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MECHANISMS OF RESISTANCE

Emergence and Within-Host Genetic Evolution of Methicillin-Resistant *Staphylococcus aureus* Resistant to Linezolid in a Cystic Fibrosis Patient

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ABSTRACT Methicillin-resistant *Staphylococcus aureus* (MRSA) infection has increased in recent years among cystic fibrosis (CF) patients. Linezolid (LZD) is one of the antistaphylococcal antibiotics widely used in this context. Although LZD resistance is rare, it has been described as often associated with long-term treatments. Thirteen MRSA strains isolated over 5 years from one CF patient were studied for LZD resistance emergence and subjected to whole-genome sequencing (WGS). Resistance emerged after three 15-day LZD therapeutic regimens over 4 months. It was associated with the mutation of G to T at position 2576 (G2576T) in all 5 *rrl* copies, along with a very high MIC (>256 mg/liter) and a strong increase in the generation time. Resistant strains isolated during the ensuing LZD therapeutic regimens and until 13 months after LZD stopped harbored only 3 or 4 mutated *rrl* copies, associated with lower MICs (8 to 32 mg/liter) and low to moderate generation time increases. Despite these differences, whole-genome sequencing allowed us to determine that all isolates, including the susceptible one isolated before LZD treatment, belonged to the same lineage. In conclusion, LZD resistance can emerge rapidly in CF patients and persist without linezolid selective pressure in colonizing MRSA strains belonging to the same lineage.

KEYWORDS 23S rRNA, MRSA, *Staphylococcus aureus*, cystic fibrosis, linezolid, oxazolidinones, ribosomal resistance, WGS

With 70.6% of patients colonized/infected and a median age at the first infection of 3.6 years, *Staphylococcus aureus* is one of the major and earliest bacteria detected in infants and children with cystic fibrosis (CF) (1). Despite antibiotic intervention, *S. aureus* colonization or chronic infection persists in the lung for many years. Longitudinal studies have shown that in the majority of cases, patients are chronically colonized/infected with the same clone (2–4). With age, a decreasing incidence of *S. aureus* has been shown to coincide with an increased incidence of *Pseudomonas aeruginosa* colonization in CF patients (1). However, *S. aureus* is still present in 50% of patients with *P. aeruginosa* infection (5). CF lung colonization with methicillin-resistant *S. aureus* (MRSA) has increased in recent years, with MRSA being detected in 26% of the CF patients in the United States in 2015 (1). Several studies have reported that MRSA infection is associated with an increased rate of lung function decline and worse clinical outcomes (6, 7). The airways of CF patients provide a niche for bacteria in a hostile

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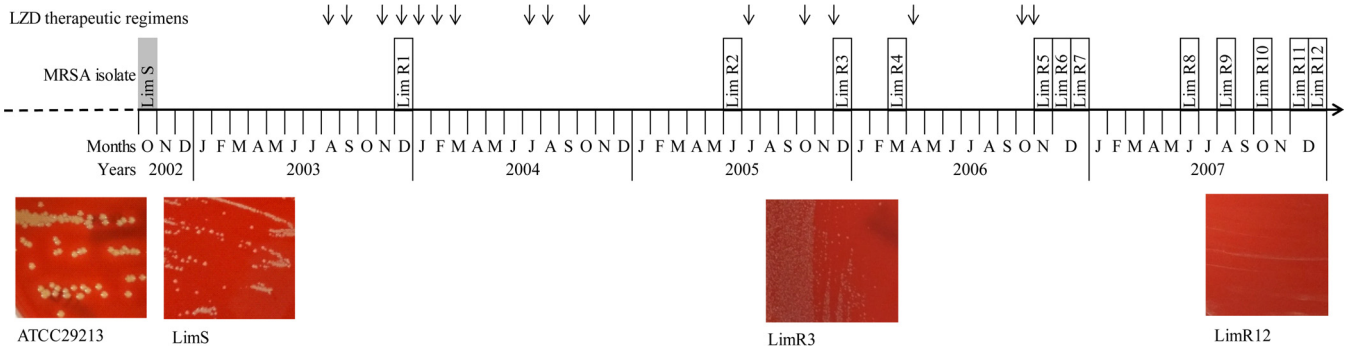


FIG 1 Timeline of MRSA strain isolation over the 5-year period, along with the dates of the 15-day periods of the 600-mg twice-daily (b.i.d.) LZD therapeutic regimen (black arrows). A total of 13 MRSA isolates were included in the study. The name of the MRSA isolate susceptible to LZD is highlighted in gray, and those resistant to LZD are framed in black. Images of isolates cultured on Colombia blood agar plates are presented for 3 isolates, with *S. aureus* reference strain ATCC 29213 shown for comparison.

environment where the challenges include host immune response, antibiotics, interspecies competition, hypoxia, and starvation, which trigger various forms of *S. aureus* adaptations (8). These adaptations include the emergence of small-colony variants (SCVs), hypermutator phenotypes, multidrug resistance, and increased ability to form biofilm (4, 9, 10).

Linezolid (LZD) is widely used in CF patients for the treatment of MRSA infections (11). It is the first member of the oxazolidinone class of antibiotics, which has been available in France since 2002. LZD disrupts the beginning of the protein synthesis by binding to domain V of the 23S rRNA in the 50S subunit of the bacterial ribosome, specifically in the peptidyl transferase center (PTC) at site A (12). Clinical LZD-resistant *S. aureus* (LRSA) is still rarely isolated globally (<1%) (13). However, resistance in *S. aureus* emerged in CF patients several years ago, often associated with long-term treatments. The mutations implicated are mostly alterations in the 23S rRNA, especially the mutation G2576U (*Escherichia coli* numbering) (14). Alterations in ribosomal proteins L3 and L4, as well as the *cfr* gene, were also reported (15–20). Suboptimal dosing has been demonstrated to play an important role in resistance emergence due to the variation of the pharmacokinetic profile of LZD among children with CF, which requires dosing adjustment. LZD resistance has also emerged in patients without LZD exposure, which is probably due to transmission of LRSA between CF patients (20).

Our objective was to explore the dynamic of LZD resistance in MRSA isolates recovered over a long period of time from one CF patient and, in particular, to describe the isolates' genetic evolution and relatedness.

RESULTS

LZD treatment, MRSA isolates, and emergence of LZD resistance. One CF patient was followed up at the CF unit of Limoges teaching hospital. Chronic MRSA colonization was diagnosed at 3 years of age. Thirteen frozen MRSA strains (LimS, which was LZD susceptible, and LimR1 to R12, which were LZD resistant) isolated from sputum samples over a 5-year period (October 2002 to December 2007) were included. The timeline of LZD treatments and the concomitant MRSA isolates are presented in Fig. 1. An initial therapeutic regimen of LZD (600 mg twice daily) was introduced in August 2003 over a 15-day period. After this one, successive therapeutic regimens of LZD (600 mg twice daily) were used alternately with intravenous glycopeptides until November 2006. Thirteen MRSA strains isolated either before (LimS), during (LimR1 to -R5), or after LZD treatment (LimR6 to -R12) were studied (Fig. 1). LZD resistance was detected in December 2003 after three 15-day LZD therapeutic regimens over 4 months (LimR1). Resistance was determined to be associated with the mutation of G to T at position 2576 (G2576T) in the 23S rRNA gene (*rrl*) by Sanger sequencing. All isolates harbored 5 rRNA operons (*rrn*), and the mutation was observed in 3 to 5 copies of *rrl*. Moreover, *rplC* and *rplD* genes were not mutated and the *cfr* gene was absent as

TABLE 1 Characterization of LZD resistance mechanisms and correlation with generation times

Isolate	LZD MIC (mg/liter)[a]	LZD resistance mechanism[b]				Minimum generation time (mean ± SD)
		23S rRNA gene (no. of mutated *rrl* alleles/total no. of *rrl* alleles)	L3 protein (*rplC* gene)	L4 protein (*rplD* gene)	*cfr* gene	
LimS	0.75	WT	WT	WT	—	49.9 ± 0.7
LimR1	>256	G2576T (5/5)	WT	WT	—	260.7 ± 38.5[c]
LimR3	8	G2576T (3/5)	WT	WT	—	54.9 ± 1.9[c]
LimR6	8	G2576T (4/5)	WT	WT	—	158.0 ± 11.4
LimR8	32	G2576T (4/5)	WT	WT	—	158.9 ± 16.4
LimR9	24	G2576T (4/5)	WT	V142A	—	65.5 ± 4.2[c]
LimR12	16	G2576T (4/5)	WT	WT	—	67.8 ± 4.8[c]

[a]LZD resistance is defined by a MIC of >4 mg/liter by EUCAST and CLSI for *S. aureus*.
[b]Determined with WGS data. WT, wild type; —, absent.
[c]$P \leq 0.05$, Student's test in comparison with the results for the first isolate LimS.

determined by PCR amplification. LZD resistance levels were different during persistence, with MICs between 8 and >256 mg/liter. Those MIC variations were correlated with the number of mutated copies of the *rrl* gene (Table 1; Table S1 in the supplemental material). The LZD resistance was still unchanged 1 year after the last LZD therapeutic regimen, with MICs of 8 to 32 mg/liter, and was associated with mutations in 4 *rrl* copies.

Multilocus sequence type (MLST) and *spa* type analysis classified 10 isolates as sequence type 72 (ST72) and *spa* type t148 (Table S1). No classification could be obtained for the remaining 3 isolates (LimR6, -R8, and -R11) due to point mutations leading to changes in alleles for MLST or in repeats for *spa* type. Consequently, new ST and *spa* types were submitted in the corresponding databases (Table S1). Nevertheless, LimR6, which belongs to the new ST4898, also belongs to *spa* type t148, and conversely, LimR8 and -R11, with new *spa* types, belong to ST72. Accordingly, these data suggested that all isolates belong to the same lineage.

Whole-genome comparison. Among the 13 isolates, 7 (LimS, LimR1, LimR3, LimR6, LimR8, LimR9, and LimR12) were selected according to LZD resistance and isolation timeline for whole-genome sequencing (WGS). All had a genomic content compatible with *S. aureus* species with draft genomes size of 2.74 Mb and a mean GC content of 32.72%. Genomic content comparison of LimS, LimR1, and LimR12 with the FORC_012 MRSA strain (the closest ST72 strain in the database) is represented in Fig. 2a. The virulomes of all isolates were identical, similar to those of other ST72 strains (FORC_012 and 2148) with the absence of the Panton-Valentine leukocidin (PVL) and TSST-1-coding genes (Table S2). All Lim isolates contained a small plasmid of 3,332 bp, similar to pC55 (GenBank accession number AY048756.1), carrying 2 genes responsible for cadmium resistance (*cadB* and *cadX*, coding, respectively, a cadmium resistance protein and an efflux system) (Fig. 2b). Another plasmid (2,473 pb), identical to p19321-P01 (GenBank accession number CP002148.1) and coding for the *ermC* gene, was detected in all isolates except LimR6 (Fig. 2c).

Antimicrobial resistance pattern and resistome. All Lim isolates shared the same antimicrobial resistance pattern and were resistant to methicillin, kanamycin, tobramycin, ciprofloxacin, rifampin, and fusidic acid. Almost all were also resistant to erythromycin and clindamycin (except LimR6 and LimR7) (Fig. 3a). Comparison of genomic resistance markers, obtained by WGS, correlated well with the antimicrobial susceptibility profiles and showed similar resistance genes or point mutations (Fig. 3b). Resistance to methicillin was conferred by a *mecA* gene in a staphylococcal cassette chromosome *mec* element (SCC*mec*) of type IV. The joining region J3 of the SCC*mec* element additionally encodes aminoglycosides (*aaD* gene) and bleomycin resistance on a pUB110 region. A constitutive macrolide-lincosamide-streptogramin B (MLS_B) phenotype was present in all but 2 strains (LimR6 and -R7) due to the *ermC* gene on the p19321-P01-like plasmid. LimR6 susceptibility to erythromycin and clindamycin correlated with the loss of this plasmid. Resistance to fluoroquinolones, associated with a

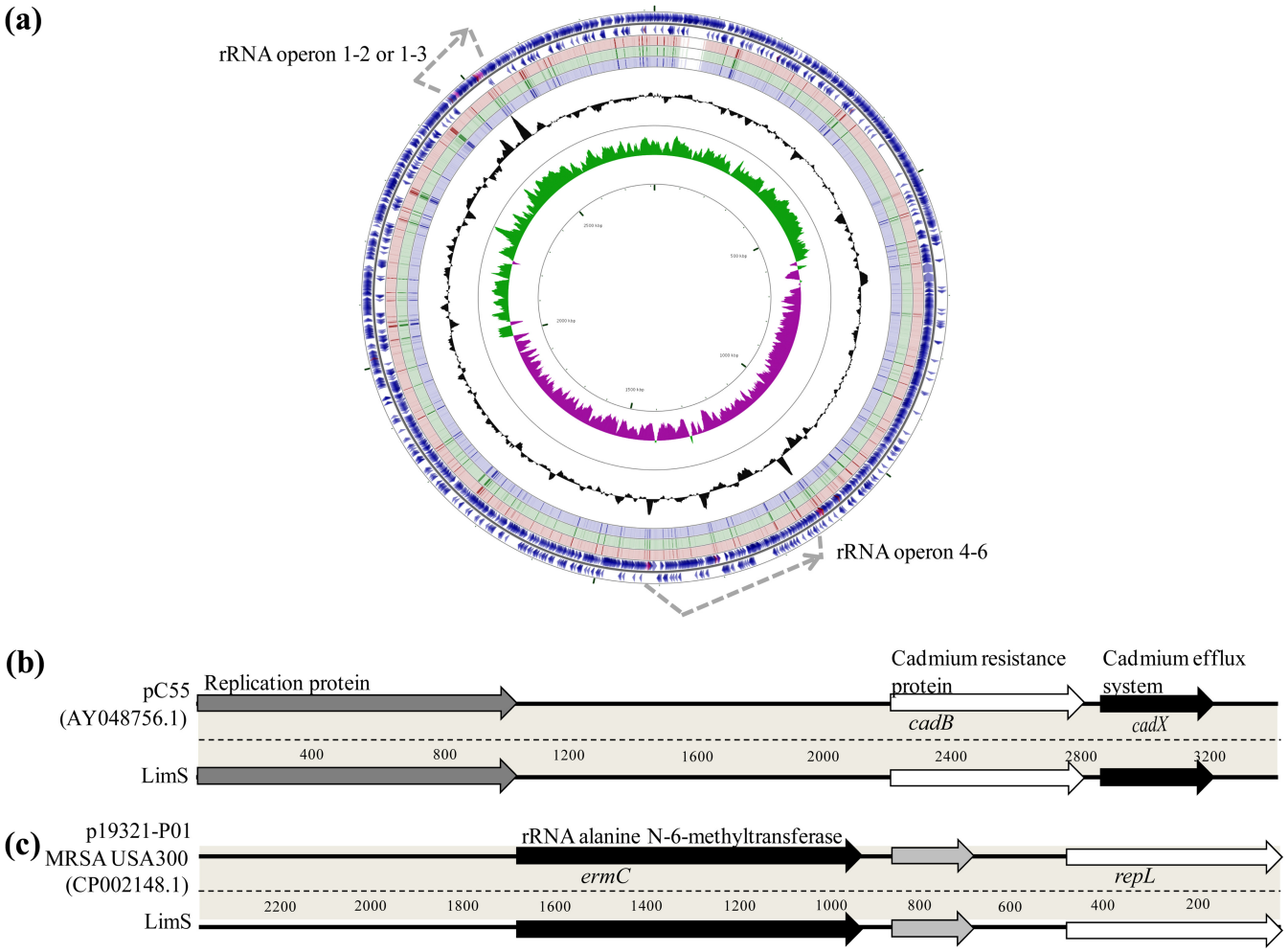


FIG 2 Genome features of the chromosome (a) and the 2 plasmids (b and c) identified in Lim isolates. (a) Comparison by BLAST of the coding sequences (CDS) of the FORC_012 (blue arrows of the 2 outer rings) and LimS (red), LimR1 (green), and LimR12 (purple) genomes (corresponding to the 3rd, 4th, and 5th rings, respectively, from outside to inside) (CGview server [56]). GC content is represented on the plot inside the ring. The major difference between the FORC_012 genome and those of Lim isolates is the number of rRNA operons (gray dotted-line arrows), with 6 *rrl* genes for FORC_012 conversely to 5 for Lim isolates, corresponding to the loss of the 3rd operon. The other difference is the presence of a supplementary prophage in the FORC_012 genome. (b and c) Genes are labeled on the coding sequences for the 2 small cryptic plasmids of isolate LimS.

mean MIC of 2 mg/liter for ciprofloxacin, was linked to 2 different point mutations (E84G and S80F) previously reported in *parC* (21, 22). High-level resistance (MIC > 32mg/liter) was observed for rifampin associated with the point mutation S486L in *rpoB* (23). Finally, the H457Q mutation in *fusA* was responsible for low-level resistance to fusidic acid, as already described (24).

Phylogenetic relatedness and SNP analysis. Phylogenetic relatedness was first evaluated with whole-genome MLST (wgMLST) analysis on 3,897 loci (core and accessory genes). It confirms a clonal relationship between the isolates (Fig. 4), with pairwise distances (MLST loci of wgMLST) of 21 to 46 different alleles between LimS, -R1, -R3, -R9, and -R12. ST72 isolates FORC_012 and 2148 were more distant, with 82 and 156 different loci, respectively, compared to those of LimS. On the other hand, LimR6 and -R8 were genetically divergent, with 662 and 311 different alleles, respectively, compared with LimR9 (the closest Lim isolate). To further assess phylogenetic links, whole-genome single-nucleotide polymorphism (wgSNP) analysis was performed and indicated that the isolates had a common ancestor. Comparison with FORC_012 indicated a total of 91 common strain-specific SNPs among the 7 isolates, indicating clonal diversification. Comparison with LimS (the first isolate) showed that pairwise distances

(a)

Phenotypic antimicrobial susceptibility testing

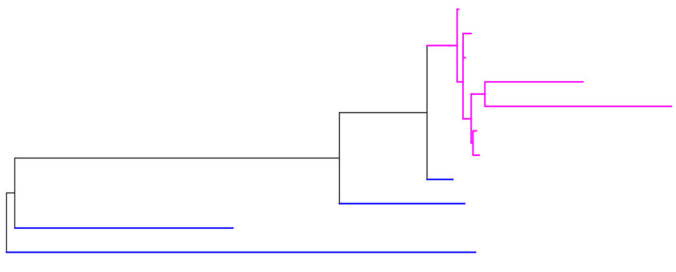
Isolate	PEN	MET	KAN	TOB	GEN	ERY	CLI	SXT	CIP	TET	FA	RIF	VAN	LZD
Lim S	R	R	R	R	S	R	R	S	R	S	R	R	S	S
Lim R1	R	R	R	R	S	R	R	S	R	S	R	R	S	R
Lim R2	R	R	R	R	S	R	R	S	R	S	R	R	S	R
Lim R3	R	R	R	R	S	R	R	S	R	S	R	R	S	R
Lim R4	R	R	R	R	S	R	R	S	R	S	R	R	S	R
Lim R5	R	R	R	R	S	R	R	S	R	S	R	R	S	R
Lim R6	R	R	R	R	S	S	S	S	R	S	R	R	S	R
Lim R7	R	R	R	R	S	S	S	S	R	S	R	R	S	R
Lim R8	R	R	R	R	S	R	R	S	R	S	R	R	S	R
Lim R9	R	R	R	R	S	R	R	S	R	S	R	R	S	R
Lim R10	R	R	R	R	S	R	R	S	R	S	R	R	S	R
Lim R11	R	R	R	R	S	R	R	S	R	S	R	R	S	R
Lim R12	R	R	R	R	S	R	R	S	R	S	R	R	S	R

	blaZ	SCC*mec*	*mecA*	*aadD*	*ermC*	*par C*	*fusA*	*rpoB*
Lim S	+	IV	+	+	+	E84G	H457Q	S486L
Lim R1	+	IV	+	+	+	S80F	H457Q	S486L
Lim R3	+	IV	+	+	+	S80F	H457Q	S486L
Lim R6	+	IV	+	+	-	S80F	H457Q	S486L
Lim R8	+	IV	+	+	+	S80F	H457Q	S486L
Lim R9	+	IV	+	+	+	S80F	H457Q	S486L
Lim R12	+	IV	+	+	+	S80F	H457Q	S486L

(b) Resistome

FIG 3 Comparison of phenotypic antimicrobial susceptibility patterns and resistomes inferred from WGS data through the bioMérieux EpiSeq analysis pipeline. (a) Antimicrobial susceptibilities of the 13 isolates included in the study. Isolate names and susceptibility results (R, resistance; S, susceptibility) are displayed in black for sequenced isolates and gray for nonsequenced isolates. (b) Resistome associated with each antimicrobial drug shown in panel a (except for LZD, which is detailed in Table 1) for the 7 sequenced isolates. At the top are the antibiotic resistance genes. +, present; −, absent. The enzyme encoded by the *aaD* gene is ANT(4′)-Ia. Antibiotic resistance associated with single point mutations in the drug target is indicated by the observed amino acid change conferring resistance. PEN, penicillin; MET, methicillin; TOB, tobramycin; KAN, kanamycin; GEN, gentamicin; ERY, erythromycin; CLI, clindamycin; SXT, trimethoprim-sulfamethoxazole; CIP, ciprofloxacin; TET, tetracycline; FA, fusidic acid; RIF, rifampin; VAN, vancomycin; LZD, linezolid.

varied between 28 and 58 SNPs over 5 years. Moreover, 21 and 36 SNPs were shared among the first 6 LZD-resistant isolates (LimR1, -R3, -R6, -R8, -R9, and -R12) and the last 4 isolates (LimR6, -R8, -R9, and -R12), respectively. Pairwise distances were not identical throughout the 5 years, and the mutation rate was more elevated between LimS and LimR1, as well as between LimR9 and -R12. LimR6 and -R8 showed more unique SNPs than the remaining 5 isolates (Fig. 5). A hypermutator phenotype was suspected. A premature termination of the *mutL* gene, part of the pathway for DNA proofreading mismatch repair, was observed in the last 4 isolates (LimR6, -R8, -R9, and -R12). This was



	Lim S	Lim R1	Lim R2	Lim R8	Lim R6	Lim R9	Lim R12	FORC 012	2148	USA 300	N315
LimS	0										
LimR1	41	0									
LimR2	26	27	0								
LimR8	335	334	326	0							
LimR6	691	687	682	804	0						
LimR9	61	59	46	311	662	0					
LimR12	62	61	47	314	666	21	0				
FORC012	82	112	97	380	743	127	130	0			
2148	156	175	166	381	662	192	192	159	0		
USA300	1851	1846	1848	1817	1926	1852	1843	1834	1582	0	
N315	1907	1901	1903	1871	1951	1903	1898	1897	1611	1963	0

FIG 4 Results of phylogenetic analysis of the 7 MRSA isolates analyzed by wgMLST. A maximum parsimony tree (Bionumerics) with default parameters was used for the analysis of all loci (wgMLST and MLST alleles). The root position in the tree was assigned to the deepest branch measured by average branch length. *S. aureus* strains FORC_012 (ST72, t664), 2148 (ST72), N315 (ST5), and USA300-FPR3757 (ST8) were used for reference genomes (blue). Lim isolate branches are depicted in purple. Numbers in the body of the table represent the pairwise wgMLST allelic differences between isolates.

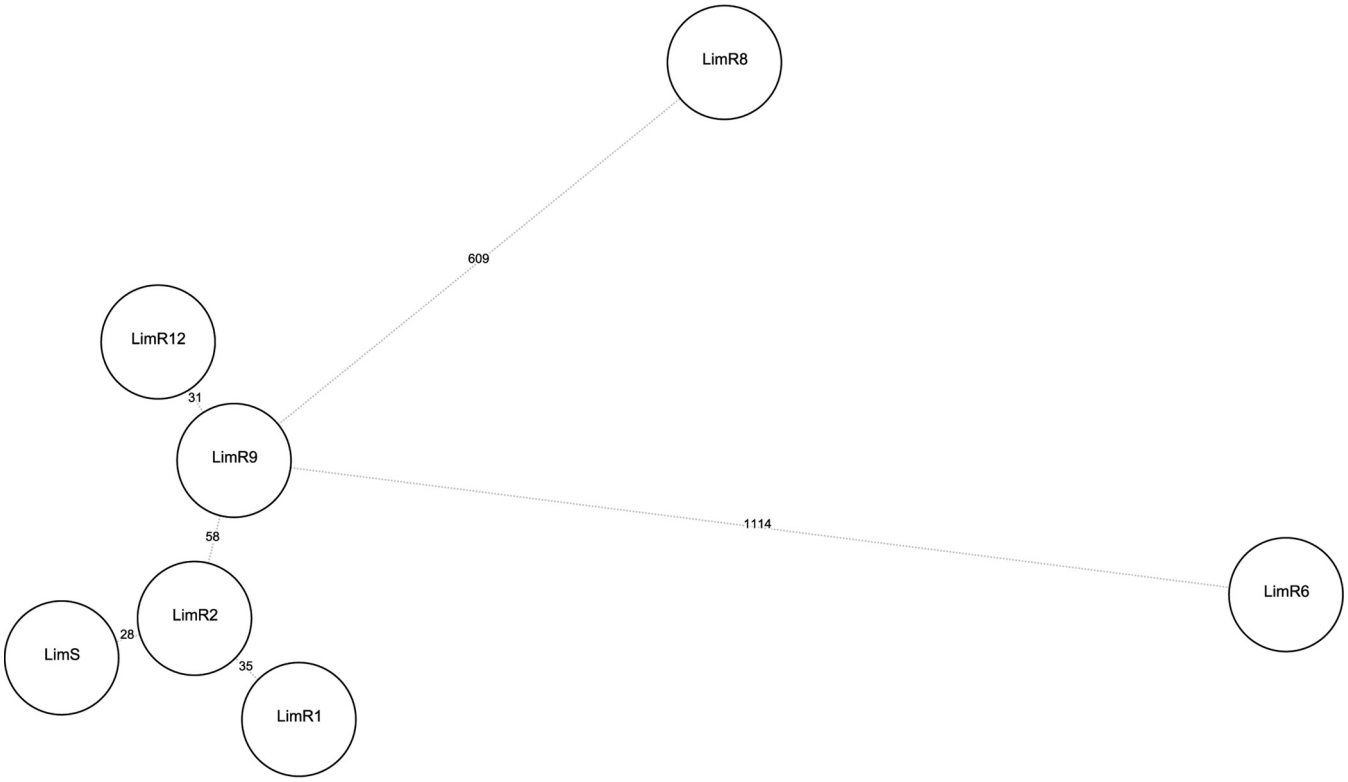


FIG 5 Evolution of the pairwise distances (SNPs) in comparison with the LZD-susceptible Lim isolate, LimS, based on SNP analysis. A minimum spanning tree (BioNumerics version 7.6) with default parameters (priority rule1, maximum number of *N* locus variants [*N* = 1]; weight, 10,000) was used. Numbers on the tree branches indicate SNP distances between genomes (circles). Genome codes refer to Lim strain numbers, which are chronologically ordered as shown in Fig. 1.

linked to a 4-bp deletion which resulted in the frameshift Tyr501stop. Additionally, in isolates LimR1 and LimR8, a stop codon appeared in the *recQ* gene, involved in DNA replication and repair, following a 1-bp deletion.

Mutations that could be associated with LZD resistance, linked to ribosomal modification, were also sought in LimR isolates compared to LimS. No potentially compensatory mutation was observed in 23S rRNA genes. The mutation Lys716Glu in the C-terminal domain, for ribosomal fixation of the *relA/spoT* enzyme, could be a consequence of the ribosomal modification, as well as the mutation Gly57Glu observed in the SSU ribosomal protein S10p.

Phenotype. As all isolates shared a common ancestor, the phenotypical consequences of LZD resistance acquisition were investigated. The first isolate, LimS, presented SCV features, with pinpoint-size colonies without beta hemolysis and absence of pigmentation observed after 24 h of growth on a blood agar plate at 37°C in ambient atmosphere (Fig. 1). However, no auxotrophy for thymidine, hemin, menadione, or CO_2 dependency was observed and, concordantly, no previously described mutations in corresponding genes were present. LZD-resistant isolates presented even smaller colonies than LimS, with LimR6 and -R8 being the smallest even after 48 h of growth. However, they contained various SNPs that could be responsible for this phenotype and for the high generation time increase. The generation times of all other LZD-resistant isolates correlate well with the number of *rrl* copies with the G2576T mutation, as already described for the G2576T mutation *in vitro* (Table 1) (25).

DISCUSSION

We studied the dynamics of LZD resistance among related MRSA isolates over a 5-year period in one CF patient who had been colonized for 15 years. LZD was used for the first time in 2003 when the patient was 18 to treat multidrug-resistant *S. aureus*

infection. Unfortunately, LZD resistance emerged rapidly after three 15-day therapeutic regimens. LZD resistance emergence in CF patients has been mainly associated with more LZD therapeutic regimens, longer duration of treatment, and transmission of a resistant isolate from another patient (16, 18). In the study by Endimiani et al., over 10% of patients developed resistance after prolonged treatment (18). However, as in our patient, rapid acquisition of resistance has also been described. It has been suggested that reduced bioavailability of LZD in CF could be responsible (17, 20). However, in contrast with other reports, resistance emerged immediately and with a high level (MIC $>$ 256 mg/liter) in our patient (17). Resistance was conferred by the G2576T mutation in domain V of the 23S rRNA gene, which is the most frequently described mutation in *Staphylococcus* clinical strains (26). The high resistance level was linked to the mutation of all *rrl* copies. All isolates were shown to have 5 copies of the ribosomal operon, which is more frequent in hospital strains, favoring easier antibiotic pressure adaptation, while community strains often have 6 copies (27). Thus, the presence of only 5 *rrn* copies could have facilitated the mutation of all copies. Concordantly, so far, no isolate with the G2576T mutation in 6 *rrl* copies has been described. Moreover, very few clinical *S. aureus* strains with mutation in all 5 copies have been mentioned (28). We looked for other events that could have facilitated the mutation of all *rrn* copies. The DNA helicase RecQ is involved in DNA replication and repair. It has been hypothesized that a mutation in this gene could contribute to rapid spread of domain V mutations after acquisition of the first mutation by increasing the frequency of short sequence recombination and, thus, potentially facilitating recombination among *rrn* loci (29). Isolate LimR1 has a truncated *recQ* gene, so we hypothesize that *recQ* deletion could have facilitated the mutation of all of the *rrn* copies. The G2576T mutation in 5 copies is associated with a high fitness cost in our isolate, as already described *in vitro* (25). This high increase in generation time could also explain the disappearance of this isolate over time. The ensuing isolates had 3 or 4 mutated copies, as generally described in clinical strains. Those isolates could have emerged from another subpopulation, as the presence of variants of a same *S. aureus* clone has been described in CF patients (30). They persisted without LZD treatment for 1 year, as was described for this mutation *in vitro* (17, 31, 32).

Further epidemiological comparison showed that all isolates belonged to ST72 and/or *spa* type t148. All strains carried a type IV SCC*mec* element commonly found in community-acquired MRSA (CA MRSA), which correlates with the ST72 lineage, also known as a CA MRSA (33). Contrary to the high rifampin resistance level observed in our isolates, the mutation S486L has been previously described as conferring low-level resistance and is not frequent in clinical strains because of its high fitness cost (23). Two different mutations in *parC* were observed, E84G for LimS (first strain) and S80F for all other isolates. The first has already been described but is infrequent, unlike the second (22). Finally the loss of the constitutive MLS_B phenotype observed in 2 strains, along with the *ermC* gene, has already been described during acquisition of LZD resistance (34).

The duration and dynamics of *S. aureus* persistence in the airways of individual CF patients over extended periods have been conclusively attributed to one single clone in 63% to 80% of patients by different typing methods (pulsed-field gel electrophoresis [PFGE] and variable-number tandem repeat–multilocus variable-number tandem-repeat analysis [VNTR-MLVA]) (2, 3). However, variation in the same lineage has been observed over the years with the MLVA technique (2). WGS- and SNP-based methods surpass all previous methods used for typing in terms of discriminatory power and were used to precisely analyze our isolates' relatedness (35). Moreover, wgMLST has recently been reported as the method of choice for surveillance of food-borne bacterial pathogens by PulseNet International (36). In our study, wgMLST analysis confirmed the clonality of all the isolates. Strain-specific SNPs indicated clonal diversification of our isolates compared with other ST72 strains. The pairwise distance between isolates varied over time between 28 and 58 SNPs (for LimS, -R1, -R3, -R9, and -R12). If changes were accumulating according to a molecular clock (i.e., at a constant mutation rate of

3.10^{-6} per site per year as described for *S. aureus*), we could expect about 8.4 mutations per year (37). However, the mutation rates varied over time between 17.5 and 93 SNPs/year, with phylogeny showing LimR2 to be closer to other isolates than to the first susceptible isolate (LimS). The explanation could be that at sampling times, there were multiple strains in the population, as already proposed (38). Moreover, the maximum SNP distance between 2 isolates of the same strain is still debated and will likely depend on the organism and even the strain under investigation, as well as on the setting. SNP analysis could be very useful for the follow-up of persistent infection in patients over several years and the evolution of strains under different pressures (39–41). However, the sampling of one *S. aureus* colony per sputum sample for SNP analysis may be considered a limitation of our study. Indeed during long-term colonization, different evolutions of a same isolate may be found (42). In a recent study with intra-CF patient SNP comparison, isolates with 74 SNPs or fewer were considered to be the same strain (38), which is higher than the 40 SNPs considered in some outbreak studies but could permit us to hypothesize that our isolates have the same ancestor (43). In CF patients, the ability of *S. aureus* colonizing strains to present a high mutation rate in response to environmental stress arises through mutations in mutator genes and could lead to high levels of SNP differences (44). Accordingly, mutations in *recQ* (LimR1 and LimR6) and *mutL* (LimR6, -R8, -R9, and -R12) could have increased the mutation rate and, thus, SNP differences. However, this cannot by itself explain the very high numbers of unique SNPs of LimR6 and LimR8 compared to the sequences of LimR9 and -R12, which harbored the same truncated *mutL* gene. Of note, this high mutation rate could also explain the unexpected appearance of point mutations in 2 MLST alleles of LimR6, leading to a new *gmk* allele and, consequently, to a new ST. The same applies to the new *spa* type for LimR8 and LimR11. However, this adaptation, with minor changes in *spa* type, has already been reported in CF patients (5, 45).

In conclusion, physicians should be aware that LZD resistance can emerge rapidly and at a high level in CF patients and persist for a long time without linezolid selective pressure in MRSA colonizing strains belonging to the same lineage. All in all, this also reflects the strong bacterial adaptability over years to minimize fitness cost and keep resistance at the required level.

MATERIALS AND METHODS

Bacterial growth conditions and antimicrobial susceptibility testing. Bacterial colonies were grown on Columbia blood agar plates (bioMérieux, Marcy l'Étoile, France) for 24 to 48 h at 37°C in ambient atmosphere. Antimicrobial susceptibility testing was performed using the agar disk diffusion method, and MICs were determined by Etest (bioMérieux) on Mueller-Hinton medium (MH; Bio-Rad, Hercules, CA) with or without blood at 5% and NAD (MHF; Bio-Rad) (46).

Whole-genome sequencing. DNA libraries were prepared using the Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA) and Nextera XT index kit (Illumina). WGS was performed with a MiSeq (Illumina) instrument to generate 200-bp paired-end reads. An average sequencing depth of 204.0 (106.2 to 258.8) was achieved. *De novo* assembly was performed with a SPAdes *de novo* assembler on the BioNumerics (version 7.6) (Applied Maths, Sint-Martens-Latem, Belgium) cloud-based calculation engine (47). Gene prediction and annotation of the contigs were done using BioNumerics. Molecular resistance and virulence determinants were obtained from WGS data through the bioMérieux EpiSeq knowledge base and with VirulenceFinder (https://cge.cbs.dtu.dk/services/VirulenceFinder/) for reference strains (48).

Phylogenetic relatedness. MLST and *spa* typing were inferred from WGS through the bioMérieux EpiSeq data analysis workflow for sequenced isolates. For other isolates and unknown ST or *spa* type from WGS data, Sanger sequencing was performed for determination or verification, respectively. New ST assignment was obtained by submission to the MLST online database (https://pubmlst.org/saureus/), and for the new *spa* type, submission to the RIDOM web server (http://spaserver.ridom.de/) was performed (49, 50).

To assess phylogenetic relatedness, whole-genome MLST (wgMLST) was performed on 3,897 loci (core and accessory genes) with the BioNumerics version 7.6 plugin (47). Two independent algorithms were used, an assembly-free *k*-mer-based method ($k = 35$) and a second, BLAST-based allele detection algorithm on *de novo* assemblies. Only the second algorithm was applied to reference strains. The closest ST72 strains, chosen among publicly available genomes by alignment of the largest contigs using BLAST, were FORC_012 and 2148 (GenBank accession no. CP010998.1 and CP016856.1, respectively). Strains with more distant genomes, MRSA N315 (ST5) and USA300-FPR3757 (ST8) (GenBank accession no. NC_002745.2 and CP000255.1, respectively), were also used. To further determine variation among isolates, whole-genome SNP analysis was performed. Two references were used for mapping with a

Bowtie2 algorithm (BioNumerics). The *de novo* assembled and annotated genome of the first isolate (LimS) was used to assess distances between isolates, and the FORC_012 strain was used to assess clonal diversification. Strict SNP filtering was applied with the following conditions: extraction at positions shared by all strains, at least one variant within the strain set detected, minimum total coverage of 5 reads, at least 1 supporting read in each direction, and minimum distance between retained SNP positions of 12 bp. Clustering of the wgMLST and wgSNP results was performed with a maximum parsimony tree and a minimum spanning tree, respectively, based on pairwise wgMLST allelic differences between isolates using BioNumerics version 7.6.

Linezolid resistance determinants. Specific LZD resistance determinants (23S rRNA genes *rrl*, *rplC*, *rplD*, and *cfr*) were searched by PCR and Sanger sequencing as previously described, confirmed from WGS data, and compared with reference *S. aureus* strain N315 using BLAST (51–53). For mutations in 23S rRNA genes, the mutated copy number was estimated by the coverage, with mutated reads divided by the total coverage (alternate and reference allele) according to the rRNA gene copy number determined. The latter was determined with a specific PCR as previously described (27).

Auxotrophy complementary testing and growth studies. Auxotrophy was assessed by disc diffusion on MH agar using hemin disks (Oxoid, Waltham, MA, USA), 1.5 μg thymidine (Sigma-Aldrich, St. Louis, MO), or 1.5 μg menadione (Sigma-Aldrich), as already described (54). Growth studies were performed in triplicate by inoculating brain heart infusion (BHI) broth with an overnight BHI culture on a microplate. The optical density (OD) at 620 nm was measured every hour for 14 h. The generation times were calculated from the growth rates in the exponential growth phase as previously described (55).

Accession number(s). The sequencing raw data for each isolate were submitted to GenBank with BioProject record number PRJNA434495.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00720-18.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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