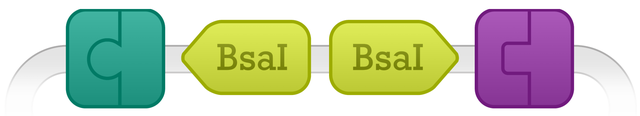
Iterative cloning using a methylation protected oligonucleotide duplex

Bob Van Hove, Chiara Guidi, Jo Maertens & Marjan De Mey

Metabolic Engineering Group - Laboratory of Industrial Biotechnology and Biocatalysis - Ghent University - Belgium

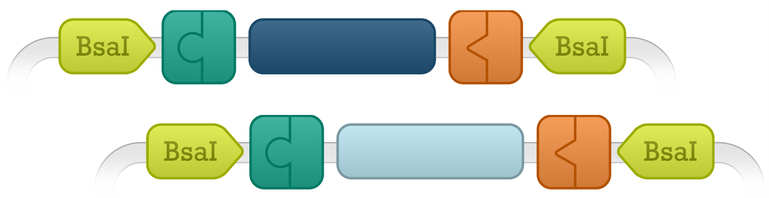
Introduction

Golden Gate cloning enables one-pot, high efficiency assembly of multi-part constructs, while avoiding the introduction of PCR-derived mutations[1]. However, because the resulting designs should be devoid of the restriction enzyme's recognition site, additional parts cannot easily be cloned into the same plasmid. As a solution, we present **PODAC: Protected Oligonucleotide Duplex Assisted Cloning**.



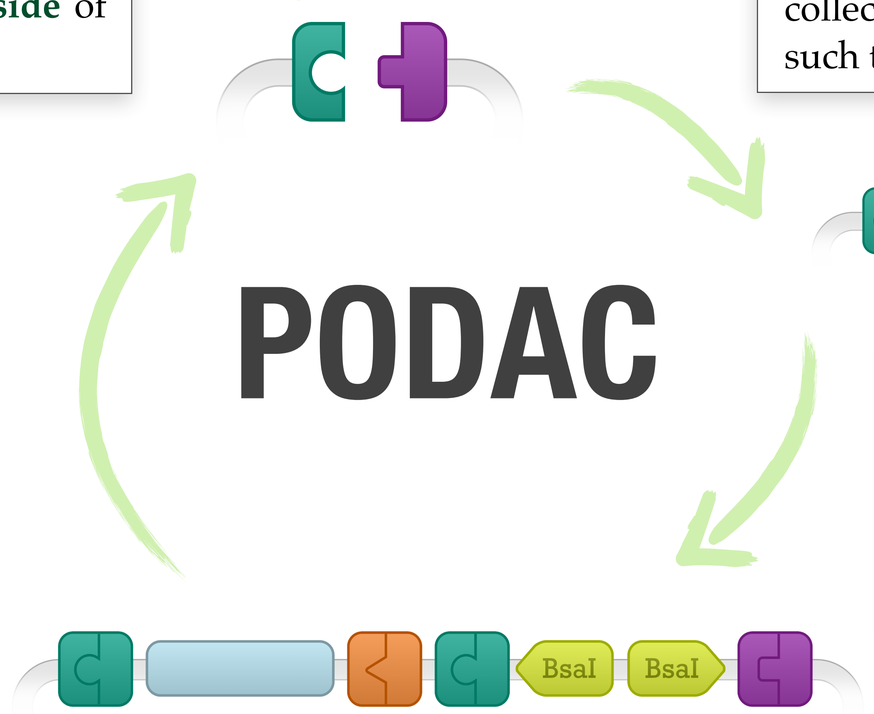
Acceptor

The Acceptor vector contains recognition sites for *BsaI*, a restriction enzyme that cleaves **outside** of its binding site.



Donor

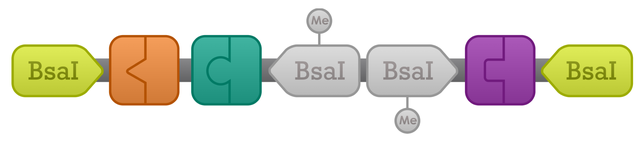
Genetic parts to be cloned are obtained from a collection of Donor plasmids containing *BsaI* sites such that complementary sticky ends are created.



Reactivation

Restriction and ligation are performed simultaneously and the product is transformed into *E. coli*. The main idea behind PODAC is that DNA replication in *E. coli* does not maintain the introduced methylation pattern.

E. coli **activates** the *BsaI* sites, resulting in a new Acceptor that can receive another part in the next iteration. Donor vectors and duplex are reusable because the sticky ends do not change.



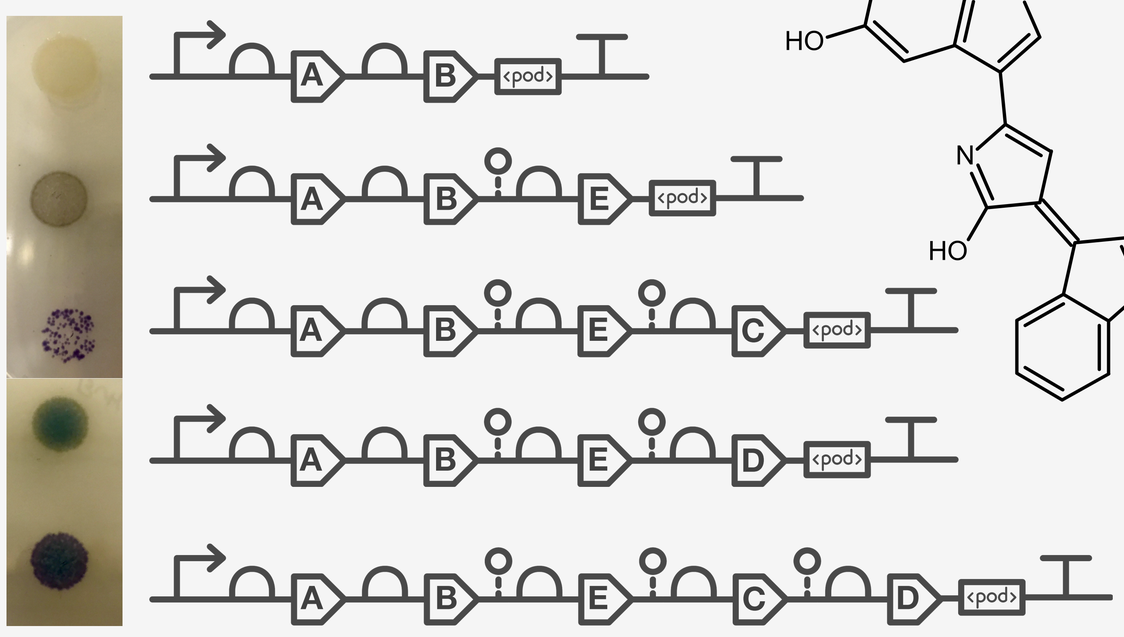
Protected duplex

PODAC introduces a new cloning site in the form of annealed oligonucleotides. The internal *BsaI* cloning site has been **deactivated** due to cytosine methylation, protecting them from digestion during the Golden Gate reaction[4].

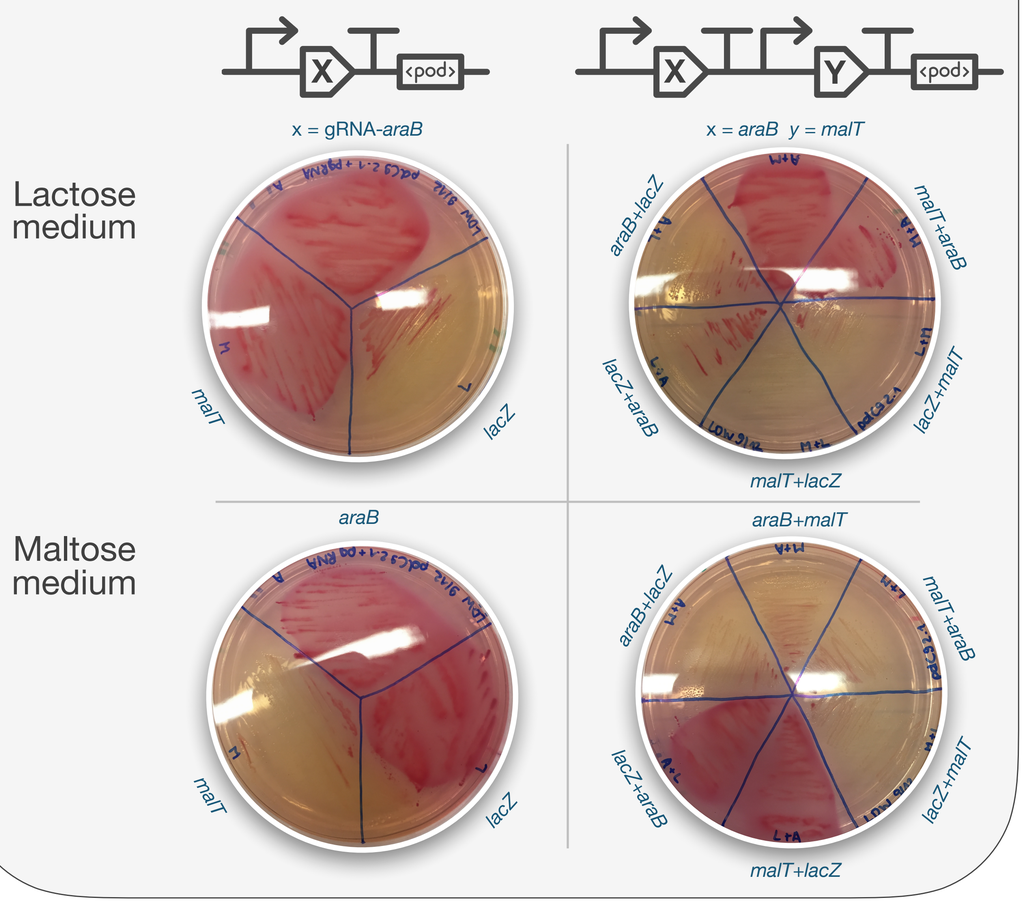
Test Cases

We chose the **violacein biosynthetic pathway** from *Chromobacterium violaceum* as a first test case for PODAC. This branched pathway consists of 5 enzymes (VioA,B,C,D,E) leading up to the pigment violacein, which shows potential as an antibiotic, antifungal and anticancer agent[3].

VioAB and regulatory sequences were present in the initial acceptor vector. Donor vectors supplied *vioC*, *D* or *E* preceded by a ribozyme insulator[2]. Successful assembly of consecutive PODAC iterations could be monitored by the various colours of pathway intermediates.



As a demonstration of its broad applicability, we used PODAC to create artificial **CRISPR guide RNA arrays** for use in transcriptional roadblock mediated knockdown[5]. Multiple genes related to carbohydrate metabolism were targeted, enabling a visual screen based on acidification of medium to which the specific sugar was added. Absence of red colour indicates a successful gene knockdown.



References

(1) Engler, C., et al. (2008) A One Pot, One Step, Precision Cloning Method with High Throughput Capability. *PLoS ONE 3*, 3647–3654.
(2) Lou, C., et al. (2012) Ribozyme- based insulator parts buffer synthetic circuits from genetic context. *Nature Biotechnology 30*, 1137–1142.
(3) Balibar, C. J., and Walsh, C. T. (2006) In Vitro Biosynthesis of Violacein from l- Tryptophan by the Enzymes VioA–E from *Chromobacterium violaceum*. *Biochemistry 45*, 15444–15457.
(4) Storch, M., et al. (2015) BASIC: A New Biopart Assembly Standard for Idempotent Cloning Provides Accurate, Single-Tier DNA Assembly for Synthetic Biology *4*, 781– 787.
(5) Qi, L. S., et al. (2013) Repurposing CRISPR as an RNA-Guided Platform for Sequence- Specific Control of Gene Expression. *Cell 152*, 1173–1183.

This research was supported by the Fund for Scientific Research-Flanders (FWO) through project G.0321.13N and a Ph.D. grant for BVH.

bob.vanhove@ugent.be
bit.ly/atgtaa