Canadian Journal of Microbiology

OPEN ACCESS | Methods

Sequential markerless genetic manipulations of species from the *Neisseria* genus

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Abstract

The development of simple and highly efficient strategies for genetic modifications is essential for postgenetic studies aimed at characterizing gene functions for various applications. We sought to develop a reliable system for *Neisseria* species that allows for both unmarked and accumulation of multiple genetic modifications in a single strain. In this work, we developed and validated three-gene cassettes named RPLK and RPCC, comprising of an antibiotic resistance marker for positive selection, the phenotypic selection marker *lacZ* or mCherry, and the counterselection gene *rpsL*. These cassettes can be transformed with high efficiency across the *Neisseria* genus while significantly reducing the number of false positives compared with similar approaches. We exemplified the versatility and application of these systems by obtaining unmarked luminescent strains (knock-in) or mutants (knock-out) in different pathogenic and commensal species across the *Neisseria* genus in addition to the cumulative deletion of six loci in a single strain of *Neisseria elongata*.

Key words: *Neisseria*, deletion, gene, insertion, markerless

Résumé

Le développement de stratégies simples et hautement efficaces en matière de modifications génétiques est essentiel pour les études post-génétiques visant à caractériser les fonctions de gènes en vue de diverses applications. Les auteurs ont cherché à développer un système fiable pour les espèces de *Neisseria* qui permet à la fois l'obtention de transformants non marqués et l'accumulation de multiples modifications génétiques dans une seule souche. Dans ce travail, ils ont développé et validé des cassettes à trois gènes identifiées RPLK et RPCC, comprenant un marqueur de résistance aux antibiotiques pour la sélection positive, le marqueur de sélection phénotypique *lacZ* ou mCherry, et le gène de contre-sélection *rpsL*. Ces cassettes peuvent être transformées avec une grande efficacité dans tout le genre *Neisseria* tout en réduisant significativement le nombre de faux positifs par rapport à des approches similaires. Ils illustrent la polyvalence et l'application de ces systèmes en obtenant des souches luminescentes (knock-in) ou des mutants (knock-out) non marqués chez différentes espèces pathogènes et commensales du genre *Neisseria*, en plus de la délétion cumulative de six loci dans une seule souche de *Neisseria elongata*. [Traduit par la Rédaction]

Mots-clés : Neisseria, délétion, gène, insertion, sans marqueur

Introduction

The *Neisseria* genus consists of commensal species that reside in the mammalian mucosa, mainly in the oral cavity, but also two major human pathogens namely *Neisseria meningitidis* and *Neisseria gonorrhoea* (Hitchcock 1989; Perrin et al. 1999; Marri et al. 2010; Brynildsrud et al. 2018). *Neisseria meningitidis* causes invasive meningococcal disease with an annual global incidence of 500 000–120 000 and 10% case fatality rate (Jafri et al. 2013; Deghmane et al. 2022). *Neisseria gonorrhoea* is the causative agent of gonorrhea, a sexually transmitted disease accounting for 87 million new infections in 2016 (Rowley et al. 2019). Both species are highly related (subspecies) as they have emerged from a common commensal symbiont ancestor (Tacconelli et al. 2018). Several groups have already compared *Neisseria* species, focusing mainly on the last step of pathogenic emergence (Bennett et al. 2010; Joseph et al. 2011; Putonti et al. 2013; Maiden and Harrison 2016; Brynildsrud et al. 2018). Others look for stepwise ancestral events at different nodes of evolution that may have drastic consequences on the pathogens as we know them today (Veyrier et al. 2015; Nyongesa et al. 2022). This includes the evolutionary events not directly linked to pathogen speciation that could help clarify ecological niche adaptation, enhanced colonization, and (or) virulence of the pathogenic species. These type of studies require multiple successive genetic modifications of both commensal and pathogenic species (gain-of-function or loss-of-function). Although several molecular tools have been developed over the last decades for pathogenic *Neisseria*, only a

few attempts have been made to genetically modify commensal species (Higashi et al. 2011; Veyrier et al. 2015; Anonsen et al. 2016; Custodio et al. 2020). Advancements in molecular cloning such as the CRISPR–Cas systems have limitations, such as the associated cytotoxicity due to continuous expression of foreign CRISPR in the bacterial cells (Yan and Fong 2017; Arroyo-Olarte et al. 2021). In the case of the *Neisseria* genus, a functional endogenous CRISPR–Cas9 system has only been identified in a few species and thus requires optimization and implementation efforts to be used in other species (reviewed in Zhang 2017).

Neisseria species are naturally competent, they undergo frequent intra- and interspecies exchange of genetic material through horizontal gene transfers (HGTs). During HGT, exogenous DNA is acquired, translocated across the membranes, and eventually recombined with homologous regions of the chromosome (Frye et al. 2013; Mell and Redfield 2014). Natural competence is enhanced by the presence of factors such as type IV pili and 10–12 bp *Neisseria* specific DNA uptake sequence (DUS) repeats (Goodman and Scocca 1988). Of note, due to strong restriction barriers, the processing of large plasmids into smaller pieces, and the translocation of a single strand of DNA through the inner membrane, replicative plasmids are scarce and of little use for the genetic manipulation of *Neisseria* species (Hamilton and Dillard 2006; Budroni et al. 2011; van Dam and Bos 2012). Integrative DNA constructions are therefore preferred. Natural competence has facilitated genetic manipulation studies to obtain gene deletions, insertions, and point mutations in both pathogenic and commensal *Neisseria* species (Dillard 2011; van Dam and Bos 2012; Veyrier et al. 2015). For example, a marked gene editing strategy, consisting of an antibiotic-resistant marker flanked on both ends by short DNA sequences homologous to the upstream (5′) and downstream (3′) regions of the targeted gene, allows for targeted gene modification through double-crossover homologous recombination. Although seemingly straightforward, this approach is limited by the available antibiotic options for *Neisseria* species (Dillard 2011). The creation of unmarked mutants is advantageous because it allows for antibiotic recycling and further eliminates polar effects associated with the presence of large cassettes that may affect expression of the downstream genes in an operon (Bailey et al. 2019). Unmarked mutants are obtained through a second transformation step that introduces DNA comprising of the flanking 5′ and 3′ regions of the previously edited gene to the marked mutants, thereby removing the resistance marker and associated cassette through double homologous recombination. Screening for the correct unmarked transformants can be laborious without a system that limits the growth of false positive clones. Thus, negative selection markers such as tetracycline sensitivity *tetAR*, sucrose sensitivity *sacB*, and streptomycin sensitivity *rpsL* are employed for counterselection (Reyrat et al. 1998). These systems however have some shortfalls. For example, *tetAR* system is applicable to mostly *Escherichia coli* strains, while *sacB* system is limited by the low selection stringency and need for optimization of strain-specific selection conditions (Reyrat et al. 1998; Li et al. 2013; Li et al. 2014). On the other hand, the *rpsL* system is dependent on the dominance of the wild-type streptomycin sensitive (Sm^S) allele over the streptomycin-resistant (Sm^R) allele, and such a system requires prior genetic modification of the bacteria (Trindade et al. 2009).

There is a never-ending need for the development of new and improved methods that can be easily and cheaply employed for gene editing purposes in bacterial species. In this work, we sought to develop an efficient system for generating unmarked mutants across any *Neisseria* species. Through the use of *lacZ* (blue-white screening) or mCherry (fluorescence) in combination with antibiotic selection markers and the counterselection gene *rpsL*, we created three-gene cassettes named RPLK and RPCC and demonstrated the efficiency and applicability of these systems for genetic editing of different *Neisseria* species.

Materials and methods

Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are listed in Tables S1 and S2. *Escherichia coli* DH5α cells were cultured at 37 °C on lysogeny broth media (Difco) supplemented with either ampicillin (100 μg/mL) for pUC plasmids, kanamycin (50 μg/mL), and X-gal (20 μg/mL) for RPLK-based plasmids, or chloramphenicol (25 μg/mL) for RPCC-based plasmid transformations. *Neisseria* strains were cultured at 37 °C with 5% CO_2 on gonococcal base (GCB) agar (Oxoid) supplemented with Kellogg's supplements as previously described (Kellogg et al. 1963). When required, X-gal (20 μg/mL), kanamycin (100 μg/mL), chloramphenicol (5 μg/mL), and streptomycin (100 μg/mL) were added to the GCB agar.

Generation of streptomycin-resistant *Neisseria* strains

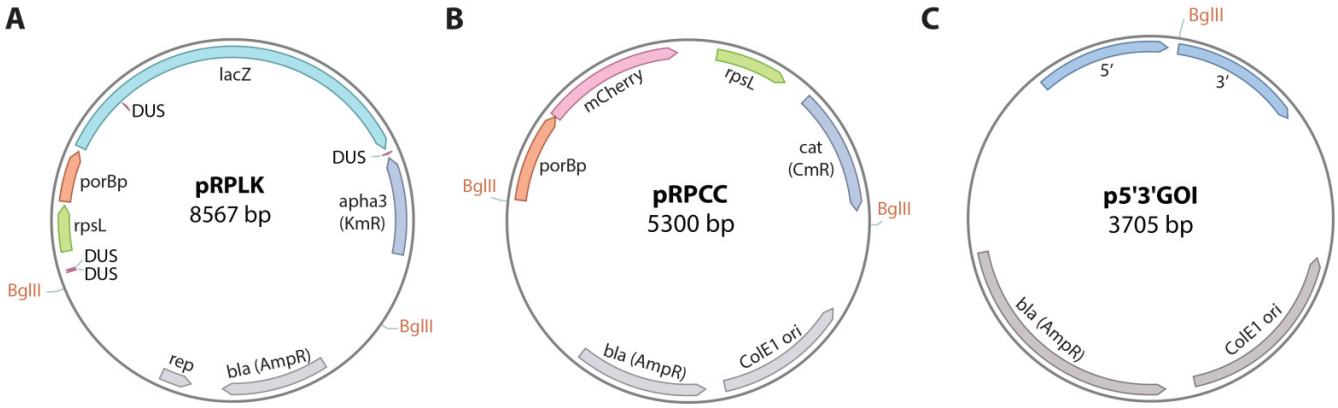
Streptomycin-resistant *Neisseria elongata* strains were obtained by plating wild-type *N. elongata* subsp. *glycolytica* (ATCC 29315) cells on GCB agar containing 20 μg/mL streptomycin for 2 days. DNA was extracted from the resulting clones, and their *rpsL* gene was amplified and sequenced using primers rpsLXbaI_F/rpsLNheI_R to confirm the streptomycin resistance mutation K43R. Subsequently, *N. meningitidis* LNP20553 and *Neisseria musculi* CCUG68283 were transformed with the resulting $rpsL^{K43R}$ PCR product as described previously (Dillard 2011; Veyrier et al. 2015). The selection of *rpsL*-mutated clones was done on GCB plates supplemented with streptomycin.

Construction of pRPLK and pRPCC plasmids

Manipulations involving DNA extraction, PCR amplification, restriction enzyme digestion, and ligation were done using standard protocols according to the manufacturers' specifications. Unless otherwise indicated, Phusion polymerase (NEB) was used for the PCR reactions. Restriction enzymes and T4 DNA ligase were purchased from NEB, while the plasmid extraction, PCR, and gel purification kits were from Qiagen. Primers used in the study are listed in Table S3.

The RPLK construct (Fig. 1A) was assembled with the wild-type *N. elongata rpsL*, the constitutive *N. meningitidis* promoter *porB*p controlling the selection markers *lacZ*

Fig. 1. Plasmids for the markerless modification of *Neisseria* species. Circular maps of (A) pRPLK (Addgene 184282) and (B) pRPCC (Addgene 184283) plasmids containing the selection–counterselection cassettes used in this study, as well as (C) p5′3′GOI plasmid for the integration of such cassettes, which is a theoretical construct containing homology regions flanking any gene of interest (GOI). BglII restriction sites are shown, which are used to extract the RPLK or RPCC cassette and subclone it into any *Neisseria* integrative plasmid, herein p5′3′GOI.



(encoding β-galactosidase) and *apha3* (encoding a kanamycin resistance protein). Promoter *porBp* was amplified from the gDNA of *N. meningitidis* MC58 using primers porBpF/porBpbluntR, *lacZ* was amplified from mini-CTX-lacZ with primers porBplacZF/lacZRKm7up, while the primers Km7up/Km6 were used to amplify *apha3* from pGEM::Km (Becher and Schweizer 2000; Veyrier et al. 2011). Purified PCR products were mixed in equimolar concentrations and fused through a subsequent PCR reaction using primers porBpF and Km6. The resulting 5.5 kb amplicon of porBp-lacZ-Km^R was gel purified, ligated to the pCR4blunt-TOPO vector (Thermo), and subsequently transformed in *E. coli* DH5α cells to generate pPCR3 plasmid. Genomic DNA from *N. elongata* was used to amplify *rspL*^{wt} together with a 250 bp intergenic region upstream containing its promoter and a DUS using primer pair rpsLXbaI_F and rpsLNheI_R. Plasmid pPCR3 and the *rspL*^{wt} amplicon were digested using NheI and XbaI–NheI restriction enzymes respectively, before ligation and transformation in *E. coli* DH5α cells to obtain pRPLK plasmid. The RPLK cassette was then extracted with BglII digestion for subcloning into *Neisseria* integrative plasmids.

The RPCC cassette was obtained by first synthesizing the *porB*p, mCherry, and *rpsL*^{wt} in the pUC57 vector (Biobasic), resulting in pUC57::RPC. The *cat* gene conferring chloramphenicol resistance was amplified by PCR with primers CmR_SpeI_F and CmR_PpuMI_R, and then inserted into the synthesized plasmid by conventional restriction-ligation with SpeI and PpuMI enzymes, generating pRPCC (Fig. 1B). Of note, in this construct, the native *rpsL* locus from *N. lactamica* was used to show the cross-species applications of our strategy.

Both plasmids have been deposited in Addgene repository (pRPLK, Addgene No. 184282; pRPCC, Addgene No. 184283).

Construction of *Neisseria* integrative plasmids for markerless modifications

Approximately 500 bp regions (5′ and 3′) flanking each gene of interest (GOI) were synthesized in pUC57 by Biobasic with a central BglII site to generate various p5′3′GOI constructs (where 5′3′GOI represent the flanking regions of each GOI) (Fig. 1C). The RPLK or RPCC cassette was then inserted at the BglII site by conventional restriction-ligation, resulting in p5′3′GOI::RPLK or p5′′3′′GOI::RPCC (Table S2). As a cheaper and sometimes quicker alternative, 5′3′ regions can be generated by overlap extension PCR with overlapping inner primers containing a BglII site, then inserted into pUC57 by blunt ligation into the EcoRV site.

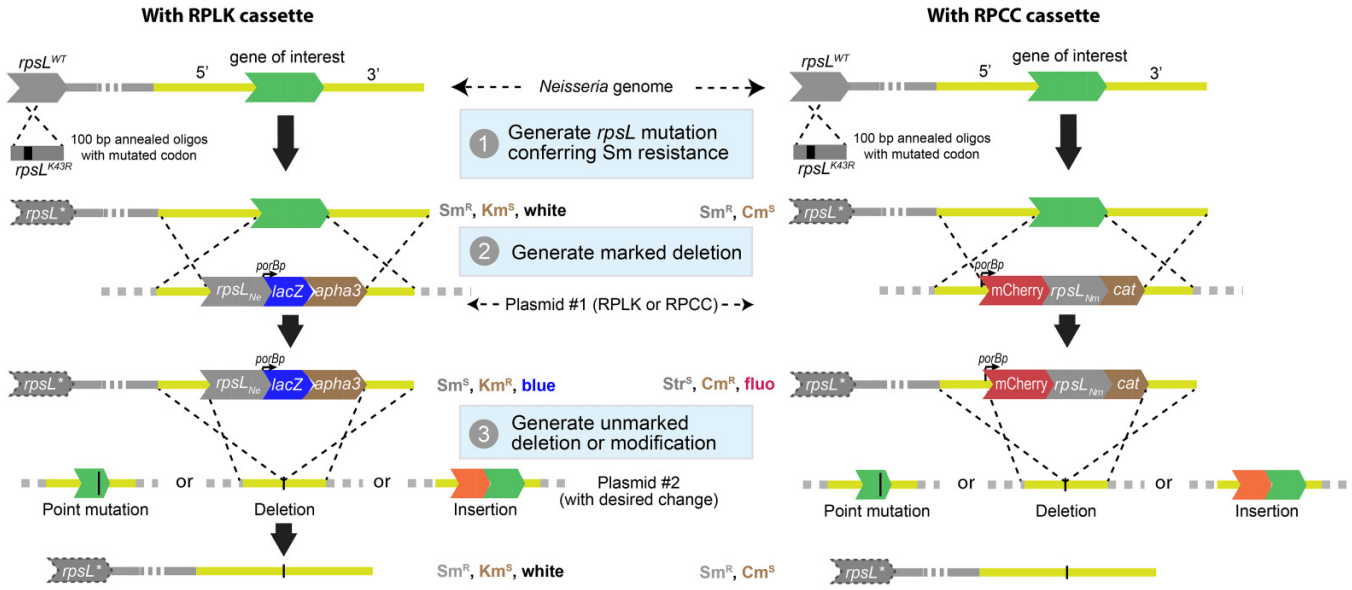
Generation of marked and markerless gene modified *Neisseria* strains

Transformations were done as previously described (Dillard 2011; Veyrier et al. 2015). Briefly p5′3′GOI::RPLK or p5′3′GOI::RPCC constructs were linearized using ScaI. Five to ten microlitres (500 ng) of the linearized plasmid DNA was deposited on a fresh streak of streptomycin-resistant *Neisseria* cells and cultured for 6 h on GCB agar plates containing 10 mmol/L $MgCl_2$. For the RPLK transformations, subculturing was done on GCB plates supplemented with Km and X_gal to obtain blue, Km^R, and Sm^S clones. For the RPCC transformations, subculturing was done on GCB plates supplemented with Cm and fluorescence was verified on a Typhoon FLA9500 imager (GE Healthcare).

To obtain unmarked deletions, p5′3′GOI without RPLK or RPCC were subsequently used to transform the marked *Neisseria* mutants, resulting in the loss of the selection–counterselection cassette (Fig. 2). When removing the RPLK cassette, transformants were selected on GCB agar supplemented with Sm and X_gal to obtain white Sm^R clones. When removing the RPCC cassette, nonfluorescent transformants were selected on GCB agar supplemented with Sm after fluorescence imaging. Unmarked mutants were verified by PCR.

Here, the markerless deletion of MtgA in *N. elongata* was done using the RPLK cassette, and verified with primers RTMtgA-F/MtgAKpnI-R, 5KOmtgA_F/3KOmtgA_R, and Km7up/Km6. Markerless deletion of BolA was also

Fig. 2. Markerless genetic modification workflow. First, the *rpsL* allele in the target *Neisseria* strain is mutated to render it streptomycin resistant (*rpsL*∗ or *rpsL*^{K43R}), which can be done by transformation with annealed oligos containing the K43R mutation. Second, a marked deletion strain is generated using a plasmid containing the RPLK (left panel) or RPCC (right panel) cassette within sequences homologous to the flanking regions surrounding the GOI or area to modify. Third, a plasmid containing the same homology regions with the desired modification in between is used to transform the previous marked strain. In this illustration, a gene deletion workflow is shown. The selection markers to use are indicated. Steps 2 and 3 can be repeated to cumulate a virtually unlimited number of modifications within a single strain. Sm, streptomycin; Cm, chloramphenicol; Km, kanamycin.



done, this time using the RPCC cassette and verified using primers BolANe_F/BolANe_R, BolA_ExtNe_F/BolA_ExtNe_R, and CmR_SpeI_F/CmR_PpuMI_R. As a species specific control for *N. elongata*, *mraZ* gene was amplified by 5MraZ_F/3MraZ_R primers in both deletions.

Multiple gene deletions were obtained by the sequential transformation of p5′3′GOI::RPLK into the previous unmarked Sm^R *Neisseria* mutant, followed by removal of the cassette. Each marked and unmarked mutant was confirmed by PCR using primers RTRapZ_R/RapZKpn1_R, Ne_PbP3_F/Ne_PbP3_R, Ne_gloB_F/Ne_gloB_R, Ne_07135_F/Ne_07135_R, RTMtgA_F/MtgAKpnI_R, RTMraZ_F/MraZKpnI_R, and Km7up/Km6.

Generation of markerless luminescent *Neisseria* strains

The luminescence operon *luxCDABE* was amplified from a luminescent mutant of *N. meningitidis* LNP24198 (Guiddir et al. 2014) using LuxCNcoIF/LuxEPstIR and cloned in p*pilE*pLuc (Veyrier et al. 2015) digested with NcoI and PstI. The *pilE*p promoter that was present in this plasmid was subsequently replaced by *N. meningitidis porB*p amplified with porBp_NheI_F/porBp_NcoI_R using NheI and NcoI to generate pporBLuxCDABE::Km. The luminescence operon along with the promoter was amplified using porBp_EcoRI_F/luxE_EcoRI_R and subcloned in pCR4blunt-TOPO with the Zero Blunt PCR Cloning Kit (Invitrogen) resulting in pCR_porbplux from which the luminescence cassette can be extracted using EcoRI. 1000 bp sequences centered on intergenic regions of *N. meningitidis*, *N. musculi*, and *N. elongata* were synthesized with MfeI and BglII restriction sites in the middle (Biobasic), resulting in pNm, pNmus, and pNelon. The BglII site was used to insert the RPLK cassette, while the MfeI site was used to insert the EcoRI-flanked luminescence cassette, resulting in plasmids pNm::RPLK, pNmus::RPLK, pNelon::RPLK, pNm::lux, pNmus::lux, and pNelon::lux. Each strain was transformed first with the RPLK-containing plasmid, followed by a second transformation with the luminescence-cassette-containing plasmid. Luminescent clones were selected by directly imaging the culture plates with an IVIS Lumina III (PerkinElmer), and their antibiotic susceptibility was assessed to confirm successful removal of RPLK.

Results

Strategy for the markerless deletion and insertion of genes

Our approach is based on three-gene cassette constructs RPLK and RPCC. These cassettes include antibiotic-resistance selection markers (Km or Cm), phenotypic selection markers (lacZ or mCherry) for blue-white screening or fluorescence selection respectively, in addition to the *Neisseria* species wild-type streptomycin sensitive *rpsL*^{wt} gene for streptomycin sensitivity selection of the mutants. The first critical step of this strategy consists of mutating the native *rpsL* locus in the tar-

get strain, making it resistant to high levels of streptomycin. This can be achieved by transforming any *Neisseria* species with 100 bp annealed oligonucleotides containing *rpsL* with the K43R mutated codon (Fig. 2), or by culturing the parental strain in gradually increasing concentrations of streptomycin and confirming the proper mutation by sequencing. The second step involves transformation of the RPLK or RPCC cassette into a streptomycin-resistant strain as demonstrated (Fig. 2). Since the selection cassettes are inserted within sequences homologous to the *Neisseria* genomic locus to be modified (5′ and 3′), a double homologous recombination results in the replacement of the gene of interest with the corresponding cassette, thus generating a marked deletion mutant. Transformed clones are easily selected by their ability to grow as blue colonies on media supplemented with kanamycin and X-gal (for RPLK), or fluorescent colonies on media with chloramphenicol (for RPCC). Because of the dominant effect of $rpsL^{wt}$ over the native $rpsL^{K43R}$ locus, the transformed clones become streptomycin sensitive, which must be verified to minimize false positives in subsequent steps. The third step of this strategy involves removing the selection cassette RPLK or RPCC and replacing it with the desired modification, thus generating markerless mutants. This is achieved by transforming the marked strain from step 2 with a plasmid harboring the same homology regions (p5′3′GOI), with or without the desired DNA sequence inside (Fig. 2). Transformants lose the RPLK cassette and are selected on media supplemented with streptomycin and X-gal as white, Sm^R and Km^S clones. False positives that have not lost the selection cassette will remain blue, avoiding the need for additional screening tests such as verifying for kanamycin sensitivity.

Gene deletion using the RPLK cassette

We used the RPLK cassette to demonstrate the viability of this approach in obtaining marked and unmarked mutants, first through the deletion of *mtgA* in *N. elongata* (Fig. 3A), a gene encoding a peptidoglycan transglycosylase. The RPLK-containing strain (marked deletion) is the only one that grew as blue clones in the presence of kanamycin and X_gal, while only the $rpsL^{K43R}$ and the markerless deletion strains could grow in the presence of streptomycin. Two blue colonies were visible on GCB media with streptomycin for the RPLK strain, indicative of natural Sm^R revertants. Correct deletion of *mtgA* was confirmed by PCR using primers amplifying within and around the *mtgA* gene, the kanamycin resistance gene and the control gene *mraZ* (Fig. 3B). Sequencing of the deletion region confirmed that no unwanted modifications were introduced during the cloning steps (Fig. S1).

Gene deletion using the RPCC cassette

To exemplify the versatility of this markerless genetic manipulation method, we designed another cassette named RPCC ($rpsL_{wt}$, $porB_p$, cat, mCherry). Instead of using *lacZ* for blue-white screening and a kanamycin resistance gene, we used a chloramphenicol resistance gene (cat) coupled to a fluorescence marker. To demonstrate the cross-species potential of our approach, the RPCC cassette contains the *rpsL* gene from *N. lactamica*. In this example, the RPCC cassette was used to delete the *bolA* gene in *N. elongata* (Fig. 4A), which encodes a putative regulator (Santos et al. 2002; Freire et al. 2009). As expected, only the marked mutant is fluorescent and Cm^R, while both the $rpsL^{K43R}$ and the unmarked deletion strains are Sm^R. Each strain was verified by PCR using a similar approach as with *mtgA* (Fig. 4B). Sequencing of the deletion region confirmed that no unwanted modifications were introduced during the cloning steps (Fig. S2).

Markerless gene insertions in *Neisseria* species (luminescent strains)

To demonstrate the use of our method for gene insertions, we introduced the 6.3 kb *porb*p-*luxCDABE* luminescence cassette into three *Neisseria* species: *N. meningitidis* LNP20553, *N. elongata* subsp. *glycolytica* ATCC29315, and *N. musculi* CCUG68283 (Fig. 5). The expression of the *lux* operon allows luminescence measurement without the need for exogenous luciferin since it encodes both the luciferase enzyme and the proteins needed to synthesize its substrate. The markerless strains obtained here emitted a persistent luminescent signal. Of note, luminescent strains of *N. meningitidis* have been previously used successfully in murine infection models to measure bacterial burden (Alonso et al. 2003; Zarantonelli et al. 2007; Bernet et al. 2020) and the markerless gene modification option is an added advantage.

Multiple markerless deletions in *N. Elongata*

The most impactful advantage of the strategy described here is the fact that it allows for unlimited genetic modifications in a single strain, since selection markers are used transiently and do not accumulate. To exemplify the endless possibilities offered by such methodology, we used the RPLK cassette to cumulatively delete six genes in *N. elongata* (Fig. 6). Starting with an $rpsL^{K43R}$ streptomycin-resistant strain, we replaced one gene at a time with the selection cassette before removing the cassette to make the strain Sm^R once again for the deletion of subsequent genes (Fig. 6A). PCRs were done at each step to confirm the presence of the cassette for marked deletions and its absence for markerless deletions (Fig. 6B).

To determine the frequency of false positives when removing the selection cassette, we quantified both white and blue streptomycin-resistant clones from two independent gene deletions (Table 1). Around one-third of the obtained Sm^R clones were false positives still carrying the RPLK cassette (blue on X-gal), supporting the necessity of adding another selection marker (*lacZ*) to the traditional two-gene cassettes often used for similar purposes.

Discussion

Strategies that allow efficient and accurate genetic modifications such as gene deletions, insertions, and point mutations are crucial for the study of protein functions. This is particularly true in the context of evolutionary studies when multiple genetic events are implicated in the emergence of novel phenotypes (Veyrier et al. 2015). In bacterial mutagenesis studies, mutants are mainly obtained by artificial transformation involving the introduction of DNA containing a

Fig. 3. *mtgA* deletion in *N. elongata* using the RPLK cassette. The methodology described above was used to generate a markerless Δ*mtgA N. elongata* strain. (A) Growth of *N. elongata* at different stages of the deletion strategy: 1, WT strain; 2, *rpsL*∗ strain; 3, RPLK deletion strain; and 4, markerless deletion strain. All plates contain X-gal. (B) PCR confirmation of the genetic manipulations of *N. elongata* throughout the strategy (left), where the colored boxes match the primers imaged on the right. The control gene is *mraZ*. Primers for *mtgA* (green): RTMtgA-F, 3KOMtgA_R. For flanks (orange): 5KOMtgA_F, 3KOMtgA_R. For Km^R (blue): Km7up, Km6. For control (black): 5MraZF, 5MraZR. No amplification is seen with flanking primers for the RPLK strain since the insert is too large for the PCR conditions we used. GCB, GC base agar; Km, kanamycin; Sm, streptomycin.

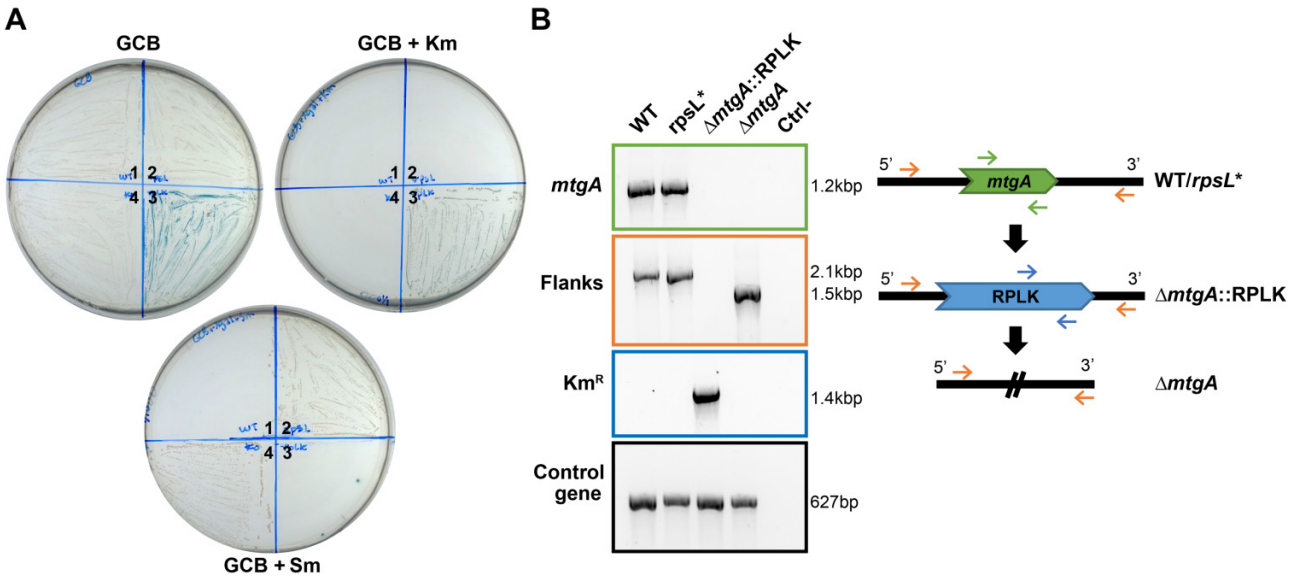


Fig. 4. *bolA* deletion in *N. elongata* using the RPCC cassette. The RPCC cassette was used to generate a markerless Δ*bolA N. elongata* strain. (A) Growth of *N. elongata* at different stages of the deletion strategy: 1, WT strain; 2, *rpsL*∗ strain; 3, RPCC deletion strain; and 4, markerless deletion strain. Plates were photographed (left panel) and imaged for fluorescence with a Typhoon FLA9500 imager (right panel). (B) PCR confirmation of the genetic manipulations of *N. elongata* throughout the strategy (left), where the colored boxes match the primers imaged on the right. The control gene is *mraZ*. GCB, GC base agar; Cm, chloramphenicol; Sm, streptomycin.

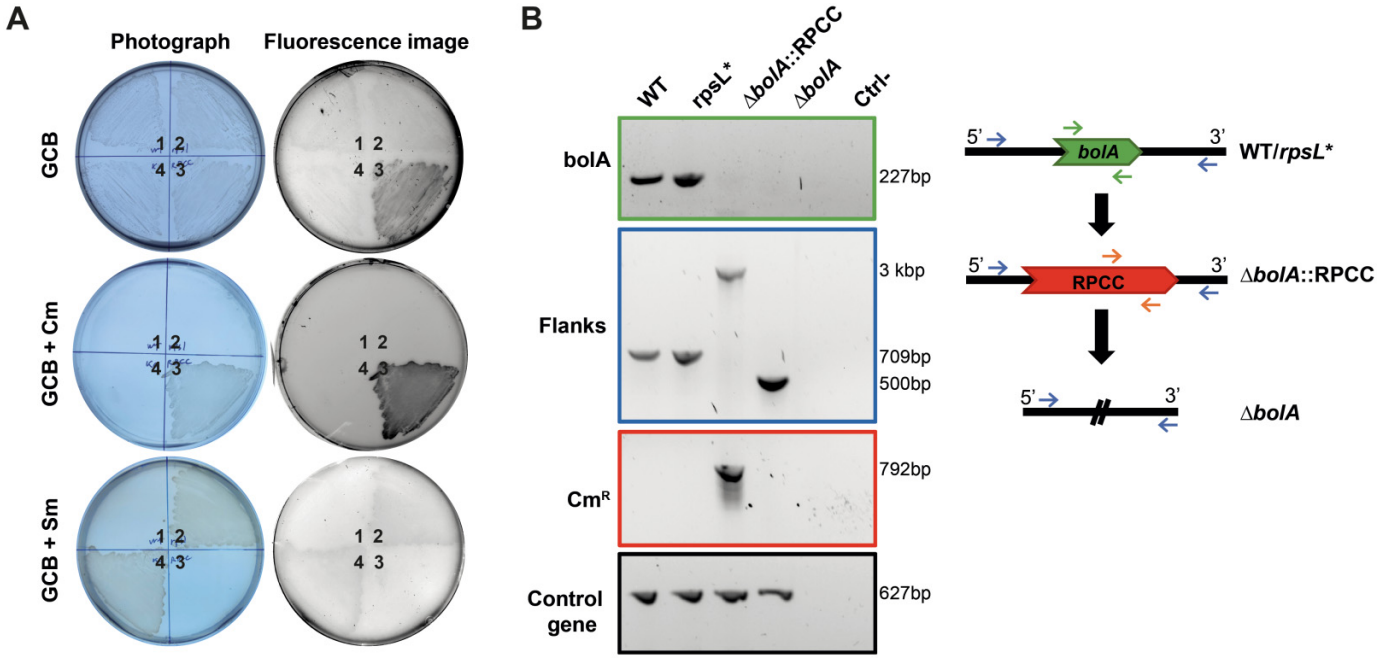
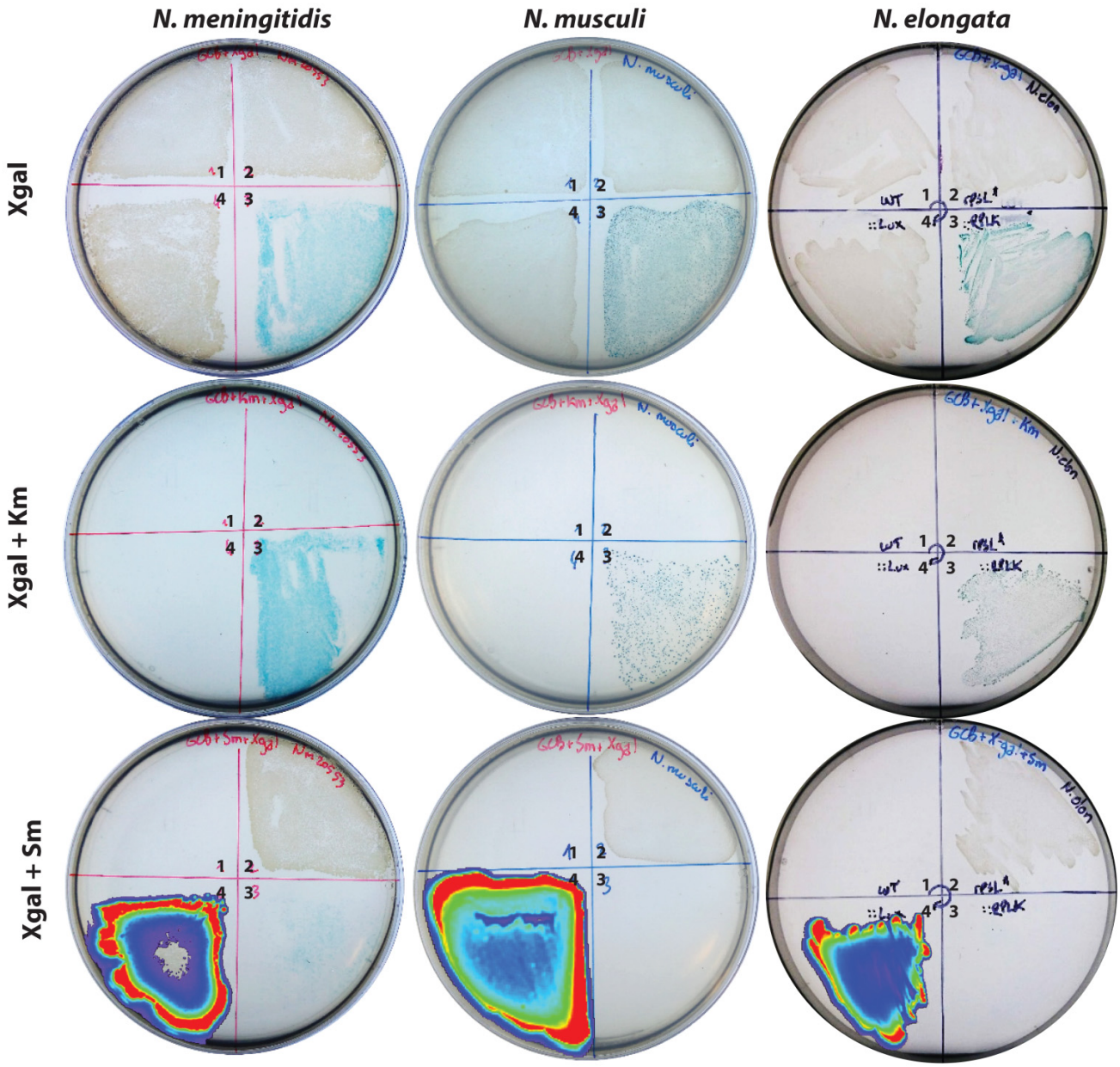


Fig. 5. Generation of markerless luminescent *Neisseria* species. The RPLK cassette was used to introduce the *porBp-luxCDABE* luminescence cassette in *N. meningitidis, N. musculi*, and *N. elongata*. Suspensions from each step of the cloning strategy were plated on X-gal-supplemented GCB, GCB+Km, and GCB+Sm. Luminescence was assayed for the GCB+Sm plates with an IVIS Lumina III (PerkinElmer), for which overlaid images are shown. 1, WT strains; 2, *rpsL** strains; 3, RPLK-insertion strains; 4, markerless luminescent strains. GCB, GC base agar; Km, kanamycin; Sm, streptomycin.



selection marker that facilitates the selection of transformants. Despite the progress made in this field, some of the major challenges include low transformation efficiency that necessitates laborious screening of many clones, the difficulty in creating unmarked mutants, and the inability to reliably modify multiple genes in the same strains (Bosse et al. 2014; Yan and Fong 2017; Arroyo-Olarte et al. 2021). Therefore, the need to develop new methods and continuously improve the existing ones cannot be overemphasized.

Natural competence of *Neisseria* species allows them to be transformed repeatedly with high efficiency, making them a great model for complex or cumulative genetic modifications. Because of an impressive repertoire of restriction-modification systems, replicative plasmids are of limited use (Budroni et al. 2011). Instead, homologous recombination to the genome is favored, requiring sufficient homology with the transforming DNA. Here, we demonstrate an improved three-gene cassette system for markerless and multiple genetic modification of virtually any *Neisseria* species. While most studies focus on only two pathogenic *Neisseria* species in humans, *N. meningitidis* and *N. gonorrhoeae*, we wanted to showcase the versatility of our cloning strategy by using it in multiple species. We performed markerless gene deletions and insertions in several commensal species such as *N. elongata* and *N. musculi* in addition to *N. meningitidis*. Our strategy combined the use of an antibiotic resistance cassette as the

Fig. 6. Multiple markerless deletions in *N. elongata*. Using the RPLK cassette with the methodology described in this work, sequential cumulative gene deletions were performed in *N. elongata*. Six deletions were performed in a single strain. (A) Workflow for multiple deletions. (B) PCR verification of the deleted loci and kanamycin resistance gene (from RPLK) at each step of the process. For simplicity, gene names were replaced with letters (Locus A = *rapZ*, B = *pbp3*, C = *gloB*, D = NELON_RS07135, E = *mtgA* and F = *mraZ*). Km^R, kanamycin resistance gene.

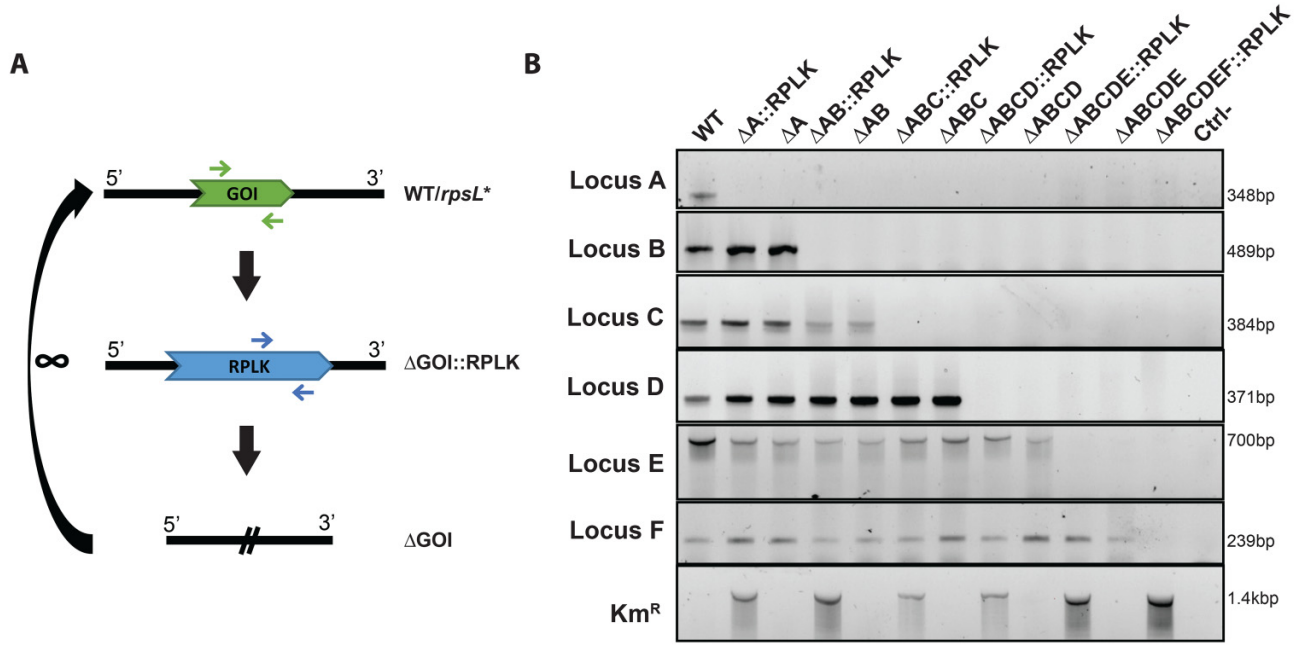


Table 1. False positive rates from two independent gene deletions.

Gene deleted	False positive rate (blue/total CFUs)
Locus C	30% ± 4%
Locus E	33% ± 3%

Notes: The last transformation step was performed in biological triplicates for two independent gene deletions from *N. elongata* made with the RPLK cassette (cf. Fig. 6). Blue and white transformants were counted from GCB plates supplemented with streptomycin and X-gal to determine the rate of Sm^R clones still carrying the RPLK cassette.

positive selection marker, *lacZ* alpha subunit or mCherry as phenotypic selection markers, and streptomycin-sensitive $rpsL^{wt}$ allele for the counterselection of transformants. A previous study showed that the *rspL* from *E. coli* is highly inefficient at reversing streptomycin resistance in *N. gonorrhoeae*, raising concerns that a cross-species barrier may exist (Johnston and Cannon 1999). In our gene deletion example with the RPCC cassette, we demonstrated that the *rpsL* allele from *N. meningitidis* or *N. lactamica* could both be used to modify *N. elongata*, suggesting that such a barrier does not exist within the *Neisseria* genus. Finally, as shown in Fig. 2, our approach can be used to generate markerless deletions and insertions (as demonstrated here), but can also be used to generate markerless complementation strains of a previous deletion by inserting the gene in another locus (Nyongesa et al. 2022).

Similar approaches for markerless modifications of bacteria employ the use of two-gene cassettes comprising a resistance gene for selection and $rpsL^{wt}$ for counterselection (Johnston and Cannon 1999; Sander et al. 2001; Sung et al. 2001; Bird et al. 2011; Kaczmarczyk et al. 2012). The main drawback of these strategies is the frequent occurrence of Sm^R clones carrying both *rpsL* alleles, leading to a significant proportion of false positives in the last transformation step (Sung et al. 2001; Kohler et al. 2005; Dillard 2011). In our hands, over 30% of transformants were false positives upon removal of the RPLK cassette. Moreover, Sm^R clones were naturally arising even in pure cultures of the RPLK mutants previously screened for Sm sensitivity. It was shown in *N. gonorrhoeae* that this phenomenon is not due to incomplete dominance of the inserted $rpsL^{wt}$ allele over the native $rpsL^{K43R}$ allele, but rather due to the spontaneous mutation of the inserted allele and recombination events between both alleles (Kohler et al. 2005). The relevance of using additional counterselection markers in reducing the false positivity rate associated with allelic conversion was previously shown (Li et al. 2014). To solve this problem, we added *lacZ* and mCherry under the strong *Neisseria* promoter *porB*p to the RPLK and RPCC selection cassettes, respectively. These elements allow for direct blue-white and fluorescence screening of the Sm^R clones obtained at the last step, therefore increasing the reliability of the strategy besides reducing the need for PCR screening and kanamycin susceptibility testing. Overall, assuming the plasmid constructs are ready, a full markerless gene modification workflow can be accomplished in as little as 5 days, including proper verifications and stock preparation. The efficiency of our cassettes can be improved further by limiting recombination between *rpsL* alleles in the merodiploid strain by using streptomycin-sensitive *rpsL* with low sequence homology to the resistant *Neisseria rspL* gene as demonstrated

previously (Bird et al. 2011). Similarly, the use of other antibiotic resistances and phenotypic selection markers in place of the ones mentioned in the study could improve the versatility of this method.

Although the RPLK and RPCC cassettes are fairly big, 5 and 2.5 kb, respectively, the insert size is not an issue in *Neisseria* species. In fact, using the improved methodology described above, dilution of the transformed cells is often needed to get individual colonies, showcasing the high efficiency of this approach. Insertion of the 6.2 kb *porB*p-*luxCDABE* luminescence cassette was also not an issue. Generating markerless luminescent or fluorescent strains as exemplified here can be of great use in animal infection model studies, which are severely lacking for commensal *Neisseria* (Weyand et al. 2013; Weyand 2017; Ma et al. 2018). We further demonstrated the strength of our strategy by performing six cumulative gene deletions in *N. elongata*. With the recent leap of genomic and bioinformatics studies, such a tool is invaluable when trying to recreate ancestral evolutionary events leading to speciation, since these events often include several gene insertions, deletions, and point mutations.

To summarize, we improved previous methods of markerless gene modifications by developing three-gene selection–counterselection cassettes that can be transformed with high efficiency into multiple *Neisseria* species. Using this method, we performed large gene insertions and deleted up to six loci in a single strain.

Article information

History dates

Received: 28 January 2022
Revised: 26 March 2022
Accepted: 31 March 2022
Accepted manuscript online: 5 May 2022
Version of record online: 20 July 2022

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Funding information

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) discovery grant (RGPIN-2016-04940) (F.J.V.), the Fonds De Recherche du Quebec Nature et technologies (FRQNT) Établissement de la relève professorale (205027) (F.J.V.), and the Canadian Institutes of Health Research (CIHR) (CFJA-184531, F.J.V.). E.B. received a Ph.D. Fellowship from the Fondation Armand-Frappier. F.J.V. received a Junior 1 and Junior 2 research scholar salary award from the Fonds de Recherche du Québec—Santé. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Supplementary material

Supplementary data are available with the article at https://doi.org/10.1139/cjm-2022-0024.

References

Alonso, J.M., Guiyoule, A., Zarantonelli, M.L., Ramisse, F., Pires, R., Antignac, A., et al. 2003. A model of meningococcal bacteremia after respiratory superinfection in influenza A virus-infected mice. FEMS Microbiol. Lett. **222**: 99–106. doi:10.1016/S0378-1097(03)00252-0. PMID: 12757952.

Anonsen, J.H., Vik, A., Borud, B., Viburiene, R., Aas, F.E., Kidd, S.W., et al. 2016. Characterization of a unique tetrasaccharide and distinct glycoproteome in the O-linked protein glycosylation system of *Neisseria elongata* subsp. *glycolytica*. J. Bacteriol. **198**: 256–267. doi:10.1128/JB.00620-15. PMID: 26483525.

Arroyo-Olarte, R.D., Bravo Rodriguez, R., and Morales-Rios, E. 2021. Genome editing in bacteria: CRISPR–Cas and beyond. Microorganisms, **9**: 844. doi:10.3390/microorganisms9040844.

Bailey, J., Cass, J., Gasper, J., Ngo, N.D., Wiggins, P., and Manoil, C. 2019. Essential gene deletions producing gigantic bacteria. PLoS Genet. **15**: e1008195. doi:10.1371/journal.pgen.1008195. PMID: 31181062.

Becher, A., and Schweizer, H.P. 2000. Integration-proficient *Pseudomonas aeruginosa* vectors for isolation of single-copy chromosomal *lacZ* and *lux* gene fusions. Biotechniques, **29**: 948–950, 952. doi:10.2144/00295bm04. PMID: 11084852.

Bennett, J.S., Bentley, S.D., Vernikos, G.S., Quail, M.A., Cherevach, I., White, B., et al. 2010. Independent evolution of the core and accessory gene sets in the genus *Neisseria*: insights gained from the genome of *Neisseria lactamica* isolate 020-06. BMC Genomics, **11**: 652. doi:10.1186/1471-2164-11-652. PMID: 21092259.

Bernet, E., Lebughe, M., Vincent, AT., Haghdoost, M.M., Golbaghi, G., Laplante, S., et al. 2020. Sodium tetraphenylborate displays selective bactericidal activity against *N. meningitidis* and *N. gonorrhoeae* and is effective at reducing bacterial infection load. Antimicrob. Agents Chemother. **65**: e00254–e00220.

Bird, A.W., Erler, A., Fu, J., Heriche, J.K., Maresca, M. Zhang, Y., et al. 2011. High-efficiency counterselection recombineering for site-directed mutagenesis in bacterial artificial chromosomes. Nat. Methods, **9**: 103–109. doi:10.1038/nmeth.1803. PMID: 22138824.

Bosse, J.T., Soares-Bazzolli, D.M., Li, Y., Wren, B.W., Tucker, A.W., Maskell, D.J., et al. 2014. The generation of successive unmarked mutations and chromosomal insertion of heterologous genes in *Actinobacillus pleuropneumoniae* using natural transformation. PLoS One, **9**: e111252. doi:10.1371/journal.pone.0111252. PMID: 25409017.

Brynildsrud, O.B., Eldholm, V., Bohlin, J., Uadiale, K., Obaro, S., and Caugant, D.A. 2018. Acquisition of virulence genes by a carrier strain gave rise to the ongoing epidemics of meningococcal disease in West Africa. Proc. Natl. Acad. Sci. USA, **115**: 5510–5515. doi:10.1073/pnas.1802298115. PMID: 29735685.

Budroni, S., Siena, E., Dunning Hotopp, J.C., Seib, K.L., Serruto, D. Nofroni, C., et al. 2011. *Neisseria meningitidis* is structured in clades associated with restriction modification systems that modulate homologous recombination. Proc. Natl. Acad. Sci. USA, **108**: 4494–4499. doi:10.1073/pnas.1019751108. PMID: 21368196.

Custodio, R., Johnson, E., Liu, G., Tang, C.M., and Exley, R.M. 2020. Commensal *Neisseria cinerea* impairs *Neisseria meningitidis* microcolony development and reduces pathogen colonisation of epithelial cells. PLoS Pathog, **16**: e1008372. doi:10.1371/journal.ppat.1008372. PMID: 32208456.

Deghmane, A.E., Taha, S., and Taha, M.K. 2022. Global epidemiology and changing clinical presentations of invasive meningococcal disease: a

narrative review. Infect. Dis. (Lond). **54**: 1–7. doi:10.1080/23744235.2021.1971289. PMID: 34459329.

Dillard, J.P. 2011. Genetic manipulation of *Neisseria gonorrhoeae*. Curr. Protoc. Microbiol. **4**: Unit4A.2. PMID: 22045584.

Freire, P., Moreira, R.N., and Arraiano, C.M. 2009. BolA inhibits cell elongation and regulates MreB expression levels. J. Mol. Biol. **385**: 1345–1351. doi:10.1016/j.jmb.2008.12.026. PMID: 19111750.

Frye, S.A., Nilsen, M., Tonjum, T., and Ambur, O.H. 2013. Dialects of the DNA uptake sequence in Neisseriaceae. PLoS Genet. **9**: e1003458. doi:10.1371/journal.pgen.1003458. PMID: 23637627.

Goodman, S.D., and Scocca, J.J. 1988. Identification and arrangement of the DNA sequence recognized in specific transformation of *Neisseria gonorrhoeae*. Proc. Natl. Acad. Sci. USA, **85**: 6982–6986. doi:10.1073/pnas.85.18.6982. PMID: 3137581.

Guiddir, T., Deghmane, A.E., Giorgini, D., and Taha, M.K. 2014. Lipocalin 2 in cerebrospinal fluid as a marker of acute bacterial meningitis. BMC Infect. Dis. **14**: 276. doi:10.1186/1471-2334-14-276. PMID: 24885531.

Hamilton, H.L., and Dillard, J.P. 2006. Natural transformation of *Neisseria gonorrhoeae*: from DNA donation to homologous recombination. Mol. Microbiol. **59**: 376–385. doi:10.1111/j.1365-2958.2005.04964.x. PMID: 16390436.

Higashi, D.L., Biais, N., Weyand, N.J., Agellon, A., Sisko, J.L., Brown, L.M., and So, M. 2011. *N. elongata* produces type IV pili that mediate interspecies gene transfer with *N. gonorrhoeae*. PLoS One, **6**: e21373. doi:10.1371/journal.pone.0021373. PMID: 21731720

Hitchcock, P.J. 1989. Unified nomenclature for pathogenic *Neisseria* species. Clin. Microbiol. Rev. **2 Suppl**: S64–S65. doi:10.1128/CMR.2.Suppl.S64. PMID: 2720633.

Jafri, R.Z., Ali, A., Messonnier, N.E., Tevi-Benissan, C., Durrheim, D. Eskola, J., et al. 2013. Global epidemiology of invasive meningococcal disease. Popul. Health Metr. **11**: 17. doi:10.1186/1478-7954-11-17. PMID: 24016339.

Johnston, D.M., and Cannon, J.G. 1999. Construction of mutant strains of *Neisseria gonorrhoeae* lacking new antibiotic resistance markers using a two gene cassette with positive and negative selection. Gene, **236**: 179–184. doi:10.1016/S0378-1119(99)00238-3. PMID: 10433979.

Joseph, B., Schwarz, R.F., Linke, B., Blom, J., Becker, A. Claus, H., et al. 2011. Virulence evolution of the human pathogen *Neisseria meningitidis* by recombination in the core and accessory genome. PLoS One, **6**: e18441. doi:10.1371/journal.pone.0018441. PMID: 21541312.

Kaczmarczyk, A., Vorholt, J.A., and Francez-Charlot, A. 2012. Markerless gene deletion system for sphingomonads. Appl. Environ. Microbiol. **78**: 3774–3777. doi:10.1128/AEM.07347-11. PMID: 22427496.

Kellogg, D.S., Jr., Peacock, W.L., Jr., Deacon, W.E., Brown, L., and Pirkle, D.I. 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. J. Bacteriol. **85**: 1274–1279. doi:10.1128/jb.85.6.1274-1279.1963. PMID: 14047217.

Kohler, P.L., Cloud, K.A., Hackett, K.T., Beck, E.T., and Dillard, J.P. 2005. Characterization of the role of LtgB, a putative lytic transglycosylase in *Neisseria gonorrhoeae*. Microbiology (Reading), **151**: 3081–3088. doi:10.1099/mic.0.28125-0. PMID: 16151218.

Li, X.T., Thomason, L.C., Sawitzke, J.A., Costantino, N., and Court, D.L. 2013. Positive and negative selection using the tetA-sacB cassette: recombineering and P1 transduction in *Escherichia coli*. Nucleic Acids Res. **41**: e204. doi:10.1093/nar/gkt1075. PMID: 24203710.

Li, Y., Thompson, C.M., and Lipsitch, M. 2014. A modified Janus cassette (Sweet Janus) to improve allelic replacement efficiency by high-stringency negative selection in *Streptococcus pneumoniae*. PLoS One, **9**: e100510. doi:10.1371/journal.pone.0100510. PMID: 24959661.

Ma, M., Powell, D.A., Weyand, N.J., Rhodes, K.A., Rendon, M.A., Frelinger, J.A., and So, M. 2018. A natural mouse model for *Neisseria* colonization. Infect. Immun. **86**: e00839–17. doi:10.1128/IAI.00839-17.

Maiden, M.C., and Harrison, O.B. 2016. Population and functional genomics of Neisseria revealed with gene-by-gene approaches. J. Clin. Microbiol. **54**: 1949–1955. doi:10.1128/JCM.00301-16. PMID: 27098959.

Marri, P.R., Paniscus, M., Weyand, N.J., Rendon, M.A., Calton, C.M. Hernandez, D.R., et al. 2010. Genome sequencing reveals widespread virulence gene exchange among human *Neisseria* species. PLoS One, **5**: e11835. doi:10.1371/journal.pone.0011835.

Mell, J.C., and Redfield, R.J. 2014. Natural competence and the evolution of DNA uptake specificity. J. Bacteriol. **196**: 1471–1483. doi:10.1128/JB.01293-13. PMID: 24488316.

Nyongesa, S., Weber, P., Bernet, E., Pullido, F., Nieckarz, M., Delaby, M., et al. 2022. Evolution of multicellular longitudinally dividing oral cavity symbionts (Neisseriaceae). Research Square. doi:10.21203/rs.3.rs-1200288/v1.

Perrin, A., Nassif, X., and Tinsley, C. 1999. Identification of regions of the chromosome of *Neisseria meningitidis* and *Neisseria gonorrhoeae* which are specific to the pathogenic *Neisseria* species. Infect. Immun. **67**: 6119–6129. doi:10.1128/IAI.67.11.6119-6129.1999. PMID: 10531275.

Putonti, C., Nowicki, B., Shaffer, M., Fofanov, Y., and Nowicki, S. 2013. Where does *Neisseria* acquire foreign DNA from: an examination of the source of genomic and pathogenic islands and the evolution of the Neisseria genus. BMC Evol. Biol. **13**: 184. doi:10.1186/1471-2148-13-184. PMID: 24007216.

Reyrat, J.M., Pelicic, V., Gicquel, B., and Rappuoli, R. 1998. Counterselectable markers: untapped tools for bacterial genetics and pathogenesis. Infect. Immun. **66**: 4011–4017. doi:10.1128/IAI.66.9.4011-4017.1998. PMID: 9712740.

Rowley, J., Vander Hoorn, S., Korenromp, E., Low, N., Unemo, M., Abu-Raddad, L.J., et al. 2019. Chlamydia, gonorrhoea, trichomoniasis and syphilis: global prevalence and incidence estimates, 2016. Bull. World Health Organ. **97**: 548–62P. doi:10.2471/BLT.18.228486. PMID: 31384073.

Sander, P., Springer, B., and Bottger, E.C. 2001. Gene replacement in *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG using *rpsL* as a dominant negative selectable marker. Methods Mol. Med. **54**: 93–104. doi: 10.1385/1-59259-147-7:093. PMID:21341071.

Santos, J.M., Lobo, M., Matos, A.P., De Pedro, M.A., and Arraiano, C.M. 2002. The gene *bolA* regulates dacA (PBP5), dacC (PBP6) and ampC (AmpC), promoting normal morphology in Escherichia coli. Mol. Microbiol. **45**: 1729–1740. doi:10.1046/j.1365-2958.2002.03131.x. PMID: 12354237.

Sung, C.K., Li, H., Claverys, J.P., and Morrison, D.A. 2001. An *rpsL* cassette, Janus, for gene replacement through negative selection in *Streptococcus pneumoniae*. Appl. Environ. Microbiol. **67**: 5190–5196. doi:10.1128/AEM.67.11.5190-5196.2001. PMID: 11679344.

Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D.L., et al. 2018. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect. Dis. **18**: 318–327. doi:10.1016/S1473-3099(17)30753-3. PMID: 29276051.

Trindade, S., Sousa, A., Xavier, K.B., Dionisio, F., Ferreira, M.G., and Gordo, I. 2009. Positive epistasis drives the acquisition of multidrug resistance. PLoS Genet. **5**: e1000578. doi:10.1371/journal.pgen.1000578. PMID: 19629166.

van Dam, V., and Bos, M.P. 2012. Generating knock-out and complementation strains of *Neisseria meningitidis*. Methods Mol. Biol. **799**: 55–72. doi:10.1007/978-1-61779-346-2_4. PMID: 21993639.

Veyrier, F.J., Boneca, I.G., Cellier, M.F., and Taha, M.K. 2011. A novel metal transporter mediating manganese export (MntX) regulates the Mn to Fe intracellular ratio and *Neisseria meningitidis* virulence. PLoS Pathog. **7**: e1002261. doi:10.1371/journal.ppat.1002261. PMID: 21980287.

Veyrier, F.J., Biais, N., Morales, P., Belkacem, N., Guilhen, C., Ranjeva, S., et al. 2015. Common cell shape evolution of two nasopharyngeal pathogens. PLoS Genet. **11**: e1005338. doi:10.1371/journal.pgen.1005338. PMID: 26162030.

Weyand, N.J. 2017. Neisseria models of infection and persistence in the upper respiratory tract. Pathog. Dis. **75**: ftx031. doi: 10.1093/femspd/ftx031. PMID: 28369241.

Weyand, N.J., Wertheimer, A.M., Hobbs, T.R., Sisko, J.L., Taku, N.A., Gregston, L.D., et al. 2013. Neisseria infection of rhesus macaques as a model to study colonization, transmission, persistence, and horizontal gene transfer. Proc. Natl. Acad. Sci. USA, **110**: 3059–3064. doi:10.1073/pnas.1217420110. PMID: 23382234.

Yan, Q., and Fong, S.S. 2017. Challenges and advances for genetic engineering of non-model bacteria and uses in consolidated bioprocessing. Front. Microbiol. **8**: 2060. doi:10.3389/fmicb.2017.02060. PMID: 29123506.

Zarantonelli, M.L., Szatanik, M., Giorgini, D., Hong, E., Huerre, M., Guillou, F., et al. 2007. Transgenic mice expressing human transferrin as a model for meningococcal infection. Infect. Immun. **75**: 5609–5614. doi:10.1128/IAI.00781-07. PMID: 17893132.

Zhang, Y. 2017. The CRISPR–Cas9 system in *Neisseria* spp. Pathog. Dis. **75**: ftx036. doi:10.1093/femspd/ftx036.