Identification of the Novel Lincosamide Resistance Gene *lnu*(E) Truncated by ISE*nfa*-cfr-ISE*nfa5* 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 ISE*nfa*-cfr-ISE*nfa5* 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<sub>B</sub> 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 *lna*) (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, *lna*(A), has been detected in many staphylococcal species (2), and plasmids seem to play an important role in the interspecies transfer of *lna*(A) (3, 12).

In a previous study, we identified the cfr-carrying plasmid pStcrf, isolated from porcine *Streptococcus suis* S10 in Beijing, China (13). In this plasmid, cfr was bracketed by two copies of insertion sequence ISE*nfa5*, 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 ISE*nfa*-cfr-ISE*nfa5* 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 *Streptococcus 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. AJ928180) 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. AJ238229) or Lnu(F) from *Salmonella enterica* serovar Stanley (GenBank accession no. EU118119).

Since the original *lnu*(E) gene in *S. suis* was truncated by ISE*nfa*-cfr-ISE*nfa5* in *S. suis*, a PCR-directed search (primers LnuE-F [5’T-AGACCTCCAAATACCTTA-3’T] and LnuE-R [5’T-ATGACCTTTTTTCTTTTA-3’T]; 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...
ing the "empty" shuttle vector (Table 1). In addition, no MIC
for lincomycin (8 mg/liter) but only 2-fold increases in the MICs for
lincomycin by Lnu(C) (9) or Lnu(D) (10). These results suggest that
the L phenotype, with resistance to lincomycin but susceptibility to clindamycin and borderline sus-
ceptibility to pirlimycin, conferred by this novel nucleotidyltrans-
ferase was similar to the phenotypes observed with the lincos-
amide 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. au-
reus RN4220+pAM401 cells (control) were tested for their ability
to inactivate lincomycin. The assay was conducted in liquid me-
dium 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 dis-
sociation (CID) with argon as the collision gas. The most abun-
dant ions were detected at the collision energy of 25 eV. The spec-
tra 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 nucleotidyldlated 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 moeity or the adenosine moiety of the compound,
respectively, as previously described in the inactivation of lincos-
amycin by Lnu(C) (9) or Lnu(D) (10). These results suggest that
the inactivation products represent nucleotidyldlated lincomycin as
previously described (9, 10) and confirm that the Lnu(E) protein
represents in fact a novel type of lincosamide nucleotidyldtrans-
ferase.

In conclusion, a novel lincomycin resistance gene, lnu(E), con-
ferring low-level lincomycin resistance in staphylococci, was de-
tected. Since this gene was nonfunctional in the original por-

![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 (532 bp) and its promoter region (223 bp) used for functional confirmation of lnu(E) as a lincosamide resistance gene.](image1)

![FIG 2 Homology tree of Lnu proteins based on multiple sequence alignment, produced by using DNAMAN software (Lynnon-BioSoft, Ontario, Canada).](image2)

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>MIC (mg/liter) of drug:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lincomycin</td>
</tr>
<tr>
<td>S. aureus RN4220</td>
<td>0.5</td>
</tr>
<tr>
<td>S. aureus RN4220+pAM401</td>
<td>0.5</td>
</tr>
<tr>
<td>S. aureus RN4220+pAM401-lnu(E)*</td>
<td>8</td>
</tr>
</tbody>
</table>

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

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

all 94 enterococci and 97 streptococci (including 58 S. suis iso-
lates) of food animal origin isolated during 2008 to 2012, suggest-
ing 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 Biotech-
nology Company (Jiangsu, China). The sequence of this syn-
thetic 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 seg-
ment 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 orig-
inal 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 Staphy-
llococcus 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 carry-
ing the “empty” shuttle vector (Table 1). In addition, no MIC
changes were observed for erythromycin, streptomycin, gentami-
cin, kanamycin, vancomycin, cefazolin, oxacillin, ciprofloxacin,
enrofloxacin, sulfamethoxazole-trimethoprim, spiramycin, tilmici-
sin, 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 nucleotidylation
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 lincos-
amide 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 sus-
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cine S. suis isolate due to the insertion of an IS\textsubscript{Enfa5}-cfr-IS\textsubscript{Enfa5} segment, \textit{de novo} synthesis was employed to confirm its functionality. Although we could not detect a naturally occurring, functionally active \textit{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 \textit{S. suis} plasmid (13). Routine surveillance for \textit{lnu}(E) in bacteria of human and animal origin is warranted and might help to clarify whether \textit{S. suis} is the original host, in which bacteria this gene is present, and how widespread it is.

\textbf{Nucleotide sequence accession number.} The sequence of the \textit{lnu}(E) gene was deposited in GenBank under the accession no. KF287643.

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\textbf{REFERENCES}

