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Novel Tn*916*-like elements confer aminoglycoside/macrolide co-resistance in clinical isolates of *Streptococcus gallolyticus* ssp. *gallolyticus*

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Background: *Streptococcus gallolyticus* ssp. *gallolyticus* (*Sgg*) is a commensal bacterium and an opportunistic pathogen. In humans it has been clinically associated with the incidence of colorectal cancer (CRC) and epidemiologically recognized as an emerging cause of infective endocarditis (IE). The standard therapy of *Sgg* includes the administration of a penicillin in combination with an aminoglycoside. Even though penicillin-resistant isolates have still not been reported, epidemiological studies have shown that this microbe is a reservoir of multiple acquired genes, conferring resistance to tetracyclines, aminoglycosides, macrolides and glycopeptides. However, the underlying antibiotic resistance mobilome of *Sgg* remains poorly understood.

Objectives: To investigate the mobile genetic basis of antibiotic resistance in multiresistant clinical *Sgg*.

Methods: Isolate NTS31106099 was recovered from a patient with IE and CRC at Nantes University Hospital, France and studied by Illumina WGS and comparative genomics. Molecular epidemiology of the identified mobile element(s) was performed using antibiotic susceptibility testing (AST), PCR, PFGE and WGS. Mobility was investigated by PCR and filter mating.

Results: Two novel conjugative transposons, Tn*6263* and Tn*6331*, confer aminoglycoside/macrolide co-resistance in clinical *Sgg*. They display classical family Tn*916*/Tn*1545* modular architecture and harbour an *aph*(3′)-III→*sat4*→*ant*(6)-Ia→*erm*(B) multiresistance gene cluster, related to pRE25 of *Enterococcus faecium*. These and/or closely related elements are highly prevalent among genetically heterogeneous clinical isolates of *Sgg*.

Conclusions: Previously unknown Tn*916*-like mobile genetic elements conferring aminoglycoside/macrolide co-resistance make *Sgg*, collectively with other gut Firmicutes such as enterococci and eubacteria, a potential laterally active reservoir of these antibiotic resistance determinants among the mammalian gastrointestinal microbiota.

Introduction

Streptococcus gallolyticus ssp. *gallolyticus* (*Sgg*, formerly *Streptococcus bovis* biotype I) is a common Gram-positive gut commensal in animals and humans as well as an opportunistic pathogen causing a variety of clinical entities.[1] In humans this bacterium has been clinically associated with the incidence of colorectal cancer (CRC) for more than half a century[2] and, due to being among the five most commonly isolated microbes in Europe,[3] has been epidemiologically recognized as an emerging cause of infective endocarditis (IE).[4]

The standard therapy of *Sgg* includes the administration of a penicillin in combination with an aminoglycoside.[5,6] Strikingly, while penicillin-resistant isolates have still not been reported, epidemiological surveys and WGS have demonstrated very different landscapes regarding the antibiotic resistance mobilome of *Sgg*. On one hand, the former have suggested elevated presence of mobile genetic elements, owing to the high detected prevalence of acquired tetracycline, aminoglycoside and macrolide resistance genes as well as co-localization of these determinants, owing to the observed highly correlated resistance phenotypes.[4,5] On the other hand, while the number of public *Sgg* genomes continues to grow (20 assemblies as of December 2017), comparative genomics studies have accounted for just a few elements, conferring tetracycline and glycopeptide resistance.[7–9] Hence studying the genetic elements underlying acquired aminoglycoside and/or

macrolide resistance is necessary in order to shed light on the current epidemiological situation and the contribution of this species to the horizontal spread of such determinants in the mammalian gut.[10] The aim of this work was to investigate the mobile genetic basis of antibiotic resistance in multiresistant clinical *Sgg*.

Materials and methods

Isolate collection and antibiotic susceptibility testing (AST)

All clinical isolates were recovered at Nantes University Hospital (Table S1, available as Supplementary data at *JAC* Online) and identified as *Sgg* using VITEK® MS MALDI-TOF technology (bioMérieux, Marcy l'Etoile, France). AST was performed with VITEK 2 AST ST01 cards (bioMérieux) according to the EUCAST recommendations (www.eucast.org).

WGS and comparative analysis

WGS is explained elsewhere.[11,12] Comparative analysis software and visualization is described at the end of the first Supplementary data file.

Molecular epidemiology, typing and determination of macrolide resistance genes

Tn*6263* prevalence was determined by PCR as explained in Figure S1.[13] Primers and amplification conditions are given in Table S2. Clonal relatedness was investigated by PFGE. Macrolide resistance genes were determined by PCR.[5]

Mobility analysis

Excision of Tn*6263* was investigated by PCR[13] as follows: total DNA was extracted from isolate NTS31106099 grown on antibiotic-free Brain Heart Infusion (BHI) agar plates, as well as on BHI agar plates containing either tetracycline or erythromycin (both at 16 mg/L) followed by amplification and sequencing of its reconstituted target site and circular intermediate junction (Figure S2). Filter mating[14] was performed between erythromycin-resistant isolate NTS31106099 as a donor and vancomycin-resistant *Sgg* LMG17956 as a recipient.

Results and discussion

Recently, we reported the draft genome of multiresistant *Sgg* isolate NTS31106099 which was recovered in 2011 at Nantes University Hospital, France from positive blood culture of a patient with IE and CRC.[11]

During routine AST, NTS31106099 displayed susceptibility to ampicillin (MIC = 0.125 mg/L) and gentamicin (MIC = 4 mg/L) as well as resistance to tetracyclines (>256 mg/L), aminoglycosides [kanamycin (>256 mg/L), streptomycin (>256 mg/L)] and macrolides [erythromycin (>256 mg/L), clindamycin (>256 mg/L)].

Automated identification of acquired antibiotic resistance genes indicated the presence of *tet*(M), *aph*(3′)-III, *ant*(6)-Ia, *sat4* and *erm*(B) determinants, which confer resistance to tetracyclines, aminoglycosides, nucleosides and macrolides, respectively (Table S3). Interestingly, while all genes were found to be chromosomally located, we observed a lack of genetic linkage between the *tet*(M) and the other determinants (see column Contig in Table S3). Opposite to what was expected,[5] this result suggested that they are harboured by different mobile genetic elements.

Indeed, in agreement with previous studies[7,9] we found that the *tet*(M) gene is harboured by a putative Tn*916*-like element (coordinates 10766.28821 on contig JYKU01000009, 99% identity to Tn*916* of *Enterococcus faecalis*[15]) inserted into a conserved hypothetical membrane protein homologous to GALLO_0777 in *Sgg* UCN34.[7] However, the other four genes are co-localized on contig JYKU01000013, thus forming a characteristic 5.4 kb multiresistance gene cluster *aph*(3′)-III→*sat4*→*ant*(6)-Ia→*erm*(B) harboured by a 44.6 kb isolate-specific genomic island (coordinates 186444.231098 on contig JYKU01000013). The element disrupts a putative RNA methyltransferase gene corresponding to GALLO_1429 in *Sgg* UCN34. Strikingly, an exact match to this sequence was not found in any publicly available *Sgg* (or other) genome (Figure S1). However, we identified three highly related (>95% identity) non-characterized elements in draft genomes of *Enterococcus faecium* and *Eubacterium contortum* (Figure S3).

In order to elucidate its genetic organization, we compared the island of NTS31106099 to the 29 kb conjugative transposon CTn*7* of *Clostridium difficile* 630[16] (51% coverage, 85% identity) and to Tn*916* (21% coverage, 71% identity) (Figure 1). The former belongs to the Tn*916*/Tn*1545* family of mobile genetic elements and is the closest well-characterized element available (as determined by BLAST), while the latter is the archetype of the Tn*916*/Tn*1545* family. In accordance with the established guidelines,[17] we found that the island of NTS31106099 displays a characteristic Tn*916*-like modular architecture. Firstly, a terminally situated recombination module is identifiable, which is represented by a single gene that encodes a large serine recombinase (*orf1*, 99% coverage and 86% identity to the serine recombinase of CTn*7*). Secondly, *orf3* and *orf4* might constitute a regulation module since they appear homologous to the regulatory *orf7* and *orf8* of Tn*916*.[17] Finally, the *orf28*.*orf40* module is homologous to the recombination modules of CTn*7* and Tn*916*. However, similarly to CTn*7* and unlike Tn*916*, it contains additional coding sequences (CDS, *orf35*, *orf36*, *orf41*.*orf46*) and lacks an *orf24* homologue. In addition to the identified *aph*(3′)-III→*sat4*→*ant*(6)-Ia→*erm*(B) cluster, automated annotation revealed 10 genes, encoding conserved hypothetical proteins and 19 accessory functionally-assigned CDS (Table S4). Interestingly, 69% of them have received functional annotation based on similarity to clostridial homologues, which might point to their probable origin. Owing to these results, we concluded that the genomic island of isolate NTS31106099 is a putative conjugative transposon of the Tn*916*/Tn*1545* family which was registered as Tn*6263* in the transposon registry.[18]

BLAST analysis of the *aph*(3′)-III→*sat4*→*ant*(6)-Ia→*erm*(B) cassette identified highly conserved sequences mostly in Gram-positive species (coverage 37%–99%, identity 98%–100%). Notably, the sequence is highly related (43% coverage, 99% identity) to the aminoglycoside/streptothricin resistance cluster described in multiresistant *E. faecium*.[19] Furthermore, it seems to be assembled from various conserved regions of the 50 kb conjugative multi-resistance plasmid pRE25 of *E. faecalis*[20] (Figure 2). Interestingly, the same observation has been reported about the highly related macrolide/aminoglycoside/streptothricin (MAS) element of Tn*6003* (48% coverage, 100% identity), described in *Streptococcus pneumoniae*.[21] Collectively these findings suggest common ancestry of these multi-resistance clusters.

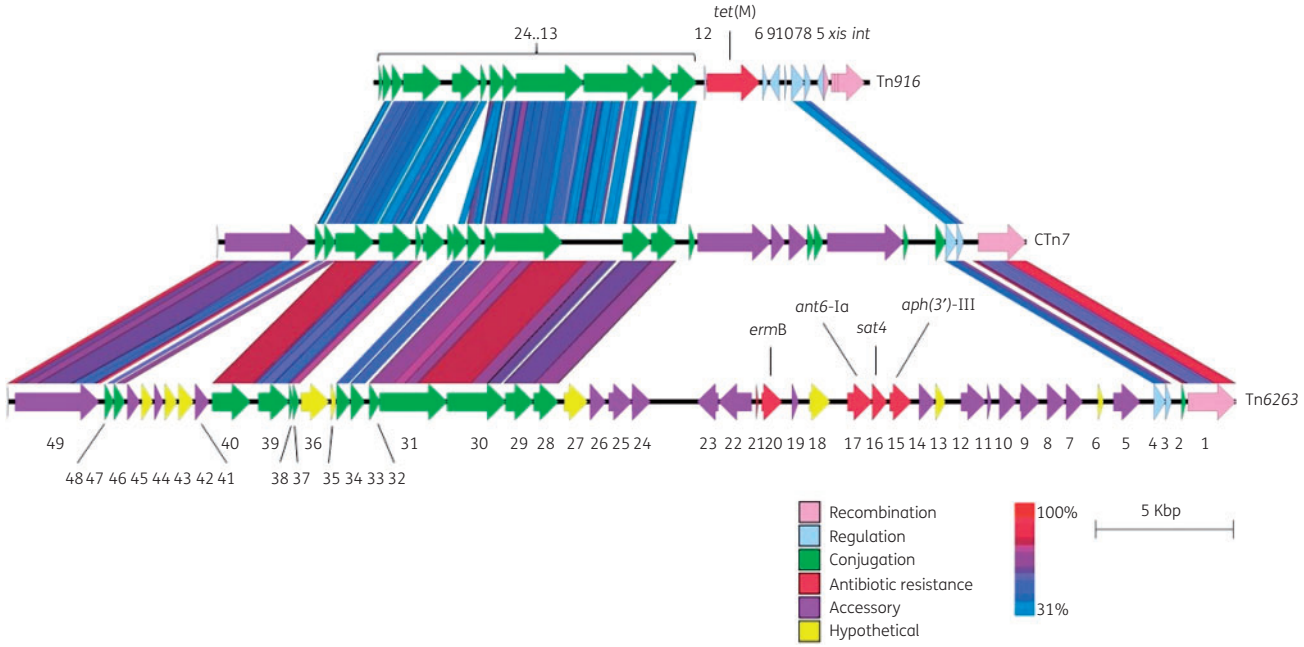

Figure 1. Comparison of Tn*6263* to Tn*916* of *Enterococcus faecalis* and CTn*7* of *Clostridium difficile*. Tn*916* is the archetype of the Tn*916*/Tn*1545* family of mobile genetic elements. CTn*7* is the closest characterized element to Tn*6263*, as determined by BLAST. The classical Tn*916*-like modular architecture of Tn*6263* is evident: recombination, regulation and conjugation modules are clearly distinguishable. More detailed description of the annotated genes is presented in Table S4. Comparison was performed with the WebACT resource using the TBlastX algorithm and visualized using Easyfig. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

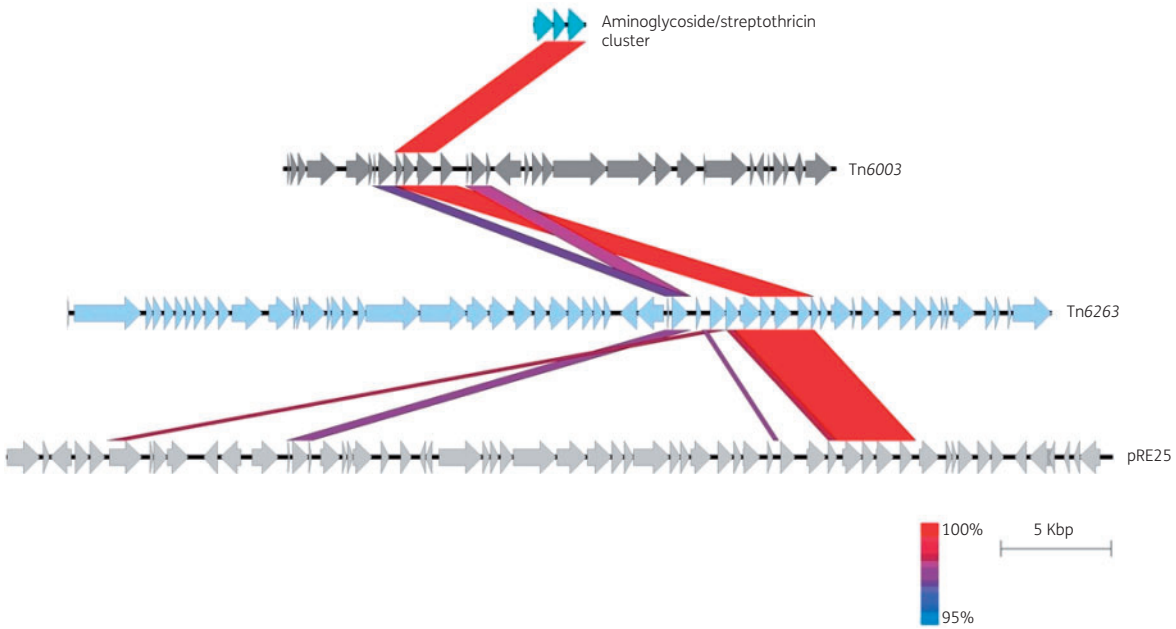

Figure 2. Relatedness of the *aph*(3′)-III→*sat4*→*ant*(6)-Ia→*erm*(B) cluster of Tn*6263* to the aminoglycoside/streptothricin cluster of *Enterococcus faecium*, Tn*6003* of *Streptococcus pneumoniae* and the multiresistant plasmid pRE25 of *Enterococcus faecalis*. Comparison was performed with the WebACT resource using the TBlastX algorithm and visualized using Easyfig. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Taking into account its low abundance in the public nucleotide databases, we investigated the prevalence of Tn*6263* by AST and PCR among 60 clinical isolates recovered at our clinical centre (Figure S2 and Table S5). Accordingly, no amoxicillin-resistant isolates were detected. However, consistent with previous studies,[5,10] we detected elevated levels of tetracycline and macrolide resistance [67% ($n = 40$) and 59% ($n = 35$), respectively, with 85% co-resistance relative to the former] as 92% ($n = 33$) of the

macrolide-resistant isolates tested positive for the *ermB* gene. Strikingly, 69% ($n = 24$) of them tested positive for the recombinase gene of Tn*6263* and for its disrupted target gene. Two of these isolates, NTS31301958 and NTS31307655, displayed different Tn*6263* right-end amplicons (Figure S4a) and the only indistinguishable PFGE profiles (Figure S4b). Subsequent WGS[12] revealed that they contain another novel element, named Tn*6331*, which is highly related to Tn*6263* (Figure S4c). Collectively, these results suggest that: (i) Tn*6263* and/or related transposons are highly prevalent among the tested genetically heterogeneous *Sgg* isolates; (ii) they are the major *ermB*-carrying elements in the studied collection and; (iii) an integration hot spot for these elements is present in the chromosome of *Sgg*.

Intracellular mobility analysis indicated that the excision of Tn*6263* from the chromosome of NTS31106099 is upregulated by inhibitors of protein synthesis, such as tetracycline and erythromycin (Figure S5a), which is in agreement with the recent study of Scornec *et al.*[22] and additionally supports its classification as a Tn*916*/Tn*1545* family element. Interestingly, while CTn*7* and Tn*6263* both appear to have integrated into putative rRNA methyltransferase genes (*rum*A in *C. difficile* 630 and UG96_07300 in *Sgg* NTS31106099), they seem to insert into different target sequences[13] (Figure S5b). Regarding its intercellular mobility, we did not observe any transfer of Tn*6263* between isolates NTS31106099 and LMG17956. However, PCR screening, molecular typing and WGS collectively suggest multiple acquisition of Tn*6263* rather than a mere clonal expansion due to the observed isolate and element genetic heterogeneity.

Future work including more isolates from multiple clinical centres as well as from non-human sources will focus on elucidating the underlying reasons for the high prevalence of these elements, since neither aminoglycosides nor macrolides are generally used against *Sgg* infections. Thus, the selective pressure driving their dissemination cannot be attributed to these antibiotics.

In conclusion, we have found that previously unknown Tn*916*-like mobile genetic elements conferring aminoglycoside/macrolide co-resistance make *Sgg*, collectively with other gut Firmicutes such as enterococci and eubacteria, a potential laterally active reservoir of these antibiotic resistance determinants among the mammalian gastrointestinal microbiota.

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Transparency declarations

None to declare.

Supplementary data

Figures S1 to S5 and Tables S1 to S5 as well as details about the comparative analysis are available as Supplementary data at *JAC* Online.

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