DNA Microarray-Based Typing of *Streptococcus agalactiae* Isolates

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Streptococcus agalactiae frequently colonizes the urogenital tract, and it is a major cause of bacterial septicemia, meningitis, and pneumonia in newborns. For typing purposes, a microarray targeting group B streptococcus (GBS) virulence-associated markers and resistance genes was designed and validated with reference strains, as well as clinical and veterinary isolates. Selected isolates were also subjected to multilocus sequence typing. It was observed that putative typing markers, such as alleles of the alpha-like protein or capsule types, vary independently of each other, and they also vary independently from the affiliation to their multilocus sequence typing (MLST)-defined sequence types. Thus, it is not possible to assign isolates to sequence types based on the identification of a single distinct marker, such as a capsule type or *alp* allele. This suggests the occurrence of frequent genomic recombination. For array-based typing, a set of 11 markers (*bac*, *alp*, *pil1* locus, *pepS8*, *fbsB*, capsule locus, *hylB*, *abiG*-I/-II plus Q8DZ34, *pil2* locus, *nss* plus *srr* plus *rogB2*, and *rgfC/A/D/B*) was defined that provides a framework for splitting the tested 448 *S. agalactiae* isolates into 76 strains that clustered mainly according to MLST-defined clonal complexes. There was evidence for region- and host-specific differences in the population structure of *S. agalactiae*, as well as an overrepresentation of strains related to sequence type 17 among the invasive isolates. The arrays and typing scheme described here proved to be a convenient tool for genotyping large numbers of clinical/veterinary isolates and thus might help obtain insight into the epidemiology of *S. agalactiae*.

*S*treptococcus agalactiae, also commonly referred to as group B streptococcus (GBS), belongs to the gastrointestinal and genitourinary flora in humans and can cause, e.g., urinary tract infections. More importantly, it can be found in the vagina, and up to 20 to 40% of pregnant women are colonized (1–4). *S. agalactiae* is a major cause of septicemia, meningitis, and pneumonia in newborns (5). Mother-to-child transmission may lead to neonatal infection in 1 to 2 infants per 1,000 live births, with mortality rates ranging from 10 to 15% for early onset disease and 2 to 6% for late-onset disease (6–8). For prevention, it is strongly recommended to collect vaginal and rectal swabs between 35 and 37 weeks after gestation for screening and to eradicate GBS if present. The recommended antibiotics for decolonization and therapy include penicillin G, ampicillin, or first-/second-generation cephalosporins. Resistance to the β-lactams has rarely been observed (9). Other options, e.g., in case of allergy, are erythromycin or clindamycin, although there is a considerable risk of resistance (10–14). Tetracyclines can be given to nonpregnant adults only, and under some conditions, other compounds, like vancomycin or linezolid, may be considered.

S. agalactiae is also an important pathogen in animals, especially in cattle, in which it causes bovine mastitis. It may also cause meningoencephalitis and septicemia in fish (15).

Several typing techniques have been described. These include serotyping (16–20), restriction fragment length polymorphism analysis (21), ribotyping (22), pulsed-field gel electrophoresis (23), multilocus enzyme electrophoresis (24, 25), analysis of randomly amplified polymorphic DNA (26), amplified *cps* restriction polymorphism analysis (27) and multilocus sequence typing (MLST) (28).

For the present work, a microarray for the typing of GBS was designed and validated with reference strains and clinical isolates.

MATERIALS AND METHODS

Bacterial isolates. Eight fully sequenced strains, i.e., *S. agalactiae* NEM316 (GenBank accession no. AL732656.1), A909 (CP000114.1), 2603V/R (AE009948), COH1 (AAJR00000000.1), strain 515 (AAJP00000000.1), H36B (AAJS00000000.1), 18RS21 (AAJO00000000.1), and CJB111 (AAJQ00000000.1) were tested and used for quality control purposes and protocol optimization.

One hundred thirty-two isolates from the Dresden University Hospital were collected between 2008 and mid-2010. This mainly included routine vaginal and rectal swabs but also other clinical samples, like swabs from the ears, eyes, and throats of newborns ($n = 8$), as well as swabs from (chronic) wounds ($n = 18$), blood cultures ($n = 9$), urine ($n = 14$), breast milk samples ($n = 4$), and swabs from the male genital tract ($n = 4$).

Additionally, 97 isolates (courtesy of R. Berner, Freiburg/Dresden) from invasive infections of newborns, i.e., septicemia, meningitis, and pneumonia, were collected across Germany as part of the ESPED (i.e., the Surveillance Unit for Rare Pediatric Diseases in Germany) study and were genotyped. Furthermore, surveillance isolates from different parts of Germany (courtesy of M. van der Linden, National Reference Centre for Streptococci, Aachen, Germany [$n = 119$], and S. Weber, Bioscientia MVZ, Saarbrücken, Germany [$n = 36$]) and from the hospital of the University of West Indies, St. Augustine, Trinidad and Tobago (courtesy of P. Akpaka; $n = 34$), were processed. One isolate originated from a laboratory proficiency test (supplied by Instand e.V. [http://www.instandev.de/en.html]). Finally, 21 isolates from bovine mastitis cases

were included (courtesy of K. Kadlec, Friedrich-Loeffler-Institute, Neustadt-Mariensee, Germany).

Ethics statement. The isolates were obtained during routine diagnostics and were analyzed anonymously. No patient data beyond classification as infant or adult and sample type were stored. Ethics approval and informed consent were thus not required.

Preparation of genomic DNA. The isolates were incubated for 12 to 24 h at 37°C on Columbia blood agar. One inoculation loop of colony material was harvested and placed in 100 μl of lysis buffer comprising 10 μl of achromopeptidase (catalog no. A3547-100KU; Sigma-Aldrich; 100 units dissolved in 5 ml of phosphate-buffered saline [PBS] and stored frozen in small aliquots), 2 mg of lysozyme (Sigma-Aldrich), 2 mg of RNase A (Sigma-Aldrich), 2 μl of 20 mM Tris-HCl (pH 8.0), 2 μl of 2 mM EDTA, and 1 μl of Triton X-100. After incubation (at 37°C and 550 rpm on a thermomixer), 10 μl of proteinase K and 100 μl of AL buffer (Qiagen, Hilden, Germany) were added, and another incubation step (55°C without mixing) followed. After adding 100 μl of ethanol, DNA was purified using Qiagen spin columns. Finally, DNA was redilted in 50 μl of PCR-grade distilled water and heated (for 10 min at 70°C) to evaporate any traces of ethanol from the washing buffers.

MLST. Representative isolates were characterized by MLST using protocols and software provided at http://pubmlst.org/sagalactiae/.

Microarray design and protocol optimization. The microarray covered typing markers, such as capsule- and pilus-associated genes and *alp* genes. Additionally, macrolide/lincosamide, tetracycline, and heavy metal resistance genes, genes associated with phages and gene motility, and various genes coding for virulence factors, digestive enzymes, and certain metabolic functions were included, with variability in the presence or sequence being the main inclusion criterion (Tables 1 to 4; see also Tables S1 and S2 in the supplemental material).

For each target, all relevant entries were retrieved from GenBank. One entry was selected as the reference, and its coding sequence was excised. All BLAST hits for that sequence were downloaded into a local database excising and aligning all coding sequences related to each target gene. These alignments were inspected visually. Based on sequence similarities, the sequences were classified into paralogues and allelic variants. The consensus regions in the alignments of all available sequences of each target were chosen for the probe design. The probe sequences were selected for specificity and, in order to yield similar signal intensities, for having similar G+C contents, lengths, and melting temperatures. The resulting probe sequences were again BLASTed against all available sequences to theoretically exclude false-positive reactions due to cross-reactivity or false-negative reactions due to sequence variations.

Two hundred thirteen probes were spotted on arrays that were mounted into ArrayStrips (Alere Technologies, Jena, Germany). The probe lengths ranged from 25 to 36 bases, with a median of 30 bases. The probes were designed to have approximately identical binding temperatures. There were 208 primers with lengths ranging from 18 to 28 bases; their median length was 23 bases. The primers were designed to have similar annealing temperatures in order to be used in a multiplex reaction.

Probes and primers (see Table S1 in the supplemental material) were synthesized and purified by Metabion (Martinsried, Germany). The DNA microarrays were based on the ArrayStrip platform (Alere Technologies), with arrays being mounted in standard 8-well strips. The buffers and reagents were taken from the HybPlus kit (Alere Technologies), unless stated otherwise.

For validation and protocol optimization, completely sequenced strains (*S. agalactiae* COH1, A909, 515, H36B, 18RS21, CJB 111, 2603VR, and CIP 8245; for GenBank accession numbers, see above) were used. First, hybridization experiments under high-, medium-, and low-stringency conditions were simulated by mapping the probe sequences against known genome sequences. Next, real experiments were performed, and the results were compared to those from the simulations. The hybridization temperature was kept at 55°C, but washing temperatures varied between 25°C and 55°C until the experiments yielded results matching the high-stringency prediction (Table 2; see Table S2 in the supplemental material).

Microarray procedures. A linear and thermally synchronized primer elongation reaction was used. For one labeling reaction, 1.5 μl of a primer mixture containing one primer per target sequence (0.135 μmol/liter each), an oligonucleotide mixture, and biotin-16-dUTP, as well as 1 to 2 μg of unfragmented RNA-free target DNA, were utilized. Amplification was performed using a standard thermocycler with initial denaturation at 96°C for 5 min, followed by 55 cycles (60 s at 96°C, 20 s at 50°C, and 40 s at 72°C) and final storage at 4°C.

First, the arrays were prewashed with 200 μl of double-distilled water and 200 μl of hybridization buffer 1 (both steps were 5 min at 55°C and 550 rpm on the thermomixer). Labeled DNA samples were diluted with 90 μl of hybridization buffer 1 and then transferred into the ArrayStrip well. After that, the samples were incubated at 55°C and 550 rpm for 60 min and then removed completely. The arrays were washed twice with 200 μl of washing buffer C2 (for 2 min and for 5 min, at 35°C and 550 rpm). Next, the microarrays were incubated with 100 μl of horseradish peroxidase-streptavidin (15 min at 30°C and 550 rpm). After removal of this solution, the arrays were washed twice with 200 μl of washing buffer C5 at 30°C and 550 rpm for 2 min and 5 min, respectively. Finally, 100 μl of precipitating substrate was added. After 5 min of incubation at room temperature (without shaking), the substrate was removed and the arrays were scanned and analyzed using the ArrayMate reader (Alere Technologies). The signal intensities were calculated as previously described (29). For breakpoint determination, the median values of the intensities of those probes of species markers [*fbsB* (consensus), *femH* (locus 2), *gtfB* (variant 1), *gtfB* (variant 2), *nss1*, and *nss2*] that yielded a raw value of >0.2 and the biotin control were calculated. Each probe that yielded a signal of >2/3 of this median value was considered positive, and signals between 1/3 and 2/3 of this median value were considered ambiguous. If the median was <0.5, the entire experiment was considered invalid. If mutually exclusive alleles of species markers [*gtfB* (variant 1) and *gtfB* (variant 2) or *nss1* and *nss2*] were detected together, the experiment was considered invalid, assuming contamination.

eBURST. Analysis of clonal complexes was performed using the software provided on eBURST (see http://eburst.mlst.net/v3/enter_data/single/). The MLST profiles were downloaded from http://pubmlst.org/perl/bigsdb /bigsdb.pl?db=pubmlst_sagalactiae_seqdef&page=downloadP rofiles&scheme_id=1 (see Table S3 in the supplemental material). For an eBURST analysis of the array hybridization profiles, 11 markers (see below and Table 2) were selected, and their presence or their alleles were coded numerically.

RESULTS

A full list of the data sets for the tested strains and isolates can be found in Table S2 in the supplemental material, while the abridged results are provided in Tables 2 to 4.

Typing GBS by MLST. Thirty-six isolates that were representative of the common array hybridization patterns were subjected to MLST, and the MLST types for the sequenced reference strains were deduced from published genome sequences (Table 2). For further analysis, clonal complexes (CC) were introduced in analogy to *Staphylococcus aureus* terminology using the eBURST algorithm (http://eburst.mlst.net/). CC19 was the most common and diverse CC. It was quite inhomogeneous with regard to both the MLST- and array-based (see below) typing approaches. CC19 included sequence type 1 (ST1), ST2, ST5, ST6, ST7, ST8, ST10, ST12, ST14, ST17, ST19, ST22, ST44/178, ST110, ST193, ST291, ST297, ST425, and ST569, as well as a single-locus variant (SLV) of ST579. eBURST shows that CC19 comprises several distinct clusters that were recognized as independent clonal complexes (CC01, CC17, CC19, and CC22) before linking STs were identified. These clusters were designated CC19/01, CC19/17, etc. (Table 2). Addi-

TABLE 1 Key markers for array-based strain classification, their average positions in the *S. agalactiae* genome sequence, and known alleles

Median position based on NEM316, A909, and 2603V/R	Gene(s)	Presence/allele (GenBank accession no. or strain)	Representative GenBank accession no. (positions)	Code no.
145000	*bac*	Negative		B-0
		Positive	CP000114.1 (145690–149184)	B-1
459000	*alp*	Negative		A-0
		alp-2	AL766845.1 (114012–117392)	A-2
		alp-3	AF245663.1 (365–2962)	A-3
		alp-4	DQ629924.1 (1–1095)	A-4
		alp-5	DQ629925.1 (1–1347)	A-5
		alp-bca	CP000114.1 (459256–462318)	A-B
		alp-rib2	AM050558.1 (1–735; partial)	A-R2
		alp-rib (R4)	AE009948.1 (447507–451676)	A-R4
647000	*pil1* locus	Negative		P1-0
		pil1A/C		P1–2
		pil1A/B/C	AE009948.1 (A: 636431–639103)/(B: 631950–633614)/(C: 633703–634626)	P1–3
700000	*pepS8*	Negative		E-0
		Positive	CP000114.1 (740435–743092)	E-1
877000	*fbsB*	*fbsB* (AM050623)	AM050623 (1–1293)	F-3
		fbsB (AM050624)	AM050624 (1–1992)	F-4
		fbsB (515)	AAJP01000006.1 (11614–13521)	F-5
		fbsB (AY326423)	AY326423 (167–2320)	F-6
		fbsB (A909)	CP000114.1 (910378–912639)	F-9
1171000–1235000	Capsule locus	No capsule		C-0
		Capsule type IA	For *capsH* Ia, CP000114.1 (1226719–1227858)	C-1A
		Capsule type IB	For *capsH* Ib, AAJS01000021.1 (16021–17160)	C-1B
		Capsule type II	For *capsH* II, AAJO01000077.1 (1918–3024)	C-2
		Capsule type III	For *capsH* III, AL732656.1 (1279631–1280776)	C-3
		Capsule type IV	For *capsH* IV, AF355776.1 (6895–7992)	C-4
		Capsule type V	For *capsH* V, AE009948.1 (1172623–1173717)	C-5
		Capsule type VI	For *capsH* VI, AF337958.1 (6907–8064)	C-6
		Capsule type VII	For *capsH* VII, AY376403.1 (3881–4975)	C-7
		Capsule type VIII	For *capsH* VIII, AY375363.1 (4219–5367)	C-8
		Capsule type IX	For *capsH* IX, EF157290.1 (1–330; partial)	C-9
1256000	*hylB*	Lacking *hylB*		H-0
		hylB (NEM316)	AL732656.1 (1308821–1311775)	H-3
		hylB (A909)	CP000114.1 (1256012–1258993)	H-9
1353000	*abiG*-I/-II, Q8DZ34	Lacking *abiG*-I/-II, Q8DZ34		Q-0
		abiG-I/-II, Q8DZ34 (NEM316)	AL732656.1 (1408678–1409268)/(1409265–1410113)/(1410217–1413012)	Q-3
		abiG-I/-II, Q8DZ34 (H36B)	AAJS01000027.1 (10052–10642)/(10639–11483)/(11569–14370)	Q-6
1419000–1422000	*pil2* locus	Lacking *pil2*		P2-0
	pil2 A/B/C	*pil2a* (18RS21)	AAJO01000002.1 (A: 21274–23979)/(B: 19027–21144)/(C: 16227–17153)	P2-A1
		pil2a (NEM316)	AL732656.1 (A: 1528058–1530763)/(B: 1525911–1527935)/(C: 1523114–1524040)	P2-A3
		pil2a (ABC020013424)	A: EU929280.1 (12346–15051)/B: EU929899.1 (1–2082)	P2-A4
		pil2a (515)	AAJP01000012.1 (A: 12346–15051)/(B: 15181–17208)/(C: 19079–20005)	P2-A5
		pil2a (H36B)	AAJS01000017.1 (A: 21508–24198)/(B: 19308-21389)/(C: 16511–17437)	P2-A6
		pil2a (ABC020016680)	A: EU929258.1 (1–2691)/B: EU929876.1 (1–2115)	P2-A8
		pil2a (ABC020005369)	A: EU929282.1 (1–2691)/B: EU929901.1 (1–2049)	P2-A9
		pil2b (COH1)	AAJR01000022.1 (B: 7660–11964)/(C: 14416–15087)	P2-B1
		pil2b (527.25)	A: AM051289.1 (1–1365)	P2-B5
		pil2b (A909)	CP000114.1 (A: 1420792–1422300)/(B: 1422341–1426641)/C:(1419218–1419889)	P2-B9
1482000–1487000	*nss, srr* *rogB2*	Lacking *nss/srr/rogB2*		N-0
		nss (COH1) + *srr* (COH1)	AAJR01000012.1 (16899–17828)/(28428-3168)	N-1
		nss + *srr* + *rogB2* (NEM316)	AL732656.1 (1587233–1588240)/(1588571–1592503)/(1531047–1532576)	N-3
		nss + *srr* + *rogB2* (A909)	CP000114.1 (1482334–1483341)/(1483672–1487448)/(1487831–1489323)	N-9
1944000	*rgfC/A/D/B*	Lacking *rgf*		R-0
		rgf (COH1)	AAJR01000004.1 (A: 44577 −45329)/(B: 45545–46360)/(C: 43246–44580)/ (D: 45304–45432)	R-1
		rgf (515)	AAJP01000002.1 (A: 18747 −19499)/(B: 19776–20591)/(C: 18379–18739)	R-5
		rgf (A909)	CP000114.1 (A: 1920710–1921462)/(B: 1921678–1922493)/(C: 1919379–1920713)/(D: 1921437–1921565)	R-9

tional CC19/02 and CC19/05 clusters were designated because their MLST profiles link CC19/01, or CC19/10, respectively, to CC19/19. CC23 was the second most common CC, with ST23, ST24, and ST144. To CC26 and CC130, only one ST each (ST26 and ST130) was assigned. For CC103, two ST314 isolates were identified by MLST.

Genotyping GBS by microarray hybridization. Putative typing markers (e.g., *alp* alleles and capsule types) vary independently of each other and independently of their ST/CC affiliation (Table 2). Thus, it is not possible to assign an isolate to an ST/CC based on a single marker.

For array-based typing, 11 markers (*bac*, *alp*, *pil1* locus, *pepS8*, *fbsB*, capsule locus, *hylB*, *abiG*-I/-II plus Q8DZ34, *pil2* locus, *nss* plus *srr* plus *rogB2*, and *rgfC/A/D/B*; Table 2) were selected that provide a framework for splitting GBS organisms into strains. These markers were selected based on variability affecting either sequences, allowing assignment to clear-cut alleles or their presence/absence. In general, the typing markers are distributed rather

TABLE 2 Array-based typing results

Hybridization pattern	CC	ST	Reference strain (GenBank accession no.)	Identical genome sequences, GenBank accession no, and predicted ST (in silico analysis only)	Germany (clinical)	Trinidad and Tobago (clinical)	Germany (cattle)	bac	alp	pil1	pepS8	fbsB	Capsule	hylB	abiGI Q8DZ34	pil 2	nss/srr/ rogB2	rgf
HP-01	CC19/01	ST1	CJB111 (AAJQ00000000.1)	CCUG 44140, ANDL01, ST1; CCUG 49072, ANDP01, ST524; CCUG 49100, ANDR01, ST1; FSL S3-023, ANCP01, ST1; Gottschalk 13227, ANEZ01, ST1; Gottschalk 2864, ANFA01, ST1	56	2	2	B-0	A-03	P1-3	E-1	F-9	C-05	H-3	Q-0	P2-A3	N-9	R-9
HP-02				09mas018883, HF952104.1, ST1	1	0	0	B-0	A-03	P1-3	E-1	F-9	C-05	H-3	Q-6	P2-A3	N-9	R-9
HP-03	CC19/01	ST1			0	0	1	B-0	A-03	P1-0	E-1	F-9	C-05	H-3	Q-0	P2-A3	N-9	R-9
HP-04	CC19/01	ST1			1	0	0	B-0	A-03	P1-3	E-1	F-9	C-07	H-3	Q-0	P2-B9	N-9	R-9
HP-05	CC19/01	ST14			1	0	0	B-0	A-05	P1-3	E-1	F-9	C-1B	H-3	Q-0	P2-A3	N-9	R-9
HP-06	CC19/01	ST297			1	0	0	B-0	A-05	P1-3	E-1	F-9	C-X	H-3	Q-0	P2-B9	N-9	R-9
HP-07	CC19/01	ST425			0	0	2	B-0	A-BC	P1-2	E-1	F-9	C-04	H-3	Q-0	P2-B9	N-9	R-1
HP-08					0	6	0	B-0	A-BC	P1-0	E-1	F-5	C-04	H-3	Q-0	P2-B1	N-1	R-5
HP-09					0	1	0	B-0	A-BC	P1-0	E-1	F-5	C-00	H-3	Q-0	P2-B1	N-1	R-5
HP-10	CC19/02	ST2			1	0	0	B-0	A-05	P1-3	E-0	F-9	C-04	H-3	Q-3	P2-A3	N-9	R-X
HP-11	CC19/02			CCUG 28551, ANDA01, ST2	1	0	1	B-0	A-05	P1-3	E-0	F-9	C-04	H-3	Q-0	P2-A3	N-9	R-9
HP-12	CC19/04	ST5		GB00901, ALUK01, ST459	3	0	0	B-0	A-05	P1-3	E-1	F-9	C-04	H-3	Q-6	P2-A4	N-9	R-9
HP-13	CC19/10	ST6			1	0	0	B-0	A-05	P1-2	E-1	F-9	C-1A	H-3	Q-0	P2-B9	N-9	R-9
HP-14	CC19/10	ST6			1	0	0	B-1	A-03	P1-3	E-1	F-9	C-02	H-3	Q-3	P2-A5	N-3	R-9
HP-15	CC19/10	ST7	H36B (AAJS00000000.1)	STIR-CD-28, ALRT01, ST500	0	0	0	B-1	A-BC	P1-3	E-1	F-9	C-1B	H-9	Q-6	P2-A6	N-3	R-9
HP-16			A909 (CP000114.1)	CF01173, CAQB01, ST7	1	0	0	B-1	A-BC	P1-3	E-1	F-9	C-1A	H-9	Q-0	P2-B9	N-9	R-9
HP-17	CC19/10	ST8		FSL S3-014, ANCR01, ST8; Gottschalk 999B, ANFD01, ST8	19	0	2	B-1	A-BC	P1-3	E-1	F-9	C-1B	H-9	Q-0	P2-A3	N-3	R-9
HP-18					1	0	0	B-1	A-BC	P1-3	E-1	F-9	C-1B	H-9	Q-0	P2-A5	N-3	R-9
HP-19					1	0	0	B-1	A-BC	P1-3	E-1	F-9	C-1B	H-9	Q-0	P2-A9	N-3	R-9
HP-20	CC19/10	ST12			10	0	0	B-1	A-BC	P1-3	E-1	F-9	C-02	H-9	Q-0	P2-A3	N-3	R-9
HP-21	CC19/10	ST10			9	0	0	B-1	A-BC	P1-0	E-1	F-9	C-05	H-3	Q-0	P2-A3	N-3	R-9
HP-22	CC19/10	ST12			0	0	1	B-1	A-BC	P1-3	E-1	F-9	C-05	H-3	Q-0	P2-A9	N-3	R-9
HP-23	CC19/10	ST12			2	0	0	B-1	A-BC	P1-0	E-1	F-9	C-02	H-3	Q-3	P2-A4	N-9	R-9
HP-24					1	0	0	B-1	A-BC	P1-3	E-1	F-9	C-02	H-3	Q-3	P2-A4	N-9	R-9
HP-25					1	0	0	B-1	A-BC	P1-3	E-1	F-9	C-02	H-3	Q-3	P2-A4	N-9	R-X
HP-26	CC19/10	ST569			3	0	1	B-1	A-BC	P1-3	E-1	F-9	C-02	H-3	Q-0	P2-A5	N-3	R-9
HP-27	CC19/10	ST579slv			1	0	0	B-1	A-BC	P1-3	E-1	F-9	C-04	H-3	Q-0	P2-A5	N-3	R-9
HP-28					1	0	0	B-1	A-BC	P1-3	E-1	F-9	C-02	H-3	Q-0	P2-A8	N-3	R-9
HP-29					1	0	0	B-1	A-BC	P1-3	E-1	F-9	C-02	H-3	Q-0	P2-X	N-3	R-9
HP-30	CC19/17	ST17	COH1 (AAJR00000000.1)	FSL S3-102, ANCS01, ST31; GB00112, AKXO01, ST17; M732, ALQW01, ST17; Melin 455511, ALRA01, ST17	62[a]	2	0	B-0	A-R4	P1-3	E-1	F-5	C-03	H-3	Q-0	P2-B1	N-1	R-1
HP-31	CC19/17	ST291			1	0	0	B-0	A-R4	P1-3	E-1	F-5	C-03	H-3	Q-0	P2-X	N-1	R-1
HP-32					5	0	0	B-0	A-R4	P1-3	E-1	F-5	C-04	H-3	Q-0	P2-B1	N-1	R-1
HP-33	CC19/17	ST17			1	0	0	B-0	A-R4	P1-0	E-1	F-5	C-03	H-3	Q-0	P2-B1	N-1	R-1
HP-34	CC19/19	ST44 or 178			1	1	0	B-0	A-R4	P1-3	E-1	F-5	C-03	H-3	Q-6	P2-B1	N-1	R-1
HP-35	CC19/19	ST19	18RS21 (AAJO00000000.1)	Gottschalk 1003A, ALSI01, ST19	42	2	0	B-0	A-R4	P1-3	E-1	F-9	C-03	H-3	Q-6	P2-A1	N-3	R-5
HP-36				CCUG 91, ANCY01, ST28; GB00588, ALTQ01, ST447	20	2	0	B-0	A-R4	P1-3	E-1	F-9	C-02	H-3	Q-6	P2-A1	N-3	R-5
HP-37	CC19/19	ST28			2	0	0	B-0	A-R4	P1-3	E-1	F-9	C-02	H-3	Q-0	P2-A1	N-3	R-5
HP-38					4	1	0	B-0	A-R4	P1-3	E-1	F-9	C-02	H-3	Q-6	P2-A1	N-0	R-5
HP-39					0	1	0	B-0	A-R4	P1-3	E-1	F-9	C-02	H-3	Q-6	P2-X	N-0	R-5
HP-40	CC19/19	ST193		FSL S3-003, ALQJ01, ST19	12	0	0	B-0	A-R4	P1-3	E-1	F-9	C-03	H-3	Q-0	P2-A1	N-3	R-5

	CC	ST	Strain														
HP-41				1	0	0	B-0	A-R4	PI-x	E-1	F-9	C-03	H-3	Q-0	P2-A1	N-3	R-5
HP-42	CC19/19	ST110	2603V/R (AE009948)	4	1	0	B-0	A-R4	PI-3	E-1	F-9	C-05	H-3	Q-6	P2-A1	N-3	R-5
HP-43				2	0	0	B-0	A-R4	PI-0	E-1	F-9	C-05	H-3	Q-6	P2-A1	N-3	R-5
HP-44	CC19/19	ST19		4	0	0	B-0	A-05	PI-3	E-1	F-9	C-05	H-3	Q-6	P2-A1	N-3	R-5
HP-45				1	0	0	B-0	A-R4	PI-0	E-0	F-9	C-03	H-3	Q-0	P2-A9	N-3	R-5
HP-46	CC19/22	ST22		6	0	0	B-0	A-BC	PI-0	E-0	F-6	C-02	H-3	Q-6	P2-A9	N-3	R-5
HP-47				1	0	0	B-0	A-BC	PI-0	E-0	F-6	C-02	H-3	Q-3	P2-A5	N-3	R-5
HP-48	CC23	ST23	Strain 515 (AAJP00000000.1), ANCQ01, ST23; FSL S3-090, "RV2"[b] Gottschalk 31825, ANFB01, ST23	53[c]	3	1	B-0	A-05	PI-0	E-0	F-5	C-1A	H-3	Q-0	P2-A5	N-3	R-5
HP-49	CC23	ST24		3	9	0	B-0	A-BC	PI-0	E-0	F-5	C-1A	H-3	Q-0	P2-A5	N-3	R-5
HP-50	CC23	ST144		1	5	0	B-0	A-R4	PI-0	E-0	F-5	C-1A	H-3	Q-0	P2-A5	N-3	R-5
HP-51				1	0	0	B-0	A-05	PI-0	E-0	F-5	C-1A	H-3	Q-3	P2-A5	N-3	R-5
HP-52				1	0	0	B-0	A-05	PI-0	E-0	F-5	C-05	H-3	Q-0	P2-A5	N-3	R-5
HP-53				2	0	0	B-0	A-05	PI-x	E-0	F-5	C-1A	H-3	Q-0	P2-A5	N-3	R-5
HP-54	CC23	ST23	NEM316 (AL732656.1)	5	0	0	B-0	A-02	PI-3	E-0	F-5	C-03	H-3	Q-3	P2-A3	N-3	R-5
HP-55	CC23	ST23		2	0	0	B-0	A-02	PI-3	E-0	F-5	C-03	H-3	Q-3	P2-A5	N-3	R-5
HP-56				0	0	1	B-0	A-02	PI-3	E-0	F-5	C-03	H-3	Q-3	P2-A8	N-3	R-5
HP-57	CC23	ST23		14	0	0	B-0	A-02	PI-0	E-0	F-5	C-1A	H-3	Q-3	P2-A8	N-3	R-5
HP-58				1	0	0	B-0	A-02	PI-0	E-0	F-5	C-1A	H-3	Q-3	P2-A8	N-3	R-5
HP-59				1	1	0	B-0	A-02	PI-0	E-0	F-5	C-1A	H-3	Q-X	P2-A8	N-3	R-5
HP-60	CC26	ST26		1	0	0	B-0	A-00	PI-0	E-0	F-4	C-05	H-3	Q-6	P2-A9	N-3	R-5
HP-61				1	0	0	B-0	A-00	PI-0	E-0	F-4	C-05	H-3	Q-0	P2-A9	N-3	R-5
HP-62				0	0	2	B-0	A-05	PI-0	E-1	F-3	C-1A	H-9	Q-6	P2-B5	N-9	R-9
HP-63				0	0	1	B-0	A-00	PI-0	E-1	F-3	C-03	H-9	Q-0	P2-B5	N-9	R-9
HP-64	CC103	ST314		1	0	2	B-0	A-05	PI-0	E-1	F-3	C-1A	H-9	Q-0	P2-B5	N-9	R-9
HP-65				0	0	1	B-0	A-05	PI-0	E-1	F-3	C-03	H-9	Q-0	P2-B5	N-9	R-9
HP-66	CC103	ST314		0	0	0	B-0	A-X	PI-0	E-1	F-3	C-1A	H-9	Q-6	P2-B5	N-9	R-9
HP-67				0	0	1	B-0	A-BC	PI-0	E-1	F-3	C-1A	H-9	Q-6	P2-X	N-9	R-9
HP-68	CC130	ST130	GB00300, ALTJ01, ST130	3	0	0	B-1	A-BC	PI-0	E-1	F-3	C-09	H-3	Q-6	P2-A1	N-3	R-9
HP-69				1	0	0	B-0	A-03	PI-3	E-1	F-9	C-02	H-3	Q-3	P2-A3	N-9	R-9
HP-70				1	0	0	B-0	A-05	PI-0	E-1	F-9	C-02	H-3	Q-3	P2-X	N-9	R-9
HP-71				0	0	1	B-0	A-05	PI-X	E-1	F-9	C-1A	H-3	Q-6	P2-B9	N-9	R-5
HP-72				1	0	0	B-0	A-R4	PI-2	E-1	F-9	C-02	H-3	Q-3	P2-B9	N-9	R-9
HP-73				1	0	0	B-0	A-R4	PI-3	E-1	F-5	C-03	H-9	Q-X	P2-A1	N-3	R-5
HP-74				1	0	0	B-1	A-BC	PI-0	E-1	F-9	C-1B	H-9	Q-0	P2-X	N-3	R-9
HP-75				0	0	0	B-1	A-BC	PI-0	E-1	F-9	C-02	H-3	Q-6	P2-A1	N-3	R-9
HP-76				1	0	1	B-1	A-BC	PI-3	E-1	F-9	C-02	H-3	Q-3	P2-A3	N-3	R-9

[a] Includes two isolates from clinically/epidemiologically established transmission.

[b] A strain from the proficiency testing program for German diagnostic microbiology laboratories, 2010/Febr./RV4, obtained from INSTAND eV.

[c] Includes two isolates from another clinically/epidemiologically established transmission event.

evenly across the genome sequence. For each marker locus, the presence or absence or a small number of different alleles was interrogated. This allowed, in analogy to MLST, a conversion of the hybridization results for these markers into 11 numerical codes (Table 2). Ubiquitous genes, such as *cylD/cylE* (β-hemolysin locus), were not suitable as typing markers. Highly mobile plasmid-borne (such as resistance genes) or phage-borne genes were also not considered, although they can be detected by the array. Their use for further discerning the variants within strains still needs to be evaluated.

Details on the 11 markers will be discussed below in the order of their localizations in the *S. agalactiae* genome that are conserved among known genomes (30). The gene positions in the following paragraphs are provided as medians of the starting positions from the fully and conventionally sequenced genomes of NEM316, A909, and 2603V/R.

The *bac* gene encodes a surface protein binding the Fc part of IgA (31). Because of an extremely variable repeat region (32), only its presence or absence, rather than specific alleles, was interrogated. Its presence apparently correlated with alleles 4, 9, and 10 of the MLST gene *adhP*, or roughly with affiliation to CC19/10 and CC130.

Genes encoding alpha, alpha-like (33), Bca, and Rib proteins are all regarded as alleles of the *alp* gene (for GenBank accession numbers, see Table 1). The alleles *alp-2*, *alp-3*, *alp-4* (= *alp*-6), and *alp-5* (= *alp-7*, *alp*-epsilon), *alp-bca* (= alpha C or C-alpha), *alp-rib*(*R4*) (= R4, rib), and *alp-rib2* were covered. The alleles *alp-rib*(*R4*) and *alp-bca* were the most common *alp* alleles in human isolates; in the cattle group, *alp-5* was most prevalent. Three isolates, including both CC26 isolates, were *alp* negative (or carried an alternative undetectable allele). There was no correlation between *alp* and alleles of the neighboring MLST markers *tkt*, *glck*, and *atr*.

S. agalactiae pili (34) are composed of PilB and the accessory pilus proteins PilA and PilC (35, 36). There are two loci. The first one (*pilA/B/C-1*) is situated around position 650000 in the genome sequence. In a vast majority of isolates, either the three *pil1* genes together or no *pil1* genes at all were detected. Four isolates yielded only signals for *pilA/C-1*. For the second pilus locus, there are two mutually exclusive genomic islands (*pil2a* and *pil2b*) at identical positions in the genome sequence. Eighteen probes recognized seven *pil2a* alleles, while another eight probes yielded three *pil2b* patterns, one of which matches only an incompletely known sequence (GenBank accession no. AM051289.1). In the human isolates, the *pil2a* genes were generally more common (Table 2). In the cattle isolates, *pil2b* was as common as *pil2a*. Three isolates yielded unassignable patterns. There were no isolates lacking *pil2*. The loci adjacent to *pil2* are *gtfA/B* (encoding glycosyltransferase, around position 1466000) and r*ogB1* (encoding the transcriptional regulator RogB, locus 1, position 1475000). Two alleles of *gtfA/B* were discerned to correlate with *pil2a* or *pil2b*, respectively. The presence of *rogB1* is associated with *pil2a*. Thus, *gtfA/B* and *rogB1* yield no additional typing information.

Another variable genomic island is detected by probes for *pepS8* (peptidase S8). All CC19/22, CC23, and CC26 isolates, as well as a part of CC19/02, did not contain this island.

The gene *fbsB* encodes a fibrinogen binding protein (37, 38). Five hybridization patterns were generated by eight probes; isolates lacking *fbsB* were not observed.

A cluster of 16 genes encodes the capsule, of which there are 10

types (Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX). The different prevalences of the German, Caribbean, cattle, and human isolates are shown in Table 3. Capsule type III was overrepresented among the invasive isolates. Types VI, VII, and IX were very rare, and type VIII was not found (although the array can identify it; therefore, a type VIII reference isolate was kindly provided by K. Zürn and F. Lander, Dresden University Medical Centre). One Caribbean isolate yielded signals with a part of the capsule IV probes (*cpsJ*-IV, *cpsM*-IV, and *cpsM*-IV) only.

Another typing factor was *hylB* (hyaluronate lyase), with two distinct alleles.

Two variants of a gene cluster comprising *abiG*-I, *abiG*-II (encoding the abortive infection proteins I and II, respectively), and Q8DZ34 (CHAP domain containing protein/N-acetylmuramoyl-L-alanine amidase) were discerned. An NEM316-like variant was more common than an H36B-like variant; two isolates were unassignable. Most isolates did not contain these variants, and there were no major differences between the different populations tested.

Another variable gene cluster comprises *nss*, *srr*, and *rogB2*, encoding a putative nucleotide sugar synthetase, a serine-rich repeat 1 glycoprotein mediating the penetration of the blood-brain barrier (39), and a second locus of the transcriptional regulator, respectively. Three variants of this genomic island were discerned.

The GBS genome sequence comprises several regulatory loci, including the *rgf* quorum-sensing locus (55). Three different variants were identified, with *rgfB* being conserved, *rgfA* and *rgfC* having two or three alleles, respectively, and *rgfD* being absent from several lineages and from the genome sequence of *S. agalactiae* strain 515 (GenBank accession no. AAJP01000002.1).

Analyzing the hybridization results for these 11 markers, 76 distinct strain profiles were distinguished (notated as hybridization profiles [HP], followed by sequential numbers). When applying eBURST on these markers, some of the HPs clustered. Generally, the HPs showed some correlation with the MLST-defined CCs/STs (Table 2 and Fig. 1). The isolates that showed that identical or related HPs belonged to the same CC, but isolates that are related by MLST sometimes show dissimilar hybridization patterns.

The highest diversity (46 HPs) was observed in CC19. Many isolates displaying similar HPs belonged to the same cluster within CC19 (Fig. 1) having the same STs or single locus variants thereof.

Nine HPs were not assigned to CCs/STs, as they yielded patterns that were dissimilar to any isolates with known STs.

Population structure. CC19 was the largest CC, accounting for 75% of the human isolates from Germany, 50% of the human isolates from Trinidad and Tobago, and 32% of the bovine isolates from Germany. For CC23, the corresponding figures were 22%, 50%, and 6%, respectively. HP-08 and HP-09 (CC19/01-ST425) and HP-49 and HP-50 (CC23-ST24 and -144, respectively) appeared to predominate in Trinidad and Tobago. The cattle isolates belonged to diverse lineages, but CC103 was more common in this group than in the other groups, with 23% of the bovine isolates being associated with this CC. With regard to clinical background (Fig. 1), the highest proportion of invasive isolates was observed for CC19/17, followed by CC19/01, CC19/10, and CC23.

Detection of antibiotic resistance markers. Table 4 shows the data for the antibiotic resistance genes. The isolates were screened for *erm*(A), *erm*(B), and *erm*(C), which code for methylases conferring macrolide/clindamycin resistance by methylation of the

TABLE 3 Prevalences of marker alleles

Presence/allele (accession no. or strain)	Code no.	Total		Human, Germany		Human, Trinidad and Tobago		Veterinary, Germany		Human, invasive infection		Human, localized infection		Human, carriage	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
bac negative	B-0	384	85,7	327	85.2	34	100.0	16	76.2	100	87.0	42	87.5	219	85.9
bac positive	B-1	64	14.3	57	14.8	0	0.0	5	23.8	15	13.0	6	12.5	36	14.1
alp negative	A-0	3	0.7	2	0.5	0	0.0	1	4.8	1	0.9	1	2.1	0	0.0
alp-2	A-2	25	5.6	23	6.0	0	0.0	1	4.8	4	3.5	9	18.8	10	3.9
alp-3	A-3	67	15.0	61	15.9	2	5.9	3	14.3	19	16.5	9	18.8	35	13.7
alp-4	A-4	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
alp-5	A-5	86	19.2	70	18.2	3	8.8	11	52.4	20	17.4	8	16.7	45	17.6
alp-bca	A-B	89	19.9	66	17.2	16	47.1	5	23.8	18	15.7	6	12.5	58	22.7
alp-R2	A-R2	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
alp-rib (R4)	A-R4	177	39.5	161	41.9	13	38.2	0	0.0	53	46.1	15	31.3	106	41.6
alp atypical/unidentified	A-X	1	0.2	1	0.3	0	0.0	0	0.0	0	0.0	0	0.0	1	0.4
pil1 negative	P1-0	120	26.8	83	21.6	24	70.6	10	47.6	24	20.9	9	18.8	74	29.0
pil1A/C	P1-2	4	0.9	2	0.5	0	0.0	2	9.5	0	0.0	0	0.0	2	0.8
pil1A/B/C	P1-3	320	71.4	296	77.1	10	29.4	8	38.1	89	77.4	39	81.3	178	69.8
pil1 atypical/unidentified	P1-X	4	0.9	3	0.8	0	0.0	1	4.8	2	1.7	0	0.0	1	0.4
pepS8 negative	E-0	118	26.3	95	24.7	17	50.0	3	14.3	29	25.2	16	33.3	67	26.3
pepS8 positive	E-1	330	73.7	289	75.3	17	50.0	18	85.7	86	74.8	32	66.7	188	73.7
fbsB (AM050623)	F-3	12	2.7	5	1.3	0	0.0	7	33.3	0	0.0	1	2.1	4	1.6
fbsB (AM050624)	F-4	2	0.4	2	0.5	0	0.0	0	0.0	1	0.9	1	2.1	0	0.0
fbsB (515)	F-5	187	41.7	155	40.4	26	76.5	2	9.5	61	53.0	22	45.8	98	38.4
fbsB (AY326423)	F-6	7	1.6	7	1.8	0	0.0	0	0.0	1	0.9	0	0.0	6	2.4
fbsB (A909)	F-9	240	53.6	215	56.0	8	23.5	12	57.1	52	45.2	24	50.0	147	57.6
Capsule type:															
Ia	C-1A	107	23.9	80	20.8	17	50.0	7	33.3	25	21.7	14	29.2	58	22.7
Ib	C-1B	26	5.8	23	6.0	0	0.0	2	9.5	10	8.7	0	0.0	13	5.1
II	C-2	64	14.3	57	14.8	4	11.8	2	9.5	11	9.6	4	8.3	46	18.0
III	C-3	137	30.6	129	33.6	3	8.8	3	14.3	47	40.9	14	29.2	71	27.8
IV	C-4	20	4.5	11	2.9	6	17.6	3	14.3	1	0.9	4	8.3	12	4.7
V	C-5	88	19.6	79	20.6	3	8.8	4	19.0	21	18.3	11	22.9	50	19.6
VI	C-6	1	0.2	1	0.3	0	0.0	0	0.0	0	0.0	0	0.0	1	0.4
VII	C-7	1	0.2	1	0.3	0	0.0	0	0.0	0	0.0	0	0.0	1	0.4
VIII	C-8	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
IX	C-9	3	0.7	3	0.8	0	0.0	0	0.0	0	0.0	1	2.1	2	0.8
No capsule/truncated *cap* locus	C-0	1	0.2	0	0.0	1	2.9	0	0.0	0	0.0	0	0.0	1	0.4
hylB negative	H-0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
hylB (NEM316)	H-3	401	89.5	348	90.6	34	100.0	12	57.1	102	88.7	46	95.8	234	91.8
hylB (A909)	H-9	47	10.5	36	9.4	0	0.0	9	42.9	13	11.3	2	4.2	21	8.2
abiG/Q8DZ34 negative	Q-0	309	69.0	261	68.0	28	82.4	15	71.4	92	80.0	28	58.3	169	66.3
abiG/Q8DZ34 (NEM316)	Q-3	36	8.0	34	8.9	0	0.0	1	4.8	4	3.5	10	20.8	20	7.8
abiG/Q8DZ34 (H36B)	Q-6	101	22.5	87	22.7	6	17.6	5	23.8	17	14.8	10	20.8	66	25.9
abiG/Q8DZ34 (unidentified)	Q-X	2	0.4	2	0.5	0	0.0	0	0.0	2	1.7	0	0.0	0	0.0
pil2 negative	P2-0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
pil2a (18RS21)	P2-A1	104	23.2	96	25.0	5	14.7	1	4.8	19	16.5	9	18.8	73	28.6
pil2a (NEM316)	P2-A3	115	25.7	105	27.3	2	5.9	6	28.6	32	27.8	13	27.1	62	24.3
pil2a (ABC020013424)	P2-A4	7	1.6	7	1.8	0	0.0	0	0.0	1	0.9	3	6.3	3	1.2
pil2a (515)	P2-A5	88	19.6	67	17.4	17	50.0	2	9.5	24	20.9	7	14.6	53	20.8
pil2a (H36B)	P2-A6	1	0.2	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
pil2a (ABC020016680)	P2-A8	18	4.0	17	4.4	0	0.0	1	4.8	2	1.7	8	16.7	7	2.7
pil2a (ABC020005369)	P2-A9	11	2.5	10	2.6	0	0.0	1	4.8	2	1.7	1	2.1	7	2.7
pil2a (unidentified)	P2-AX	6	1.3	5	1.3	1	2.9	0	0.0	2	1.7	0	0.0	4	1.6
pil2b (COH1)	P2-B1	79	17.6	69	18.0	9	26.5	0	0.0	33	28.7	7	14.6	38	14.9
pil2b (527.25)	P2-B5	8	1.8	2	0.5	0	0.0	6	28.6	0	0.0	0	0.0	2	0.8
pil2b (A909)	P2-B9	9	2.0	5	1.3	0	0.0	3	14.3	0	0.0	0	0.0	5	2.0
pil2b (unidentified)	P2-BX	2	0.4	1	0.3	0	0.0	1	4.8	0	0.0	0	0.0	1	0.4
nss/srr/rogB2 negative	N-0	6	1.3	4	1.0	2	5.9	0	0.0	1	0.9	0	0.0	5	2.0

(Continued on following page)

TABLE 3 (Continued)

Presence/allele (accession no. or strain)	Code no.	Total		Human, Germany		Human, Trinidad and Tobago		Veterinary, Germany		Human, invasive infection		Human, localized infection		Human, carriage	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
nss/srr (COH1)	N-1	80	17.9	70	18.2	9	26.5	0	0.0	33	28.7	7	14.6	39	15.3
nss/srr/rogB2 (NEM316)	N-3	268	59.8	234	60.9	21	61.8	7	33.3	62	53.9	29	60.4	164	64.3
nss/srr/rogB2 (A909)	N-9	94	21.0	76	19.8	2	5.9	14	66.7	19	16.5	12	25.0	47	18.4
rgf negative	R-0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
rgf (COH1)	R-1	75	16.7	70	18.2	2	5.9	2	9.5	33	28.7	7	14.6	32	12.5
rgf (515)	R-5	233	52.0	188	49.0	30	88.2	10	47.6	48	41.7	24	50.0	146	57.3
rgf (A909)	R-9	138	30.8	124	32.3	2	5.9	9	42.9	34	29.6	17	35.4	75	29.4
rgf atypical/unidentified	R-X	2	0.4	2	0.5	0	0.0	0	0.0	0	0.0	0	0.0	2	0.8

binding site at the ribosome. No *erm* genes were detected in the Caribbean isolates. In Germany, the most common *erm* gene was *erm*(B). *erm*(A) and *erm*(C) were less common, and they were not found among the cattle isolates. The tetracycline resistance marker *tet*(M) was common among human isolates but less common among the veterinary isolates.

All isolates harbored *emrB/qacA* (multidrug resistance transporter).

Detection of heavy metal resistance markers. Table 4 shows the data for the heavy metal resistance markers. Two genes associated with cadmium resistance (*cadC*, encoding a cadmium efflux system accessory protein, and *cadD*, encoding cadmium resistance protein D) were interrogated. These genes are not associated with each other, and *cadD* was much more common. The mercury resistance genes *merA* (encoding a mercuric reductase) and *merR* (encoding a mercuric resistance operon regulatory protein) were always found together. Nearly half of the human isolates from Germany and 88% of the isolates from Trinidad and Tobago but only a single veterinary isolate were found positive. While *merA/R* were common in CC19 and CC23, they were rare in other CCs.

DISCUSSION

S. agalactiae is a common cause of illness in infants and is transferred vertically. Besides establishing such a transmission in individual cases, molecular typing of this organism should also address whether there are hypervirulent clones. It would be relevant to know the geographic distribution of such clones, so as to identify regions where a risk for infants might be above average. Moreover, it would be interesting to know if there is a possible zoonotic risk or if, conversely, livestock can be infected with GBS from humans.

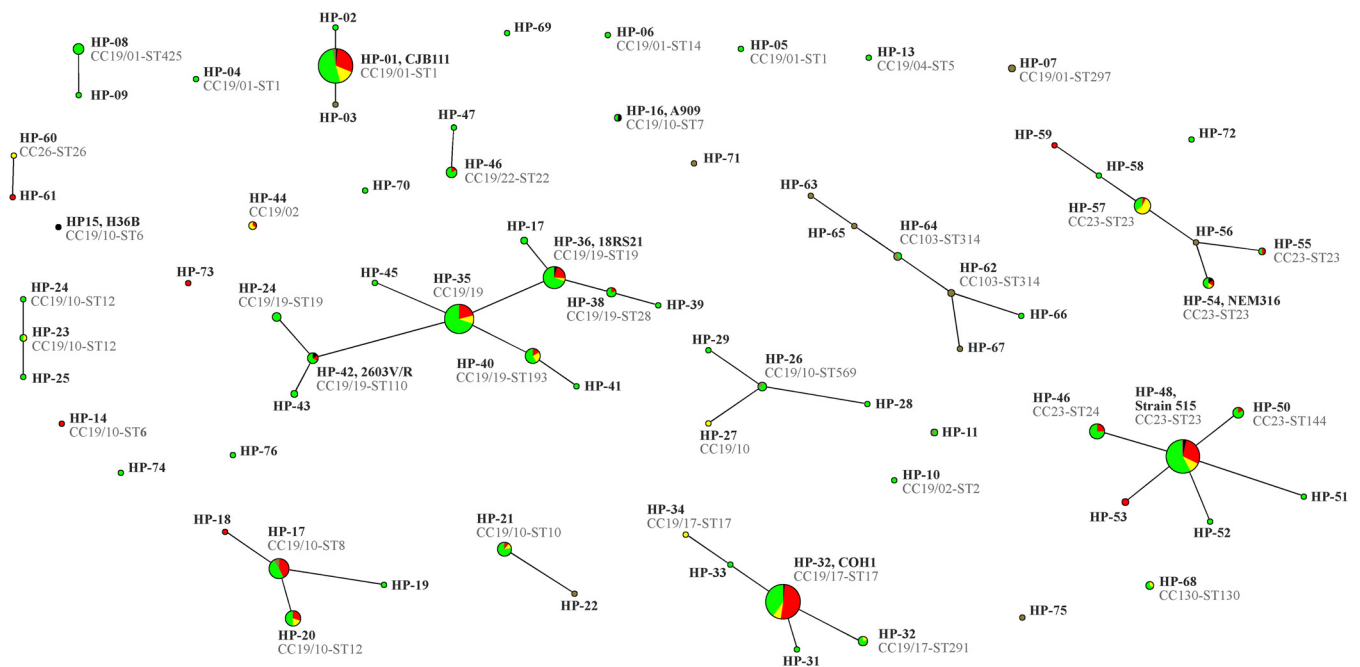FIG 1 eBURST population snapshot graph based on hybridization patterns for 11 variable marker genes. The sizes of the circles correlate with the numbers of isolates. The color codes indicate provenance: red are invasive isolates, yellow are isolates from local infections, and green are from carriage. Black indicates reference strains, and brown are isolates from local infections (mastitis) in cattle.

TABLE 4 Prevalence of resistance genes in clinical isolates

Resistance gene(s)	Prevalences for isolates by type:							
	Total		Clinical, Germany		Clinical, Trinidad and Tobago		Veterinary, Germany	
	No. resistant	%	No. resistant	%	No. resistant	%	No. resistant	%
erm(A)	26	5.8	26	6.8	0	0.0	0	0.0
erm(B)	47	10.5	44	11.5	0	0.0	3	14.3
erm(C)	8	1.8	8	2.1	0	0.0	0	0.0
tet(M)	345	77.0	306	79.7	24	70.6	10	47.6
emrB/qacA	448	100.0	384	100.0	34	100.0	21	100.0
cadC	95	21.2	86	22.4	6	17.6	0	0.0
cadD	400	89.3	352	91.7	32	94.1	10	47.6
merR + *merA*	213	47.5	176	45.8	30	88.2	1	4.8

With regard to population structure, we observed that CC19 predominated among the human isolates from Germany, whereas in Trinidad and Tobago, CC19 and CC23 were equally common. In cattle, CC19 was also common, but another prevalent lineage is CC103 (40), which is rare among human isolates. These observations might indicate both geographic- and host-specific differences in population structure. With regard to host specificity, one virulence factor, *scpB* (encoding a complement-inactivating C5a peptidase), might be involved (see Table S2 in the supplemental material). It was present in 412 of 418 human isolates (98.6%) but in only 10 of 21 cattle isolates (48%). All isolates belonging to the bovine-associated CC103 (including two human isolates) lacked *scpB*. It was also absent in some bovine isolates belonging to human lineages (one of two HP-07, CC19/01-ST297 isolates, one HP-56, and a CC23 isolate).

When studying the population structure, a major disadvantage of MLST/eBURST became visible. Some previously described CCs (CC1, CC17, and CC22) are not recognized as CCs anymore but lumped together into CC19. Similar observations were made for *S. aureus*, as nowadays, e.g., distinct CC5 and CC8 are merged together by eBURST (41). Strains linking two distinct CCs can theoretically be common ancestors of both, but they also might be recent hybrids or recombinants. MLST/eBURST is not able to visualize the difference so that distinct CCs are merged together when linking strains are found. In *S. aureus*, for instance, the hybrid strain ST239 links CC8 to the unrelated CC30 (42). Both lineages became linked to a third unrelated CC (CC45), with the identification of a recombinant strain (ST2249) that emerged from ST239 and CC45 parents (43). Thus, with a broadening database, the current concept of CCs will obscure phylogenetic patterns and, eventually, an entire species might be regarded as a single CC, especially if it is a species in which horizontal gene transfer is as common as it is in *S. agalactiae* (44). Hence, clusters within CC19 (such as CC19/01 and CC19/17) were distinguished (Table 2) to visualize the relationships within the large CC19.

With DNA microarrays, it is possible to genotype high numbers of isolates, with the method being less cumbersome than MLST and providing additional information on specific virulence and resistance determinants.

Similarly to what has been shown for *S. aureus*, hybridization patterns appear to correspond to STs and CCs (29). Compared to

S. aureus, this correlation in GBS is poor. It is possible to predict an ST/CC affiliation from a hybridization pattern if it is identical or similar to a previously MLS-typed strain. However, isolates belonging to the same CC/ST might yield different hybridization patterns, and it not possible to predict a hybridization pattern from a known ST. Otherwise, we frequently observed (Table 2) that similar strains differed in single features, such as capsule genes, *alp*, or pilus genes, and that these genes vary independently of each other. Some ubiquitous genes or gene clusters that apparently always reside at the same position in the genome sequence (30) show a number of distinct alleles, and the identity of an actual allele at a given position is of a random nature. This might be explained by a high number of past recombination events (30, 44) or, theoretically, also by convergent evolution.

In both cases, typing by array and by MLST would not reflect a true phylogeny in terms of providing information on the evolutionary history and provenance of a given strain but just create a random pattern to be compared with other random patterns. The identities of two isolates might then indicate, e.g., a transmission event, but a mere similarity between two isolates would not necessarily mean they were related, i.e., have a recent common ancestor. The resolution of a typing method might be increased with a greater number of interrogated markers. The markers described here could be combined with the MLST scheme, creating an 18-marker framework. This approach could be used experimentally or, in the near future, purely *in silico* by extracting handy information out of the abundance of data generated by next-generation sequencing. Besides, *erm*, *cad*, *mer*, or *tet* genes could also be used to prove the identity or nonidentity of isolates, provided that data on the frequency of their acquisition or loss becomes available.

In order to understand the possible differences in virulence within *S. agalactiae*, future studies should focus not only on ST/CC affiliations but also on the alleles of relevant genes or gene clusters, because these might vary within an ST/CC. No single factor was identified that distinguishes between invasive and carriage isolates. However, it was observed that invasive isolates are less likely to carry *abiG*-I/II and Q8DZ34, but they are more likely to show the presence of the *pil1* locus, *fbsB* (515), and capsule type III (13, 45), as well as COH1-like alleles of *pil2b*, *nss/srr*, and *rgf* (Table 3). This does not necessarily mean that these factors are involved in invasiveness. They also just might be surrogate markers being linked to invasive strains. Several of these markers appear together in CC19/17 isolates. Previously, CC19 (including ST01, ST17, ST19, and ST28) was found in neonatal sepsis cases from very diverse geographic origins (30, 46–48), and the CC19/17 cluster has also frequently been observed in invasive isolates (49, 50).

Other virulence factors include *speM* (encoding exotoxin M) and the *cyl* operon (see Table S2 in the supplemental material). The *speM* gene was found in only seven isolates. The *cyl* operon was always found, and thus, its detection cannot be helpful for predicting virulence. However, *in vivo* expression might be a very different issue, and possibly, the key to understanding the difference between invasive and carriage isolates is not the mere presence or absence of specific genes or alleles but their expression.

Generally, it is not possible to safely distinguish invasive from carriage isolates and to predict an individual fate based on available typing data. Even if some strains are associated with an increased risk of infection, other strains cannot be considered harmless. For practical purposes, this warrants screening for and

eradication of any *S. agalactiae* colonization prior to delivery, regardless of the genotyping data. Vaccination might become an additional tool for preventing infection, and a vaccine covering capsule types Ia, Ib, and III is currently being tested (51). Capsule types are geographically unevenly distributed, as the comparison between the German and Caribbean isolates and other work (50, 52, 53) indicate. Thus, the efficacy of a capsule-based vaccination might vary in different countries and also might change over time (54), emphasizing a need for typing GBS.

For the sake of statistical significance, future studies should target more isolates from defined clinical conditions and healthy controls and aim to compare *S. agalactiae* populations from different regions and host species. The arrays described here provide a convenient tool for typing large numbers of clinical/veterinary isolates and thus might help to obtain insights in GBS epidemiology.

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