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Construction of isogenic mutants in *Streptococcus gallolyticus* based on the development of new mobilizable vectors

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Abstract

Streptococcus gallolyticus is an emerging cause of infective endocarditis that has been epidemiologically linked to colorectal cancer. *S. gallolyticus* is poorly transformable using electroporation and no defined mutant has been published yet. Hence, we used mobilization to introduce plasmid DNA from *Streptococcus agalactiae* into *S. gallolyticus* using the transfer origin of the conjugative element TnGBS1 (*oriT_{TnGBS1}*), followed by a classical homologous recombination technique. Two isogenic mutants of *S. gallolyticus* UCN34, one deleted for the *pil1* pilus operon and another for the sortase A gene, were constructed and characterized. This genetic tool should help in unravelling virulence mechanisms of this bacterium.

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1. Introduction

Streptococcus gallolyticus, formerly known as *Streptococcus bovis* biotype I, is present asymptotically in the gastrointestinal tract of 2.5–15% of the human population (Klein et al., 1977). In the elderly or immunocompromised persons, this commensal bacterium can turn into a deadly pathogen responsible for septicemia and infective endocarditis. Intriguingly, epidemiological studies revealed a strong association, up to 65%, between endocarditis due to *S. gallolyticus* and colorectal malignancies (Abdulmir et al., 2011; Boleij et al., 2009; Klein et al., 1977). Whether *S.*

gallolyticus infection is a cause or a consequence of colon cancer development remains to be investigated (Boleij et al., 2011). Genome analysis of strain UCN34, isolated from a patient suffering from infective endocarditis and colon cancer, revealed the existence of three pilus loci, namely *pil1*, *pil2* and *pil3* (Rusniok et al., 2010). Pili are long filamentous organelles, extending from the bacterial surface, which play key roles in adhesion and colonization of host tissues. We previously characterized the *pil1* locus, which is composed of three genes, encoding a major pilin, PilB (Gallo2178), a collagen binding adhesin, PilA (Gallo2179), and a sortase C (Gallo2177) that covalently binds pilin subunits one to another. Importantly, Pil1 pilus was shown to be involved in development of infective endocarditis in a rat model (Danne et al., 2011). At that time, due to the lack of genetic tools allowing the construction of targeted deletion mutants in

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Table 1
Bacterial strains, plasmids and primers.

Bacterial strains	Description	Reference
<i>E. coli</i> DH5 α	F' ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR</i> 17($r_k^- m_k^+$) <i>phoA</i> <i>supE</i> 44 <i>thi-1 gyrA</i> 96 (Nal) <i>relA1</i> λ^-	Invitrogen
<i>S. gallolyticus</i> UCN34	Tn916 (Tc), infectious endocarditis and colon cancer	R. Leclercq, (5)
UCN34 Δ <i>pill</i>	UCN34 with in frame deletion of the <i>pill</i> genes (<i>gallo2179</i> , <i>gallo2178</i> , <i>gallo2177</i>)	This work
UCN34 Δ <i>srtA</i>	UCN34 with in frame deletion of the <i>srtA</i> gene (<i>gallo1127</i>)	This work
<i>S. agalactiae</i> NEM316	MLST-23, serotype III isolated from neonate blood culture	(11)
Plasmids	Description	Reference
pG1	Em; <i>oriR</i> pUC, <i>oriR</i> ^{ts} pWV01; MCS pUC18	Poyart and Trieu-Cuot, unpublished
pTCVerm	Em, Km, Mob+ (IncP); <i>oriR</i> pACYC184; <i>oriR</i> pAM β 1 (low copy number); MCS <i>lacZ</i> α +	Poyart and Trieu-Cuot, unpublished
Primers	Sequences 5' $\rightarrow$ 3' ^a	Amplified fragment
<i>oriT</i>_{TnGBS1} cloning		
CD65	CGAAGCTTCGGTAAACTCTATGTTTCAGAT	769 pb
CD66	CGAAGCTTCGATGGTCCGATGTCAATTTC	769 pb
UCN34Δ<i>pill</i>		
CD Δ <i>pill</i> -5'	CGGAATTCTCAACACACCAAGGGAG	942 pb
CD Δ <i>pill</i> -5' ^c	GTAATCTGCGGTAAACATTTGGATCCCCGGGTACCACTTTATCACTCCCTTT	942 pb
CD Δ <i>pill</i> -3' ^c	AGGGAGTGATAAAGTGGTACCCGGGGATCCAAATGTTTACCGCAGAT	977 pb
CD Δ <i>pill</i> -3'	CCCGGATCCACCATATGAGCCGTGAC	977 pb
UCN34Δ<i>srtA</i>		
CD111	GCGAATTCACCTTGTGTGAATAGTCTTAAC	878 pb
CD112	CTGGAGAAGACCTAGAGTAGTGGTAAAGCTGGGACAACCCACAGC	878 pb
CD113	GCTGGGGTGTGCCAGCTTTTACCACTACTCTAGGTCTTCTCCAG	855 pb
CD114	GTGGATCCGTATCAACAAGCACTCATGAC	855 pb
Complementation		
pTCVermΩPtet-<i>pill</i>		
CD <i>pill</i> -Bam	ATTAGGGATCCAGGGAGTGATAAAGTGGTTGCT	4752 pb
CD <i>pill</i> -Xba	GCATTCTAGATACCGTCGCCCAAACAGT	4752 pb
Transcriptional analyses		
gallo2178-fwd	ACTGTTGAGAACGGTGGTAGTGGA	125 pb
gallo2178-rev	GTTTGACCAGCTGTAGTGATGCCA	125 pb
gallo2179-fwd	CACTATTGAGGTCACCTGGTCGAT	173 pb
gallo2179-rev	CCCACCTGATACATTTTCCATTG	173 pb
gallo16SRNA-fwd	CAGGTCTTGACATCCCGATGCTAT	169 pb
gallo16SRNA-rev	CGCTAGAGTGCCCAACTGAATGAT	169 pb

^a Restriction sites are in bold.

S. gallolyticus, we used recombinant lactococcal strains expressing or not the *pill* locus (Danne et al., 2011).

The incidence of natural transformation varies greatly among the six recognized groups of species within the genus streptococcus. Many species in the *Streptococcus anginosus*, *Streptococcus mitis*, and *Streptococcus salivarius* groups are recognized as naturally transformable (Martin et al., 2006). For the *S. bovis* group of species, only a single case of natural genetic transformation was reported for *S. bovis* strain JB1 (Mercer et al., 1999). However, peptide-induced competence was recently reported in two species of the *S. bovis* group (*Streptococcus infantarius* and *Streptococcus macedonicus*) (Morrison et al., 2013). Our attempts to transform *S. gallolyticus* using various protocols of electroporation were very poorly efficient and plasmid-dependent. We took advantage of a recent observation that transposon TnGBS1 is able to transfer from *Streptococcus agalactiae* to *S. gallolyticus* (Guerillot et al., 2013) to construct a mobilizable vector containing the origin *oriT*_{TnGBS1} for the construction of deletion mutants.

In this study, we developed a new strategy to transfer plasmids into *S. gallolyticus* strains and construct targeted

deletion mutants in the *pill* operon (*gallo2179-2178-2177*) and in the *srtA* gene (*gallo1127*). Phenotypic analyses of Δ *pill* and Δ *srtA* are presented as examples.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

Bacterial strains, plasmids and oligonucleotide primers used in this work are listed in Table 1. *S. gallolyticus* and *S. agalactiae* strains were grown at 37 °C in Todd-Hewitt (TH) broth in filled flasks under static conditions. Erythromycin was used at 10 μ g/mL; for *S. gallolyticus* selection after conjugation, tetracycline was added at 10 μ g/mL.

2.2. Construction of the mobilizable pG1 and pTCVerm vectors

The *oriT*_{TnGBS1} was cloned into the thermosensitive shuttle plasmid pG1 related to pGhost5 (Biswas et al., 1993) (Table 1). pG1 was digested with *Hind*III, 5'-overhangs were filled

in using T4 DNA polymerase and dephosphorylated. The *oriT_{TnGBS1}* blunt-ended product amplified with Phusion High Fidelity DNA Polymerase was phosphorylated with T4 polynucleotide kinase and cloned into pG1. Similarly, the *oriT_{TnGBS1}* has been cloned into pTCVerm as described above into the *EagI* restriction site.

2.3. Mating experiments

pG1-*oriT_{TnGBS1}* and its derivatives were introduced into *S. agalactiae* NEM316 by electroporation as described (Biswas et al., 1993). Conjugation between *S. agalactiae* NEM316 carrying pG1-*oriT_{TnGBS1}* and *S. gallolyticus* UCN34 was realized as described (Guerillot et al., 2013). Briefly, 6 ml of overnight cultures of donor and recipient strains were diluted in 30 mL of TH broth each, supplemented with erythromycin 10 µg/mL for *S. agalactiae* NEM316 pG1-*oriT_{TnGBS1}* strain, and incubated at 30 °C until reaching exponential phase ($OD_{600nm} = 0.6$). Then cultures were centrifuged and pellets were recovered in 150 µl of TH broth. The donor and recipient strains were mixed together on hydrophobic edge membrane filter (Millipore, Billerica, MA) with a donor-to-recipient ratio of 1:1. After incubation at 30 °C for 8–16 h, bacteria were gently recovered in 500 µl of TH broth and spread on a TH agar plate supplemented with erythromycin and tetracycline at 10 µg/mL each and then incubated at 30 °C for 36 h to select the *S. gallolyticus* transconjugants that have received the plasmid pG1-*oriT_{TnGBS1}*. Of note, the *S. gallolyticus* UCN34 strain harbours Tn916 and is naturally resistant to tetracycline but not *S. agalactiae* strain NEM316. The conjugative transfer efficiencies were obtained by dividing the number of transconjugants by the number of donor cells after the mating period.

2.4. Construction of mutants

The primers used to carry out chromosomal in-frame deletions of the *pill* operon (*gallo2179-2178-2177*) and of the *srtA* gene (*gallo1127*) are listed in Table 1. These deletion mutants were obtained using splicing-by-overlap extension PCR as described (Biswas et al., 1993; Dramsi et al., 2006). Briefly, 1 kb-fragments generated by PCR corresponding to 5' and 3' end of the chromosomal region to delete, here *pill* or *srtA*, were digested by the restriction enzymes *EcoRI/KpnI* and *BamHI/PstI*, respectively, and cloned into pG1-*oriT_{TnGBS1}*. The resulting recombinant vector pG1-*oriT_{TnGBS1}*- Δ *pill* or pG1-*oriT_{TnGBS1}*- Δ *srtA* were introduced by electroporation into *S. agalactiae* NEM316 (2–5 µg DNA) and the plasmid was transferred into *S. gallolyticus* UCN34 by mobilization. UCN34 cells in which pG1-*oriT_{TnGBS1}*- Δ had integrated into the chromosome were selected by growth of the transformants at 38 °C in TH in the presence of erythromycin 10 µg/mL. Integrants were serially passaged in TH broth at 30 °C without antibiotic to facilitate the excision of the plasmid pG1-*oriT_{TnGBS1}*- Δ by homologous recombination, resulting either in gene deletion or back to the WT gene (bWT). In-frame deletions were identified by PCR and confirmed by sequence analysis on chromosomal DNA.

2.5. Mutant complementation

For complementation of the Δ *pill* mutant, the three genes constituting the *pill* operon (*gallo2179-2178-2177*) were amplified using the Phusion High-Fidelity DNA polymerase from UCN34 and cloned into the low-copy number mobilizable plasmid pTCVerm-*oriT_{TnGBS1}* under the control of a strong constitutive promoter *Ptet* (promoter of tetracycline resistance gene *tet(M)* from the conjugative transposon Tn916) resulting in the plasmid pTCVerm-*oriT_{TnGBS1}*-*Ptet-pill* (Table 1).

2.6. Immunogold electron microscopy

For scanning electron microscopy (SEM) analysis, bacteria were collected after overnight growth, fixed and stained with rabbit anti-PilB IgG followed by anti-rabbit secondary antibody conjugated to 10 nm colloidal gold as described (Kontogiorgi et al., 2009).

2.7. Cell wall protein extracts and Western-blot

Bacteria were grown in TH medium at 37 °C and harvested for protein analysis during late exponential phase of culture. Cell wall extracts were prepared as described (Dramsi et al., 2006). For analysis of PilB expression by Western blot, cell wall proteins were boiled in Laemmli sample buffer, resolved on Tris-Acetate Criterion XT gradient gels 4–12% SDS-PAGE gels and transferred to nitrocellulose membrane (Trans-Blot Turbo Transfer Pack, Bio-Rad). PilB was detected using specific polyclonal antibodies and horseradish peroxidase (HRP)-coupled anti-rabbit secondary antibodies (Zymed) and the Western pico chemiluminescence kit (Pierce). Image capture and analysis were done on GeneGnome imaging system (Syngene).

2.8. Quantitative reverse transcriptase polymerase chain reaction

Total RNA (15 µg) were extracted and treated as described (Dramsi et al., 2006). Quantitative RT-PCR analysis was performed as previously described (Dramsi et al., 2006) with gene-specific primers (Table 1).

2.9. Adherence assay

For adherence assay, 96-well polystyrene plates were coated with 0.1 mg/mL collagen I (rat tail; BD Biosciences) diluted in phosphate-buffered saline (PBS) overnight at 4 °C. Overnight cultures of *S. gallolyticus* strains grown in TH broth were washed and resuspended in phosphate-buffered saline (OD_{600} 2 per mL), and 100 µL of cell suspension was dispensed into 96-well plates and incubated at 37 °C for 2 h. After three harsh washings with PBS, adherent bacteria were stained with 0.1% crystal violet as described (Dramsi et al., 2006). The assay was performed in quadriplate and repeated in 3 independent experiments. Asterisks represent *P* values (***P* < 0.05) using a Mann–Whitney test.

3. Results and discussion

3.1. Obtention of isogenic $\Delta pill$ and $\Delta srtA$ deletion mutants in *S. gallolyticus* UCN34

As shown previously, the transposon *TnGBS1* is able to transfer from *S. agalactiae* NEM316 to *S. gallolyticus* UCN34

at a frequency of 3.2×10^{-8} (Guerillot et al., 2013). Hence, we constructed a mobilizable pG1 vector containing the identified 731-bp long transfer origin *oriT_{TnGBS1}* to delete genes in *S. gallolyticus* by using splicing-by-overlap extension PCR as described (Biswas et al., 1993; Dramsi et al., 2006). The resulting plasmid pG1-*oriT_{TnGBS1}* could be transferred by mobilization from *S. agalactiae* NEM316 to *S. gallolyticus*

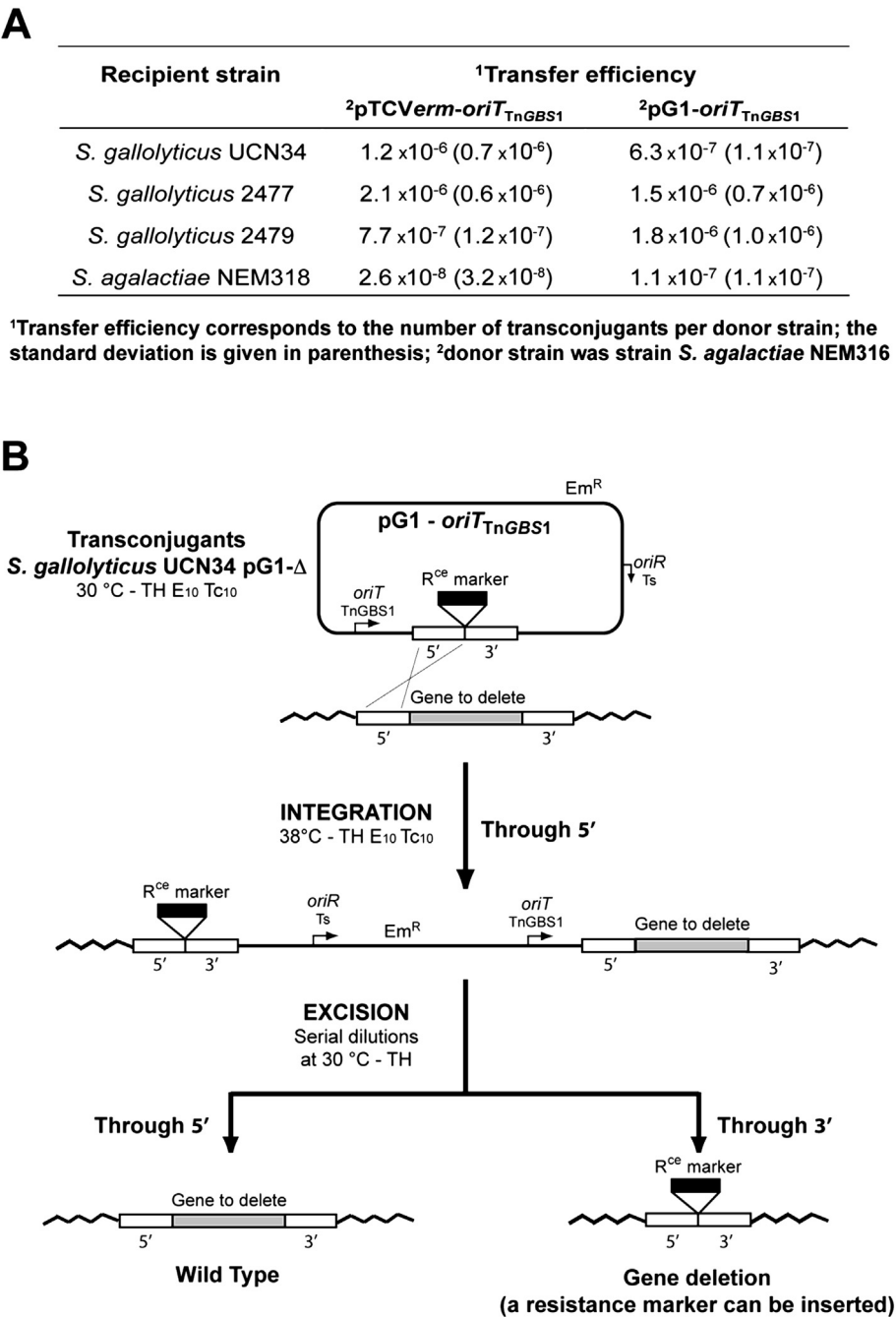


Fig. 1. Mobilization efficiencies and double homologous recombination strategy in *S. gallolyticus*. (A) Mobilization efficiency (number of transconjugants per donor cell) of pTCVerm-*oriT_{TnGBS1}* and pG1-*oriT_{TnGBS1}* between *S. agalactiae* NEM316 and different recipient strains. The standard deviations for three independent experiments are given in parenthesis. (B) General strategy for double homologous recombination. The first recombination event (integration by simple crossing-over) was selected at 38 °C, i.e. a non- permissive temperature for replication (*ori^{ts}*) in gram-positive bacteria, in the presence of antibiotics. Integrants were re-isolated and cultivated at 30 °C to enable plasmid replication, a feature that stimulates the recombination event leading to plasmid excision (Noirot et al., 1987). Theoretically, fifty percent of the excisants should display the parental genotype (back to the WT strains) and the remaining clones the expected deletion. Symbols: straight line, plasmid backbone; wavy line, chromosome; white boxes, regions of homology between the chromosome and the plasmid; grey boxes, gene to delete; black boxes, resistance marker.

UCN34 at a frequency of 6.3×10^{-7} ($\pm 1.1 \times 10^{-7}$) (Fig. 1A). Similar values were obtained with the plasmid pTCVerm-oriT_{TnGBS1}, which allowed complementation of the mutant strains (Fig. 1A). We also transferred these two mobilizable plasmids into other *S. gallolyticus* clinical isolates (strains 2477 and 2479) and into the non-electroporable *S. agalactiae* strain NEM318 (Fig. 1A).

A schematic representation of the mutagenesis protocol is shown in Fig. 1B. Upstream (5') and downstream (3') regions of equal sizes, 0.7 kb for Δ srtA and 0.9 kb for Δ pilI were cloned in the pG1-oriT_{TnGBS1} plasmid (Fig. 1B). In *S. gallolyticus* UCN34, the excision event (second homologous recombination) occurred rapidly, after only 2 passages at 30 °C without selection pressure. PCR analyses of isolated colonies obtained from the second culture at 30 °C revealed that about 30% of the clones underwent gene deletion (mutants) and the remaining 70% returned to a WT genotype (bWT back to the WT).

3.2. Phenotypic analysis of UCN34 Δ pilI and Δ srtA mutants

Preliminary tests including growth in TH broth at various temperatures, colony morphology using Gram-staining, phase-

contrast microscopy on fresh culture did not reveal any differences between WT UCN34 and isogenic UCN34 Δ pilI and UCN34 Δ srtA mutants (data not shown). Immunogold electron microscopy confirmed the absence of PilI synthesis in UCN34 Δ pilI as shown in Fig. 2A. Then deletion of pilI was also confirmed at the transcriptional level by quantitative RT-PCR (Fig. 2B). Consistently, the bWT strain UCN34 Δ pilI_bWT expressed the same amount of pilB transcript than the WT strain. Complementation of the Δ pilI mutant with pTCVerm-oriT_{TnGBS1}-Ptet-pilI restored pilI expression at a higher level than in the WT strain as shown in Fig. 2B. The other strains Δ srtA and Δ srtA_bWT were not different from UCN34 for pilB expression (Fig. 2B). Western blot analyses of cell wall protein extracts confirmed the absence of the major pilin PilB in the Δ pilI mutant, WT level of PilB in the Δ pilI_bWT and higher level of PilB in the complemented strain (Fig. 2C left part). Similar Western blot analyses showed a reduced quantity of PilI pilus in the cell wall extracts of Δ srtA compared to WT or Δ srtA_bWT and conversely a higher amount of PilI polymers were released in the supernatant of Δ srtA compared to the parental strains (Fig. 2C). These results indicate that the housekeeping Sortase A of *S. gallolyticus* is responsible to a large extent for PilI anchoring to the peptidoglycan as previously shown in *S. agalactiae*

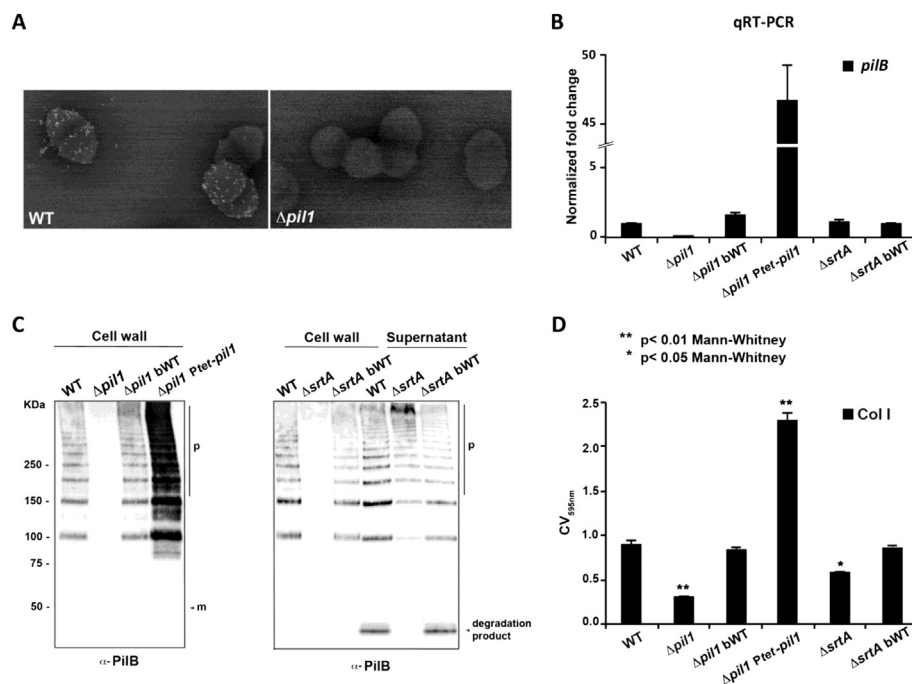


Fig. 2. Phenotypic analyses of *S. gallolyticus* UCN34 Δ pilI and Δ srtA mutants. (A) Scanning immunogold electron microscopy of *S. gallolyticus* UCN34 WT and Δ pilI strains. Pili were revealed with an anti-PilB polyclonal antibody (pAb) coupled to 10 nm gold beads. (B) Quantitative RT-PCR performed on RNAs extracted from UCN34 WT, Δ pilI, Δ pilI_bWT, Δ pilI/pTCVerm-oriT_{TnGBS1}ΩPtet-pilI, Δ srtA and Δ srtA_bWT strains with primers specific to pilB gene. The expression levels were normalized using 16S rRNA as an internal standard and are indicated as the n-fold change with respect to untreated WT strain UCN34, expressed as means and standard deviations of at least three independent experiments. Asterisks represent *P* values (**P* < 0.05) evaluated using a Student *t* test. (C) Western blot analysis of cell wall protein extracts isolated from *S. gallolyticus* UCN34 WT, Δ pilI, Δ pilI_bWT and Δ pilI pTCVerm-oriT_{TnGBS1}ΩPtet-pilI strains (on the left), and of cell wall and supernatant proteins of UCN34 Δ srtA and Δ srtA_bWT strains (on the right) using anti-PilB antibody. (D) Adherence of *S. gallolyticus* UCN34 WT, Δ pilI, Δ pilI_bWT, Δ pilI pTCVerm-oriT_{TnGBS1}ΩPtet-pilI, Δ srtA and Δ srtA_bWT strains binding to immobilized collagen type I. Microtiter wells were coated with 10 μ g of collagen I, and 10^7 bacterial colony-forming units were added. The wells were washed, and bound bacteria were detected using crystal violet (CV) staining. Optical density at 595 nm (OD_{595nm}) values are presented as means $\pm$ standard deviations for 3 independent experiments performed in triplicate. Asterisks represent *P* values (***P* < 0.01, **P* < 0.05) evaluated using a Mann–Whitney test. Abbreviations: bWT, back to the WT; Col I, collagen type I; m, monomer; p, polymers.

(Konto-Ghiorghi et al., 2009). However we cannot rule out that the pilus-specific SrtC sortase, encoded by the *pilI* operon (*gallo2177*), also participates to the Pil1 pilus anchoring, as observed in *Streptococcus pneumoniae* (El Mortaji et al., 2012).

We previously showed that Pil1 conferred collagen-binding capacity to *S. gallolyticus* (Danne et al., 2011). Therefore, we tested the various mutants for their ability to bind to collagen type I on 96-wells microtiter plates using crystal violet staining (Fig. 2D). The non-piliated mutant UCN34 Δ *pilI* is strongly reduced in its ability to bind to collagen I, with an OD_{595nm} value corresponding to negative control stained with crystal violet. The back to WT strain UCN34 Δ *pilI*_bWT was similar to the parental strain UCN34 whereas the complemented strain UCN34 Δ *pilI* pTCVerm-oriT_{TnGBS1}-Ptet-*pilI* that expressed higher level of Pil1 adhered better to collagen (Fig. 2D). As expected from the western blot analysis, the UCN34 Δ *srtA* mutant was decreased in its ability to bind to collagen (Fig. 2B), but not as much as the UCN34 Δ *pilI* mutant. Indeed, some pili polymers could remain associated to the bacterial membrane even if not covalently anchored by the SrtA sortase.

4. Conclusions

This work reports the first genetic tool enabling construction of targeted deletions in *S. gallolyticus*. This should help in elucidating the molecular mechanisms of this opportunistic pathogen responsible for endocarditis and associated to colorectal cancer. Finally, these mobilizable plasmids could be used for the genetic analysis of non-transformable streptococci and enterococci.

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