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Modular Cloning by Golden Gate Assembly and Possible Application in Pathway Design

Zrinka Raguz Nakic and Christin Peters*

Abstract: Preparation of expression vectors using conventional cloning strategies is laborious and not suitable for the design of metabolic pathways or enzyme cascades, which usually requires the preparation of a vector library to identify productive clones. Recently, Modular Cloning as a novel cloning technique in synthetic biology has been developed. Modular Cloning relies on Golden Gate assembly and supports preparation of individual expression vectors in one-step and one-pot reactions, thus allowing rapid generation of vector libraries. A number of Modular Cloning toolkits for specific applications has been established, providing a collection of distinct genetic elements such as promoters, ribosome binding sites and tags, that can be combined individually in one-step using defined fusion sites. Modular Cloning has been successfully applied to generate various strains for producing value-added compounds. This was achieved by orchestrating complex pathways involving up to 20 enzymes. Due to the novelty of the genetic approach, industrial applications are still rare. In addition, some applications are limited due to the lack of high-throughput screening methods. This shifts the bottleneck from library preparation to screening capacity and needs to be addressed by future developments to pave the path for the establishment of Modular Cloning in industrial applications.

Keywords: Expression control · Golden Gate assembly · Modular Cloning · Pathway design

Zrinka Raguz Nakic obtained her Master's degree in Biotechnology at the ESBS in Strasbourg, France. Subsequently, she completed her PhD thesis on the role of protein phosphorylation in regulating microbial metabolism at the ETH in Zürich, Switzerland, under the supervision of Prof. Uwe Sauer. Afterwards, she joined the Biosystems Technology group of Christin Peters at the Zurich University of Applied Science, Switzerland, as a postdoctoral researcher. Since then, her main research focus is on genetic and strain engineering for whole-cell enzyme cascades optimization as well as process development.

Christin Peters studied biochemistry at the University of Greifswald, Germany. Her PhD thesis (under supervision of Prof. Uwe Bornscheuer, 2015) focused on establishing enzyme cascades by using ene reductases in whole-cell biocatalysis. She then joined the Biocatalysis group of Prof. Buller at the Zurich University of Applied Science as a postdoc. Since 2018, she is leading the Biosystems Technology group, which concentrates on enzyme cascade reactions and process optimization of large-scale biotransformations.

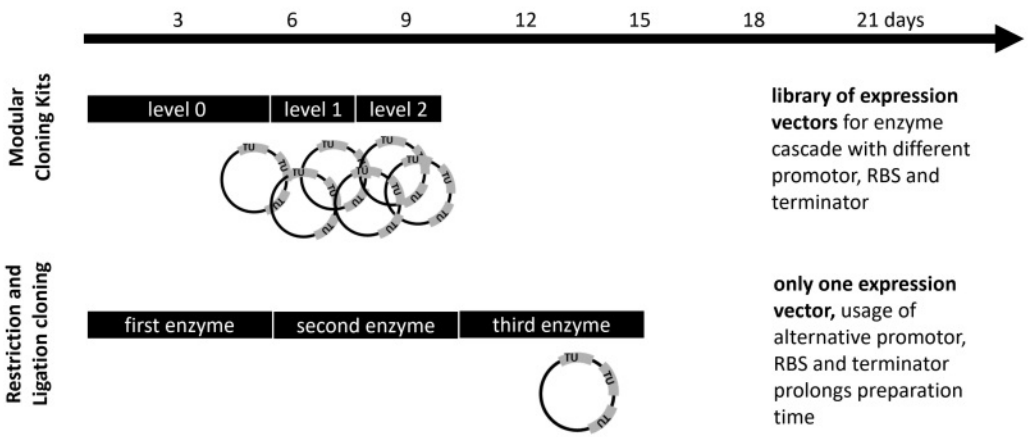
1. Introduction

The field of synthetic biology has developed tools and frameworks to design the pathways of life. Without synthetic biology, efficient biotechnological production of value-added compounds is not conceivable anymore.[1] A multitude of products, including soy leghemoglobin for simulating meat tasting and meat color,[2,3] sitagliptin,[4] engineered bacteria to be used as nitrogen fertilizer,[5] CAR (chimeric antigen receptor) T cell therapy,[6] or genetically modified soy[7] would not be commercially available. In the last decade, novel toolboxes with distinct genetic elements (*e.g.* promoters or ribosome binding sites (RBS)) have been developed. This allows combinations thereof in complex circuits with regulatory parts like logic switches or riboswitches.[8] Nevertheless, in applied synthetic biology, the production of value-added compounds remains challenging. The expression strength of enzymes in metabolic pathways or cascades must be well chosen to account for the differences in activities of the individual enzymes. Balanced enzyme activities are essential to avoid rerouting of flux towards undesired by-products or accumulation of (toxic) intermediates, and hence for efficient turnover.[9] In the last decade, different methods and tools that address this challenge have been developed by designing individual transcription units with distinct promoters, ribosome binding sites or terminators that allow to control expression strengths.[10] Toolboxes, such as CIDAR,[11] BioBrickTM[12] or CRISPR-CLONInG,[13] use standardized vector parts that allow multi hierarchical insert assembly. Our article gives an overview of existing toolboxes for Modular Cloning by Golden Gate assembly for the biotechnological workhorses *E. coli* and yeast. The underlying principles will be described and recent applications to produce value-added compounds will be highlighted.

2. Golden Gate Cloning

Traditionally, single genes are cloned by restriction site digestion and ligation. Here, Type IIp restriction enzymes from the palindromic family are used, which recognize and cleave within symmetric DNA sequences. Especially for more complex applications such as cloning of enzyme cascades or entire pathways this method reaches its limit as it is highly labor-intensive and of limited flexibility (Scheme 1). To avoid the time-consuming

*Correspondence: Dr. C. Peters, E-mail: christin.peters@zhaw.ch
Biosystems Technology Group, Institute of Chemistry and Biotechnology, Zurich University of Applied Sciences, CH-8820 Wädenswil



Scheme 1. Timeline for cloning an expression vector library to establish a three-enzyme cascade with Golden Gate assembly or classical restriction and ligation cloning.

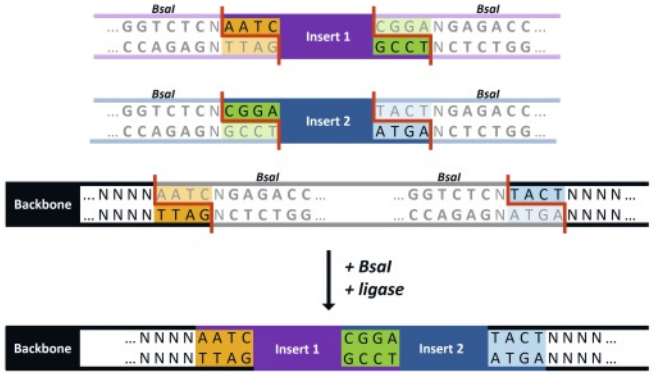
digestion and ligation steps, new cloning methods have been developed, some of which exploit Type IIs restriction endonucleases, which cleave DNA outside their recognition sequence. The first use of these restriction endonucleases was described by Fromme and Klingenspor in 2007[20] who combined Type II and Type IIs endonucleases and by Kotera and Nagai in 2008[21] who used Type IIs endonucleases in combination with polymerase inhibitors. Only a few months later, Marillonnet and coworkers[22,23] published a protocol for the one-step and one-pot Golden Gate cloning method which relies on Type IIs endonucleases, establishing the basis for present Golden Gate protocols. In contrast to classical Type IIp endonucleases, the restriction site of Type IIs endonucleases is located at a defined distance outside the recognition sequence. Due to the shift in the position of the restriction site, subsequent ligation will result in loss of the recognition site in new fragments, thus omitting any undesired further digestion. This allows digestion and ligation to be carried out simultaneously in one-pot reactions, thereby significantly simplifying and accelerating the overall workflow.[24] Importantly, the restriction site is sequence-independent, allowing overhangs of any desired sequence.[25] The frequently applied Type IIs endonuclease BsaI, for instance, follows the recognition pattern 5'-GGTCTC(N1)/(N5)-3' and generates individual designed sticky ends with four base overhangs (Scheme 2).[26] Taking advantage of the option to individually configure sticky ends, Pryore and coworker succeeded in one-pot assemblies of up to 35 DNA fragments with 71% correct clones.[27] Two years later the same group demonstrated the power of Golden Gate assembly by assembling the 40 kb T7 bacteriophage genome from 52 fragments.[28]

3. Hierarchical Assembly

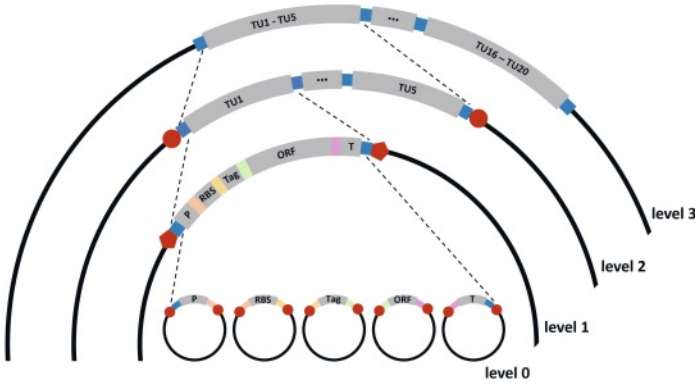
Yet, the efficiency of Golden Gate cloning by itself is not sufficient to disentangle the challenge of balanced expression of enzymes in metabolic pathways or cascades. To this end, the ability to perform hierarchical assemblies is exploited in the so-called Modular Cloning strategies. Here, cloning is performed in several modular assembly steps to create different structural stages that increase in complexity, also called levels. The core for gene assembly are libraries of standard modules (level 0), each of them containing a specific genetic element.[24] The genetic elements typically include different promoters, RBS, protein tags and fluorescent proteins, signal peptides, coding sequences and terminators, all of which are flanked by characteristic fusion sites that allow controlled assembly in a logical predefined order into a backbone, resulting in a level 1 transcription unit (TU) (Scheme 3). The TUs themselves are flanked with inverse sites of a second type IIs endonuclease, enabling in a second step the assembly of up to five such TUs into higher level expression constructs.[24] Importantly, the vector backbones or genes must be free of additional cleavage sites for the type IIs endonucleases. Particularly when ordering synthetic genes, attention should be paid to avoiding the most important type IIs recognition sites in the gene sequences during codon usage optimization. For the implementation of the genetic method, careful and precise planning regarding compatibility of flanking and restriction sites is required to allow flexible shuffling of genetic elements and to ensure correct assembly of the desired constructs. For this purpose, kits with standardized vectors and genetic elements have been designed by various research groups.[10,24]

4. Toolkits for Golden Gate Cloning

The toolboxes developed based on Golden Gate cloning are as diverse as the questions addressed in biotechnology. In Table 1 and 2, examples are listed for toolkits for assemblies in *E. coli* and yeast, respectively.[8,10,24,29] These toolkits contain different plasmid backbones and all elements necessary for combining the genetic fragments into TUs and entire pathways. Nevertheless, the toolkits have important differences. For example, the MoClo Yeast Toolkit[30] contains 96 parts consisting of 5' and 3' assembly connectors, promoters, coding sequences, terminators, markers, origin and homology markers, whereas the YeastFab[31] toolkit contains over 2000 different promoters. Not only the number of



Scheme 2. Example of Golden Gate assembly using the Type IIs endonuclease BsaI. Two genomic parts (insert 1/ 2) are integrated into the backbone of a target vector. Compatible sticky ends ensuring directed assembly are depicted with the same color.



Scheme 3. Overview of the vector levels of a typical Modular Cloning kit. Level 0 vectors contain the individual genetic elements required for directional cloning into a level 1 vector transcription unit (TU). This process is illustrated here with a construct consisting of a promoter (P), a ribosome binding site (RBS), a His-tag (Tag), an open reading frame (ORF) and a terminator (T). Compatible sticky ends ensuring directed assembly of level 0 vectors are depicted with the same color. Red circles and pentagons represent the recognition sequence for different type IIs endonucleases. Subsequently, several level 1 TU vectors (TU1-TU5 in this example) can be digested by a second type IIs endonuclease and ligated into a level 2 vector to enable expression of several proteins from a single vector. Level 2 vectors can be further digested with the first type IIs endonuclease for cloning of 20 or more transcription units into a level 3 vector for large metabolic pathway engineering.

genetic elements can vary strongly. Some toolkits contain in addition specific parts for designated applications, *e.g.* COMPASS (COMbinatorial Pathway ASSembly) enables integration of DNA parts into the yeast genome and utilizes artificial plant-derived transcription factors to construct orthogonal plant based transcription regulatory units.[32] Besides, the number of proteins targeted for expression may play a critical role in the choice of a certain toolkit. This is especially crucial when designing larger metabolic pathways or cascades. When working with *E. coli*, the CIDAR MoClo toolkit offers a flexible system, but it can combine only 4 TUs.[11] In contrast, the EcoFlex toolkit allows the assembly of up to 20 TUs, thus supporting flexible generation of larger metabolic pathways.[16] However, EcoFlex is not compatible with other toolkits since it does not follow the standard fusion site syntax[33] as CIDAR MoClo.[11] or MoClo Toolkit[34] do.

5. Applications

Over the last 15 years, more than 30 different Modular Cloning toolkits have been developed for a wide variety of organisms.[24] However, the number of applications reported remains far behind the numerous toolkits available. The Ecoflex kit was primarily applied to optimize the production of violacein as a bisindole pigment.[16] Violacein is of notable interest due to its antitumor effects and serves as a suitable model metabolic pathway as its purple color allows for easy screening. Combinations of different promoter and RBS that support diversity in expression strengths of the five pathway enzymes were examined regarding violacein formation in *E. coli*. The best variant created using Modular Cloning resulted in a conversion of 66.3 ± 5.6 mg/L while the least productive strain achieved a yield of 3.6 ± 1.7 mg/ml. Interestingly, the positive control with all violacein genes under the control of the standard J23114 promoter, pET RBS and standard BBa_B0015 terminator per gene produced violacein in a comparable range at 69.1 ± 7.4 mg/L.[16] Thus, Modular Cloning using EcoFlex was capable to generate a pathway with superior performance compared to the initial metabolic pathway. In contrast, significantly higher production of violacein with 1829 ± 46 mg/L was achieved with the Modular Cloning toolkit ePathBrick *via* monocistronic assembly.[17] Here, the metabolic pathway was constructed using IPTG inducible promoters instead of constitutive promoters. A first screening evaluating the strengths of the different promoters showed that constructs with weak to medium strength promoters resulted in good conversions. Thus, only the three weakest promoters were used to screen a larger number of variants to identify the most promising candidate.[17] To avoid a potential infection risk of *E. coli* by bacteriophages, the production of violacein in *Yarrowia lipolytica* was also investigated. Here, the violacein pathway was optimized by combining three promoters with different expression strength to establish the five pathway enzymes and to achieve a titer of 70.04 mg/L.[39]

Besides violacein, production of a variety of other compounds was optimized by Modular Cloning approaches. The recently developed Modular Cloning toolkit YALIcloneNHEJ for non-homologous end-joining (NHEJ)-mediated random integration in *Yarrowia lipolytica* enabled construction of a high production strain of the sesquiterpene (–)-α-bisabolol with 4.4 g/L by balancing the expression strength.[40] Using the Ecoflex kit, the production of the raspberry ketone (main aroma component of raspberries) was recently also successfully demonstrated. Here, a design-build-test-learn cycle approach was used to fine tune the enzyme expression on the promoter site, thereby increasing the productivity from 0.2 mg/L to 12.9 mg/L.[41]

Previous applications of pathway design by Modular Cloning focused largely on the production of substances that cause coloring of colonies, which greatly simplifies screening. Development of additional screening approaches for the identification of productive clones will broaden the applicability of Modular Cloning

Table 1. Examples of reported toolkits for assembly in E. coli (not exhaustive)

Toolbox	Most characteristic genetic parts	Details
CIDAR MoClo[11]	39 promoters, 6 RBS, 11 CDS, 4 terminators	3 level pathway assembly, maximum 4 TUs
CRIMoCLO[14]	5 promoters, 1 RBS, 8 CDS, 3 terminators, 4 att sites	Allows a rapid route to chromosomal integration
EcoFlex[15,16]	15 promoters, 15 RBS, 2 N-terminal tag, 3 CDS, 13 terminators	4 level pathway assembly, maximum 20 TUs
ePathOptimize[17]	5 promoters, 5 CDS	T7 promoter library
Mobius Assembly Vector Toolkit[18]	16 vectors, genetic parts can be used from MoClo, Golden Braid, and Phytobricks	Use of AarI as alternative IIs endonuclease, alternative plasmid backbones
OLMA[19]	Different promoters, 20 RBS, 3 CDS	Oligo-linker (coding rbs) mediated assembly

Table 2. Examples of reported toolkits for assembly in yeast (not exhaustive)

Toolbox	Most characteristic genetic parts	Details
COMPACTER[35]	Different promoters (circa 30)	1 level pathway assembly
COMPASS[32]	Promoters, 9 inducible artificial transcription factors / binding site, CDS, terminator	3 level pathway assembly, plant-derived artificial transcription factors and homologous recombination-based cloning
COSPLAY[36]	7 promoters, 4 CDSs, 4 degrons, 1 terminators, 5 selection markers	Properties of the pRS plasmids
GoldenPiCS[37]	20 promoters, 10 terminators, 4 genome integration loci, 4 resistance marker cassettes	Up to 8 transcription units per plasmid
Modular Yeast Toolkit[30]	7 5'- and 7 3'-assembly connectors, 23 promoters, 5 coding sequences, 6 terminators, 7 markers, 5 origin and 9 homology markers	2 level pathway assembly
VEGAS[38]	10 promoter, 5 terminator, 18 VEGAS (Versatile genetic assembly system) adapters	For performing homologous recombination with terminal homology, 3 level pathway assembly
YeastFab[31]	Over 2 000 promoters, 2 terminators	3 level pathway assembly

techniques. For example, using the COMPASS toolkit and applying biosensors, the scientists around Gita Naseri have recently been able to develop a strain for the production of colorless β-ionone.[32]

6. Outlook and Conclusion

The applications described illustrate that the benefits of Modular Cloning have not yet been fully exploited in industrial application. There are several reasons for this. Importantly, most of the toolboxes are novel and have only been developed in the last 10 years. On the other hand, the creation of a large combinatorial library requires screening of the individual clones to identify the best variant. Depending on the product, there is often a large discrepancy in the pace at which the expression libraries are created using Golden Gate assembly and the screening that is usually time-consuming and complex. The bottleneck of library evaluation is no longer on the side of the cloning but shifts to the screening of the clones. Due to the variety of elements that can be combined into TUs or pathways, the number of possible combinations rises rapidly. The construction of a library for a 5-step pathway with only 10 different expression levels per enzyme already results in 100'000 possible combinations. Notably, the number of combinatorial elements is usually much higher. While the applicability of the reported toolkits was mostly demonstrated with colored products that simplify screening, most added-value molecules are colorless, and alternative high-throughput screening approaches are required. In order to enable high-throughput screening or even ultrahigh-throughput screening,[42] synthetic biology itself offers solutions with integrated biosensors[43] based on transcription factors,[44] fluorescence resonance energy transfer (FRET),[45] RNA-based biosensors[46] or synthetic genetic circuits.[47] Yet, the application of biosensors increases the complexity of the system and thus the susceptibility to errors and the development time of the system. One possibility to decrease the screening effort is the reduction of the number of clones to be screened by smart and rational libraries. This reduction can be achieved by exploiting knowledge of theoretical expression levels of individual elements as well as specific enzyme activities. Here, the recent developments in machine learning and deep learning can be of great benefit, provided that suitable data sets for the training of the system are available.[48] For example, Panke and coworker recently developed RedLibs (Reduced Libraries), an algorithm that allows rational design of smart combinatorial libraries for pathway optimization.[49] Furthermore, automated Design-Built-Test-Learn (DBTL) pipelines for the rapid design and optimization of biochemical pathways will be of increasing relevance as for example applied in the production of the flavonoid (2*S*)-pinocembrin in *E. coli*.[50] In any future applications of Modular Cloning techniques, knowledge and compatibility of the individual elements for plasmid design will be essential. An important contribution to this is made by the Standard European Vector Architecture (SEVA), a web-based resource and material clone repository that supports the selection of optimal plasmid vectors for the de- and reconstruction of complex prokaryotic phenotypes.[51]

Less than 15 years after the development of the Golden Gate assembly, there is already an enormous variety of Modular Cloning toolkits not only for *E. coli* and yeast, but also for plants, mammalian cells and baculovirus, amongst others.[24] Rapid progress is still ongoing in this dynamic field, and it is expected that the applications of Modular Cloning will expand even further, so that Modular Cloning can also be increasingly used in industry in the future.

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