Host specificity in the diversity and transfer of Isa resistance genes in group B Streptococcus

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Objectives: In group B Streptococcus (GBS), cross-resistance to lincosamides, streptogramin A and pleuromutilins (LSAP) is mediated by the acquisition of Isa genes. Here, we characterized the diversity, mobility and ecology of Isa genes in this species.

Methods: Isa variants were systematically identified by BLAST searches in the genomes of 531 GBS strains from different hosts and geographical origins. The associated phenotypes were determined by a microdilution MIC method. Acquisition of resistance genes was deduced from comparative genomics and phylogeny. Their mobility was tested by conjugation experiments.

Results: Isa(E) and three variants of Isa(C) were identified in GBS strains. Two Isa(C) variants had not been previously reported. All four variants conferred LSAP phenotypes. Isa(E) was located in a multiresistance gene cluster of a single human strain. This gene was transferred by a high-frequency recombination-type mechanism between GBS strains. Two Isa(C) variants are carried in six unrelated human strains by two similar elements specifically integrated in the oriT site of four different classes of integrative and conjugative elements (ICEs). Strikingly, the acquisition of the resistance gene always occurred by the integration of the element into a resident ICE. The third Isa(C) variant was located at the same site in the core genome of 11 genetically distant bovine strains and was likely propagated by horizontal transfer of the corresponding chromosomal region.

Conclusions: Isa genes in GBS show distinct host specificities and modes of transfer. In general, their dissemination is mediated by recombination rather than by the transfer of conjugative elements.

Introduction

Streptococcus agalactiae or group B Streptococcus (GBS) is a major cause of neonatal infections worldwide and an important pathogen in immunocompromised patients and elderly people.1,2 However, GBS is primarily a commensal organism colonizing the human genitourinary and gastrointestinal tracts.3 Both intrapartum prophylaxis and treatment of invasive infections are usually based on β-lactam antibiotics (penicillin and ampicillin), whereas macrolides (erythromycin) and lincosamides (clindamycin) provide alternative therapies to penicillin-allergic patients and carriers. During the last decade, a significant and increasing resistance to erythromycin and clindamycin in GBS strains has been reported in several countries, with prevalence values from 4% to 54% and from 3% to 33%, respectively.4,5

GBS is also a pathogenic agent of bovine mastitis, although the incidence of GBS mastitis considerably decreased in herds in Europe with the appropriate use of antibiotics.6 The strains that cause mastitis are susceptible to β-lactam antibiotics and the resistance rate to macrolides and lincosamides is similar to that observed for human GBS.7,8

Different resistance mechanisms to macrolides and lincosamides conferring distinctive phenotypes have been reported in GBS. The ribosomal alteration of the antibiotic target site, which is mediated by methylases encoded by erm genes, confers resistance to macrolides, lincosamides and streptogramin B (MLSβ phenotype).7 In contrast, the expression of efflux pumps encoded by mef genes confers resistance only to macrolides (M phenotype).9 An additional mechanism of resistance is the enzymatic inactivation of lincosamides (L phenotype) mediated by the acquisition of Inu genes encoding nucleotidyltransferases.10 More recently, another phenotype conferring cross-resistance to lincosamides, streptogramin A and pleuromutilins (LSAP phenotype) has been described in GBS. This particular resistance is mediated by two genes of the Isa family, namely Isa(C) and Isa(E).10,11

Isa genes encode ATP-binding proteins that have been classified as class 2 ABC transporters.12 These proteins have duplicated...
ATP-binding domains, each containing the Walker A and B motifs and the conserved ABC signature motifs, but lack any obvious membrane-spanning domain. Resistance might be a result of an efflux mechanism of the antibiotics. However the absence of transmembrane domains and the structural diversity of the substrate argue for an alternative mechanism. The Iṣa(C) gene was found to be carried by an integrative element, whereas the determinant Iṣa(E) is located in a multiresistance cluster along with the Inu(B) gene. Two other Iṣa variants, Iṣa(A) and Iṣa(B), have been characterized in Firmicutes and confer different phenotypes. The chromosomal Iṣa(A) gene confers intrinsic resistance to lincosamides and streptogramin A (LSₐ phenotype) in Enterococcus faecalis and the plasmidic Iṣa(B) gene is responsible for clindamycin resistance (L phenotype) in Staphylococcus sciuri and Staphylococcus haemolyticus. Except for Iṣa(A), present in the core genome of E. faecalis, all other Iṣa determinants have been found to be carried by mobile genetic elements (MGEs), implying putative mobility. So far, Iṣa(C) has only been identified in GBS while Iṣa(B) seems to be restricted to staphylococci. In contrast, the multiresistance cluster carrying Iṣa(E) is shared between streptococci, enterococci and staphylococci.

MGEs, which carry antibiotic resistance genes, contribute to bacterial evolution by disseminating new genes and conferring new phenotypes on their recipient. Access to a broad collection of 531 GBS genomes from different hosts and different geographical origins provides a unique set of information to characterize the diversity, mobility and ecology of MGEs carrying antibiotic resistance determinants in this species. Here, combining comparative genomics, phylogeny and conjugation experiments allowed us to reconstruct the evolutionary history of the acquisition of Iṣa resistance genes in GBS, revealing profoundly different behaviours of Iṣa genes between isolates from human and bovine hosts.

Materials and methods

Bacterial strains

Bacterial strains used in this study are listed in Table S1 (available as Supplementary data at JAC Online; http://jac.oxfordjournals.org/). Seven strains carrying Iṣa genes and a control strain were used for antibiotic susceptibility testing. A collection of 72 bovine GBS strains, isolated from different regions of France, was used to screen the presence of Iṣa genes. Spontaneous rifampicin- and fusidic acid-resistant derivative of strains BM110 and COH1 were selected as described by Puymège et al. The resulting strains BM110-RF and COH1-RF and strain A909-RF were used as recipients in mating assays.

Antibiotic susceptibility

MICs of lincomycin, clindamycin, virginiamycin M, synergicin, tiamulin and valnemulin were determined in triplicate by the microbroth dilution method according to the CLSI guidelines (tested range 0.015–64 mg/L). Lincomycin (L6004) and clindamycin (C5269) were purchased from Sigma and tiamulin (BIA-T1321) and valnemulin (BIA-V1492) from Bioastralis. Virginiamycin M and synergicin (a mixture of quinupristin and dalfopristin in a ratio of 30:70) were obtained from Sanofi.

Mating experiments

Conjugal transfer experiments were performed using the LSₐP resistant strains (CCH172, CCH620, CCH006 and CCH615) as the donors and rifampicin- and fusidic acid-resistant strains as the recipients. Donor and recipient strains were mated on a hydrophobic-edge membrane filter (Millipore) with a donor-to-recipient ratio of 1:1 as previously described. After incubating the plates overnight at 37°C, bacterial cells were resuspended in Todd–Hewitt (TH) broth and diluted. Dilutions were then spread on TH agar plates containing 0.5 mg/L lincomycin, 50 mg/L rifampicin and 10 mg/L fusidic acid to select for transconjugants (TCs) and plates containing 0.5 mg/L lincomycin to select for donor strains. The conjugation frequency was determined by dividing the number of TCs by the number of donor cells.

PCR amplification and DNA cloning

Oligonucleotides used in this study were designed by primer3 (http://gmdslab.shgmo.org/primer3/) and are listed in Table S2. To investigate the antimicrobial resistance encoded by Iṣa(C) variant 2, the gene product was overexpressed by using the inducible expression vector pJM2246-ptet. The complete ORF of this gene was amplified by PCR using primers modified to include NotI and SacII restriction sites. The fragment was then cloned into the pJM2246-ptet vector digested by NotI and SacII. The recombinant plasmid, after verification by sequencing, was transformed by electroporation into BM132. Transformants were selected on agar plates containing chloramphenicol (4 mg/L). Expression from the xyl/tet(O) promoter was induced by the addition of 1 μM hydroxytetracycline (aTET) in the medium as previously described.

GBS genomes

The sequences of 531 GBS genomes were screened for the presence of Iṣa genes. Of these, 228 genome sequences were from a previous study on the diversity of GBS strains and 303 genome sequences (16 complete and 287 draft) were retrieved from the NCBI FTP site (http://www.ncbi.nlm.nih.gov/ftp/). Among these strains, 430 were isolated from human and 101 from animal sources, of which 58 were bovine.

Diversity of GBS strains

MLST was performed by extracting the sequences of the seven housekeeping genes of the MLST scheme and comparing them with the known STs from the GBS MLST database (http://pubmlst.org/gosa56-lactate/). A whole-genome phylogeny was built for the strains belonging to the same clonal complex (CC) by using the CSI Phylogeny service from the Center for Genomic Epidemiology server (https://cge.cbs.dtu.dk/services/CSIPhylogeny/). Eight complete genomes belonging to eight different CCs (CC1, CC7, CC17, CC19, CC22, CC23, CC26 and CC552) were used for comparative analysis (accession numbers HF952104, NC_007432, HG939456, NC_004116, CP007570, NC_004368, CP006910 and NC_019048, respectively).

Sequence analysis of Iṣa genes and MGEs carrying these genes

Iṣa genes were searched by BLASTx using as query the protein sequences of four previously identified Lsa variants Lsa(A) (AAW30455), Lsa(B) (NP_899166), Lsa(C) (AEA37904) and Lsa(E) (AAH31491) and a cut-off of 80% identity. Related proteins conferring resistance to macrolides and related compounds, such as Msr-like (BAD85637, AY004350, AF274302 and JF769133) and Vga-like (YP_002332257, AAB95639, CAY33094, ACX92986 and CBY88983) proteins, were also searched by BLASTx analysis.

Multiple sequence alignments were performed using MUSCLE and the corresponding phylogenetic tree was built using the maximum likelihood method in MEGA6.06 version. Genomic islands encoding Iṣa genes were analysed by extracting the sequence flanking the resistance genes using a Biopython script. Annotation of genomic islands was first performed by the RAST server and subsequently refined by Uniprot and Pfam analyses.
The nucleotide sequences of the lsa(C) variants 1 and 2 have been deposited in the EMBL database under the accession numbers LN849945 and LN849946.

Results

GBS genomes encode four different Lsa proteins

Twelve lsa genes displaying >80% identity with lsa(C) and lsa(E) variants were identified by BLASTx analysis in 12 GBS isolates (Table 1). The phylogenetic tree in Figure 1 represents their genetic relatedness at the amino acid level.

Eleven predicted proteins were related to the Lsa(C) protein encoded in the chromosome of GBS UCN70. Two proteins, present in an ST19 and a ST23 strain were considered as identical (one and two amino acid changes). The other nine represent two variants of Lsa(C): four proteins encoded by strains from three different lineages (ST1, ST19 and ST23) showed 93% identity to Lsa(C) and five proteins detected in GBS strains of five different STs showed 90% identity to Lsa(C) (Table 1). These two variants have not been described previously. Based on the nomenclature for macrolide–lincosamide–streptogramin resistance determinants, we designated them Lsa(C) variant 1 and variant 2, respectively. The Lsa protein encoded in the ST19 strain CCH615 genome was identical to the Lsa(E) protein identified in SGB76.

Isa(C) variant 2 is a bovine-associated resistance determinant

Among the five GBS strains carrying lsa(C) variant 2, four were associated with bovine mastitis. Variant 2 from bovine strains were identical at the nucleotide level, whereas the only one in a human strain was frameshifted at position 487, leading to a premature stop codon at residue 172 and likely to a non-functional protein. Comparison of the sequence flanking these genes with GBS reference genomes revealed that they are inserted at the same position in the core genome, inside a two-component regulatory system-encoding locus (gbs1908 and gbs1909 in NEM316). The

Table 1. Characteristics of GBS strains carrying lsa genes

<table>
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<tr>
<th>Strain</th>
<th>Host</th>
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<th>ST</th>
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<th>Reference</th>
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</tbody>
</table>

*alsa(C) variant 2 is inactivated by a frameshift mutation.*

Sequence accession numbers

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Figure 1. Unrooted phylogenetic tree based on the protein sequence alignment of 11 Lsa proteins detected in 11 GBS strains and four Lsa proteins (*AEA37904, NP_899166, AHH31491 and AAW30455) used as query for the similarity search. The phylogenetic tree was built using the maximum likelihood method in MEGA6.06 version and was edited using FigTree (http://tree.bio.ed.ac.uk/software/figtree/).
insertion, probably by non-homologous recombination, led to an 804 bp deletion encompassing the 3' end of the sensor histidine kinase gene and the 5' end of the transcriptional regulator (Figure S1). The presence of this intrinsic resistance gene in five genetically distinct strains suggests that this variant was acquired through horizontal gene transfer. Pairwise alignments of the regions directly surrounding the lsa genes in these five strains revealed a low density of SNPs compared with the far-off regions (data not shown). This observation strongly suggests that variant 2 was acquired through a high-frequency recombination (Hfr)-type mechanism of conjugal transfer between GBS isolates as previously described.21 The presence of this gene in 6.9% of the sequenced bovine strains prompted us to search for this gene in additional bovine isolates. We further screened by PCR and sequencing a collection of 72 GBS isolates causing mastitis in different areas in France7 for the presence of the four lsa genes. Seven strains (9.7%) from five different STs (ST420, 421, 427, 428, 429) were positive for variant 2, but none for the three other lsa genes. In the seven strains, lsa(C) variant 2 was inserted at the same position as the five genes identified in silico (Table 1). These results strengthen our hypothesis of the spreading of the lsa(C) variant 2 by horizontal transfer of the corresponding chromosomal region.

**lsa(C) and lsa(C) variant 1 are carried by integrative elements targeting the oriT site of diverse ICEs**

The two lsa(C) genes and the four closely related lsa(C) variant 1 genes are carried by two genetic elements displaying 90% identity in six human isolates belonging to different STs (Figure S2). These elements belong to a new family of integrative and mobilizable elements (IMEs) recently described as specifically inserted at the oriT site from different ICEs (IME_\textit{oriT}s).15 Comparison of the flanking regions of these two related IME_\textit{oriT}s in the six strains showed that they are integrated in six different ICEs, which are depicted in Figure 2(a). They therefore result from six different acquisition events. The ICEs in strains GB-NI-015, CCH006, GB00984 and

![Figure 2](image_url)
GB00957 are identical to the previously described elements ICE_2603_tRNAlys, ICE_515_tRNAlys, Tn916_2603 and Tn5801_COH1, respectively. The ICE identified in strain CCH172 is closely related to ICE_515_tRNAlys, but lacks the region harbouring CAMP factor II. In contrast, the ICE present in strain CCH620 did not show any similarity with well-characterized MGEs. This novel ICE is 34 kb long and encodes 35 genes, including relaxase genes and a type IV secretion system. It is integrated at the 3' end

**Figure 3.** Phylogenetic trees of a sub-set of strains belonging to CC19 (a), CC23 (b) and CC1 (c). The strains carrying lsa(C) and lsa(C) variant 1 and the associated ICEs are underlined. The acquisition of ICEs is indicated by arrows. A whole-genome phylogeny was built for the strains belonging to CC1 (n=99), CC19 (n=79) and CC23 (n=84) by using the CSI Phylogeny service from the Center for Genomic Epidemiology server (https://cge.cbs.dtu.dk/services/CSIPhylogeny/). Complete GBS genomes 09mas018883 (HF952104), 2603V/R (NC_004116) and NEM316 (NC_004368) were used as a reference for the CC1, CC19 and CC23 phylogenies.
of the \( rpsl \) gene and was designated as ICE CCH620 \( rpsl \). The \( lsa(C) \) elements were integrated at the predicted \( oriT \) site of four classes of ICEs (Figure 2b).

The observed insertion inside an ICE in GBS strains could result either from the transfer of the \( lsa(C) \) elements and their integration into the \( oriT \) site of a resident ICE or from the conjugative transfer of an ICE carrying the integrative element. To distinguish between these two scenarios, we reconstructed the most likely evolutionary history of the acquisition of the resistance genes by combining the analysis of these ICE-carrying \( lsa(C) \) genes with phylogenetic analysis. In CC19, strain GB00984 belongs to the clone ‘Tn916-17’, which derived from the insertion of Tn916 at 923 kb (strain 2603V/R).\(^{25}\) Analysis of ICE Tn916 showed that strain GB00984 shares the same ICE (<10 SNPs), integrated at the same position in the genome as the other isolates of this lineage, implying that the IME_\( oriT \) in this strain was acquired by integration into the already inserted Tn916 (Figure 3a). A similar conclusion was drawn for the acquisition of \( lsa(C) \) genes in the five other strains. Indeed, in these five cases, the elements are inserted into an ICE shared by related strains and therefore already present in the common ancestor of these isolates, as depicted in Figure 3(a–c). In the six cases the most parsimonious scenario is therefore the insertion of the integrative element in an ICE already present in the GBS strain.

The transferability of the two \( lsa(C) \) genes was examined by conjugation experiments between GBS strains, using three \( LSSP \) resistant strains (CCH172, CCH620, CCH006) as donor and A909-RF, BM110-RF and COH1-RF as recipient strains. Lincomycin or virginiamycin M was used to select the transfer of \( lsa(C) \) genes. In repetitive experiments only a single transconjugant was obtained using strain CCH620 as donor. The insertion site of the \( lsa(C) \) variant 1 and the presence of ICE_\( rpsl \) were tested by PCR and sequencing. Strikingly, this TC contains two copies of the \( lsa(C) \) element integrated at the \( oriT \) sites of the ICEs ICE_Tn916 and ICE_\( trRNA^{psl} \), but we did not observe the transfer of ICE_\( rpsl \). Therefore, the insertion of the \( lsa(C) \) element likely does not depend on the mobility of the ICE following its insertion, but once transferred it efficiently inserts into \( oriT \) sites.

**Figure 3. Continued**

\( lsa(E) \) gene

The \( lsa(E) \) gene of the CCH615 strain is carried by an 8.5 kb element (nine ORFs) composed of a multiresistance gene cluster [\( aadE \), \( spw \) and \( lnu(B) \) genes, conferring resistance to streptomycin, spectinomycin and lincosamides, respectively] flanked by two copies of the transposase IS1216. This element exhibited similar structure to the \( lsa(E) \)-containing plasmids previously described in \( E.\ faecium \), \( E.\ faecalis \) and \( S.\ aureus \) and to the \( lsa(E) \)-carrying segment recently described in GBS (Figure S3). The sequences of the central parts of the six elements encompassing the multiresistance cluster are almost identical (maximum of five SNPs) among the six strains, whereas the sequences on either side of the cluster are different even between the two GBS isolates. This suggests that recombination events can result in the emergence of new elements that can spread by horizontal dissemination in different species. In CCH615 the insertion of the \( lsa(E) \) element in the core genome between \( asp3 \) and \( rga \) led to a 16 kb deletion in the secA2 locus (Figure S4).

We then investigated the mobility of the \( lsa(E) \)-carrying element by conjugation into strains A909-RF, BM110-RF and COH1-RF. The element was only mobilized into the A909-RF GBS strain with a transfer efficiency of \( 10^{-8} \) and for all five TCs tested it was inserted at the same chromosomal locus as in the donor. To differentiate the transfer of the element with the transfer of a chromosomal region containing the element, we analysed the SNP of the two regions flanking the \( lsa(E) \) element and found that they were identical to the donor strain. We conclude that the \( lsa(E) \)-encoding multiresistance element was transferred by an Hfr-type mechanism and did not involve the two copies of IS1216.

**Antibiotic susceptibility/MIC determination**

The MICs of lincosamides, streptogramin A and pleuromutilins for seven strains carrying \( lsa \) genes and the control strain BM132 are shown in Table 2. Compared with the control strain, MICs of lincosamides were increased by an 8- to 256-fold dilution factor in all
Table 2. MICs of lincosamides, streptogramin A and pleuromutilins for GBS strains carrying lsa genes

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<th>TH+tet</th>
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<th>pJM2246</th>
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<td>0.12</td>
<td>0.25</td>
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Discussion

Access to an extensive set of 531 genomes of GBS strains from broad origins provides a unique resource to analyse antibiotic resistance gene distribution and mobility. Here, we have mined these genomes for the presence of lsa resistance determinants and related genes conferring the L5P phenotype. We have identified lsa variants in 12 GBS strains (2.25%), whereas none of the Vga-like and Msr-like proteins was detected. The previously described lsa genes lsa(C) and lsa(E) were present in two strains and one strain, respectively. We identified two new variants, namely lsa(C) variant 1 (found in four strains) and lsa(C) variant 2 (found in five strains). lsa(C), lsa(C) variant 1 and lsa(E) were observed only in strains of human origin, whereas variant 2 was present in 6.9% of the strains responsible for bovine mastitis. A similarity search against the 9231 Firmicutes genomes available from the NCBI database shows that lsa(C) variants were restricted to GBS, and lsa(C) was identified in only two other streptococcal isolates (Streptococcus sp. SR1 and Streptococcus sp. SR4). By contrast, lsa(E) was found in 64 Firmicutes genomes from nine different species. The presence of lsa(E) in a single isolate is possibly incidental, whereas the three other variants occurring independently in different isolates revealed a specificity for S. agalactiae.

The antimicrobial susceptibility testing showed that the four lsa variants found in GBS confer an L5P phenotype. The phenotype associated with the lsa(C) gene in strain CCH172 shows a similar resistance profile to strain UN70. However, the 256-fold increase in the MIC of clindamycin in this strain might be due to the presence of the erm(B) gene. Compared with lsa(C), the new lsa(C) variant 1 in the CCH620 strain provides enhanced resistance to lincosamycin, virginiamycin M and valnemulin by a factor of 2 (Table 2). The strongest L5P phenotype was observed in strain CCH615, carrying the lsa(E) and InuB genes. The observed resistance is probably mediated by the lsa(E) gene, as the InuB gene is frameshifted at the 208th codon out of 267 and is likely non-functional. The new lsa(C) variant 2 provides a low level of resistance to the different antibiotics (Table 2). However, when overexpressed, the most
significant increase in MIC was obtained for tiamulin (8-fold) and virginiamycin M (16-fold).

Isa(C) and Isa(E) variant 1 are carried by MGEs, whereas Isa(C) variant 2 and Isa(E) are inserted in the core genome. Integrative elements are usually inserted into conserved genes from the core genome, such as tRNA genes or genes, encoding ribosomal proteins. The specificity of the Isa(C) elements for conserved oriT sites represents a novel strategy of integration contributing to the dissemination of antibiotic resistance. This insertion alters the GC-rich inverted repeat sequence by changing three to five nucleotides (Figure 2b). Lee and Grossman found that point mutations in the inverted repeat sequence of the oriT of the ICEBS1 in Bacillus subtilis dramatically reduced mating efficiency. Therefore, the alteration of the oriT site could explain the absence of observed conjugal transfer for the two ICE_tRNA variants tested and for the ICE_rpsL. Targeting the oriT site, which is conserved in diverse and broadly distributed ICEs from Firmicutes, provides a safe and widespread place for integration. However, it does not seem to be associated with gain of mobility by inserting into a conjugative element, although this was an appealing hypothesis. In line with this result, comparative analysis with closely related strains points in all cases to integration into a resident ICE and not the mobility of the ICE carrying the IME oriT. This was confirmed by the analysis of the single TC we have obtained for this IME.

This study demonstrates that L5P resistance in GBS is a result of horizontal gene transmission and not the clonal expansion of specific resistant clones. Two different mechanisms of acquisition were observed between the different variants. Isa(C) and Isa(C) variant 1 were acquired by the integration of the resistance element into a resident ICE. The donor species for these two elements remain unknown. The Isa(E) gene is found in a multiresistance non-mobile genomic island. We showed that it could be transferred between GBS isolates by an Hfr-type mechanism. In staphylococci and enterococci isolates the Isa(E) variants are carried by different plasmids. In one strain of E. faecalis and one strain of E. faecium these plasmids were shown to be transferable by conjugation. In GBS, the Isa(E) gene might have been acquired by a plasmidic conjugative transfer and the subsequent insertion of the Isa(E) element into the chromosome by recombination. Strikingly, Isa(C) variant 2 inserted in the genomic backbone was horizontally disseminated among bovine strains through transfer of the corresponding chromosomal region. The transfer of this variant between GBS of bovine origin probably reflects the ecology of the udder, where only a few bacteria are present, increasing the probability of co-infection by GBS and genetic exchanges. It is generally assumed that MGEs contribute to the dissemination of antibiotic resistance genes, but in the case of Isa genes in GBS the most frequent mechanisms of transfer appear to have involved chromosomal recombination.

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Transparency declarations
None to declare.

Supplementary data
Tables S1 and S2 and Figures S1, S2, S3 and S4 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References
Isa genes in group B Streptococcus


