAG-3′	897	3	1 cycle of 5 min at 95°C; 35 cycles of 15 s at 94°C, 35 s at 50°C, and 50 s at 72°C; 1 cycle of 5 min at 72°C
rplV[a,b]	L22_F L22_R	5′-GCATAACGCAAAGACCAC-3′ 5′-AGCAATACCTTCAGCCACCA-3′	834	3	1 cycle of 5 min at 95°C; 35 cycles of 15 s at 94°C, 35 s at 50°C, and 50 s at 72°C; 1 cycle of 5 min at 72°C
rrl[c] (copies no. 1, 2, and 3)	F1	5′-GGAAAGTTGGCCGTAGAGG-3′			
rrl[c] (copy no. 1)	R1.2	5′-TCCTTCGCCATCGGAAAGTC-3′	3,440	1.5	1 cycle of 1 min at 98°C; 35 cycles of 15 s at 98°C, 30 s at 58°C, and 120 s at 72°C; 1 cycle of 8 min at 72°C
rrl[c] (copy no. 2)	R2.2	5′-AGGGCAAGGAATCACGGTAG-3′	3,302	1.5	1 cycle of 1 min at 98°C; 35 cycles of 15 s at 98°C, 30 s at 58°C, and 120 s at 72°C; 1 cycle of 8 min at 72°C
rrl[c] (copy no. 3)	R3.5	5′-AGTGTTCTGCAAGTGGACAACTC-3′	3,505	1.5	1 cycle of 1 min at 98°C; 35 cycles of 15 s at 98°C, 30 s at 58°C, and 120 s at 72°C; 1 cycle of 8 min at 72°C
rplD[c]	L4_MF L22_R	5′-CAGCAGCTATTACAACGA-3′ 5′-AGCAATACCTTCAGCCACCA-3′	4,855	3	1 cycle of 1 min at 98°C; 35 cycles of 15 s at 98°C, 30 s at 58°C, and 150 s at 72°C; 1 cycle of 8 min at 72°C
rplV[c]	L22_MF L22_MR	5′-CACGAGGTGAGTGATGGA-3′ 5′-AACACCATTACTCTCAAGA-3′	4,674	3	1 cycle of 1 min at 98°C; 35 cycles of 15 s at 98°C, 30 s at 58°C, and 150 s at 72°C; 1 cycle of 8 min at 72°C

[a]Primers used for DNA amplification.
[b]Primers used for DNA sequencing (dynamics of substitutions).
[c]Primers used for natural transformation.

Determination of MICs. For each *L. pneumophila* clone to be tested, the MICs of antibiotics were determined in duplicate by a broth microdilution method previously described (59).

Accession number(s). WG sequences were deposited in the European Nucleotide Archive (ENA) under BioProject accession number PRJEB14949.

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We declare that we have no conflicts of interest.

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