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Virulence Gene Pool Detected in Bovine Group C *Streptococcus dysgalactiae* subsp. *dysgalactiae* Isolates by Use of a Group A *S. pyogenes* Virulence Microarray▽

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A custom-designed microarray containing 220 virulence genes of *Streptococcus pyogenes* (group A *Streptococcus* [GAS]) was used to test group C *Streptococcus dysgalactiae* subsp. *dysgalactiae* (GCS) field strains causing bovine mastitis and group C or group G *Streptococcus dysgalactiae* subsp. *equisimilis* (GCS/GGS) isolates from human infections, with the latter being used for comparative purposes, for the presence of virulence genes. All bovine and all human isolates carried a fraction of the 220 genes (23% and 39%, respectively). The virulence genes encoding streptolysin S, glyceraldehyde-3-phosphate dehydrogenase, the plasminogen-binding M-like protein PAM, and the collagen-like protein SclB were detected in the majority of both bovine and human isolates (94 to 100%). Virulence factors, usually carried by human beta-hemolytic streptococcal pathogens, such as streptokinase, laminin-binding protein, and the C5a peptidase precursor, were detected in all human isolates but not in bovine isolates. Additionally, GAS bacteriophage-associated virulence genes encoding superantigens, DNase, and/or streptodornase were detected in bovine isolates (72%) but not in the human isolates. Determinants located in non-bacteriophage-related mobile elements, such as the gene encoding R28, were detected in all bovine and human isolates. Several virulence genes, including genes of bacteriophage origin, were shown to be expressed by reverse transcriptase PCR (RT-PCR). Phylogenetic analysis of superantigen gene sequences revealed a high level (>98%) of identity among genes of bovine GCS, of the horse pathogen *Streptococcus equi* subsp. *equi*, and of the human pathogen GAS. Our findings indicate that alpha-hemolytic bovine GCS, an important mastitis pathogen and considered to be a nonhuman pathogen, carries important virulence factors responsible for virulence and pathogenesis in humans.

Alpha-hemolytic Lancefield group C *Streptococcus dysgalactiae* subsp. *dysgalactiae* (GCS) is a pathogen frequently associated with clinical and subclinical bovine mastitis, a disease that causes major economic losses in the dairy industry (51, 67). Virulence determinants have been identified for this pathogen, such as surface proteins which specifically interact with plasma or extracellular matrix proteins of the host, such as alpha-2-macroglobulin, plasminogen, albumin, fibrinogen, fibronectin, vitronectin, and collagen (30, 35, 46, 64), and genes coding for proteins assumed to play a role in mastitis, such as the alpha-2-macroglobulin-, immunoglobulin G-, or immunoglobulin A-binding protein Mig (25, 55); the alpha 2-macroglobulin- or immunoglobulin G-binding protein Mag (24); and a fibrinogen-binding M-like protein (65).

Recently, *S. dysgalactiae* subsp. *dysgalactiae* was reported to be associated with toxic shock-like syndrome in cattle (9), suppurative polyarthritis in lambs (28), bacteremia in dogs (66), and systemic granulomatous inflammatory disease and severe septicemia in fish (16) and in ascending upper limb cellulitis in humans in contact with raw fish (27). The presence of the streptococcal pyrogenic exotoxin G gene (*spegg*) and streptolysin S structural gene (*sagA*), which have been associated with invasive disease in the exclusively human pathogen *Streptococcus pyogenes* (group A *Streptococcus* [GAS]), has been documented for fish isolates of *S. dysgalactiae* subsp. *dysgalactiae* (1). We have previously reported the presence of GAS phage-carried virulence genes among alpha-hemolytic *S. dysgalactiae* subsp. *dysgalactiae* isolates from bovine mastitis, namely, the streptococcal pyrogenic exotoxin genes *speK*, *speC*, *speL*, and *speM*; the phage DNase I gene *spd1*; and other genes encoding antimicrobial resistance determinants located on mobile genetic elements (MGEs) (47). So far, no more information is available regarding the presence of GAS virulence genes among *S. dysgalactiae* subsp. *dysgalactiae* strains, and nothing is known regarding the presence of GAS prophages in *S. dysgalactiae* subsp. *dysgalactiae*. However, the exchange of lysogenic phages among GAS and other human and animal species, particularly group C *Streptococcus dysgalactiae* subsp. *equisimilis* (a pathogen that colonizes and infects humans with a clinical spectrum of diseases resembling those caused by GAS),

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Streptococcus equi subsp. *equi* (exclusively horse pathogen), and *S. equi* subsp. *zooepidemicus* (a zoonotic pathogen) isolates, was previously reported (19, 68).

The aim of the present work was to use a microarray of genes encoding GAS virulence factors (41) to have a better insight into the virulence gene pool (encoded or not by mobile genetic elements) shared between GAS and alpha-hemolytic *S. dysgalactiae* subsp. *dysgalactiae* isolates associated with bovine mastitis in comparison with beta-hemolytic *S. dysgalactiae* subsp. *equisimilis* isolates associated with human disease.

MATERIALS AND METHODS

Bacterial isolates and identification. A total of 18 alpha-hemolytic *S. dysgalactiae* subsp. *dysgalactiae* field isolates of Lancefield group C (GCS), one of the causative agents of bovine subclinical mastitis in dairy herds in Portugal, were used in the present study. Detailed information regarding these field isolates, including identification and molecular typing data, was described previously (47). In addition, six nonduplicated beta-hemolytic *S. dysgalactiae* subsp. *equisimilis* isolates of Lancefield group G (group G *Streptococcus* [GGS]) ($n = 5$) and group C (GCS) ($n = 1$) collected in Portugal, causing pharyngitis ($n = 5$) and invasive disease ($n = 1$) in humans, were included in the study for the purpose of comparison. The identification of *S. dysgalactiae* subsp. *equisimilis* isolates was based on colony morphology, hemolysis in blood agar plates, and Lancefield grouping using the Streptex kit (Remel Europe Ltd., Dartford, England) and PCR amplification of the 16S rRNA gene and sequencing (71).

We have included two invasive GCS alpha-hemolytic *S. dysgalactiae* subsp. *dysgalactiae* strains in the study, which were analyzed for the detection of selected virulence genes by PCR only (see below). One of these strains was associated with toxic shock-like syndrome in cattle (9). The other strain caused ascending upper limb cellulitis in humans in contact with raw fish (27). The latter strain was previously identified (27) by PCR amplification of the 16S rRNA and *sodA* genes and sequencing (GenBank accession numbers EU693902 and EU719068, respectively). We confirmed the identification of the strain associated with the toxic shock-like syndrome in cattle by PCR amplification of the 16S rRNA (71) and *sodA* (1) genes and sequencing. Sequences were analyzed by using the BioEdit sequence alignment editor (17) and compared with sequences from the National Center for Biotechnology Information (NCBI) database (Bethesda, MD) by using the BLAST alignment tool (www.ncbi.nlm.nih.gov/BLAST).

PFGE profiles for clonal characterization. A description of the bovine *S. dysgalactiae* subsp. *dysgalactiae* GCS field strains ($n = 18$), including typing by pulsed-field gel electrophoresis (PFGE), was reported previously (47). The typing of the human *S. dysgalactiae* subsp. *equisimilis* GGS/GCS isolates ($n = 6$) by PFGE was carried out in this study as described previously for the bovine pathogen *Streptococcus uberis* (48). SmaI-digested DNA banding patterns obtained by PFGE were analyzed visually according to previously established criteria (61).

Microarray design and hybridization. The microarray was described previously (41) and was used with minor modifications. In brief, the array consists of 70-mer oligonucleotides from the conserved regions of all "classical" GAS virulence factors and orthologues of virulence factors found in other bacterial species as well as putative virulence genes present in the M1, M3, and M18 genomes. In total, 220 virulence factor/extracellular protein genes, 10 housekeeping genes (positive controls), and 10 negative controls were randomly spotted in six locations on the chip.

Genomic DNA was extracted by using zirconicum beads in combination with the Qiagen DNeasy kit. Genomic DNA was partially digested with AluI, yielding fragments of between 500 and 1,000 bp, and labeled with biotin. The labeled DNA was purified (PCR purification kit; Qiagen), and the labeling efficiency was verified by gel electrophoresis. Array hybridization was performed at 42°C for 16 h, followed by incubation with streptavidin-Cy5 using a SlideBooster SB800 instrument (Advalytix). Fluorescence signals were obtained with a DNA microarray scanner (G2565CA; Agilent Technologies) at a 633-nm excitation wavelength and quantified by using ImaGene software (BioDiscovery).

Microarray data processing. The raw data were corrected for background and transformed to a log scale. A two-component normal-mixture model (39) was fitted to the corrected data by a maximum likelihood method adapted from the mclust package (12). A discriminant function was calculated to represent the propensity of a gene for being present or absent. Discriminant values were stored in a signal probability matrix and colored for presentation purposes using the following scheme: black indicates state 0 (not present), green indicates state 1 (present), and yellow indicates an indecisive measurement.

Confirmation of array data by PCR screening. In order to confirm the results obtained with the GAS array, PCR was carried out on several genes (*speH*, *speC*, *speA*, *speL*, *speK*, *speI*, *speM sdn*, *ssa*, *smeZ*, *sla*, *drs*, *prtf2*, *speG*, *ska*, *dppA*, *lbp*, *scpA*, *emm*, *isp*, SpyM3_1736, *slo*, *nga*, *spegg*, and *sagA*). The primer sequences, gene description, and amplification length of each reaction are described in Table 1. Samples without DNA and strains lacking (negative) or carrying (positive) specific genes were used as controls in the PCR.

Sequence data and phylogenetic analysis of bacteriophage-associated virulence superantigen genes. Sequences of the superantigen-encoding genes *speC*, *speK*, *speL*, and *speM* of the bovine mastitis isolates under study, with high levels of identity among them, were chosen to generate an alignment of DNA sequences of the alleles of those genes and homologous sequences deposited in the NCBI database, in particular sequences of the *speC*, *speK*, *speL*, and *speM* genes of *S. pyogenes*; the *seeL* and *seeM* genes of *S. equi* subsp. *equi*; the *szeL* and *szeM* genes of *S. equi* subsp. *zooepidemicus*; and the *sdm* gene of *S. dysgalactiae* subsp. *dysgalactiae*.

The alignment (380 bp) was used to construct a phylogenetic tree by using MEGA, version 4, software (60). The *p*-distance parameter and neighbor-joining method were used. Bootstrap values were calculated from 1,000 replicates. Deduced amino acid sequences from these bovine alleles were compared with similar sequences from the NCBI database by using the BLAST alignment tool (www.ncbi.nlm.nih.gov/BLAST). Sequencing was performed by STAB-Vida (Lisbon, Portugal), using the same primers used for amplification. Nucleotide sequences were analyzed by using the BioEdit sequence alignment editor (17).

***emm* typing.** Determination of *emm* gene (coding the M protein) types was performed as described by the CDC (http://www.cdc.gov/ncidod/biotech/strep/M-ProteinGene_typing.htm).

Gene expression assays by RT-PCR. For RNA extraction, all isolates were grown in Todd-Hewitt broth (Oxoid Limited, Basingstoke, England) supplemented with 1% yeast extract (BD, Franklin Lakes) (THY) at 37°C until the mid-exponential phase was reached (optical density [OD] at 600 nm of 0.5), and the NucleoSpin RNAII kit (Macherey-Nagel, Dueren, Germany) was used according to the manufacturer's instructions, followed by the addition of 2 U/μl of DNase I (Applied Biosystems/Ambion). RNA quality was confirmed by 1% agarose gel electrophoresis, and images were captured by using the Gel Doc XR system and Quantity One 1-D analysis software (Bio-Rad). To confirm that no remaining DNA was present in RNA samples, PCR assays were performed by targeting the housekeeping genes *rpsL* and *rpsB* and the genes under study, *speM*, *speK*, *speL*, *speC*, *spd1*, *sdn*, *slo scpA*, *ska*, *nga*, *lmb*, *isp*, *dppA*, *emm*, and SpyM3_1736, using RNA as a template. Reverse transcriptase (RT) reactions for cDNA synthesis were performed by using the SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen, New Zealand). PCR targeting of those genes under study was carried out again by using cDNA as a template.

Growth curve analysis. In order to ascertain if growth curves (using the same media and conditions of growth) of six of the bovine mastitis GCS *S. dysgalactiae* subsp. *dysgalactiae* isolates from the present study (chosen according to their genotype) were comparable or not, an automated growth curve analysis system (BioScreen C, Piscataway, NJ) was used as described previously (6). Briefly, these isolates were grown overnight in THY, and the cells were centrifuged in order to collect 10^9 CFU in the pellet. After being resuspended and washed in phosphate-buffered saline (PBS) (10 mM, pH 7.0), the pellet was resuspended in 100 μl of PBS, and 1% (vol/vol) of this bacterial suspension was used to inoculate each of the wells of the BioScreen plaque containing growth media. Three different growth media were tested: THY, bovine blood serum (Probiologica), and bovine milk serum freshly prepared in the laboratory as previously described (42). The growth media were prepared at different pHs (6.0, 6.6, and 7.4), and three different incubation temperatures (37°C, 38°C, and 40°C) were tested for each growth medium, which represent different environmental conditions in the bovine udder (e.g., the body temperature of a healthy cow is around 38°C, which may rise to 40°C during a mastitis infection).

Virulence gene profiling of invasive *Streptococcus dysgalactiae* subsp. *dysgalactiae*. Eighteen virulence genes (*sagA*, *sla*, *sdn*, *spd1*, *speI*, *speC*, *speA*, *speB*, *speK*, *speF*, *speM*, *speH*, *speL*, *speJ*, *ssa*, *smeZ*, *spegg*, and *prtf1*) were searched for by PCR (Table 1) in the two invasive GCS strains that were included in the study.

Microarray data accession number. Microarray data have been deposited in the public ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MEXP-3168.

Nucleotide sequence accession numbers. Sequence data have been deposited in GenBank under the following accession numbers: JF789447, JF789445,

TABLE 1. Genes and PCR primers used for screening of virulence determinants among group C *Streptococcus dysgalactiae* subsp. *dysgalactiae* and group C and G *Streptococcus dysgalactiae* subsp. *equisimilis* isolates

Gene description (origin)	Primer target	Primer sequence	Product size (bp)	Source or reference
Antiphagocytic M protein (chromosome)	*emm* (forward) *emm* (reverse)	TATTCGCTTAGAAAATTAA GCAAGTTCTTCAGCTTGTTT	Variable	—[a]
Streptolysin S (chromosome)	*sagA* (forward) *sagA* (reverse)	TACTTCAAATATTTTAGCTACT GATGATACCCCGATAAGGATAA	487	1
Pyrogenic exotoxin H precursor (phage)	*speH* (forward) *speH* (reverse)	AGATTGGATATCACAGG CTATTCTCTCGTTATTGG	416	50
Pyrogenic exotoxin F (chromosome)	*speF* (forward) *speF* (reverse)	TACTTGGATCAAGACG GTAATTAATGGTGTAGCC	782	50
Pyrogenic exotoxin B (chromosome)	*speB* (forward) *speB* (reverse)	TTCTAGGATACTCTACCAGC ATTTGAGCAGTTGCAGTAGC	300	23
Pyrogenic exotoxin C precursor (phage)	*speC* (forward) *speC* (reverse)	GCAGGGTAAATTTTTCAACGACACACA TGTGCCAATTTCGATTCTGCCGC	407	This study
Pyrogenic exotoxin A precursor (phage)	*speA* (forward) *speA* (reverse)	ATGGAAAACAATAAAAAAGTATTG TTACTTGGTTGTTAGGTAGACTTC	755	36
Pyrogenic exotoxin L precursor (phage)	*speL* (forward) *speL* (reverse)	CTGTTAGGATGGTTTCTGCGGAAGAG AGCACCTTCCTCTTTCTCGCCT	605	This study
Pyrogenic exotoxin K precursor (phage)	*speK* (forward) *speK* (reverse)	TACAAATGATGTTAGAAATCCAAGGAAC ATATATGCT CAAAGTGACTTACTTTACTCATATCAATC GTTTC	656	This study 31
Pyrogenic exotoxin I precursor (phage)	*speI* (forward) *speI* (reverse)	AATGAAGGTCCGCCATTTTC TCTCTCTGTCACCATGTCCTG	516	36
Pyrogenic exotoxin M precursor (phage)	*speM* (forward) *speM* (reverse)	CCAATATGAAGATAACAAAGAAAATTG GCACCC CAAAGTGACTTACTTTACTCATATCAATC GTTTC	600	This study 31
Pyrogenic exotoxin J (chromosome)	*speJ* (forward) *speJ* (reverse)	ATCTTTCATGGGTACG TTTCATGTTTATTGCC	535	50
Streptodornase (phage)	*sdn* (forward) *sdn* (reverse)	ACCCCATCGGAAGATAAAGC AACGTTCAACAGGCGCTTAC	489	36
Streptococcal DNase I (phage)	*spd1* (forward) *spd1* (reverse)	CCCTTCAGGATTGCTGTCAT ACTGTTGACGCAGCTAGGG	400	15
Streptococcal superantigen SSA (phage)	*ssa* (forward) *ssa* (reverse)	TCCACAGGTCAGCTTTTACAG TGATCAAATATTGCTCCAGGTG	502	36
Phospholipase A2 (phage)	*sla* (forward) *sla* (reverse)	CTCTAATAGCATCGGCTACGC AATGGAAAATGGCACTGAAAG	440	36
Mitogenic exotoxin Z (chromosome)	*smeZ* (forward) *smeZ* (reverse)	CTTCAATATTCATTGCAATAATTTC TGTAACTGTTGTTTTGTTAGTTGAT	400	G. S. Chhatwal
Pyrogenic exotoxin G of *S. pyogenes* (chromosome)	*speG* (forward) *speG* (reverse)	TGTATCTTTAGGGATTACTGATCAG CTCGACCTAAAAGCTTATCATCCTT	389	This study
Pyrogenic exotoxin G of *S. dysgalactiae* (chromosome)	*spegg* (forward) *spegg* (reverse)	GCTTATGATGTTACTCCACTTGA ATAACGCGATTCCGAATCATAGA	420	This study
Drs protein (chromosome)	*drs* (forward) *drs* (reverse)	CAGCAGATGAAGCAAGTAATAGC CTTGTTTGTCAATTTTGCTTTACGACC	760	18
Fibronectin-binding protein F1 (chromosome)	*prtf1* (forward) *prtf1* (reverse)	TATCAAAATCTTCTAAGTGCTGAG AATGGAACACTAACTTCGGACGGG	930	59

Continued on following page

TABLE 1—*Continued*

Gene description (origin)	Primer target	Primer sequence	Product size (bp)	Source or reference
Fibronectin-binding protein F2 (chromosome)	*prtf2* (forward) *prtf2* (reverse)	ATAGGATTGTCCGGAGTATCA TTATGTTGCTTCTCACCA	2,000	G. S. Chhatwal
Streptolysin O (chromosome)	*slo* (forward) *slo* (reverse)	ACGGCAGCTCTTATCATT GACCTCAACCGTTGCTTTGT	600	G. S. Chhatwal
C5A peptidase precursor (transposon)	*scpA* (forward) *scpA* (reverse)	CCAAGACTTCAGCCACAAGG CAATTCCAGCCAATAGCAGC	591	G. S. Chhatwal
Streptokinase A precursor (chromosome)	*ska* (forward) *ska* (reverse)	CGATCAAAGGGATCATACGG AGGTTCACAGTAACGACGGC	598	G. S. Chhatwal
NAD-glycohydrolase precursor (chromosome)	*nga* (forward) *nga* (reverse)	ATAACGGGAATAAATTGGTCCTC CGCTTTCTTTGTAGACTTGTTTT	408	This study
Laminin-binding protein (transposon)	*lmb* (forward) *lmb* (reverse)	AACCCCAAACAGCCTACGCAAG TAAAACGGGATCCGTCCAGGTAT	375	This study
Immunogenic secreted protein (chromosome)	*isp.1* (forward) *isp.1* (reverse)	CAACTGAAAAAACCCCAGAGCC GGTTGAAGTCAAAGGCACCATAA	429	This study
Surface lipoprotein DppA (chromosome)	*dppA* (forward) *dppA* (reverse)	CCGTTATGGAGTCCACAATGAA ACTAGCTTTGAGTTTAATAGTAATC	1,045	This study
Putative ATP-binding cassette transporter protein (chromosome)	SpyM3_1736 (forward) SpyM3_1736 (reverse)	GAGAAGTCAAAGAGGTCTTTGTT GGTGTCATACTCTAGTTTACCTTT	392	This study

[a] http://www.cdc.gov/ncidod/biotech/strep/M-ProteinGene_typing.htm.

HQ724300, HQ724301, HQ724302, HQ724303, HQ724304, HQ724305, HQ696925, JF789444, JF789442, and JF789443.

RESULTS

Identification by 16S rRNA and *sodA* gene sequence analyses. The identification of the bovine mastitis *S. dysgalactiae* subsp. *dysgalactiae* isolates was confirmed by using 16S rRNA gene sequencing as described previously (47). The 16S rRNA gene sequences of all human GCS/GGS isolates from the present study showed 99 to 100% identity to 16S rRNA gene sequences of *S. dysgalactiae* subsp. *equisimilis* deposited in the NCBI database. Taking into account the phenotypic characteristics of the isolates together with the 16S rRNA gene sequence analysis, we could confirm the six human beta-hemolytic GGS/GCS isolates included in this study as being *S. dysgalactiae* subsp. *equisimilis* isolates.

The 16S rRNA and *sodA* gene sequences of the GCS strain associated with the toxic shock-like syndrome in cattle were deposited in the GenBank database (accession numbers JF789447 and JF789445, respectively), whereas the same above-mentioned sequences of the GCS strain isolated from a case of ascending upper limb cellulitis in humans were already available (accession numbers EU693902 and EU719068, respectively), as described previously (27). Both 16S rRNA and *sodA* gene sequences of these two strains showed 99 to 100% identity with *S. dysgalactiae* subsp. *dysgalactiae* 16S rRNA and *sodA* gene sequences available for comparison in the NCBI database.

PFGE profiles. The 18 bovine *S. dysgalactiae* subsp. *dysgalactiae* isolates had 15 PFGE patterns, as shown previously (47). The six *S. dysgalactiae* subsp. *equisimilis* isolates had six different PFGE patterns with more than six band differences and were therefore considered unrelated according to established criteria (61).

***emm* typing.** None of the 18 alpha-hemolytic group C *S. dysgalactiae* subsp. *dysgalactiae* bovine isolates were typed by *emm* typing, since no amplification for this gene could be obtained. The *emm* gene types of the six human *S. dysgalactiae* subsp. *equisimilis* isolates were as follows: *stC839* (GCS isolate; $n = 1$), *stG485*, *stG480*, *stG6792*, and *stG4831* (GGS isolates; $n = 5$).

Microarray data. Of the 220 GAS virulence genes on the array (41), 44 genes (20%) were present in all bovine mastitis GCS (*S. dysgalactiae* subsp. *dysgalactiae*) and in all human GGS/GCS (*S. dysgalactiae* subsp. *equisimilis*) isolates, whereas 66 genes (30%) were not present in any of the isolates tested. The remaining genes (50%) showed variable distributions among isolates of both origins. Relevant genes present in at least one isolate (bovine or human) and genes present in all the bovine isolates (with the exception of hypothetical proteins) are shown in Table 2. Nine genes (*ska*, *dppA*, *lmb*, *scpA*, *emm*, *isp*, *nga*, *slo*, and SpyM3_1736) were present in all the human isolates and absent in the bovine isolates, whereas only one gene (SpyM3_0345), encoding an uncharacterized protein, was detected in all bovine isolates and absent in all human isolates.

If we restricted the comparison to bacteriophage genes on the array, we observed that 65% of the phage-related genes

TABLE 2. Distribution of group A *Streptococcus pyogenes* virulence factors of the array in bovine mastitis group C *Streptococcus dysgalactiae* subsp. *dysgalactiae* isolates and human noninvasive and invasive group C or G *Streptococcus dysgalactiae* subsp. *equisimilis* isolates[b]

Human group A *Streptococcus pyogenes* virulence class protein (gene)	Distribution (%) (no. of isolates)	
	Bovine group C *Streptococcus dysgalactiae* subsp. *dysgalactiae* (n = 18)	Human group C/G *Streptococcus dysgalactiae* subsp. *equisimilis* (n = 6)
Adhesins		
Antiphagocytic M protein (*emm*)	0 (0)	100 (6)
Laminin-binding protein (*lmb*)[a]	0 (0)	100 (6)
Collagen-like surface protein (*scl*)[a]	89 (16)	100 (6)
PAM	94 (17)	100 (6)
Putative adhesion protein (*adcA*)	94 (17)	100 (6)
Putative extracellular matrix-binding protein (*epf*)[a]	94 (17)	100 (6)
Putative pullulanase (*pulA*)	94 (17)	83 (5)
Collagen-like protein SclB (*sclB*)	100 (18)	100 (6)
Glyceraldehyde-3-phosphate dehydrogenase (*plr*)	100 (18)	83 (5)
R28[a]	100 (18)	100 (6)
Putative choline binding protein (SpyM3_0025)	100 (18)	100 (6)
Putative collagen-like protein (Spy1054)	100 (18)	100 (6)
Putative enolase (*eno*)	100 (18)	100 (6)
Putative internalin A precursor (*inlA*)	100 (18)	100 (6)
Fibronectin-binding proteins		
SfbX	6 (1)	17 (1)
SfbI[a]	89 (16)	100 (6)
PrtF15[a]	100 (18)	100 (6)
Putative fibronectin-binding protein-like protein A (SpyM3_0652)	100 (18)	67 (4)
Proteases		
C5A peptidase precursor (*scpA*)[a]	0 (0)	100 (6)
Putative C3-degrading proteinase (*cppA*)	22 (4)	0 (0)
Putative exfoliative toxin (SpyM3_0632)	94 (17)	67 (4)
Putative serine protease (*degP*)	94 (17)	100 (6)
Putative protease (SpyM3_0418)	100 (18)	100 (6)
Toxins		
Streptolysin O (*slo*)	0 (0)	100 (6)
Putative hemolysin III (*hlyIII*)	83 (15)	100 (6)
Putative hemolysin (*hlyX*)	94 (17)	100 (6)
Streptolysin S-associated protein (*sagA*)	100 (18)	100 (6)
Putative DNA entry nuclease (*endA*)	100 (18)	100 (6)
Putative hemolysin (*hlyA*)	100 (18)	100 (6)
Other virulence factors		
Dipeptide permease complex (*dppA*)	0 (0)	100 (6)
Immunogenic secreted protein precursor (*isp*)	0 (0)	100 (6)
Inhibitor of complement-mediated lysis (*sic*)	0 (0)	50 (3)
NAD-glycohydrolase (*nga*)	0 (0)	100 (6)
Pyrogenic exotoxin G precursor (*speG*)	0 (0)	83 (5)
Streptokinase A precursor (*ska*)	0 (0)	100 (6)
Transposase (Spy2009)[a]	22 (4)	83 (5)
Putative hyaluronidase (*hyl*)	28 (5)	100 (6)
Putative 6-phospho-beta-galactosidase (*lacG*)	39 (7)	0 (0)
Immunogenic secreted protein precursor homologue (*isp.2*)	67 (12)	100 (6)
Extracellular hyaluronate lyase (*hylA*)	78 (14)	50 (3)
Streptococcal protective antigen (*spa*)	78 (14)	50 (3)
Putative penicillin-binding protein 1A (*pbp1a*)	83 (15)	33 (2)
Putative glutathione peroxidase (SpyM3_0428)	89 (16)	100 (6)
Putative GTP-binding protein LepA (*lepA*)	89 (16)	100 (6)
Arginine deiminase (*arcA*)	100 (18)	100 (6)
Maltose/maltodextrin-binding protein (*malE*)[a]	100 (18)	100 (6)
67-kDa myosin-cross-reactive streptococcal antigen (SpyM3_0332)	100 (18)	100 (6)
Putative acid phosphatase (*lppC*)	100 (18)	100 (6)
Putative amidase (SpyM18_2055)[a]	100 (18)	100 (6)
Putative carbamate kinase (*arcC*)	100 (18)	100 (6)
Putative citrate lyase beta subunit (*citE*)[a]	100 (18)	50 (3)
Putative cytoplasmic membrane protein (*lemA*)	100 (18)	100 (6)
Putative dipeptidase (*pepD*)	100 (18)	100 (6)

Continued on following page

TABLE 2—*Continued*

Human group A *Streptococcus pyogenes* virulence class protein (gene)	Distribution (%) (no. of isolates)	
	Bovine group C *Streptococcus dysgalactiae* subsp. *dysgalactiae* ($n = 18$)	Human group C/G *Streptococcus dysgalactiae* subsp. *equisimilis* ($n = 6$)
Putative dipeptidase (SPyM3_1763)	100 (18)	100 (6)
Putative ferric uptake regulator (*spf*)	100 (18)	100 (6)
Putative fructose-1-phosphate kinase (*fruK*)	100 (18)	100 (6)
Putative lipoprotein (*atmB*)	100 (18)	100 (6)
Putative manganese-dependent inorganic pyrophosphatase (SpyM3_0278)	100 (18)	100 (6)
Putative metal-binding protein of ABC transporter (*mtsA*)	100 (18)	100 (6)
Putative peptidyl-prolyl *cis-trans* isomerase (*cypB*)	100 (18)	100 (6)
Putative protease maturation protein (*prsA*)	100 (18)	50 (3)
Putative proton-translocating ATPase subunit b (SPyM3_0495)	100 (18)	100 (6)
Putative PTS system IIB component (SPyM3_1679)	100 (18)	100 (6)
Putative sugar transporter sugar-binding lipoprotein	100 (18)	100 (6)
Putative two-component sensor histidine kinase (*yesM*)	100 (18)	100 (6)
Putative uridine kinase (*udk*)	100 (18)	100 (6)
Bacteriophage-encoded recognized virulence factors		
Streptodornase, phage associated (*sdn*)	22 (4)	0 (0)
Putative exotoxin L precursor, phage associated (*speL*)	22 (4)	0 (0)
Pyrogenic exotoxin C precursor, phage associated (*speC*)	33 (6)	0 (0)
Putative DNase, phage associated (*spd1*)	33 (6)	0 (0)
Streptococcal pyrogenic K exotoxin, phage associated (*speK*)	50 (9)	0 (0)
Putative exotoxin M precursor, phage associated (*speM*)	56 (10)	0 (0)
Other bacteriophage-encoded factors		
Putative DNase	33 (6)	50 (3)
Putative lysin, phage associated	89 (16)	100 (6)
Hypothetical phage protein	100 (18)	100 (6)
Putative endolysin, phage associated	100 (18)	100 (6)

[a] Genes associated with lateral gene transfer (non-bacteriophage related).
[b] Only genes present in at least one isolate (bovine or human) and genes present in all bovine group C *Streptococcus dysgalactiae* subsp. *dysgalactiae* isolates are listed. Hypothetical proteins are not listed in the table.

were present in at least one bovine GCS isolate, whereas 35% were detected in at least one human GCS/GGS isolate. None of the 13 bacteriophage-harbored virulence genes *speC*, *speJ*, *speI*, *speH*, *ssa*, *mf4*, *slaA*, *speA3 speK*, *speL*, *speM*, *spd1*, and *sdn* were detected in human GGS/GCS *S. dysgalactiae* subsp. *equisimilis* isolates, whereas at least one of the six genes *speC*, *speK*, *speL*, *speM*, *spd1*, and *sdn* was detected in 72% ($n = 13$) of the bovine mastitis GCS *S. dysgalactiae* subsp. *dysgalactiae* isolates.

Other GAS genes on the array, located in variable and recombinatory loci of the GAS genomes (40, 41), were detected in both human and bovine isolates. These include the Spy2009 gene, encoding a transposase (57), and *sic* (at the *mga* chromosomal location); the SpyM18_2055 gene, coding for an amidase (at the *spa* chromosomal location), *malE* (at the maltose chromosomal location); *prtF15* and *sfbI* (at the fibronectin-collagen-T-antigen [FCT]-encoding region); *citE* (at the *cit* chromosomal location); and *epf* (at the *sagA* chromosomal location). Interestingly, the gene coding for the adhesin R28 carried by a putative integrative conjugative element was present in all bovine and human isolates.

Confirmation of array data by PCR screening. All PCR results confirmed the GAS microarray data (Table 2), except for the *speM*, *speK*, and *speG* genes. The *speM* and *speK* genes share high levels of identity among their DNA sequences, which resulted in false-positive results in the array data. In addition, it was not clear how many isolates carried the *speG* gene by both the array and PCR amplification results. The latter may be due to a lack of homology between the primers used and the *speG* gene of *S. dysgalactiae* (named *spegg*), as variants of this gene may occur (73). By using other primers (designed in this study) targeting the *speM* and *speK* genes of *S. pyogenes* and *spegg* of *S. dysgalactiae* subsp. *equisimilis* (73), eight bovine isolates that carried *speK*, one bovine isolate that carried *speM*, one bovine isolate that carried *speK* and *speM*, and five human isolates that carried *spegg* were found.

Sequence data and phylogenetic analysis of bacteriophage-associated virulence superantigen genes. Three *speK* alleles (*speK-1*$_{Bov}$, *speK-2*$_{Bov}$, and *speK-3*$_{Bov}$) were identified among nine bovine isolates, two *speM* alleles (*speM-1*$_{Bov}$ and *speM-2*$_{Bov}$) were identified in two bovine isolates, one *speC* allele (*speC*$_{Bov}$) was identified in six bovine isolates, and one *speL* allele (*speL*$_{Bov}$) was identified in four bovine isolates (GenBank accession numbers HQ724300 to HQ724305 and HQ696925). The phylogenetic tree based on sequences of those alleles and of homologous sequences available in the NCBI database showed four major groups, each one comprising one of the four *spe* genes (*speK*, *speM*, *speC*, or *speL*) of bovine *S. dysgalactiae* subsp. *dysgalactiae* isolates and of *S. pyogenes*, as shown in Fig. 1. The tree also shows that *speM* and *speK* diverged more recently, as these two groups showed

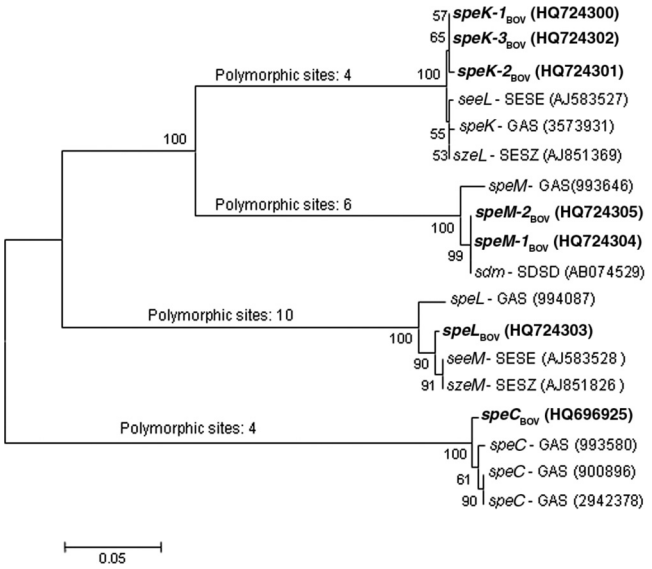

FIG. 1. Phylogenetic analysis of superantigen gene sequences of bovine group C *Streptococcus* from the present study and of sequences of *S. pyogenes*, *S. equi* subsp. *equi*, *S. equi* subsp. *zooepidemicus*, and *S. dysgalactiae* subsp. *dysgalactiae* available in the National Center for Biotechnology Information (NCBI) (Bethesda, MD) database. Bovine group C *Streptococcus* sequences from the present study are designated in the tree in boldface type. Other sequences are designated as follows: SESE, *S. equi* subsp. *equi* (*seeL* and *seeM*); SESZ, *S. equi* subsp. *zooepidemicus* (*szeL* and *szeM*); SDSD, *S. dysgalactiae* subsp. *dysgalactiae* (*sdm*); GAS, *S. pyogenes* (*speK*, *speM*, *speL*, and *speC*). GenBank accession numbers (for bovine alleles, *seeL*, *seeM*, *szeL*, *szeM*, and *sdm*) and gene ID numbers (for *speK*, *speM*, *speL*, and *speC*) are included in parentheses after the gene names.

higher identities among them than with the *speL* or *speC* groups.

Amino acid sequences deduced from the bovine GCS alleles always showed 98 to 99% identity with the homologous GAS pyrogenic exotoxin gene sequences from the NCBI database, with the exception of bovine SpeL, which showed a higher level of identity with SeeM from *S. equi* subsp. *equi* (99%) than with SpeL from *S. pyogenes* (96%). In particular, and interestingly, SpeM from bovine isolates in the present study showed 99% identity with SpeM from GAS and 100% identity with Sdm from *S. dysgalactiae* subsp. *dysgalactiae* (sequences deposited in the NCBI database).

Gene expression assays by RT-PCR. Transcriptional analysis revealed that the bacteriophage-associated virulence genes *speM*, *speK*, *speL*, *speC*, *spd1*, and *sdn*, detected in bovine GCS isolates, were transcribed and that the virulence genes *ska*, *dppA*, *lmb*, *scpA*, *emm*, *isp*, SpyM3_1736, *slo*, and *nga*, detected only in human isolates, were also transcribed.

Growth curve analysis. In the present study all the strains under analysis by BioScreen reached the highest OD values when grown in bovine blood serum medium (compared to those when grown in THY and bovine milk serum) and under infection-related conditions (at 40°C), with the end of the exponential phase being achieved relatively fast (prior to 5 h of incubation). In bovine milk serum, the end of the exponential phase was achieved in most cases after 10 h of incubation, whereas in THY, growth curves reached the end of the exponential phase prior to 10 h of growth, followed by long stationary phases (no OD decrease was observed during 30 h or more of growth). Interestingly, we have observed differences in growth curves among isolates from the present study, suggesting a strain-specific mode of growth.

Virulence gene profiling of invasive *Streptococcus dysgalactiae* subsp. *dysgalactiae*. Both strains were negative for all the genes tested ($n = 18$) by PCR, with the exception of the *sagA* gene. According to the sequences available in the NCBI database, the *sagA* gene of the animal strain (GenBank accession number JF789442) showed 95% identity either with the *sagA* gene of *S. dysgalactiae* subsp. *dysgalactiae* or with that of *S. dysgalactiae* subsp. *equisimilis*. In parallel, the *sagA* gene of the human strain (accession number JF789443) was 100% identical to the *sagA* gene of *S. dysgalactiae* subsp. *dysgalactiae* and 99% identical to the *sagA* gene of *S. dysgalactiae* subsp. *equisimilis*.

DISCUSSION

Assessment of genes associated with MGEs among *Streptococcus dysgalactiae* subsp. *dysgalactiae* and *S. dysgalactiae* subsp. *equisimilis* isolates. An array containing 220 virulence genes from *S. pyogenes* (group A *Streptococcus* [GAS]) was used to analyze the virulence gene pool among *S. dysgalactiae* subsp. *dysgalactiae* (group C *Streptococcus* [GCS]) strains, associated with bovine mastitis, and among *S. dysgalactiae* subsp. *equisimilis* (group C or group G *Streptococcus* [GCS/GGS]) isolates, associated with human pharyngitis and invasive disease episodes. We previously reported the presence of GAS virulence genes in bovine GCS isolates (47), which motivated us to further analyze the bovine strains in a search for the presence of other GAS virulence genes (either chromosomal or encoded by mobile genetic elements). The genes included in the array used in this study are from M1, M3, and M18 GAS genomes, of which M1 and M3 in particular are usually associated with severe disease in Europe and North America (33, 53). The results showed that the genes were unevenly distributed among isolates of different host origins. The bovine GCS and human GAS isolates shared 23% of all genes, and both the human GCS/GGS and GAS isolates shared 39% of all genes. A higher content of GAS virulence genes in human GGC/GGS isolates was expected, since both species share the same tissue niche in humans and cause similar spectra of diseases (11, 22, 58). Nevertheless, and most interestingly, none of the 13 bacteriophage virulence-related genes, all associated with GAS disease, were detected in the human GCS/GGS isolates. However, 6 of those 13 genes were detected in the bovine GCS isolates, and we have observed that these GAS phage-related genes (*speK*, *speL*, *speM*, *speC*, *spd1*, and *sdn*) present in bovine GCS are expressed *in vitro*, suggesting that bacteriophages may also play a role in the genetic plasticity and virulence of bovine mastitis GCS *S. dysgalactiae* subsp. *dysgalactiae* isolates. Specifically, the distribution of genes among the bovine GCS isolates ranged from one phage-related gene (*sdn*) present in three isolates to five genes (*speC-speK-speL-speM-spd1*) present in one isolate. Also, the observed linkage of genes in a same bovine GBS strain, in particular *speC-spd1* (from the M1 phage), *speM-speL* (from the M18 phage), and *speK* (from the M3 phage), indicates polylysogeny, similarly to what was de-

scribed previously for GAS (2). However, the lack of an association of sets of genes carried by complete phage genomes in our bovine isolates strongly suggests a recombinatory mosaic nature of phages, as observed previously for GAS (2).

Also interesting was the observation that "non-bacteriophage-associated" genes previously shown to be located in recombinatorial and mutational hot spots of the GAS genome and thus considered to be associated with lateral gene transfer (40, 41) were detected in both the bovine GCS and human GCS/GGS isolates from the present study (Table 2). These genes (such as *prtf15*, *epf*, *citE*, and *sic*) belong to four of the five large chromosomal regions described previously to have variable loci in the GAS genome (41). One of the chromosomal regions includes the FCT locus (63), which is considered one of the major locations of adhesins in GAS isolates. Another chromosomal region is *sagA*, whereas another region includes the maltose transport and *cit* operons. The fourth region includes the *mga* and *spa* loci.

Furthermore, the gene encoding the cell surface-anchored adhesin R28, carried by putative integrative conjugative elements in GAS which resemble genetic elements of group B *Streptococcus agalactiae* (GBS) (56), was detected in all bovine and human isolates. Also, the C5a peptidase precursor *scpA* gene as well as the *lbp* gene, encoding the laminin-binding protein, both known to be carried by a composite transposon of GBS (13), were detected in all human isolates and not in bovine isolates. Together, our data highlight the importance of MGEs mediating lateral gene transfer among different streptococcal species, including bovine GCS isolates.

Nonrandom distribution of GAS virulence genes in bovine mastitis GCS isolates. Genes of GAS encoding adhesins, such as glyceraldehyde-3-phosphate dehydrogenase, a putative enolase, PrtF15, R28, a putative internalin A precursor, and putative fibronectin-binding protein-like protein A (4, 10, 26, 56, 69), detected in all bovine GCS *S. dysgalactiae* subsp. *dysgalactiae* isolates, strongly suggest that these might represent important virulence factors in this particular subspecies. In particular, the *emm* gene, encoding the antiphagocytic M protein, was not present in the bovine isolates, although 94% of these isolates carried the gene encoding PAM, a member of the M protein family.

Also interestingly, streptolysin S, strongly associated with invasive disease caused by GAS and associated with the beta-hemolytic phenotype of GAS and GCS/GGS (*S. dysgalactiae* subsp. *equisimilis*) (3, 21), was detected in all bovine isolates, which are alpha-hemolytic (a sequence of this gene from one of the bovine mastitis isolates was deposited in GenBank under accession number JF789444). Furthermore, the presence of the streptolysin S gene (*sagA*) in alpha-hemolytic strains of group G (*S. dysgalactiae* subsp. *equisimilis*) and group C (*S. dysgalactiae* subsp. *dysgalactiae*) streptococci, from human and animal origins, respectively, was reported previously (1, 72).

Bovine GCS is known to grow well in mammary secretions, either during lactation or from dry animals (44, 55), which may be necessary for survival and establishment in the specific environmental niche that is the bovine mammary gland. The results obtained in the present study by using the BioScreen suggest the environmental adaptability of the bovine GCS strains, which demonstrated the ability to grow fast in bovine blood serum and also, although with lower levels of growth, in the remaining tested media (THY and bovine milk serum).

GAS genes related to housekeeping functions were also detected in the bovine isolates from the present study, such as arginine deiminase, a putative metal-binding protein of the ABC transporter, a putative phosphotransferase system (PTS) IIB component, maltose/maltodextrin-binding protein, and putative ferric uptake regulator (7, 29, 32, 49, 52).

The gene encoding 6-phospho-beta-galactosidase, an enzyme of glycoside hydrolase family 1 (5) associated with the capacity for carbohydrate utilization, was detected in only 39% of the bovine isolates (and absent in all human isolates). We have observed differences in growth curves among isolates from the present study, which do not seem to correlate with the presence or absence of the 6-phospho-beta-galactosidase gene. Variable growth patterns of strains in bovine mammary secretions were previously described (44) and may be related to the presence or absence of genes associated with the capacity for carbohydrate utilization but probably not of this particular gene.

Virulence genes detected only in the human GCS/GGS isolates. In epidemiologically and genetically unrelated *S. dysgalactiae* subsp. *equisimilis* strains from human disease (pharyngitis and invasive), we detected nine genes that were not detected in the bovine isolates. Out of these nine genes, five were previously described for human *S. dysgalactiae* subsp. *equisimilis*: *lbp* (encoding the adhesion Lmb, a laminin-binding protein) (62), *ska* (encoding the plasminogen-activating Ska-streptokinase A protein) (37), *slo* (encoding the cytolytic streptolysin O toxin) (54), *emm* (encoding the M protein), as well as *scpA* (encoding a C5a peptidase), with the latter two genes acting on the complement pathway of the host, inhibiting bacterial opsonization and phagocytosis (8, 20). Both the *lbp* and *scpA* genes have been found in human GBS isolates and are usually absent in bovine GBS isolates (14). These latter two genes and also *ska* are known to be carried by a bovine pathogen, *S. uberis*, although Lmb is not required for the attachment of *S. uberis* to host epithelial cells, and the Ska locus is devoid of plasminogen activator coding sequences (70). The four remaining genes were found for the first time, in our study, in human *dysgalactiae* subsp. *equisimilis* isolates: the *nga* gene (encoding a GAS extracellular enzyme NAD-glycohydrolase), associated with cytotoxicity in host cells (34); the *isp* gene (encoding an immunogenic secreted protein), which has an unknown function, although it is known to be expressed by GAS in the human host and generates antibody responses (38); the *dppA* gene (encoding a dipeptide permease complex), which is a membrane-associated transporter for dipeptides in GAS and is regulated by the multigene transcriptional regulator Mga (45); and the SpyM3_1736 gene (coding for a putative ABC transporter protein). By gene expression assays (RT-PCR), we observed that all these nine genes from GAS isolates were expressed in human GCS/GGS isolates.

These observations together with the absence of these nine genes in the bovine GCS isolates from this study suggest that they may be more important in human host streptococci than in animal host streptococci.

Sequence data and phylogenetic analysis of superantigen genes of bovine group C *S. dysgalactiae* subsp. *dysgalactiae*. The bovine alleles were distributed in the four clades according to

the *S. pyogenes* alleles (Fig. 1). In the *speK* clade, the bovine alleles are organized together and separately from the *seeL* and *szeL* genes of *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* and the *speK* gene of *S. pyogenes*, which was expected, since the streptococcal pathogens *S. equi* subsp. *equi*, *S. equi* subsp. *zooepidemicus*, and *S. pyogenes* are known to share a phage pool (19). In contrast, within the *speL* clade, the sequences of the *seeM* and *szeM* genes of *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus*, respectively, were organized closer to the bovine $speL_{Bov}$ allele from the present study and separately from the *speL* sequence of *S. pyogenes*, suggesting a common phage content among the animal species. Also, considering the *speC* clade, we may speculate that the same or a similar phage(s) is shared between bovine *S. dysgalactiae s*ubsp. *dysgalactiae* and human *S. pyogenes*.

The reason why the *seeL* and *szeL* sequences were not located in the *speL* clade and the *seeM* and *szeM* sequences were not in the *speM* clade may be due to incorrect nomenclature given to these genes of *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus*. Therefore, *seeL* and *szeL* should be named *seeK* and *szeK*, whereas *szeM* and *seeM* should be named *seeL* and *szeL*.

The bovine GCS SpeM amino acid sequence (with a length of 177 amino acid residues) showed 100% identity with the *S. dysgalactiae*-derived mitogen (Sdm) sequence available in the NCBI database. Sdm (encoded by the *sdm* gene) was the only superantigen with mitogenic activity described so far for *S. dysgalactiae* subsp. *dysgalactiae* (43). In agreement with our findings, the authors of that study (43) also noticed high levels of identity between *sdm* (from *S. dysgalactiae* subsp. *dysgalactiae*) and *speM* of *S. pyogenes*. *sdm* and *speM* are probably the same gene, with different nomenclatures.

Our findings underline the role of GAS phages (which are known to be shared with *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus*) in the genetic diversity of bovine *S. dysgalactiae* subsp. *dysgalactiae* strains. Additionally, it would be of interest to further search for the presence and expression of virulence factors not represented on the array, which can be either present or not in the genomes of the strains tested. Since several genome sequences of streptococcal species are available, whole-genome and transcriptome comparisons using a pair of *S. dysgalactiae* subsp. *dysgalactiae* strains described in the present study (the putative zoonotic strain and the bovine mastitis strain with a large pool of GAS virulence phage-related genes) would certainly give further insights into the genomic plasticity of this pathogenic subspecies.

In conclusion, the presence of GAS virulence genes, particularly genes carried by MGEs, either randomly or nonrandomly distributed among strains of bovine GCS may contribute to the increased virulence potential of these strains, namely, the possibility of dissemination to different tissues of the host and to take advantage of new niches. As we have pointed out previously, *S. dysgalactiae* subsp. *dysgalactiae* should not be disregarded as an infectious agent in humans. In fact, this subspecies was associated with invasive disease in humans (27) and here was shown to carry the *S. pyogenes* streptolysin S gene, further suggesting that *S. dysgalactiae* subsp. *dysgalactiae* is an emerging zoonotic pathogen.

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