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# pBfK, a new thermosensitive shuttle vector for *Streptococcus pyogenes* gene deletion by homologous recombination

S. Boukthir<sup>a,b,\*</sup>, P. Gaudu<sup>c</sup>, A. Faili<sup>b,d,e</sup>, S. Kayal<sup>a,b,d,f,\*\*</sup>

<sup>a</sup> CHU de Rennes, Service de Bactériologie-Hygiène Hospitalière, Rennes, France

<sup>b</sup> Inserm, CIC 1414, Rennes, France

<sup>c</sup> Micalis Institute, INRAE, AgroParisTech, Université Paris-Saclay, Jouy-en-Josas, France

<sup>d</sup> OSS- Oncogenesis, Stress, Signaling, INSERM 1242, Rennes, France

<sup>e</sup> Université de Rennes, Faculté de Pharmacie, Rennes, France

<sup>f</sup> Université de Rennes, Faculté de Médecine, Rennes, France

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## ABSTRACT

*Streptococcus pyogenes*, or Group A *Streptococcus* (GAS), is a major human pathogen for which genetic manipulation remains an ongoing challenge. We created a new temperature-sensitive plasmid pBfK expressing spectinomycin resistance adapted to homologous recombination procedure to perform a complete gene deletion in GAS. Herein the mutagenesis strategy with pBfK was performed in a highly virulent GAS *emm3* genotype.

## 1. Introduction

*Streptococcus pyogenes* or Group A *Streptococcus* (GAS) is an important human pathogen causing a wide range of pathology from simple carriage to life-threatening infections [1]. Although studied for a long time, many aspects of GAS pathophysiology remain unclear, and investigation of GAS pathogenesis still requires genetic manipulation. Most clinical isolates from invasive infections are virulent strains and are often challenging to manipulate genetically [2]. Generating mutants by homologous recombination allowed the construction of an isogenic mutant with no polar effects on the expression of other genes. Thus, the differences observed between the two strains with the same genetic background can be attributable to the deleted gene. They can be used in various experimental models, whether *in vitro*, *ex vivo* with human tissues or *in vivo* using animal models. The crossing-over method reported herein using a new thermosensitive shuttle vector in GAS was adapted from the procedure recently described by Barnett *et al.* [3]. In the present work, we constructed a new thermosensitive plasmid adapted to generate in two weeks bacterial mutant by homologous recombination procedure. We tested its effectiveness to create isogenic mutants with a complete gene deletion from a virulent *emm3*-genotype strain, one of the most prevalent GAS genotypes in high- and middle-income countries [4]. We targeted the *grab* gene for the homologous recombination procedure because we believe it plays a crucial role in the pathophysiological process of invasive skin infections.

\* Corresponding author. CHU de Rennes, Service de Bactériologie-Hygiène Hospitalière, Rennes, France.

\*\* Corresponding author. CHU de Rennes, Service de Bactériologie-Hygiène Hospitalière, Rennes, France.

E-mail addresses: [sarah.boukthir@univ-rennes.fr](mailto:sarah.boukthir@univ-rennes.fr) (S. Boukthir), [samer.kayal@univ-rennes.fr](mailto:samer.kayal@univ-rennes.fr) (S. Kayal).

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## 2. Materials and methods

### 2.1. Plasmids

We used pBSU101 plasmid [5], and the temperature-sensitive plasmid pBR322-pGhost8 [6]. All DNA plasmids, before and after construction, were amplified in *E. coli* DH5 $\alpha$  strain before being extracted using MiniPrep kit (Qiagen), according to the manufacturer's instructions.

### 2.2. GAS strains

As a wild-type GAS strain, we used the *emm3*-genotype strain STAB11064 from our previously described collection [7]. STAB11064 was isolated in 2011 in French Brittany from a 38-year-old man with necrotizing fasciitis. Briefly, this strain was identified by Matrix-Assisted Laser Desorption Ionisation-Mass Spectrometry (MALDI-TOF MS, Bruker Daltonics GmbH, Germany), stored at  $-80^{\circ}\text{C}$ , and subcultured at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  on Columbia blood agar plates containing 5% sheep blood (Biorad<sup>TM</sup>, France) before performing any experimental procedure. Bacteria were then cultured in Todd Hewitt medium supplemented with 0.2% yeast extract (THY) and spectinomycin (SPC) (S4014, Sigma-Aldrich<sup>TM</sup>) when required. All bacterial cultures of GAS were performed with 5%  $\text{CO}_2$  at indicated temperature during the crossing-over procedure.

### 2.3. Nucleic acids extraction and PCR protocols

Genomic DNA (gDNA) of bacteria was extracted using InstaGene<sup>TM</sup> Matrix (Bio-Rad), according to the manufacturer's instructions. SPC resistance gene (*spc*) was amplified by PCR from the pBSU101 plasmid [5] by using the primers: *spc*-F and *spc*-R (Table 1) with the following PCR protocol: 35 cycles  $98^{\circ}\text{C}$ -20 s;  $58^{\circ}\text{C}$ -20 s;  $72^{\circ}\text{C}$ -1 min 30 s.

The two fragments A (541 bp) and B (519 bp), flanking the upstream and the downstream part of the entire *grab* gene, were amplified with the sets of primers, P1-F/P1-R and P2-F/P2-R respectively (Table 1). Those two fragments had 50 bp overlapping sequence identity between the 3' end of the "A fragment" and the 5' end of the "B fragment". The merged sequence of A and B fragments ( $\Delta$ *grab*) was obtained by performing a new PCR using P1-F and P2-R primers.

Transformed GAS isolates selected on SPC resistance were checked for the presence of pBFK plasmid by performing a specific PCR for the shuttle vector (primers: pBFK-F and pBFK-R, Table 1). We checked the allele profile (wild-type or mutant) on GAS strains after homologous recombination and plasmid excision by PCR (primers mut-F and mut-R, Table 1; PCR protocol: 35 cycles  $98^{\circ}\text{C}$ -20 s;  $58^{\circ}\text{C}$ -20 s;  $72^{\circ}\text{C}$ -2 min).

### 2.4. DNA sequence analysis

Sequence analysis was performed using an Applied Biosystems BigDye<sup>TM</sup> terminator kit (BigDye Terminator v1.1 cycle sequencing kit, ThermoFischer), and the ABI Prism 3500 Genetic Analyzer (ThermoFischer), according to the manufacturer's instructions.

### 2.5. Bacterial transformation

We prepared chimiocompetent *E. coli* and performed their transformation as described elsewhere [8]. Electrocompetent GAS were prepared as described by Le Breton et al. [2] and transformation was performed using a pulser (Gene Pulser Xcell Electroporation System, Biorad<sup>TM</sup>, France; 2.1 kV, 200  $\Omega$  and 25  $\mu\text{F}$  in a 0.2 cm cuvette).

**Table 1**  
Primers sequences.

Primer name	Sequence
spc-F	5'-CTCTCAATTAGTAGTGTATTTTCC-3'
spc-R	5'-TAACACTACTAATTGAGAGAAGTTTC-3'
P1-F	5'-TCTAGGATCCTCTAGTGACTGCTGGCTAA-3'
P1-R	5'-CTAACGCACCTGTACTTACCATCCAAAAGCTGATCTACGC-3'
P2-F	5'-GCGTAGATCAGCTTTTGGATGGTAAGTACAGGTGCGTTAG-3'
P2-R	5'-CTTCGAATTCCTTCTATTGCCCACTGACCT-3'
pBFK-F	5'-TAAACAAATAGGGGTCCGC-3'
pBFK-R	5'-AGTCCGTAAATCGACTGGC-3'
mut-F	5'-CAACAGCCATTAACGCCCTT-3'
mut-R	5'-TTAGCTTCTGGCTGGC-3'

### 3. Results

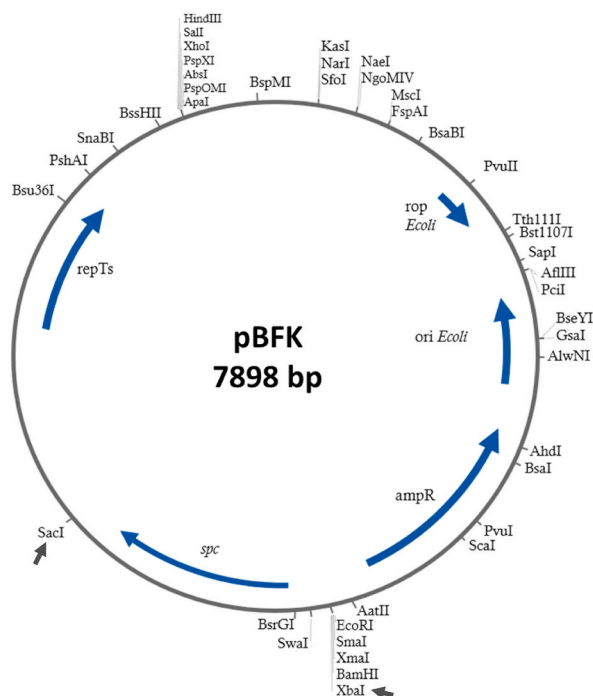
#### 3.1. Construction of the thermosensitive plasmid pBFK

Aiming to perform gene deletion on GAS isolates by homologous recombination, we wanted to build a plasmid with SPC-resistance gene which could be used in all GAS strains. At first, we based on the temperature-sensitive plasmid pBR322-pGhost8 [6] that has been used in Gram-positive bacteria like *Lactococcus lactis* and *Streptococcus agalactiae* but not in GAS [9]. In contrast to other plasmids used for homologous recombination with a growth temperature of 30 °C like the plasmid pLZT recently used in GAS [3], this plasmid has the advantage of being easily amplified at 37 °C in *E. coli*. Moreover, some plasmids used for crossing-over procedure like pJRS236 [10] carry erythromycin-resistance gene and therefore, cannot be used in some erythromycin-resistant GAS strains.

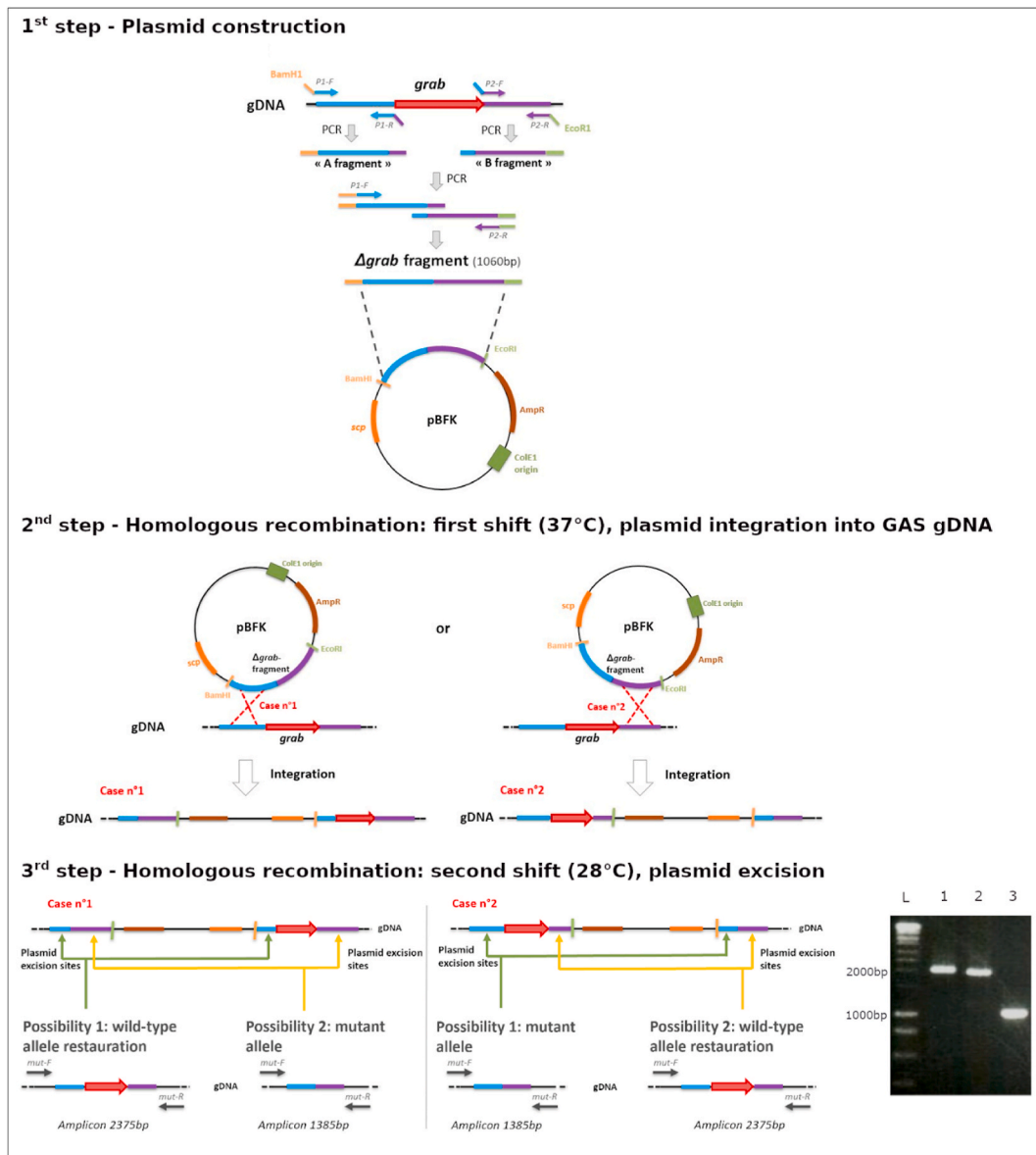
Originally, pBR322-pGhost8 expresses ampicillin-resistance (*amp<sub>E.coli</sub>*) in Gram-negative bacteria, and tetracycline resistance (*tet* gene originated from pT181 plasmid isolated in *Staphylococcus aureus* [11]) in Gram-positive bacteria. Since tetracycline resistance is frequently expressed in clinical GAS isolates [12], our first purpose was to replace *tet* gene of pBR322-pGhost8 by *spc* gene, which confers SPC-resistance when expressed in Gram-positive bacteria. The *spc* gene was formerly amplified from the pBSU101 plasmid [5], and introduced in pBR322-pGhost8 plasmid between *Xba*I and *Sac*I restriction sites as illustrated in Fig. 1. The new plasmid was designated as pBFK. Then, to check that plasmid can express in GAS strains including STAB11064 strain, we transformed GAS strains with 1 µg of plasmids and cultured on THY agar supplemented with SPC (120 µg/ml) at 28 °C, 5% CO<sub>2</sub> for three days. The presence of plasmid on all SPC-resistant colonies has been confirmed by performing a specific PCR for the shuttle vector (primers used: pBFK-F and pBFK-R, Table 1).

#### 3.2. GAS isogenic mutation by homologous recombination using pBFK plasmid

We then aimed to generate a *grab*-deleted isogenic mutant from the invasive clinical GAS isolate (STAB11064). Firstly, the fragment Δ*grab* (see “Materials and methods” and Fig. 2) was inserted into the vector pBFK, after an enzymatic digestion by *Bam*HI and *Eco*RI (as shown in Fig. 2). 1 µg of the pBFKΔ*grab* construct was used to transform electrocompetent STAB11064 strain (see “Materials and methods”). After 3 days of culture at 28 °C under SPC selection, resistant colonies were then cultured into THY broth with SPC for two successive 18–24 h subcultures (37 °C, 5% CO<sub>2</sub>, overnight) to promote plasmid integration by homologous recombination before plating on THY plate with SPC (37 °C, 5% CO<sub>2</sub>, overnight). Crossing-over was verified by PCR and sequence analysis on gDNA using paired primers hybridizing on bacterial chromosome and on a plasmid sequence respectively to ultimately amplify the plasmid-chromosome junction. Among 16 SPC-resistant GAS isolates, all integrated plasmid by crossing-over with 43% (7/16) of isolates made homologous recombination by the “A fragment” and 57% (9/16) by the “B fragment” as represented in the Fig. 2.



**Fig. 1.** Restriction map of pBFK thermosensitive plasmid used for homologous recombination in GAS strain. *spc*: spectinomycin-resistance gene expressed in Gram-positive bacteria, *ampR*: ampicillin-resistance gene expressed in Gram-negative bacteria. Black arrows represent the restriction sites used for resistance-marker replacement.



**Fig. 2.** Successive steps conducted to isogenic mutant construct. Primers are represented by small arrows with homologous regions defined by the same colour. Electrophoresis gel on the right of the third step represents amplicons obtained after PCR on GAS gDNA after plasmid excision from different strains (indicated by the number on the top of the gel): 1: STAB11064 (control), 2: Isolate with the wild-type allele, 3: Strain with the mutant allele (*grab* deleted). The letter “L” indicates the ladder used with amplicon sizes indicated on the left of the gel.

The last step consisted of plasmid curing. To this end, each crossing-over positive cell was successively subcultured for three days (28 °C, 5% CO<sub>2</sub>) in antibiotic-free THY broth (without SPC), before being plated on antibiotic-free THY agar with the same culture conditions. Each isolated colony was peaked on THY agar with and without SPC to finally selected only SPC sensitive clones. From colonies sensitive to SPC, proving plasmid excision could result in a wild-type allele restoration or a mutant allele acquisition. We checked for the mutated allele by PCR and the size of the amplicon: an amplicon of 2375bp was obtained if the wild-type allele was restored, and an amplicon of 1385bp corresponded for the mutant allele (Fig. 2). Thus, each SPC-sensitive isolate with an amplicon of 1385bp (n = 9/27 i.e. 33% of mutant frequency obtained) was then sequenced to verify the acquisition of mutant allele (n = 9/9).

Finally, to attest the absence of fitness modification in bacteria, we evaluated their growth curve *in vitro* by OD<sub>600</sub> measures (BioSpec-Mini Spectrophotometer, Shimadzu Biotech), and both curves were superimposable (mean generation time was 0.53 h [0.45–0.65] for the wild-type strain as described elsewhere [13] and 0.54 h [0.44–0.66] for the isogenic mutant, results were obtained from triplicate of three independent experiments, see growth curves in the Supplementary figure).

#### 4. Discussion

In this work, we have constructed the pBFB plasmid for creating mutants in Gram-positive bacteria by homologous recombination that, in theory, is useable for all transformable GAS. Also, in contrast to currently described protocols for homologous recombination in GAS (2), the method we described requires a smaller amount of purified plasmid for GAS; 1 µg versus 300–500 µg as described by LeBreton et al. [2] and a shorter insert A and B (500bp) for the homologous recombination (1000bp employed elsewhere (5)). After the bacterial transformation, the plasmid replicates at 28 °C for 3 days allowing more efficient homologous recombination (8), unlike when suicide plasmids are used.

Isogenic construction is a powerful tool widely used in microbiology to create a unique gene mutation or deficiency with no polar effects on the expression of other genes [14]. The mutant generated can be used in many experiments (eg, animal or human *ex vivo* tissues) to study the role of a protein expression in virulence and bacterial pathogenesis. Herein, we deleted *grab* gene in GAS, and no more than 2 weeks were required to inactivate a gene with our protocol. Moreover, SPC resistance has never been described for GAS species allowing the use of pBFB in any GAS strain. In contrast, main thermosensitive plasmids carry erythromycin resistance gene [15], and almost 10% of GAS strains are naturally resistant to erythromycin in our collection (personal data). Although we have not tested pBFB to construct isogenic mutants in bacteria other than GAS, the same methodology is most likely to apply to other Gram-positive bacteria.

Among GAS genotypes, the *emm-3* genotype is one of the most prevalent and most frequently associated with invasive infections, particularly cutaneous infections [4]. In a previous publication, we reported a closed genome sequence obtained by HiSeq 2000 technology of an *emm-3*-GAS genotype isolated from a non-invasive infection (STAB902), and the genomic analysis found a unique region deletion (300 bp) in *grab* gene suggesting a likely involvement during the cutaneous infectious process [16]. GRAB protein, known as a virulent factor, is covalently attached to the peptidoglycan, and is involved in GAS protection against human protease [17, 18].

Many strategies can be used to inactivate bacterial gene expression as altering the gene sequence by tagging or introducing base pair changes [19]; however, in some cases, the gene studied may limit the use of these techniques. Indeed, the presence of polymorphism within the *grab* gene can complicate or make the choice of the site of genetic modifications impossible, with unpredictable results of the mutagenesis. Thus, we have chosen a homologous recombination technique to completely delete the *grab* gene insofar as it contains six repeated motifs.

#### 5. Conclusion

GAS is an ancient bacterium, widely studied, and despite the considerable capabilities to analyse strain genomes from one decade, it remains many interrogations on its pathogenesis mechanism. Thus, targeted mutagenesis by homologous recombination is still required to study the role of gene expression in bacteria during the infectious process. The temperature-sensitive shuttle vector pBFB described here is an effective and practical tool to modify one or several GAS genes by homologous recombination.

#### Author contribution statement

Sarrah Boukthir: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. Philippe Gaudu: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. Ahmad Faili: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. Samer Kayal: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

#### Data availability statement

No data was used for the research described in the article.

#### Additional information

Supplementary content related to this article has been published online at [URL].

#### Declaration of competing interest

The authors declare no competing interests.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.heliyon.2023.e16720>.

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