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► To cite this version:

Sarah Khazaal, Rim Al Safadi, Dani Osman, Aurélia Hiron, Philippe Gilot. Dual and divergent transcriptional impact of IS1548 insertion upstream of the peptidoglycan biosynthesis gene *murB* of *Streptococcus agalactiae*. *Gene*, 2019, 720, 10 p. 10.1016/j.gene.2019.144094 . hal-02626494

HAL Id: hal-02626494

<https://hal.inrae.fr/hal-02626494>

Submitted on 20 Dec 2021

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**Dual and divergent transcriptional impact of IS1548
insertion upstream of the peptidoglycan biosynthesis gene
murB of *Streptococcus agalactiae***

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27 **ABSTRACT**

28

29 Fourteen different insertion sequences belonging to seven families were identified in the
30 genome of *Streptococcus agalactiae*. Among them, IS1548, a mobile element of the ISAsI
31 family, was linked to clonal complex (CC) 19 strains associated with neonatal meningitis and
32 endocarditis. IS1548 impacts *S. agalactiae* in two reported ways: i) inactivation of virulence
33 genes by insertion in an open reading frame (eg. *hylB* or *cpsD*), ii) positive modulation of the
34 expression of a downstream gene by insertion in an intergenic region (e.g *lmb*). We
35 previously identified an unknown integration site of IS1548 in the intergenic region between
36 the *folK* and the *murB* genes involved in folate and peptidoglycan biosynthesis, respectively.
37 In this work, we analyzed the prevalence of IS1548 in a large collection of nine hundred and
38 eleven *S. agalactiae* strains. IS1548 positive strains belong to twenty-nine different sequence
39 types and to ten CCs. The majority of them were, however, clustered within sequence type 19
40 and sequence type 22, belonging to CC19 and CC22, respectively. In contrast, IS1548 targets
41 the *folK-murB* intergenic region exclusively in CC19 strains. We evaluated the impact of the
42 insertion of IS1548 on the expression of *murB* by locating transcriptional promoters
43 influencing its expression in the presence or absence of IS1548 and by comparative β -
44 galactosidase transcriptional fusion assays. We found that in the absence of IS1548, genes
45 involved in folate biosynthesis are co-transcribed with *murB*. As it was postulated that a folic
46 acid mediated reaction may be involved in cell wall synthesis, this co-transcription could be
47 necessary to synchronize these two processes. The insertion of IS1548 in the *folK-murB*
48 intergenic region disrupt this co-transcription. Interestingly, we located a promoter at the right
49 end of IS1548 that is able to initiate additional transcripts of *murB*. The insertion of IS1548 in
50 this region has thus a dual and divergent impact on the expression of *murB*. By comparative
51 β -galactosidase transcriptional fusion assays, we showed that, consequently, the overall
52 impact of the insertion of IS1548 results in a minor decrease of *murB* gene transcription. This
53 study provides new insights into gene expression effects mediated by IS1548 in *S. agalactiae*.

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56 Keywords : peptidoglycan, polyamine, folate biosynthesis, mobile genetic element, ISAsI
57 family, adaptation

58 1. Introduction

59

60 Classical insertion sequences (ISs) are simple transposable elements consisting of
61 short DNA sequences ranging between 0.7 and 2.5 kb in length. They are capable of repeated
62 insertion at various genomic locations using processes independent of homologous
63 recombination between large DNA regions. ISs encode a transposase involved in the
64 transposition mechanism and sometimes regulatory proteins. They terminate in flanking
65 imperfect terminal inverted repeat sequences which are often bordered by a direct repeats
66 (DR) at each end (Mahillon and Chandler, 1988; <https://isfinder.biotoul.fr/>). ISs are important
67 actors of the plasticity of bacterial genome and of its evolution (Bennet, 2004). They allow
68 genomic rearrangements by homologous recombination between related elements inserted at
69 distinct sites of the chromosome. They can also negatively or positively modulate the
70 expression of genes by the disruption of a promoter or an open reading frame (ORF), by the
71 provision of a promoter for a neighboring ORF, or by the disruption or displacement of a
72 regulator binding site (Casacuberta and Gonzalez, 2013; Fléchart and Gilot, 2014; Siguier et
73 al, 2014; Fléchart et al., 2018).

74 *Streptococcus agalactiae* is a Gram-positive bacterium that is one of the leading cause
75 of neonatal infections. It is also an emerging pathogen in immunocompromised non-pregnant
76 adults and elderly person, where it is responsible for invasive infections, such as meningitis,
77 endocarditis or soft tissue and osteoarticular infections. In addition, it also infects or colonizes
78 bovines, fishes, aquatic mammals, horses and dogs (Farley, 2001; Evans et al., 2008; for
79 reviews see Vornhagen et al., 2017; Shabayek and Spellerberg, 2018). The population
80 structure of *S. agalactiae* was intensively studied by multilocus sequence typing (MLST), a
81 nucleotide-sequence-based method allowing the classification of strains in sequence types
82 (STs), which can be grouped in clonal complexes (CCs) based on genetic proximity. Some of
83 these STs were linked to particular invasive or colonization behavior (Jones et al., 2003;
84 Manning et al., 2009).

85 Fourteen different ISs belonging to seven families were identified in *S. agalactiae*
86 genomes (<https://isfinder.biotoul.fr/search.php>). These elements affect *S. agalactiae* by
87 inflecting the expression of several virulence genes and by modulating its capability of
88 adaptation to various environments (for a review see Fléchart and Gilot, 2014). In this
89 context, IS1548 is a transposable element that shows specific impacts on its *S. agalactiae* host
90 (Fléchart and Gilot, 2014). IS1548, a 1316-bp-element of the ISAs1 family, carries a 19 bp-

91 imperfect terminal inverted repeat and encodes a putative transposase of 377 amino acids with
92 DDE-transposase I associated superfamily motifs and similarities to H repeat-associated
93 proteins (Granlund et al. 1998; Granlund et al., 2001). *IS1548* was discovered by Granlund et
94 al. (1998) in a *Streptococcus agalactiae* strain lacking hyaluronidase activity. In this strain,
95 *IS1548* is integrated in the hyaluronidase ORF (*hylB*) and in the intergenic region between the
96 C5a-peptidase gene (*scpB*) and the laminin/Zn binding protein gene (*lmb*). In some other
97 clinical isolates, *IS1548* was also identified in the *cpsD* gene that is involved in the regulation
98 of capsular polysaccharide biosynthesis (Sellin et al., 2000). *IS1548* insertion in the virulence
99 genes *hylB* or *cpsD* unables the production of hyaluronidase or renders the strains defective
100 for capsule production, respectively (Granlund et al. 1998; Sellin et al., 2000). Conversely,
101 *IS1548* insertion in the *scpB-lmb* intergenic region, towards *lmb*, leads to an increased
102 transcriptional activity of the *lmb* gene and a stronger binding of the strain to laminin,
103 probably due to the presence of a promoter in the right end of *IS1548* (Al Safadi et al., 2010).
104 The prevalence of *IS1548* was found to correlate with *S. agalactiae* strains of clonal complex
105 (CC) 19 associated with neonatal meningitis and endocarditis but this mobile element is also
106 common in *S. pyogenes* and more recently detected *S. dysgalactiae* subsp. *equisimilis*
107 (Granlund et al., 1998; Bidet et al., 2003; Dmitriev et al., 2003; Héry-Arnaud et al., 2005;
108 Luan et al., 2005; Flécharde and Gilot, 2014). By analyzing the DNA sequences around the DR
109 generated after *IS1548* integration, we were able to identify two conserved motifs that
110 allowed the identification of a previously unidentified *IS1548* target into the *folK-murB*
111 intergenic region of some strains (Flécharde et al., 2013a; Flécharde et al., 2013b). MurB, a
112 UDP-N-acetylpyruvoylglucosamine reductase involved in peptidoglycan synthesis, is encoded
113 by an essential gene in bacteria (van Heijenoort, 2001). By examining *S. agalactiae*
114 sequenced genomes, we found that *murB* is the first gene of a putative operon comprising also
115 the *potABCD* genes encoding a polyamine ABC transporter and a gene encoding a chloride
116 channel. In *S. suis* and *S. pneumoniae*, *murB* was proved to be co-transcribed with the
117 *potABCD* genes (Ware et al., 2005; Liu, W., 2018). In *S. pneumoniae*, the *murB-potABCD*
118 operon is induced by environmental stresses and involved in fitness and pathogenicity (Shah
119 et al., 2011). Insertion of *IS1548* into the *folK-murB* intergenic region, toward the *murB* gene,
120 might thus lead to a modification of the expression of *murB* and downstream genes, and could
121 have some effects on the physiology of *S. agalactiae* and on its capacity of adaption to
122 environmental stresses.

123 In this work, we analyzed the prevalence of *IS1548* in the *folK-murB* intergenic region
124 in a collection of nine hundred and eleven *S. agalactiae* strains. The genomic sequences of

125 these strains were available as whole genome contigs or as complete genome sequences at the
126 National Center for Biotechnology Information (NCBI) database. We then analyzed strains
127 with *IS1548* insertion in the *folK-murB* intergenic region by Multilocus Sequence Typing
128 (MLST) to better perceive their correlation with particular sequence types and clonal
129 complexes. Finally, we evaluated the impact of the insertion of *IS1548* on the expression of
130 *murB* by locating active promoters in the presence or absence of *IS1548*, and by using
131 comparative β -galactosidase reporter assays.

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133 **2. Materials and methods**

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135 *2.1. Plasmids, bacterial strains and growth conditions*

136

137 Plasmids and bacterial strains used in this study are listed in Table 1. *Escherichia*
138 *coli* strains were cultured liquid LB medium (MP Biomedicals, Solon, OH, USA; Cat. n°
139 3002022) at 37°C with agitation (200 rpm) or on LB-agar plates (1.5% agar). Unless
140 otherwise stated, *S. agalactiae* strains were grown in Todd Hewitt (TH) broth with agitation
141 (200 rpm) at 37°C or on TH-agar (BD Bacto, Sparks, MD, USA; Cat. n° 249240). *S.*
142 *agalactiae* strains were also cultured (37°C, agitation at 200 rpm) in the liquid chemically
143 defined medium described by Moulin et al. (2016), with the addition of 17.4 μ M ZnSO₄
144 \cdot 7H₂O, 10.5 μ M CoCl₂·H₂O, 0.4 μ M CuSO₄·5H₂O, and 55 mM D-glucose (CDM). All CDM
145 components were from Sigma-Aldrich. When necessary, *E. coli* and *S. agalactiae* strains
146 were grown with erythromycin (150 μ g/ml for *E. coli* or 10 μ g/ml for *S. agalactiae*).

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148 *2.2. Identification of strains with IS1548 genomic insertion*

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150 The genome of 911 *S. agalactiae* strains were analysed in this study. The sequences
151 of these genomes were available as whole genome contigs or as complete genome sequences
152 at the NCBI database on the 19th of January 2018 (Supplemental Table S1,
153 <https://www.ncbi.nlm.nih.gov/genome/genomes/186/>). To identify strains with an *IS1548*
154 genomic insertion, the complete genome sequence or all of the contigs sequences of each of
155 these strains were first blasted (<https://blast.ncbi.nlm.nih.gov/>) with the *IS1548* complete
156 DNA sequence of strain Mc1 (GenBank Acc. n°Y14270). To identify strains with an *IS1548*
157 insertion in the *folK-murB* intergenic region, the genome of these strains was then blasted
158 with a 239 bp sequence of strain L29 comprising the 3'-end of *folK* and the beginning of

159 IS1548 (GenBank Acc. n° HF548341), and with a 451 bp sequence of strain L29 comprising
160 the end of IS1548 and the 5'-end of *murB* (GenBank Acc. n° HF588342).

161

162 2.3. Multi Locus Sequence Typing of *Streptococcus agalactiae* strains

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164 *S. agalactiae* strains with an IS1548 genomic insertion were typed by MLST. Seven
165 housekeeping genes were analyzed: alcohol dehydrogenase gene (*adhP*), phenylalanine tRNA
166 synthetase gene (*pheS*), amino acid transporter gene (*atr*), glutamine synthetase gene (*glnA*),
167 serine dehydratase gene (*sdhA*), glucose kinase gene (*glcK*) and transketolase gene (*tkt*). A
168 sequence type (ST), based on the allelic profile of these housekeeping genes, was assigned to
169 each strain by submitting the complete genome sequence or all of the contigs sequences of
170 each strain to the *Streptococcus agalactiae* MLST databases (<http://pubmlst.org/sagalactiae/>;
171 Jolley and Maiden, 2010). Strains were then grouped into clonal complexes (CCs) with the
172 eBURST software (<http://eburst.mlst.net/>). An eBURST clonal complex was defined as all
173 allelic profiles sharing six identical alleles with at least one other member of the group.

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175 2.4. In silico identification of transcriptional promoters and terminators

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177 To identify σ^{70} transcriptional promoters upstream of *murB*, the sequences from the
178 start of *folK* to the start of *murB* of strain SA87, from the start of *folK* of strain S10-201 to the
179 left direct repeat (DR) of IS1548, from the left to the right DR of IS1548, and from the stop of
180 the transposase of IS1548 to the start of *murB* of strain S10-201 were analysed with the
181 BProm software from the SoftBerry suite
182 (<http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb>).

183

184 To identify rho-independent transcriptional terminators upstream of *murB*, the
185 sequences of the intergenic regions between *folK* and *murB* of strain SA87, between *folK* and
186 the transposase gene of IS1548 of strain S10-201, and between the transposase gene (*tnp*) of
187 IS1548 and *murB* of strain S10-201 were analysed with the Arnold program
188 (<http://rssf.i2bc.paris-saclay.fr/toolbox/arnold/>). Similar analyses were also made with the
entire coding sequence of *tnp*, *folK* and *murB*.

189

190 2.5. Nucleic acid manipulations

191

192 Standard nucleic acid manipulation techniques were carried out as described by
193 Sambrook and Russel (2001). For genomic DNA purification, *S. agalactiae* bacteria cultured
194 overnight without agitation in 10 ml of TH broth were centrifuged and resuspended in 1 ml of
195 a lysis buffer (1 M NaCl, 5 mM EDTA, 0.5% (v/v) Tween 20, 10 mM Tris-HCl [pH 8.0]).
196 The bacteria were then lysed mechanically with glass beads in a FastPrep-24 instrument (MP
197 Biomedicals, Solon, OH, USA). After centrifugation (10 min, 12 000 x g), the DNA was
198 purified from the supernatant by the phenol-chloroform extraction method. Total RNA was
199 extracted from mid-exponential-phase cells (OD₆₀₀ of 0.5) growing in TH broth. The bacteria
200 were lysed mechanically with glass beads in a FastPrep-24 instrument (MP Biomedicals,
201 Solon, OH, USA), and total RNAs were extracted by the phenol/TRIzol-based purification
202 method as described by Lamy et al. (2004). *E. coli* plasmids were purified with a NucleoSpin
203 Plasmid kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions.
204 Nucleic acid concentrations were measured with a NanoDrop™ Lite Spectrophotometer
205 (Thermo scientific, Waltham, MA, USA). The ratio of absorbance at 260 nm and 280 nm was
206 used to check the purity of nucleic acids. Bacteria were transformed by electroporation with
207 the Micropulser apparatus (Biorad, Hercules, CA, USA) and the Ec2 conditions (2.5 kV), as
208 described by Dower et al. (1988) for *E. coli* and by Ricci et al. (1994) for *S. agalactiae*.

209
210 *2.6. Amplification of nucleic sequences by Polymerase Chain Reaction (PCR) and by Reverse*
211 *Transcription (RT)-PCR*

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213 PCR were performed with the Applied Biosystem 2720 Thermal cycler using Q5
214 High-Fidelity DNA polymerase (New England Biolab, Evry, France) for cloning and with
215 OneTaq polymerase (New England Biolab, Evry, France) for analytical PCR. For cloning, the
216 resulting PCR fragments were further purified with a NucleoSpin Gel and PCR clean-up kit
217 (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions.
218 Oligonucleotides (Sigma-Aldrich, Darmstadt, Germany) used in this study are listed in Table
219 2. For reverse transcription, a DNase (Turbo DNase, Ambion, Vilnius, Lithuania) treatment
220 of the purified RNAs was first realized. The RNAs were then reverse-transcribed by using
221 the iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA), according to the
222 manufacturer's instructions. Finally, cDNAs were amplified by PCR with appropriate primers
223 (Table 2), as described above for PCR amplification of DNA. Control RT-PCRs, omitting
224 reverse transcriptase, were performed to check for DNA contamination of the RNA
225 preparation.

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2.7. DNA sequencing

229 Cloned fragments were sequenced on both strands using the Big Dye Terminator v3.1
230 cycle sequencing kit from Applied Biosystems and the ABI Prism 310 Genetic Analyzer.

231

2.8. β -galactosidase transcriptional fusion assays

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234 Plasmid pTCV-*lacZ* (Poyart and Trieu-Cuot, 1997) was used to construct
235 transcriptional fusions between the *E. coli lacZ* reporter gene and 550 bp regions upstream of
236 the translation start of the *murB* gene. To this end, the chromosomal DNA of strain SA87 and
237 S10-201 were amplified by PCR using primers SK12 (possessing an *EcoRI* restriction site)
238 and SK13 (possessing a *BamHI* restriction site) or SK11 (possessing an *EcoRI* restriction site)
239 and SK13, respectively (Table 2). The two amplification products differ uniquely by 445 nt at
240 their 5'-end belonging either to the 3'-end of *folK* and to the 38 first nt of the *folK-murB*
241 intergenic region (SA87 amplification product) or to *IS1548* (S10-201 amplification product).
242 The corresponding purified DNA fragments were then hydrolysed with *EcoRI* and *BamHI*
243 and cloned in the pTCV-*lacZ* vector previously hydrolysed by the same restriction enzymes.
244 Cloned fragments were then sequenced on both strands to assure that no mutation had
245 occurred.

246 For quantification of the strength of the promoter(s) present in the cloned fragments,
247 CDM containing 10 $\mu\text{g/ml}$ erythromycin were inoculated to an $\text{OD}_{600 \text{ nm}}$ of 0.05 with
248 overnight cultures in the same medium of strains A909 or L29 containing the above
249 constructed plasmids. These cultures were incubated at 37°C during 10 h with agitation.
250 Bacteria were harvested (10 ml samples) at the mid-exponential ($\text{OD}_{600 \text{ nm}} = 0.45$), at the early
251 stationary ($\text{OD}_{600 \text{ nm}} = 1.2$) and at the late stationary phase of growth ($\text{OD}_{600 \text{ nm}} = 2$). Samples
252 were stored at -80°C until β -galactosidase assays. β -galactosidase assays were realized as
253 previously described by Moulin et al. (2016). All experiments were carried out in triplicate.

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2.9. Statistical analyses

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257 Data are presented as the mean \pm standard deviation for three independent
258 experiments. An unpaired Student's *t*-test was used to determine the significance of the
259 differences between means (*, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$) (Swinscow, 1978).

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3. Results and discussion

3.1. IS1548 targets the *folK-murB* intergenic region exclusively in CC19 strains

We analyzed the prevalence of IS1548 in the *folK-murB* intergenic region in a very large collection of *S. agalactiae* strains using BlastN searches (Suppl. Table S1). This collection comprised the *S. agalactiae* strains whose genomes were available as whole genome contigs or as complete genome sequences at the NCBI database on the 19th of January 2018. Our analysis indicated that one hundred and twenty-one of the nine hundred and eleven analyzed strains (13,3 % of the strains) possess IS1548 in their genomes. However, only seventy- three of them (8,0 % of the strains) have integrated IS1548 in the *folK-murB* intergenic region (Suppl. Table S1). To better perceive their correlation with sequence types and clonal complexes, we further analyzed all the strains possessing IS1548 by Multilocus Sequence Typing. These epidemiologically unrelated strains belong to twenty-nine different sequence types and to ten clonal complexes (Fig. 1). The majority of them were, however, clustered within sequence type 19 (55,3 % of the strains) and sequence type 22 (15,5 % of the strains), belonging to CC19 and CC22, respectively. In contrast, IS1548 targets the *folK- murB* intergenic region exclusively in CC19 strains (Fig.1).

Clonal complex19 strains caused infections in both neonates and immunocompromised adults (Héry-Arnaud et al., 2005; Luan et al., 2005). To understand why IS1548 targets the *folK-murB* intergenic region exclusively in CC19 strains, we compared this region in all of the strains possessing IS1548 in their genomes (Table S1). With the exception of the presence or not of IS1548, the *folK-murB* region of these strains is very similar. They all possess the conserved motif 2 (AACAACCT) that was identified close to the IS1548 insertion site and the CAGAAATTGT sequence that is duplicated after its insertion. The most apparent difference is the presence of an A at position – 52 upstream of *murB* in all of the strains with an IS1548 insertion, whereas all of the other strains possess a G at that position. (Fig. 2, Flécharde et al., 2013a). The nucleotide at position -52 seems thus important for IS1548 transposition into the *folK-murB* intergenic region. Furthermore, as the strains

294 possessing *IS1548* are not in equivalent proportion in each sequence type identified in Fig. 1,
295 this might involve that *IS1548* was not acquired by a common ancestor of the main clonal
296 complexes of *S. agalactiae*, but that independent integrations occurred firstly in CC19, then in
297 CC22, and later in CCs 1, 7, 10, 12, 17, and 23. This hypothesis is strengthened by the
298 analysis of the *IS1548* insertion sites in the full sequenced genomes of *S. agalactiae* [the one
299 CC10 strain (B507), the six CC19 strains (2603 V/R, SAG27, SAG158, SG-M25, H002, and
300 HU-GS5823), and the three CC22 strains (GBS2-NM, GBS1-NY, and GBS6)]. The average
301 number of *IS1548* insertion sites in CC19 strains is twelve (from six in strain 2603 V/R to 15
302 in strain H002), whereas it is eight in CC22 strains (from seven in strain GBS2-NM to ten in
303 strain GBS6) and six in the CC10 strain. This suggests a temporal acquisition of *IS1548* in
304 these three clonal complexes. Moreover, although *IS1548* inserted in seven identical sites in
305 the three CC22 strains and in five identical sites in the six CC19 strains, *IS1548* inserted only
306 in one site, the *scpB-lmB* site, common to all of the ten analyzed strains. This confirms the
307 fact that the *scpB-lmB* site is the preferential insertion site of *IS1548*. It can thus be presumed
308 that the *scpB-lmB* site is the first site in which *IS1548* integrate in each of the above cited
309 clonal complexes and that it jumps then more aleatory in the other potential integration sites.
310 Due to the presumably more recent acquisition of *IS1548* by strains belonging not to CC19, it
311 appears thus that, although *IS1548* should have the capability to insert in the *folK-murB* site,
312 these strains have not yet evolved, by substituting the G at position -52 upstream of *murB* by a
313 A, to allow its insertion at that site. Nevertheless, we cannot exclude that this substitution is a
314 consequence and not a prerequisite of the insertion of *IS1548*, as our blastN searches did not
315 revealed the existence of any strain without the insertion of *IS1548* in the *folK-murB*
316 intergenic region and with a A at position -52 upstream of *murB*.

317

318

319 2. *Transcription patterns of murB in the presence or absence of an upstream IS1548 element*

320

321 To evaluate if the insertion of *IS1548* upstream of the *murB* gene affects its
322 expression, we first searched *in silico* for the presence of putative σ^{70} promoters and rho-
323 independent transcriptional terminators in the *folK-murB* region of a strain without (SA87) or
324 with (S10-201) an *IS1548* insertion in the *folK-murB* intergenic region (Fig. 3). Two
325 promoters were predicted to be present in the *folK-murB* region of strain SA87 : P_{murB} in the
326 intergenic region (-10 box, TGGTATAAT; -35 box, TCGTCA) and P_1 in the *folK* coding
327 sequence (-10 box, TTGAATTAT; -35 box, TAGAGA). A potential Rho independant

328 transcriptional terminator was also identified at position – 36 to – 73 upstream of *murB* by the
329 Arnold program. This hairpin structure ($\Delta G = -5.9$ kcal/mol) overlaps the -10 box of P_{murB}
330 (Fig. 3). However, it is surprising to identify a terminator structure at this position
331 downstream the *murB* promoter, as this would mean that there is no possible transcription of
332 the *murB* gene. This hairpin structure could perhaps be an attenuator or a binding site for a
333 regulator protein. Yet, to our knowledge, nothing is known about the regulation of *murB* in *S.*
334 *agalactiae* or in other species of the genus *Streptococcus*. In strain S10-201, four additional
335 putative promoters, brought by IS1548, were identified in this region: P_{mp} just upstream the
336 IS1548 transposase gene (-10 box, ATTCATAAT; -35 box, TTGTTT), P_2 (-10 box,
337 GGTCATCAT; -35 box, TTGTAA) and P_3 (-10 box, CGTTATTTT; -35 box, TGGTCA) in
338 the transposase coding sequence, and P_4 downstream the transposase gene (-10 box,
339 TTTCAAAAT; -35 box, TTGATT). The transcriptional terminator predicted to be present in
340 strain SA87 was not identified in strain S10-210 due to a single nucleotide substitution in the
341 intergenic region of these two strains, that affects the stability of the hairpin (the G to A
342 substitution at position -52 upstream of *murB* reported above) (Fig. 3).

343 We next tested by RT-PCR the capability of these predicted promoters to initiate the
344 transcription of *murB*. In strain SA87, RT-PCR, performed with reverse primers annealing
345 into the *murB* gene (SK5 or SK17, Fig. 3) and forward primers annealing in the *folK* gene
346 (SK18, SK16 or SK15, Fig. 3), indicate that *folK* is co-transcribed with *murB* (Fig. 4, lanes
347 2A, 4A, and 6A). This co-transcription is, at least in part, due to the activity of a promoter
348 localized upstream of P_1 , as an amplification product was obtained by using the SK18 forward
349 primer that anneals upstream of P_1 (Fig. 3; Fig. 4, lane 2A). It can, nevertheless, not be
350 excluded at this stage that the P_1 promoter does not also initiate a *murB* transcript. The *folK*
351 gene is the last gene of an operon of five genes (*folCEPBK*) involved in folate biosynthesis.
352 Folate derivatives are essential cofactors in the biosynthesis of purines, pyrimidines, and
353 amino acids, as well as formyl-tRNA. The dihydropteroate synthase encoded by *folP* is the
354 target of sulfonamide antibiotics (Brochet et al., 2008). It was postulated that a folic acid
355 mediated reaction may be involved, directly or indirectly in cell wall synthesis (Dulaney and
356 Marx, 1971). The co-transcription of genes of the *fol* operon with *murB* could thus be
357 necessary to synchronize two processes involved in cell wall synthesis. The presence of an
358 internal promoter inside the *folB* gene upstream of *folK* was predicted by BProm (-10 box,
359 TGTTATTGT; -35 box, TTAATA). This promoter could be responsible for the *murB*
360 transcripts initiated upstream of P_1 . We also noticed that, as in *S. suis* and in *S. pneumoniae*,
361 the *murB* gene of *S. agalactiae* is also co-transcribed with *potABCD* (unpublished results). In

362 strain S10-201, no amplicon was obtained when RT-PCR was performed with the SK5
363 reversed primer (annealing into the *murB* gene, Fig. 3) and the SK1 forward primer
364 (annealing between the P₃ and P₄ promoters of IS1548, Fig. 2 and Fig. 3), indicating that all
365 the promoters upstream of P₄, are unable to initiate the transcription of *murB* (Fig. 4, lane 2B).
366 On the contrary, amplicons were obtained when RT-PCRs were performed with the SK5
367 reversed primer and the forward primers SK2 (annealing 11 nt downstream of the P₄
368 promoter, Fig. 2 and Fig. 3) or SK3 (annealing at the P_{murB} promoter, Fig. 2 and Fig. 3),
369 indicating that the P₄ promoter initiates *murB* transcripts (Fig. 4, lanes 4B and 6B). We
370 localized experimentally P₄ on a stretch of 46 nt (between the end of primer SK1 and the
371 beginning of primer SK2, Fig. 3). This P₄ promoter should be the one that was responsible for
372 the increased transcriptional activity of the *lmb* gene after the insertion of IS1548 in the *scpB*-
373 *lmb* intergenic region (Al Safadi et al., 2010).

374 Therefore, the insertion of IS1548 in the *folK-murB* intergenic region as a dual effect :
375 1) this insertion prevents the co-transcription of genes of the *fol* operon with *murB*, 2) this
376 insertion provides an additional promoter for the transcription of *murB*.

377

378 *3. Impact of IS1548 insertion in the folK-murB intergenic region on the expression of*
379 *downstream genes*

380

381 To determine if the presence of IS1548 in the *folK-murB* intergenic region could
382 modulate the expression of *murB*, we cloned a 550-bp DNA fragment upstream of the *murB*
383 translation start of strain SA87 (containing P₁ and P_{murB}, Fig. 3) or of strain S10-201
384 (containing P₄ and P_{murB}, Fig.3) in front of the β-galactosidase gene (a *spoVG-lacZ* fusion) of
385 the promoter probe plasmid pTCV-*lacZ*. Although, each cloned fragment comprises two
386 promoters, one of these two promoters is common to the two regions. This strategy allows the
387 preservation of the original genetic context of the DNA regions upstream of *murB*. The single
388 nucleotide substitution described above at position -52 upstream of *murB* was corrected
389 during the amplification of the SA87 fragment that was cloned in pTCV-*lacZ*. The SA87 and
390 S10-201 cloned fragments differ thus uniquely by 445 nt at their 5'-end belonging or not to
391 IS1548. The nucleotide sequence of this 445 nt region of SA87 is identical to the one of strain
392 S10-201.

393 The recombinant plasmids (pTCV-P₁P_{murB}:: *lacZ*) or (pTCV-P₄P_{murB}:: *lacZ*) were then
394 inserted in strains A909 (CC7) or L29 (CC19) to test if the exclusive insertion of IS1548 in
395 the *folK-murB* intergenic region of CC19 strains has an influence on the expression of *murB*

396 in this particular background, only. We first took advantage of the pTCV-P₁P_{murB}:: *lacZ*
397 construction to check the activity of the P₁ internal promoter of *folK*. To this end, RT-PCR
398 experiments were performed with RNAs of strain A909 (pTCV-P₁P_{murB}:: *lacZ*) by using the
399 SK19 reverse primer annealing at the 5'-end of the *spoVG-lacZ* fusion and forward primers
400 annealing either upstream (SK18) or downstream (SK15 and SK16) of the P₁ promoter (Table
401 2). Amplicons were only obtained with the two forward primers annealing downstream P₁
402 indicating that the P₁ promoter is active (Fig. 5). We localized experimentally P₁ on a stretch
403 of 112 nt (between the end of primer SK18 and the beginning of primer SK16, Fig.3)

404 We then quantified the strength of the promoters present in the two cloned fragments
405 by measuring the β-galactosidase activity during growth of strains A909 and L29. As shown
406 in figures 6, these promoters are similarly active during all the growth phase of the CC7 strain
407 A909 (Fig. 6A) and slightly upregulated during the stationary growth phase of the CC19
408 strain L29 (Fig. 6B). Regardless of the genetic background of the strain and of its growth
409 phase, the activities of the P₁P_{murB} promoters (black rectangles) are always identical or slightly
410 higher than the activities of the P₄P_{murB} (white rectangles) promoters.

411

412 **4. Conclusion**

413 The insertion of *IS1548* in the *folK-murB* intergenic region has dual and divergent
414 effects on *murB* expression. Although, *IS1548* brings an additional promoter able to initiate
415 *murB* transcription, the insertion of this element unable also the co-transcription of *murB* with
416 genes of the folate pathway. The overall impact of the insertion of *IS1548* results in a minor
417 negative modulation of the expression of *murB*. This dual role of *IS1548* differed from the
418 two previously reported effects of this mobile genetic element on the expression of *S.*
419 *agalactiae* genes: i) inactivation of virulence genes by insertion in an open reading frame (eg.
420 *hylB* or *cpsD*), ii) positive modulation of the expression of a downstream gene by insertion in
421 an intergenic region (e.g *lmb*).

422

423 **Acknowledgments**

424

425 SK was supported by PhD fellowships of the Lebanese University and AZM & SAADÉ and
426 of the Lebanese Association for Scientific Research (LASeR). A. Almeida, P. Glaser and N.
427 van der Mee-Marquet are thanked for the gift of strains.

428

429 **References**

430

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554

555 **LEGEND TO FIGURES**

556





557

558 Fig. 1. *IS1548* targets the *folK-murB* intergenic region exclusively in CC19 strains. One
559 hundred and twenty-one epidemiologically unrelated strains of *S. agalactiae* containing
560 *IS1548* were analyzed by Multilocus Sequence Typing. Black rectangles represent the
561 proportion of strains carrying *IS1548*, regardless of its position in the genome. White
562 rectangles represent the proportion of strains with an *IS1548* insertion in the *folK-murB*
563 intergenic region.

564

565 Fig. 2. Nucleotide sequence of the *murB* upstream region of strain S10-201. The stop codon
566 (TAA) of the *IS1548* transposase gene and the start codon of *murB* (ATG) are represented in
567 bold italic. Minus 35 and minus 10 boxes of the predicted P₃, P₄ and P_{*murB*} promoters are
568 framed. The motif 2 present close to *IS1548* insertion sites and the sequence that is duplicated
569 after its insertion (DR) are indicated. The A always present in place of a G in strains with
570 *IS1548* integration in the *folK-murB* region is circled. Primers used for RT-PCR experiments
571 are indicated by arrows.

572

573 Fig. 3. Schematic representation of the *folK-murB* region of *Streptococcus agalactiae* in two
574 genomic contexts. Putative transcriptional promoters () and rho-independent terminators
575 () were identified *in silico* by the BPPROM and the Arnold software, respectively. Open
576 reading frames (open arrows), direct repeats (DR), CAGAAATTGT sequence duplicated after
577 *IS1548* insertion (), and primers used for RT-PCR experiments () are indicated.
578 The position of the DNA fragments cloned in pTCV-*lacZ* is represented by black rectangles.

579

580 Fig. 4. Experimental analysis of the influence of *in silico* predicted promoters on the
581 expression of *murB*. Identification of co-transcripts between *murB* and upstream DNA regions
582 was revealed by RT-PCR performed with reverse primers annealing in the *murB* gene (SK5
583 and SK17) and various forward primers annealing upstream of the *murB* ORF. Amplification
584 products (SK18/SK17, lanes 2A and 3A; SK16/SK17, lanes 4A and 5A; SK15/SK5, lanes 6A
585 and 7A; SK1/SK5, lanes 2B and 3B; SK2/SK5, lanes 4B and 5B; SK3/SK5, lanes 6B and 7B;
586 SK4/SK5, lanes 8B and 9B) were electrophoresed in a 1% agarose gel containing ethidium
587 bromide and visualized under UV light (260 nm). As positive controls, cDNAs and
588 chromosomal DNA of strain S10-201 were amplified with primers SK4/SK5 (lane 8B) or

589 SK1/SK5 (lane 11B), respectively. RT-PCRs were performed in the absence of reverse
590 transcriptase to check for DNA contamination (lanes 3A, 5A, 7A, 3B, 5B, 7B, and 9B).
591 Molecular weight markers (Quick-Load Purple, 100 bp DNA Ladder, New England Biolabs)
592 of the indicated size are in lanes 1A, 1B, 10B, and 12B.

593

594 Fig. 5. The predicted internal P₁ promoter of *folK* is functional. A 550-bp DNA fragment
595 upstream of the *murB* translation start of strain SA87 (containing P₁) was cloned in pTCV-
596 *lacZ*. The recombinant plasmid (pTCV-P₁P_{murB}::*lacZ*) was inserted in strain A909.
597 Transcription of the *spoVG-lacZ* was revealed by RT-PCR performed with a reverse primer
598 (SK19) annealing in the *spoVG-lacZ* fusion and forward primers annealing upstream (SK18)
599 or downstream (SK15 and SK16) of the P₁ promoter. Amplification products (SK18/SK19,
600 lanes 2 and 3; SK15/SK19, lanes 5 and 6; and SK16/SK19, lanes 7 and 8 were
601 electrophoresed in a 1% agarose gel containing ethidium bromide and visualized under UV
602 light (260 nm). As a positive control, chromosomal DNA of strain A909 (pTCV-
603 P₁P_{murB}::*lacZ*) was amplified with primers SK18 and SK19 (lane 4). RT-PCRs were
604 performed in the absence of reverse transcriptase to check for DNA contamination (lanes 3, 6,
605 and 8). Molecular weight marker (Quick-Load® Purple, 100 bp DNA Ladder, New England
606 Biolabs) of the indicated size are in lanes 1 and 9.

607

608 Fig. 6. Comparison of the activity of the P₁P_{murB} and P₄P_{murB} promoters in two genetic
609 background. A 550 bp region upstream of the translation start of *murB* was amplified in strain
610 SA87 (P₁P_{murB}, black rectangles) and in strain S10-201 (P₄P_{murB}, white rectangles), and cloned
611 upstream of the β -galactosidase gene of the promoter probe plasmid pTCV-*lacZ*. The CC7
612 strain A909 (A) and the CC19 strain L29 (B) were transformed by these constructions and
613 grown in CDM containing 10 μ g/ml erythromycin at 37°C, with agitation. Bacteria were
614 harvested at the mid-exponential, at the early stationary and at the late stationary phase of
615 growth and the β -galactosidase specific activity was measured. Results are presented as the
616 mean and standard deviation of three independent cultures. The significance of differences
617 between the activity of the P₁ P_{murB} promoters (absence of IS1548 in the *folK-murB* intergenic
618 region) and the P₄P_{murB} promoters (presence of IS1548 in the *folK-murB* intergenic region)
619 were estimated by a Student's t-test (**, 0.001 > P > 0.01; *, 0.01 > P > 0.05). Values of β -
620 galactosidase activity (arbitrary unit) of negative controls performed with the empty plasmid
621 pTCV-*lacZ* were 0.0 \pm 0.0, 23.0 \pm 2.0, and 46.7 \pm 7.8 for the mid-exponential, the early
622 stationary and the late stationary phase of growth, respectively.

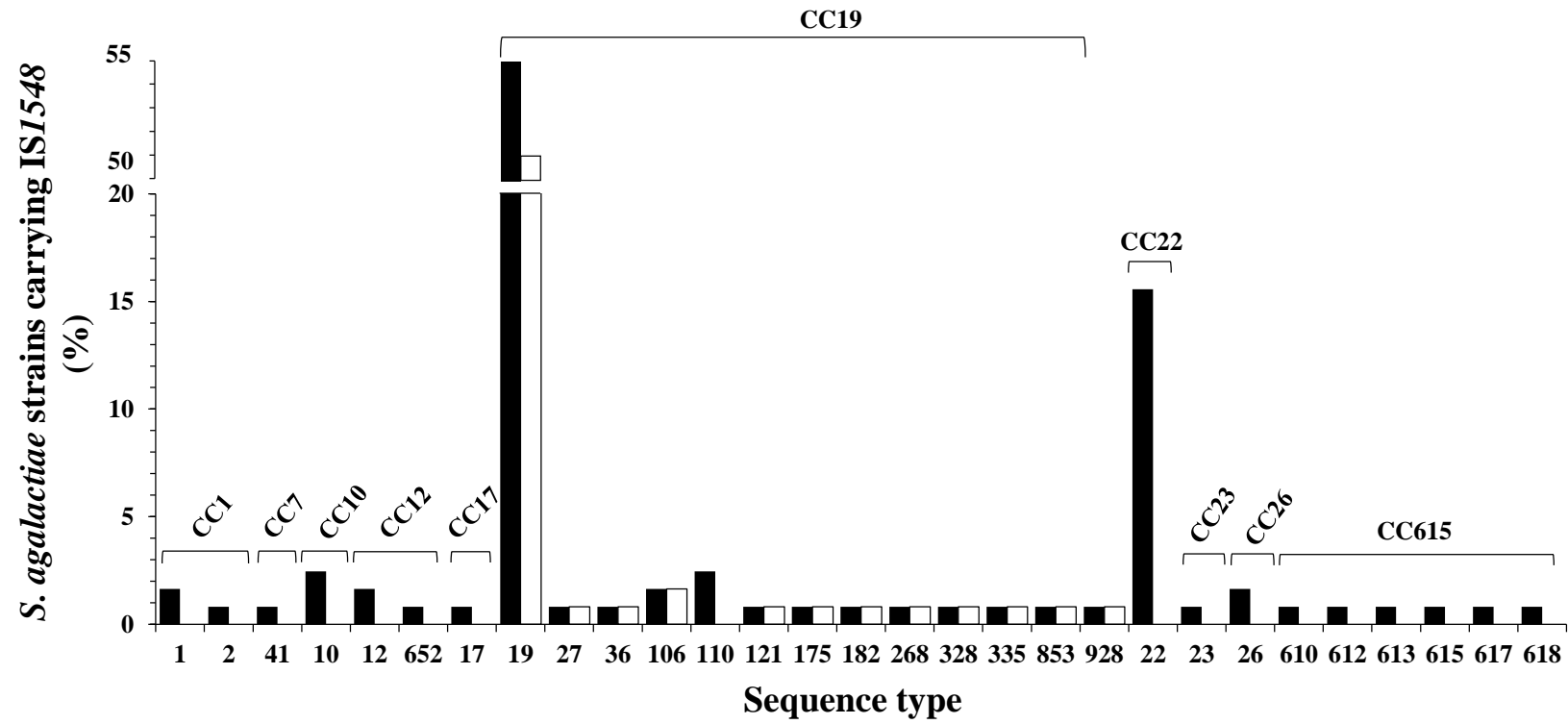


Figure 1

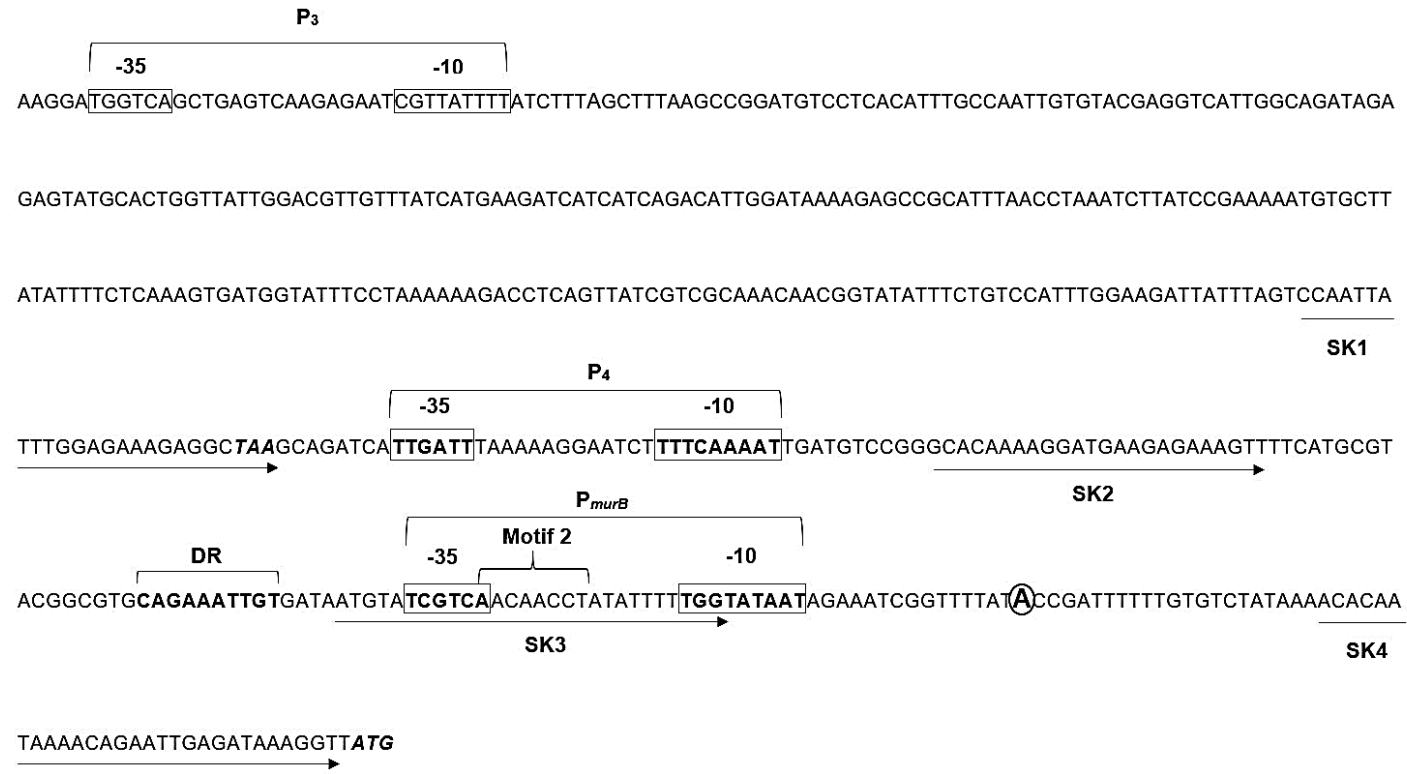


Figure 2

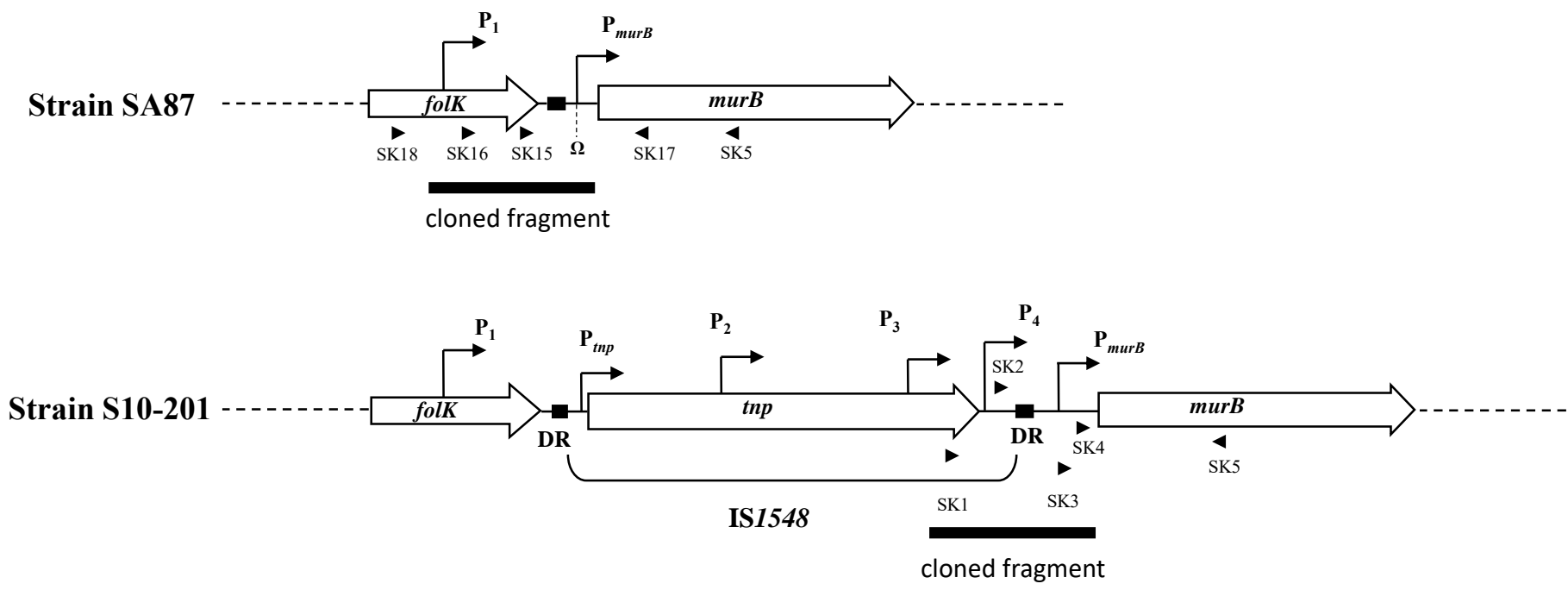


Figure 3

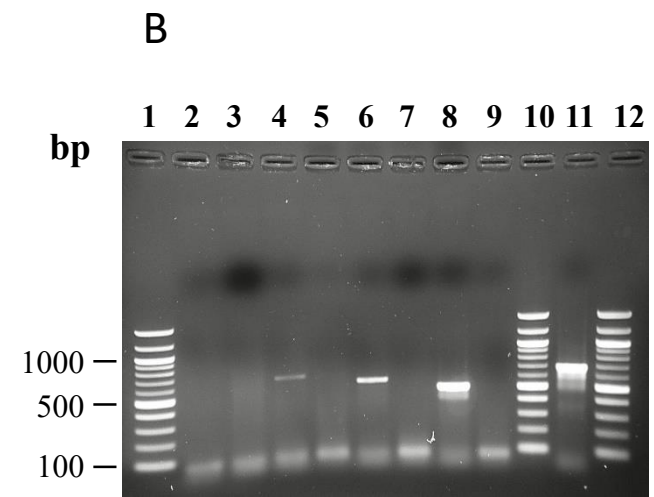
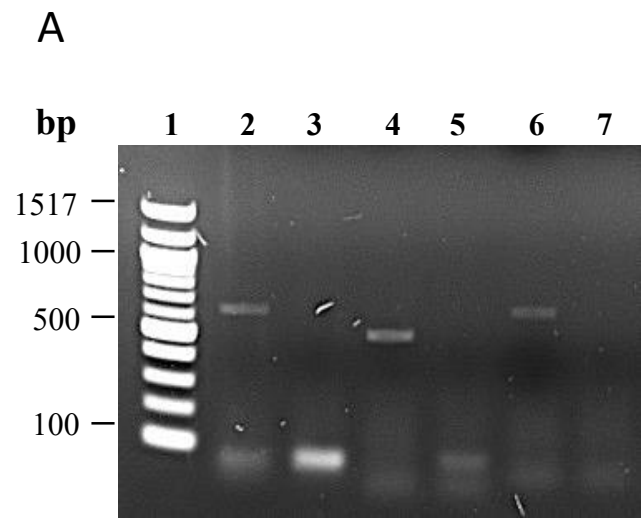


Figure 4

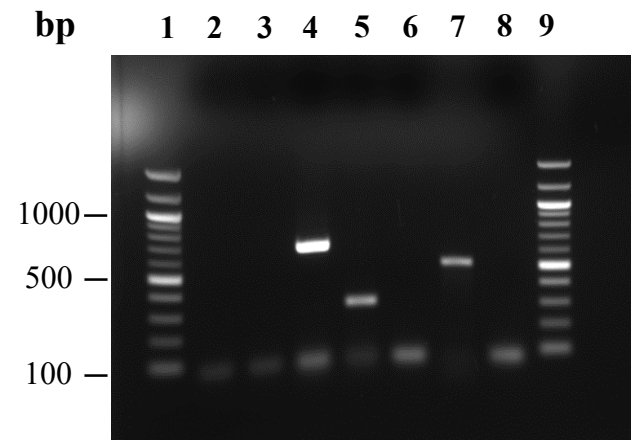


Figure 5

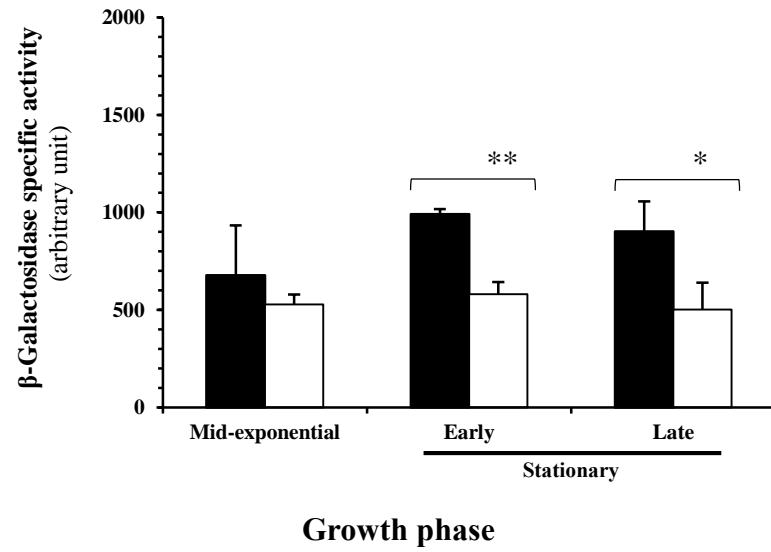
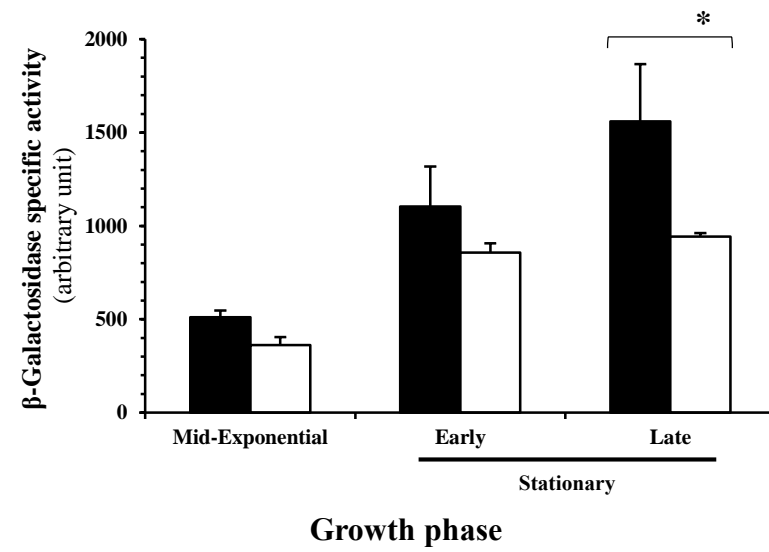
A**B****Figure 6**

Table 1

Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
<u><i>E. coli</i> strains</u>		
XL1-blue	<i>endA1 gyrA96</i> (Nal ^R) <i>thi-1 recA1 relA1 lac glnV44 hsdR17</i> (r _K - m _{K+}) F' [::Tn10 (Tet ^R) <i>proAB</i> ⁺ <i>lacI</i> ^q <i>ZAM15</i>]	Stratagene
<u><i>S. agalactiae</i> strains</u>		
A909	Isolated from a septic human neonate in 1934 (ST 7, CC 7)	Tettelin et al., (2005)
L29	Isolated from cerebrospinal fluid of a neonate suffering from meningitis (ST 19, CC 19)	Quentin et al., (1995)
S10-201	Isolated from the blood of an early onset neonate (ST 19, CC 19)	Van der Mee-Marquet et al., (2017)
SA87	Isolated from mastitic bovine milk (ST 61, CC 17)	Almeida et al., (2016)
<u>Plasmids</u>		
pTCV- <i>lacZ</i>	Promoter probe plasmid carrying the <i>ermB</i> gene (Ery ^R) and a <i>lacZ</i> gene devoided of a promoter	Poyart and Trieu-Cuot, (1997)
pTCV-P ₁ P _{<i>murB</i>} :: <i>lacZ</i>	pTCV- <i>lacZ</i> containing a 550 bp region upstream the start of the <i>murB</i> gene of <i>S. agalactiae</i> SA87 in the <i>EcoRI/BamHI</i> site upstream of <i>lacZ</i>	This study
pTCV-P ₄ P _{<i>murB</i>} :: <i>lacZ</i>	pTCV- <i>lacZ</i> containing a 550 bp region upstream the start of <i>murB</i> gene of <i>S. agalactiae</i> S10-201 in the <i>EcoRI/BamHI</i> site upstream of <i>lacZ</i>	This study

Table 2

Primers used in this study

Name ^a	Sequence ^b	Location ^c
<u>Primers used for RT-PCR</u>		
SK1 _{fw}	CCAATTATTTGGAGAAAGAGGCTAA	nt 1110 to 1182 of <i>IS1548 tnp</i> (strain S10-201)
SK2 _{fw}	GCACAAAAGGATGAAGAGAAAGT	nt -156 to -134 upstream of <i>murB</i> (strain S10-201)
SK3 _{fw}	ATGTATCGTCAACAACCTATATTTTTGG	nt -101 to -74 upstream of <i>murB</i> (strain S10-201)
SK4 _{fw}	ACACAATAAAACAGAATTGAGATAAAGGT	nt -30 to -2 upstream of <i>murB</i> (strain S10-201)
SK5 _{rv}	TTGAGTTCACCTGTGGTGT	nt 476 to 457 of <i>murB</i> (strain S10-201 and SA87)
SK15 _{fw}	TAGGAGAAGTTCATTATTTCAAGCC	nt 455 to 479 of <i>folK</i> (strain SA87)
SK16 _{fw}	GCGTGAGACATGAGCACTG	nt 248 to 266 of <i>folK</i> (strain SA87)
SK17 _{rv}	GCGATAGTTCAAGGCGATTG	nt 136 to 117 of <i>murB</i> (strain SA87)
SK18 _{fw}	CGCTATTTATGAAACAGCTGCT	nt 114 to 135 of <i>folK</i> (strain SA87)
SK19 _{rv}	AGGCGATTAAGTTGGGTAACG	nt 68 to 48 of the <i>spoVG-lacZ</i> fusion (pTCV- <i>lac</i>)
<u>Primers used for transcriptional fusion</u>		
SK11 _{fw}	<u>ATTAGAAATTC</u> GTATTGGGATGACTCGTAA CACGATTGATAAGGATGGTC	nt 785 to 823 of <i>IS1548 tnp</i> (strain S10-201)
SK12 _{fw}	<u>AAAATGAAATTC</u> TCAATTTGCAAAAGACAAAA GTCGCGCAAATTC	nt 81 to 113 of <i>folK</i> (strain SA87)
SK13 _{rv}	<u>TTAGGATCC</u> CTTTATCTCAATTCTGTTTTATT GTGTTTTATAGACACAAAAAATCGGTATAAAACCG	nt -4 to -61 upstream of <i>murB</i> (strains S10-201 and SA87)
<u>Primers used for sequencing</u>		
Vlac-1 _{fw}	GTTGAATAACACTTATTCCTATC	nt -63 to -41 upstream of the <i>EcoRI</i> site of the pTCV- <i>lac</i> polylinker
Vlac-2 _{rv}	CTTCCACAGTAGTTCACCACC	nt 60 to 40 downstream of the <i>BamHI</i> site of the pTCV- <i>lac</i> polylinker

^a fw, forward primer; rv, reverse primer. ^b Tails containing a restriction site (in bold) are underlined. ^c Nucleotide (nt) position with respect to the first coding nt of the gene of interest or to a polylinker restriction site.