Journal of Antimicrobial Chemotherapy

J Antimicrob Chemother 2011; **66:** 1174–1191
doi:10.1093/jac/dkr037
Advance Access publication 17 February 2011

The new genetic environment of *cfr* on plasmid pBS-02 in a *Bacillus* strain

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Keywords: florfenicol resistance, IS*256*, restriction-modification system

Sir,

The gene *cfr*, encoding a 23S rRNA methyltransferase, confers resistance to five chemically unrelated antimicrobial classes, including phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A ($PhLOPS_A$), and has been observed mainly in staphylococcal isolates over the past decade.[1–3] Our previous study reported a *cfr*-carrying plasmid, pBS-01, in a novel strain (BS-01) of a *Bacillus* species isolated from a pig farm in China.[4] In our routine surveillance study on antimicrobial resistance in farm animals in 2010, another florfenicol and chloramphenicol-resistant *Bacillus* species strain (named BS-02) from swine faeces was identified. Gram staining, sequence analysis of the 16S rRNA and API 50CH testing associated with the API 20E system (bioMérieux, France) showed that BS-02 had an identical profile to that of strain BS-01, which suggested that BS-02 also belongs to a novel *Bacillus* species. Despite their identical profiles, more than six PFGE band differences were observed between BS-01 and BS-02 (data not shown), indicating that they belong to different clonal types of the same species.

Strain BS-02 was further screened for *cfr* and *fexA* genes, which contribute to florfenicol resistance, using primers described previously.[4] Both the *cfr* and *fexA* genes were amplified and confirmed by sequencing. A single plasmid, of ~16 kb and designated pBS-02, was extracted from BS-02 using a DNA midi kit (Qiagen, Germany) and subsequently transformed into a *Staphylococcus aureus* recipient strain (RN4220) by electroporation. Southern blotting analysis showed that the *cfr* gene hybridized to pBS-02 in the original strains and transformants. However, the result of Southern blotting suggested only a chromosomal location of the *fexA* gene in the BS-02 strain. The pBS-02-harbouring transformant exhibited elevated MICs (≥4-fold increase) of chloramphenicol, florfenicol, clindamycin, linezolid and tiamulin, which was in line with the properties of the transformant carrying pBS-01, and demonstrated that pBS-02 also conferred the same resistance phenotypes.

To characterize pBS-02 in detail, the sequence of the 16543 bp plasmid (GenBank accession no. HQ128580) was obtained by primer walking, starting from the amplified *cfr*

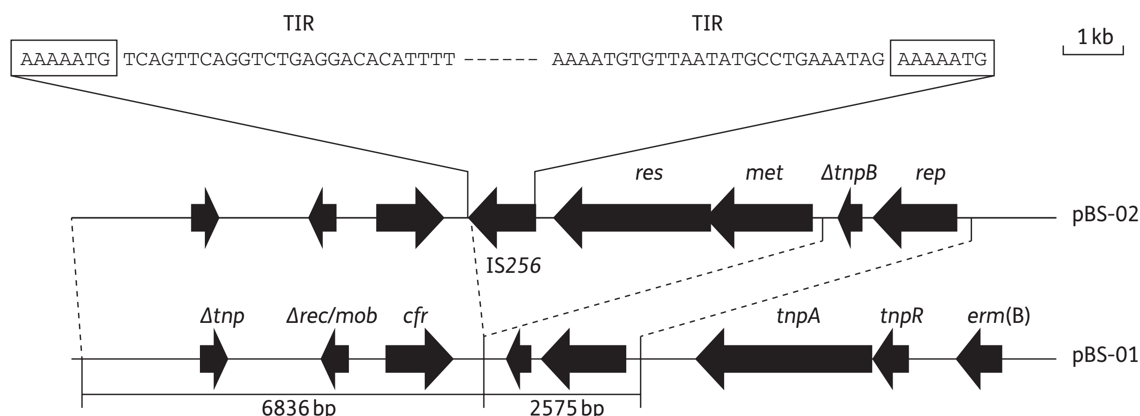

Figure 1. Comparison of plasmids pBS-02 and pBS-01 derived from *Bacillus* strains BS-01 and BS-02, respectively. Open reading frames are shown with solid arrows and the regions of homology on these two plasmids are connected by dashed lines. The 7 bp direct target site (GTAAAAA) duplications at the junctions of the IS*256* element in pBS-02 are highlighted in boxes. The imperfect terminal inverted repeats (TIRs) are also displayed between the target site duplications. The dashed line between the TIRs represents the sequence between them.

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gene. Eight predicted coding sequences for proteins of ≥100 amino acids were identified in pBS-02 using GLIMMER software (Figure 1). Similar to pBS-01, pBS-02 has eight open reading frames (ORFs); however, compared with pBS-01, pBS-02 has a three gene deletion and a three gene insertion. The deleted genes are *tnpA*, *tnpR* and *erm*(B), while the inserted genes are IS*256*, *res* and *met*. The five ORFs shared between pBS-01 and pBS-02 are either identical or nearly identical, as only seven nucleotide differences were found between the two plasmids in the region containing *cfr* and its upstream sequences.

A unique feature of pBS-02 is its carriage of an insertion element. A complete IS*256* element, which was 1324 bp and consisted of a single ORF encoding a transposase protein flanked by non-coding regions (NCRs) harbouring 26 bp imperfect terminal inverted repeats (TIRs), was located directly downstream of the *cfr* gene (Figure 1). This single element showed 99.9% identity (1 bp different on the left NCR) to that in the *Enterococcus faecalis* plasmid, pWZ1668 (GenBank accession no. GQ484956). Typically, IS*256* generates 8 or 9 bp direct target site duplications (TSDs) upon insertion;[5] however, a novel 7 bp (5′-GTAAAAA-3′) TSD was found in pBS-02. Interestingly, pBS-01 appears to contain remnants of IS*256*, as the C terminus of the transposase (32 bp) and the intact right NCR (24 bp) were present downstream of *cfr* in pBS-01. This finding suggested that the IS*256* element, probably along with the genes *res* and *met*, was originally integrated into the site between *cfr* and Δ*tnp* in pBS-01, but later vanished from this plasmid. Although very little is known about the transposition mechanism of IS*256*, it is widespread in the genomes and plasmids of *Enterococcus* and *Staphylococcus*.[6] In addition, the extrachromosomal IS*256* circles obtained by inverse PCR in RN4220 containing pBS-02 indicates the active transposition of this element (data not shown). The placement of IS*256* directly downstream of the *cfr* gene, which was distinctly different from the insertion sequence IS*21-558* involved in the dissemination of the *cfr* gene and frequently detected as a *cfr* flanking region in staphylococci,[7,8] suggests that this insertion element may have played a role in the acquisition and dissemination of this resistance gene in *Bacillus*.

A putative restriction-modification (RM) system was found downstream of IS*256* in pBS-02. The *res* next to IS*256* showed 45% amino acid sequence identity to the restriction enzyme LlaFI of *Lactococcus lactis* (AAD15793). The *met* gene, with 7 bp overlapping *res*, presented 47% amino acid sequence identity to the DNA methylase N-4/N-6 domain protein of *Bacillus coagulans* 36D1 (EEN91760). As the insertion of an RM gene complex into a plasmid increases the stability of plasmid maintenance in bacterial cells,[9] the RM system in pBS-02 may play a key role in plasmid stabilization in *Bacillus* spp. Moreover, the RM system in pBS-02 is similar to the RM elements that are often linked to mobile genetic elements, such as transposons, integrons, plasmids and viruses.[10] This result further suggested that both the IS*256* element and RM system, which may come from bacterial species other than *Bacillus*, could be inserted together into the sequence between *cfr* and Δ*tnpB*.

In summary, this study describes the presence of the multidrug resistance gene *cfr* in pBS-02 from *Bacillus* in a new genetic context. Given that the *cfr* gene and IS*256* are highly homologous to the corresponding genes in *Staphylococcus* or *Enterococcus*, it is plausible to speculate that the *cfr* gene was first introduced from other Gram-positive organisms into *Bacillus* by an insertion element. The carriage of a mobile *cfr* gene by *Bacillus* species and their common association with foods and environments will facilitate the spread of $PhLOPS_A$ resistance to other bacterial organisms in various ecosystems, including animal hosts.

Funding

This work was supported by grants both from the National Natural Science Foundation of China (No. 31001087) and from the Program for Chang Jiang Scholars and the Innovative Research Team at the University of China (No. IRT0866).

Transparency declarations

J. H. is supported through the Oak Ridge Institute for Science and Education. All other authors: none to declare.

Disclaimer

The work presented in this manuscript does not necessarily reflect the views of the US Food and Drug Administration.

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