

Identification of the Novel Lincosamide Resistance Gene *lnu(E)* Truncated by *ISEnfa5-cfr-ISEnfa5* Insertion in *Streptococcus suis*: *De Novo* Synthesis and Confirmation of Functional Activity in *Staphylococcus aureus*

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The novel lincosamide resistance gene *lnu(E)*, truncated by insertion of an *ISEnfa5-cfr-ISEnfa5* segment, was identified in *Streptococcus suis*. The gene *lnu(E)* encodes a 173-amino-acid protein with ≤69.4% identity to other lincosamide nucleotidyltransferases. The *lnu(E)* gene and its promoter region were *de novo* synthesized, and *Staphylococcus aureus* RN4220 carrying a shuttle vector with the cloned *lnu(E)* gene showed a 16-fold increase in the lincomycin MIC. Mass spectrometry experiments demonstrated that Lnu(E) catalyzed the nucleotidylation of lincomycin.

To date, the three lincosamides, lincomycin, clindamycin, and pirlimycin, are widely used to treat staphylococcal and streptococcal infections in veterinary medicine. Lincomycin alone or in the combination lincomycin-spectinomycin is licensed in the European Union for the control of a number of bacterial pathogens involved in infections of the respiratory, gastrointestinal, and urinary/genital tracts as well as wound and skin infections in cattle, swine, horses, dogs, cats, and poultry (1). In contrast, pirlimycin is approved only for the control of staphylococci and streptococci from cases of bovine mastitis (2). Clindamycin is widely used in human medicine (3) but is also approved for use in cats and dogs, mainly for the treatment of skin and soft tissue infections, periodontitis, and osteomyelitis (4).

Resistance to lincosamides can be due to a number of different genes which specify different resistance mechanisms (<http://faculty.washington.edu/marilynr/>). The highest MICs of lincosamides are commonly seen in the presence of rRNA methylases which modify the ribosomal binding site for macrolides, lincosamides, and streptogramin B antibiotics (MLS_B phenotype) and thereby confer cross-resistance to these antimicrobial agents (5). Resistance to lincosamides only (L phenotype) is exclusively based on the inactivation of lincosamides by nucleotidylation via O-nucleotidyltransferases (6). Since the first report of a lincosamide nucleotidyltransferase gene, *lnu(A)* (formerly *linA*) (7), a number of *lnu* genes have been described in different bacteria from animal, human, and environmental sources, including *lnu(B)* (8), *lnu(C)* (9), *lnu(D)* (10), and *lnu(F)* (11). Most of these *lnu* genes confer resistance to lincomycin and pirlimycin, but their MICs for clindamycin are often below the breakpoints for resistance (2, 9, 10). Of all Lnu proteins, Lnu(A) is the best-studied enzyme (6). The corresponding gene, *lnu(A)*, has been detected in many staphylococcal species (2), and plasmids seem to play an important role in the interspecies transfer of *lnu(A)* (3, 12).

In a previous study, we identified the *cfr*-carrying plasmid pStrcfr, isolated from porcine *Streptococcus suis* S10 in Beijing, China (13). In this plasmid, *cfr* was bracketed by two copies of insertion sequence *ISEnfa5*, located in the same orientation, and direct target site duplications of 3 bp (GAT) were found immediately upstream and downstream of this segment (13). Further

analysis identified a 522-bp open reading frame into which this *ISEnfa5-cfr-ISEnfa5* segment was inserted (Fig. 1). The intact reading frame possibly coded for a 173-amino-acid (aa) protein that showed 69.4% identity to the lincosamide nucleotidyltransferase Lnu(A) from *Staphylococcus haemolyticus* (GenBank accession no. YP_758631.1) (12). Since an amino acid identity of <80% is considered indicative of a novel type of Lnu protein, the designations *lnu(E)* and Lnu(E) were tentatively provided by the nomenclature center for MLS resistance genes (<http://faculty.washington.edu/marilynr/>) for the new gene and the corresponding protein, respectively. Figure 2 shows a homology tree of the Lnu(E) protein with other lincosamide nucleotidyltransferases deposited in the GenBank database. As assumed from the initial amino acid comparison, Lnu(E) was most closely related to Lnu(A). In a multisequence alignment, Lnu(E) exhibited distinctly lower identities of 24.3% and 25.9% to Lnu(C) from *Streptococcus agalactiae* (GenBank accession no. AY928180) and Lnu(D) from *Streptococcus uberis* (GenBank accession no. EF452177), respectively. No significant homology was found between Lnu(E) and Lnu(B) from *Enterococcus faecium* (GenBank accession no. AJ238249) or Lnu(F) from *Salmonella enterica* serovar Stanley (GenBank accession no. EU118119).

Since the original *lnu(E)* gene in *S. suis* was truncated by *ISEnfa5-cfr-ISEnfa5* in *S. suis*, a PCR-directed search (primers *lnuE-F* [5'-AGACCTCCAAATAACTTA-3'] and *lnuE-R* [5'-AAATGACCTTCTTTCTTA-3']; annealing temperature, 48°C; amplicon size, 450 bp) for an intact and functionally active *lnu(E)* gene was conducted among unrelated streptococcal and enterococcal isolates. Unfortunately, negative results were obtained for

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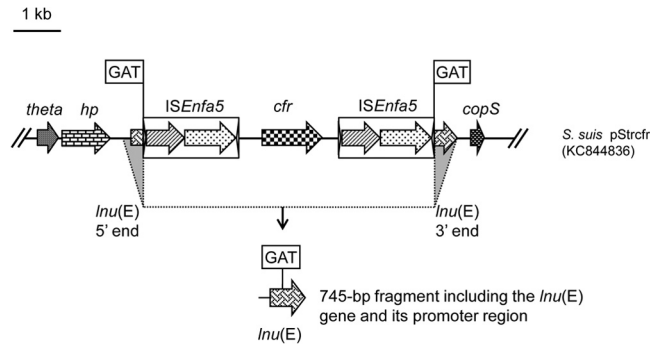


FIG 1 Schematic presentation of the *lnu(E)* gene truncated by insertion of an *ISEnfa5-cfr-ISEnfa5* segment in plasmid pStrcfr from *S. suis* S10 and the *de novo*-synthesized 745-bp segment which included the *lnu(E)* gene (522 bp) and its promoter region (223 bp) used for functional confirmation of *lnu(E)* as a lincosamide resistance gene.

all 94 enterococci and 97 streptococci (including 58 *S. suis* isolates) of food animal origin isolated during 2008 to 2012, suggesting a low prevalence of *lnu(E)* in these bacteria (data not shown). To investigate whether an intact *lnu(E)* gene confers lincosamide resistance, a 745-bp fragment (Fig. 1) which included the *lnu(E)* coding and promoter regions was synthesized by Genewiz Biotechnology Company (Jiangsu, China). The sequence of this synthetic fragment corresponded exactly to that found in plasmid pStrcfr from *S. suis* S10 but, however, (i) without the *ISEnfa5-cfr-ISEnfa5* segment and (ii) without one of the two 3-bp direct repeats generated during insertion of the *ISEnfa5-cfr-ISEnfa5* segment into the *lnu(E)* reading frame, but (iii) with XbaI and BamHI restriction sites in the noncoding regions at the 5' and 3' termini. Sequence analysis was conducted to confirm that the sequence of this synthetic segment corresponds exactly to the original *S. suis* sequence. This synthetic fragment was ligated into the XbaI- and BamHI-digested *Escherichia coli-Enterococcus faecalis* shuttle plasmid vector pAM401, which also replicates in *Staphylococcus aureus*. The recombinant plasmid pAM401-*lnu(E)* was then transformed into *S. aureus* RN4220. Susceptibility testing of RN4220+pAM401-*lnu(E)* exhibited 16-fold increases in the MICs for lincomycin (8 mg/liter) but only 2-fold increases in the MICs for clindamycin (0.12 mg/liter) and pirlimycin (1 mg/liter), compared to the recipient strain *S. aureus* RN4220 and *S. aureus* RN4220 carrying the “empty” shuttle vector (Table 1). In addition, no MIC changes were observed for erythromycin, streptomycin, gentami-

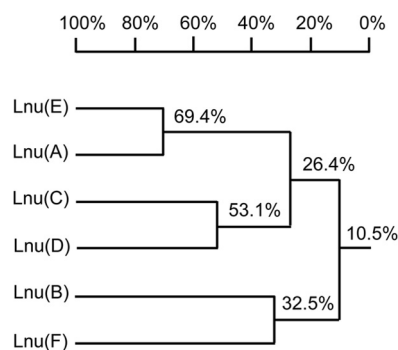


FIG 2 Homology tree of Lnu proteins based on multiple sequence alignment, produced by using DNAMAN software (Lynnon-BioSoft, Ontario, Canada).

TABLE 1 MICs of *S. aureus* RN4220 and strains containing the *lnu(E)*-carrying recombinant plasmid or the corresponding vector-only plasmids

Bacterial strain	MIC (mg/liter) of drug:		
	Lincomycin	Pirlimycin	Clindamycin
<i>S. aureus</i> RN4220	0.5	0.5	0.06
<i>S. aureus</i> RN4220+pAM401	0.5	0.5	0.06
<i>S. aureus</i> RN4220+pAM401- <i>lnu(E)</i> ^a	8	1	0.12

^a This intact *lnu(E)* gene and its promoter region were *de novo* synthesized by Genewiz Biotechnology Company.

cin, kanamycin, vancomycin, ceftazolin, oxacillin, ciprofloxacin, enrofloxacin, sulfamethoxazole-trimethoprim, spiramycin, tilimycin, and chloramphenicol (data not shown).

In a comparison between Lnu(E) and Lnu(A), two domains involved in the binding of the cofactor for the nucleotidyltransferase reaction, ATP or GTP, were found to be well conserved (6). The first domain in Lnu(E), consisting of Val-Asp-Val-Leu-Thr-Gly-Lys, was identical to that in Lnu(A), while the second domain, Lys-Leu-Val-Lys-Lys, was very similar to the Lys-Val-Ile-Gln/Lys-Lys sequence in Lnu(A), with Leu, Val, and Ile representing nonpolar and hydrophilic amino acids with similar biological functions. Whether these amino acid exchanges in the second functional domain have an impact on the relatively low lincosamide MICs conferred by Lnu(E) in *S. aureus* RN4220 remains to be determined. However, the L phenotype, with resistance to lincomycin but susceptibility to clindamycin and borderline susceptibility to pirlimycin, conferred by this novel nucleotidyltransferase was similar to the phenotypes observed with the lincosamide nucleotidyltransferases Lnu(A), Lnu(C), and Lnu(D) (2, 9, 10, 12). These results suggested that the intact *lnu(E)* reading frame—even if synthesized *de novo*—might represent a novel, functionally active *lnu* gene.

To analyze the mechanism of resistance conferred by Lnu(E), *S. aureus* RN4220+pAM401-*lnu(E)* cells in comparison to *S. aureus* RN4220+pAM401 cells (control) were tested for their ability to inactivate lincomycin. The assay was conducted in liquid medium by ultra-high-performance liquid chromatography coupled with Quattro LC triple quadrupole tandem mass spectrometry (UPLC-MS/MS; Waters, Milford, MA, USA) (Fig. 3). Spectra were acquired in a full MS scan (mass range from *m/z* 200 to 800; maximum ion time, 100 ms), followed by a collision-induced dissociation (CID) with argon as the collision gas. The most abundant ions were detected at the collision energy of 25 eV. The spectra showed the presence of the nonmodified lincomycin (407.0 Da) in Fig. 3A (control) whereas the 735.9-Da and the 717.9-Da peaks in Fig. 3B represent the nucleotidylated lincomycin with and without one H₂O molecule, respectively. Other fragments of 600.8, 468.9, and 249.9 Da are believed to result from leakage of the adenine moiety or the adenosine moiety of the compound, respectively, as previously described in the inactivation of lincomycin by Lnu(C) (9) or Lnu(D) (10). These results suggest that the inactivation products represent nucleotidylated lincomycin as previously described (9, 10) and confirm that the Lnu(E) protein represents in fact a novel type of lincosamide nucleotidyltransferase.

In conclusion, a novel lincomycin resistance gene, *lnu(E)*, conferring low-level lincomycin resistance in staphylococci, was detected. Since this gene was nonfunctional in the original por-

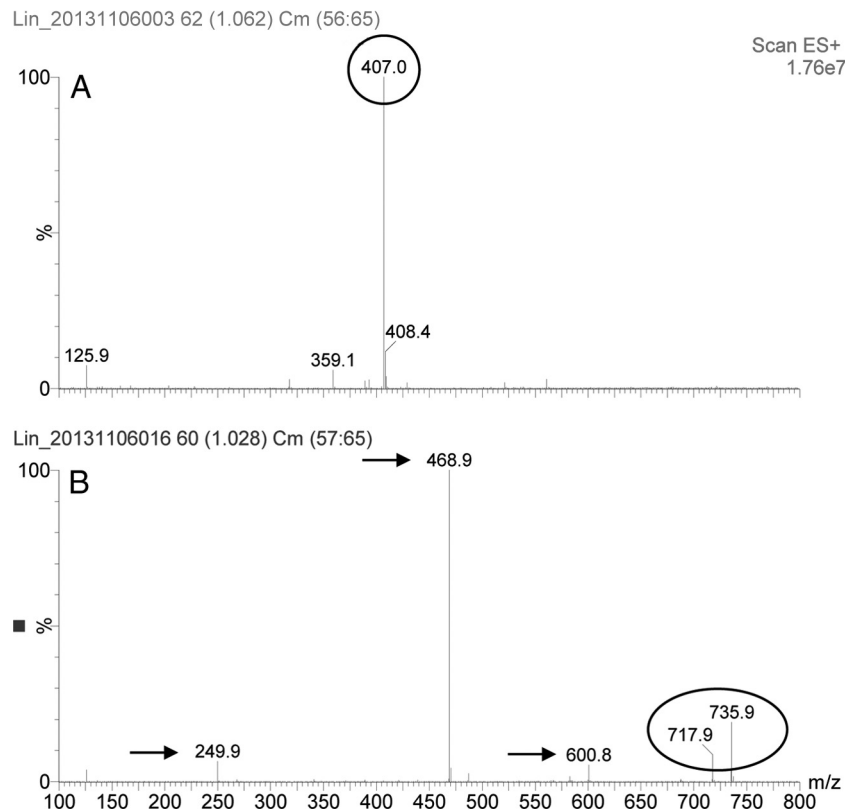


FIG 3 UPLC-MS/MS analysis of *S. aureus* RN4220+pAM401 cells (control sample) (A) and *S. aureus* RN4220+pAM401-*lnu(E)* cells (B). The 407.0-Da peak in panel A represents the nonmodified lincomycin, whereas the 735.9-Da and the 717.9-Da peaks in panel B represent the nucleotidylated lincomycin and the nucleotidylated lincomycin which has lost one H₂O molecule, respectively. Results are shown for fragments observed after CID. Fragments indicated by arrows are believed to result from leakage of the adenine or the adenosine moiety of the compound.

cine *S. suis* isolate due to the insertion of an *ISEnfa5-cfr-ISEnfa5* segment, *de novo* synthesis was employed to confirm its functionality. Although we could not detect a naturally occurring, functionally active *lnu(E)* gene in our strain collections, it is nevertheless most likely that such a gene exists since its inactivated version was present on a naturally occurring *S. suis* plasmid (13). Routine surveillance for *lnu(E)* in bacteria of human and animal origin is warranted and might help to clarify whether *S. suis* is the original host, in which bacteria this gene is present, and how widespread it is.

Nucleotide sequence accession number. The sequence of the *lnu(E)* gene was deposited in GenBank under the accession no. [KF287643](https://www.ncbi.nlm.nih.gov/nuccore/KF287643).

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