Complete Nucleotide Sequence of cfr-Carrying IncX4 Plasmid pSD11 from Escherichia coli

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We report the complete nucleotide sequence of a plasmid carrying the multiresistance gene cfr. This plasmid was isolated from an Escherichia coli strain of swine origin in 2011. This 37,672-bp plasmid, pSD11, had an IncX4 backbone similar to those of the IncX4 plasmids obtained from the United States and Australia, in which the cfr gene was flanked by two copies of IS26 and a truncated Tn1331 was inserted.

The transferable multiresistance gene cfr is of great concern, due to the fact that it encodes resistance to five different classes of antimicrobials—phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A—and each of them is widely used in the treatment of bacterial infections in both human and veterinary medicine (1). Since initial identification in a bovine Staphylococcus sciuri isolate in 1997 (2), the cfr gene has been well documented worldwide (3–7). Although mainly found in Gram-positive bacteria, such as Staphylococcus spp. (3–5), Bacillus spp. (8–10), Enterococcus spp. (11, 12), and Macrococcus spp. and Jeotgalicoccus spp. (13), this gene has also been sporadically detected in Gram-negative bacteria, including Escherichia coli (14, 15) and Proteus vulgaris (16).

The interspecies and even intergeneric distribution of the cfr gene is mainly attributed to the transfer of mobile genetic elements, such as plasmids and insertion sequences. Cfr-encoding plasmids from Gram-positive bacteria have been widely described (7); however, there is little information available about the characteristics of the plasmids carrying the cfr gene in Gram-negative bacteria. To date, only two cfr-positive plasmids from E. coli isolates have been reported, namely, pEC-01 and pSCEC2 (14, 15). pSCEC2 is an IncA/C plasmid, with a size of 135,615 bp, and has been well characterized and completely sequenced (14). pEC-01 is ~110 kb in size, but it has not been typed by PCR-based replicon typing (PBRT) (15). Recently, an ~30-kb plasmid carrying the cfr gene was identified by our group (17). Here, we report the complete nucleotide sequence of this plasmid, pSD11.

Plasmid pSD11 was extracted from the E. coli electroporant DH10B using the Qiagen large construct kit (Qiagen, Courtaboeuf, France). Sequencing was performed using the Illumina platform, and data were assembled by SOAPdenovo (18).

FIG 1 Linear comparison of IncX4 plasmids pSD11, pSEEH1578_02, and pJEG012. The arrows represent the positions and transcriptional directions of the ORFs. The IS26 elements are shown as boxes. Regions of homology are shaded in gray, and the functional regions of the plasmids are shown above the linear maps.

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gaps between the contigs were closed by PCR, and respective amplicons were sequenced. Gene prediction and annotation were performed using the RAST tools (19). The sequence comparison and map generation were performed using BLAST (http://blast.ncbi.nlm.nih.gov) and Easyfig version 2.1 (20).

Plasmid pSD11 is a circular molecule of 37,672 bp, with an average GC content of 41.8%, and harbors 52 predicted open reading frames (ORFs). A BLASTn comparison against the NCBI database revealed that aside from the cfr-harboring variable region, pSD11 has a typical IncX4 plasmid backbone of 33.7 kb, with predicted regions involved in plasmid replication, partition, maintenance, and transfer (see Fig. 1)—closely related to the other completely sequenced IncX4 plasmids, including pSEEH1578_02 (GenBank accession no. CP004088) from Salmonella enterica, pSAM7 and pJIE143 (GenBank accession no. JX981514 and JN194214, respectively) from E. coli, and pJEG012 (GenBank accession no. KC354802.1) from Klebsiella pneumoniae. Of these IncX4 plasmids, the backbone of pSD11 is highly homologous to pSEEH1578_02 (99% coverage and 99% nucleotide identity). The replication region of pSD11 includes a rep gene similar to that on pSEEH1578_02 but quite different from the pir gene on pJEG012. parA and a resolvase gene are involved in the partition of pSD11. The conjugation region contains a VirB4/D4 type IV secretion system. Plasmid pSD11 also possesses two toxin/antitoxin (TA) addiction systems, hicAB and stbDE, which are likely to be involved in the stability of this plasmid.

The cfr-harboring variable region contains the following components (Fig. 1). (i) There is a truncated transposon, of which a 621-bp fragment of ΔtnpA and a putative 38-bp inverted repeat (IR) show 100% identity with transposon Tn1331 in pJEG012. Immediately upstream of the IR, a 5-bp direct repeat (AATTA), characteristic of Tn1331 transposition, is also present. This truncated Tn1331 transposon in pSD11 is inserted 129 bp upstream of the resolvase gene res, while in pJEG012, Tn1331 is inserted 387 bp upstream of res, indicating a different insertion site of Tn1331 in the similar backbone. (ii) There is an IS26 composite element, which is known to be critical for the mobilization of the cfr gene (15, 16). However, no characteristic 8-bp direct repeats (DRs) are present immediately upstream or downstream of these IS26 elements. This could have resulted from insertion of an additional copy of IS26 in a close position, followed by homologous recombination between two copies of IS26 (21). Interestingly, this region was identical to the one found on plasmid pEC-01 from an E. coli strain (GenBank accession no. JN982327.1) (22), implying that
the element containing the gene cfr, two IS26 elements, and the truncated Tn1331 have integrated into diverse plasmid backbones.

Based on the structure of the cfr-harboring variable region in pSD11, we speculate that this region may arise following multiple events (Fig. 2). (i) It appears that the Tn1331-like element was inserted into a region between res and hp1 of the IncX4 plasmid backbone as in pSEEH1578_02, generating a 5-bp DR (AATTA). (ii) Two copies of IS26 were inserted into tnpA of Tn1331 and hp2, respectively. Homologous recombination between the two IS26 elements could result in a deletion of the intervening part, with only one IS26 left. (iii) A recombination between this IS26 element, a minicircle-comprising cfr gene, and another IS26 copy has occurred. This finally led to the integrated cfr gene being flanked by two copies of IS26 that are located in the same orientation. The segment containing cfr and one copy of an insertion sequence could easily be excised from chromosomes or plasmids and formed minicircles. A large number of insertion sequences have been described to be involved in the formation of minicircles, such as IS26 that are located in the same orientation. The segment containing cfr and one copy of an insertion sequence could easily be excised from chromosomes or plasmids and formed minicircles. A large number of insertion sequences have been described to be involved in the formation of minicircles, such as IS2126 in Enterococcus spp., ISEfa5 in Streptococcus spp., and IS21-558 or ISEnfa4 in Staphylococcus spp., as well as the more recently reported IS26 in E. coli (7, 14). Moreover, the PCR-based stability test also revealed that the minicircle-comprising cfr and one IS26 element could easily be excised from plasmid pSD11 (17), suggesting that cfr may be transferable by IS26-mediated recombination.

The pSD11-like plasmids in the six cfr-positive E. coli strains previously described were further analyzed by PCR-restriction fragment length polymorphism (RFLP) (17). Four pairs of primers were designed to amplify four partially overlapping regions covering the complete sequence of pSD11. Subsequently, the PCR products were purified with the Takara DNA fragment purification kit (TakaRa, Tokyo, Japan) and digested with corresponding restriction enzymes (TakaRa, Tokyo, Japan) according to the manufacturer’s instructions (Table 1). After digestion, RFLP patterns were analyzed on 1% agarose with a DNA marker (see Fig. S1 in the supplemental material). The entire regions of all cfr-positive plasmids showed highly similar RFLP patterns, except for the plasmid in strain 1ZF13D, in which only one different band was observed on region 1 (position 128.07635). Comparison of these isolates by pulsed-field gel electrophoresis (PFGE) showed that they were not related to each other (17), implying that the pSD11-like plasmids were moving between genetically uncorrelated E. coli isolates.

The IncX plasmids are narrow-host-range plasmids of Enterobacteriaceae, such as E. coli, Salmonella, Shigella, Citrobacter, and Klebsiella, and include at least five subtypes, IncX1 to IncX5 (22). IncX plasmids have been implicated in the spread of many resistance genes, such as aqxAB, qnrS1, blaCTX-M, and blaOXA-9, as well as blaKPC and blapNDM (22, 23). Among them, blaCTX-M and blaOXA-9 were carried by IncX4 plasmids. Moreover, IncX4 plasmids seem to be efficiently transferred at different temperatures and different lack-of-fit burdens among bacterial hosts, which may facilitate the transfer of cfr among Enterobacteriaceae and highlight its important role in dissemination of antimicrobial resistance (23).

In conclusion, we report the first complete sequence of an IncX4 plasmid carrying cfr. This finding expands the range of replicons into which cfr is able to transfer and contributes to explaining the dissemination of cfr among E. coli isolates. Further surveillance is necessary to determine the prevalence of cfr-carrying IncX plasmids among the other Gram-negative bacteria from animals.

**Nucleotide sequence accession number.** The complete DNA sequence of plasmid pSD11 was assigned GenBank accession no. KM212169.

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