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Acquisition of Insertion Sequences and the GBSi1 Intron by *Streptococcus agalactiae* Isolates Correlates with the Evolution of the Species

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The prevalence of insertion sequences IS*1548*, IS*861*, IS*1381*, and ISSa4 and of the group II intron GBSi1 within *Streptococcus agalactiae* human isolates strongly correlates with the genetic lineages obtained by multilocus sequence typing. Our results yielded an evolutionary scheme for the acquisition of these genetic elements linked to the ecosystems from which the isolates were obtained.

Mobile genetic elements drive bacterial evolution and adaptation via recombination and horizontal transfer events (12), and they can influence the virulence of bacteria by modulating gene expression (9). These mobile genetic elements include bacteriophages, transposons, superintegrons, insertion sequences (ISs), and group II introns. ISs are small genetic elements, usually less than 2.5 kb in size, encoding only their own mobility (15), whereas group II introns have a more complicated mechanism and transpose via an RNA intermediate that acts like a ribozyme (17). *Streptococcus agalactiae* has emerged, during the last 35 years, as the most common pathogen implicated in neonatal infectious diseases (24). It harbors several mobile genetic elements. Five are well characterized: four ISs, IS*1548* (8), IS*861* (18), IS*1381* (26), and ISSa4 (24), and one group II intron, GBSi1 (7). Some of these elements have been reported to be associated with virulence genes: IS*1548* with the hyaluronate lyase gene (8), IS*1548* and GBSi1 with the C5a-peptidase gene and the gene encoding the laminin-binding protein Lmb (7), IS*861* and IS*1548* with the capsule cluster *cps* gene (21, 22), and ISSa4 with the *cylB* gene, which encodes the membrane-spanning domain of a putative hemolysin transporter (25). Given the influence of these repeat elements on genome plasticity, the aim of this study was to correlate the presence of IS*1548*, IS*861*, IS*1381*, ISSa4, and GBSi1 with the evolution of the *S. agalactiae* species analyzed by multilocus sequence typing (MLST).

Our collection was composed of 52 epidemiologically unrelated *S. agalactiae* isolates, representative of the various human sites where *S. agalactiae* is found. They were collected in France from 1986 to 2003: 20 were isolated from the cerebrospinal fluid of neonates, 17 from the blood cultures of patients suffering from endocarditis, 10 from the vaginas of asymptomatic women, and 5 from the gastric fluids of neonates. Serotyping was performed with a Pastorex rapid latex agglutination test (Bio-Rad, Hercules, Calif.) and by a molecular serotyping process as described by Kong et al. (13). Thirty isolates were from serotype III (57.7% of isolates), seven from serotype Ib (13.5%), four from serotype II (7.7%), three from serotype Ia (5.8%), two from serotype V (3.8%), and one from serotype IV (1.9%). Five isolates were nontypeable (9.6%).

We evaluated the prevalence of IS*1548*, IS*861*, IS*1381*, ISSa4, and GBSi1 within the 52 isolates by PCR using previously described primers and amplification conditions (1, 4). PCR products corresponding to each IS and GBSi1 were checked by sequencing. Forty of the 52 isolates possessed IS*861* (76.9%), 37 possessed IS*1381* (71.2%), 19 possessed IS*1548* (36.5%), 16 possessed GBSi1 (30.8%), and 4 possessed ISSa4 (7.7%). Based on the presence or absence of each of the five elements, nine genetic variants, designated V1 to V9, were defined among the 52 isolates in our collection (Table 1).

The genomic positions of the copies of each IS and GBSi1 were determined by Southern blot analysis for each of the 52 isolates. Briefly, DNA was digested with EcoRI, for which there were no cleavage sites in the four ISs or GBSi1. Hybridizations were carried out as previously described (6), with probes specific for each IS and GBSi1. Vector NTI v 9.0 software was employed to estimate the number and the size of EcoRI fragments by using the whole genome sequence of *S. agalactiae* NEM316 (GenBank accession number AL732656). The Smart Ladder molecular-size standard (Eurogentec), which was run in parallel, allowed us to locate the copies of the ISs and of GBSi1 on the restriction fragments generated in silico.

Phylogenetic analysis was performed by the MLST technique with seven housekeeping genes (*adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK*, and *tkt*) as described by Jones et al. (11). Nucleotide sequences were compared with those available on the MLST database (http://pubmlst.org/sagalactiae/). After assigning an allele number to each locus, the sequence type (ST), which takes into account the allele combinations of the seven

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TABLE 1. Nine genetic variants defined by combination of the presence or absence of each of the four ISs and of the group II intron GBSi1[a]

Genetic variant	No. of different ISs and/or group II intron	Result for:					No. (%) of isolates	Serotype (no. of isolates)[b]
		IS*1548*	GBSi1	IS*861*	IS*1381*	ISSa4		
V1	0	−	−	−	−	−	2 (4)	III (1), NT (1)
V2	1	−	−	−	+	−	6 (11)	NT (2), IV (1), V (1), Ia (1), Ib (1)
V3	1	−	−	−	−	+	3 (6)	III (3)
V4	2	−	+	+	−	−	10 (9)	III (10)
V5	2	−	−	+	+	−	8 (15)	Ib (6), Ia (1), NT (1)
V6	3	+	−	+	+	−	17 (33)	III (15), II (1), NT (1)
V7	3	−	+	+	+	−	3 (6)	Ia (1), II (1), III (1)
V8	3	−	+	−	+	+	1 (2)	II (1)
V9	4	+	+	+	+	−	2 (4)	II (1), V (1)

[a] Serotypes of the isolates are indicated in the last column.
[b] NT, nontypeable.

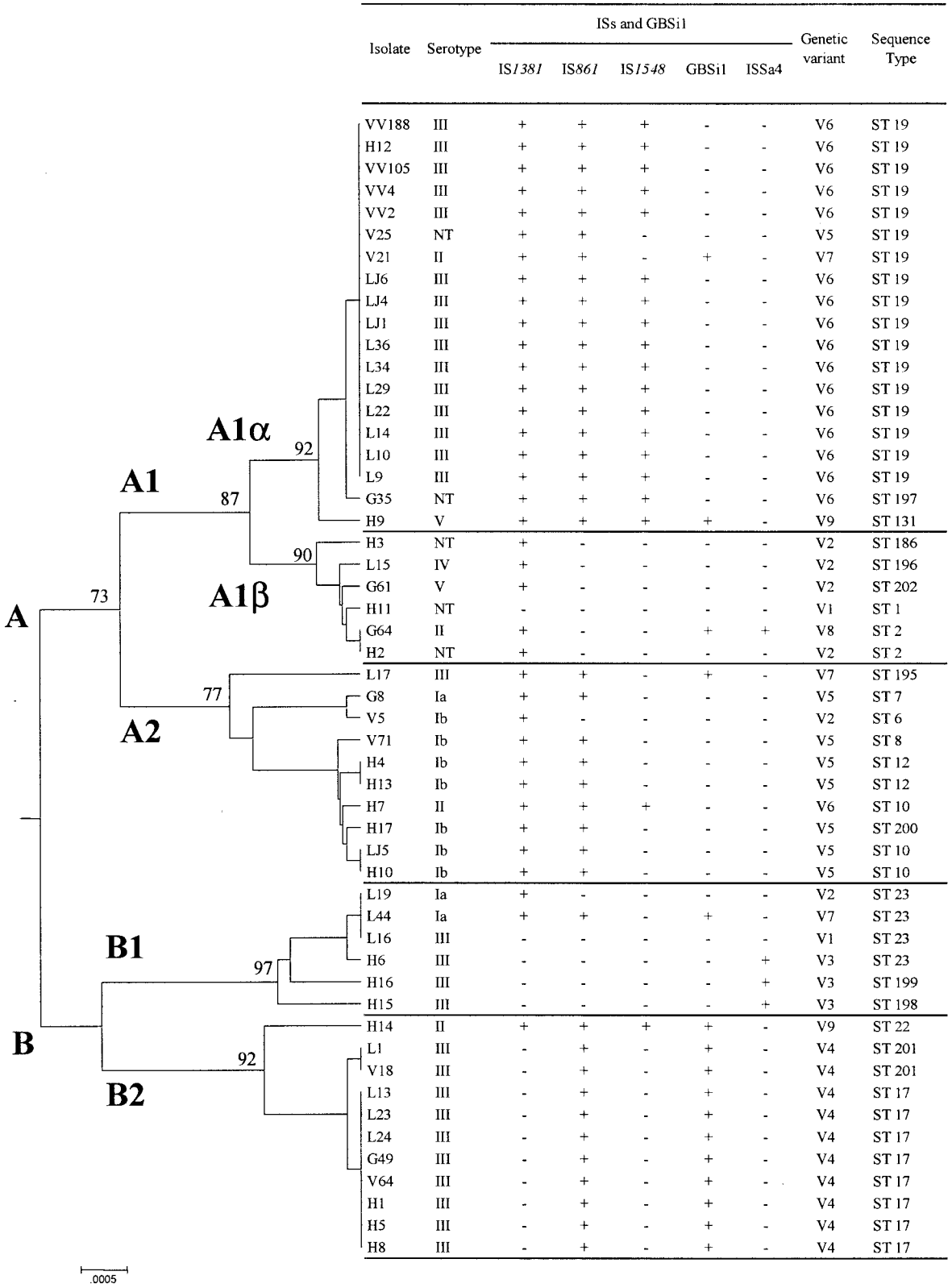
loci, was determined for each isolate. Between three (genes *pheS* and *glnA*) and eight (gene *adhP*) alleles were present for each locus (Table 2). Based on the combinations of the alleles for the seven loci, 21 STs were identified, of which 14 corresponded to a single isolate; three STs, ST 19 ($n = 17$), ST 17 ($n = 8$), and ST 23 ($n = 4$), accounted for 55.8% of the whole collection. These STs corresponded to three of the four major STs previously defined by Jones et al., confirming the worldwide spread of ST 17, ST 19, and ST 23. These results also confirm the close correlation of ST 19 and ST 17 with serotype III (11). Moreover, nucleotide sequences of the seven genes were concatenated into a single "supergene" (3,456 bp), which allows the construction of a phylogenetic tree by use of the MEGA software version 2.1 (http://www.megasoftware.net) with the UPGMA algorithm and the Kimura two-parameter mutation model of genetic distance. The level of statistical support for the nodes on the tree was evaluated by examining their percentage of recovery in 1,000 resampled trees by use of the bootstrap test (23). The MEGA software grouped the isolates into two main genomic divisions: division A, which could be subdivided into A1α, A1β, and A2, and division B, which could be subdivided into B1 and B2 (Fig. 1).

A strong correlation was observed between the distribution of the ISs and GBSi1 and the divisions obtained by MLST (Fig. 1). IS*1381* was significantly associated with division A (34 of the 37 isolates [92%] harboring IS*1381* were distributed in

TABLE 2. STs assigned on the basis of allelic profiles[a]

ST	Allelic profile	No. (%) of:			Total no. (%) of isolates ($n = 52$)	Serotype (no. of isolates)[d]
		CSF[b] isolates ($n = 20$)	Endocarditis isolates ($n = 17$)	Colonizing isolates[c] ($n = 15$)		
19	1, 1, 3, 2, 2, 2, 2	10 (50)	1 (5.9)	6 (40)	17 (32.7)	III (15), NT (1), II (1)
17	2, 1, 1, 2, 1, 1, 1	3 (15)	3 (17.7)	2 (13.3)	8 (15.4)	III (8)
23	5, 4, 6, 3, 2, 1, 3	3 (15)	1 (5.9)	0 (0)	4 (7.7)	Ia (2), III (2)
10	9, 1, 4, 1, 3, 3, 2	1 (5)	2 (11.8)	0 (0)	3 (5.81)	Ib (2), II (1)
12	10, 1, 4, 1, 3, 3, 2	0 (0)	2 (11.8)	0 (0)	2 (3.85)	Ib (2)
2	1, 1, 3, 1, 1, 2, 2	0 (0)	1 (5.9)	1 (6.6)	2 (3.85)	II (1), NT (1)
201	2, 1, 25, 2, 1, 1, 1	1 (5)	0 (0)	1 (6.6)	2 (3.85)	III (2)
1	1, 1, 2, 1, 1, 2, 2	0 (0)	1 (5.9)	0 (0)	1 (1.92)	NT (1)
6	9, 1, 2, 1, 3, 2, 2	0 (0)	0 (0)	1 (6.6)	1 (1.92)	Ib (1)
7	10, 1, 2, 1, 3, 2, 2	0 (0)	0 (0)	1 (6.6)	1 (1.92)	Ia (1)
8	4, 1, 4, 1, 3, 3, 2	0 (0)	0 (0)	1 (6.6)	1 (1.92)	Ib (1)
22	13, 3, 1, 3, 1, 1, 1	0 (0)	1 (5.9)	0 (0)	1 (1.92)	II (1)
131	1, 1, 3, 2, 2, 3, 2	0 (0)	1 (5.9)	0 (0)	1 (1.92)	V (1)
186	1, 1, 2, 1, 1, 2, 5	0 (0)	1 (5.9)	0 (0)	1 (1.92)	NT (1)
195	9, 1, 1, 1, 3, 2, 1	1 (5)	0 (0)	0 (0)	1 (1.92)	III (1)
196	1, 1, 3, 1, 1, 12, 2	1 (5)	0 (0)	0 (0)	1 (1.92)	IV (1)
197	1, 1, 3, 1, 2, 2, 2	0 (0)	0 (0)	1 (6.6)	1 (1.92)	NT (1)
198	13, 4, 6, 3, 2, 1, 3	0 (0)	1 (5.9)	0 (0)	1 (1.92)	III (1)
199	5, 4, 6, 3, 2, 1, 2	0 (0)	1 (5.9)	0 (0)	1 (1.92)	III (1)
200	9, 1, 4, 1, 3, 3, 5	0 (0)	1 (5.9)	0 (0)	1 (1.92)	Ib (1)
202	40, 1, 3, 1, 1, 2, 2	0 (0)	0 (0)	1 (6.6)	1 (1.92)	V (1)

[a] The allelic profiles, available on the MLST database website (http://pubmlst.org/sagalactiae/), were obtained by combination of each of the seven alleles, in the following order: *adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK*, and *tkt*. The ecological origins and serotypes of the *S. agalactiae* isolates are also indicated for each ST.
[b] CSF, cerebrospinal fluid.
[c] Isolated from vaginas of asymptomatic women and from the gastric fluids of neonates.
[d] NT, nontypeable.



Isolate	Serotype	ISs and GBSi1					Genetic variant	Sequence Type
		IS*1381*	IS*861*	IS*1548*	GBSi1	ISSa4		
VV188	III	+	+	+	-	-	V6	ST 19
H12	III	+	+	+	-	-	V6	ST 19
VV105	III	+	+	+	-	-	V6	ST 19
VV4	III	+	+	+	-	-	V6	ST 19
VV2	III	+	+	+	-	-	V6	ST 19
V25	NT	+	+	-	-	-	V5	ST 19
V21	II	+	+	-	+	-	V7	ST 19
LJ6	III	+	+	+	-	-	V6	ST 19
LJ4	III	+	+	+	-	-	V6	ST 19
LJ1	III	+	+	+	-	-	V6	ST 19
L36	III	+	+	+	-	-	V6	ST 19
L34	III	+	+	+	-	-	V6	ST 19
L29	III	+	+	+	-	-	V6	ST 19
L22	III	+	+	+	-	-	V6	ST 19
L14	III	+	+	+	-	-	V6	ST 19
L10	III	+	+	+	-	-	V6	ST 19
L9	III	+	+	+	-	-	V6	ST 19
G35	NT	+	+	+	-	-	V6	ST 197
H9	V	+	+	+	+	-	V9	ST 131
H3	NT	+	-	-	-	-	V2	ST 186
L15	IV	+	-	-	-	-	V2	ST 196
G61	V	+	-	-	-	-	V2	ST 202
H11	NT	-	-	-	-	-	V1	ST 1
G64	II	+	-	-	+	+	V8	ST 2
H2	NT	+	-	-	-	-	V2	ST 2
L17	III	+	+	-	+	-	V7	ST 195
G8	Ia	+	+	-	-	-	V5	ST 7
V5	Ib	+	-	-	-	-	V2	ST 6
V71	Ib	+	+	-	-	-	V5	ST 8
H4	Ib	+	+	-	-	-	V5	ST 12
H13	Ib	+	+	-	-	-	V5	ST 12
H7	II	+	+	+	-	-	V6	ST 10
H17	Ib	+	+	-	-	-	V5	ST 200
LJ5	Ib	+	+	-	-	-	V5	ST 10
H10	Ib	+	+	-	-	-	V5	ST 10
L19	Ia	+	-	-	-	-	V2	ST 23
L44	Ia	+	+	-	+	-	V7	ST 23
L16	III	-	-	-	-	-	V1	ST 23
H6	III	-	-	-	-	+	V3	ST 23
H16	III	-	-	-	-	+	V3	ST 199
H15	III	-	-	-	-	+	V3	ST 198
H14	II	+	+	+	+	-	V9	ST 22
L1	III	-	+	-	+	-	V4	ST 201
V18	III	-	+	-	+	-	V4	ST 201
L13	III	-	+	-	+	-	V4	ST 17
L23	III	-	+	-	+	-	V4	ST 17
L24	III	-	+	-	+	-	V4	ST 17
G49	III	-	+	-	+	-	V4	ST 17
V64	III	-	+	-	+	-	V4	ST 17
H1	III	-	+	-	+	-	V4	ST 17
H5	III	-	+	-	+	-	V4	ST 17
H8	III	-	+	-	+	-	V4	ST 17

FIG. 1. UPGMA dendrogram showing the genetic relationships between the 52 *S. agalactiae* isolates. The dendrogram was constructed with the MEGA 2.1 software by using the nucleotide sequences of the seven housekeeping genes (*pheS*, *atr*, *tkt*, *glcK*, *sdhA*, *glnA*, and *adhP*) concatenated into a single "supergene." Bootstrap values are shown at the major nodes. Isolates can be divided into two divisions (A and B) and five subdivisions (A1α, A1β, A2, B1, and B2). For each isolate, the serotype, ST, presence or absence of the four ISs and of the group II intron GBSi1, and genetic variants (deduced from the combination of the four ISs and GBSi1 and named V1 to V9) are indicated. L and LJ refer to meningitis isolates, H to endocarditis isolates, G to gastric fluid isolates, and V and VV to vaginal isolates.

division A), whereas this IS was almost completely absent from division B ($P < 0.0001$, chi-square test) (Fig. 1). IS*861* was significantly associated with subdivisions A1α, A2, and B2 (39 of the 40 isolates [98%] harboring IS*861* were distributed in subdivisions A1α, A2, and B2) ($P < 0.0001$) (Fig. 1). IS*1548* was significantly associated with subdivision A1α (17 of 19 [89%] isolates harboring IS*1548* were distributed in subdivision A1α) ($P < 0.0001$) (Fig. 1). Similarly, GBSi1 was significantly associated with subdivision B2 (11 of 16 [69%] isolates harboring GBSi1 were distributed in subdivision B2) ($P < 0.0001$); conversely, 100% of the isolates from subdivision B2 were GBSi1 positive (Fig. 1). Finally, ISSa4 was significantly associated with subdivision B1 (3 of 4 isolates harboring ISSa4 were distributed in subdivision B1) ($P < 0.001$), although the small number of isolates in B1 and the small number of ISSa4-positive isolates must be taken into account (Fig. 1). The rare exceptions we noticed in the correlation of IS distribution within MLST lineages were probably due to horizontal gene transfers.

Therefore, based on the prevalence of each IS and GBSi1 in each MLST group, an evolutionary scheme corresponding to the acquisition of these mobile genetic elements by *S. agalactiae* during its evolution can be proposed. Indeed, IS*1381* may have been acquired during the differentiation of the common ancestor (Fig. 2). IS*1548*, GBSi1, and ISSa4 seem to have been acquired more recently by subdivisions A1α, B2, and B1, respectively. The hypothesis that ISSa4 was acquired recently is consistent with the empirical observation of Spellerberg et al., in that all ISSa4-positive isolates in their study were isolated after 1996 (25). IS*861*, which is present in both divisions A and B but not in every subdivision, may have been acquired recently by subdivisions A1α, A2, and B2 (Fig. 2a). Alternatively, the earliest common ancestor may have harbored IS*861*, which was subsequently lost by subdivisions A1β and B1 (Fig. 2b).

One striking finding, already pointed out by Granlund et al. (8) and Bohnsack et al. (3), is the rarity of IS*1548*$^+$ GBSi1$^+$ isolates (only two isolates in our collection harbored both). To date, three insertion sites on the *S. agalactiae* chromosome are known for IS*1548*, named site X, site Y, and site Z (7, 14, 22). Site X corresponds to the intergenic fragment located between the *scpB* and *lmb* genes, encoding the C5a-peptidase and the laminin-binding protein, respectively. As IS*1548* and GBSi1 may share this insertion site, the simultaneous insertion of IS*1548* and GBSi1 at the same insertion site is unlikely. Indeed, in our two IS*1548*$^+$ GBSi1$^+$ isolates, IS*1548* was not found at site X or within *hylB*. The IS*1548*-free site X could therefore be explained by the presence of GBSi1 at site X, as confirmed by Southern blot analysis.

Interestingly, the evolutionary scheme presented here suggests another explanation for the mutually exclusive distribution of IS*1548* and GBSi1. In fact, subdivisions A1α (carrying IS*1548*) and B2 (carrying GBSi1) separated early during the evolutionary process (when the ancestors A and B separated) (Fig. 1 and Fig. 2). Furthermore, GBSi1 was associated with IS*1381* only once within subdivision B2 isolates (Fig. 1); similarly, ISSa4 was associated with other ISs only once (Fig. 1), in agreement with Dmitriev et al. (4). Conversely, GBSi1 and IS*1548* were always associated with IS*861*, except for one isolate (Fig. 1). Such a preferential association has already been observed in the *Escherichia coli* reference (ECOR) collection

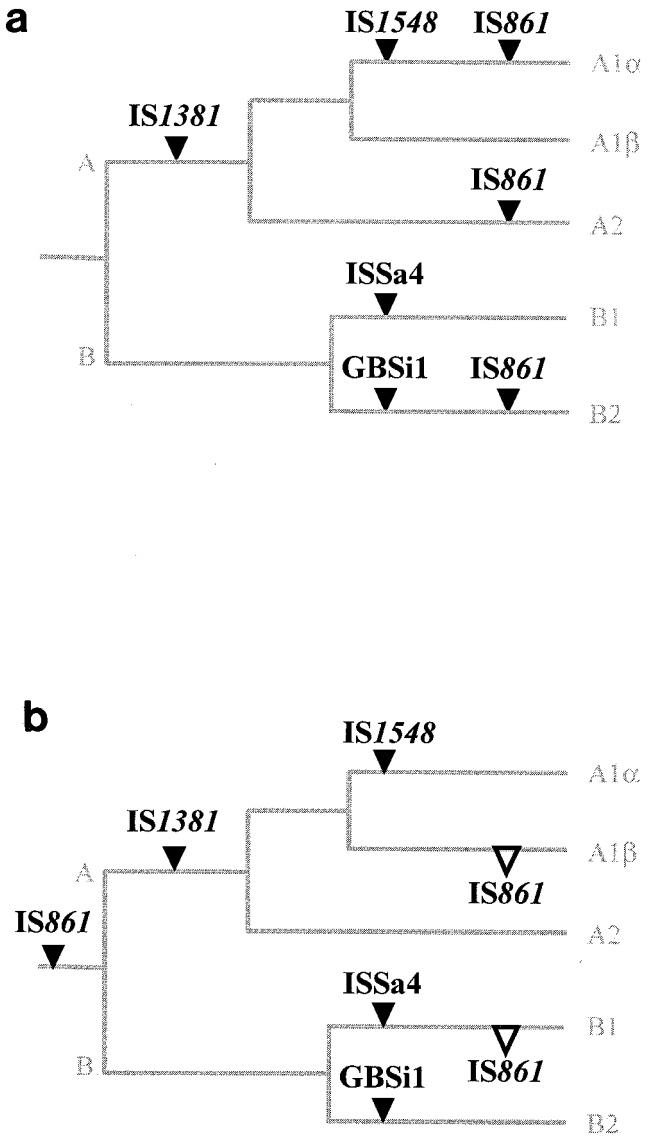


FIG. 2. Evolutionary scheme showing the chronology of acquisition of the four ISs and of the group II intron GBSi1 during *S. agalactiae* evolution. This scheme was deduced from the prevalence of each mobile genetic element within the divisions and subdivisions obtained by MLST as indicated in Fig. 1. IS*1381* may have been acquired (acquisition of ISs or GBSi1, ▼) by division A, IS*1548* by subdivision A1α, ISSa4 by subdivision B2, and GBSi1 by subdivision B2. IS*861* may have been acquired by subdivisions A1α, A2, and B2 (Fig. 2a), or it may have been acquired by the common ancestor and then lost (loss of ISs or GBSi1, ▽) by subdivisions A1β and B1 (Fig. 2b). Branch values are not representative of genetic distances.

(19). The mutually exclusive distribution of ISs, as well as the preferential association of ISs, is consistent with the delivery of ISs by a vector carrying multiple ISs. Although transmission of ISs by phages has not yet been reported for *S. agalactiae*, it has been observed for *E. coli*, *Pseudomonas aeruginosa*, and *Bacillus thuringiensis* (16). Despite the low prevalence of plasmids within the species *S. agalactiae* (10), many of the genomic islands flanked by ISs in *S. agalactiae* are associated with genes related to phages, integrative plasmids, and conjugative trans-

posons (5, 27). Therefore, the peculiar distribution of ISs (mutually exclusive mode and preferential association mode) could be explained by the link between the isolates and the ecosystems from which they were isolated. It is tempting to consider that ISs and GBSi1 reflect the original ecosystem of the isolates and that many of the IS-mediated mutations contribute to the adaptation of bacterial populations to their environment (20). This hypothesis is in accordance with a recent study reporting that the ST 17 complex, that is, our subdivision B2 characterized by GBSi (Fig. 1), was closer to bovine allelic profiles, whereas the ST 19 complex, i.e., our subdivision A1α characterized by IS*1548* (Fig. 1), was closer to allelic profiles initially described for *S. agalactiae* human isolates (2).

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