ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 2005, p. 2716–2719 Vol. 49, No. 7
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.7.2716–2719.2005

New *lnu*(C) Gene Conferring Resistance to Lincomycin by Nucleotidylation in *Streptococcus agalactiae* UCN36

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Received 19 February 2005/Returned for modification 27 March 2005/Accepted 28 March 2005

***Streptococcus agalactiae* UCN36 was resistant to lincomycin (MIC = 16 μg/ml) but susceptible to clindamycin (MIC = 0.12 μg/ml) and erythromycin (MIC = 0.06 μg/ml). A 4-kb HindIII fragment was cloned from *S. agalactiae* UCN36 total DNA on plasmid pUC18 and introduced into *Escherichia coli* AG100A, where it conferred resistance to lincomycin. The sequence analysis of the fragment showed the presence of a 1,724-bp element delineated by imperfect inverted repeats (22 of 25 bp) and inserted in the operon for capsular synthesis of *S. agalactiae* UCN36. This element carried two open reading frames (ORF). The deduced amino acid sequence of the upstream ORF displayed similarity with transposases from anaerobes and IS*1*. The downstream ORF, *lnu*(C), encoded a 164-amino-acid protein with 26% to 27% identity with the $LnuA_{N2}$, LnuA, and LnuA′ lincosamide nucleotidyltransferases reported for *Bacteroides* and *Staphylococcus*, respectively. Crude lysates of *E. coli* AG100A containing the cloned *lnu*(C) gene inactivated lincomycin and clindamycin in the presence of ATP and $MgCl_2$. Mass spectrometry experiments demonstrated that the LnuC enzyme catalyzed adenylylation of lincomycin.**

Streptococcus agalactiae (group B streptococcus) is a major cause of invasive infection in neonates and pregnant women. It has also been increasingly recognized as a significant pathogen in nonpregnant adults, especially among patients with underlying conditions (13, 26). *S. agalactiae* is responsible for acute and chronic diseases, such as respiratory tract infections, endocarditis, sepsis, meningitis, pyelonephritis, and neurological problems (12). Penicillin G and ampicillin, which are always active against this pathogen, are the therapy of choice for *S. agalactiae* infections. However, in the case of intolerance to penicillins or lack of clinical response, clindamycin and macrolides are major alternatives (2).

Lincosamide antibiotics include lincomycin and clindamycin. Their structure consists of a hygric acid alkylated in position 4 and linked to a 6 amino-thio-octopyranoside residue. Clindamycin is a semisynthetic derivative obtained by chlorination of lincomycin (4). The spectrum of activity of lincosamides include gram-positive cocci, with some exceptions, such as *Enterococcus faecalis* (25). They prevent the protein synthesis by inhibition of the peptidyltransferase in binding mainly the A2058 of the 23S rRNA in the 50S subunit of the bacterial ribosome (9).

The most common mechanism of resistance to lincosamides involves N^6 dimethylation of a specific adenine residue (A2058) of the 23S rRNA molecule (17, 28). This alteration of the antibiotic target site is invariably catalyzed by an rRNA methyltransferase encoded by *erm* genes. This resistance mechanism confers cross-resistance to macrolides, lincosamides, and streptogramin B (MLS phenotype) (17, 28). In contrast to the MLS phenotype, specific resistance to lincosamides is due to enzymatic inactivation of those antibiotics. Phosphorylation and nucleotidylation of the hydroxyl group at position 3 of lincosamides have been detected in several species of *Streptomyces* (1, 21). Lincosamide nucleotidyltransferases encoded by *lnu* genes (formerly *lin*) was observed in both animal and human strains (8, 10, 11, 18). In clinical isolates, six *lnu* genes have been described: *lnu*(A), *lnu*(A′), *lnu*(B), *lnu*(B-like), *lnu*(A_{N2}), and *linF* (3, 4, 5, 15, 27). The *O*-nucleotidyltransferases encoded by these genes inactivate lincosamides by adenylylation (3, 5). *lnu*(A) and *lnu*(A′) have been reported in *Staphylococcus haemolyticus* and *Staphylococcus aureus*, respectively (4, 5). They encode two isoenzymes of 161 amino acids differing by 14 amino acids. An *lnu*(A_{N2}) gene homologous to *lnu*(A) and *lnu*(A′) (55% of identity) was evidenced in *Bacteroides* spp. (27). This gene would be carried by a mobilizable transposon. The *lnu*(B) gene from *Enterococcus faecium* has been described (3). This gene does not display homology with the other *lnu* genes and is carried by a large conjugative plasmid. More recently, an *lnu*(B-like) gene [79% identity with *lnu*(B)] and an *linF* gene [34.9% identity with *lnu*(B)] were identified in *Eubacterium* and *Escherichia coli*, respectively (15). In *E. coli*, the *linF* gene confers cross-resistance to lincomycin and clindamycin, whereas in the other organisms, the *lnu* genes confer resistance to lincomycin but not to clindamycin. However, the bactericidal activity of clindamycin, which is already weak against susceptible strains, is totally abolished (18). In this study, we characterized the function and genetic support of a new *lnu*(C) gene that confers resistance to lincomycin in a clinical strain of *S. agalactiae* UCN36.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. agalactiae* UCN36 was isolated in our laboratory from the vaginal tract of a pregnant woman. *S. haemolyticus*

BM4610 [*lnu*(A) gene], *S. aureus* BM4611 [*lnu*(A′) gene], and *E. coli*(pVMM25) [containing a cloned *lnu*(B) gene] were used as controls in PCR experiments (3, 18). *E. coli* AG100A, kindly provided by Hiroshi Nikaido was used in cloning experiments (23). *E. coli* AG100A is a mutant susceptible to lincosamides resulting from inactivation of the AcrAB pump responsible for active efflux of lincosamides by transposon Tn*903* harboring a kanamycin resistance gene. Strains were grown in brain heart infusion broth and agar incubated aerobically at 37°C. *S. agalactiae* BM132 was used as a negative control in lincosamide inactivation experiments (16).

Antibiotic susceptibility testing. Susceptibility to antibiotics was determined by the disk diffusion technique (6). MICs of antibiotics were determined by the agar dilution method with Mueller-Hinton medium supplemented with 5% sheep blood and incubated at 37°C under an aerobic atmosphere (6). Erythromycin was from Aventis Pharma (Romainville, France). Lincomycin and clindamycin were from Pfizer (Groton, Conn.).

Lincosamide inactivation. The kinetics of lincomycin inactivation by resting cells were determined in liquid medium as previously described (19). *S. agalactiae* UCN36 cells, suspended in 0.01 M phosphate buffer (pH 7) containing 14 μg of lincomycin per ml, were incubated at 37°C for various periods of time. The pH of this suspension remained constant. Inactivation of lincomycin was followed by a bioassay with *Micrococcus luteus* ATCC 9341 as an indicator organism.

For preparation of modified lincomycin, *E. coli* AG100A cells containing the cloned *lnu*(C) gene were lysed by sonication. Cell debris were removed by centrifugation at 40,000 × *g* for 45 min. Lincomycin (200 μg/ml) was added in the supernatants and then incubated at 37°C for 18 h in the presence of ATP (2.5 mM) and $MgCl_2$ (50 mM). Inactivation of antibiotics was monitored as indicated above. Aliquots of inactivated lincomycin were freeze-dried.

Mass spectrometry. Samples were analyzed by using an electrospray ion trap mass spectrometer (LCQ Deca XP; Thermofinnigan, San Jose, CA) coupled on line with high-performance liquid chromatography (HPLC) (Surveyor LC). They were separated by reverse-phase HPLC on a C_{18} capillary column (ThermoHyPurity C_{18} 150 by 0.18). A linear gradient (flow rate, 5 μl/min) from 5 to 95% B was used, where solvent A was a 2 mM ammonium acetate aqueous solution and B was a 2 mM ammonium acetate solution in methanol. The electrospray ionization parameters were as follows: spray voltage, 4.5 kV; spray current, 80 μA; sheath gas flow rate, 35; auxiliary gas flow rate, 10; capillary temperature, 250°C; capillary voltage, 10 V; tube lens offset, −5 V. These parameters were issued from an optimization of the detection of lincomycin. Spectra were acquired in a mode that alternated a full mass spectrometry (MS) scan (mass range from *m*/*z* 200 to 1,000; 3 microscans; maximum ion time, 100 ms), followed by a collision-induced dissociation (CID)-MS2 and a CID-MS3 (3 microscans; maximum ion time, 400 ms; collision energy, 35%) of the most abundant ion detected in the previous spectra.

PCR conditions. The primers used for the amplification of *lnu*(A), *lnu*(A′), and *lnu*(B) genes were previously described (3, 18). The PCR consisted of denaturation (95°C, 30 s), annealing (50°C, 30 s), and extension (72°C, 30 s to 3 min). The products were stored at 4°C until ready for analysis. The *Taq* DNA polymerase was obtained from Eurobio (Les Ulis, France).

Cloning and sequencing of a DNA fragment conferring resistance to lincosamides. Chromosomal DNA from *S. agalactiae* strain UCN36 was digested with various restriction enzymes and ligated at 4°C to plasmid vector pUC18 digested with the corresponding restriction enzymes. Recombinant plasmids were transformed by electroporation (Gene Pulser; Bio-Rad, Ivry-sur-Seine, France) into electrocompetent *E. coli* AG100A cells. *E. coli* AG100A transformants were selected on media containing clindamycin (6 μg/ml), ampicillin (100 μg/ml), and kanamycin (20 μg/ml). Both DNA strands were sequenced in an automated ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA). Nucleotide and amino acid sequences were analyzed by using the BLAST and FASTA software available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). Multiple-sequence alignments were performed with the ClustalX program available at the Centre de Ressources Infobiogen website (http://www.infobiogen.fr/).

Nucleotide sequence accession number. The nucleotide sequence of the 1,724-bp element from *S. agalactiae* UCN36 has been deposited in the GenBank database under accession no. AY928180.

RESULTS

Lincomycin resistance in *S. agalactiae* UCN36. *S. agalactiae* UCN36 displayed an unexpected phenotype of resistance to antibiotics. The strain was resistant to lincomycin (MIC = 16 μg/ml) but susceptible to erythromycin (MIC = 0.06 μg/ml), clindamycin (MIC = 0.12 μg/ml), quinupristin (MIC = 16 μg/ml), and dalfopristin (MIC = 2 μg/ml). This phenotype contrasted with the usual phenotype of cross-resistance between macrolides, lincosamides, and streptogramins B and was similar to the phenotype due to nucleotidylation of lincosamides conferred by the *lnu* class of genes in gram-positive cocci (3, 18). However, PCR experiments failed to detect any DNA sequence related to *lnu*(A), *lnu*(A′), *lnu*(B), or *lnu*(B-like) genes.

To investigate the mechanism of resistance, a lincomycin inactivation bioassay was implemented. The kinetics of lincomycin inactivation by resting cells of *S. agalactiae* UCN36 showed that the concentration of native lincomycin (14 μg/ml) decreased within 6 h to 2.5 μg/ml, which is the limit of detection of the method in contrast to the negative-control *S. agalactiae* BM132. Clindamycin was similarly inactivated despite a low MIC. This result suggested the presence of a lincosamide-inactivating enzyme in *S. agalactiae* UCN36.

Characterization and localization of the *lnu*(C) gene. Restricted DNA fragments from *S. agalactiae* UCN36 were cloned on plasmid pUC18, and recombinant plasmids were introduced into the lincosamide-sensitive *E. coli* mutant strain AG100A. The plasmid content of 10 transformants resistant to ampicillin, kanamycin, and clindamycin was analyzed by agarose gel electrophoresis of crude bacterial lysates. The smallest recombinant plasmid, pUV14, with a 4-kb HindIII insert was studied further. Acquisition of the recombinant plasmid by *E. coli* AG100A led to an increase in the MICs of clindamycin and lincomycin from 2 to 32 μg/ml and 64 to 256 μg/ml, respectively. The transformant was found able to inactivate lincomycin and clindamycin.

The insert was entirely sequenced. Analysis of the sequence revealed homology with known genes. The 5′ end sequence of the insert was identical to that of a chromosomal fragment of *S. agalactiae* NEM316 bearing the 3′ end of the *cpsD* gene and the first 272 nucleotides of the *cpsE* gene. The *cpsD* and *cpsE* genes belong to the capsular synthesis operon and encode two galactosyl transferases (14). The open reading frame of the *cpsE* gene of *S. agalactiae* UCN36 was interrupted after nucleotide 272 by the insertion of a 1,724-bp fragment and continued after the insertion. As a consequence, the strain was nontypeable. Two open reading frames (ORFs) in the same orientation, ORF 1 and ORF 2 of 1,038 and 495 bp, respectively, were identified within the inserted fragment (Fig. 1). Both translation ATG start codons were preceded at 9 bp by ribosome binding site-like sequences, 5′-CCGAAGGAGG-3′ and 5′-TTTCTGGAGA-3′, complementary at 7 and 4 bases (underlined) to the 3′-OH-terminal (5′-UCUUUCCUCC-3′) sequence of *Bacillus subtilis* 16S rRNA, respectively (22).

The deduced amino acid sequence (164 amino acids) obtained from ORF 2 showed 26 to 27% of identity with lincosamide nucleotidyltransferases, including $LnuA_{N2}$ from *Bacteroides* sp. and LnuA and LnuA′ from staphylococci. The *lnu*-related gene of *S. agalactiae* UCN36 was thus designated *lnu*(C) (designation provided by Marilyn Roberts [http://faculty.washington.edu/marilynr/]). ORF 1 was located 23 bp upstream from *lnu*(C). The deduced protein sequence (345 amino acids) of ORF 1 was distantly related to several transposases described for anaerobic strains and IS*1*. The protein displayed 35% identity with the IS*1* transposase of *E. coli* (24)

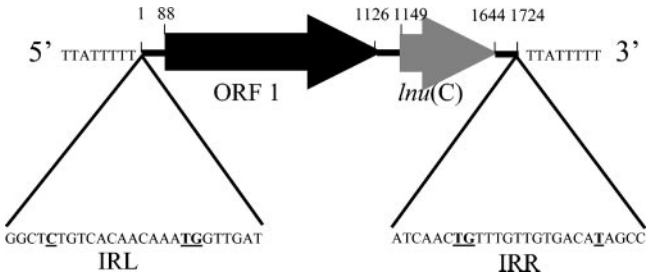

FIG. 1. Schematic map of the 1,724-bp genetic element bearing the *lnu*(C) gene. The element contains ORF 1 (black arrow), putatively encoding a homologue of IS*1* transposase, and the *lnu*(C) gene (grey arrow). The 25-bp imperfect inverted repeats (IRL and IRR) are shown, and the mismatches are in bold and underlined. The element is flanked by 8-bp direct repeats.

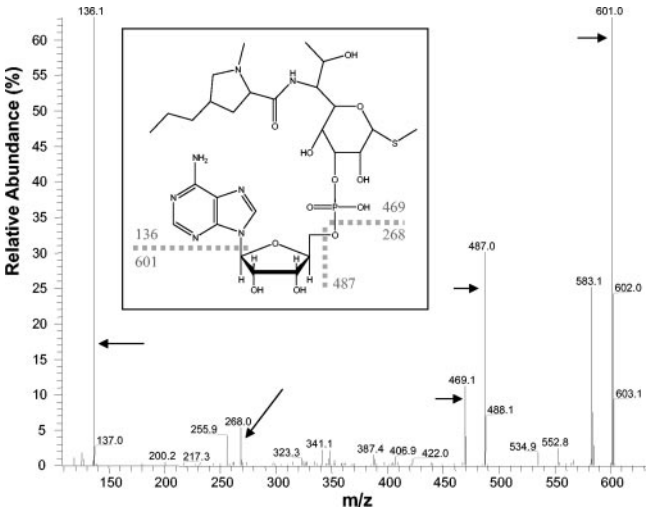

FIG. 2. CID-MS2 analysis of the 736.4-amu product resulting from lincomycin modification. (Inset) Proposed structure for adenylyl lincomycin, as deduced from fragments observed after CID. Positions of fragments are indicated by arrows. Note that the ester bond was arbitrarily positioned on C-3 of lincomycin on the figure but could also involve the hydroxyl group of C-2 or that of C-4.

and 33 to 42% identity with putative transposases reported for *Clostridium sordellii*, *Clostridium tetani*, and *Clostridium acetobutylicum* (GenBank accession numbers BAC57548.1, NP_782873.1, and NP_348122.1, respectively). A conserved domain shared with transposases belonging to the IS*1* family was detected between amino acid 52 and amino acid 126.

The 1,724-bp fragment bearing ORF 1 and *lnu*(C) was bound at both extremities by 25-bp imperfect inverted repeats. Twenty-two nucleotides of 25 were complementary (Fig. 1). This organization suggested that *lnu*(C) was borne by a transposon-like structure. The element was flanked by an AT-rich 8-bp sequence which could correspond to the duplication of the target during transposition.

Mechanism of resistance to lincosamides. Inactivation of 200 μg of lincomycin per ml was obtained when crude extracts of *E. coli* AG100A/pUV14 were incubated with ATP and $MgCl_2$ but not when cells were incubated in the absence of ATP. HPLC analysis of the treated samples revealed a single peak eluted at 8.6 min. MS analysis of this fraction revealed three major peaks, displaying *m/z* of 736.4, 369.4, and 758.3. These 3 peaks corresponded to a unique 736.4-atomic mass unit (amu) compound and to its doubly charged and sodium adduct forms, respectively. The mass observed for this compound was in agreement with adenylylation of lincomycin (+329). The structure of this compound was further characterized by CID-tandem mass spectrometry of both the singly and the doubly charged forms (Fig. 2). Spectra revealed major fragments at 601 and 136 amu, resulting from leakage of the adenine moiety of the compound, and fragments at 469 and 268 amu, resulting from the leakage of the adenosine moiety.

DISCUSSION

Isolated resistance to lincosamides which defines the L phenotype, appears to be rare in *S. agalactiae*. Recently, *S. agalactiae* isolates intermediate or resistant to clindamycin and lincomycin but susceptible to erythromycin have been reported from New Zealand (20). However, resistance to lincosamides was combined with high MICs of dalfopristin (4 to 32 μg/ml), a streptogramin A antibiotic, defining the so-called LSA phenotype. The biochemical and genetic basis for this resistance remained unknown. In Canada, a single clindamycin-resistant and erythromycin-susceptible strain has been reported which contained an *lnu*(B) [*lin*(B)] gene similar to that initially reported for *E. faecium* and responsible for lincosamide nucleotidylation (3, 7). In this study, we report a new lincosamide resistance gene called *lnu*(C) distantly related to the other *lnu* genes. Mass spectrometry experiments showed that lincomycin resistance was due to nucleotidylation of the antibiotic. The precise site of nucleotidylation of lincomycin and clindamycin was not characterized in this study. The LnuA nucleotidyltransferase modifies a hydroxyl group of clindamycin and lincomycin at positions 3 and 4, respectively. By contrast, LnuB modifies a hydroxyl at position 3 in both clindamycin and lincomycin.

In the original gram-positive host and in the *E. coli* transformant, both lincomycin and clindamycin were inactivated. However, resistance to lincomycin only was detected in *S. agalactiae* and resistance to both lincomycin and clindamycin was detected in *E. coli*. The reason for the difference in phenotypic expression of the resistance determinant in the two backgrounds remains unexplained. Hypothetically, the difference between the two lincosamides, which was also reported for the *lnu*(A) gene, might be related to differences in relative affinities of clindamycin and lincomycin for the ribosomes of gram-positive and gram-negative organisms and for the LnuC enzyme: clindamycin might have better affinity for the gram-positive ribosomes than for LnuC.

The *lnu*(C) gene was located on a genetic element which bore a homologue of the IS*1* transposase gene and which was delineated by imperfect inverted repeats. The structure of the genetic element displayed characteristics similar to those of a transposon. However, it differed from the classical insertion sequences, which are small and compact DNA sequences encoding only functions involved in their translocation, and from the classical transposons, which contain resolvase genes. Further characterization of this putative transposon is currently in progress.

On a practical point of view, lincomycin resistance may be misidentified in strains with the L phenotype if only erythromycin is tested. In addition, the test of clindamycin does not predict for lincomycin resistance. Although the activity of clindamycin against *S. agalactiae* is only weakly affected by the mechanism of resistance, a 100-fold increase in the bacterial inoculum led to a 3-dilution increase in the MIC of clindamycin for *S. agalactiae* UCN36 (data not shown). This inoculum effect was greater than that observed for the lincosamide-susceptible strain, and the clinical significance of this modest increase in the MIC of clindamycin remains to be evaluated.

ACKNOWLEDGMENTS

We thank Hiroshi Nikaido for the gift of *E. coli* AG100A, the Fondation pour la Recherche Médicale for financial support, and Marilyn Roberts for providing the designation for the *lnu*(C) gene.

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