Quick molecular techniques to generate mutants in the Neisseria genus

Ingrid Spielman[1], Dustin Higashi[2], Gregory Boel[3] and Nicolas Biais[1]
1: Brooklyn College CUNY, 2: University of Notre Dame, 3: Columbia University.

Brooklyn College — The City University of New York

Abstract

We have developed simple and highly efficient mutagenesis protocols based on PCR products to obtain mutants in multiple Neisseria species. Thus far, generation of mutants in pathogenic Neisseria has relied on the construction of plasmids bearing flanking sequences of the gene or region targeted for deletion. These homologous regions enable recombination at desired genomic loci. Similar to techniques used to obtain gene deletions in S. cerevisiae and cholerae, our techniques bypass the need for subcloning into plasmid vectors by using PCR products to directly transform bacteria. We have designed vectors bearing various antibiotic resistance cassettes under the control of a consensus promoter and include a Neisseria DNA uptake sequence. PCR utilizing long primers containing upstream and downstream regions of the gene targeted for deletion along with complementary sequences to the antibiotic resistance vector produces a PCR product that can be directly transformed into Neisseria by spot transformation on agar plates. With the typical one day of selection on antibiotic plates, mutants can be obtained in as little as two days. As few as 80 base pairs of homology on both the upstream and downstream sequences were sufficient to obtain mutants, but homology of 150 base pairs was sometimes necessary. We have successfully applied these techniques to generate mutants in both Neisseria gonorrhoeae, the causative agent of gonorrhea, and the human commensal Neisseria elongata. In addition, as an alternate approach to the use of long primers, we have fused multiple DNA blocks using Gibson assembly protocols and used this DNA to transform Neisseria. These protocols provide both modularity and speed to Neisseria mutagenesis, allowing for the development of complex systematic mutant libraries in the Neisseria genus.

Introduction

Neisseria are gram negative bacteria that are **naturally competent** for transformation. Neisseria bacteria such as N. *elongata*, N. *gonorrhoeae,* or N. *meningitides* all use type IV pili (Tfp) for horizontal gene transfer and can homologously recombine the DNA they take up into their own genome.

Type IV pili are used by bacteria to move, tether themselves to surfaces, interact with other bacteria, as well as transfer DNA between one another. Many of these bacteria are pathogenic in nature. We study both pathogenic and commensal Neisseria species to learn how to control /prevent disease and how they use their Tfp for all these different functions. The focus of our lab is to understand the role of retraction forces of Tfp in their multiple functions.

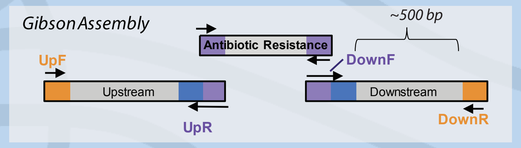
In order to dissect the role of the Tfp machinery across the Neisseria genus, we needed to be able to have an easy method to generate mutants that would work across the Neisseria genus. Up to now, Neisseria *gonorrhoeae* and Neisseria *meningitidis* mutants were mostly generated by partial gene deletions and insertional inactivation in which antibiotic cassettes are inserted into the middle of the gene of interest. This requires the use of restriction enzymes which imposes constraints on the design of the mutants.

We present here a set of techniques inspired by recent development in molecular biology that enables the rapid and modular construction of mutants across the Neisseria genus. We have designed plasmids bearing antibiotic resistance cassettes along with the Neisseria DNA uptake sequence that can be used as the basis to obtain in a day in-frame gene deletions, gene reporters, complementations or fluorescent fusion proteins. This technique was used to produce the first mutants in the commensal Neisseria elongata and can be used to create a library of mutants in a Neisseria species.

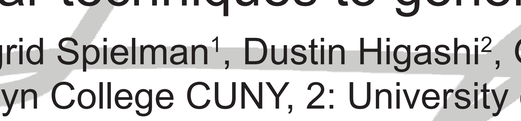
Steps

1. Design the primers

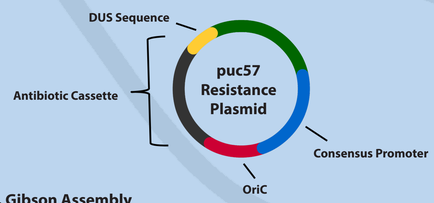
Gibson assembly
The upstream and downstream sequences (400bp fragments) of the gene of interest are amplified by PCR using primer pairs UpF / UpR and DownF / DownR where UpR contains a 20 base pair overlap to the antibiotic resistance cassette 5' end (reverse complement) and the DownF contains a 20 base pair overlap with the antibiotic resistance cassette 3' end, as shown below. Primers were designed such that each had a Tm of ~60C (not including overhang segments). The UpR primer was chosen from just upstream of the ORF for the gene to be deleted. The DownF primer is designed to begin immediately downstream of the gene to be deleted.



Single step
Design primers for the antibiotic resistance cassette that contain long 40 bp overhangs that confer the up- and downstream sequences flanking the gene of interest. At least 80 base pairs of homology on both the upstream and downstream sequences were sufficient to obtain mutants through natural homologous recombination.



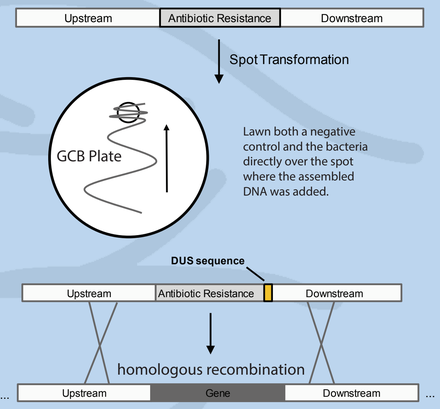
2. PCR of individual pieces
We designed a plasmid with a DUS sequence downstream of antibiotic resistance cassette (either Kanamycin or Chloramphenicol). We amplify out this resistance cassette via PCR using primers such that there is homology between the antibiotic resistance and downstream DUS sequence.



3. Gibson Assembly
In the case of Gibson assembly, we add **2ul of Gibson assembly mix** to 2ul of 0.1pmol total mixture of overlapping DNA fragments. We follow the Gibson Assembly protocol provided by NEB but reduce the total reaction volume to 4ul to reduce cost. Upstream and downstream sequences are designed to overlap with the antibiotic resistance sequence. Therefore it is not necessary to redesign the antibiotic resistance piece and they can be used in each subsequent mutant assembly.

4. PCR of Gibson Assembly
In order to obtain enough product for transformation we amplify the **unpurified Gibson assembly mixture** via PCR using the UpF and DownR primers. We confirm the fragments assembled correctly using this amplification product.

5. Spot Transformation
After confirming the size of the assembled DNA sequence or the PCR amplified resistance cassette 15ul of the **unpurified PCR product** (0.5 - 1ug) is added directly to a GCB (gonococcal broth) plate and let to dry under sterile conditions. In the case where there are side products in addition to the correct product, we found that it is best to add the product directly for spot transformation instead of attempting to isolate the product. Streak the bacteria onto a GCB plate over the dried spot of DNA and let the bacteria grow for 8 - 15 hours before the next step.



6. Selection Plates Lawn bacteria from spot to a selection plate using a dacron swab**.** Carefully swirl the swab towards the center of the DNA spot so as to not spread the bacteria and collect as much bacteria as possible from the plate. Use bacteria that were not streaked over the DNA spot as a negative control.

After 8-16 hours, transfer the bacteria onto resistance plates. The colonies are visible after 24-48 hours. Colonies are streaked and then store in 20% glycerol stocks (-80C) as well as diH20 for sequence amplification.

7. Confirm modified sequence (*Sanger sequencing*)
PCR modified sequence using read primers that extend the homologously recombined piece by 50bp on both sides. We use the diH20 colony stocks for this PCR. There is **no need for DNA purification** and there is enough lysed cells in the water for free DNA to be present in the PCR reaction.

Advantages

SIMPLE: Designing the primers for gene deletion is simple. No need to check whether or not restriction sites exist within the gene segment or flanking regions. Allows for in-frame deletion of entire gene. Primer design can be automated.
- 60c Tm of all primers,
- ~400bp flanking sequences
- UpR and DownF primers are designed from immediate flanking sequences up- and downstream of gene with overhangs that complement the resistance cassette.
- Incorporation of DUS sequence downstream of resistance cassette

FAST: Mutants strains can be completed within a week. With DNA fragment building, assembly, and amplification only taking half a day, mutants can be generated in as few as 2 days.

MODULARITY: DNA fragments can be build and used for multiple mutant assemblies

SCALABLE: Potential to build arrays of in-frame gene deletions, gene reporters, complementations or fluorescent fusion proteins.

LOW COST:

	Single Step Deletion	Gibson Assembly
Assembly mix	--	2ul - $3.10 / rxn
Primers ($0.18/bp)	100bp x 4 - $72.00	~40bp x 4 - $28.00
PCR mix (goTaq)	$0.30 / mutant	$0.30 / mutant
Total	$72.30 / mutant	$31.40 / mutant

Use & Future Directions

- We have the tools to develop a high throughput method for designing and building a mutant library consisting of in-frame single-gene deletions of every ORF within each Neisseria species. Neisseria *gonorhhoeae* and Neisseria *elongata* are the prime candidates.
- Large-scale construction of mutants across the Neisseria genus in a systematic manner that is simple enough for implementation in an undergraduate laboratory class.
- Potential to build gene reporter libraries and fluorescent fusion protein libraries quickly and cost effectively.
- Building a Neisseria genus wide mutant library will allow for researchers to screen for genes that are essential to Tfp function and bacteria pathogenicity.

Example Case

Name	Sequence	Tm
Up_F	TTACCGACCAAATCGGCTAC	58.4
Up_R	TAAAGTGTCAAGCCCTCGAGtttgacacttccatttgttagtaaaag	58.4/60
Down_F	GCCGTCTCAAGTTTAAACATCGA Tattagtttttaaatcactattcaaaatatttag	61.8/60.8
Down_R	GCAGTCTGGTCGGGTTGACA	62.5

Designed Deletion Mutant

1. The DNA uptake sequence (DUS - GCCGTCTGAA) is designed into the resistance plasmid and can be seen in the DownF primer.
2. The upstream and downstream pieces are both ~500bp in size.
3. **Building the mutant took a single day.**

Contact: ingrid@mechano-micro-biology.org
Website: https://sites.google.com/a/mechano-micro-biology.org/mmbl/