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Original Article

Molecular characterization of lugdunin inactivation mechanisms and their association with *Staphylococcus lugdunensis* genetic types

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Abstract *Background and purpose:* Our previous studies showed that lugdunin activities are associated with *Staphylococcus lugdunensis* genotypes, and most isolates do not exhibit lugdunin activity. As a continuation of our previous analysis, we focused on the reasons for defects in lugdunin production in *S. lugdunensis* clinical isolates.
Methods: A comparative analysis of 36 *S. lugdunensis* whole genome sequencing data revealed three major mutation types, unknown deletion mechanism that caused most of *lug* operon genes lost, mobile genetic element (MGE) insertion, and nonsense mutations, which potentially damaged lugdunin production. A total of 152 *S. lugdunensis* clinical isolates belonging to lugdunin nonproducers were further examined for the above three mutation types. PCR products were sequenced to examine these variations.
Results: Forty-six of the 152 isolates were CRISPR-Cas IIC isolates, including 26 ST27, 14 ST4, and 6 ST29 isolates; further investigation confirmed that all of their *lug* operons had lost almost all *lug* operon genes except *lugM*. An IS*256* insertion in *lugA* was identified in 16 isolates, and most isolates (15 over 16) belonged to ST3. In addition, three nonsense mutations caused by

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single nucleotide substitutions (an adenine deletion in *lugB* at the 361th and 1219th nucleotides and an adenine deletion in *lugC* at the 1612nd nucleotide) that were frequently observed among 36 *S. lugdunensis* whole genome sequencing data were further observed in our clinical isolates. These three nonsense mutations were frequently found in most of CRISPR-Cas IIIA strains, especially in ST6 isolates.

Conclusion: Our findings suggest that the mechanisms affecting lugdunin production are associated with *S. lugdunensis* molecular types.

Introduction

Staphylococcus lugdunensis is a commensal coagulase-negative *Staphylococcus*. It is known for the high mortality rate because it causes infectious endocarditis[1] and other infections such as skin and soft tissue infection[2] or prosthetic joint infection[3]; thus, it is emerging as an important pathogen.[1] Epidemiological investigations through pan-genomic sequencing analyses showed that low diversities among *S. lugdunensis* genomic sequences may be caused by various restriction-modification systems, implying a conserved genome composition among this population.[4] This finding suggests that few horizontal gene transfers occur among this population. In contrast, different molecular types with unique traits may exist to differentiate between the strains. Our previous studies support this hypothesis that molecular type specificities, such as virulence factors,[5] drug-resistant genotypes and phenotypes,[6,7] and CRISPR-Cas systems,[8] occur in *S. lugdunensis* populations.

As a commensal, *S. lugdunensis* can produce and secrete a novel thiazolidine-containing cyclic peptide called "lugdunin" to suppress *Staphylococcus aureus* in a ferric-deficient environment.[9] In addition to interference with *S. aureus* growth, *S. lugdunensis* IVK28, a sequence type 3 (ST3) isolate, has a broad bioactivity against several gram-positive species, such as *Listeria monocytogenes*, *Enterococcus* spp., and *Streptococcus pneumoniae*.[9,10] We observed that lugdunin production is associated with strain molecular types and the *agr* system[11] and surprisingly found that most clinical isolates are lugdunin nonproducers.

According to the original lugdunin study, the *lug* operon was hypothesized to be horizontally transferred into *S. lugdunensis* and to function properly.[9] A functional *lug* operon is assumed to be beneficial for environmental competence; however, the study showed that most *S. lugdunensis* strains belong to lugdunin nonproducers, implying that other issues may play more important roles in the *lug* operon becoming dysfunctional than species competition. Since our previous study showed that lugdunin production is associated with the strain's genotype, it is reasonable to speculate that lugdunin nonproduction may be related to the strain's genotype. Thus, our present study aimed to focus on characterizing lugdunin nonproducing *S. lugdunensis* strains and to investigate the association of lugdunin nonproduction with the genetic types of these strains.

Materials and methods

Bacterial strains

A total of 202 *S. lugdunensis* clinical isolates were collected during 2003—2014 from Chang Gung Memorial Hospital (Linkou), Taiwan, which were also used in our previous study.[11] Among them, 152 clinical isolates were confirmed to be lugdunin nonproducers. All strains underwent molecular typing using multilocus sequence typing (MLST) and CRISPR-Cas typing, as previously described.[8] In the present study, CGMH-SL131 (SAMN13870883) was used as the lugdunin producer control and CGMH-SL118 (SAMN13870882) as the lugdunin nonproducer control.

Pairwise comparison of whole genome sequence data

A total of 36 complete whole genome sequence (WGS) data of the reference strains in GenBank were downloaded and analyzed. The accession numbers and information of all strains are listed in Table S1. The molecular types of each strain were further verified through Pasteur Institute's MLST database (https://bigsdb.pasteur.fr/staphlugdunensis/). The originally reported lugdunin producer IVK28[9] was used as the positive control, and its *lug* operon sequence was compared to those of the other 35 strains. Any sequence variations observed through a comparison with IVK28 that could lead to the dysfunction of the *lug* operon gene were considered as potential reasons causing lugdunin nonproduction. All potential reasons were further verified by examination of 152 clinical lugdunin nonproducers.

Lugdunin operon and gene cluster genotyping

To verify the variations among the 36 strains' *lug* operon-related genes, the IVK28 strain reported in the original lugdunin activity analysis[9] was considered as the wild-type strain, and the genomes sequences of the others were compared to its genome sequence. We verified that our CGMH-SL131 strain harbored identical *lug* operon sequences to the IVK28 strain. Further sequence variation examination was performed using the CGMH-SL131

Table 1 Primers used in this study.

Primer	Sequence (5′—3′)	Location (from the beginning of each gene's 5′ end)	Purpose	PCR Product Size (bp)
rpsI-F	gaatatagaggtacaggccg	*rpsI* 19-38	PCR to verify *lug* operon existence	*rpsI-F* + *cobB-R*: 2453
cobB-R	tcagtagcaagtggcatacc	*cobB* 82-101		*lugM-F* + *cobB-R*: 1182
lugM-F	gcagaagacgatgtacttgc	*lugM* 433-452		
lugA-1-F3	ggaaatcgagttctctgatg	*lugA* 1137-1156	PCR and sequencing to verify	1933 (with IS256 insertion)
lugA-1-R3	cagacagttttcatcttccc	*lugA* 1718-1737	IS256 insertion in *lugA*	601 (without IS256 insertion)
lugB-F	ttgagtccacagcagaaagg	*lugB* 22-41	PCR and sequencing to verify SNPs in *lugB*	1574
lugB-R1	aggagcgggtttgattcttc	*lugB* 1576-1595		
lugC-1-F2	gagctccgtatgcaatatg	*lugC* 1475-1493	PCR and sequencing to verify SNPs in *lugC*	1181
lugC-2-R2	agggaacttgttagctaaag	*lugC* 2636-2655		

sequence, and specific primer sets were designed to target the *lug* operon and lugdunin gene clusters: *lugA*, *B*, and *C*. All primers are listed in Table 1. The PCR conditions for each sequence variation examination are described as follows: *lugA*: 1x (98 °C, 5 s), 35x (98 °C, 5 s), (53 °C, 10 s), (72 °C, 30 s), 1x (72 °C, 5 min), *lugB:* 1x (98 °C, 5 s), 35x (98 °C, 5 s), (57 °C, 10 s), (72 °C, 30 s), 1x (72 °C, 5 min), *lugC:* 1x (98 °C, 5 s), 35x (98 °C, 5 s), (52 °C, 10 s), (72 °C, 30 s), 1x (72 °C, 5 min), *lug* operon: (98 °C, 5 s), 35x (98 °C, 5 s), (55 °C, 10 s), (72 °C, 30 s), 1x (72 °C, 5 min). To verify the structural integrity of the *lug* operon, three gene-specific primers were designed for *rpsI*, *lugM*, and *cobB*; the primer set of *rpsI-F* with *cobB-R* was designed to check whether the *lug* operon had complete structural integrity, and a primer set of *lugM-F* with *cobB-R* was designed as the internal control. The PCR conditions for *rpsI-F* with *cobB-R* were 1x (98 °C, 5 s), 35x (98 °C, 5 s), (53 °C, 10 s), (72 °C, 30 s), 1x (72 °C, 5 min), and the conditions for *lugM-F* with *cobB-R* were 1x (98 °C, 5 s), 35x (98 °C, 5 s), (57 °C, 10 s), (72 °C, 30 s), 1x (72 °C, 5 min). Agarose gel electrophoresis was performed to confirm the presence and size of the PCR products (Table 1), which were then sequenced to identify variations in these genes. Sixteen ST3 strains containing IS*256* inserted into *lugA* were sequenced and submitted to GenBank under the accession number BankIt2611873.

Results

Three variations were identified from a pairwise comparison of 36 *lug* operons

To elucidate the possible reasons leading to the lugdunin nonproducing activities, we performed a comparative investigation of the gene clusters of the *lug* operon using WGS data of 36 strains (Table S1). Three types of variation were identified when the *lug* operons of these 36 strains were compared: the unknown deletion mechanism caused most of *lug* operon genes lost, mobile genetic element (MGE) insertion, and nonsense mutations caused by frameshift mutations by a single nucleotide deletion or insertion (Table 2). Three strains, one ST5 (VISLISI_25, CP020763) and two ST27 (C_33, CP020768; RMLUG2, CP084434) isolates, were found to have lost most of their *lug* operon genes. A MGE insertion was found in JICS135 (ST3, AP021848), in which IS*256* was inserted in *lugA*. The comparative gene analysis found several single nucleotide variations (SNVs), and most of them caused silent and missense mutations; only a few led to nonsense mutations. A total of 24 SNVs were identified that led to nonsense mutations (20 deletions and four insertions) distributed in various *lug* operon genes (Table 2), and the most frequent deletions were adenine deletions at the following positions: *lugB* 360, *lugB* 1219, and *lugC* 1612.

CRISPR-Cas types are associated with an unknown deletion mechanism which caused most of the *lug* operon genes to be lost

One ST5 (VISLISI_25) and two ST27 isolates (C_33 and RMLUG2) were found that most of their *lug* operon genes were lost except *lugM*. Interestingly, in our lugdunin activity test, we found that all our ST27 isolates were lugdunin nonproducers, which led us to further analyze whether this unknown deletion mechanism is one of the possible reasons for lugdunin nonproduction. Fig. 1 shows the complete *lug* operon of the ST3 CGMH-SL131 strain (Fig. 1A). Specific primer sets between *rpsI* and *cobB* can only amplify fragments from isolates containing *lugM* and relative flanking region (Fig. 1B). Our results confirmed that all ST27 isolates' *lug* operon was lost for most of their genes, except *lugM*. In addition, all ST4 and ST29 isolates were lugdunin nonproducers, as reported previously[11] (Table S2); both of these STs and ST27 were CRISPR-Cas IIC isolates. We therefore examined all isolates of ST4 and ST29 and confirmed that all isolates of ST4 and ST29 have the same situation with ST27 that all their *lug* operons had lost most of their genes except *lugM*. We applied the same approach to the other STs isolates and found that this situation only existed in CRISPR-Cas IIC isolates.

Through WGS data analysis, we found that all *lug* operons were located between two genes, *rpsI* and *cobB*. According to the different GC contents between *lug* operon and whole genome, the original study of lugdunin suspected that this region may be horizontal transferred into *S. lugdunensis*.[9] Following this rationale, we speculated that the sequences near the 5′- and 3′-flanking regions of the *lug* operon may exhibit similarities with other species. A sequence BLAST analysis revealed that the 3′- and 5′-flanking regions of the

Table 2 Variations in the whole genome sequences of 36 strains and a comparison of their *lug* operons with that of IVK28.

Mutation types		Condition (frequency)	Sequence types (Strain name)
Incomplete operon		All lug genes were deleted except *lugM* (3)	ST5 (SL5) ST27 (C_33) ST27 (RMLUG2)
MGE insertion	IS*256*	inserted in *lugA* (1)	ST3 (JICS135)
Nonsense mutations	**Deletion**		
	lugA	A820 (1)	ST3 (CGMH-SL138)
		A6240 (1)	ST3 (FDAAGROS_381)
		A6241 (1)	ST34 (RMLUG4)
	lugB	A120 (1)	ST6 (CGMH-SL118)
		A361 (5)	ST1 (HKU09) ST1 (N920143) ST6 (CGMH-SL118) ST6 (RMLUG5) ST6 (VISLISI_33)
		A1219 (7)	ST3 (JICS135) ST3 (RMLUG6) ST6 (RMLUG5) ST6 (SL13) ST12 (VISLISI_27) ST28 (NCTC7990) ST34 (RMLUG4)
		A2592 (2)	ST1 (HKU09) ST34 (RMLUG4)
		A2942 (1)	ST3 (FDAAGROS_381)
		A3589 (1)	ST24 (SL118)
		A3628 (1)	ST3 (APC3758)
		T3901 (3)	ST6 (RMLUG5) ST6 (SL13) ST28 (NCTC7990)
	lugC	A149 (1)	ST34 (RMLUG4)
		A802 (1)	ST3 (FDAAGROS_381)
		C920 (1)	ST6 (RMLUG5)
		A1612 (3)	ST6 (CGMH-SL118) ST6 (SL13) ST6 (VISLISI_33)
		A7680 (2)	ST3 (FDAAGROS_381) ST6 (CGMH-SL118)
	lugR	A386 (1)	ST1 (HKU09)
	lugT	G219 (1)	ST3 (FDAAGROS_381)
	lugZ	A388 (1)	ST6 (CGMH-SL118)
		A498 (1)	ST3 (K93G)
	Insertion		
	lugC	A3569 (1)	ST24 (NCTC12217)
		T5256 (1)	ST24 (NCTC12217)
	lugT	G219 (1)	ST24 (NCTC12217)
	lugZ	A425 (1)	ST1 (HKU09)

lugM of the CGMH SL-131 (complete *lug* operon) and C_33 strains (*lug* operon lost most of its genes) were almost identical (99 % identities) (Fig. 2), which partially supported our hypothesis. Both ST27 strains of C_33 and CGMH SL-35 have the same *rpsI* and *lugM* sequences, which were identical in the 5′ and 3′-flanking regions of *lugM*. In addition, we found that the sequence of the 5′-flanking region of the *lug* operon was similar to that in other species, including one bacteriophage (*Caudoviricetes* sp. isolate ctmtq1) and two *Streptococcus* spp. (*Streptococcus agalactiae* YZ1605 and *Streptococcus thermophilus* STH_CIRM_65). This region can be divided into two parts: (1) highly conserved sequences near the 3′ region of *rpsI*, including the sequence of bacteriophage ctmtq1 (Fig. 2, red box). One exception was the CGMH SL-131sequence, in which the 3′-flanking region of *rpsI* differed from that of the others. (2) The partial sequences of the 5′-flanking region of the *lug* operon that were similar to the 3′-flanking sequences of the integrase in bacteriophage ctmtq1 and two *Streptococcus* spp. (Fig. 2, blue box).

IS*256* inserted in *lugA* has molecular type specificity

The MGE insertion event led us to consider whether these events may be widely present in our clinical isolates. Considering that IS*256* was inserted into *lugA* of ST3 strain JICS135, we designed specific primer sets to verify our hypothesis (Fig. 3A). In 16 of the 202 isolates, the existing IS*256* was inserted into *lugA*, and most of them (15 over 16) were ST3 lugdunin nonproducers; only one isolate was a ST6 lugdunin nonproducer. Furthermore, analysis of the sequencing data of flanking regions of the IS*256* insertion showed that all 16 isolates contained the same inverted repeat on the left (IRL) and inverted repeat on the right (IRR). In addition, an eight-nucleotide direct repeat (DR, TTTAATTC) belonging to *lugA* sequences (1396th-1403rd) was found in addition to both IRL and IRR in 15 isolates and was identical to the IS*256* inserted into *lugA* of JICS135 (Fig. 3B); only one isolate contained a different DR sequence (TAAAGATT) (Fig. 3A). According to the WGS data, 16 IS*256* copies were inserted into the genome of JICS135, and the DR sequences of the other IS*256* copies inserted differed from the IS*256* sequence inserted into *lugA* (Fig. 3B and Fig. S1).

Nonsense mutations show gene and molecular type specificities

Twenty-four positions with nonsense mutations were identified through the *lug* operon sequence comparison (Table 2). Twenty nonsense mutations were caused by single nucleotide deletion, and four locations contained an extra nucleotide, leading to frameshift nonsense mutations. Most nonsense mutations were observed in either *lugB* or *lugC*, and *lugB* 361 and 1219 and *lugC* 1612 had the highest frequency for mutations, with adenine deletions observed at these three locations. We therefore examined the above three locations among our isolates, and our results showed that these mutations were associated with molecular type and STs of *S. lugdunensis* (Table 3). First, in addition to ST3 and ST6, most STs with fewer isolates exhibited at least one mutation among these three spots. Among the 28 ST3 lugdunin nonproducers, only few isolates containing at least one of these three mutations were observed. In contrast, most ST6 lugdunin nonproducers contained more than one

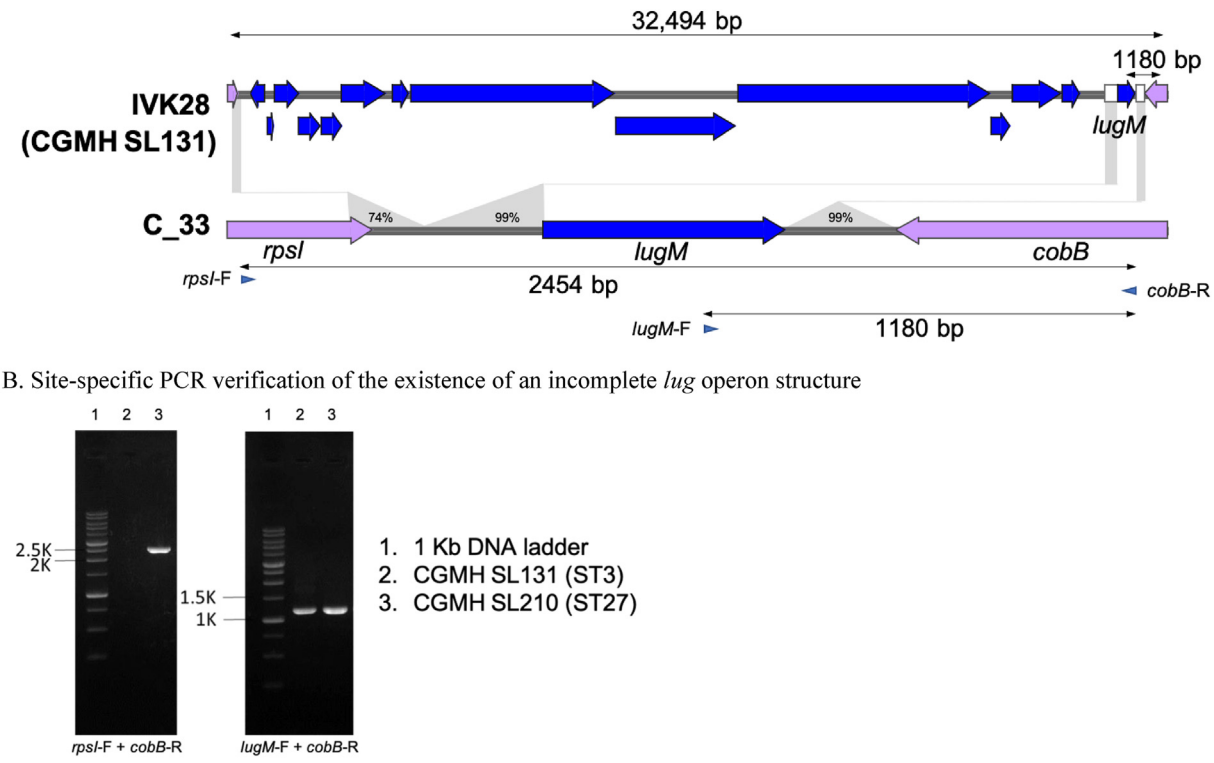

Figure 1. An illustration showing the differences between strains with or without the *lug* operon (A) and following PCR examination (B). The *lug* operon is located between *rpsI* and *cobB*, which are shown in purple. The complete *lug* operon is represented by the IVK28 strain and is identical to that of the CGMH-SL131 strain. All *lug* operon genes are shown in blue. An incomplete *lug* operon was found in *S. lugdunensis* C_33, which only contained *lugM* (blue). The specific primers targeting *rpsI-F* with *cobB-R* were designed for verification of the existence of the *lug* operon, and those targeting *lugM-F* with *cobB-R* were designed as an internal control to verify the PCR reaction. The gray area indicated similar sequences between IVK28 and C_33, and the number represents the identity percentage.

mutation (52 over 64) in both *lugB* 361 and *lugC* 1612, and half of ST6 isolates even contained all of these three mutations (32 over 64).

Discussion

In our recent study, we demonstrated that the ability of *S. lugdunensis* to produce lugdunin is specifically associated with molecular types of the strains.[11] However, only 51 lugdunin-producers were observed among all our 202 clinical isolates, and most isolates showed lugdunin nonproducing activities, which inspired us to further investigate whether lugdunin nonproducers were associated with strain-specific genetic types similar to lugdunin-producers. In the present study, we found possible mechanisms underlying lugdunin nonproduction with their distribution among various CRISPR-Cas and MLST types (Table 4). IS*256* insertion events were mostly discovered in ST3 isolates without the CRISPR-Cas system, while the unknown deletion mechanisms caused that most of the *lug* operon's genes lost only in CRISPR-Cas IIC isolates and the frameshift mutations caused by SNPs which led to the nonsense mutations were mostly distributed in CRISPR-Cas IIIA strains, especially in ST6 isolates.

The unknown deletion mechanism was the first genetic variation examined and was only observed in ST4, ST27, and ST29 isolates. All the above STs were characterized as CRISPR-Cas IIC strains previously.[8] Notably, all isolates did not exhibit lugdunin producing activities. We were curious about this phenomenon, which was not observed in strains without the CRISPR-Cas system or CRISPR-Cas IIIA isolates. Further investigations showed that the *lug* operon in CRISPR-Cas IIC isolates showed sequence similarities with that in other species, providing alternative evidence that the *lug* operon may be horizontally transferred from other species (Fig. 2). This analysis provided several interesting findings; first, all similar sequences from this comparison were accompanied by an integrase gene; second, these similar sequences (sequences in red box and blue box) were found in two nearby copies in the *S. thermophilus* isolate STH_CIRM_65; finally, except the lugdunin producer *S. lugdunensis* CGMH-SL131, all other bacterial species shown in Fig. 2 were CRISPR-Cas II strains.[12,13] We compared these integrase sequences which showed high similarities, and UniProt (https://www.uniprot.org) analysis suggested that the sequence was potentially a tyrosine-type integrase. Seven consensus residues identical to a recently identified tyrosine integrase were observed (Fig. S2). In our previous study on the CRISPR-Cas system in *S. lugdunensis*, we found

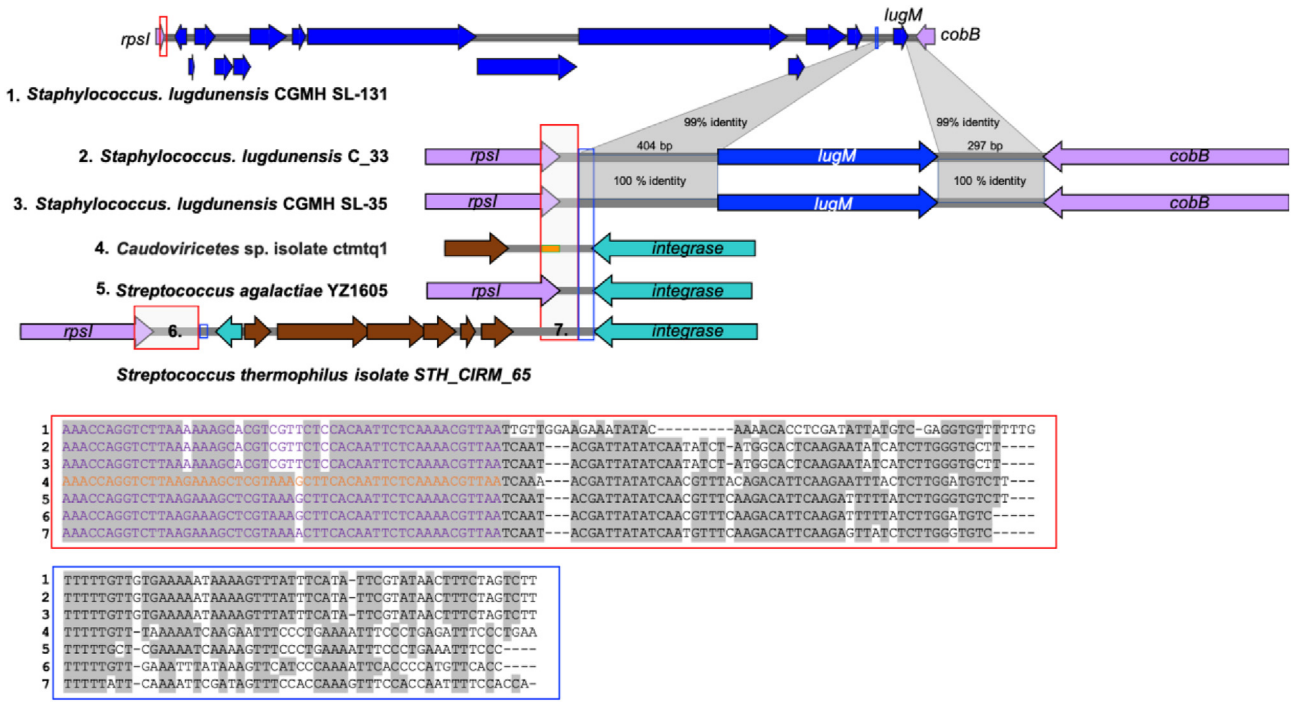

Figure 2. Comparison of the 5′-flanking region of the *lug* operon of *S. lugdunensis* C_33 with three other highly similar sequences An illustration showing the NCBI megablast result of the 5′ flanking region of the *lug* operon of two *S. lugdunensis* ST27 strains C_33 (No. 2) and CGMH SL-35 (No.3) with other strains containing similar sequences. Two ST27 strains share identical sequence region between *rpsI* and *lugM*. *S. lugdunensis* CGMH SL-131 (No. 1) is shown as the control with its complete *lug* operon. A phage (*Caudoviricetes* sp. isolate ctmtq1, No. 4) and two *Streptococcus* spp., *Streptococcus agalactiae* YZ1605 (No. 5) and *Streptococcus thermophilus* STH_CIRM_65 (No. 6 and No. 7), were found to have sequences (red and blue box) similar to the 5′ flanking region of the *lug* operon of *S. lugdunensis* CGMH SL-131 (No. 1), *S. lugdunensis* C_33 (No. 2) and CGMH SL-35 (No. 3). Each unique gene is represented by a different color; purple represents *rpsI* and *cobB*, brown represents a hypothetical protein, dark blue represents genes belonging to the *lug* operon, and blue green represents integrase genes. The sequence in No. 4 which is similar to the 3′ end sequence of *rpsI* is represented in orange. The grey regions represent the highly similar sequences between No. 1, No. 2, and No. 3, which was measured by Clustal Omega alignment. The numbers in these grey regions represent their sequence identities. The red box represents the sequences near *rpsI* in five bacterial strains and the similar sequence in phage ctmtq1. The blue box represents sequences that are similar to the 5′ flanking sequence of *lugM* in CGMH-SL131. Both red and blue box show the sequence alignment results in below the two columns.

that most spacers belonging to MGEs in CRISPR-Cas II isolates originated from phages.[8] Further analysis of the origin of these phages showed that some phages contained tyrosine integrases. Our data suggests that phages containing tyrosine integrase may be associated with horizontal gene transfer in these CRISPR-Cas II isolates.

Previous lugdunin research has proposed that the *lug* operon is horizontally transferred from other bacterial species.[9] Although the *lug* operon is located between *rpsI* and *cobB* in both CRISPR-Cas IIIA strains and in strains without the CRISPR-Cas system, our data analysis showed that sequences flanking *rpsI* were different between CRISPR-Cas IIC strain C_33 and non-CRISPR-Cas system strain CGMH-SL131(ST3) (red box, Fig. 2). Due to the different working mechanisms between CRISPR-Cas IIC and IIIA, we hypothesized that the CRISPR-Cas system may participate in the horizontal gene transfer process mediated by phages, consequently resulting in the excised *lug* operon, leading to most of the *lug* operon genes deletion. However, further experiments are required to verify this hypothesis.

Investigations in the present study showed that half ST3 lugdunin nonproducers all harbored the IS*256* insertion in *lugA* (Fig. 3). IS*256* is commonly distributed in enterococci and staphylococci[14–16] and plays a role in biofilm formation and the spread of aminoglycoside resistance.[15,17] IS*256* comprises a transposase accompanied by two imperfect inverted repeat sequences (IRR and IRL) at the both 5′ and 3′ ends, which responds to transposase binding.[18] In addition to the IR, 8–9 base pair DR sequences on the host genome were generated during transposition, which is crucial for targeting recognition.[19] A previous study showed that these DR sequences had varied AT-rich combinations,[20] and reports have shown that IS*256* has multiple copies in the bacterial genome.[15,21,22] Through WGS analyses, we found that IS*256* was inserted in *lugA* in the ST3 strain JICS135 of *S. lugdunensis* (Table 2), and further investigations found that IS*256* had 16 copies in the genome of JICS135 (Fig. S1). All these IS*256* copies harbored AT-rich DRs, and one of the DR sequences was identical to *lugA*. Almost all lugdunin nonproducers in which the ability to produce lugdunin was inhibited by insertion of IS*256* into

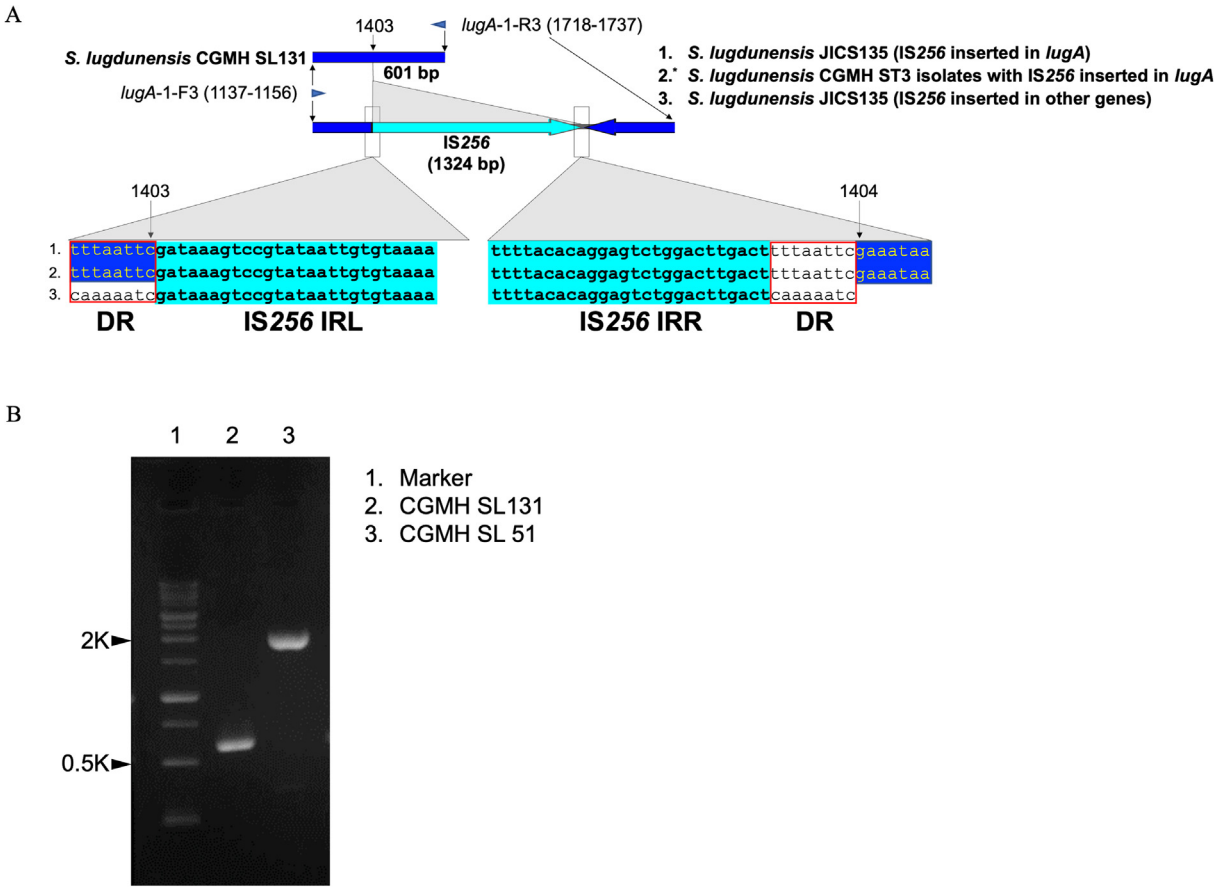

Figure 3. An illustration showing the differences between strains with the *lugA* gene with or without IS*256* insertions (A) and followed by PCR examination (B). Two *S. lugdunensis* ST3 strains with IS*256* insertion were compared to find similarities between the IS*256* insertion sequences. *S. lugdunensis* CHMH SL131 represents strains without IS*256* insertion, CHMH SL51 represents strains containing the IS*256* insertion confirmed by PCR, and *S. lugdunensis* JICS135 represents strains containing the multiple IS*256* insertion copies based on the WGS analysis. Dark blue represents *lugA* and light blue represents IS*256*. The sequences of inverted repeats on the left (IRL) and right (IRR) of IS*256* are marked with a black box, and the direct repeat is highlighted with a red box. A 1324 bp IS*256* inserted into the 1403rd position of *lugA* was observed in JICS135, which was further used to design specific primers for PCR verification. Strains with the 1933 bp PCR product indicated IS*256* inserted in *lugA*, while the 601 bp PCR product indicated strains without IS*256* insertion. All PCR products were further sequenced for verification the sequence composition of IRL, IRR and DR.

*Sixteen ST3 isolates have IS*256* inserted in *lugA*; 15 isolates within the same DR sequence are shown in Fig. 3A which were "tttaattc" except one isolate, CGMH-SL51, whose DR sequence was "taaagatt".

Table 3 Distribution of nonsense mutations in *lugB* and *lugC* in lugdunin-nonproducing *S. lugdunensis* isolates with different sequence types.

Molecular types of lugdunin nonproducer (n)	Nonsense mutation distribution in *lugB* (adenine deletions at positions 361 and 1219) and *lugC* (adenine deletion at position 1612)						
	lugB 361	*lugB* 1219	*lugC* 1612	*lugB* 361 & *lugB* 1219	*lugB* 361 & *lugC* 1612	*lugB* 1219 & *lugC* 1612	*lugB* 361 & 1219 & *lugC* 1612
ST1 (2)	1	0	1	0	0	0	0
ST2 (1)	0	1	0	0	0	0	0
ST3 (28)	2	7	4	1	0	2	0
ST6 (64)	61	32	52	32	52	32	32
ST9 (4)	1	4	0	1	0	0	0
ST12 (4)	1	4	0	1	0	0	0
ST15 (3)	*3*	1	3	1	3	1	1
Total (106)	69	49	59	36	55	35	33

Table 4 Summary of possible mechanisms underlying the nonproduction of lugdunin in *S. lugdunensis* with different molecular types.

CRISPR-Cas types	Molecular types (total number)	Nonsense mutation (n)			IS*256* insertion	Incomplete *lug* operon	Unknown variations
		lugB		lugC			
		A361	A1219	A1612			
Strains without CRISPR-Cas system	ST2 (1)	1	0	1	0	0	0
	ST3 (28)	2	6	4	15	0	6
	ST9 (4)	1	4	0	0	0	0
CRISPR-Cas IIC	ST4 (14)	ND*			0	14	0
	ST27 (26)				0	26	0
	ST29 (6)				0	6	0
CRISPR-Cas IIIA	ST1 (2)	1	0	1	0	0	0
	ST6 (64)	61	32	52	1	0	3
	ST12 (4)	1	4	0	0	0	0
	ST15 (3)	3	1	3	0	0	0
Total lugdunin non-producers (152)		68	46	61	16	46	9

lugA belonged to ST3 type isolates, which suggested that IS*256* insertion in *lugA* may be associated with molecular type specificities. Since our previous study showed that ST3 isolates do not possess the CRISPR-Cas system,[8] it is reasonable to speculate that IS*256* or other MGE were translocated in *S. lugdunensis* ST3 strains without the interference from CRISPR-Cas system. Thus, we hypothesized that the CRISPR-Cas system probably restricted such events in ST6 or other strains with this system.

The last genetic variation that inhibited the ability to produce lugdunin was nonsense mutations caused by SNP-triggered frameshift mutations. According to the WGS analysis data, we found several SNVs distributed in various ST strains, and most led to silent and missense mutations. Insertions at 20 spots caused nonsense mutations. Most silent mutations that existed in different STs suggested these SNVs may represent clonal lineages (data not shown). Although amino acid substitutions caused by missense mutations may have possibly inhibited lugdunin production, it is difficult to evaluate which amino acid change may consequently lead to the inhibition of lugdunin production ability without experimental evidence. In addition, the frequency of nonsense mutations among these 20 spots was not equal, and most mutations were observed only once in one ST, implying that these spots may represent independent mutation events among these 36 *S. lugdunensis* strains (Table 2). Therefore, we only focused on those spots with the highest frequency around these 36 *S. lugdunensis* strains. Interestingly, adenine deletions in poly A sequences occurred coincidently in the 5′ region of *lugB* and *lugC*, suggesting there may exist a potential mechanism that regulates lugdunin production activity. Studies on the homopolymeric tracts showed that insertion deletion (INDEL) frameshift mutations in homopolymeric sequences, which normally exist in the 5′ region sequences of genes, may represent a regulatory mechanism in prokaryotes.[23,24] Therefore, adenine insertions or deletions in *lugB* and *lugC* may play an important role in regulating lugdunin production. We observed that most nonsense mutations in homopolymeric tracts were found in the CRISPR-Cas IIIA strain, especially in ST6 isolates. The correlation between the CRISPR-Cas system and this phenomenon is unclear; however, this result suggested that the CRISPR-Cas system may participate in regulating these nonsense mutations in homopolymeric tracts.

Irrespective of the type of variations that lead to lugdunin nonproduction, a fundamental question is what forces drive most *S. lugdunensis* strains to become lugdunin non-producers. Mutations and recombination are known to be a driving force in driving population evolution and generating clonalities.[25,26] Bacteria can undergo mutations to adjust their metabolism functions, which may help them to adapt to nutrient limiting environments.[27,28] According to a previous study of "bacterial adaptation through loss of function"; loss of function mutations occur with a much higher frequency than gains of specific functionality due to the larger mutational target area available,[27] and this point may be appropriate to explain why most *S. lugdunensis* isolates were lugdunin nonproducers. Our previous study found that lugdunin production was associated with the strain's genotype and showed that the *agr* system may be associated with lugdunin production.[11] The *agr* quorum sensing is a global regulation system, which mainly controls the strain's pathogenesis and is responsible for adaptation to environmental changes.[29–31] A recent study showed that the *agr* system is involved in the regulation of the levels of a metalloprotease lugdulysin[32] whose activity is affected by the metal chelator EDTA.[33,34] Although the correlation between lugdunin and lugdulysin is unclear, their functional metal-requirement differed; lugdunin functions in iron-deficient environments while lugdulysin function requires iron. Since both lugdulysin and lugdunin are potentially regulated by the *agr* system, *agr* may not properly regulate both of them in the same metal environment. As lugdulysin plays a key role in pathogenesis, there is a possible trade-off selection that leads to a loss in lugdunin activity that assures the *agr* system can properly regulate the strain's lugdulysin activity during pathogenesis. Since we currently lack direct evidence that lugdunin production is regulated by *agr* system, further experiments will be needed to verify this hypothesis.

In conclusion, our study revealed that various lugdunin nonproduction mechanisms were associated with specific

molecular types. One of the mechanisms may be evidence for horizontal transfer of the *lug* operon from other species. Since *S. lugdunensis* is an opportunistic pathogen, we hypothesize that losing the lugdunin functional trait may be related to its pathogenesis and cause clonal evolution.

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Declaration of competing interest

We have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2024.01.005.