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

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Dual and divergent transcriptional impact of IS*1548* insertion upstream of the peptidoglycan biosynthesis gene *murB* of *Streptococcus agalactiae*

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ABSTRACT

Fourteen different insertion sequences belonging to seven families were identified in the genome of *Streptococcus agalactiae*. Among them, IS*1548*, a mobile element of the IS*As1* family, was linked to clonal complex (CC) 19 strains associated with neonatal meningitis and endocarditis. IS*1548* impacts *S. agalactiae* in two reported ways: i) inactivation of virulence genes by insertion in an open reading frame (eg. *hylB* or *cpsD*), ii) positive modulation of the expression of a downstream gene by insertion in an intergenic region (e.g *lmb*). We previously identified an unknown integration site of IS*1548* in the intergenic region between the *folK* and the *murB* genes involved in folate and peptidoglycan biosynthesis, respectively. In this work, we analyzed the prevalence of IS*1548* in a large collection of nine hundred and eleven *S. agalactiae* strains. IS*1548* positive strains belong to twenty-nine different sequence types and to ten CCs. The majority of them were, however, clustered within sequence type 19 and sequence type 22, belonging to CC19 and CC22, respectively. In contrast, IS*1548* targets the *folK-murB* intergenic region exclusively in CC19 strains. We evaluated the impact of the insertion of IS*1548* on the expression of *murB* by locating transcriptional promoters influencing its expression in the presence or absence of IS*1548* and by comparative β-galactosidase transcriptional fusion assays. We found that in the absence of IS*1548*, genes involved in folate biosynthesis are co-transcribed with *murB*. As it was postulated that a folic acid mediated reaction may be involved in cell wall synthesis, this co-transcription could be necessary to synchronize these two processes. The insertion of IS*1548* in the *folK-murB* intergenic region disrupt this co-transcription. Interstingly, we located a promoter at the right end of IS*1548* that is able to initiate additional transcripts of *murB*. The insertion of IS*1548* in this region has thus a dual and divergent impact on the expression of *murB*. By comparative β-galactosidase transcriptional fusion assays, we showed that, consequently, the overall impact of the insertion of IS*1548* results in a minor decrease of *murB* gene transcription. This study provides new insights into gene expression effects mediated by IS*1548* in *S. agalactiae*.

Keywords : peptidoglycan, polyamine, folate biosynthesis, mobile genetic element, IS*AS1* family, adaptation

1. Introduction

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

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

Fourteen different ISs belonging to seven families were identified in *S. agalactiae* genomes (https://isfinder.biotoul.fr/search.php). These elements affect *S. agalactiae* by inflecting the expression of several virulence genes and by modulating its capability of adaptation to various environments (for a review see Fléchard and Gilot, 2014). In this context, IS*1548* is a transposable element that shows specific impacts on its *S. agalactiae* host (Fléchart and Gilot, 2014). IS*1548*, a 1316-bp-element of the IS*As1* family, carries a 19 bp-

imperfect terminal inverted repeat and encodes a putative transposase of 377 amino acids with DDE-transposase I associated superfamily motifs and similarities to H repeat-associated proteins (Granlund et al. 1998; Granlund et al., 2001). IS*1548* was discovered by Granlund et al. (1998) in a *Streptococcus agalactiae* strain lacking hyaluronidase activity. In this strain, IS*1548* is integrated in the hyaluronidase ORF (*hylB*) and in the intergenic region between the C5a-peptidase gene (*scp*B) and the laminin/Zn binding protein gene (*lmb*). In some other clinical isolates, IS*1548* was also identified in the *cpsD* gene that is involved in the regulation of capsular polysaccharide biosynthesis (Sellin et al., 2000). IS*1548* insertion in the virulence genes *hylB* or *cpsD* unables the production of hyaluronidase or renders the strains defective for capsule production, respectively (Granlund et al. 1998; Sellin et al., 2000). Conversely, IS*1548* insertion in the s*cpB-lmb* intergenic region, towards *lmb,* leads to an increased transcriptional activity of the *lmb* gene and a stronger binding of the strain to laminin, probably due to the presence of a promoter in the right end of IS*1548* (Al Safadi et al., 2010). The prevalence of IS*1548* was found to correlate with *S. agalactiae* strains of clonal complex (CC) 19 associated with neonatal mengitis and endocarditis but this mobile element is also common in *S. pyogenes* and more recently detected *S. dysgalactiae* subsp. *equisimilis* (Granlund et al., 1998; Bidet et al., 2003; Dmitriev et al., 2003; Héry-Arnaud et al., 2005; Luan et al., 2005; Fléchard and Gilot, 2014). By analyzing the DNA sequences around the DR generated after IS*1548* integration, we were able to identify two conserved motifs that allowed the identification of a previously unidentified IS*1548* target into the *folK-murB* intergenic region of some strains (Fléchard et al., 2013a; Fléchard et al., 2013b). MurB, a UDP-N-acetylpyuvoylglucosamine reductase involved in peptidoglycan synthesis, is encoded by an essential gene in bacteria (van Heijenoort, 2001). By examining *S. agalactiae* sequenced genomes, we found that *murB* is the first gene of a putative operon comprising also the *potABCD* genes encoding a polyamine ABC transporter and a gene encoding a chloride channel. In *S. suis* and *S. pneumoniae, murB* was proved to be co-transcribed with the *potABCD* genes (Ware et al., 2005; Liu, W., 2018). In *S. pneumoniae*, the *murB-potABCD* operon is induced by environmental stresses and involved in fitness and pathogenicity (Shah et al., 2011). Insertion of IS*1548* into the *folK-murB* intergenic region, toward the *murB* gene, might thus lead to a modification of the expression of *murB* and downstream genes, and could have some effects on the physiology of *S. agalactiae* and on its capacity of adaption to environmental stresses.

In this work, we analyzed the prevalence of IS*1548* in the *folK-murB* intergenic region in a collection of nine hundred and eleven *S. agalactiae* strains. The genomic sequences of

these strains were available as whole genome contigs or as complete genome sequences at the National Center for Biotechnology Information (NCBI) database. We then analyzed strains with IS*1548* insertion in the *folK-murB* intergenic region by Multilocus Sequence Typing (MLST) to better perceive their correlation with particular sequence types and clonal complexes. Finally, we evaluated the impact of the insertion of IS*1548* on the expression of *murB* by locating active promoters in the presence or absence of IS*1548*, and by using comparative β-galactosidase reporter assays.

2. Materials and methods

2.1. Plasmids, bacterial strains and growth conditions

Plasmids and bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains were cultured liquid LB medium (MP Biomedicals, Solon, OH, USA; Cat. n° 3002022) at 37°C with agitation (200 rpm) or on LB-agar plates (1.5% agar). Unless otherwise stated, *S. agalactiae* strains were grown in Todd Hewitt (TH) broth with agitation (200 rpm) at 37°C or on TH-agar (BD Bacto, Sparks, MD, USA; Cat. n° 249240). *S. agalactiae* strains were also cultured (37°C, agitation at 200 rpm) in the liquid chemically defined medium described by Moulin et al. (2016), with the addition of 17.4 µM $ZnSO_4$.$7H_2O$, 10.5 µM $CoCl_2$.H_2O, 0.4 µM $CuSO_4$.$5H_2O$, and 55 mM D-glucose (CDM). All CDM components were from Sigma-Aldrich. When necessary, *E. coli* and *S. agalactiae* strains were grown with erythromycin (150 µg/ml for *E. coli* or 10 µg/ml for *S. agalactiae*).

2.2. Identification of strains with IS1548 genomic insertion

The genome of 911 *S. agalactiae* strains were analysed in this study. The sequences of these genomes were available as whole genome contigs or as complete genome sequences at the NCBI database on the 19th of January 2018 (Supplemental Table S1, https://www.ncbi.nlm.nih.gov/genome/genomes/186?). To identify strains with an IS*1548* genomic insertion, the complete genome sequence or all of the contigs sequences of each of these strains were first blasted (https://blast.ncbi.nlm.nih.gov/) with the IS*1548* complete DNA sequence of strain Mc1 (GenBank Acc. n°Y14270). To identify strains with an IS*1548* insertion in the *folK-murB* intergenic region, the genome of these strains was then blasted with a 239 bp sequence of strain L29 comprising the 3'-end of *folK* and the beginning of

IS*1548* (GenBank Acc. n° HF548341), and with a 451 bp sequence of strain L29 comprising the end of IS*1548* and the 5'-end of *murB* (GenBank Acc. n° HF588342).

2.3. Multi Locus Sequence Typing of Streptococcus agalactiae strains

S. agalactie strains with an IS*1548* genomic insertion were typed by MLST. Seven housekeeping genes were analyzed: alcohol dehydrogenase gene (*adhP*), phenylalanine tRNA synthetase gene (*pheS*), amino acid transporter gene (*atr*), glutamine synthetase gene (*glnA*), serine dehydratase gene (*sdhA*), glucose kinase gene (*glcK*) and transketolase gene (*tkt*). A sequence type (ST), based on the allelic profile of these housekeeping genes, was assigned to each strain by submitting the complete genome sequence or all of the contigs sequences of each strain to the *Streptococcus agalactiae* MLST databases (http://pubmlst.org/sagalactiae/; Jolley and Maiden, 2010). Strains were then grouped into clonal complexes (CCs) with the eBURST software (http://eburst.mlst.net/). An eBURST clonal complex was defined as all allelic profiles sharing six identical alleles with at least one other member of the group.

2.4. In silico identification of transcriptional promoters and terminators

To identify σ^{70} transcriptional promoters upstream of *murB*, the sequences from the start of *folK* to the start of *murB* of strain SA87, from the start of *folK* of strain S10-201 to the left direct repeat (DR) of IS*1548*, from the left to the right DR of IS*1548*, and from the stop of the transposase of IS*1548* to the start of *murB* of strain S10-201 were analysed with the BProm software from the SoftBerry suite (http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb).

To identify rho-independent transcriptional terminators upstream of *murB*, the sequences of the intergenic regions between *folK* and *murB* of strain SA87, between *folK* and the transposase gene of IS*1548* of strain S10-201, and between the transposase gene (*tnp*) of IS*1548* and *murB* of strain S10-201 were analysed with the Arnold program (http://rssf.i2bc.paris-saclay.fr/toolbox/arnold/). Similar analyses were also made with the entire coding sequence of *tnp*, *folK* and *murB*.

2.5. Nucleic acid manipulations

Standard nucleic acid manipulation techniques were carried out as described by Sambrook and Russel (2001). For genomic DNA purification, *S. agalactiae* bacteria cultured overnight without agitation in 10 ml of TH broth were centrifuged and resuspended in 1 ml of a lysis buffer (1 M NaCl, 5 mM EDTA, 0.5% (v/v) Tween 20, 10 mM Tris-HCl [pH 8.0]). The bacteria were then lysed mechanically with glass beads in a FastPrep-24 instrument (MP Biomedicals, Solon, OH, USA). After centrifugation (10 min, 12 000 x g), the DNA was purified from the supernatant by the phenol-chloroform extraction method. Total RNA was extracted from mid-exponential-phase cells (OD_{600} of 0.5) growing in TH broth. The bacteria were lysed mechanically with glass beads in a FastPrep-24 instrument (MP Biomedicals, Solon, OH, USA), and total RNAs were extracted by the phenol/TRIzol-based purification method as described by Lamy et al. (2004). *E. coli* plasmids were purified with a NucleoSpin Plasmid kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. Nucleic acid concentrations were measured with a NanoDropTM Lite Spectrophotomer (Thermo scientific, Waltham, MA, USA). The ratio of absorbance at 260 nm and 280 nm was used to check the purity of nucleic acids. Bacteria were transformed by electroporation with the Micropulser apparatus (Biorad, Hercules, CA, USA) and the Ec2 conditions (2.5 kV), as described by Dower et al. (1988) for *E. coli* and by Ricci et al. (1994) for *S. agalactiae.*

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

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

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227 *2.7. DNA sequencing*
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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.
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232 *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 *Eco*RI restriction site)
238 and SK13 (possessing a *Bam*HI restriction site) or SK11 (possessing an *Eco*RI 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 IS*1548* (S10-201 amplification product).
242 The corresponding purified DNA fragments were then hydrolysed with *Eco*RI and *Bam*HI
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 µg/ml erythromycin were inoculated to an $OD_{600\ nm}$ 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 ($OD_{600\ nm}$ = 0.45), at the early
251 stationary ($OD_{600\ nm}$ = 1.2) and at the late stationary phase of growth ($OD_{600\ nm}$ = 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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255 *2.9. Statistical analyses*
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257 Data are presented as the mean ± 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).

3. Results and discussion

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

We analyzed the prevalence of IS*1548* 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 IS*1548* in their genomes. However, only seventy- three of them (8,0 % of the strains) have integrated IS*1548* 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 IS*1548* 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, IS*1548* 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 IS*1548* targets the *folK-murB* intergenic region exclusively in CC19 strains, we compared this region in all of the strains possessing IS*1548* in their genomes (Table S1). With the exception of the presence or not of IS*1548*, the *folK-murB* region of these strains is very similar. They all possess the conserved motif 2 (AACAACCT) that was identified close to the IS*1548* 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 IS*1548* insertion, whereas all of the other strains possess a G at that position. (Fig. 2, Fléchard et al., 2013a). The nucleotide at position -52 seems thus important for IS*1548* transposition into the *folK-murB* intergenic region. Furthermore, as the strains

possessing IS*1548* are not in equivalent proportion in each sequence type identified in Fig. 1, this might involve that IS*1548* was not acquired by a common ancestor of the main clonal complexes of *S. agalactiae*, but that independent integrations occurred firstly in CC19, then in CC22, and later in CCs 1, 7, 10, 12, 17, and 23. This hypothesis is strengthened by the analysis of the IS*1548* insertion sites in the full sequenced genomes of *S. agalactiae* [the one CC10 strain (B507), the six CC19 strains (2603 V/R, SAG27, SAG158, SG-M25, H002, and HU-GS5823), and the three CC22 strains (GBS2-NM, GBS1-NY, and GBS6)]. The average number of IS*1548* insertion sites in CC19 strains is twelve (from six in strain 2603 V/R to 15 in strain H002), whereas it is eight in CC22 strains (from seven in strain GBS2-NM to ten in strain GBS6) and six in the CC10 strain. This suggests a temporal acquisition of IS*1548* in these three clonal complexes. Moreover, although IS*1548* inserted in seven identical sites in the three CC22 strains and in five identical sites in the six CC19 strains, IS*1548* inserted only in one site, the *scpB-lmB* site, common to all of the ten analyzed strains. This confirms the fact that the *scpB-lmB* site is the preferential insertion site of IS*1548*. It can thus be presumed that the *scpB-lmB* site is the first site in which IS*1548* integrate in each of the above cited clonal complexes and that it jumps then more aleatory in the other potential integration sites. Due to the presumably more recent acquisition of IS*1548* by strains belonging not to CC19, it appears thus that, although IS*1548* should have the capability to insert in the *folk-murB* site, these strains have not yet evolved, by substituting the G at position -52 upstream of *murB* by a A, to allow its insertion at that site. Nevertheless, we cannot exclude that this substitution is a consequence and not a prerequisite of the insertion of IS*1548*, as our blastN searches did not revealed the existence of any strain without the insertion of IS*1548* in the *folK-murB* intergenic region and with a A at position -52 upstream of *murB*.

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

To evaluate if the insertion of IS*1548* upstream of the *murB* gene affects its expression, we first searched *in silico* for the presence of putative σ^{70} promoters and rho-independent transcriptional terminators in the *folK-murB* region of a strain without (SA87) or with (S10-201) an IS*1548* insertion in the *folK-murB* intergenic region (Fig. 3). Two promoters were predicted to be present in the *folK-murB* region of strain SA87 : P_{murB} in the intergenic region (-10 box, TGGTATAAT; -35 box, TCGTCA) and P_1 in the *folK* coding sequence (-10 box, TTGAATTAT; -35 box, TAGAGA). A potential Rho independant

transcriptional terminator was also identified at position – 36 to – 73 upstream of *murB* by the Arnold program. This hairpin structure (ΔG= -5.9 kcal/mol) overlaps the -10 box of P_{murB} (Fig. 3). However, it is surprising to identify a terminator structure at this position downstream the *murB* promoter, as this would mean that there is no possible transcription of the *murB* gene. This hairpin structure could perhaps be an attenuator or a binding site for a regulator protein. Yet, to our knowledge, nothing is known about the regulation of *murB* in *S. agalactiae* or in other species of the genus *Streptococcus*. In strain S10-201, four additional putative promoters, brought by IS*1548*, were identified in this region: P_{tnp} just upstream the IS*1548* transposase gene (-10 box, ATTCATAAT; -35 box, TTGTTT), P_2 (-10 box, GGTCATCAT; -35 box, TTGTAA) and P_3 (-10 box, CGTTATTTT; -35 box, TGGTCA) in the transposase coding sequence, and P_4 downstream the transposase gene (-10 box, TTTCAAAAT; -35 box, TTGATT). The transcriptional terminator predicted to be present in strain SA87 was not identified in strain S10-210 due to a single nucleotide substitution in the intergenic region of these two strains, that affects the stability of the hairpin (the G to A substitution at position -52 upstream of *murB* reported above) (Fig. 3).

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

strain S10-201, no amplicon was obtained when RT-PCR was performed with the SK5 reversed primer (annealing into the *murB* gene, Fig. 3) and the SK1 forward primer (annealing between the P_3 and P_4 promoters of IS*1548*, Fig. 2 and Fig. 3), indicating that all the promoters upstream of P_4, are unable to initiate the transcription of *murB* (Fig. 4, lane 2B). On the contrary, amplicons were obtained when RT-PCRs were performed with the SK5 reversed primer and the forward primers SK2 (annealing 11 nt downstream of the P_4 promoter, Fig. 2 and Fig. 3) or SK3 (annealing at the P_{murB} promoter, Fig. 2 and Fig. 3), indicating that the P_4 promoter initiates *murB* transcripts (Fig. 4, lanes 4B and 6B). We localized experimentally P4 on a stretch of 46 nt (between the end of primer SK1 and the beginning of primer SK2, Fig. 3). This P_4 promoter should be the one that was responsible for the increased transcriptional activity of the *lmb* gene after the insertion of IS*1548* in the *scpB-lmb* intergenic region (Al Safadi et al., 2010).

Therefore, the insertion of IS*1548* in the *folK-murB* intergenic region as a dual effect : 1) this insertion prevents the co-transcription of genes of the *fol* operon with *murB*, 2) this insertion provides an additional promoter for the transcription of *murB*.

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

To determine if the presence of IS*1548* in the *folK-murB* intergenic region could modulate the expression of *murB*, we cloned a 550-bp DNA fragment upstream of the *murB* translation start of strain SA87 (containing P_1 and P_{murB}, Fig. 3) or of strain S10-201 (containing P_4 and P_{murB}, Fig.3) in front of the β-galactosidase gene (a *spoVG-lacZ* fusion) of the promoter probe plasmid pTCV-*lacZ*. Although, each cloned fragment comprises two promoters, one of these two promoters is common to the two regions. This strategy allows the preservation of the original genetic context of the DNA regions upstream of *murB*. The single nucleotide substitution described above at position -52 upstream of *murB* was corrected during the amplification of the SA87 fragment that was cloned in pTCV-*lacZ*. The SA87 and S10-201 cloned fragments differ thus uniquely by 445 nt at their 5'-end belonging or not to IS*1548*. The nucleotide sequence of this 445 nt region of SA87 is identical to the one of strain S10-201.

The recombinant plasmids (pTCV-P_1P_{murB}:: *lacZ*) or (pTCV-P_4P_{murB}:: *lacZ*) were then inserted in strains A909 (CC7) or L29 (CC19) to test if the exclusive insertion of IS*1548* in the *folK-murB* intergenic region of CC19 strains has an influence on the expression of *murB*

in this particular background, only. We first took advantage of the pTCV-P_1P_{murB}:: *lacZ* construction to check the activity of the P_1 internal promoter of *folK*. To this end, RT-PCR experiments were performed with RNAs of strain A909 (pTCV-P_1P_{murB}:: *lacZ*) by using the SK19 reverse primer annealing at the 5'-end of the *spoVG-lacZ* fusion and forward primers annealing either upstream (SK18) or downstream (SK15 and SK16) of the P_1 promoter (Table 2). Amplicons were only obtained with the two forward primers annealing downstream P_1 indicating that the P_1 promoter is active (Fig. 5). We localized experimentally P1 on a stretch of 112 nt (between the end of primer SK18 and the beginning of primer SK16, Fig.3)

We then quantified the strength of the promoters present in the two cloned fragments by measuring the β-galactosidase activity during growth of strains A909 and L29. As shown in figures 6, these promoters are similarly active during all the growth phase of the CC7 strain A909 (Fig. 6A) and slightly upregulated during the stationary growth phase of the CC19 strain L29 (Fig. 6B). Regardless of the genetic background of the strain and of its growth phase, the activities of the P_1P_{murB} promoters (black rectangles) are always identical or slightly higher than the activities of the P_4P_{murB} (white rectangles) promoters.

4. Conclusion

The insertion of IS*1548* in the *folK-murB* intergenic region has dual and divergent effects on *murB* expression. Although, IS*1548* brings an additional promoter able to initiate *murB* transcription, the insertion of this element unable also the co-transcription of *murB* with genes of the folate pathway. The overall impact of the insertion of IS*1548* results in a minor negative modulation of the expression of *murB*. This dual role of IS*1548* differed from the two previously reported effects of this mobile genetic element on the expression of *S. agalactiae* genes: i) inactivation of virulence genes by insertion in an open reading frame (eg. *hylB* or *cpsD*), ii) positive modulation of the expression of a downstream gene by insertion in an intergenic region (e.g *lmb*).

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554

LEGEND TO FIGURES

Fig. 1. IS*1548* targets the *folK-murB* intergenic region exclusively in CC19 strains. One hundred and twenty-one epidemiologically unrelated strains of *S. agalactiae* containing IS*1548* were analyzed by Multilocus Sequence Typing. Black rectangles represent the proportion of strains carrying IS*1548*, regardless of its position in the genome. White rectangles represent the proportion of strains with an IS*1548* insertion in the *folK-murB* intergenic region.

Fig. 2. Nucleotide sequence of the *murB* upstream region of strain S10-201. The stop codon (TAA) of the IS*1548* transposase gene and the start codon of *murB* (ATG) are represented in bold italic. Minus 35 and minus 10 boxes of the predicted P_3, P_4 and P_{murB} promoters are framed. The motif 2 present close to IS*1548* insertion sites and the sequence that is duplicated after its insertion (DR) are indicated. The A always present in place of a G in strains with IS*1548* integration in the *folK-murB* region is circled. Primers used for RT-PCR experiments are indicated by arrows.

Fig. 3. Schematic representation of the *folK-murB* region of *Streptococcus agalactiae* in two genomic contexts. Putative transcriptional promoters ( ) and rho-independent terminators (Ω) were identified *in silico* by the BPROM and the Arnold software, respectively. Open reading frames (open arrows), direct repeats (DR), CAGAAATTGT sequence duplicated after IS*1548* insertion ( ), and primers used for RT-PCR experiments ( ) are indicated. The position of the DNA fragments cloned in pTCV-l*acZ* is represented by black rectangles.

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

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

Fig. 5. The predicted internal P_1 promoter of *folK* is functional. A 550-bp DNA fragment upstream of the *murB* translation start of strain SA87 (containing P_1) was cloned in pTCV-*lacZ*. The recombinant plasmid (pTCV-P_1P_{murB}::*lacZ*) was inserted in strain A909. Transcription of the *spoVG-lacZ* was revealed by RT-PCR performed with a reverse primer (SK19) annealing in the *spoVG-lacZ* fusion and forward primers annealing upstream (SK18) or downstream (SK15 and SK16) of the P_1 promoter. Amplification products (SK18/SK19, lanes 2 and 3; SK15/SK19, lanes 5 and 6; and SK16/SK19, lanes 7 and 8 were electrophoresed in a 1% agarose gel containing ethidium bromide and visualized under UV light (260 nm). As a positive control, chromosomal DNA of strain A909 (pTCV-P_1P_{murB}::*lacZ*) was amplified with primers SK18 and SK19 (lane 4). RT-PCRs were performed in the absence of reverse transcriptase to check for DNA contamination (lanes 3, 6, and 8). Molecular weight marker (Quick-Load® Purple, 100 bp DNA Ladder, New England Biolabs) of the indicated size are in lanes 1 and 9.

Fig. 6. Comparison of the activity of the P_1P_{murB} and P_4P_{murB} promoters in two genetic background. A 550 bp region upstream of the translation start of *murB* was amplified in strain SA87 (P_1P_{murB}, black rectangles) and in strain S10-201 (P_4P_{murB}, white rectangles), and cloned upstream of the *β-galactosidase* gene of the promoter probe plasmid pTCV-*lacZ*. The CC7 strain A909 (A) and the CC19 strain L29 (B) were transformed by these constructions and grown in CDM containing 10 µg/ml erythromycin at 37°C, with agitation. Bacteria were harvested at the mid-exponential, at the early stationary and at the late stationary phase of growth and the β-galactosidase specific activity was measured. Results are presented as the mean and standard deviation of three independent cultures. The significance of differences between the activity of the P_1 P_{murB} promoters (absence of IS*1548* in the *folK-murB* intergenic region) and the P_4P_{murB} promoters (presence of IS*1548* in the *folK-murB* intergenic region) were estimated by a Student's t-test (**, 0.001> P> 0.01; *, 0.01> P> 0.05). Values of β-galactosidase activity (arbitrary unit) of negative controls performed with the empty plasmid pTCV-*lacZ* were 0.0 ± 0.0, 23.0 ± 2.0, and 46.7 ± 7.8 for the mid-exponential, the early stationary and the late stationary phase of growth, respectively.

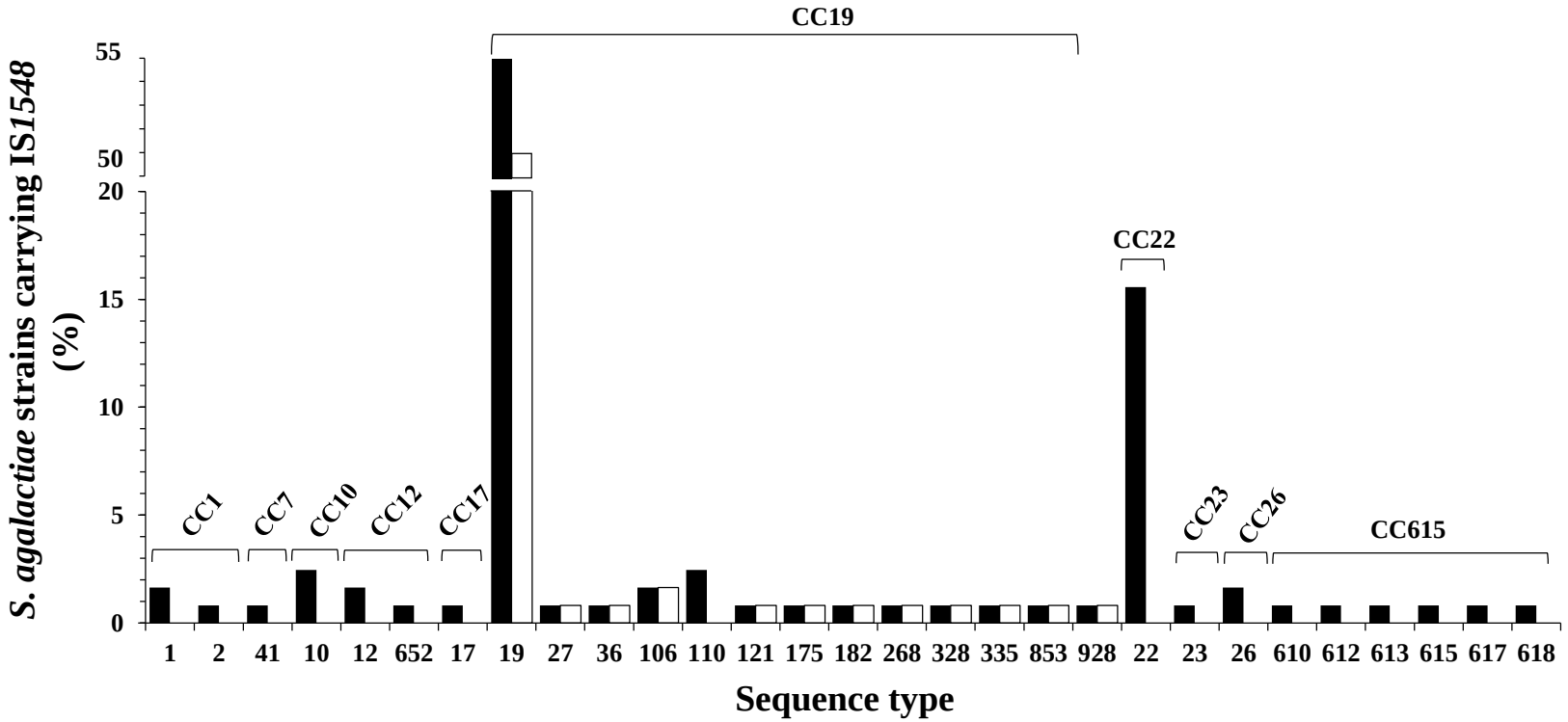
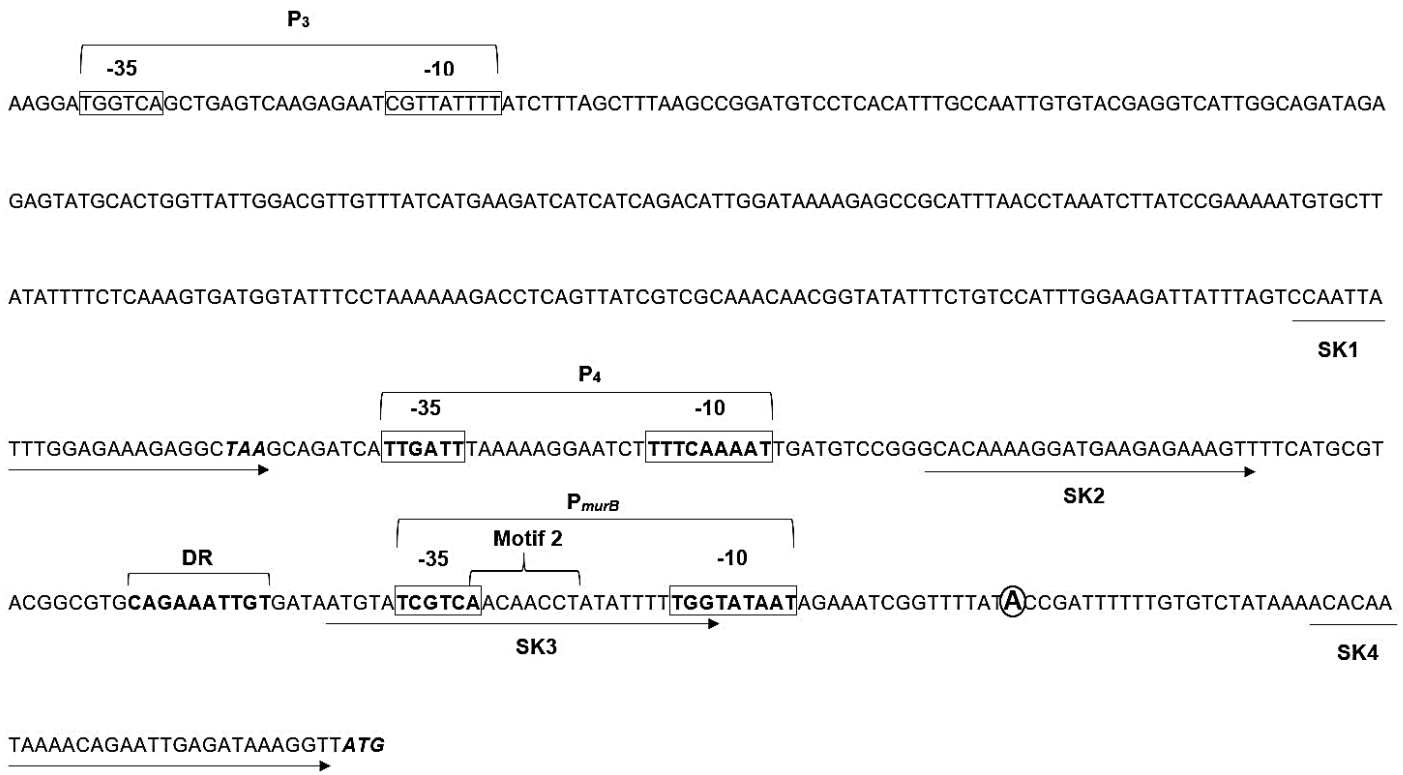

CC19
CC22
CC1
CC7
CC10
CC12
CC17
CC23
CC26
CC615
55
50
20
15
10
5
0
S. agalactiae strains carrying IS1548 (%)
1 2 41 10 12 652 17 19 27 36 106 110 121 175 182 268 328 335 853 928 22 23 26 610 612 613 615 617 618
Sequence type

Figure 1


P3
-35
-10
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GAGTATGCACTGGTTATTGGACGTTGTTTATCATGAAGATCATCATCAGACATTGGATAAAAGAGCCGCATTTAACCTAAATCTTATCCGAAAAATGTGCTT
ATATTTTCTCAAAGTGATGGTATTTCCTAAAAAAGACCTCAGTTATCGTCGCAAACAACGGTATATTTCTGTCCATTTGGAAGATTATTTAGTCCAATTA
SK1
P4
-35
-10
TTTGGAGAAAGAGGCTAAGCAGATCATTGATTTAAAAAGGAATCTTTTCAAAATTGATGTCCGGGCACAAAAGGATGAAGAGAAAGTTTTCATGCGT
SK2
PmurB
Motif 2
DR
-35
-10
ACGGCGTGCAGAAATTGTGATAATGTATCGTCAACAACCTATATTTTTGGTATAATAGAAATCGGTTTTATACCGATTTTTTGTGTCTATAAAACACAA
SK3
SK4
TAAAACAGAATTGAGATAAAGGTTATG

Figure 2

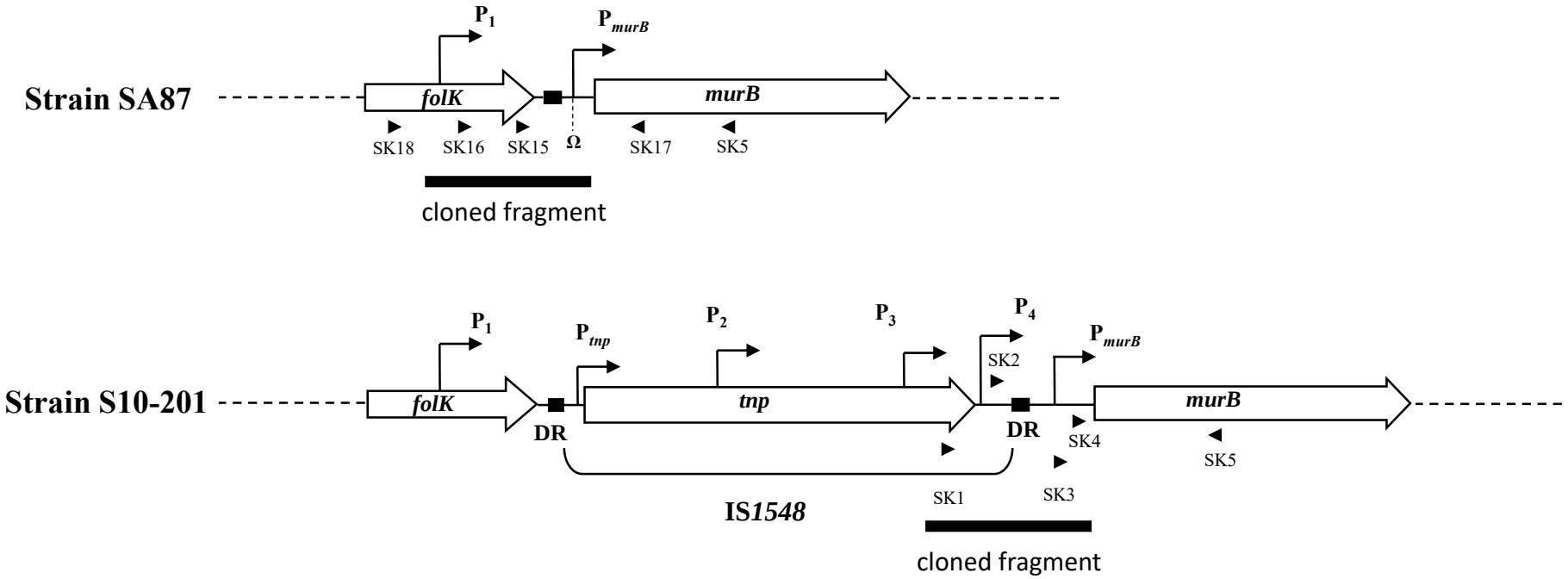
Strain SA87
P1
PmurB
folK
murB
SK18
SK16
SK15
Ω
SK17
SK5
cloned fragment
Strain S10-201
P1
Ptnp
P2
P3
P4
PmurB
SK2
folK
tnp
murB
DR
DR
SK4
SK5
SK1
SK3
IS1548
cloned fragment

Figure 3

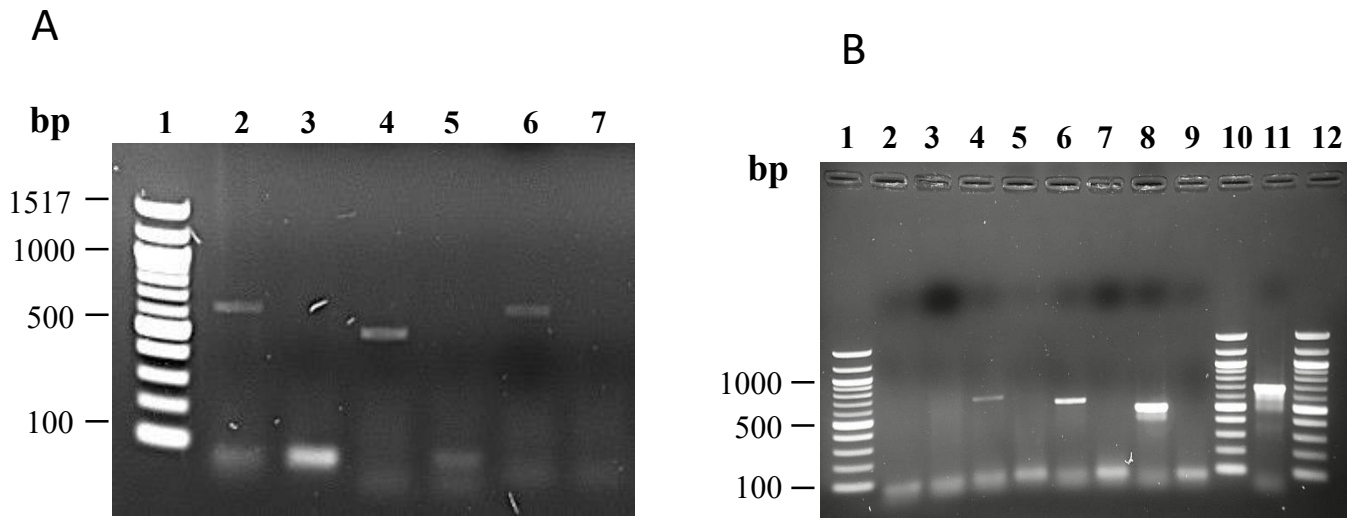
A
bp
1 2 3 4 5 6 7
1517
1000
500
100
B
1 2 3 4 5 6 7 8 9 10 11 12
bp
1000
500
100

Figure 4

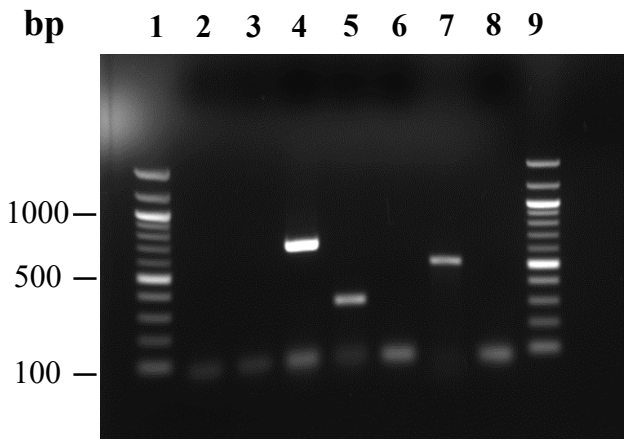

Figure 5

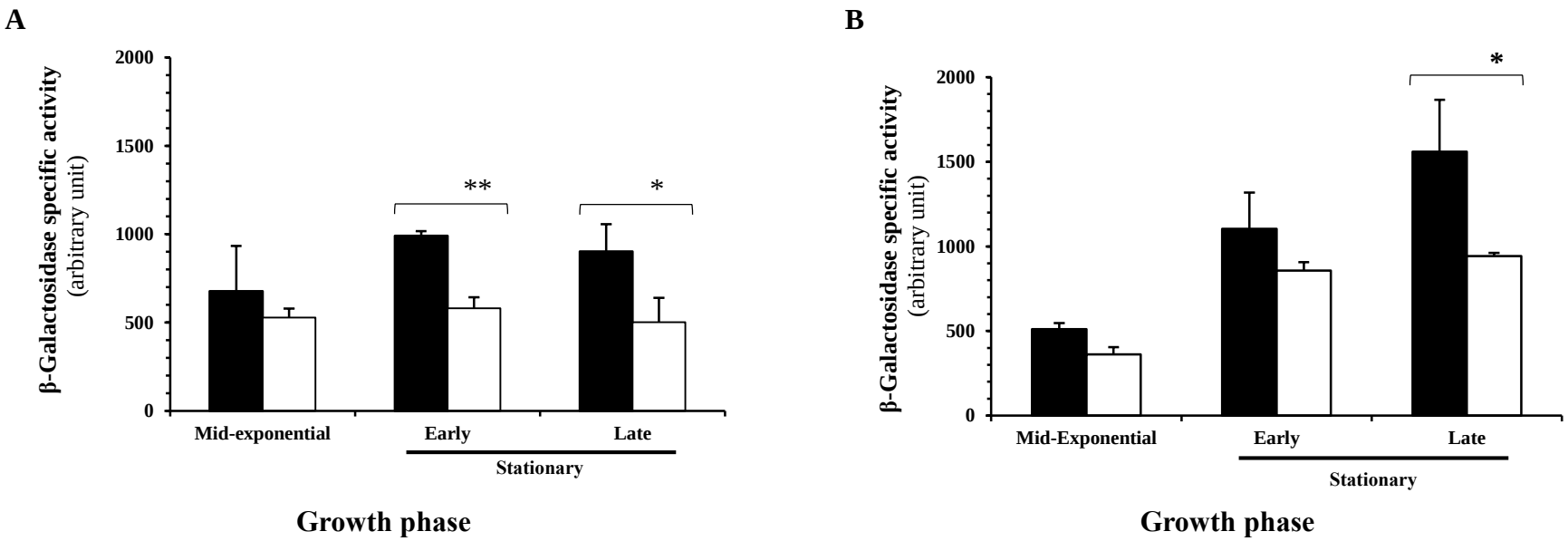
A
β-Galactosidase specific activity
(arbitrary unit)
2000
1500
1000
500
0
**
*
Mid-exponential
Early
Late
Stationary
Growth phase
B
β-Galactosidase specific activity
(arbitrary unit)
2000
1500
1000
500
0
*
Mid-Exponential
Early
Late
Stationary
Growth phase

Figure 6

Table 1

Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
E. coli strains		
XL1-blue	*endA1 gyrA96* (Nal^R) *thi-1 recA1 relA1 lac glnV44 hsdR17*(r_{K-} m_{K+}) F' [::Tn*10* (Tet^R) *proAB*$^+$ *lacI*^{q} *ZΔM15*]	Stratagene
S. agalactiae strains		
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)
Plamids		
pTCV-*lacZ*	Promoter probe plasmid carrying the *ermB* gene (Ery^R) and a *lacZ* gene devoided of a promoter	Poyart and Trieu-Cuot, (1997)
pTCV-P_1P_{murB}::*lacZ*	pTCV-*lacZ* containing a 550 bp region upstream the start of the *murB* gene of *S. agalactiae* SA87 in the *Eco*RI/*Bam*HI site upstream of *lacZ*	This study
pTCV-P_4P_{murB}::*lacZ*	pTCV-*lacZ* containing a 550 bp region upstream the start of *murB* gene of *S. agalactiae* S10-201 in the *Eco*RI/*Bam*HI site upstream of *lacZ*	This study

Table 2
Primers used in this study

Name [a]	Sequence [b]	Location [c]
Primers used for RT-PCR		
$SK1_{fw}$	CCAATTATTTGGAGAAAGAGGCTAA	nt 1110 to 1182 of IS*1548 tnp* (strain S10-201)
$SK2_{fw}$	GCACAAAAGGATGAAGAGAAAGT	nt -156 to -134 upstream of *murB* (strain S10-201)
$SK3_{fw}$	ATGTATCGTCAACAACCTATATTTTTGG	nt -101 to -74 upstream of *murB* (strain S10-201)
$SK4_{fw}$	ACACAATAAAACAGAATTGAGATAAAGGT	nt -30 to -2 upstream of *murB* (strain S10-201)
$SK5_{rv}$	TTGAGTTCACCCTGTGGTGT	nt 476 to 457 of *murB* (strain S10-201 and SA87)
$SK15_{fw}$	TAGGAGAAGTTCATTATTTCAAGCC	nt 455 to 479 of *folK* (strain SA87)
$SK16_{fw}$	GCGTGAGACATGAGCACTG	nt 248 to 266 of *folK* (strain SA87)
$SK17_{rv}$	GCGATAGTTCAAGGCGATTC	nt 136 to 117 of *murB* (strainSA87)
$SK18_{fw}$	CGCTATTTATGAAACAGCTGCT	nt 114 to 135 of *folK* (strain SA87)
$SK19_{rv}$	AGGCGATTAAGTTGGGTAACG	nt 68 to 48 of the *spoVG-lacZ* fusion (pTCV-*lac*)
Primers used for transcriptional fusion		
$SK11_{fw}$	ATTTA**GAATTC**GTATTGGGATGACTCGTAA CACGATTGATAAGGATGGTC	nt 785 to 823 of IS*1548 tnp* (strain S10-201)
$SK12_{fw}$	AAAAT**GAATTC**TCATTTGCAAAAGACAAAA GTCGCGCAAATTTC	nt 81 to 113 of *folK* (strain SA87)
$SK13_{rv}$	TTA**GGATCC**CTTTATCTCAATTCTGTTTTATT GTGTTTTATAGACACAAAAAATCGGTATAAAACCG	nt -4 to -61 upstream of *murB* (strains S10-201 and SA87)
Primers used for sequencing		
$Vlac\text{-}1_{fw}$	GTTGAATAACACTTATTCCTATC	nt -63 to -41 upstream of the *EcoR1* site of the pTCV-*lac* polylinker
$Vlac\text{-}2_{rv}$	CTTCCACAGTAGTTCACCACC	nt 60 to 40 downstream of the *BamH1* site of the pTCV-*lac* 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.