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Multi-Virulence-Locus Sequence Typing of *Staphylococcus lugdunensis* Generates Results Consistent with a Clonal Population Structure and Is Reliable for Epidemiological Typing

Jennifer Didi,[a] Ludovic Lemée,[a,b] Laure Gibert,[a,b] Jean-Louis Pons,[c] Martine Pestel-Caron[a,b]

Laboratoire G.R.A.M. (EA 2656 IRIB), University of Rouen, Rouen, France[a]; Service de Microbiologie, Rouen University Hospital, Rouen, France[b]; Laboratoire Ecosystème Intestinal, Probiotiques, Antibiotiques (EA 4065 IMTCE), University of Paris Descartes, Paris, France[c]

***Staphylococcus lugdunensis* is an emergent virulent coagulase-negative staphylococcus responsible for severe infections similar to those caused by *Staphylococcus aureus*. To understand its potentially pathogenic capacity and have further detailed knowledge of the molecular traits of this organism, 93 isolates from various geographic origins were analyzed by multi-virulence-locus sequence typing (MVLST), targeting seven known or putative virulence-associated loci (*atlL*$_{R2}$, *atlL*$_{R3}$, *hlb*, *isdJ*, SLUG_09050, SLUG_16930, and *vwbl*). The polymorphisms of the putative virulence-associated loci were moderate and comparable to those of the housekeeping genes analyzed by multilocus sequence typing (MLST). However, the MVLST scheme generated 43 virulence types (VTs) compared to 20 sequence types (STs) based on MLST, indicating that MVLST was significantly more discriminating (Simpson's index [*D*], 0.943). No hypervirulent lineage or cluster specific to carriage strains was defined. The results of multilocus sequence analysis of known and putative virulence-associated loci are consistent with a clonal population structure for *S. lugdunensis*, suggesting a coevolution of these genes with housekeeping genes. Indeed, the nonsynonymous to synonymous evolutionary substitutions (d_N/d_S) ratio, the Tajima's D test, and Single-likelihood ancestor counting (SLAC) analysis suggest that all virulence-associated loci were under negative selection, even *atlL*$_{R2}$ (AtlL protein) and SLUG_16930 (FbpA homologue), for which the d_N/d_S ratios were higher. In addition, this analysis of virulence-associated loci allowed us to propose a trilocus sequence typing scheme based on the intragenic regions of *atlL*$_{R3}$, *isdJ*, and SLUG_16930, which is more discriminant than MLST for studying short-term epidemiology and further characterizing the lineages of the rare but highly pathogenic *S. lugdunensis*.**

Staphylococcus lugdunensis is a coagulase-negative staphylococcus (CoNS) belonging to the normal human skin flora (1) that is increasingly recognized as a virulent pathogen in both community-acquired and nosocomial infections (2–4). It colonizes several distinct niches primarily in the lower part of the body (such as perineal and inguinal areas) but also the lower extremities (including feet and nails) (1). Described as a "wolf in sheep's clothing," *S. lugdunensis* resembles *Staphylococcus aureus* in its clinical presentation and course of infection (tissue destruction and aggressive clinical course) (2). Even if it can cause many types of infections ranging from localized to systemic (2, 5, 6), this opportunistic pathogen has been mainly associated with serious infections, such as skin and soft tissue infections (SSTI) (3, 4, 7, 8), infective endocarditis (5, 6, 9, 10), abscesses (11, 12), and bone and joint infections (13, 14). *S. lugdunensis* SSTI have been reported to be superficial, painful, often prolonged, and recurrent in patients with skin diseases or after trauma or surgery (7, 15). *S. lugdunensis* infective endocarditis can occur in native or prosthetic valves as well as on cardiac implantable electronic devices (10), and surgery with valve replacement is often indicated (16, 17). Endocarditis is typically characterized by an aggressive and a rapidly progressive clinical course, with mortality rates higher than those for *S. aureus* (14.5%) or *Staphylococcus epidermidis* (20%), ranging from 18.2% (10) to 75% (5, 18). Of note, the prevalence of *S. lugdunensis* infections is low but may be underestimated due to a frequent lack of reliable identification of staphylococcal species (2, 7, 14) before the matrix-assisted laser desorption ionization–time of flight mass spectrometry-based approach was used (19).

Although this CoNS species was first described >2 decades ago (20), remarkably little is currently known about the virulence factors and molecular mechanisms underpinning the virulence of *S. lugdunensis*. Furthermore, genetic tools for the manipulation of *S. lugdunensis* were limited until very recently, when efficient protoplast transformation (21) and electroporation (22) were reported. So, in contrast to *S. aureus*, only very few pathogenicity factors have been characterized. Among them, the fibrinogen binding protein Fbl is similar in structure and organization to *S. aureus* clumping factor A (ClfA) (23–25) and is probably the only fibrinogen-binding surface protein of *S. lugdunensis* (21). A von Willebrand factor-binding protein, denoted vWbl, has also been described (26). The ability of *S. lugdunensis* to bind von Willebrand factor may play a critical role in the bacterial attachment to vascular lesions, and thereby to colonization preceding infections (22). In 1997, Donvito et al. (27) observed synergistic hemolytic activity of *S. lugdunensis* with the *S. aureus* β-hemolysin. This synergistic hemolysis is a function of three small peptides, named *S. lugdunensis* synergistic hemolysins A (SLUSH-A), SLUSH-B, and SLUSH-C, encoded by the *slush* operon (27), which is con-

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Editor: D. J. Diekema
Address correspondence to Martine Pestel-Caron, martine.pestel-caron@chu-rouen.fr.

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trolled by *agr* activity (28). As phenol-soluble modulin β-like peptides, SLUSH peptides attract and stimulate human leukocytes in a formyl peptide receptor 2-dependent manner (29) and thereby play a proinflammatory role. Besides, *S. lugdunensis* is unique among the CoNS in that it contains a gene encoding an iron-regulated surface determinant (Isd) system (30), which shares significant structural and functional homology with the Isd receptors of *S. aureus*. Under iron-restricted conditions, the Isd proteins cooperate to capture heme and transfer it across the wall into the cytoplasm, where it is degraded to iron for use in cellular processes (31, 32).

Interestingly, the genome sequencing of two strains of *S. lugdunensis*, N920143 (30) and HKU09-01 (33), revealed genes encoding putative virulence factors, such as adhesins, toxins, and hemolysins. Among them is a gene denoted SLUG_16930 in the N920143 genome that encodes a putative fibrinogen/fibronectin binding adhesin named FbpA homologue, which is homologous to FbpA of *S. aureus* (30, 34, 35). The *hlb* gene encodes a putative sphingomyelinase β-toxin of 329 amino acids (aa), which shares 85% identity with that of *S. aureus* (30, 36), and a putative hemolysin III (denoted SLUG_09050 in the N920143 genome [30]) is encoded as well.

Likewise, bifunctional autolysins may contribute to bacterial pathogenesis by releasing virulence factors (37), and they may serve as adhesins, allowing cells to bind to surfaces and/or the host extracellular matrix and plasma proteins, as described for AtlA of *S. aureus* (38) and AtlE of *S. epidermidis* (39). Like AtlA and AtlE, AtlL of *S. lugdunensis* displays two enzymatic domains separated by three repeat domains ($AtlL_{R1}$ to $AtlL_{R3}$) and undergoes proteolytic processing to generate two extracellular peptidoglycan hydrolases, an *N*-acetylmuramoyl-L-alanine amidase (AM) and an *N*-acetylglucosaminidase (GL) with two ($AtlL_{R1R2}$) and one ($AtlL_{R3}$) repeated sequences harboring glycine tryptophan motifs, respectively (40). We recently reported that a Δ*atlL* mutant showed a significant reduction in biofilm formation and virulence attenuation using the *Caenorhabditis elegans* model (41). Since the overall organization of the bifunctional precursor protein is highly conserved in all staphylococcal species (42), the virulence findings for AtlA and AtlE may thus also apply for the entire *Staphylococcus* genus (43).

In an attempt to better understand the genetic background and population structure of *S. lugdunensis*, we recently developed the first multilocus sequence typing (MLST) scheme for this species (44). This phylogenetic analysis revealed a clonal population structure, a mutational evolution of this pathogen, and a lack of hypervirulent lineages.

Due to their exposure to frequent environmental changes, e.g., immune system responses, virulence and virulence-associated genes may evolve more rapidly than housekeeping genes (45, 46). Consequently, these genes may provide a higher degree of nucleotide sequence polymorphisms, a higher discriminatory power for epidemiology studies (45, 46), and some data on the specific evolution of virulence- or putative virulence-associated loci in comparison with that of housekeeping genes. Chen et al. (47) showed that MVLST provided subtyping data to define the genetic lineages in epidemic clones of *Listeria monocytogenes*. In this study, we analyzed the polymorphisms of virulence-associated genes (*vwbl* and *isdJ*) and putative virulence-associated loci ($atlL_{R2}$, $atlL_{R3}$, *hlb*, SLUG_16930, and SLUG_09050), determined the *agr* alleles, and analyzed the presence of virulence genes (*fbl* and *slush*) by PCR for 93 *S. lugdunensis* clinical and carriage isolates. The goals of this work were to: (i) develop a multi-virulence-locus sequence typing (MVLST) approach, (ii) compare the discriminatory power of MVLST to that of MLST, and (iii) generate informative sequence data for studying the virulence of *S. lugdunensis*.

MATERIALS AND METHODS

Bacterial strains. Ninety-three *S. lugdunensis* isolates were studied. The set included 3 *S. lugdunensis* reference strains (ATCC 49576, ATCC 43809, and ATCC 700328), 84 human clinical isolates collected between 1991 and 2011 from 8 different geographic sources and 3 countries (France, Belgium, and Slovenia) (44), and 6 *S. lugdunensis* carriage strains isolated from the groin, perineum, nail, toe, and axilla of healthy subjects from Kronoberg County in Sweden (1) or from Tours in France (48). The genome sequence data of the *S. lugdunensis* N920143 strain (30) were included.

MLST. MLST was performed for the 6 carriage strains, as previously described (44). Allelic profiles and sequence types (ST) were assigned by using the international MLST database (www.pasteur.fr/mlst). The STs were clustered into different clonal complexes (CCs) using the BURST program (http://pubmlst.org/analysis/burst/burst.shtml).

Multilocus sequence analysis of virulence-associated loci. A first set of 10 isolates representative of the main clusters previously defined by MLST (44) were screened for 10 known or putative *S. lugdunensis* virulence loci: *agr*, $atlL_{R2}$ (AM cell wall-anchoring [CWA] domain), $atlL_{R3}$ (GL CWA domain), *fbl*, *hlb*, *isdJ*, locus SLUG_09050, locus SLUG_16930, *slush*, and *vwbl*.

A locus was validated as a good candidate for multilocus sequence typing analysis if it (i) was present in all strains, (ii) required a single primer pair for PCR amplification and sequencing of the internal fragment, and (iii) contributed to a high discriminatory power.

Finally, seven putative virulence-associated loci were selected ($atlL_{R2}$, $atlL_{R3}$, *hlb*, *isdJ*, SLUG_09050, SLUG_16930, and *vwbl*) for MVLST (Table 1). The genomic locations of all loci studied are shown in Fig. 1.

The genomic DNA of *S. lugdunensis* was extracted from a single bacterial colony using the InstaGene matrix kit (Bio-Rad), as recommended by the manufacturer. The PCRs were performed in a final volume of 25 μl containing 0.50 μM each primer (Table 1), 12.5 μl of GoTaq (green master mix [Promega]), and 3 μl of extracted DNA. The PCR mixtures were heated for 3 min at 95°C, followed by 35 cycles of a denaturation step at 95°C for 30 s, an annealing step for 30 s at a temperature of 55°C, an elongation step at 72°C for 30 s, and ending with an extension step at 72°C during 10 min. The PCR products were then purified with a NucleoSpin gel and PCR clean-up kit (Macherey-Nagel) and sequenced with the same primers using the GenomeLab DTCS quick start kit on a GenomeLab GeXP analyzer (both Beckman Coulter). Different sequences of a given locus were given allele numbers, and each unique combination of alleles (multilocus allelic profile) was assigned to a virulence type (VT). Single point polymorphisms were assessed by resequencing DNA from two separate PCR experiments.

Computer analysis of sequence data. Clustering of the 92 isolates and of *S. lugdunensis* N920143, whose gene sequences were extracted from the available genome, was determined using the BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html) for alignment of the nucleotide sequences, and gene trees were constructed from the sequence data using the neighbor-joining method and bootstrapping algorithms contained in the MEGA 5.1 software (49). Simpson's index of diversity was calculated as proposed by Hunter and Gaston (50). The average numbers of nucleotide differences between the alleles and the ratios of nonsynonymous to synonymous substitutions (d_N/d_S) were calculated using the S.T.A.R.T.2 program (51). The codon alignments were then submitted to a suite of phylogenetic analysis tools (http://www.datamonkey.org/) for selection analysis (52, 53). In addition, Tajima's test of neutrality was performed using DnaSP version 5 (54) for each alignment.

TABLE 1 Primers used for the amplification and sequencing of the 10 loci investigated

Gene	PCR and sequencing primers (5′→3′)	Study
atlL$_{R2}$	Forward: GCTATAAACAATGATTCATCATC Reverse: GTGCGTATGCCTGAATTATT	This study
atlL$_{R3}$	Forward: GCGTACATTAGCTGTTAAAACAA Reverse: CGTCATAGAAATAGCTATCC	This study
hlb	Forward: AAAGCGGATTATATCCAAGGTCAAG Reverse: GCGTTGCCCTCGTATTGTTTAG	This study
isdJ	Forward: TGTTAAACCAGAAACATTGGCACAA Reverse: GCTGGTTTGGTTTTTGGTG	This study
SLUG_09050	Forward: TAATGCTGTTTCGCACGGAGTTGC Reverse: GACGCCTACCCATCCCATTACAA	Szabados et al. (35)
SLUG_16930	Forward: GAGATTACTGGACAACAAACG Reverse: GTATTGTGACGTCGTTTCCTG	Szabados et al. (35)
vwbl	Forward: TGGCGGGATGATTTGGACG Reverse: CCTCATTAAGATGGAGATGAATGC	This study
slush	Forward: TCGTAGATGCAATTTCAAAAGC Forward: TTTCGTCTTTGCACACACATTTCCA[a] Reverse: ACAGCACAAAGCCTTAACTATCTCA	This study Szabados et al. (35)
fbl	Forward: TGTAACAGCTACTGCTAAAGCACC Reverse: ATCCCATGCCATAGAAACTCTAGTG	This study
agrBDC	Forward: GGTCATCATTGTTGTGCCACATTC Reverse 1: GCGCAGAACCAAAGATTGCTAAAAC[b] Reverse 2: CTGCCAAAAACAACAAATAATACG[c]	This study

[a] Primer used to amplify and sequence the complete *slush* locus for Sl9.
[b] Primer used for *agr-1*$_{Sl}$ variant (57).
[c] Primer used for *agr-2*$_{Sl}$ variant (57).

The index of association (I_A) between alleles (55) was used to test for linkage disequilibrium between the alleles of the seven loci analyzed (http://www.mlst.net).

The BURST program (http://pubmlst.org/analysis/burst/burst.shtml) was used to define groups in which every isolate shares at least five identical alleles with at least one other isolate (CC_vs) and to characterize ancestral genotypes and their corresponding single-locus variants (isolates that differ at only one of the 7 loci) within these clonal complexes.

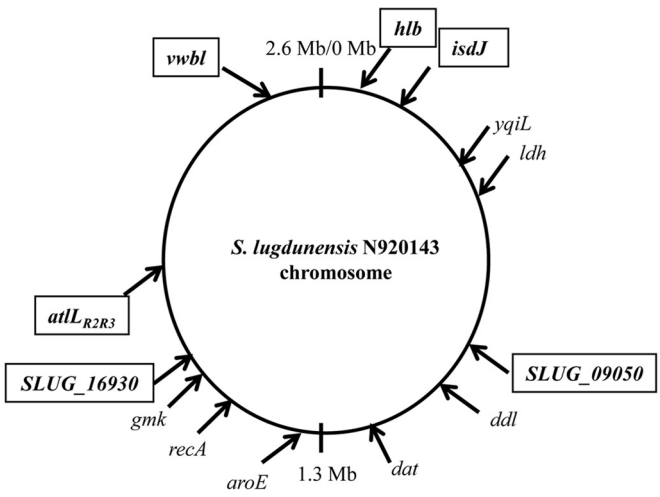


FIG 1 Genomic locations of the loci analyzed in the *S. lugdunensis* N920143 genome. The seven selected virulence loci are boxed and shown in bold type. The housekeeping genes of the MLST scheme are shown in italics (44).

Detection of *agr* alleles and virulence factor genes. All isolates were screened for *fbl* and the *slush* operon, and the *agr* alleles were determined by PCR and sequencing of an internal fragment. Genomic DNA extraction, PCR amplification, and sequencing were performed as described above, using the primers listed in Table 1. The PCR products were electrophoresed on 1% agarose gels and stained with ethidium bromide to visualize the DNA bands, whose sizes were determined using a 100-bp DNA ladder (New England BioLabs).

Synergistic δ-hemolysis. All strains were tested for synergistic hemolysis, as previously described (56) by measuring synergistic hemolysis in the presence of β-toxin secreted by *S. aureus* strain RN4220 on Trypticase soy agar plates supplemented with 5% sheep blood (bioMérieux, France). Briefly, strains of *S. lugdunensis* were streaked down perpendicular to but not touching the center streak. The test was positive when a zone of complete hemolysis was observed after incubation at 37°C for 24 h and held at room temperature for an additional 24 h (2).

Nucleotide sequence accession numbers. The nucleotide sequences of the internal fragment loci (*atlL*$_{R2}$, *atlL*$_{R3}$, *hlb*, *isdJ*, SLUG_09050, SLUG_16930, and *vwbl*) analyzed in this work have been deposited in GenBank under accession no. KJ816790 to KJ816797 for *atlL*$_{R2}$, KJ816798 to KJ816806 for *atlL*$_{R3}$, KJ816826 to KJ816835 for *hlb*, KJ816836 to KJ816849 for *isdJ*, KJ816821 to KJ816825 for SLUG_09050, KJ816807 to KJ816820 for SLUG_16930, and KJ816850 to KJ816858 for *vwbl*.

TABLE 2 Genetic polymorphisms of the seven virulence-associated genes analyzed by MVLST

Gene	Size of PCR product (bp)	Positions of the fragment analyzed within each gene[a]	No. of alleles	No. (%) of polymorphic sites	d_N/d_S[b]	d_N	d_S	Tajima's D test[c]	SLAC (95% CI)[d]
$atlL_{R2}$	650	1879–2418	8	8 (1.47)	0.416	0.0042	0.01	−0.13385	0.647 (0.277–1.250)
$atlL_{R3}$	590	2419–2859	9	9 (2.04)	0.258	0.0046	0.0179	−0.43781	0.338 (0.121–0.728)
hlb	554	274–714	10	12 (2.72)	0.024	0.0012	0.0471	0.38138	0.092 (0.015–0.285)
isdJ	498	971–1382	14	10 (2.42)	0.103	0.0034	0.033	0.90961	0.166 (0.066–0.336)
SLUG_09050	406	124–457	5	4 (1.20)	0	0	0.0282	0.95707	0 (0–0.203)
SLUG_16930	559	586–1037	14	9 (1.99)	0.256	0.0044	0.0171	0.42415	0.345 (0.158–0.643)
vwbl	461	932–1275	9	7 (2.03)	0.133	0.0025	0.0192	0.70819	0.178 (0.044–0.463)

[a] Fragment positions are based on the genome of *S. lugdunensis* N920143.
[b] d_N/d_S, ratio of nonsynonymous to synonymous substitutions.
[c] $P > 0.1$ for all Tajima's D test results.
[d] SLAC, single-likelihood ancestor counting; CI, confidence interval.

RESULTS

Distribution of *agr* alleles, *fbl* gene, and *slush* operon. All *S. lugdunensis* isolates tested were shown to be *fbl* and *slush* positive. Interestingly, the *slush* amplicon of *S. lugdunensis* strain SL9 was shorter than the others. The sequencing of this locus showed a 125-bp deletion between *slushB* and *slushC*.

The polymorphism rate of the *agr* locus was too high to design a unique primer pair for PCR amplification and double-strand sequencing. However, an internal fragment was amplified and sequenced for all strains in the study. Of the 93 strains, 53 (6 carriage strains, 3 reference strains, and 44 clinical strains) had $agr\text{-}1_{SL}$, and 40 clinical strains had $agr\text{-}2_{SL}$ (57). The genomic data available for *S. lugdunensis* N920143 allowed us to determine an $agr\text{-}1_{SL}$ allele.

Delta-like synergistic hemolytic activity. Seventy-two strains (77.4%) gave a clear zone of synergistic complete hemolysis when tested against the β-toxin of *S. aureus* RN4220. Of note, the SL9 strain was positive for this test in spite of an incomplete *slush* locus.

Allelic variation in known and putative virulence-associated loci of *S. lugdunensis*. Seven known and putative virulence-associated loci were selected ($atlL_{R2}$, $atlL_{R3}$, *hlb*, *isdJ*, SLUG_09050, SLUG_16930, and *vwbl*) for MVLST. Primers successfully amplified all seven MVLST loci (2,967 nucleotides in total) from the 93 strains. The data reporting the allelic variations in the seven virulence-associated loci studied are summarized in Table 2. The number of individual alleles for each of the seven genes ranged from 5 (for SLUG_09050) to 14 (for *isdJ* and SLUG_16930). The number of polymorphic sites on a given locus varied from 4 (1.20%) for SLUG_09050 to 12 (2.72%) for *hlb*. Polymorphisms resulted for most genes in synonymous substitutions, with a ratio of nonsynonymous to synonymous substitutions ranging from 0 (for SLUG_09050) to 0.133 (for *vwbl*). The higher d_N/d_S ratio for $atlL_{R3}$ (0.258), $atlL_{R2}$ (0.416), and SLUG_16930 (0.256) than that of *hlb* and *isdJ* might suggest a potential role of environmental selective pressure in their evolution, but in Tajima's test, no significant difference from neutral evolution was displayed for these 3 loci compared to that of the others. Single-likelihood ancestor counting (SLAC) analysis of the data (Table 2) and the mixed-effects model episodic (MEME) program (data not shown) also provided no evidence for positive selection of any amino acid of either protein.

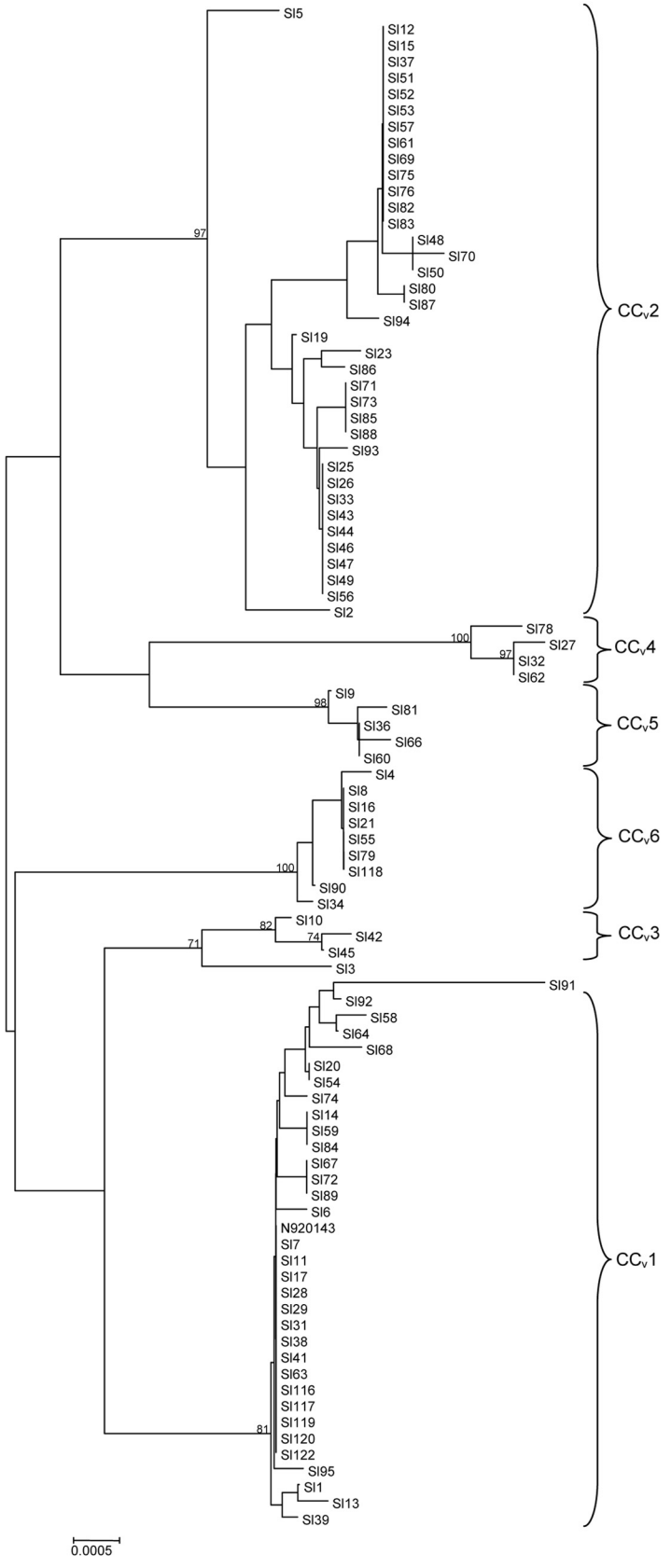
Multilocus sequence data and composite sequence-based analysis of virulence-associated loci. Multilocus allelic profile analysis based on the compilation of the seven loci, as well as the 2,967-bp composite sequence-based analysis, generated 43 different VTs among the 93 strains and the DNA sequence data of *S. lugdunensis* N920143. The phylogenetic relationships between the 93 isolates and the *in silico* data of *S. lugdunensis* N920143 are shown in Fig. 2.

Four major VTs (VT-1, VT-18, VT-31, and VT-35) were shared by five to 14 isolates, and eight VTs (VT-4, VT-5, VT-7, VT-20, VT-26, VT-36, VT-38, and VT-40) were shared by two to four isolates, but the majority (32 of 43 VTs) of these VTs were represented by a single isolate (Fig. 2), reflecting the genetic diversity of the population studied.

No correlation was found between the virulence-associated locus pattern and geographic origin, or even with clinical presentation. Indeed, 4 VTs were shared by isolates from different geographic and clinical sources; for example, for VT-1, including isolates from Belgium, France (Bordeaux, Nantes, and Rouen), and Sweden, and carriage isolates, as well as isolates collected from patients with a wide spectrum of infections (bacteremia, osteoarticular infections, SSTI, and material device-associated infections). Similarly, the carriage strains did not cluster in a separate lineage; 5 of 6 commensal strains were clustered with clinical isolates in VT-1 (Fig. 2). Of note, the 6 isolates exhibiting glycopeptide tolerance (44) belonged to 4 VTs (VT-1, VT-17, VT-25, and VT-31) and did not define a specific lineage.

The analysis using the BURST algorithm (58) defined six CC_vs, CC_v1 to CC_v6, including almost all VTs and two singletons (VT-25 and VT-29). Two majority CC_vs (CC_v1 and CC_v2) contain 27 VTs, including 71 strains. Ancestral genotypes and their single-locus variants were identified for each CC_v (Fig. 2). Of note, the strains associated with infections on material devices clustered mainly (11/12) in CC_v2.

Contribution of recombination and mutations to genomic evolution of *S. lugdunensis*. Monolocus dendrograms were globally congruent, highlighting that recombination events are scarce but can exist for the SLUG_16930 locus of *S. lugdunensis* strains SL78 and SL91. However, no statistically significant evidence for recombination was found by SplitsTree analysis and phi test (data not shown). A quantitative analysis of the linkage between the alleles from the seven loci studied was performed by calculating the index of association (I_A) to test the probability that recombination occurred (55). A significant linkage disequilibrium was



VTs	STs (ref)	Clinical Sources	Origins
34	3	NC	A
35	3	SST	A
35	3	NC	F
35	16	B	A
35	3	M	B
35	3	SST	B
35	3	M	B
35	3	OA	B
35	3	other	D
35	3	SST	D
35	3	M	C
35	20	OA	C
35	3	SST	C
35	3	OA	E
36	3	SST	B
37	3	SST	D
36	3	SST	B
38	3	SST	C
38	3	OA	E
24	3	other	A
21	2	M	A
22	2	SST	A
19	2	SST	E
20	2	OA	D
20	19	B	C
20	2	M	E
20	2	SST	E
23	2	M	A
18*	2	M	A
18*	2	M	A
18*	2	M	A
18*	2	M	G
18*	2	M	G
18*	2	SST	B
18*	2	SST	B
18*	2	SST	B
18*	14	OA	B
17	8	B	A
41	4	other	C
39	4	OA	A
40*	4	SST	A
40*	17	B	D
13	5	B	A
28	5	B	C
26*	5	SST	A
27	18	B	D
26*	5	OA	D
30	10	REF1	REF1
31*	10	REF2	REF2
31*	10	NC	F
31*	10	SST	A
31*	10	SST	B
31*	10	other	C
44	10	Ca	J
33	10	other	A
32	10	SST	A
14	13	OA	A
43*	13	OA	G
42	13	SST	G
25	9	B	A
29	1	SST	A
12	1	SST	A
9	1	OA	B
8	1	SST	D
10	7	B	D
7	1	SST	A
7	1	OA	B
6	12	SST	C
4	1	NC	F
4	6	SST	B
4	15	other	E
5	1	OA	D
5	1	OA	C
5	6	NC	H
11	6	OA	A
1*	1	SST	K
1*	15	REF3	REF3
1*	6	OA	A
1*	6	NC	A
1*	6	OA	A
1*	1	M	A
1*	12	SST	A
1*	12	NC	NC
1*	12	NC	G
1*	15	OA	D
1*	1	Ca	J
1*	1	Ca	J
1*	1	Ca	J
1*	1	Ca	J
1*	1	Ca	I
2	6	B	A
15	15	B	A
16	6	B	A
3	12	OA	A

detected when all isolates were included (I_A, 3.74) and when one isolate per VT was used (I_A, 2.56). Likewise, the G+C contents of each locus were closed to each other and similar to the G+C percentage of the whole genome, with a G+$C_{N920143}$ of 33.8%, and that of the loci as follows: G+C_{atlLR2}, 34.4%; G+C_{atlLR3}, 32.32%; G+C_{hlb}, 34.12%; G+C_{isdJ}, 34.79%; G+C_{SLUG_09050}, 34.59%; G+C_{SLUG_16930}, 32.07%; and G+C_{vwbl}, 35.48%.

The congruence of monolocus dendrograms associated with the linkage disequilibrium observed and the lack of intragenic recombination from extraspecies sources argue for a clonal population structure for *S. lugdunensis*.

Comparison of MVLST and MLST. A total of 87 strains (3 reference strains and 84 clinical isolates) investigated in this study were previously analyzed by MLST (44). In the present study, the STs of *S. lugdunensis* N920143 and of six carriage strains of *S. lugdunensis* were determined using the Institut Pasteur MLST databases (www.pasteur.fr/mlst). Five of them (4 from Sweden and one from France) clustered in ST1, whereas the sixth one from Sweden belonged to ST10 (Fig. 2). Therefore, a total of 20 STs were generated for the 93 strains and N920143.

The MVLST scheme allowed us to define 43 VTs, which is much more discriminant than the MLST scheme (44). Indeed, virulence-associated loci generated more allelic variations (5 to 14 alleles) than did the housekeeping genes (4 to 9). A slightly higher polymorphism rate (1.20 to 2.72%) was observed for the virulence-associated loci compared to that of housekeeping genes (0.9% to 1.8%). The comparison of dendrograms based on the concatenated gene sequences from the MLST gene set and the MVLST gene set showed that strains were distributed in the same clusters, leading to a congruence of dendrograms (Fig. 2). The congruence of the MLST and MVLST monolocus and multilocus dendrograms associated with a lack of recombination events (I_A was similar for both schemes) further argue for a clonal structure of *S. lugdunensis*.

However, MVLST further resolved all the clusters in which the isolates were indistinguishable by MLST, as a given ST included 3 (in STs 4, 5, 12, 13, and 15) to 8 VTs (in ST1). Isolates from a given VT belonged to 1 to 4 STs that were very closely related, and all were included in CC1 (44). Of note, the 9 isolates that represented one ST as determined by MLST were also found to be singletons by MVLST. Together, these results show that MVLST provides more discrimination than does MLST, as confirmed by the Simpson's index values (D_{MVLST}, 0.943; D_{MLST}, 0.890) (Table 3). The classification of the isolates into MVLST clonal complexes by the BURST program showed groupings that were highly concordant with those determined by MLST. In addition, CC_v2 defined by MVLST gathered isolates with STs included in two MLST-defined CCs (CC2 and CC3) (44). Furthermore, two new CC_vs (MVLST-defined CC_v3 and CC_v6) were defined; CC_v3 consists of ST13 isolates, whereas CC_v6 contains ST10 isolates.

Multilocus sequence data based on two up to six loci. In order to provide a more suitable tool for studying the virulence characteristics of *S. lugdunensis*, a multilocus allelic profile analysis based on the compilation of two up to six loci was performed for the 93 isolates and the DNA sequence data of *S. lugdunensis* N920143. The allelic variation of *isdJ* and the SLUG_16930 locus generated a maximum of 27 different VTs, which is seven more than that of the seven housekeeping gene analysis. The number of VTs identified for the 93 isolates and N920143 was 33 (of which 22 singletons) when analyzing the polymorphisms of $atlL_{R2}$ or $atlL_{R3}$ and *isdJ* and SLUG_16930, and up to 37 (of which 23 singletons) when analyzing *atlLR3*, *hlb*, *isdJ*, and SLUG_16930. The combination of five and six loci generated 41 (with $atlL_{R2}$, $atlL_{R3}$, *hlb*, *isdJ*, and SLUG_16930) and 43 VTs (with $atlL_{R2}$, $atlL_{R3}$, *hlb*, *isdJ*, SLUG_16930, and *vwbl*), respectively. In conclusion, a trilocus sequence typing scheme based on the intragenic regions of *atl* (encoding a bifunctional autolysin), *isdJ* (encoding an iron-regulated surface determinant protein), and SLUG_16930 locus (encoding an FbpA homologue) were successful for strain differentiation within *S. lugdunensis* species and may provide an alternative to MLST and MVLST for discriminating isolates ($D_{trilocus}$, 0.922) (Table 3).

TABLE 3 Comparative genetic diversity characteristics of MVLST, MLST, and trilocus analysis

Sequencing typing method	Size of concatenated nucleotidic sequences (bp)	No. (%) of polymorphic sites	Simpson's index (95% CI)[a]	*P* value
MLST	3,037	39 (1.28)	0.890 (0.862–0.917)	
MVLST	2,967	59 (1.99)	0.943 (0.918–0.967)	0.001[b]
Trilocus	1,407	27 (1.92)	0.922 (0.893–0.952)	0.075[c]/0.016[d]

[a] CI, confidence interval.
[b] *P* value between Simpson's index values of MLST and MVLST.
[c] *P* value between Simpson's index values of MLST and trilocus analysis.
[d] *P* value between Simpson's index values of MVLST and trilocus analysis.

DISCUSSION

Because *S. lugdunensis* is more virulent than other CoNS, it is worthwhile to examine the differences in the repertoire and polymorphisms of genes encoding virulence factors. The aim of the present study was thus to design a MVLST scheme based on the allelic polymorphisms of seven virulent-associated loci, which may evolve more rapidly than housekeeping genes (45, 46) and should therefore be more discriminative than MLST. We wanted to further delineate the lineages and clonal groups previously defined by MLST (44) and characterize the links between within-species genetic variations and characteristics, such as pathogenic potential, virulence, and epidemiology.

Ninety-three *S. lugdunensis* strains from diverse clinical and geographic sources were studied; 6 carriage strains and the genome sequence of N920143 (30) were added to the collection of 87 strains previously characterized by MLST (44). As systems to genetically manipulate *S. lugdunensis* succeeded only very recently (21, 22), a few virulent factors are known to date. We selected

FIG 2 Dendrogram showing genetic relationships of 93 *S. lugdunensis* isolates and the genome sequence data from *S. lugdunensis* N920143 based on the composite sequence of seven virulence-associated loci ($atlL_{R2}$, $atlL_{R3}$, *hlb*, *isdJ*, SLUG_09050, SLUG_16930, and *vwbl*). N920143 represents *S. lugdunensis* N920143 strain (*in silico* data). VTs, virulence types; STs, sequence types previously described (44). Abbreviations (for clinical sources): OA, osteoarticular; SST, skin and soft tissue; M, material device; B, blood; Ca, carriage. Abbreviations (for origins): A, Rouen (France); B, Nantes (France); C, Nancy (France); D, Bordeaux (France); E, Montpellier (France); F, Versailles (France); G, Louvain (Belgium); H, Maribor (Slovenia); I, Tours (France); J, Kronoberg County (Sweden); K, Lyon (France). CC_v, clonal complexes. The asterisks represent the ancestral VT for each CC_v. REF1 (reference sequence 1), *S. lugdunensis* ATCC 700328; REF2, *S. lugdunensis* ATCC 43809; REF3, *S. lugdunensis* ATCC 49576. NC, unknown.

genes encoding proteins that can partly explain the particular virulence of *S. lugdunensis*, such as adhesins (Fbl, vWbl, a putative fibrinogen binding protein [SLUG_16930 and FbpA homologue]) (25, 26, 35), hemolysins (SLUSH, a putative β-hemolysin [SLUG_16930], and a putative hemolysin III [SLUG_09050]) (27, 30) and IsdJ, a protein of an iron acquisition system, which of the CoNS, is only used by *S. lugdunensis* (30, 32). Besides theses virulence-associated loci, we analyzed the polymorphisms of the repeated sequences ($atlL_{R2}$ and $atlL_{R3}$) of the putative CWA domains of the AM and GL autolysins, respectively, generated by the proteolytic cleavage of the bifunctional murein hydrolase, AtlL (40). Indeed, AtlL might play a role in virulence, as it has been shown *in vitro* through biofilm formation and *in vivo* in the *C. elegans* model (41). As AtlL presents high identity percentage with all staphylococcal bifunctional autolysins (40, 42, 43), it might be involved in the internalization of the bacteria in the eukaryote cell (59). Furthermore, the *agr* quorum-sensing and signal transduction system, which has been described as a global regulator of virulence gene expression in *S. aureus*, was also studied (60).

The *fbl* gene, which was detected in all 93 isolates, was not selected for the MVLST scheme because of its very low polymorphism rate (<0.65%, data not shown). This result confirms that *fbl* is a good PCR target for the *S. lugdunensis* identification (61). The *slush* operon was also detected in all the strains of our collection, unlike in Svabados et al. (35), who detected it only in 28 strains of 58 with the same primers. Shown to mediate the synergistic δ-like hemolytic activity of *S. lugdunensis* (27) and to stimulate human leukocytes via interaction with the formyl-peptide receptor 2 (29) as phenol-soluble modulin peptides, the SLUSH peptides display an important role in the virulence of this species. Interestingly, 21 of 93 (22.6%) of the strains did not exhibit hemolytic activity on an agar plate despite the presence of the *slush* operon; of note, hemolysis was observed for the strain with an incomplete operon (SL9), suggesting that (i) the deleted portion of the *slush* gene is not directly involved in the hemolytic activity or (ii) hemolysis is regulated, implying the presence of compensatory hemolysins (62). This does not seem to involve *agr* allelic variation, as hemolysis was observed irrespective of the $agr\text{-}1_{SL}$ or $agr\text{-}2_{SL}$ variants. The detection of the only two *agr* types ($agr\text{-}1_{SL}$ and $agr\text{-}2_{SL}$) originally described by Dufour et al. (57) in this large set of strains suggests a limited allelic variation of this locus for *S. lugdunensis* compared to that of *S. aureus* (57, 60).

Thus, a total of seven loci were chosen ($atlL_{R2}$, $atlL_{R3}$, *hlb*, *isdJ*, SLUG_09050, SLUG_16930, and *vwbl*) to design the first MVLST scheme of this rare but aggressive pathogen. The analysis of an internal fragment of each virulence-associated locus showed a moderate polymorphism rate (1.20% to 2.72%), but this was higher than that of housekeeping genes (44). The low apparent rates of recombination associated with a high index of association between the alleles of the different loci and the congruence of the dendrograms of individual virulence-associated genes and of that of both schemes confirm a clonal population structure for *S. lugdunensis*. Thus, if homologous recombination does exist, it rarely contributes to the evolution of *S. lugdunensis*. The d_N/d_S ratio obtained for $atlL_{R2}$, $atlL_{R3}$, and SLUG_16930 (FbpA homologue) was higher than that observed for the other loci, but the different tests of selection performed provided no evidence for positive selection of any amino acid in either protein. Of note, the nonsynonymous mutation rates were higher in the $AtlL_{R2}$ domain (d_N/d_S, 0.416) than in the $AtlL_{R3}$ domain (d_N/d_S, 0.258), unlike the data of AtlE of *S. epidermidis*, for which the $AtlE_{R3}$ was the most polymorphic domain (63). The allelic variation in these repeated sequences might reflect differences in the target sites of both hydrolytic enzymes, AM-R1-R2 and GL-R3, at the murein structure (42), and it might affect their adhesion ability. Interestingly, Bose et al. (64) recently showed that *S. aureus* AM and GL have nonredundant functions in cell division, autolysis, and biofilm formation. By homology with *S. aureus* AtlA-AM, *S. lugdunensis* AtlL-AM might have different contributions from those of GL, but the different statistical tests used did not confirm any environmental selective pressure on the $atlL_{R2}$ locus. In this context, it would be interesting to further explore whether $AtlL_{R2}$ and $AtlL_{R3}$ are pathogenicity markers.

Forty-three VTs were defined among the 93 isolates and the genome sequence data of N920143 by MVLST, demonstrating additional resolution compared to that found in a previous MLST study (44); six divergent lineages were characterized, among which 2 (CC_v3 and CC_v6) were newly generated compared to those characterized with MLST. The distribution of the clinical strains in the MVLST dendrogram did not allow us to identify hypervirulent lineages, but the majority of strains collected from infections associated with material devices were distributed in CC_v2. The 6 carriage strains that belonged to ST1 (CC1) and ST10 were clustered with clinical strains in CC_v1 and CC_v6 by MVLST. To further characterize the virulence traits of *S. lugdunensis*, it might be interesting now to explore the allelic variation of other virulence determinants (other surface-anchored proteins, for example) or regulatory genes whose polymorphisms can affect the expression of virulence genes (65). Of note, the monolocus MVLST analysis showed that the polymorphisms of three loci, such as atl_{R2}, *isdJ*, and SLUG_16930, generated 33 different allelic profiles and therefore is well suited to short-term epidemiology. atl_{R3} was preferred to atl_{R2} because of the number of polymorphic sites.

Finally, this work provides the first MVLST scheme described for *S. lugdunensis*. This MVLST analysis (i) confirms the clonal population structure and the mutational evolution of this species and (ii) reveals a coevolution of the virulence-associated loci studied with housekeeping genes. However, MVLST offers higher resolution than MLST for *S. lugdunensis* typing for macroepidemiological purposes, and a trilocus sequence typing scheme might be proposed as a tool for microepidemiological ones. Such methods provide unambiguous and portable data, unlike pulsed-field gel electrophoresis (PFGE), which until recently was the only other typing method described (48) for this species. It would be informative then to enrich these results with sequence data from worldwide strains (in particular carriage ones) and to perform a comparative analysis of the whole-genome sequence of carriage and invasive strains of *S. lugdunensis* to further characterize the virulence determinants of this unusually aggressive CoNS.

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