Original article

Population-level transition of capsular polysaccharide types among sequence type 1 group B *Streptococcus* isolates with reduced penicillin susceptibility during their long-term hospital epidemic

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ABSTRACT

Over a 35-month period, group B *Streptococcus* isolates with reduced penicillin susceptibility (PRGBS) were detected from elderly patients at a regional hospital, accompanying population-level transition of PRGBS serotypes. Seventy-seven non-duplicate PRGBS recovered from 73 patients were analyzed to infer their genetic relatedness. Serotype III PRGBS predominated (16 serotype III/1 serotype Ib) in the first nine months (period I), then 3 serotype Ib isolates appeared transiently for the next three months (period II), which was replaced predominantly by serotype Ia (20 serotype Ia/1 serotype III/1 nontypeable) for nine months (period III). In the last 14 months (period IV), besides 25 serotype Ia, 10 serotype III isolates were also identified. Serotypes III and Ia isolates, belonging to ST1 shared G329V, G398A, V405A, and G429D in penicillin-binding protein2X. Of three strains subjected to whole-genome sequencing, serotype III strain SU12 (period I) had the higher degrees of genomic similarity with serotype Ia strain SU97 (period III) rather than serotype Ib strain SU67 (period II) based on average nucleotide identity and single nucleotide polymorphisms analyses. Analysis of the *cps* gene clusters and the upstream and downstream flanking sequences revealed that disruption of hyaluronidase gene located upstream of *cpsY* by insertion of an IS*1548* was found in strain SU12, whereas ΔIS*Sag8* was inserted between tRNA-Arg and *rpsA* genes located downstream of *cpsL* for strain SU97. Interestingly, most of serotype III PRGBS re-emerged in phase IV had this tRNA-Arg-ΔIS*Sag8-rpsA* region. Capsular switching and nosocomial transmission may possibly contribute to population-level serotype replacement among ST1 PRGBS isolates.

Keywords: PRGBS; serotype III; serotype Ia; ST1.

1. Introduction

Group B *Streptococcus* (GBS; *Streptococcus agalactiae*) is one of the most important causes of life-threatening infections such as sepsis or meningitis in neonates [1]. GBS is also associated with invasive infections in nonpregnant adults, particularly in elderly and immunocompromised individuals [2].

GBS capsular polysaccharide is a major virulence determinant for invasive infection and a target of protective immunity. GBS are serologically classified based on capsular polysaccharide synthesis (*cps*) gene cluster into ten serotypes, Ia, Ib, II to IX, as well as a nontypeable. Most of invasive GBS diseases in nonpregnant adults in the United States, Canada, France, and Taiwan have been caused by serotype V strains, especially those belonging to ST1 [3-5]. Recent increase of serotype IV among adult invasive disease strains has been reported in North America and European countries [6-8]. High diversity of genetic lineages has been observed among serotype IV isolates including ST-452 in clonal complex (CC)23, ST-459 and ST-196 in CC1, and *hvgA*-positive ST-291 which is a single locus variant of ST17 and has been originated from a capsular switching event from serotype III to IV within CC17 [9,10]. In Japan, although there are only a few studies available, the serotypes most frequently associated with invasive infections, mainly sepsis in adults has been serotype Ib, followed by serotype V [11]. Very recent studies have revealed that serotype Ib, ST10 is predominant among GBS strains from invasive infections including streptococcal toxic shock-like syndrome cases in adults [12,13].

Our study group has accumulated epidemiologic and molecular analysis findings on GBS isolates with reduced susceptibility to penicillin (PRGBS), which is the first-line antibiotic for GBS disease therapy and for intrapartum chemoprophylaxis. At least two key amino acid substitutions in penicillin-binding protein

(PBP)2X have been demonstrated to contribute to a considerable reduction in β-lactam susceptibility [14]. Besides these key substitutions, other amino acid substitutions have also been found in PBP2X, PBP2B, and PBP1A among PRGBS isolates, depending on their minimum inhibitory concentration (MIC) levels for β-lactams [14-16]. Those PRGBS from Japan has most commonly been isolated from respiratory specimens from elderly patients, and serotype VI and ST1 or ST458 (a single-locus variant of ST1 within CC1) were frequently associated with them [16,17]. PRGBS has been capable of surviving persistently at the site of infection for >three weeks, and nosocomial spread of multidrug-resistant PRGBS with serotype VI and ST458 has also been reported [15,18].

During a 35-month period, PRGBS isolates have been frequently detected from elderly patients, accompanying transition of their serotypes, mainly from serotype III in the previous period to serotype Ia in the latter period at a small-scale regional hospital in Japan. The present study examines such unique serotype transition phenomena among those PRGBS isolates.

2. Materials and methods

2.1. Bacterial isolates

A total of 77 non-duplicate PRGBS clinical isolates (penicillin MIC, ≥0.25 mg/L) recovered from 66 transtracheal aspirates (TTA), four blood cultures, three catheter urine cultures, two intravenous catheter tip cultures, and two nasal cavity cultures were collected from 73 adult patients in a 299-bed regional general hospital in Japan from April 2011 to February 2014. Those 77 isolates included two isolates each obtained from different specimens of two patients, two isolates showing different antimicrobial

susceptibility profiles of the same patient, and two isolates showing different serotypes recovered from the same specimens obtained on different days of the same patient. The 73 adult patients across eight wards (including 30 and 27 patients from two main internal medicine wards) comprised of 47 males (64.4%) and 26 females (35.6%), with the average age of 83.0 years (SD 9.0). During study period, a total of seven non-duplicate penicillin-susceptible GBS with reduced cephalosporin susceptibility (CTB^{r}PSGBS) clinical isolates recovered from five TTA, and one each of urine culture and pus culture were also collected from seven adult patients (6 males and a female at an average age of 77.9 ± 11.7 years) [19]. Identification of the GBS isolates were performed by the latex agglutination method using Seroiden Strepto Kit (Eiken Chemical, Tokyo, Japan). Those GBS isolates were grown overnight in Todd-Hewitt broth and then stored in glycerol at -80°C until use.

2.2. Serotyping assays

Capsular serotyping was performed using antisera (Denka Seiken, Tokyo, Japan) for the type-specific capsular polysaccharides, Ia, Ib, and II to VIII. Molecular capsular typing by multiplex polymerase chain reaction (PCR) was also performed as described previously [20].

Based on the shifts in predominant serotypes, 35-month isolation period (April 2011 to February 2014) was divided into four periods; periods I (April to December 2011), II (January to March 2012), III (April to December 2012), and IV (January 2013 to February 2014).

2.3. Antimicrobial susceptibility testing

MICs were determined by a broth microdilution method with a MicroScan MICroFAST panel type 5J (Beckman Coulter) and were interpreted by the CLSI guidelines [21,22]. MIC determinations were repeated independently three times for each strain to ensure the reproducibility of the MICs by using quality control strain *Streptococcus pneumoniae* ATCC 49619. Susceptibility testing with a ceftibuten disk was also performed by Kirby-Bauer's disk-diffusion method [23].

2.4. Amplification and sequencing of pbp genes

Analysis of *pbp* genes was performed as previously described [16,18]. In brief, the full-length of *pbp* genes were amplified from genomic DNAs using PCR primers specific for *pbp2x*, *pbp2b*, and *pbp1a*, and PrimeSTAR HS DNA polymerase (Takara Bio Inc., Shiga, Japan). The amplified DNA fragments were purified with a Wizard SV Gel and PCR cleanup system (Promega, Madison, WI). Sequencing analyses on both strands of purified DNA fragments were performed using consecutive primers, a BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA), and an ABI 3730x/DNA analyzer (Applied Biosystems).

The nucleotide sequences obtained were assembled into a consensus sequence, which were aligned with the published sequence of the corresponding genes of strains 2603V/R (ATCC BAA-611; GenBank accession number NC 004116) using BioEdit (version 5.0.9) software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

2.5. Multilocus sequence typing (MLST)

MLST was performed by sequencing seven housekeeping genes, *adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK* and *tkt* amplified by PCR as described previously [18,24]. Allelic profile assignment and ST determinations were made using the GBS MLST databases (http://pubmlst.org/sagalactiae).

2.6. Genome sequencing

Whole genome sequencing was performed on serotype III PRGBS strain SU12 from TTA (period I) and serotype Ia PRGBS strain SU97 from blood (period III) that were isolated one year apart from two different patients, and serotype Ib CTB[r]PSGBS strain SU66 from TTA and serotype Ib PRGBS strain SU67 from TTA (period II) that were isolated four days apart from same patient (Fig. 1A). Genomic DNA was purified using a Wizard genomic DNA purification kit (Promega), which was subjected to sequencing on an Illumina HiSeq 2500 system (Illumina, San Diego, CA, USA) generating 100 bp paired-end reads. Approximately 12 million of Illumina reads were processed and merged into contigs by using the *de novo* assembly pipeline A5-miseq [25]. Thus obtained contigs were mapped to the *S. agalactiae* NEM316 reference genome (ATCC 12403; GenBank accession number NC 004368) by using CONTIGuator2 (http://contiguator.sourceforge.net/) to create pseudocontig sequence, which was annotated by RAST (Rapid Annotation using Subsystem Technology) v2.0 [26]. For comparative analysis of *cps* gene clusters and their flanking region, EasyFig v2.2.2 were used [27].

Average nucleotide identity based on MUMmer calculation (ANIm) of paired genomes was calculated among SU12, SU66, SU67, SU97, and serotype V ST1 *S. agalactiae* strain SS1 (GenBank accession number CP010867, reference 7), serotype III ST23 *S. agalactiae* NEM316, and serotype Ia ST7

S. agalactiae A909 (GenBank accession number NC 007432) using JSpeciesWS with the default parameters (http://jspecies.ribohost.com/jspeciesws/) to assess nucleotide-level genomic similarity [28]. Phylogenetic heatmap based on pairwise comparison matrices was constructed using R (v3.5.0). SNP-based phylogeny among the above described seven strains was analyzed by using CSI Phylogeny 1.4 with default parameters (https://cge.cbs.dtu.dk/services/CSIPhylogeny/) [29]. Strain SU12 genome was used as reference sequence, and the phylogenetic tree was visualized by using FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

2.7. Analysis of insertion sequence elements

For PRGBS and CTB^{r}PSGBS clinical isolates, forward (ISSag8F, 5'-TCGAACCCCTATCCCAAGAAC-3') and reverse (ISSag8R, 5'-CGCAAGCAGAAGGTGAAAAAG-3') primers were used for determining whether or not ΔIS*Sag8* was inserted between tRNA-Arg and *rpsA* genes (1827 bp vs. 269 bp). To determine whether or not IS*1548* was inserted in hyaluronidase gene *hylB*, forward (IS1548F, 5'-ACCGCTACTTATCGTCGTTTGG-3') and reverse (IS1548R, 5'-AGCCAAGCCAGACTCTCTTT-3') primers were used (1439 bp vs. 113 bp). Insertion of ΔIS*Sag8* or IS*1548* was confirmed by sequencing all PCR products.

2.8. Nucleotide sequence accession numbers

The GenBank accession numbers for the *cps* gene clusters and their flanking region of strains SU12 and

161 SU97 in this study are LC341249 and LC341250, respectively.

162

163 **3. Results**

164 *3.1. Antimicrobial susceptibility in PRGBS isolates*

165 The MIC range, MIC values at which 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates were inhibited, and

166 percentages of susceptibility of 77 PRGBS isolates against antimicrobial agents tested are listed in Table 1.

167 The isolates had penicillin MIC_{50} of 0.25 mg/L, MIC_{90} of 0.5 mg/L, and an MIC range of 0.25 to 0.5 mg/L,

168 which is above the CLSI's susceptible MIC of ≤0.12 mg/L. The rate of nonsusceptibility of PRGBS to

169 cefotaxime and cefepime was 97% and 100%, respectively, whereas 49% of the isolates were

170 nonsusceptible to ampicillin. Though they were susceptible to meropenem, an increasing trend of MIC

171 values, 0.25 to 0.5 mg/L within the susceptibility range was observed. The rates of erythromycin,

172 clindamycin, and levofloxacin resistance were 74%, 23%, and 45%, respectively. All PRGBS and

173 CTB^{r}PSGBS isolates exhibited no growth inhibition zones around the ceftibuten disks. Those

174 CTB^{r}PSGBS isolates were susceptible to penicillin (MIC 0.06 to 0.12 mg/L) [19].

175

176 *3.2. Capsular type shift among PRGBS isolates*

177 In 74 out of 77 isolates, serotyping results were concordant with molecular serotyping results; 26 serotype

178 III, three serotype Ib, and 45 serotype Ia. Of the remaining three non-serotypeable isolates, two were

179 assigned to serotype III and Ia each by genotyping, but one remained nontypeable. A shift in the serotypes

180 was observed within those isolates collected over a 35-month period as shown in Fig. 1A. Namely,

serotype III PRGBS population predominated (16 serotype III/one serotype Ib) in the first nine months (period I), then three serotype Ib isolates appeared transiently for the next three months (period II), which was replaced predominantly by serotype Ia polulation (20 serotype Ia/one serotype III/one nontypeable) for nine months (period III). In the last 14 months, in addition to 25 serotype Ia, 10 serotype III PRGBS isolates were also identified (period IV). Among those PRGBS, two isolates recovered from TTAs of the same patient in May 2011 and May 2012 showed different serotypes, III and Ia, respectively. All of four PRGBS isolates derived from blood samples belonged to serotype Ia, and consequent poor prognosis was observed in patients. Serotype Ib CTB^{r}PSGBS was also recognized in six isolates in periods I and II as reported previously by us and in one isolate in period IV as shown in Fig. 1A [19].

3.3. Amino acid substitutions in PBPs2X, 2B, and 1A, and MLST

For analysis of amino acid sbstitutions in PBPs2X, 2B and 1A, and MLST, 25 PRGBS isolates were arbitrarily chosen depending upon their serotypes and dates of their isolation; 12 (11 serotype III and one serotype Ib) from period I, one (serotype Ib) from period II, five (serotype Ia) from period III, and seven (four serotype III and three serotype Ia) from period IV. Irrespective of periods defined, PRGBS of serotypes Ia and III shared several amino acid substitutions in PBPs (Table 2). The PBP2X amino acid substitutions included a key substitution, V405A, and three additional substitutions, G329V, G398A, and G429D. In PBP1A, one amino acid substitution, T587I was shared by serotype Ia PRGBS in period III and IV and serotype III PRGBS in period I. However, those amino acid substitutions were not detected in either serotype Ib PRGBS or serotype Ib CTB^{r}PSGBS, except for V405A in PBP2X of Ib PRGBS [19].

MLST analysis revealed that all of 25 PRGBS isolates were assigned to ST1, a founder of CC1 irrespective of their capsular types (Table 2). Serotype Ib CTB^{r}PSGBS isolates also belonged to ST1 [19].

3.4. Analyses of genetic relatedness and cps gene cluster regions among PRGBS isolates

The assembled pseudocontig sequences of four strains, serotype III PRGBS strain SU12, serotype Ia PRGBS strain SU97, serotype Ib PRGBS strain SU66 and serotype Ib CTB^{r}PSGBS strain SU67 showed approximately 2.0 - 2.2 Mb in length. Sequence similarity searching using BLAST revealed that SU12 shared 99% sequence identity with SU97 (93% query coverage), and with SU66, SU67, and SS1 (90% query coverage). SU12 showed the highest ANIm values, 99.84% with SU97, followed by 99.68, 99.67 and 99.63% with SU66, SS1 and SU67, respectively. ANIm values showed 99.96% nucleotide similarity between strains SU66 and SU67. Phylogenetic tree constructed based on ANIm analysis indicated that serotype III strain SU12 and serotype Ia strain SU97 clustered together were more closely related to serotype V strain SS1 rather than serotype Ib strains SU66 and SU67 clustered together (Fig. 2). SNP-based phylogenetic approach showed that strains SU12 and SU97, differing from each other by 957 SNPs were clustered together, whereas there were 46 SNP differences between strains SU66 and SU67 forming another cluster (Fig. 3). The strain SS1 belonging to ST1 and A909 (ST7) clustered together.

In-depth analysis results of the *cps* gene clusters and the upstream and downstream flanking sequences (approximately 55-kb region) are shown in Fig. 4. The 12.7-kb *cps* gene clusters of strains SU12 and SU97 exhibited overall 99.3% nucleotide identity, except that serotype-specific *cpsG* and *cpsH* genes had 70.5% and 28.9% nucleotide identities, respectively. An insertion sequence ΔIS*Sag8*, which was bracketed

by a 20-bp repeat sequence, "AGACTTGCTTTAGCAAGTCT" was located 9.7 kb downstream of *neuA* gene (between tRNA-Arg and *rpsA* genes) for strain SU97, whereas disruption of hyaluronidase gene *hylB* located 21 kb upstream of *cpsY* by insertion of an IS*1548* was found in strain SU12. Except for these differences, strains SU12 and SU97 shared 99.9% and 93.6% sequence identities at the region 16.0 kb upstream and 26.1 kb downstream from the *cps* gene clusters, respectively (Fig. 4A). The 55-kb *cps* gene clusters and their flanking region of strains SU66 and SU67 exhibited overall 99.8% sequence identity, where insertion of either ΔIS*Sag8* between tRNA-Arg and *rpsA* genes or IS*1548* into *hylB* structural genes were not detected (Fig. 4B). Strains SU12, SU97, SU66, and SU67 shared major virulence factors including fibrinogen-binding protein A (*fbsA*), fibrinogen-binding protein B (*fbsB*), fibronection-binding protein (*pavA*), C5a peptidase (*scpB*), laminin-binding protein (*lmb*), CAMP factor (*cfb*), hemolysin III (*spb1*), serine protease CspA (*cspA*), but did not have C-β protein (*bac*). For genes of the alpha-like protein (Alp) family, they had C-α protein (*bca*) but did not have Rib (*rib*), alpha-like protein 1 (also called epsilon; *alp1*), alpha-like protein 2 (*alp2*), alpha-like protein 3 (*alp3*), and alpha-like protein 4 (*alp4*).

3.5. Prevalence of ΔISSag8 or IS1548 insertion

As shown in Fig. 1B, the PCR and sequencing revealed that no ΔIS*Sag8* insertion combined with IS*1548* insertion was confirmed in all of 16 serotype III isolates in period I. Whereas, ΔIS*Sag8* insertion combined with no IS*1548* insertion was confirmed in 18 of 20 serotype Ia isolates in period III. The ΔIS*Sag8* insertion combined with no IS*1548* insertion was also confirmed among 23 of 27 serotype Ia

241 isolates, and notably, among nine of 10 serotype III isolates in period IV. Four serotype Ib PRGBS and
242 seven CTB[r]PSGBS isolates resulted in no insertion of both ΔIS*Sag8* and IS*1548*.

243 **4. Discussion**

244 The mortality rates have varied from three to over 30% for adult GBS invasive infection, where
245 relative higher rates are noted in elderly patients with underlying diseases [30]. Particularly, fatal outcome
246 has significantly associated with adult patients with bacteremia [31]. Thus, the development of multidrug
247 resistance including β-lactams in the GBS isolates is of clinical concern. The Japan Nosocomial Infections
248 Surveillance (JANIS) data of the Ministry of Health, Labour and Welfare (http://www.nih-janis.jp/>) show
249 a clear trend towards the increasing occurrence of penicillin nonsusceptibility among GBS isolates from
250 various clinical sources, from 4.8% (472/9,738 isolates) in 2008 to 5.7% (2,022/34,988 isolates) in 2016,
251 which is extremely higher than the rate of 0.7% (13/1975 isolates) in 2015 from CDC's Active Bacterial
252 Core surveillance [32]. Furthermore, PRGBS isolates in Japan have been characterized to be commonly
253 associated with serotype VI and CC1 (ST1 or ST458), though serotype V is predominant among ST1
254 GBS isolates in many other countries. Of note, serotypes of PRGBS isolates in this study were serotypes
255 III, Ia, and Ib which have been very rarely reported among ST1 GBS [24].

256 In the present study, dynamic change of capsular polysaccharide types mainly from serotype III to Ia
257 was confirmed in ST1 PRGBS populations which shared amino acid substitutions in PBPs in a
258 medium-sized hospital. The serotype III strain SU12 showed the highest ANIm value of 99.84% with
259 serotype Ia strain SU97, and SU12 and SU97 formed a distinct cluster from SNP-based phylogenetic
260 analysis, confirming the close genetic relatedness of these two strains sharing virulence factors. Strains

261 SU12 and SU97 were characterized by insertion of IS*1548* within *hylB* structural gene upstream of the
262 *cpsY* gene, and ΔIS*Sag8* downstream of the *neuA* gene, respectively. The disruption of *hylB* by IS*1548*
263 insertion identified in SU12 was also found among other 15 serotype III PRGBS isolates detected in
264 period I. On the other hand, the tRNA-Arg-ΔIS*Sag8-rpsA* region identified in SU97 was also found
265 among other 17 serotype Ia PRGBS detected in period III. Thus, nosocomial transmission may likely
266 contribute to the spread of serotype III PRGBS in period I and serotype Ia PRGBS in period III.

267 The *hylB* gene disrupted by IS*1548* insertion has been described in hyaluronidase-negative serotype III
268 GBS strains derived from endocarditis and septicaemia cases in adults [33]. Since the hyaluronidase, as
269 one of virulence factors produced by GBS plays an important role for evading the immune system by
270 degrading host-generated immunostimulatory hyaluronic acid fragments [34], restoring the original intact
271 *hylB* gene could become beneficial for the pathogenicity of serotype Ia PRGBS in period III. Indeed,
272 PRGBS recovered from all of four patients with poor prognosis in periods III and IV were serotype Ia
273 isolates with intact *hylB* genes. The IS*Sag8*, which is peculiar to serotype Ia is found in five copies in
274 published genome sequence of serotype Ia *S. agalactiae* A909, but is not found in that of serotype III *S.*
275 *agalactiae* NEM316. Any of those five copies of IS*Sag8* in *S. agalactiae* A909 is not located between
276 tRNA-Arg and *rpsA* genes. This tRNA-Arg-ΔIS*Sag8-rpsA* region has 99% (4750/4776 bp) sequence
277 identity to only a bovine milk isolate SA111 in France (GenBank accession number LT545678), so it may
278 be a unique region within serotype Ia PRGBS populations in periods III and IV detected in this hospital.
279 Interestingly, most (nine/10 isolates) of serotype III PRGBS re-emerged in phase IV had this
280 tRNA-Arg-ΔIS*Sag8-rpsA* region.

Population-level change in the serotype prevalence among ST1 PRGBS isolates may suggest the contribution of capsular switch event, which have so far been reported in ST1 GBS [35] and nosocomial transmission of such capsule-switched isolates. The possibility of replacement of one nosocomial PRGBS by another PRGBS of different serotype cannot be excluded. However, at the whole-genome level, the relationship between serotype III strain SU12 and serotype Ia strain SU97 showed greater genetic similarity than the relationship between serotype III strain NEM316 and serotype Ia strain A909, so capsular switching is more likely to be involved in this phenomenon. The driving force for capsular switching may be derived from bacterial stress generated by antibiotics, and/or from immune pressure by anti-capsule antibody [36,37]. Consequently, escape from immunity to the serotype III as well as advantageous characteristics such as the reversion back to normal *hylB* gene, and reduced susceptibility to β-lactams may allow serotype Ia PRGBS to achieve its successful survive and spread for long term. PRGBS has commonly been associated with multidrug resistance [18,38]. Therefore, capsular switching in PRGBS is a potential medical concern because allowing it to evade naturally occurring immunity and to resist to antimicrobial therapy, thus may cause more serious and life-threatening infections in the elderly, and immunocompromised populations.

The CTB^{r}PSGBS isolates including strain SU66, a unique serotype Ib PSGBS lineage in that they showed reduced susceptibility to several cephalosporins such as ceftibuten was detected in periods I, II, ad IV, and serotype Ib PRGBS including strain SU67 was detected in period II. Genome sequencing analysis revealed the high degree of genetic similarity between strains SU66 and SU67.

This study highlights the long-term epidemic of ST1 PRGBS among elderly patient populations.

Continuous monitoring and genetic characterization of those isolates enabled us to find the population-level serotype transition that may have been associated with capsular switching events and nosocomial transmission. The limitation of the present study is the impossibility of determining the concentration of anti-capsule antibody in the serum of patients. Nonetheless, this study including fatal outcome in four elderly patients with bacteremia provides important findings for vaccine development strategies.

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421 **Figure legends**

422 **Fig. 1.** Seventy-seven isolates of group B *Streptococcus* with reduced susceptibility to penicillin detected
423 in a regional general hospital. (a) Transition of serotypes during Periods I to IV. (b) The presence or
424 absence of ΔIS*Sag8* between tRNA-Arg and *rpsA* genes or IS*1548* in hyaluronidase gene *hylB* for each
425 isolate.

426

427 **Fig. 2.** Genetic relatedness of group B *Streptococcus* with reduced susceptibility to penicillin (PRGBS)
428 strains based on average nucleotide identity (ANI). A heatmap and a dendrogram was generated using the
429 distance matrix of ANI divergence values based on MUMmer calculation (ANIm) between each pair of
430 genomes among serotype III PRGBS strain SU12 (IIIPRGBS_SU12), serotype Ia PRGBS strain SU97
431 (IaPRGBS_SU97), serotype Ib penicillin-susceptible GBS with reduced cephalosporin susceptibility
432 (CTB^{r}PSGBS) strain SU66 (IbCTB^{r}PSGBS_SU66), serotype Ib PRGBS strain SU67 (IbPRGBS_SU67),
433 serotype V GBS strain SS1 (VGBS_SS1), serotype III GBS strain NEM316 (IIIGBS_NEM316), and
434 serotype Ia GBS strain A909 (IaGBS_A909).

435

436 **Fig. 3.** Genetic relatedness of group B *Streptococcus* with reduced susceptibility to penicillin (PRGBS)
437 strains based on single nucleotide polymorphism (SNP) differences. A phylogenetic tree was constructed
438 using the distance matrix of SNP differences between each pair of genomes among serotype III PRGBS
439 strain SU12 (IIIPRGBS_SU12), serotype Ia PRGBS strain SU97 (IaPRGBS_SU97), serotype Ib
440 penicillin-susceptible GBS with reduced cephalosporin susceptibility (CTB^{r}PSGBS) strain SU66

441 (IbCTB^{r}PSGBS_SU66), serotype Ib PRGBS strain SU67 (IbPRGBS_SU67), serotype V GBS strain SS1
442 (VGBS_SS1), serotype III GBS strain NEM316 (IIIGBS_NEM316), and serotype Ia GBS strain A909
443 (IaGBS_A909).
444
445 **Fig. 4.** Alignment of approximately 55-kp region including *cps* gene clusters and the upstream and
446 downstream flanking sequences using Easyfig v2.2.2. (a) Comparisons between serotype III group B
447 *Streptococcus* with reduced susceptibility to penicillin (PRGBS) strain SU12 and serotype Ia PRGBS
448 strain SU97. (b) Comparisons between serotype Ib penicillin-susceptible GBS with reduced cephalosporin
449 susceptibility (CTB^{r}PSGBS) strain SU66 and serotype Ib PRGBS strain SU67. Genes are indicated by
450 arrows, and the level of sequence similarity is shown in the gradient scale.
451

Table 1. MIC distributions of antimicrobial agents for 77 clinical isolates of group B streptococci with reduced penicillin susceptibility

Antimicrobials	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	%S/%R or nonsusceptible
Penicillin	0.25 - 0.5	0.25	0.5	0/100
Ampicillin	0.25 - 0.5	0.25	0.5	51/49
Cefotiam	4 - >4	4	>4	–[a]
Cefotaxime	0.5 - 1	1	1	3/97
Ceftriaxone	0.25 - 1	0.5	1	58/42
Cefditoren	0.25 - 0.5	0.5	0.5	–[a]
Cefepime	1 - >2	2	2	0/100
Cefixime	1 - >1	>1	>1	–[a]
Meropenem	0.25 - 0.5	0.25	0.5	100/0
Erythromycin	≤0.12 - >1	>1	>1	26/74
Clarithromycin	≤0.12 - >1	1	>1	26/74
Clindamycin	≤0.12 - >1	≤0.12	>1	77/23
Levofloxacin	1 - >8	2	>8	55/45
Vancomycin	0.5 - 1	0.5	0.5	100/0

MIC, minimum inhibitory concentration; MIC_{50}, MIC values at which 50% of the isolates are inhibited; MIC_{90}, MIC values at which 90% of the isolates are inhibited.

[a] –, no breakpoint available

Table 2. PBP amino acid substitutions in PRGBS and CTB^{r}PSGBS clinical isolates

Category	Capsular type	n	MLST sequence type	Amino acid substitutions in PBPs								
				PBP2X							PBP2B	PBP1A
PRGBS	Ia	8 (period III & IV)	1	G329V		G398A		V405A	G429D		ND	T587I
	III	11 (period I)	1	G329V		G398A		V405A	G429D		H633Y (7) or ND (4)	T587I
		4 (period IV)	1	G329V		G398A		V405A	G429D		ND	P385L
	Ib[a]	2 (periods I & II)	1				A400V	V405A		Q557E	T567I	ND
CTB^{r}PSGBS	Ib[a]	3 (periods I & II)	1		T394A				G429S		T567I	ND
		3 (periods I & II)	1		T394A						T567I	T145A

PBP, penicillin-binding protein; PRGBS, group B streptococci with reduced penicillin susceptibility; CTB^{r}PSGBS, penicillin-susceptible group B streptococci with reduced cephalosporin susceptibility.

[a] previously described by Nagano et al. [19]

Figure 1

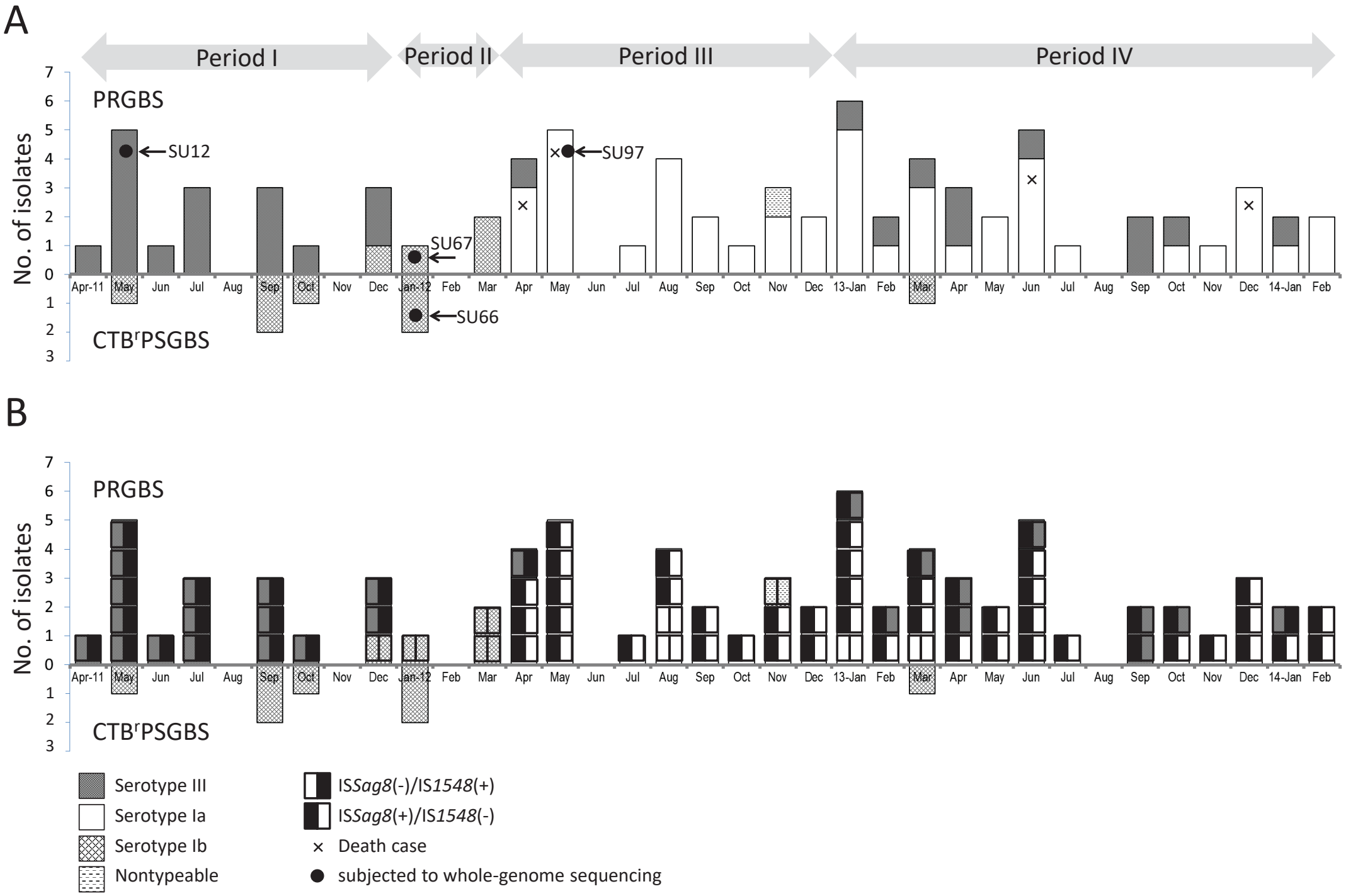
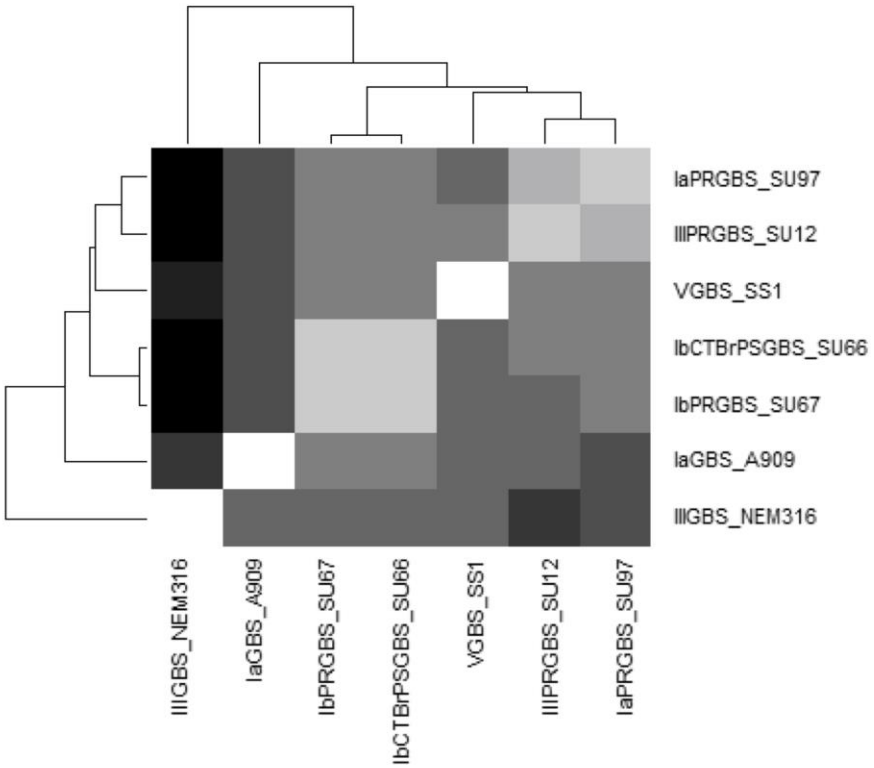

A
Period I
Period II
Period III
Period IV
PRGBS
CTB^rPSGBS
No. of isolates
SU12
SU67
SU66
SU97
Apr-11
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan-12
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
13-Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
14-Jan
Feb
B
PRGBS
CTB^rPSGBS
No. of isolates
Serotype III
Serotype Ia
Serotype Ib
Nontypeable
ISSag8(-)/IS1548(+)
ISSag8(+)/IS1548(-)
Death case
subjected to whole-genome sequencing

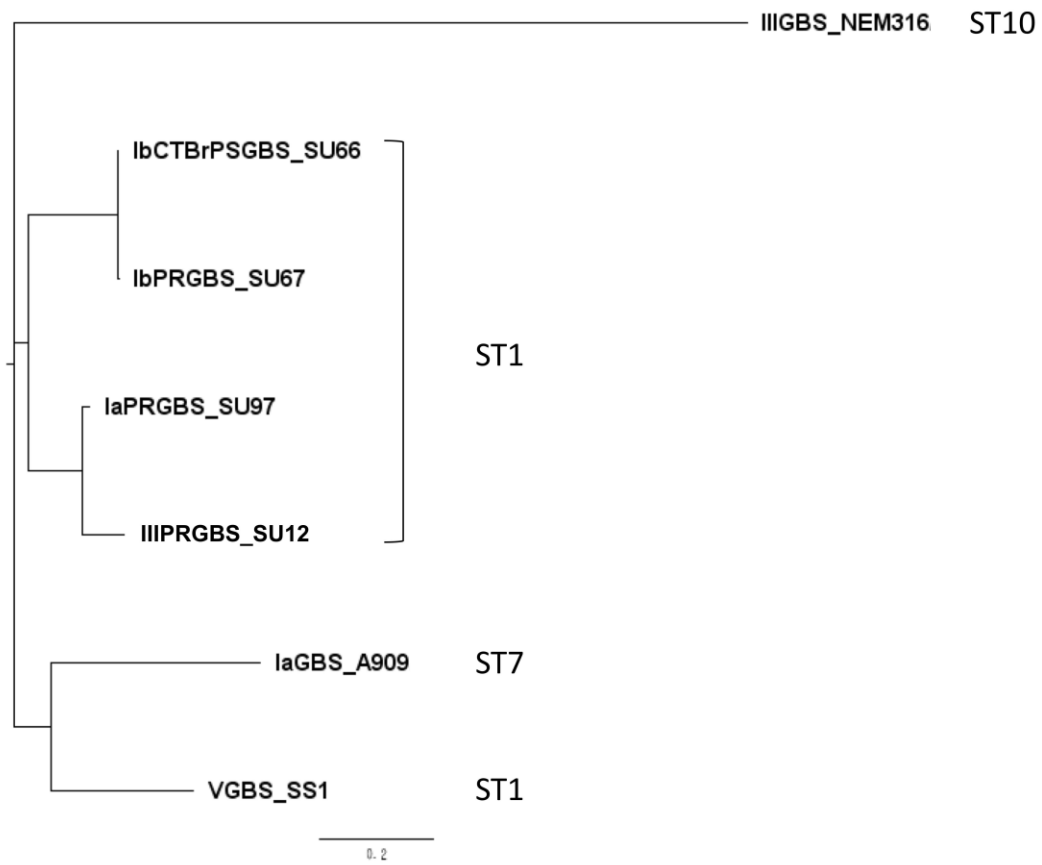
Figure 2



ANIm matrix table

	IIIPRGBS_SU12	IaPRGBS_SU97	IbCTBrPSGBS_SU66	IbPRGBS_SU67	VGBS_SS1	IIIGBS_NEM316	IaGBS_A909
IIIPRGBS_SU12	100	99.84	99.68	99.63	99.67	99.04	99.46
IaPRGBS_SU97	99.84	100	99.69	99.71	99.62	99.2	99.44
IbCTBrPSGBS_SU66	99.68	99.68	100	99.96	99.62	99.31	99.53
IbPRGBS_SU67	99.63	99.71	99.95	100	99.61	99.3	99.53
VGBS_SS1	99.67	99.62	99.62	99.61	100	99.34	99.46
IIIGBS_NEM316	99.04	99.19	99.3	99.3	99.34	100	99.33
IaGBS_A909	99.46	99.44	99.53	99.53	99.46	99.33	100

Figure 3

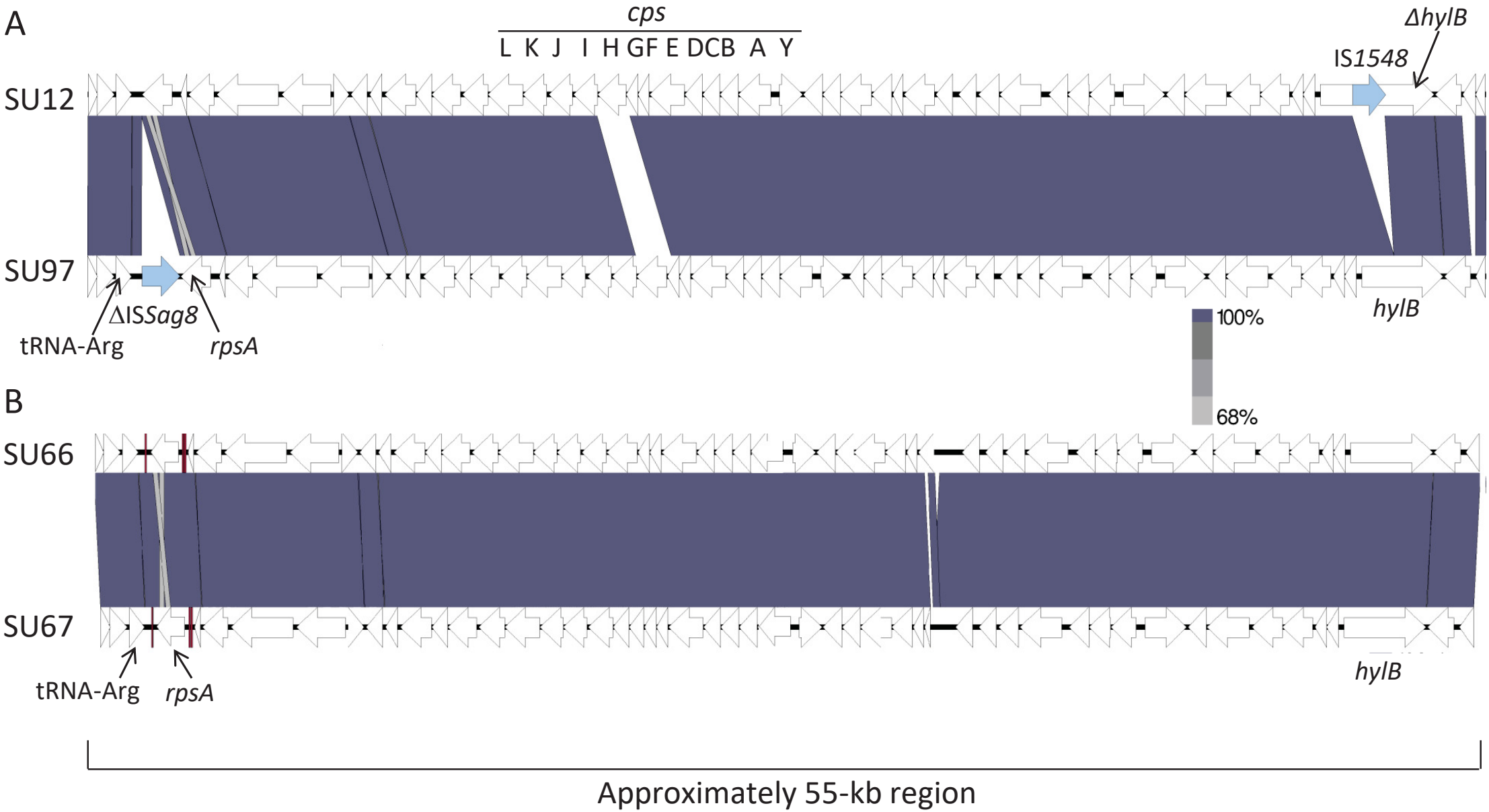


SNP difference matrix

	IIIPRGBS _SU12	Ia PRGBS _SU97	Ib CTBrPSGBS _SU66	Ib PRGBS _SU67	VGBS _SS1	IIIGBS _NEM316	Ia GBS _A909
IIIPRGBS_SU12	0	957	3022	3027	3434	7369	4920
Ia PRGBS_SU97	957	0	2436	2440	3716	7575	4315
Ib CTBrPSGBS_SU66	3022	2436	0	46	4131	7270	4535
Ib PRGBS_SU67	3027	2440	46	0	4138	7277	4542
VGBS_SS1	3434	3716	4131	4138	0	7260	4478
IIIGBS_NEM316	7369	7575	7270	7277	7260	0	7749
Ia GBS_A909	4920	4315	4535	4542	4478	7749	0

Percentage of strain SU12 reference genome covered by all isolates: 83.4709976798144
1780812 positions was found in all analyzed genomes.
Size of strain SU12 reference genome: 2133450 bp

Figure 4


A
cps
L K J I H GF E DCB A Y
ΔhylB
IS1548
SU12
SU97
ΔISSag8
tRNA-Arg
rpsA
hylB
100%
68%
B
SU66
SU67
tRNA-Arg
rpsA
hylB
Approximately 55-kb region