MANIPULATING DNA FOR NATURAL PRODUCT APPLICATIONS

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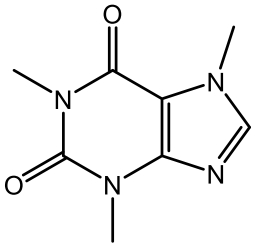
University Paris-Saclay
M2 Fundamental Microbiology

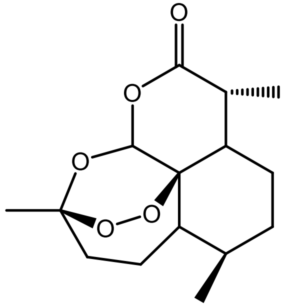
PROGRAM OF THIS SESSION

- Some knowledge on Natural Products
  - NRPS, RiPPs
- Some DNA assembly techniques
  - Mutagenesis, LCR, PCR targeting, Biobrick assembly, SLICE
- Two examples of applications from my work
  - Refactoring congocidine biosynthetic gene cluster, producing non-natural analogs of sviceucin
- Application for non natural products, the example of adipic acid

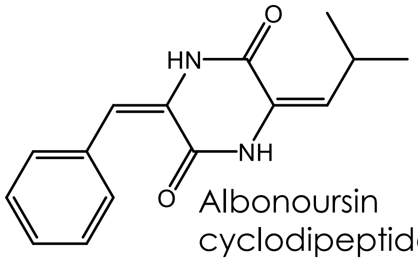
DIFFERENT CLASSES OF NATURAL PRODUCTS



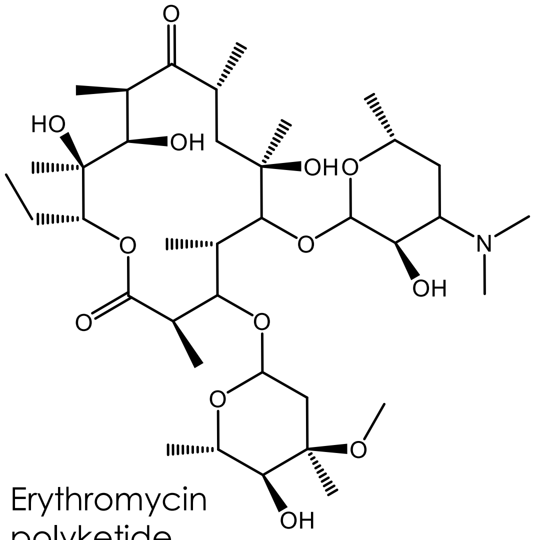
Caffeine
alcaloïde



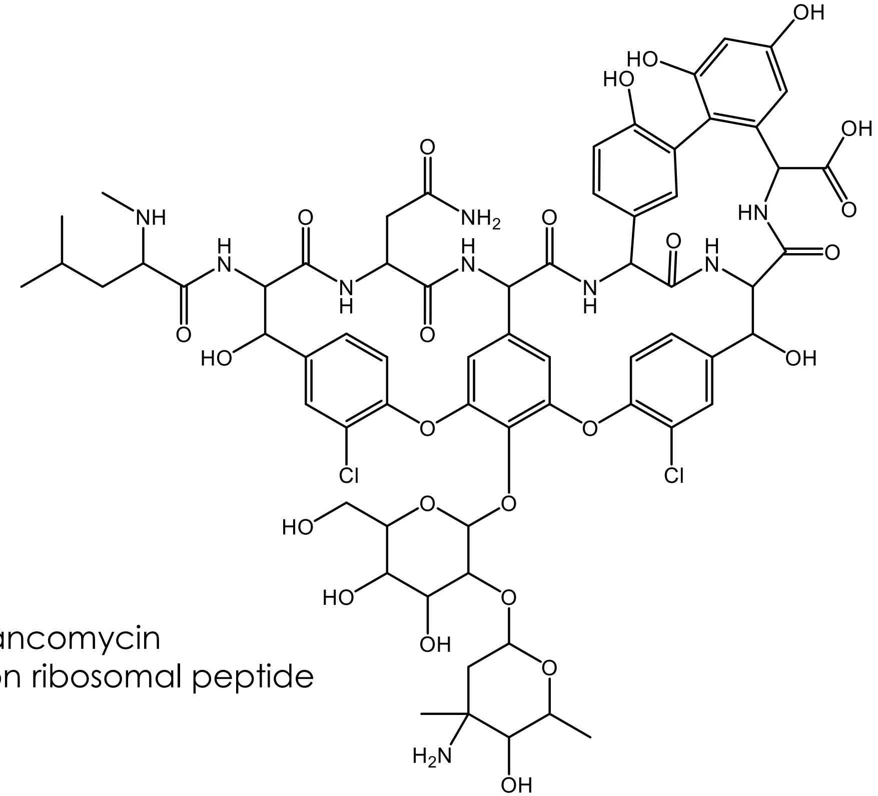
Artemisinin
terpène



Albonoursin
cyclodipeptide

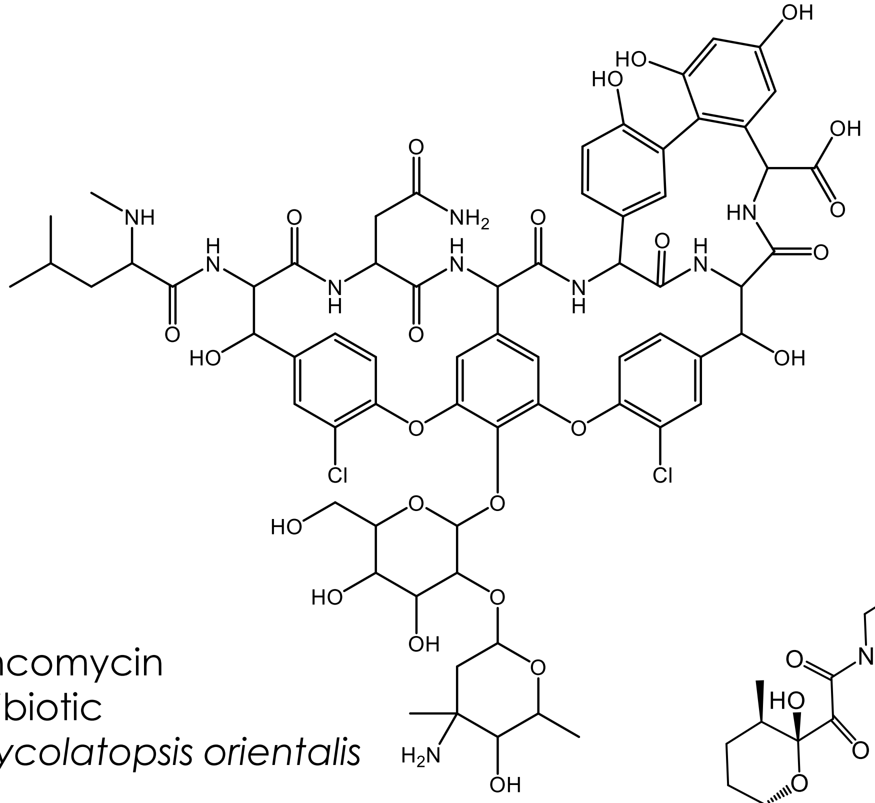


Erythromycin
polyketide

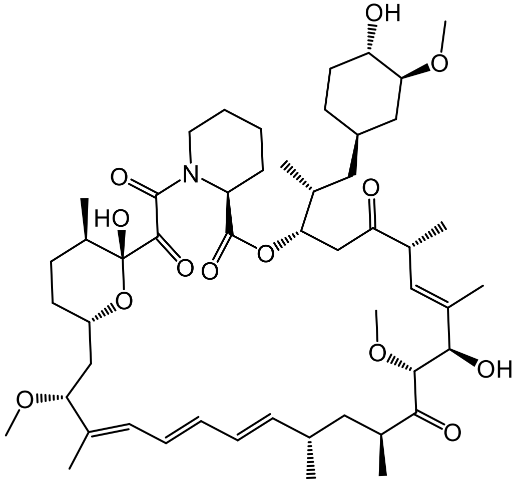


Vancomycin
non ribosomal peptide

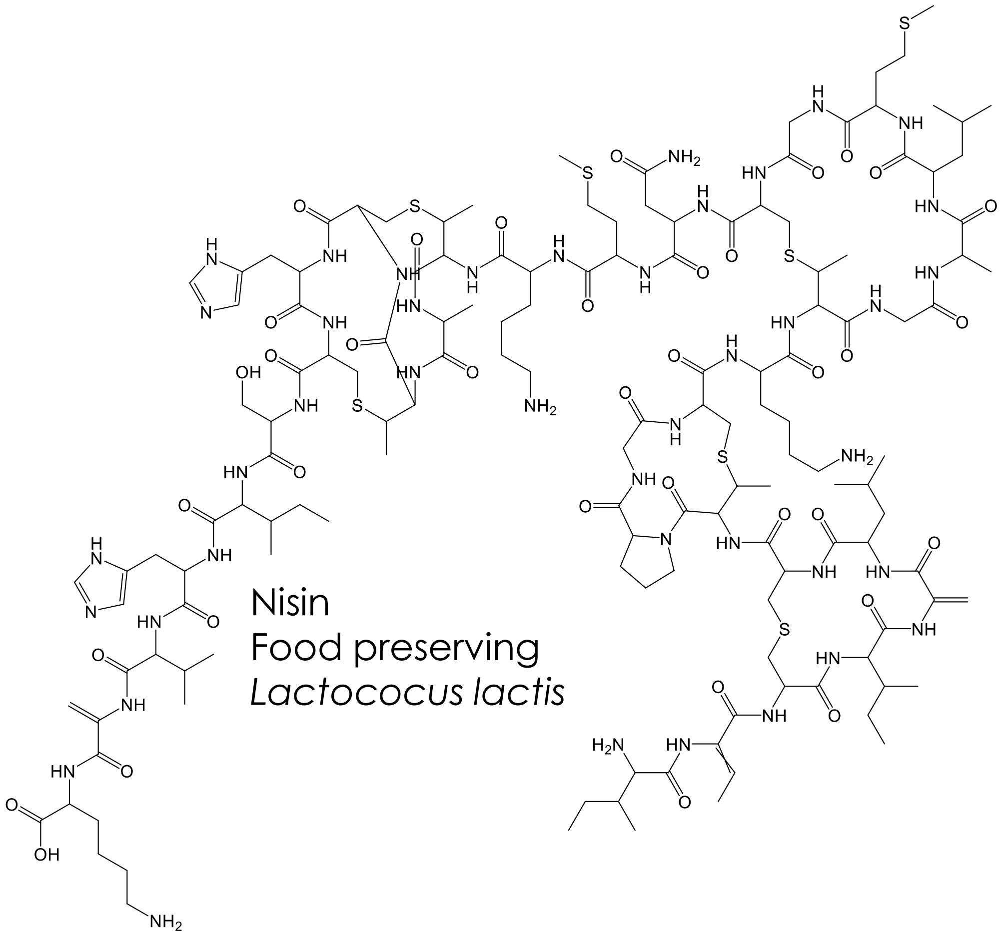
BIOLOGICAL ACTIVITIES OF NATURAL PRODUCTS



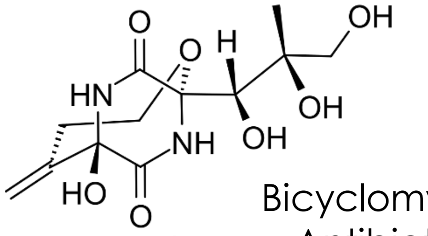
Vancomycin
Antibiotic
Amycolatopsis orientalis



Rapamycin
Immunosuppressor
Streptomyces hygroscopicus

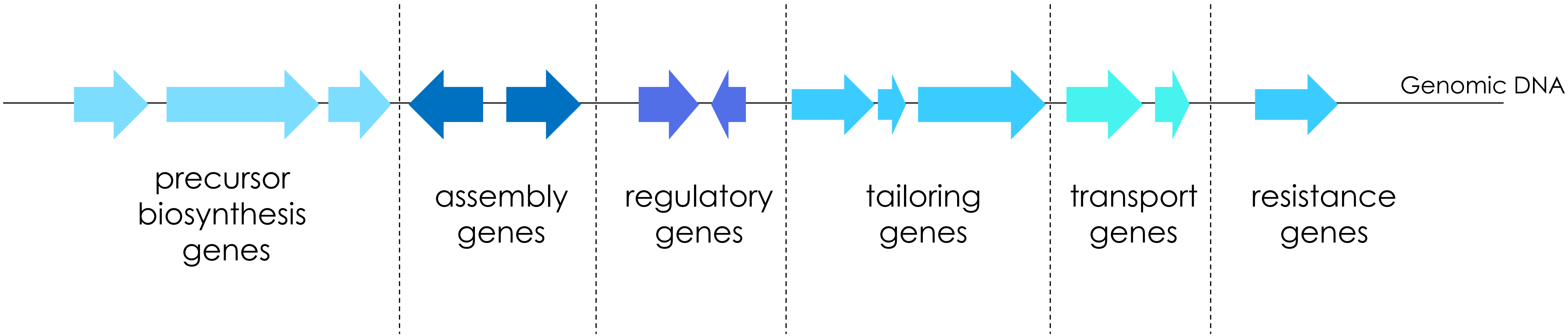


Nisin
Food preserving
Lactococus lactis



Bicyclomycin
Antibiotic
Streptomyces sapporonensis

ORGANISATION IN BIOSYNTHETIC GENE CLUSTERS



Production of one or several natural products

SYNTHETIC BIOLOGY OF NATURAL PRODUCTS

- Development of tools (strains, vectors, regulatory components)
- Reconstruction of a biosynthetic gene cluster to express a compound (refactoring)
- Combinatorial biosynthesis

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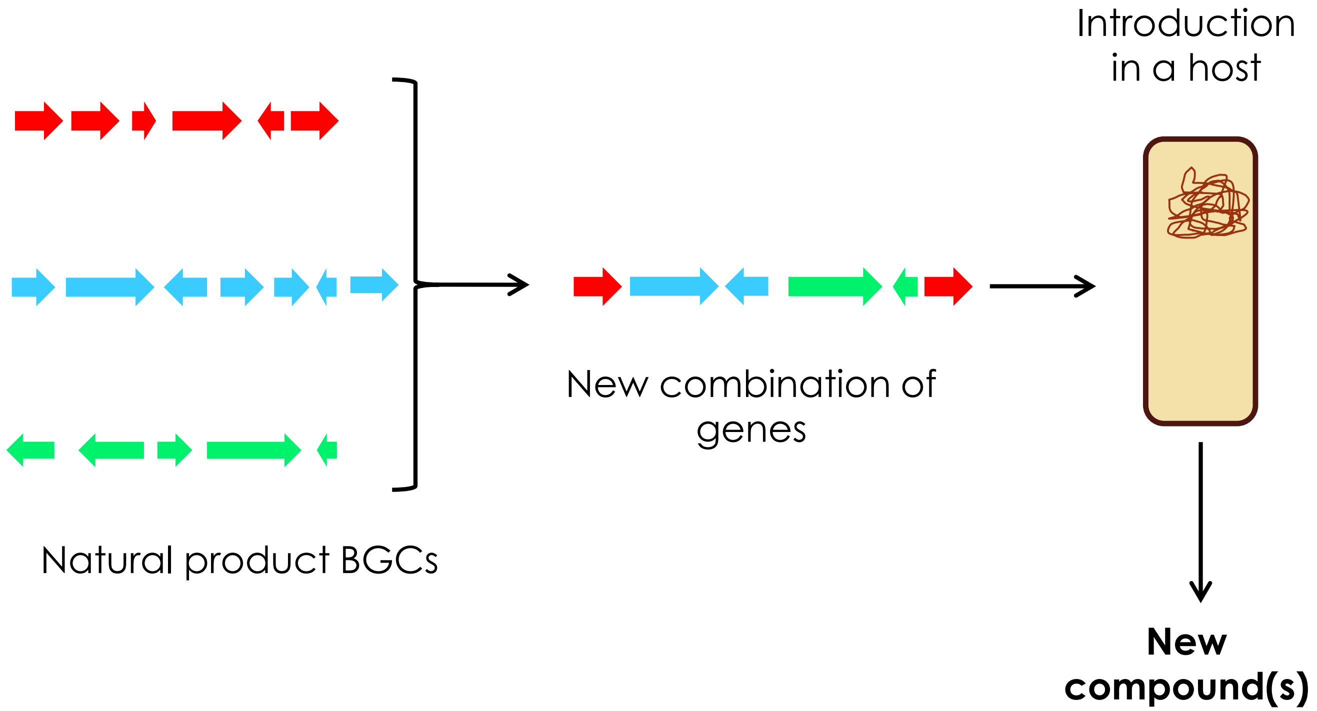
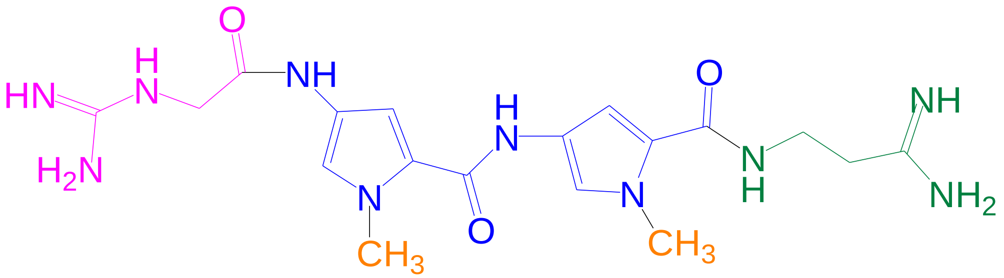
Principle of Combinatorial biosynthesis



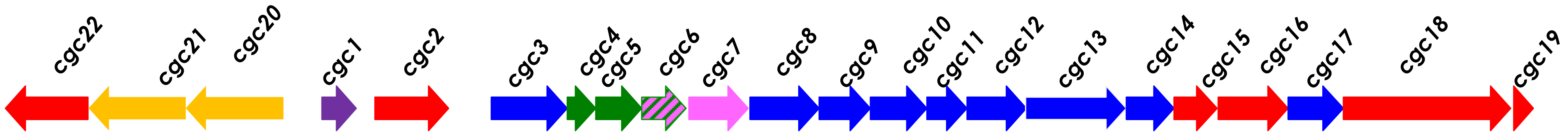
Interests:

- Understanding
- Synthesis of new compounds

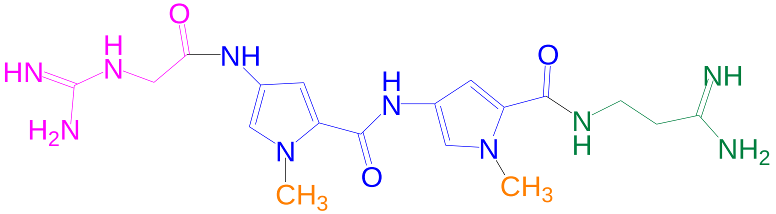


Congocidine, *S. ambofaciens*

REFACTORING OF CONGOCIDINE BIOSYNTHETIC GENE CLUSTER



THE PYRROLAMIDE FAMILY



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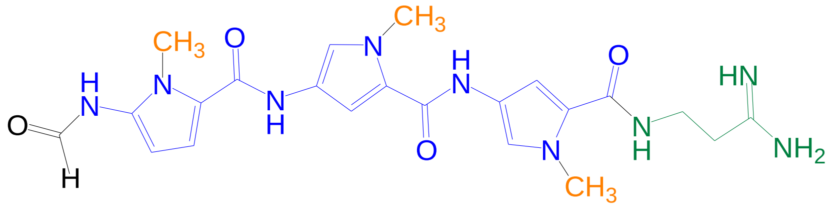
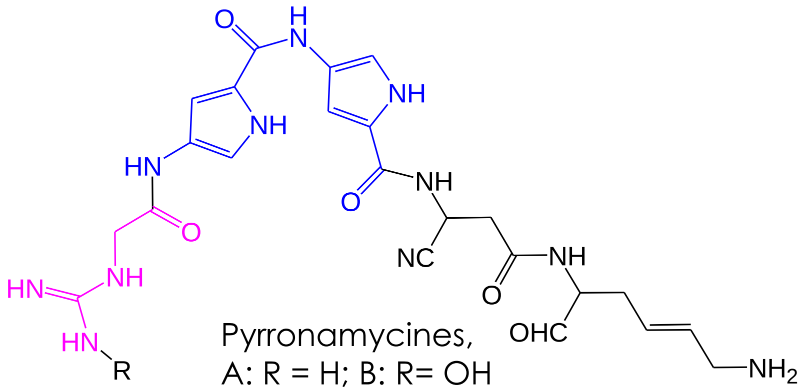
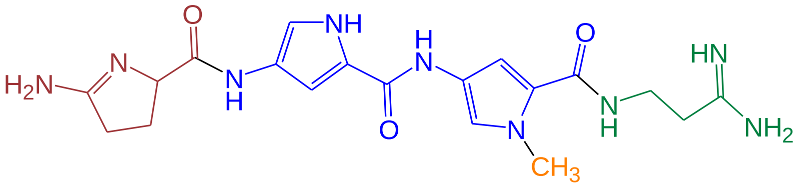


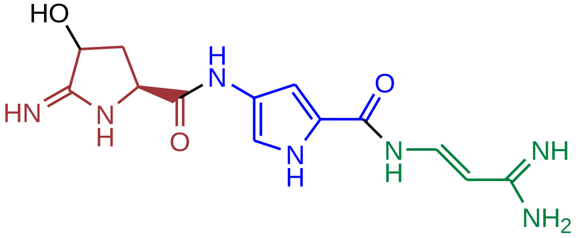
Distamycine, *S. netropsis* (*S. distallicus*)



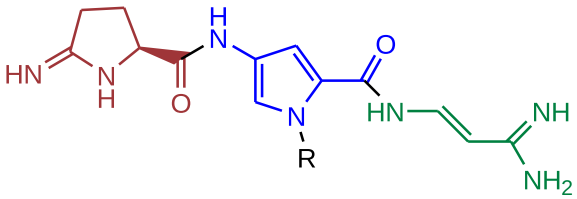
Pyrronamycines,
A: R = H; B: R= OH
(*Streptomyces* sp.)



Anthelvencine A, *S. venezuelae*



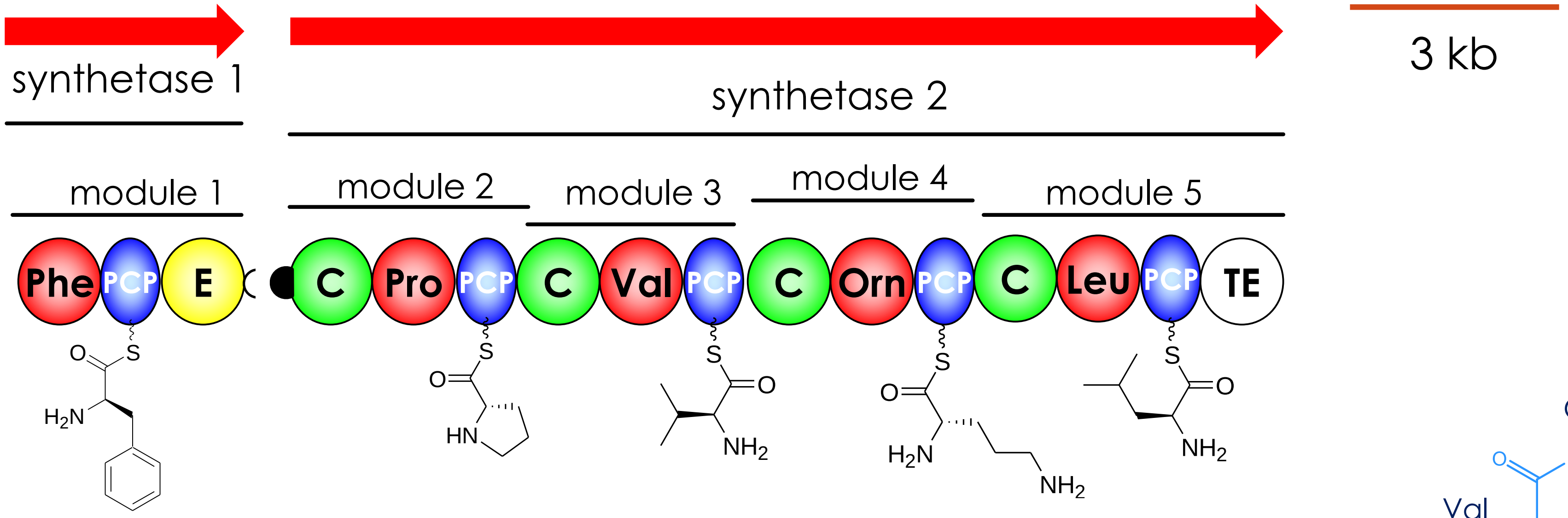
TAN 868A, *S. idiomorphus*



Kikumycines, *S. phaeochromogenes* R-719

R= H kikumycine A, R=CH3, kikumycine B

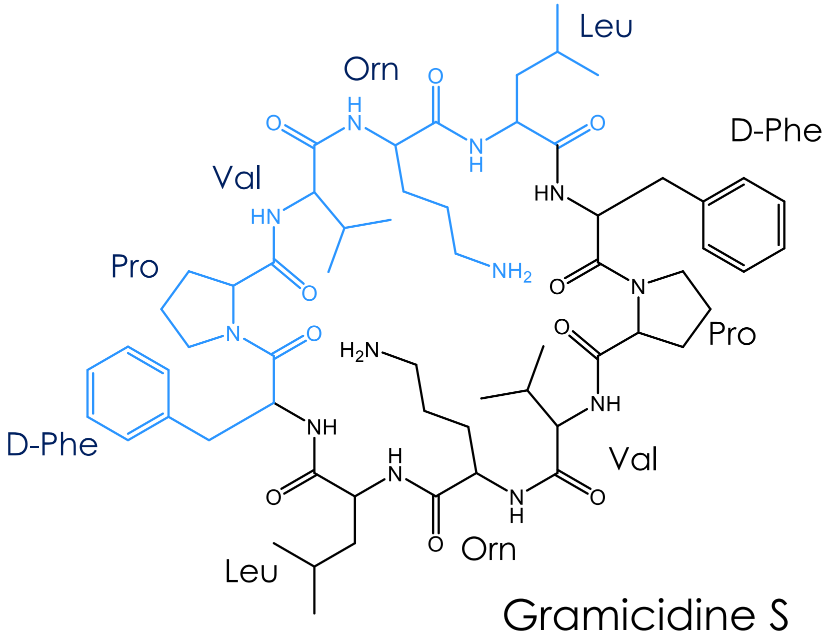
NON RIBOSOMAL PEPTIDE SYNTHETASE (NRPS)



Adenylation domain (A)

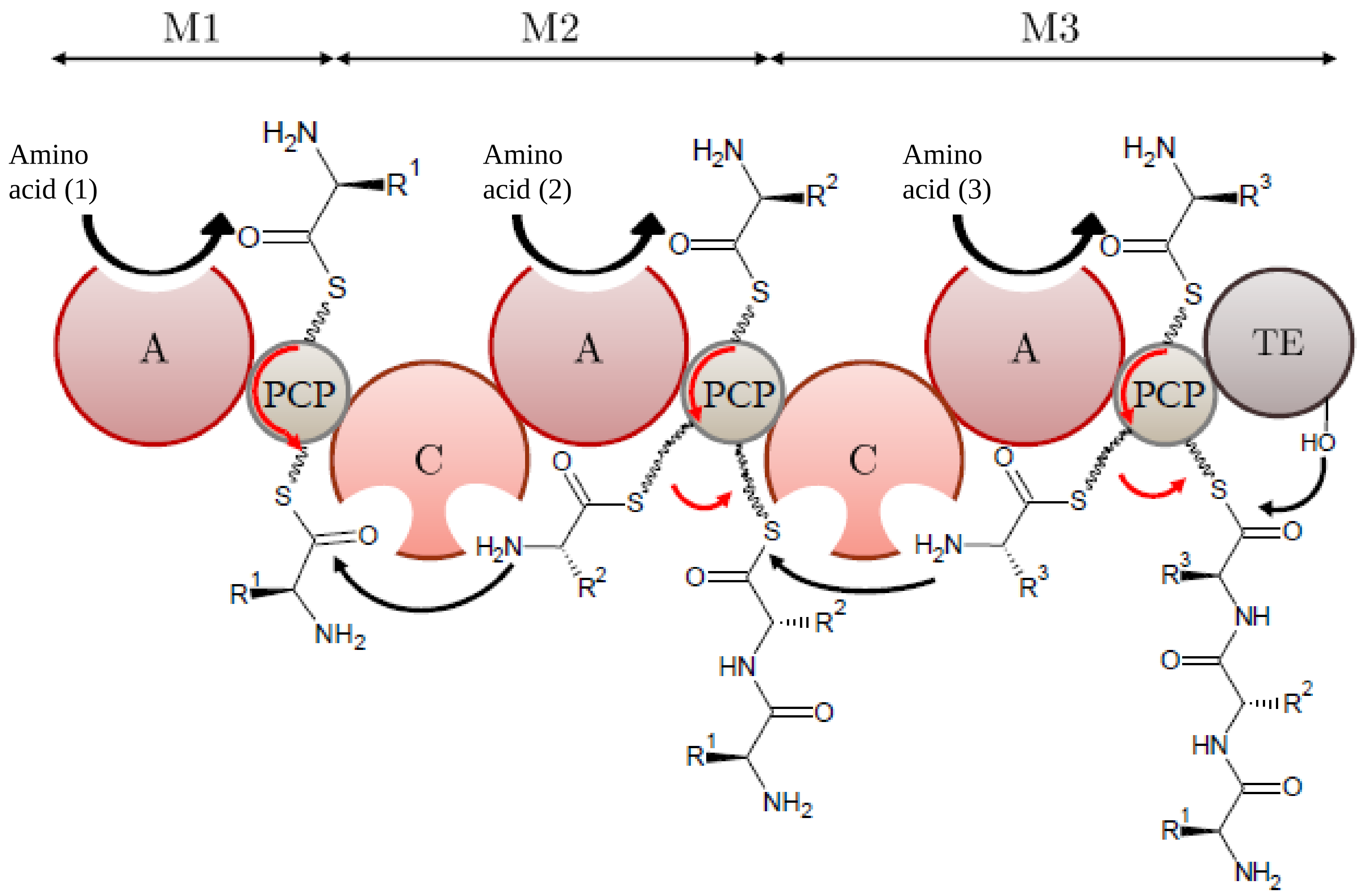
Peptidyl-Carrier Protein (PCP)

Condensation domain (C)

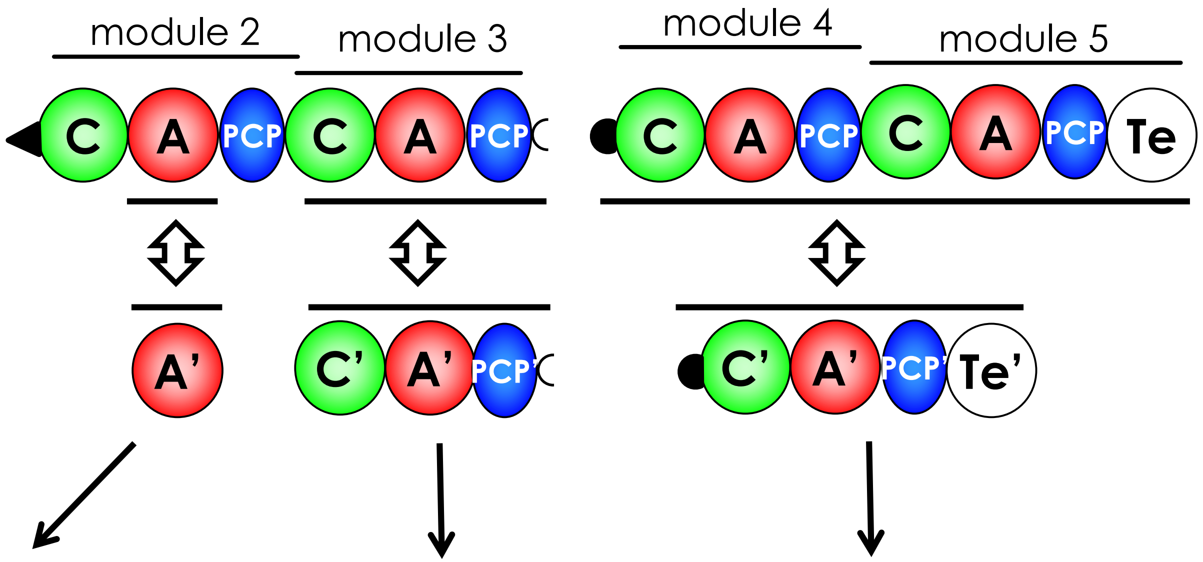


Gramicidine S

NON RIBOSOMAL PEPTIDE BIOSYNTHESIS MODEL



COMBINATORIAL BIOSYNTHESIS POSSIBILITIES OF NON RIBOSOMAL PEPTIDE SYNTHETASES (NRPS)



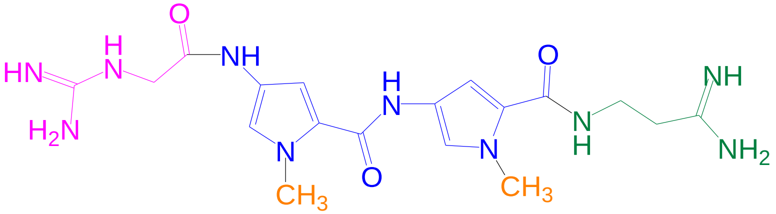
Change a A domain for another

Change the specificity of a A domain

Change a full module

Change a complete sub-unit (change the COM domain)

THE PYRROLAMIDE FAMILY



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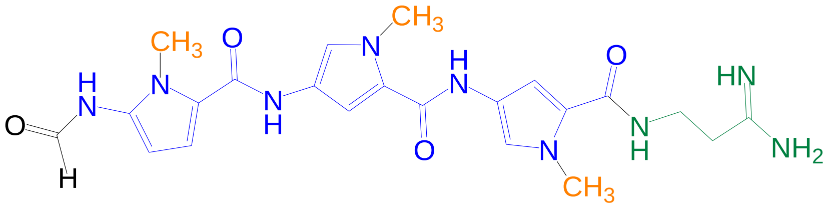

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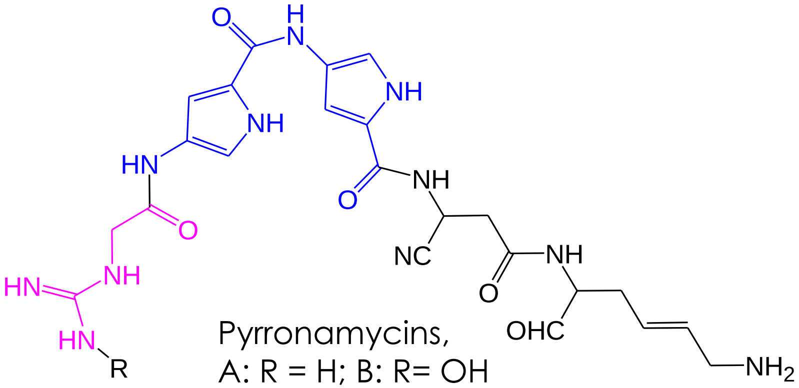

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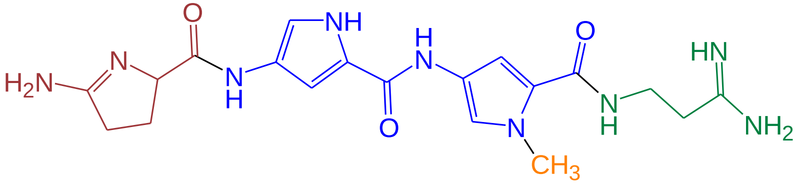

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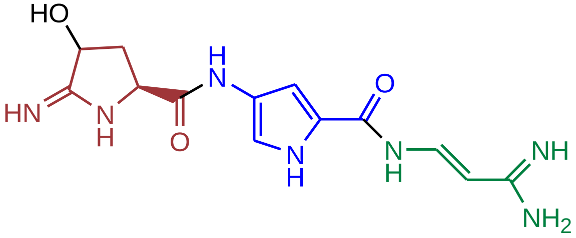

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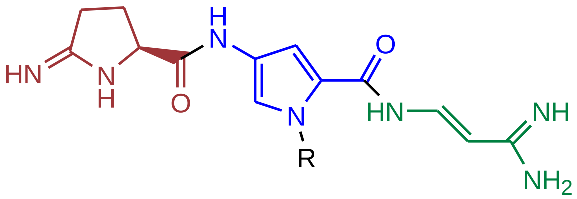

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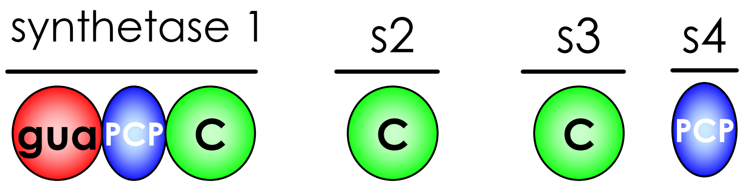
R= H kikumycin A, R=CH3, kikumycin B

NON-RIBOSOMAL PEPTIDE SYNTHETASE (NRPS) OF CONGOCIDINE

Congocidine NRPS



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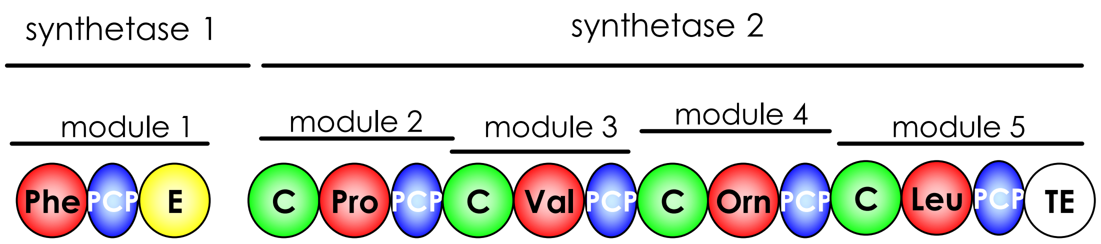
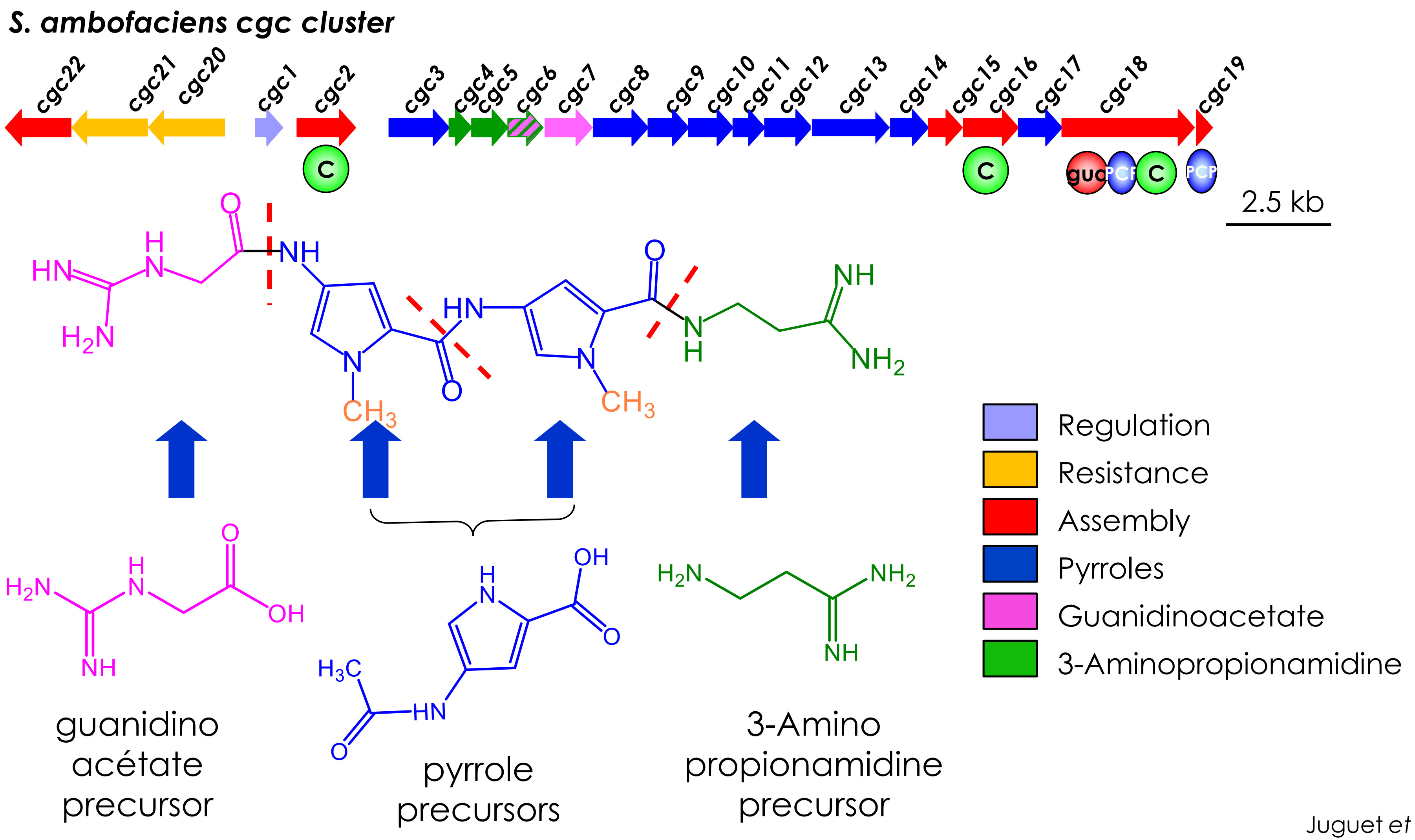
Gramicidine NRPS

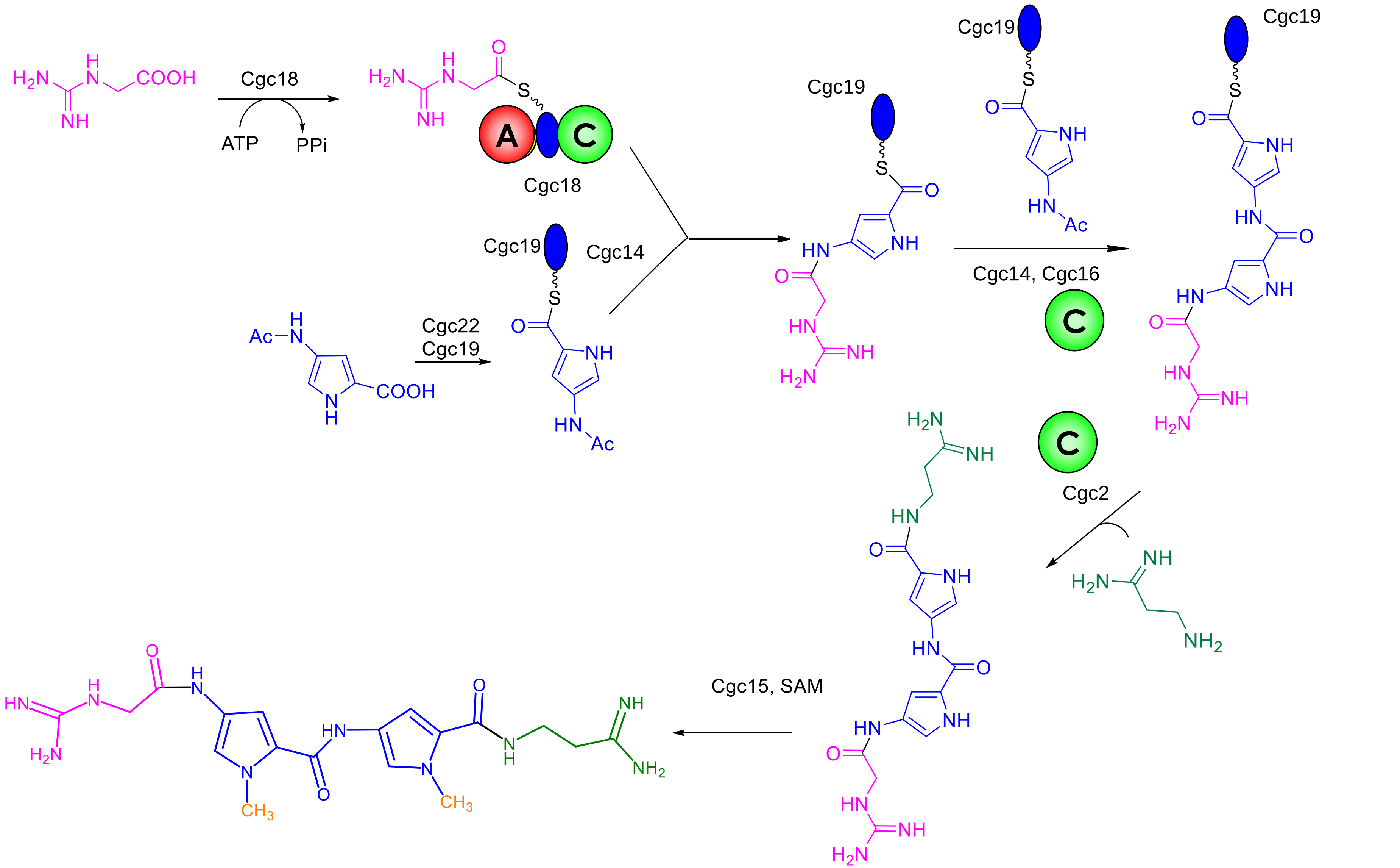


BIOSYNTHETIC GENE CLUSTER OF CONGOCIDINE FROM *STREPTOMYCES AMBOFACIENS*



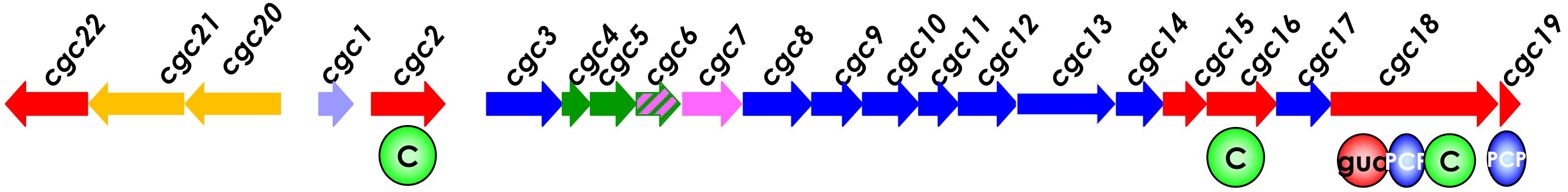
Juguet *et al*. Chem. Biol. (2009),
Vingadassalon et al., ACS Chem. Biol(2015)

MODELE OF ASSEMBLY OF CONGOCIDINE



MODIFICATION OF A BGC: PRELIMINARY QUESTIONS

S. ambofaciens cgc cluster



MODIFICATION OF A BGC: PRELIMINARY QUESTIONS

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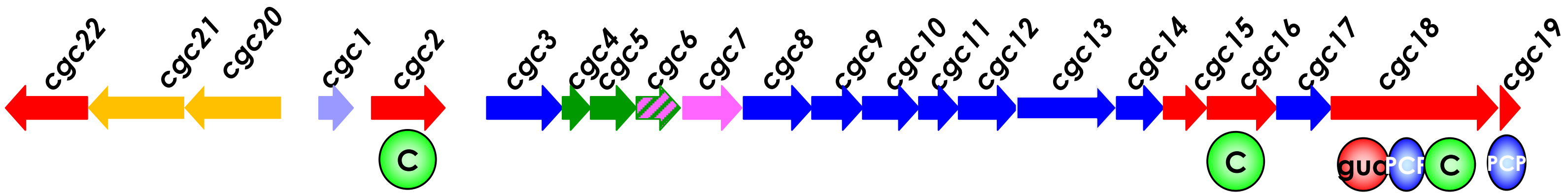
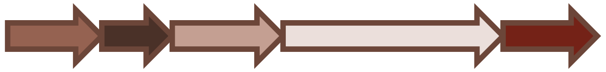


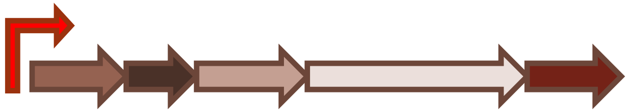
- How does the regulation work ?
- What are the limits of the genes ?
- Where are the operons ?

Decision to refactor the cluster to have a reconstructed cluster with exchangeable elements

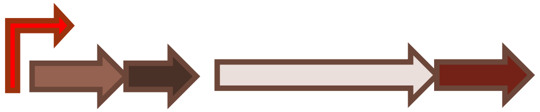
PRINCIPLE OF REFACTORING OF A BIOSYNTHETIC GENE CLUSTER



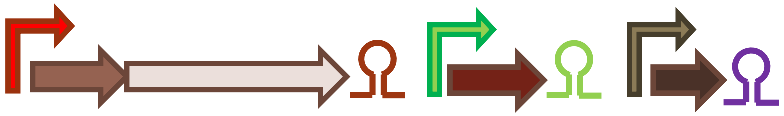
- Different levels of refactoring
  - Addition of a **promoter** upstream of the operon



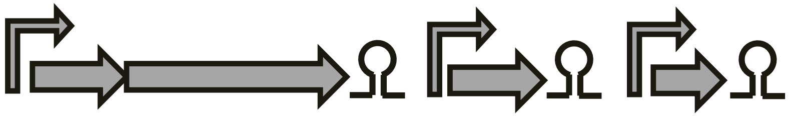
  - **Selection** of some genes, in order to evade natural transcriptional regulation



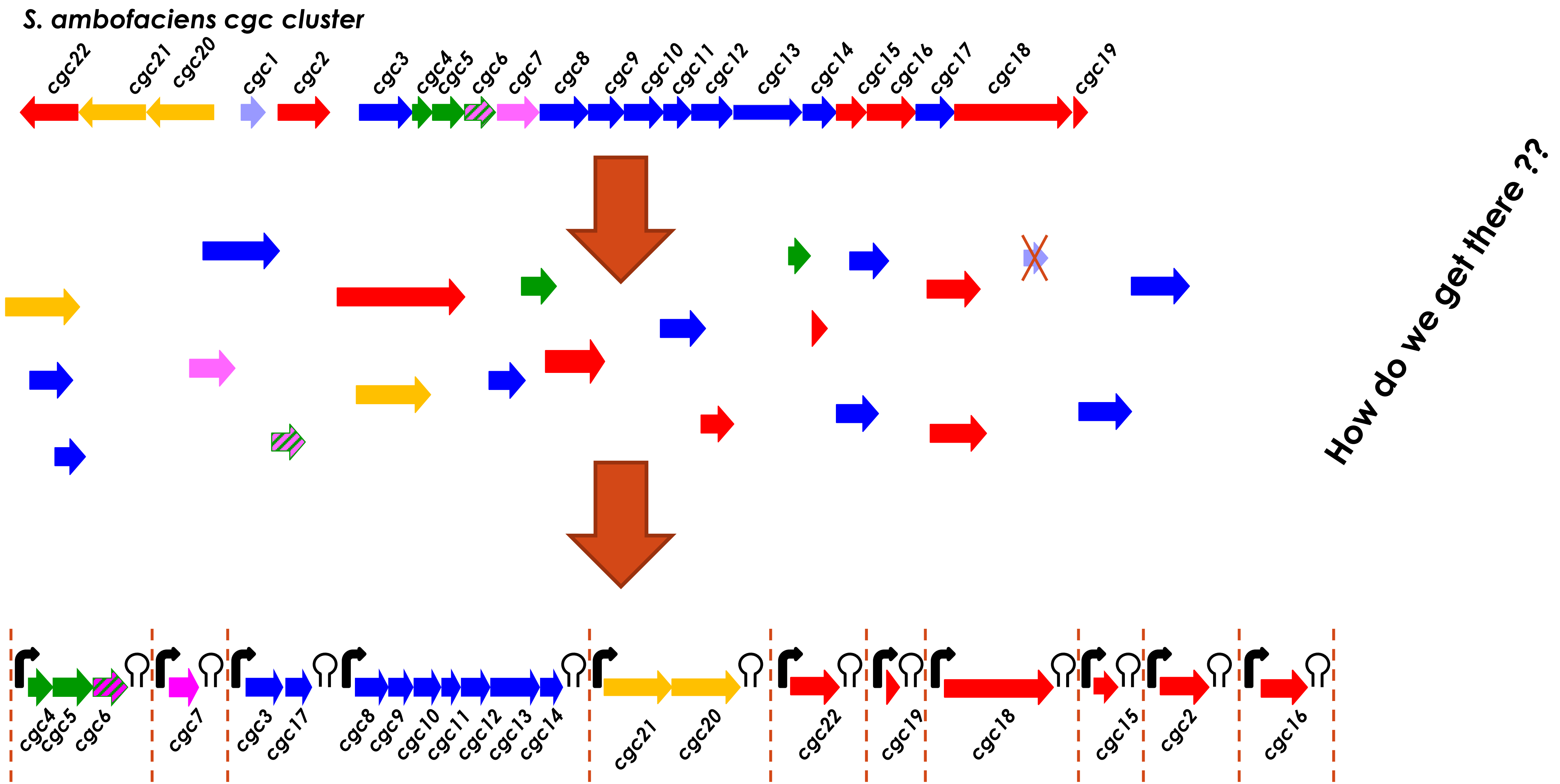
  - **Reorganisation** of the groupe of genes



  - **Synthesis** of a totally synthetic construct



REFACTORING OF THE CONGOCIDINE BGC

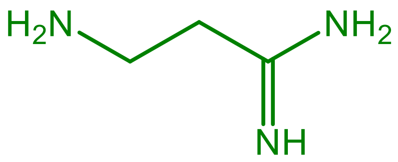


EXAMPLE OF THE SYNTHETIC CASSETTE

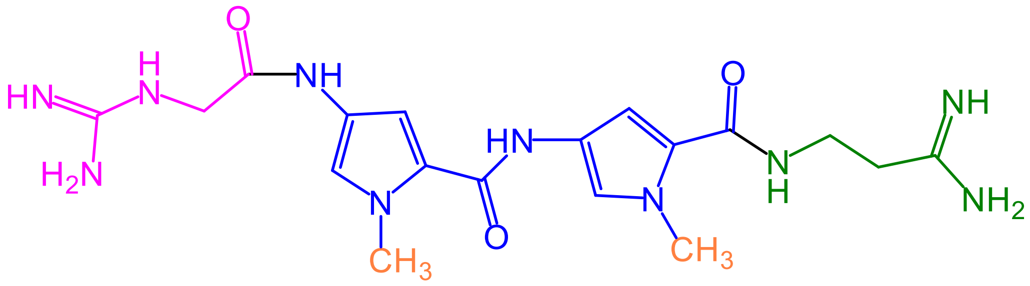
Synthetic promoter derived from *kasOp** (Bai *et al.*, 2015)

genes

terminator T4

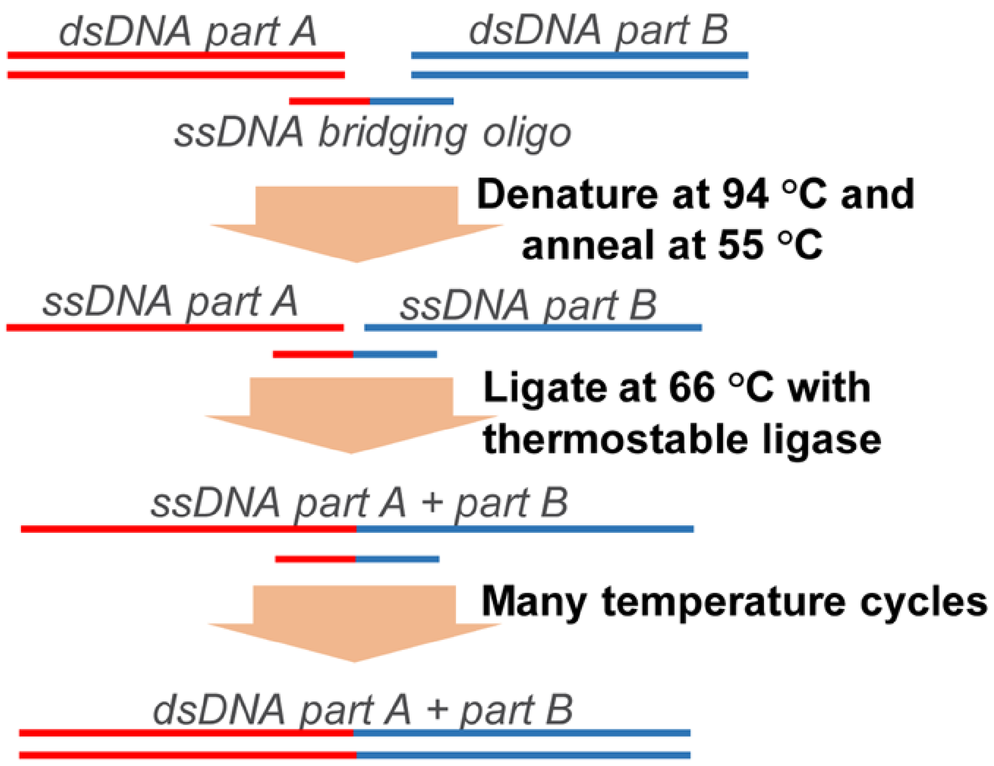


cgc4, *cgc5*, *cgc6* :
Biosynthetic genes of the precursor 3-Aminopropionamidine



congocidine

LCR ligase cycling reaction

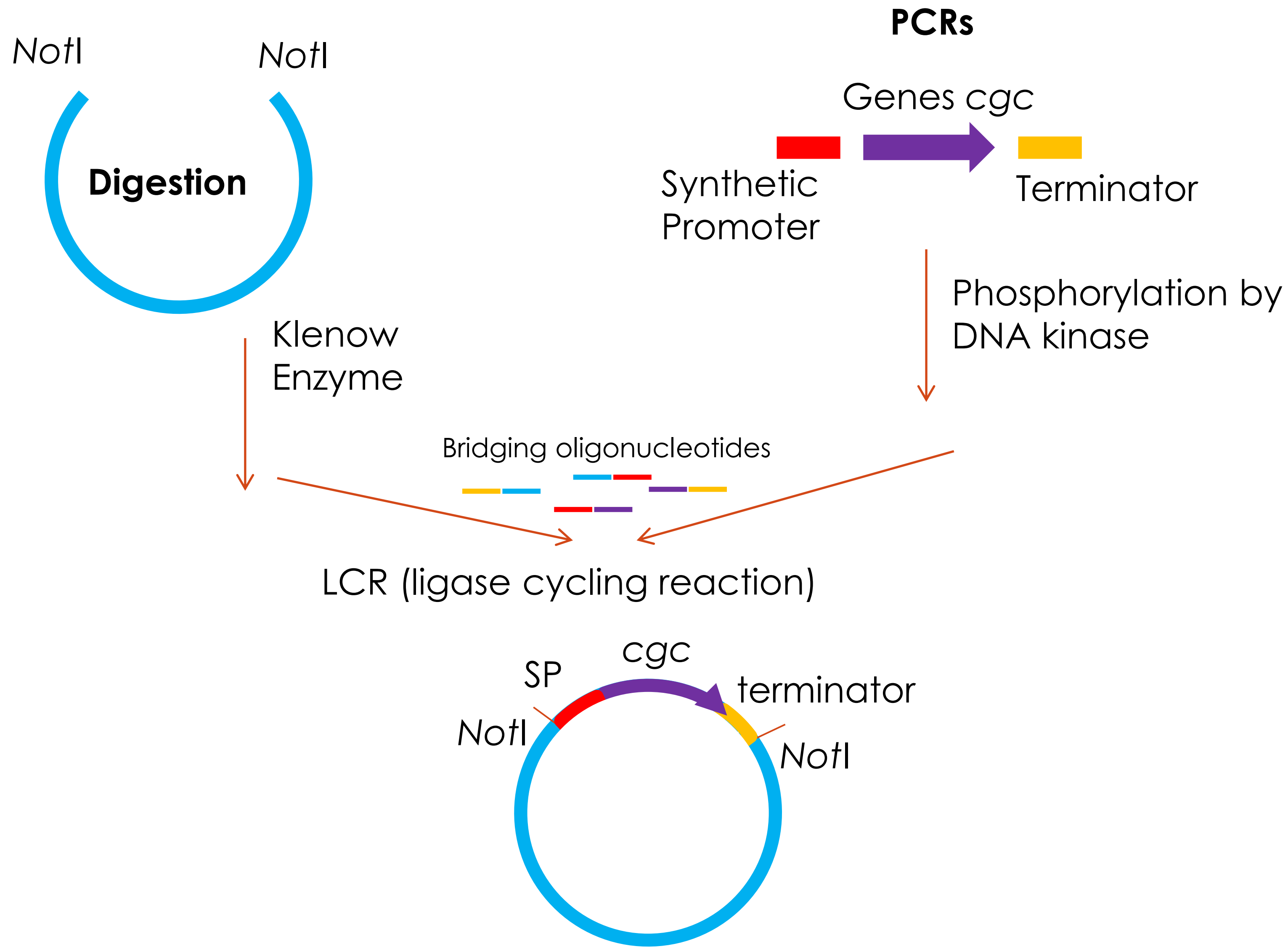


- single-stranded bridging oligos complementary to the ends of neighboring DNA parts,
- thermostable ligase to join DNA backbones,
- multiple denaturation– annealing–ligation temperature cycles

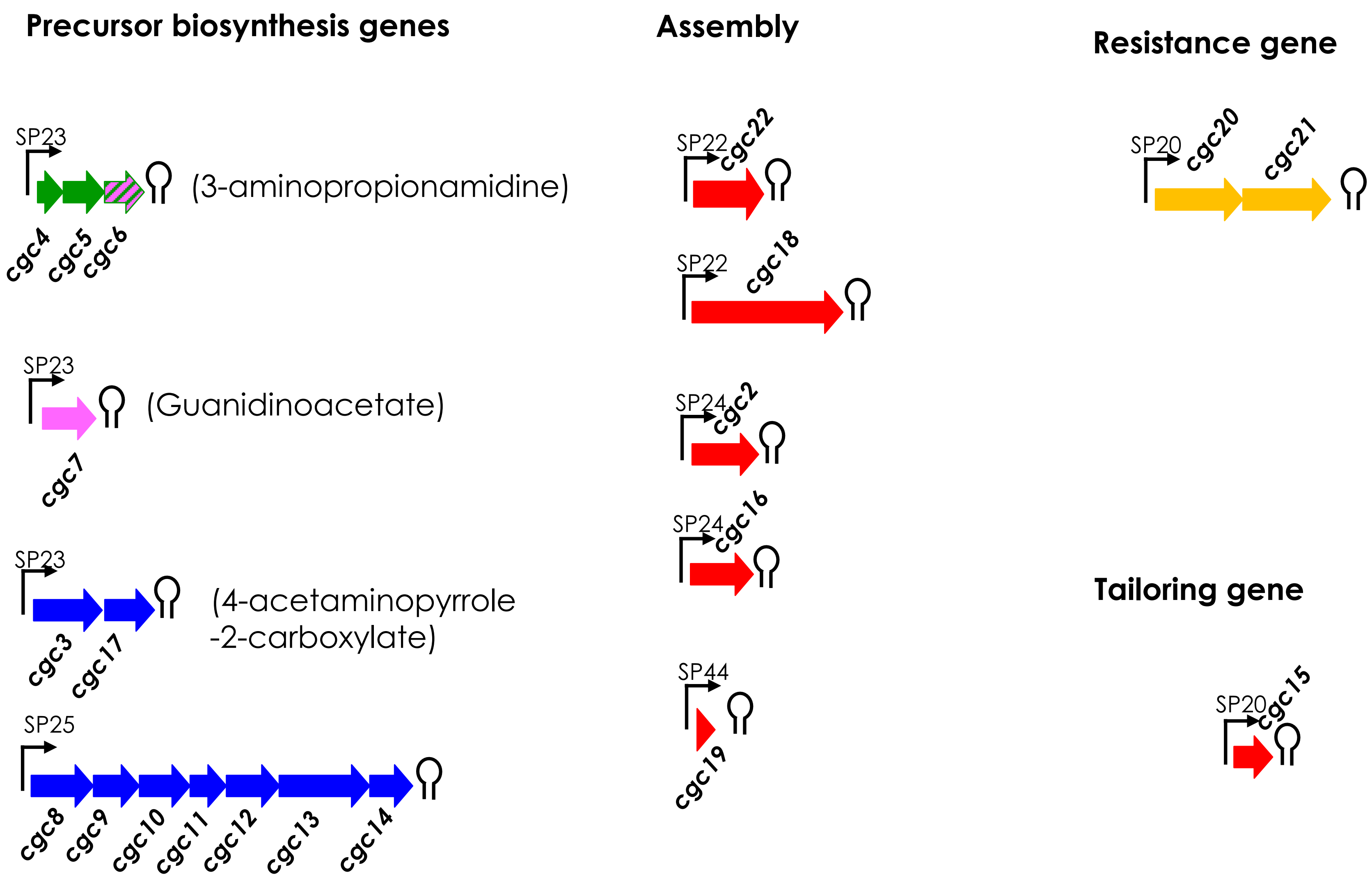
Up to 20 kb and up to 20 parts in *E. coli*

de Kok S, et al. ACS Synth Biol. 2014

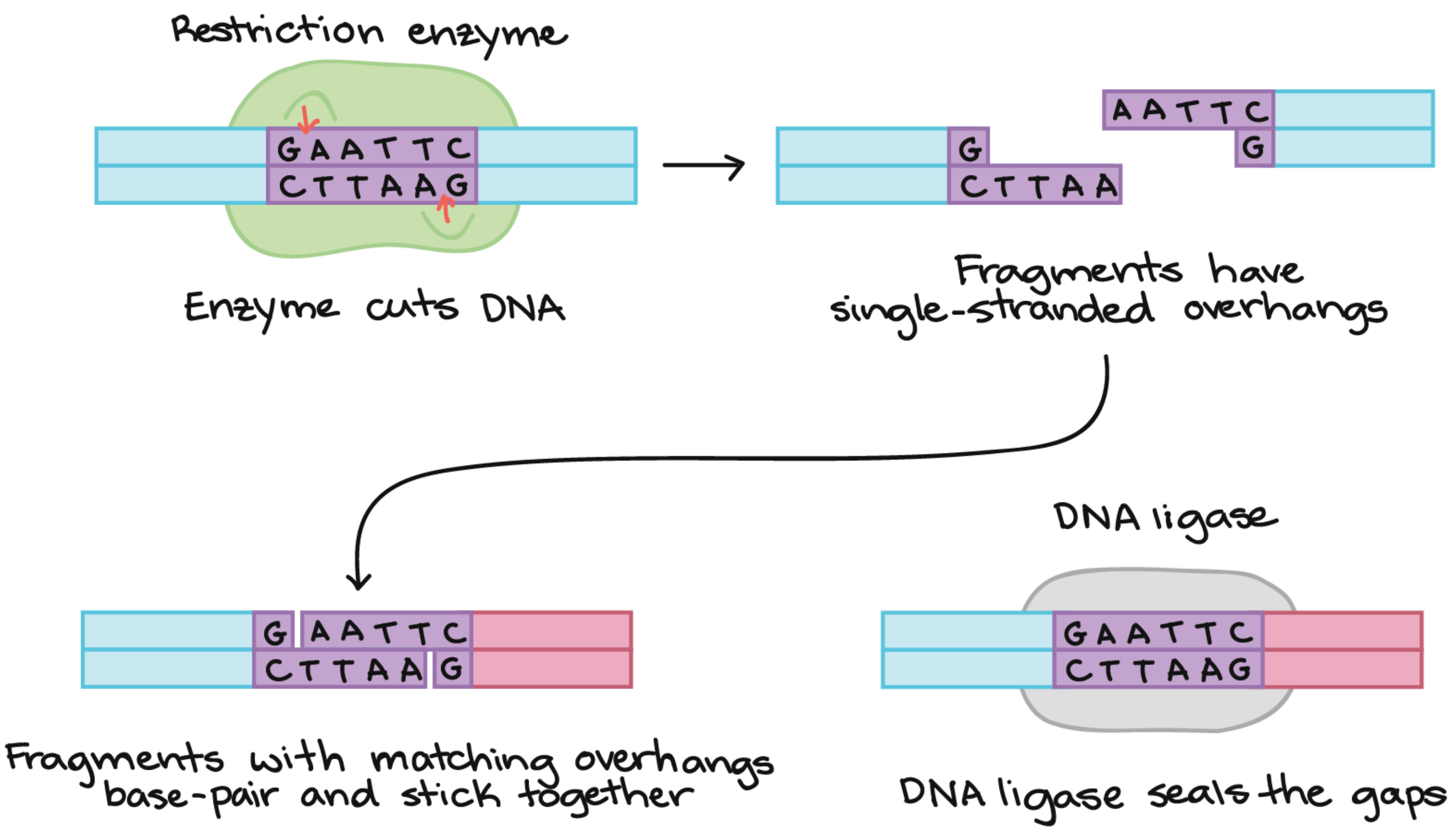
GENERAL PRINCIPLE OF A CASSETTE CONSTRUCTION



CONGOCIDINE GENE CASSETTES

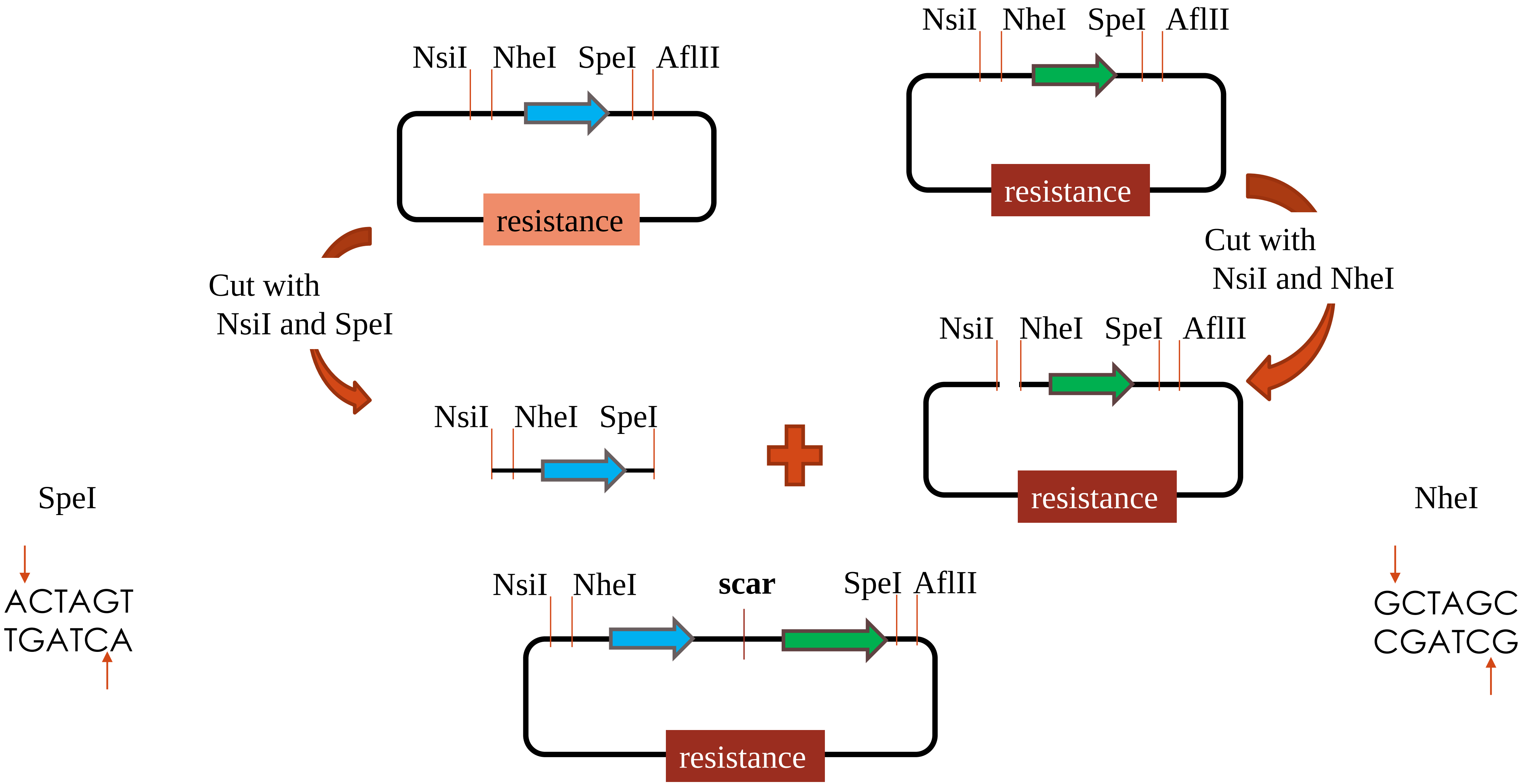


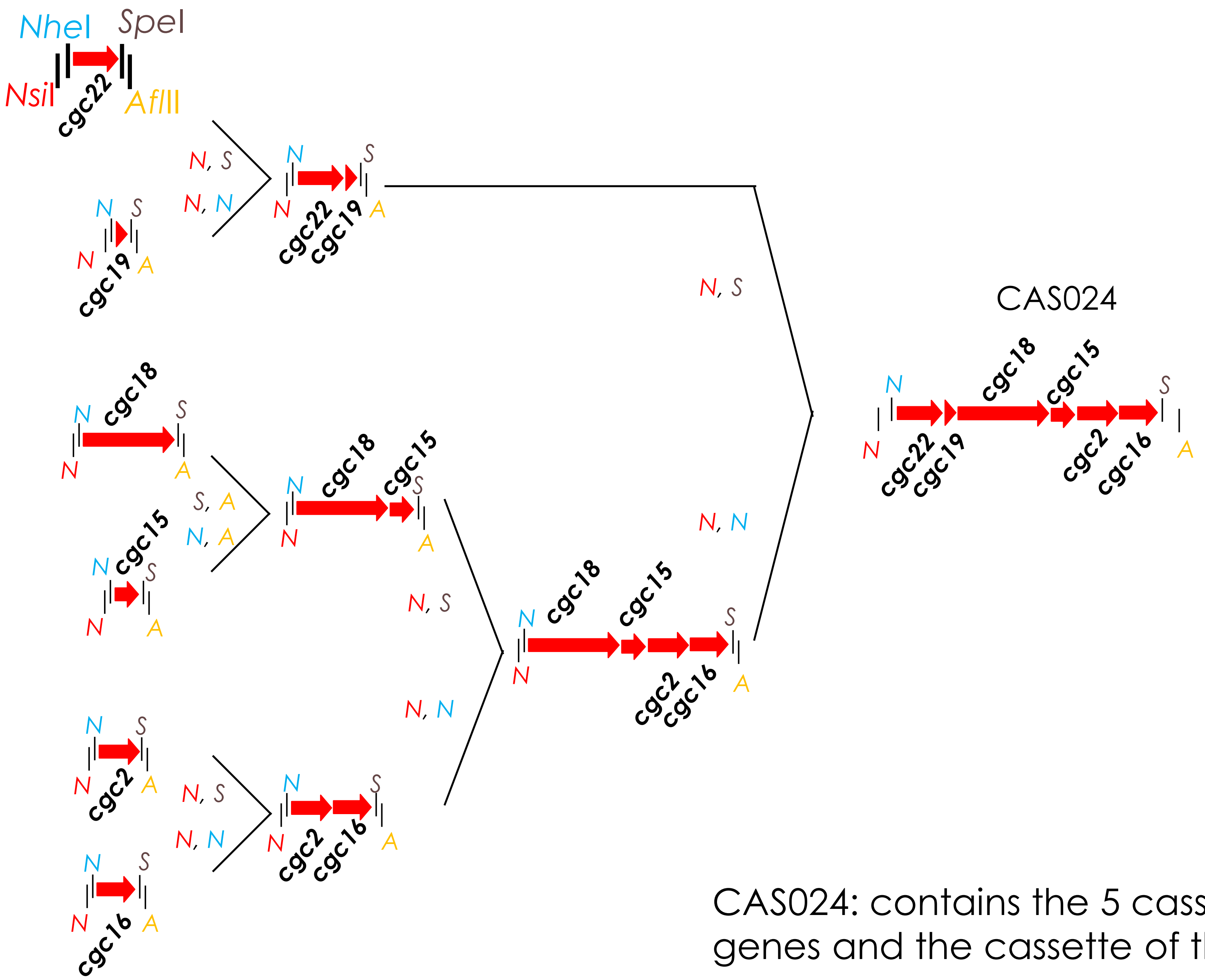
RESTRICTION ENZYMES



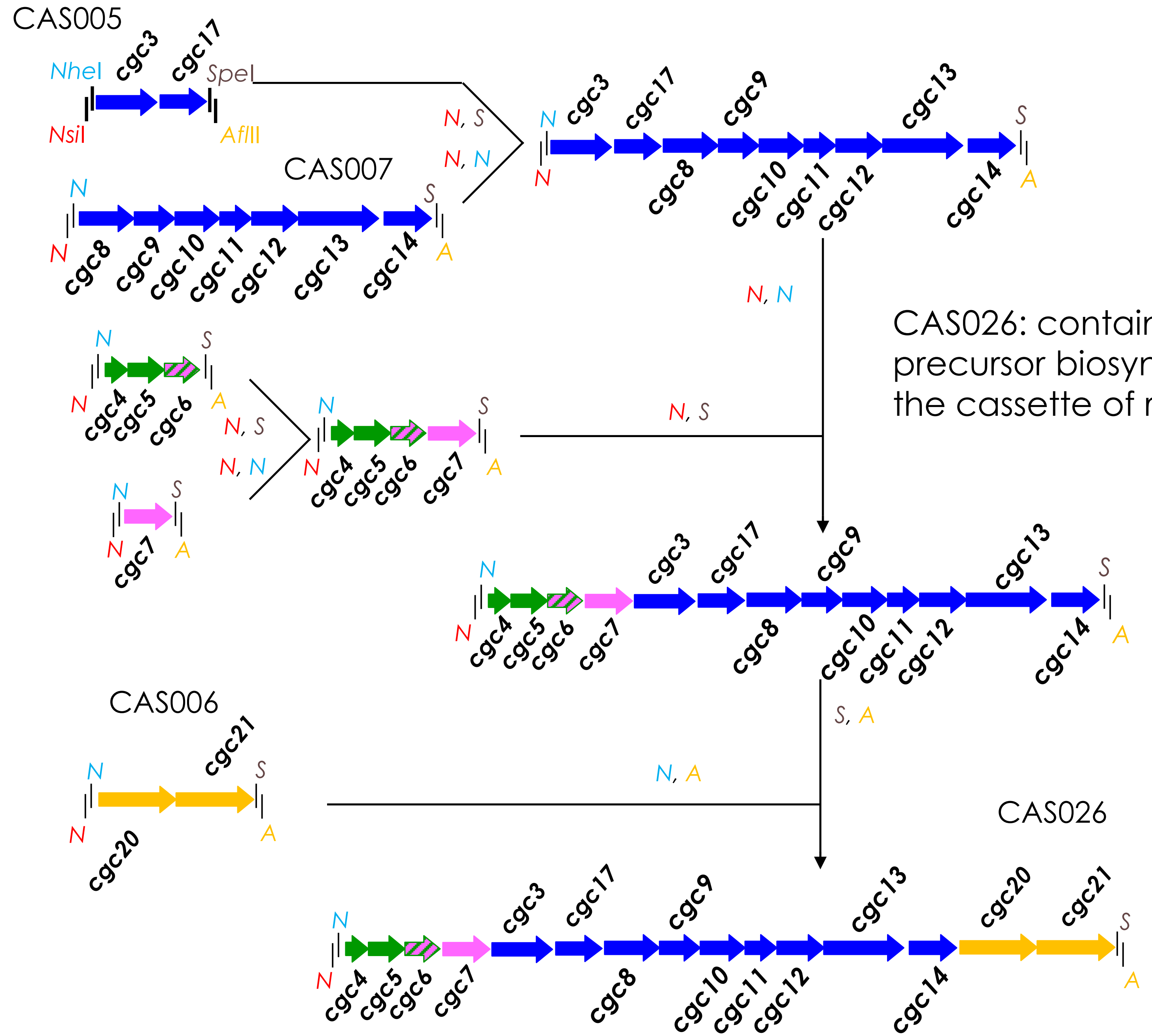
https://www.khanacademy.org/science/biology

SIMILAR TO THE BIOBRICK SYSTEM



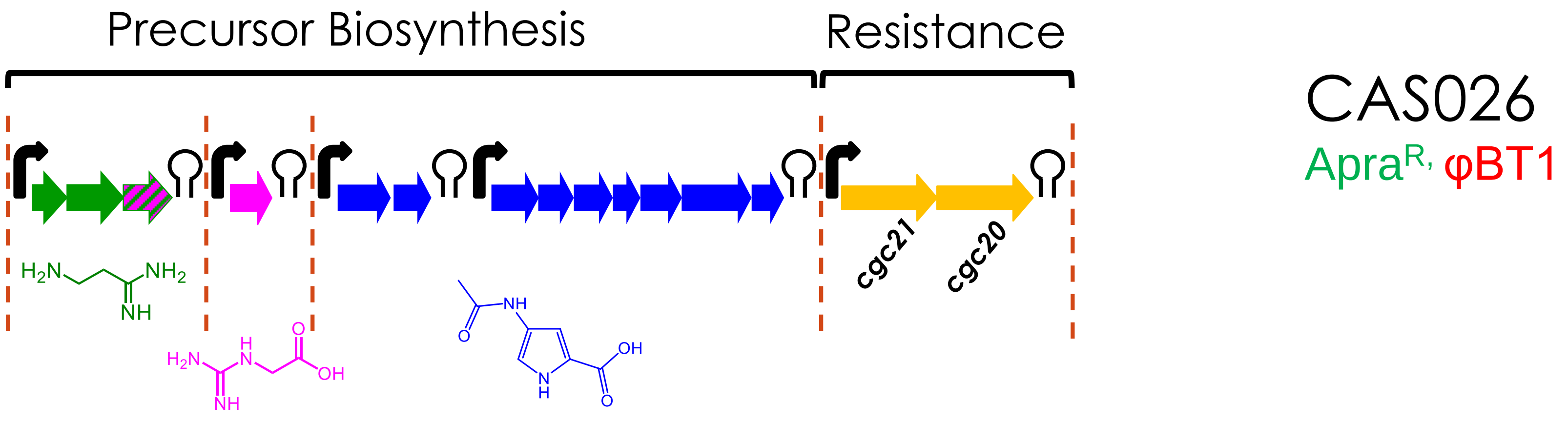


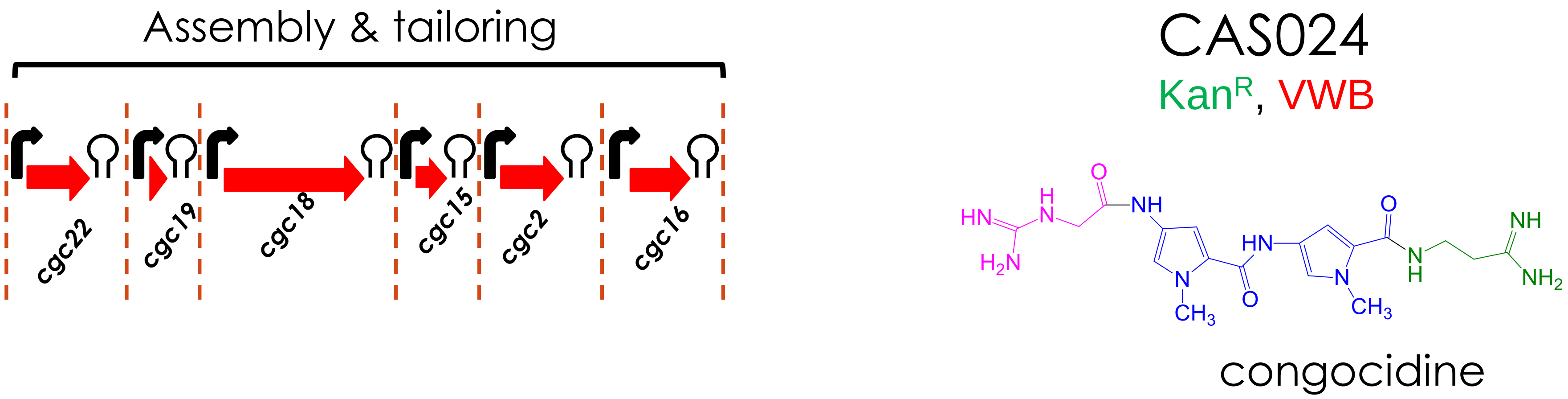
CAS024: contains the 5 cassettes of assembly genes and the cassette of the tailoring gene



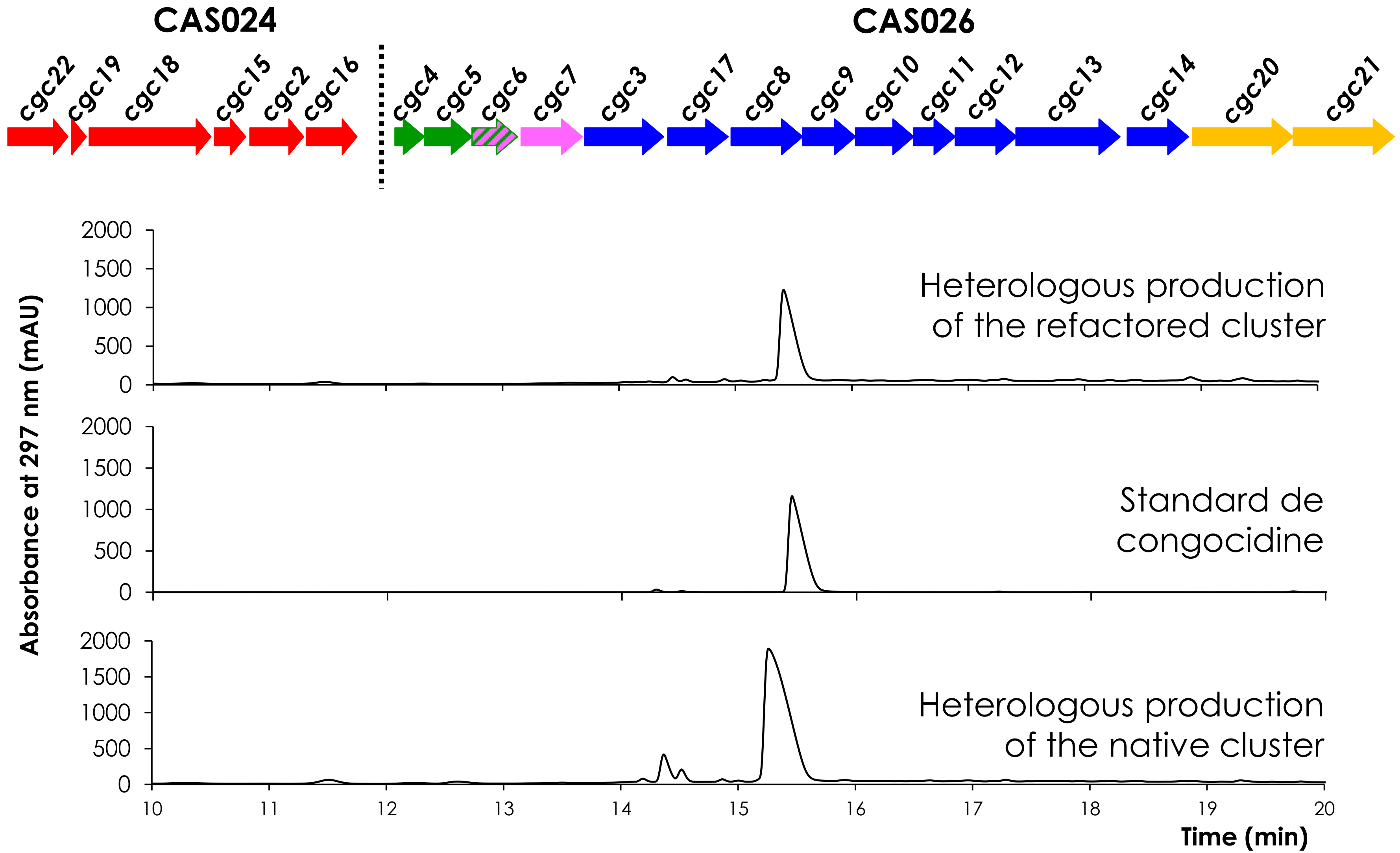
CAS026: contains the 4 cassettes of precursor biosynthesis genes and the cassette of resistance genes

OBTENTION OF TWO FINAL CONSTRUCTIONS

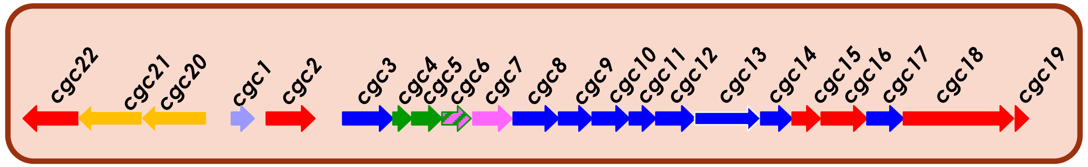




PRODUCTION OF CONGOCIDINE FROM THE REFACTORED GENE CLUSTER

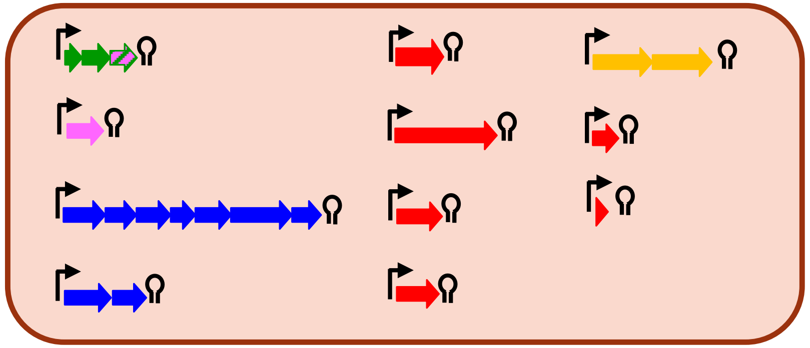


REFACTORING OF CONGOCIDINE BIOSYNTHETIC GENE CLUSTER

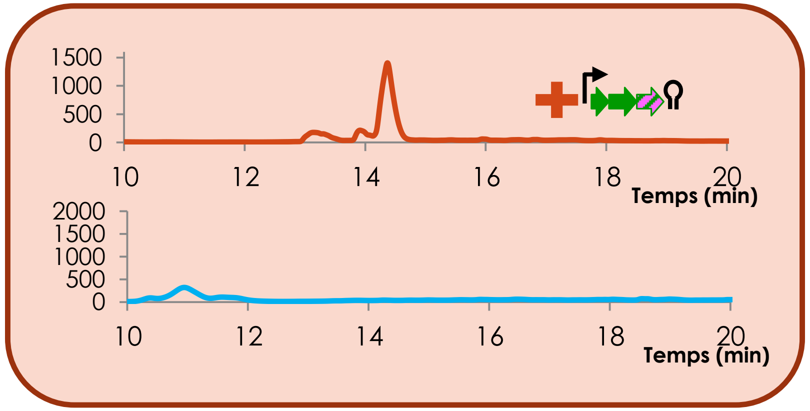


Choice of construction of synthetic operons

LCR



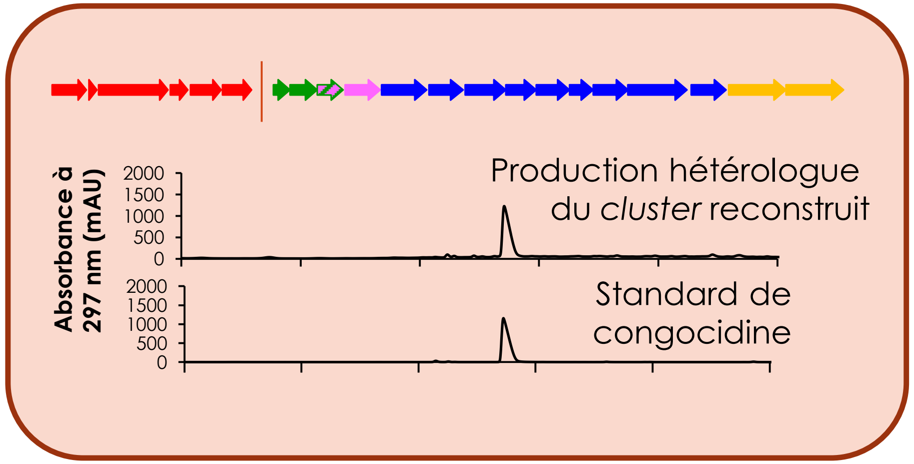
Construction of the cassettes
Confirmation of the fonctionality of the 11 gene cassettes



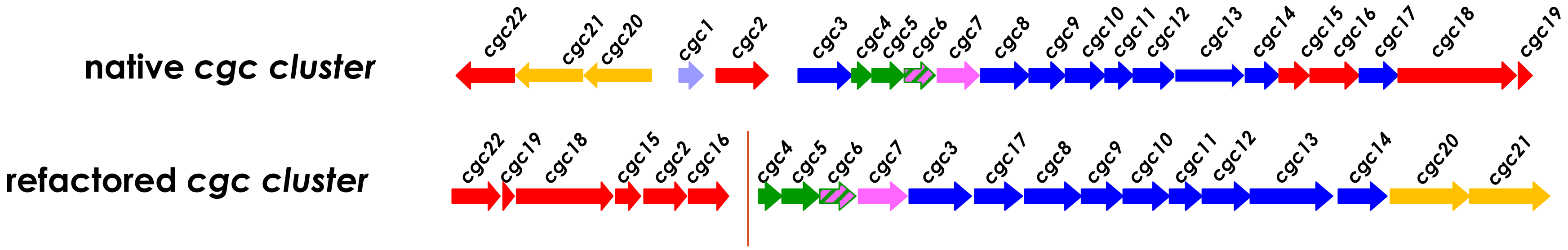
Biobrick assembly

Iterative assembly of the cassettes in 2 vectors

Heterologous expression in *S. lividans*



CONCLUSIONS ON THE REFACTORING OF THE CONGOCIDINE BIOSYNTHETIC GENE CLUSTER



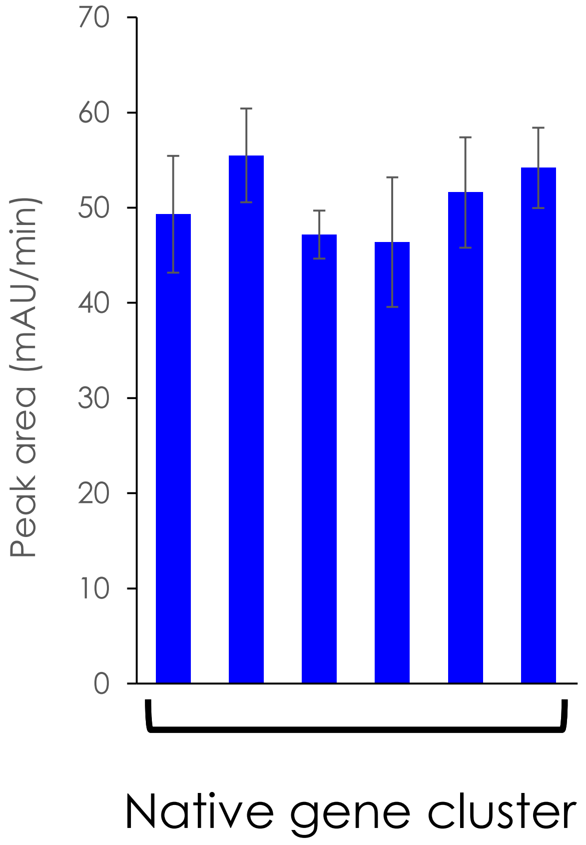
Each operon now directs:

- The biosynthesis of one precursor
- The assembly of two specific precursors
- The export of the final compound

It is then possible to replace one of the operons to produce a hybrid pyrrolamide

But… can we investigate the efficiency of production first?

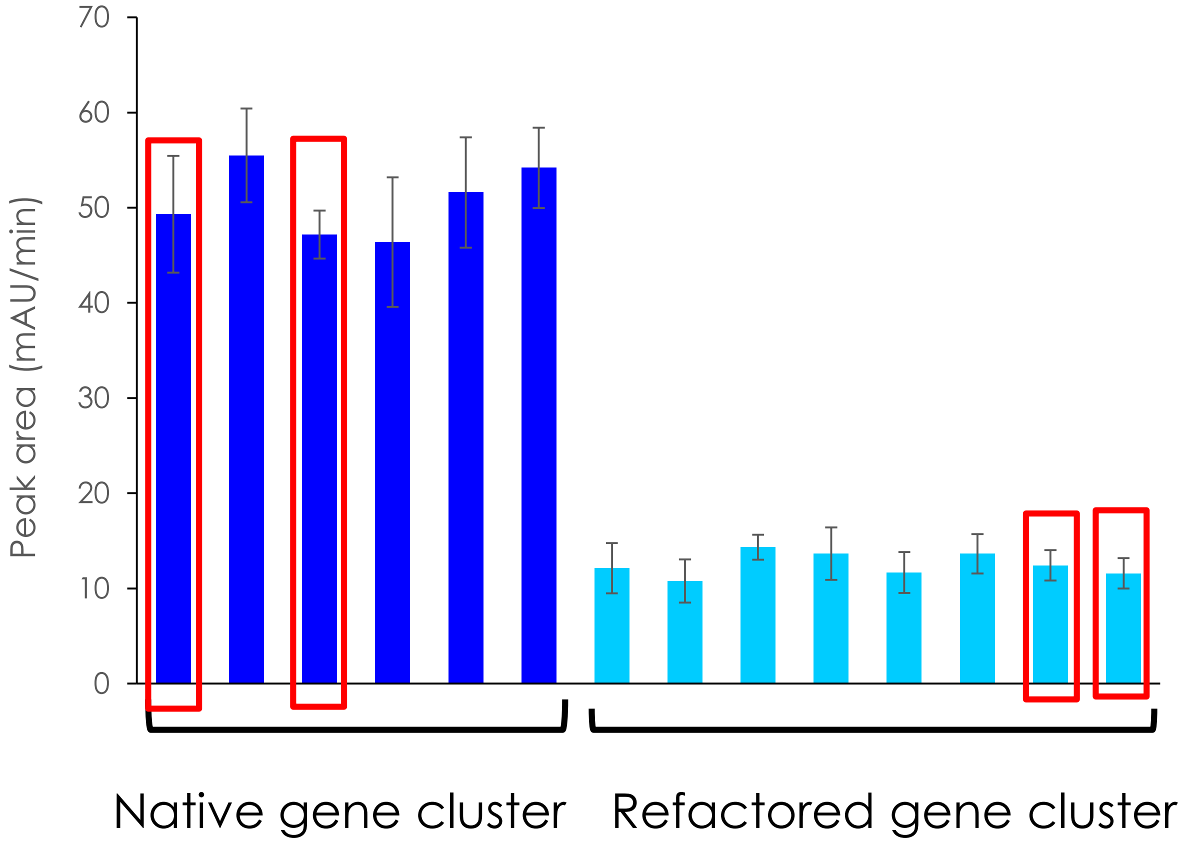
CONGOCIDINE PRODUCTION: NATIVE VS REFACTORED BGC



- Can we compare the native and the refactored clusters ?

Courtesy of S. Lautru

CONGOCIDINE PRODUCTION: NATIVE VS REFACTORED BGC



- Congocidine yield for the refactored gene cluster: about 25% of the yield of the native cluster
- Transcription levels?
- Translation levels?

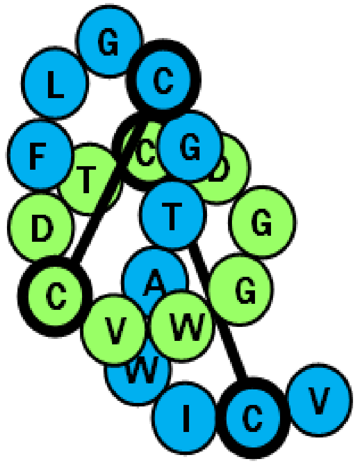
Courtesy of S. Lautru

Summary

- What we learned here:

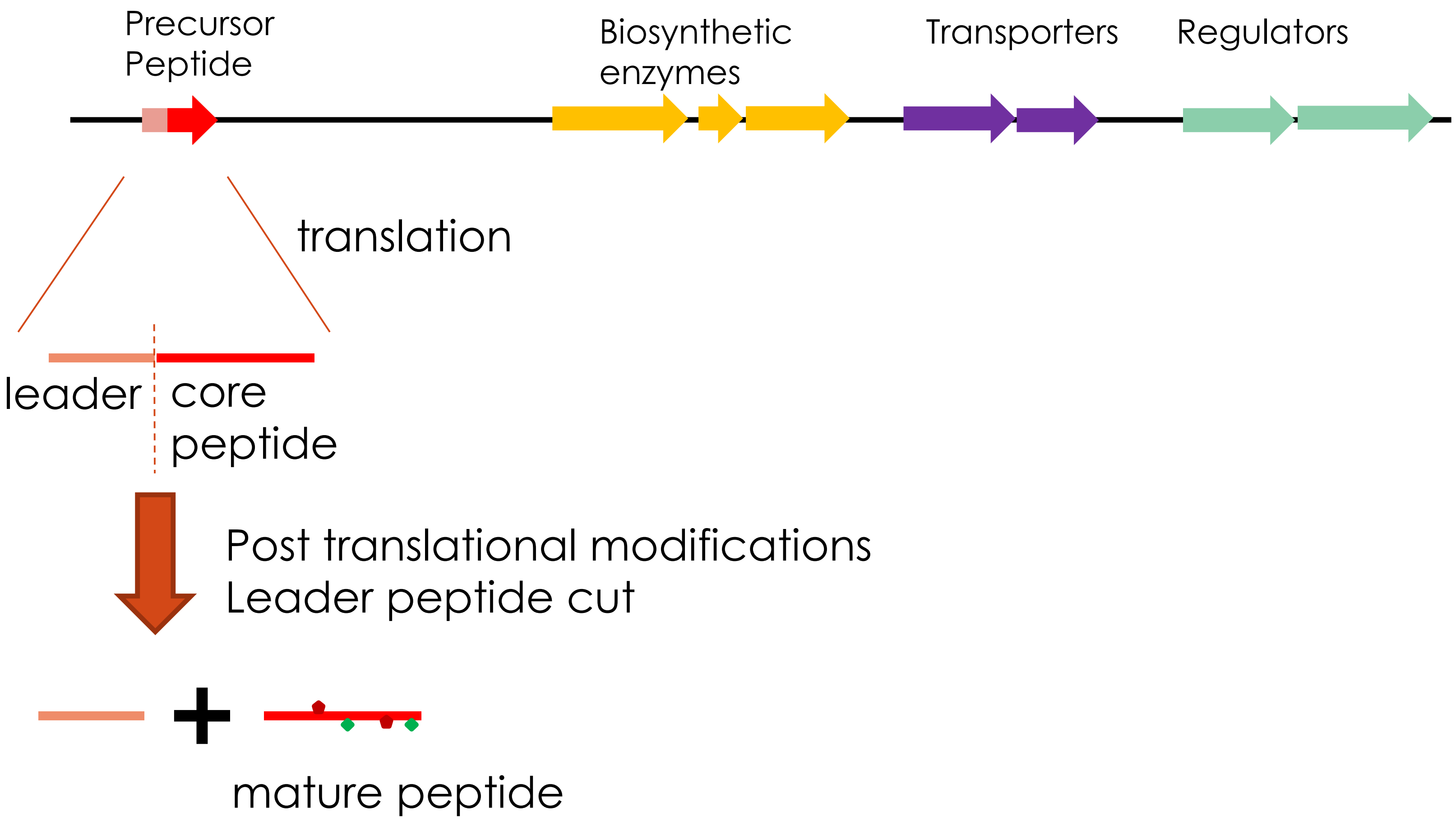
  - ❑ We should investigate the four genes that are potentially less expressed
  - ❑ Genes with the same promoter might not have the same expression
  - ❑ Promoter strength evaluated for one gene might not be transferable

All together: synthetic biology is not as well mastered as we would like it to be yet…
Plenty left to understand!!

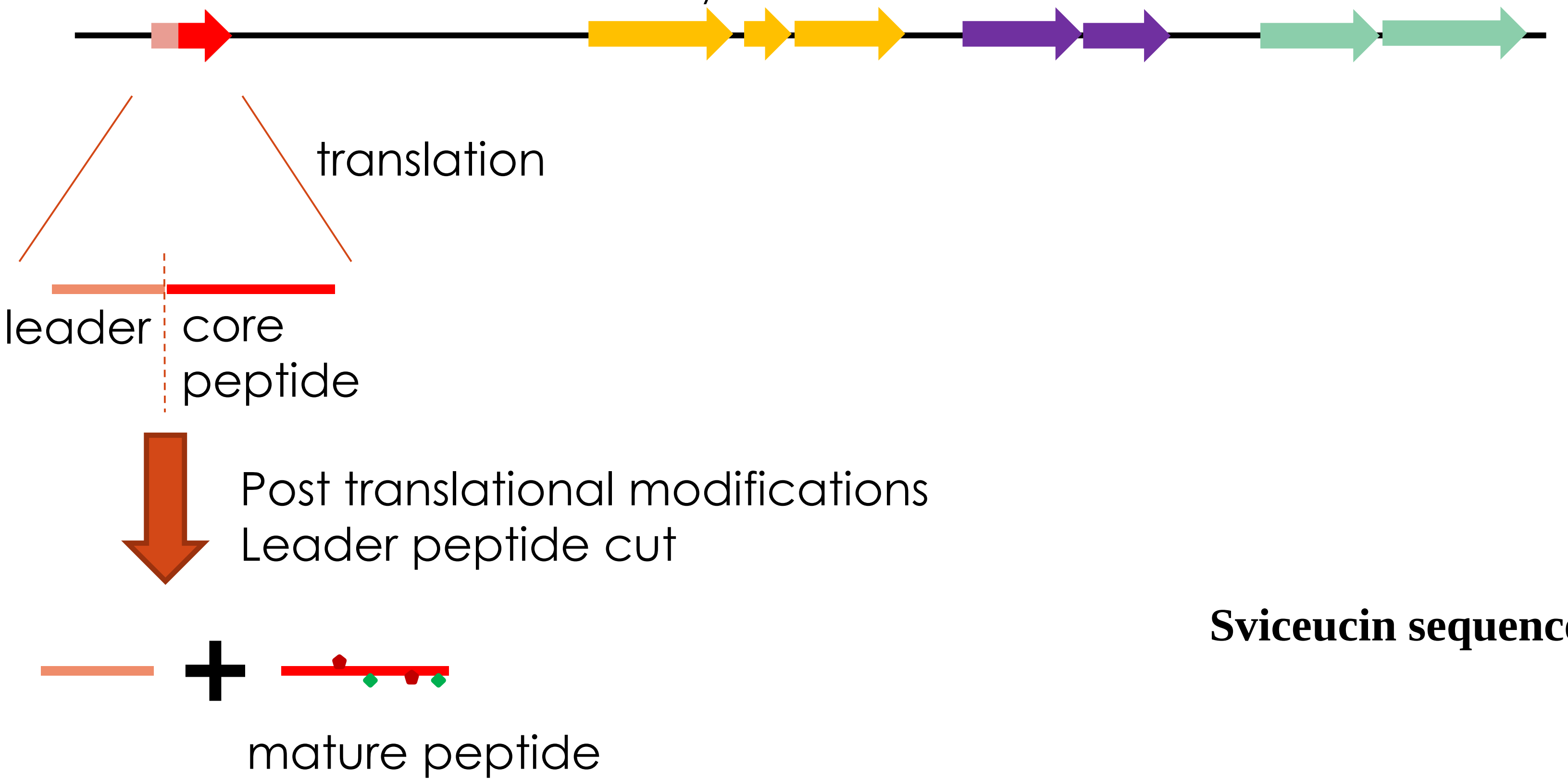


PRODUCING SVICEUCIN NON NATURAL ANALOGS

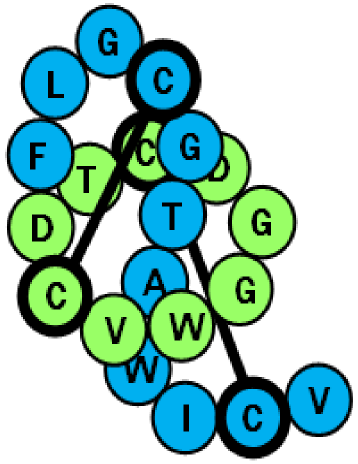
RIPPs : RIBOSOMALLY SYNTHESIZED AND POST-TRANSLATIONALLY MODIFIED PEPTIDES



RIPPs : RIBOSOMALLY SYNTHESIZED AND POST-TRANSLATIONALLY MODIFIED PEPTIDES



Sviceucin, a lasso peptide



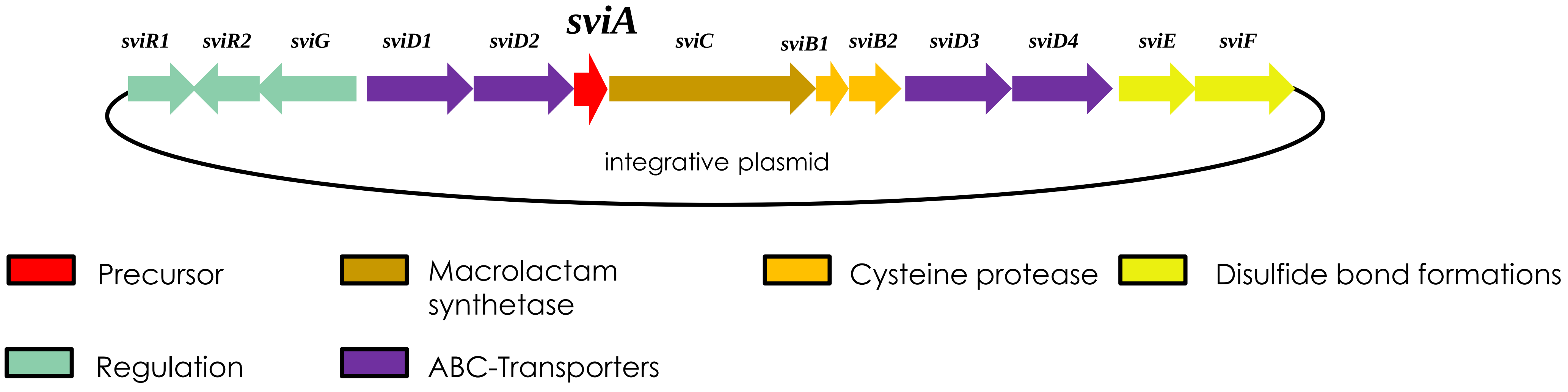
Disulfide bonds

Sviceucin sequence: **CVWGGDCTDFLGCGTAWICV**

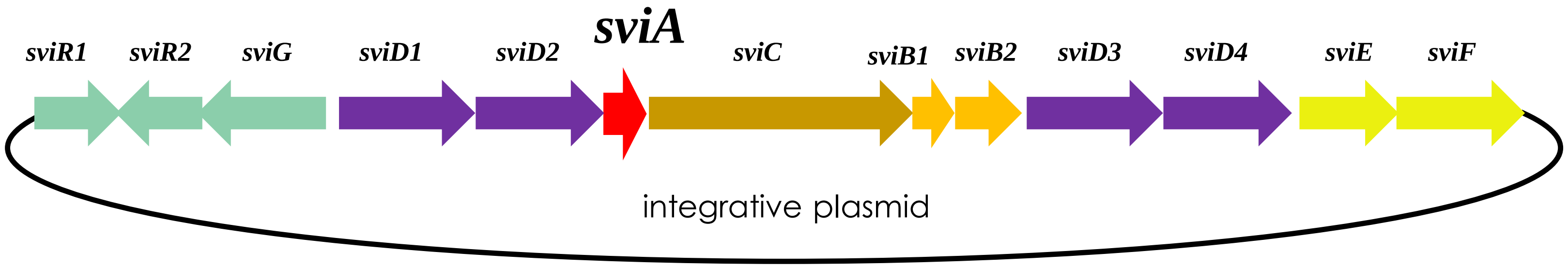
Cycle

Li et al. ACS Chem Biol. 2015

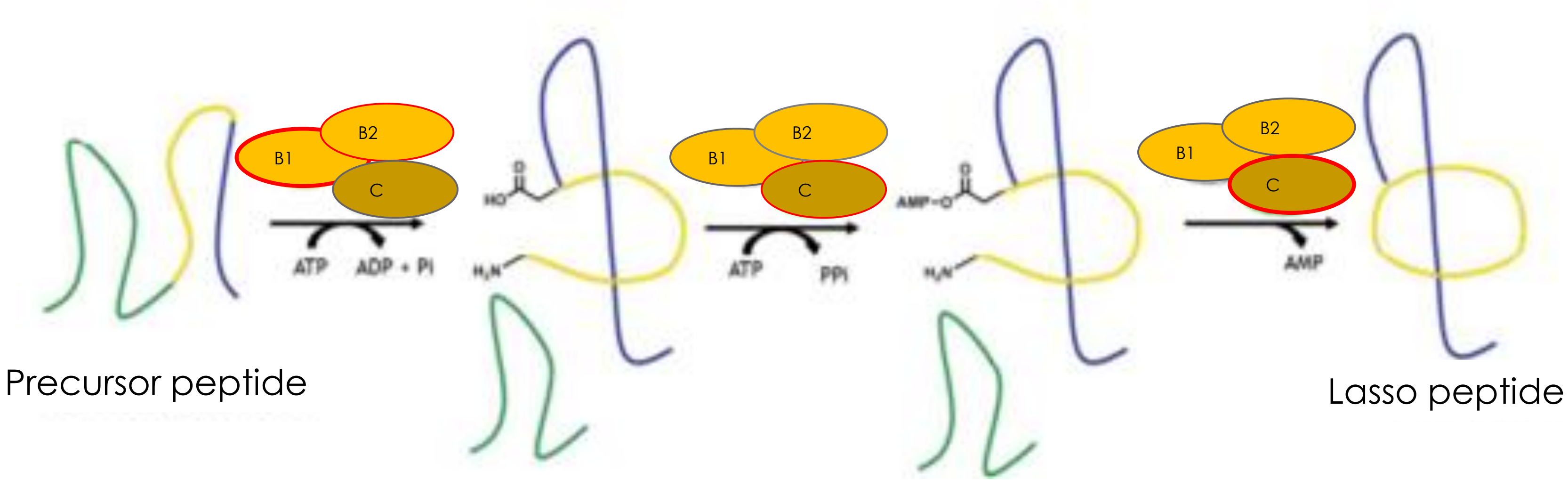
SVICEUCIN BIOYNTHETIC GENE CLUSTER

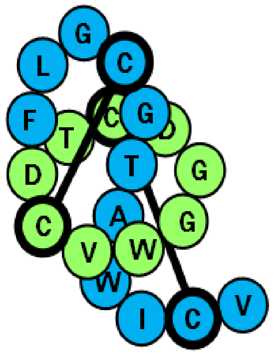


SVICEUCIN BIOYNTHETIC GENE CLUSTER



Precursor
Macrolactam synthetase
Cysteine protease
Disulfide bond formations
Regulation
ABC-Transporters

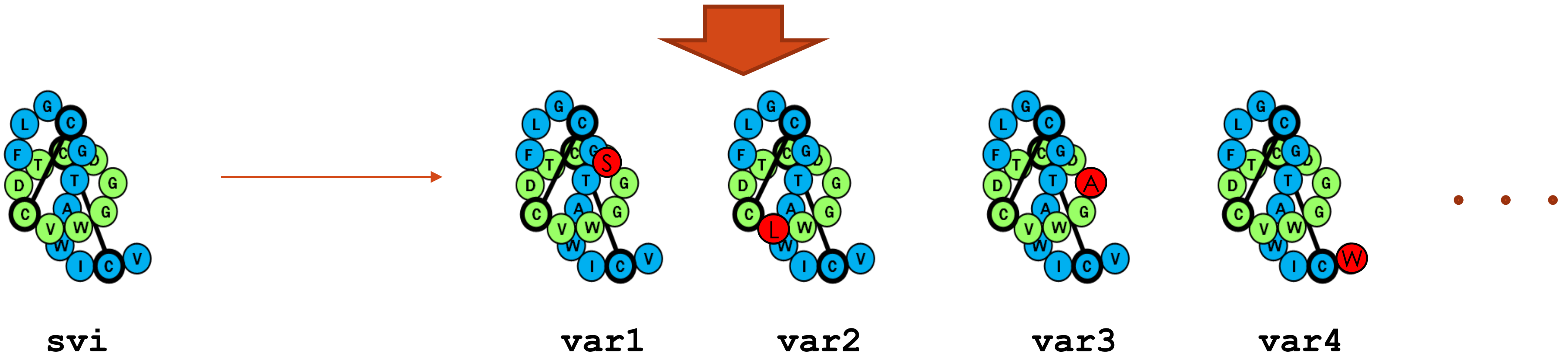




Martin-Gómez et al. Org Biomol Chem. 2018

GENERATING SVICEUCIN ANALOGS

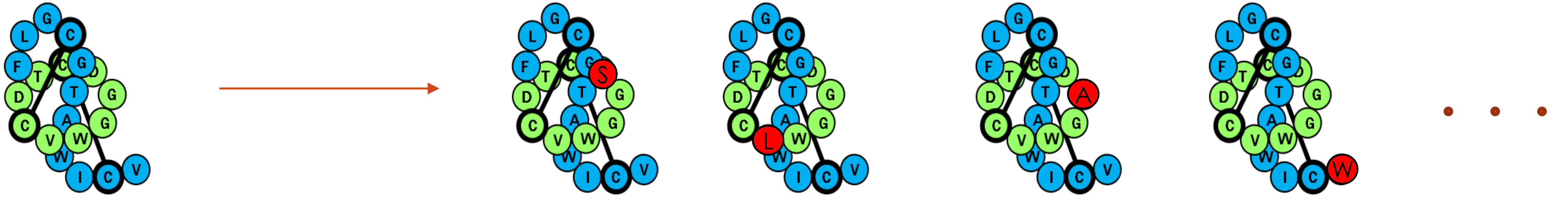
DNA Modification to generate analogs ??



GENERATING SVICEUCIN ANALOGS

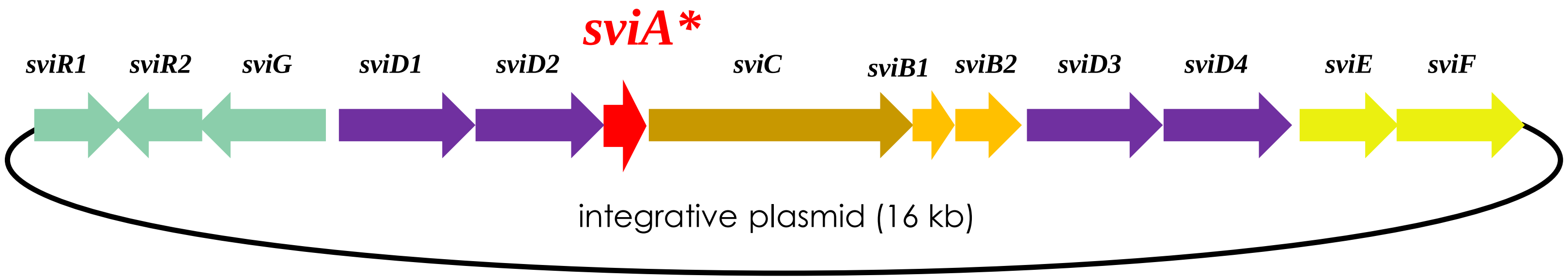
svi TGTGTGTGGGGCGGAGACTGCACCGACTTCCTCGGCTGCGGCACCGCCTGGATCTGTGTCTGA
var1 TGTGTGTGGGGCGGAAGCTGCACCGACTTCCTCGGCTGCGGCACCGCCTGGATCTGTGTCTGA
var2 TGTCTCTGGGGCGGAGACTGCACCGACTTCCTCGGCTGCGGCACCGCCTGGATCTGTGTCTGA
var3 TGTGTGTGGGGCGCCGACTGCACCGACTTCCTCGGCTGCGGCACCGCCTGGATCTGTGTCTGA
var4 TGTGTGTGGGGCGGAGACTGCACCGACTTCCTCGGCTGCGGCACCGCCTGGATCTGTTGGTGA

svi CVWGGDCTDFLGCGTAWICV
var1 CVWGGSCTDFLGCGTAWICV
var2 CLWGGDCTDFLGCGTAWICV
var3 CVWGADCTDFLGCGTAWICV
var4 CVWGGDCTDFLGCGTAWICW

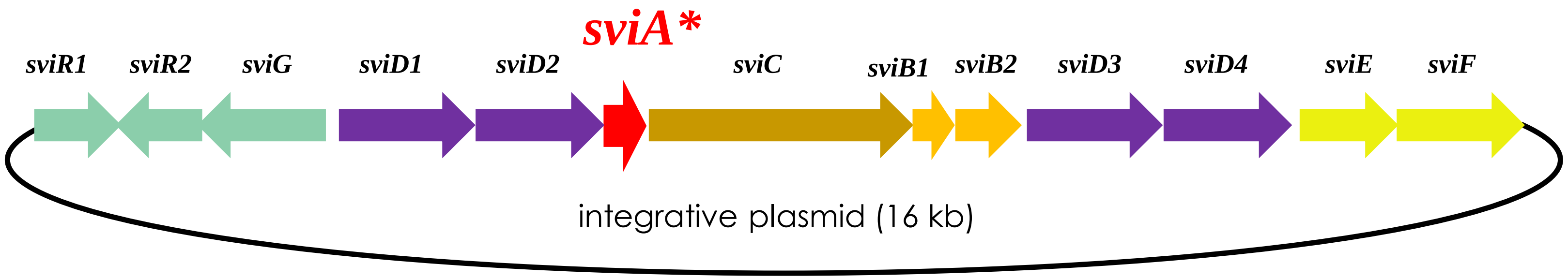


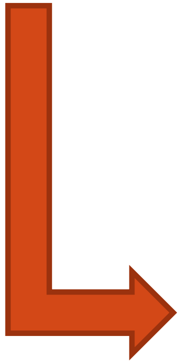
svi var1 var2 var3 var4

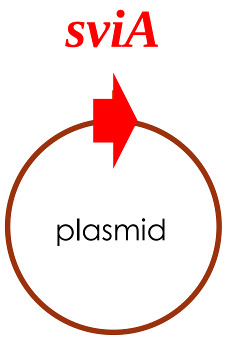
CHANGING A FEW DNA BASE PAIRS ON A PLASMID

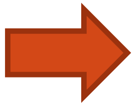


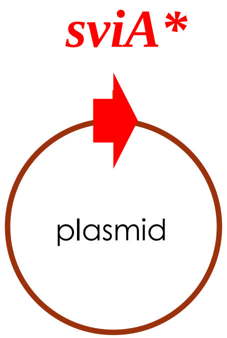
CHANGING A FEW DNA BASE PAIRS ON A PLASMID







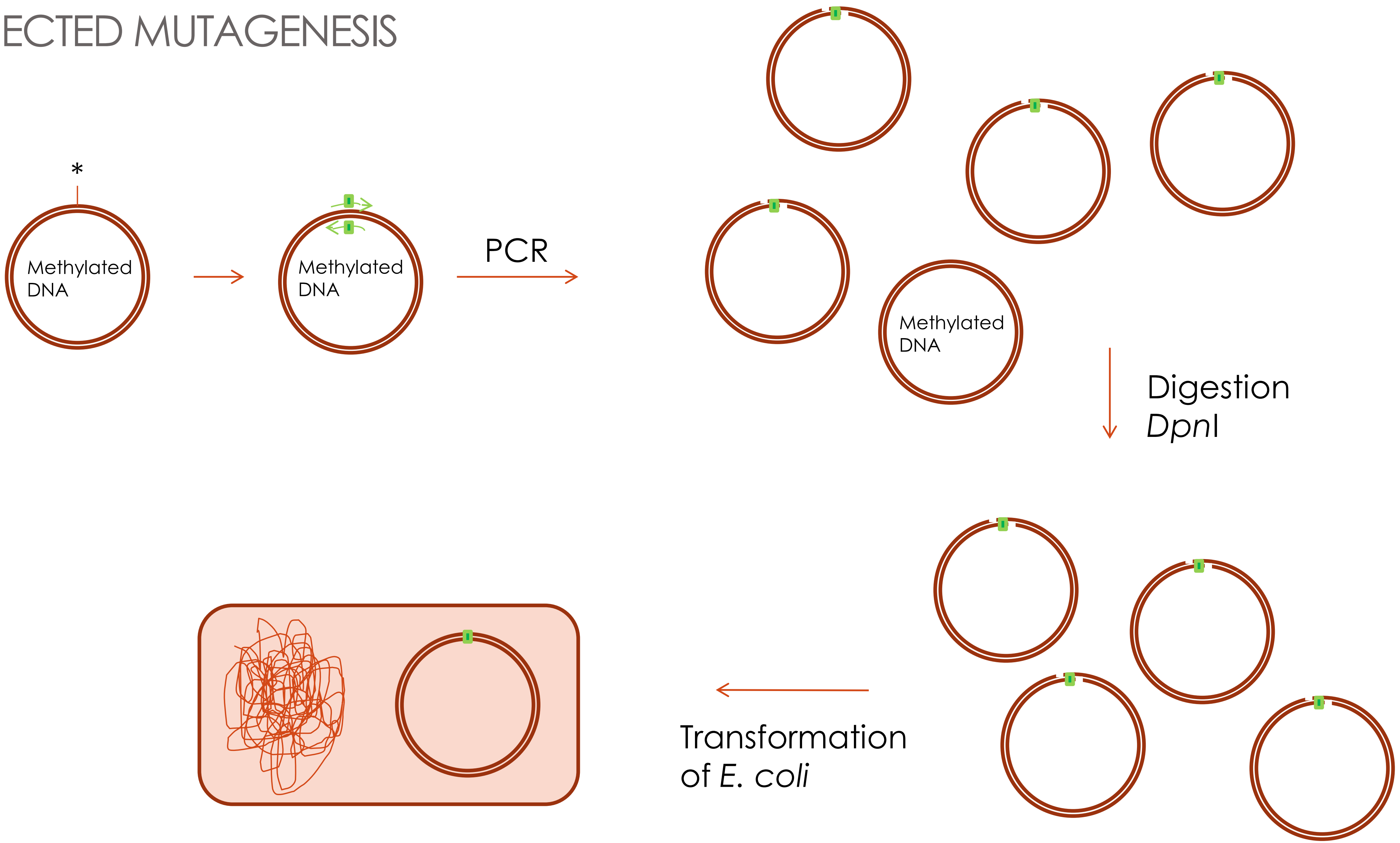




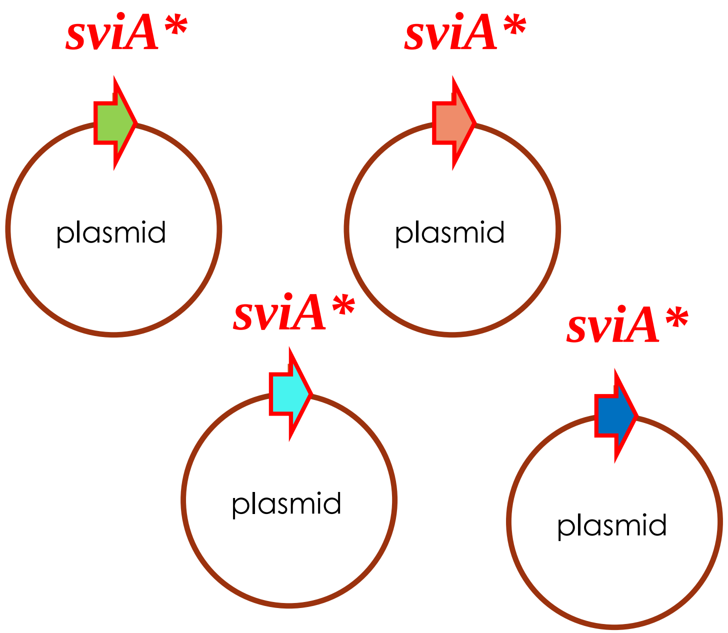
Working on
a smaller plasmid

**Method to
modify the DNA ??**

SITE DIRECTED MUTAGENESIS

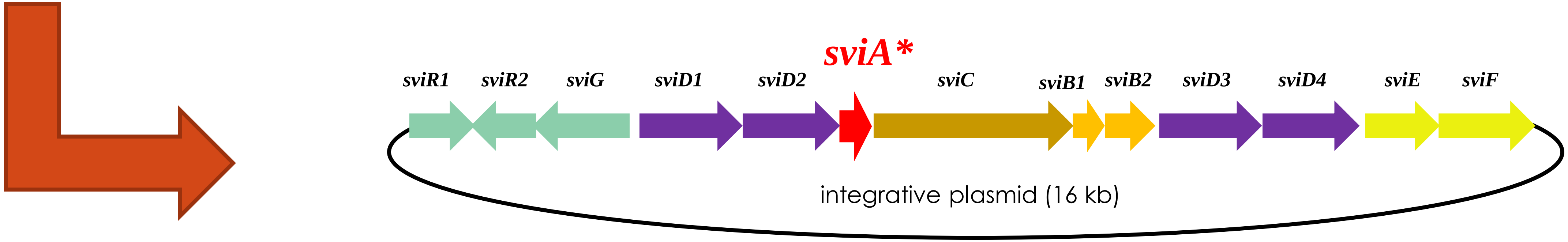


REPLACING THE MUTATED PRECURSOR GENE IN THE CLUSTER

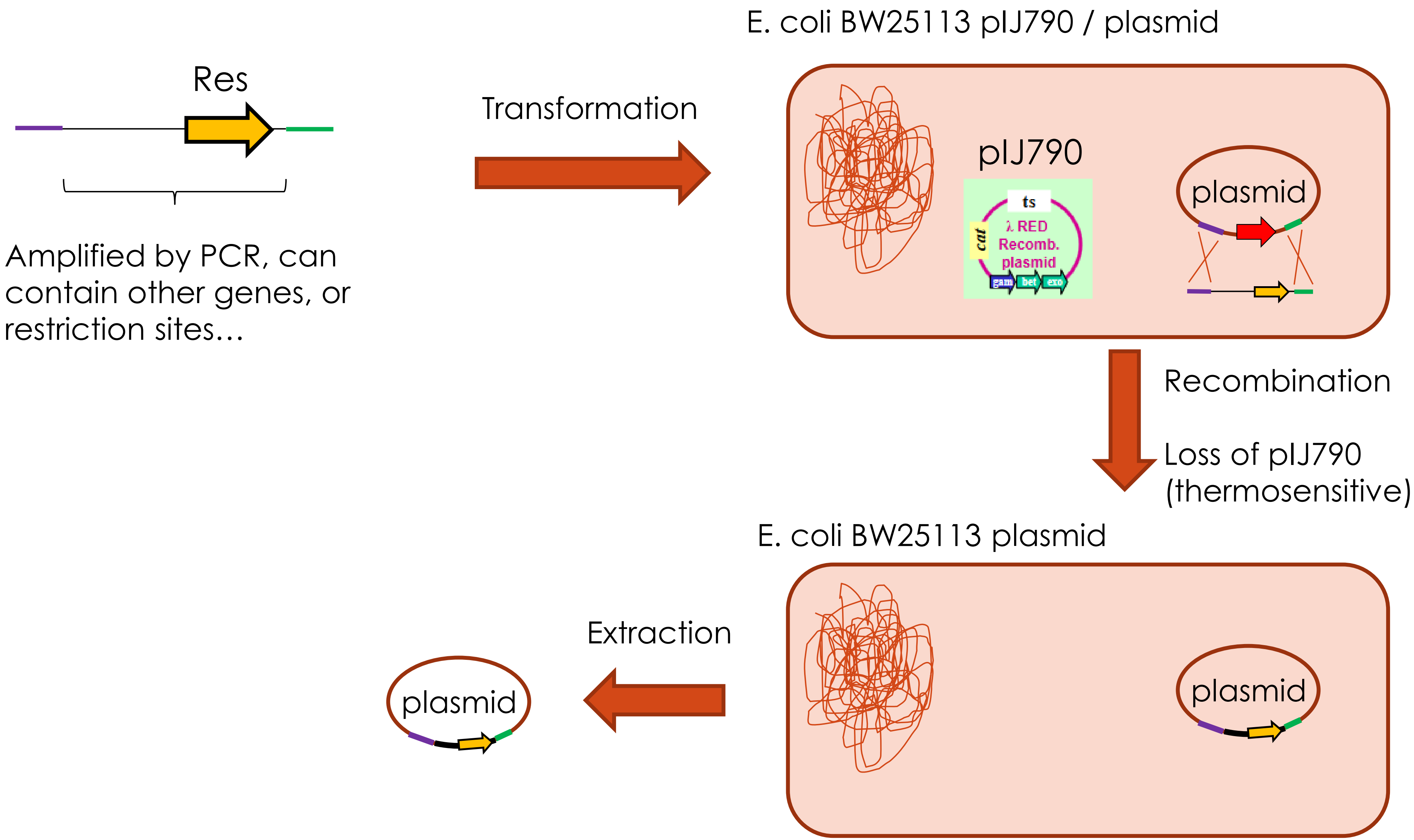


How to replace sviA* in the plasmid ??

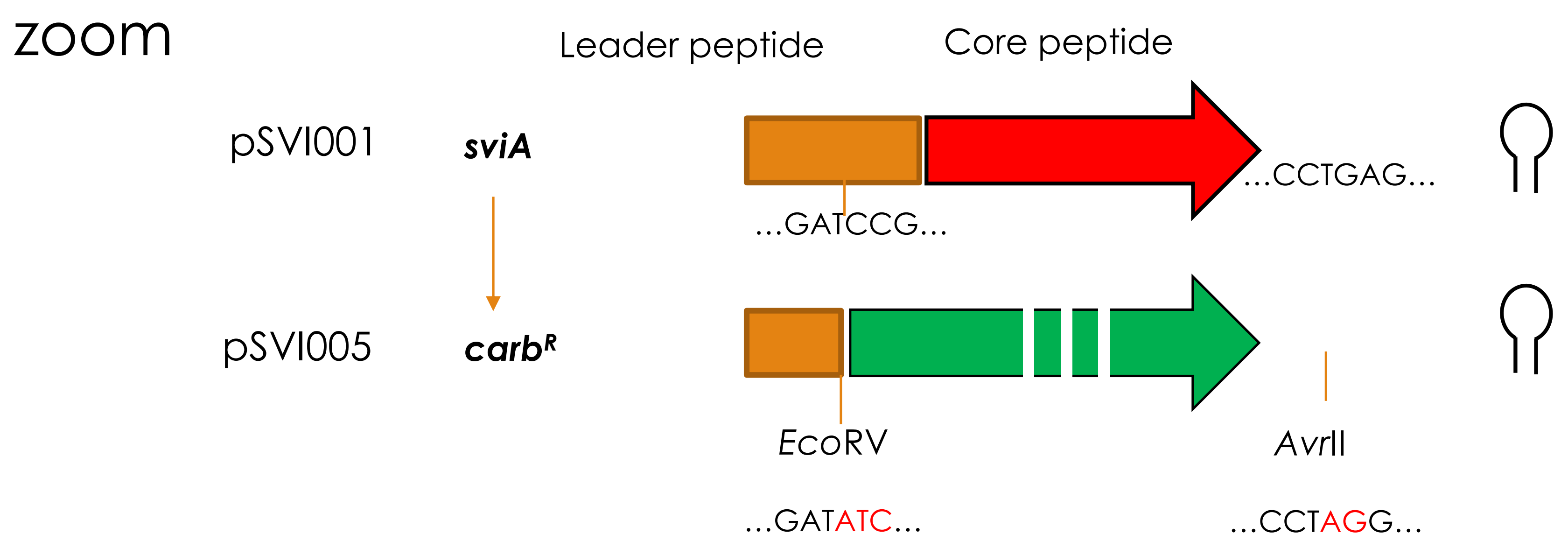
Necessary to insert restriction sites,
And to avoid scars



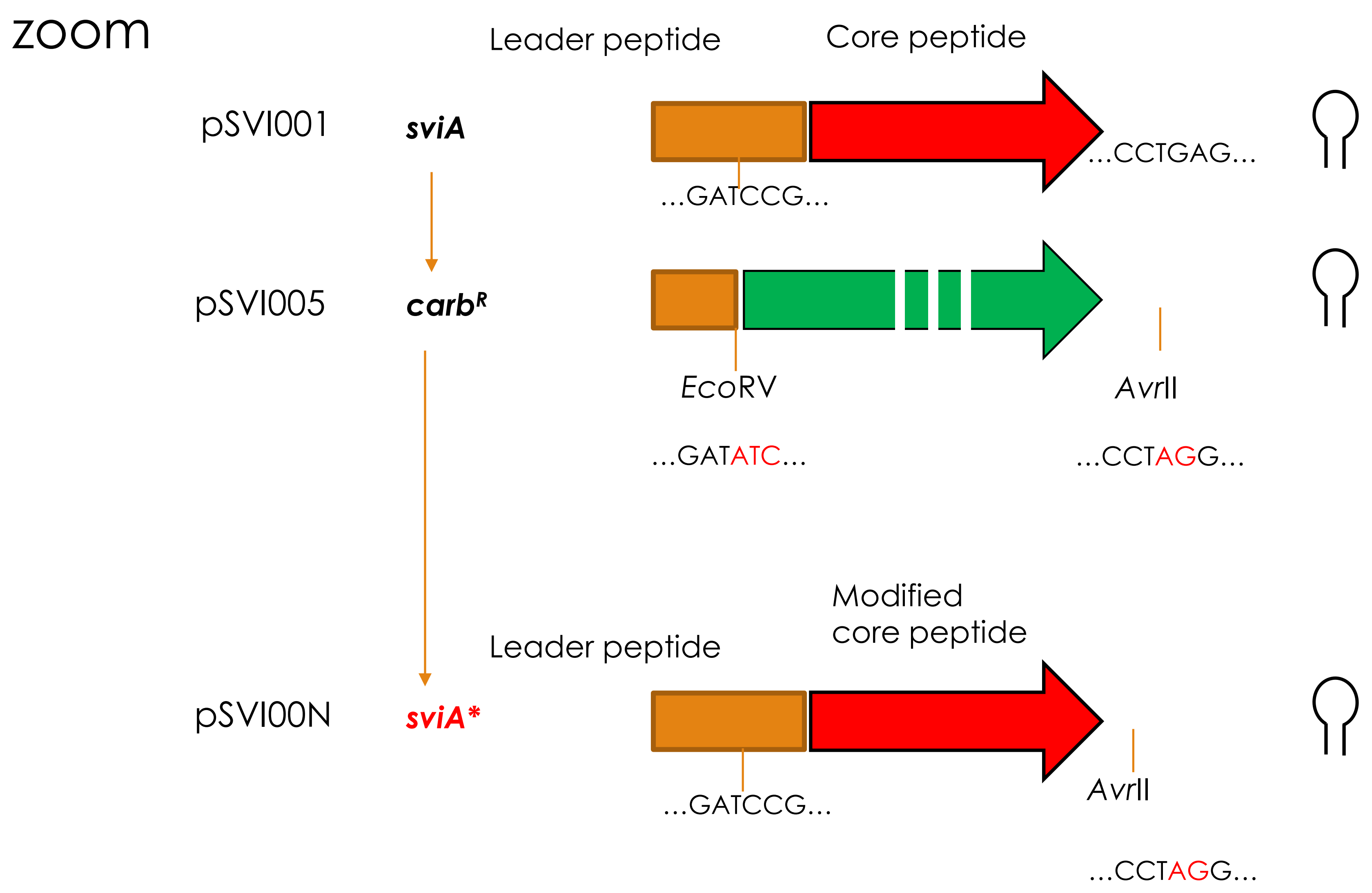
PCR TARGETING



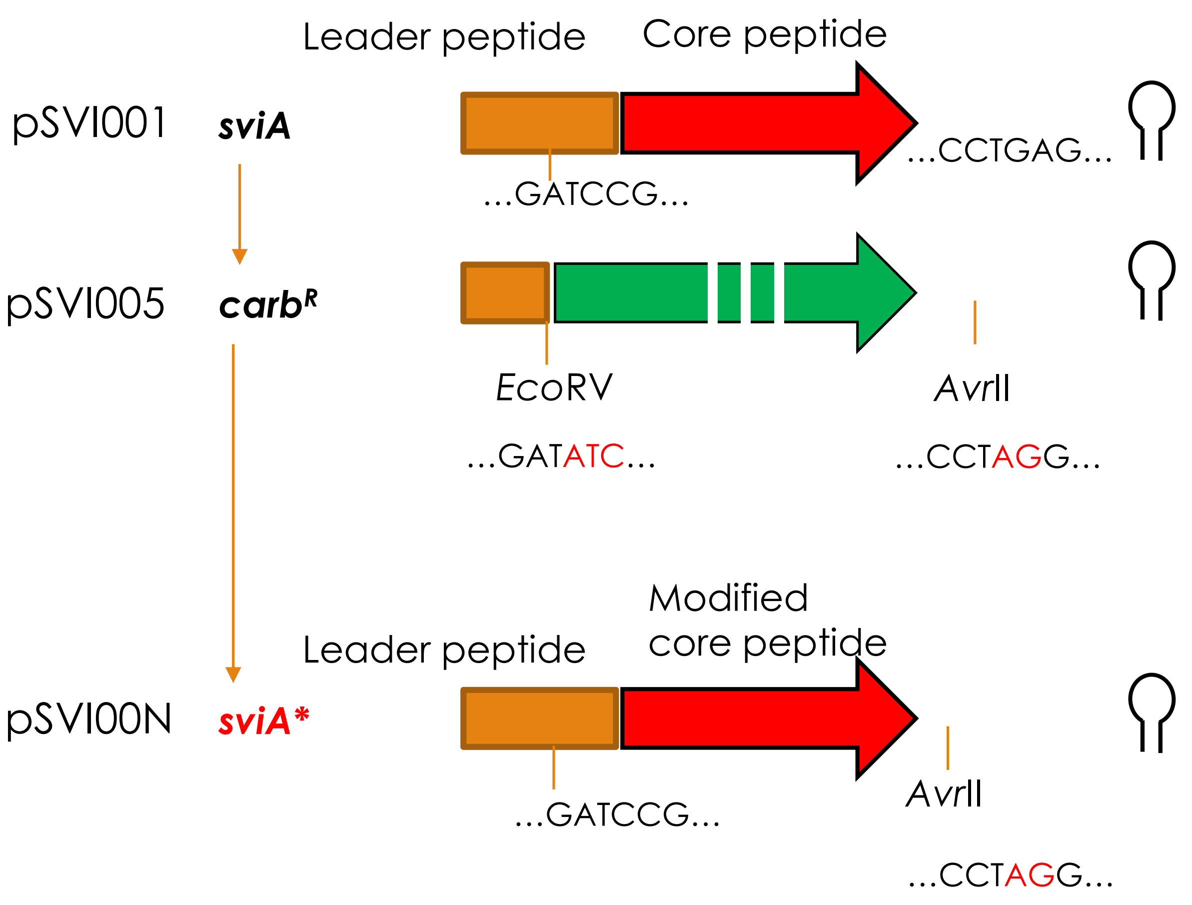
CLOSE UP LOOK ON THE EXPRESSION PLATFORM

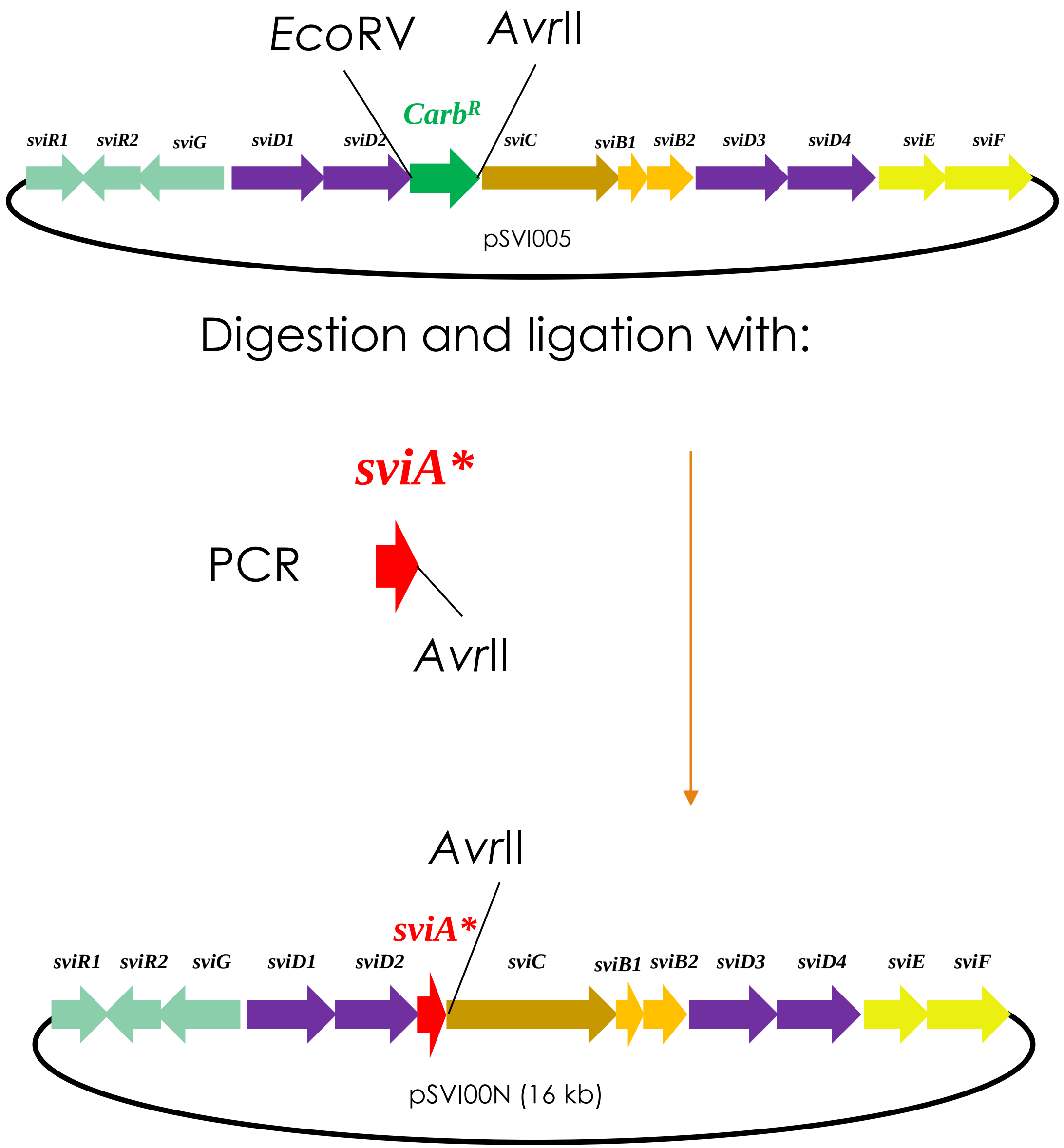


CLOSE UP LOOK ON THE EXPRESSION PLATFORM



SUMMARY OF THE CLONING STRATEGY





GENERATION SVICEUCIN VARIANTS *IN VIVO*

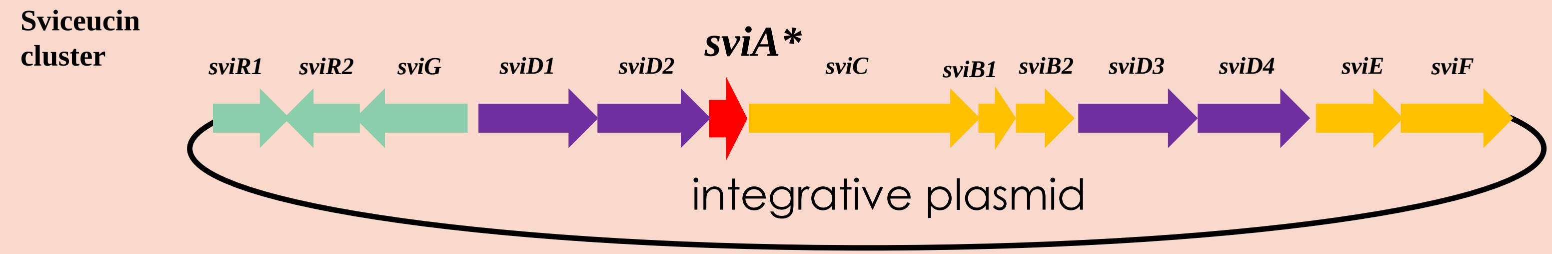
Site directed mutagenesis of the precursor peptide gene sviA

*sviA**

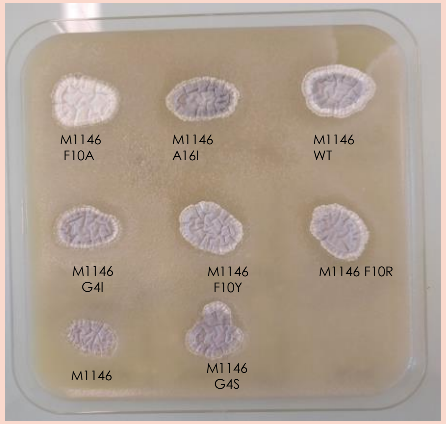
sviA CVWGGDCTDFLGCGTAWICV
sviA* CVWGGSCTDFLGCGTAWICV

50 *sviA** constructed

Ligation in sviceucin BGC

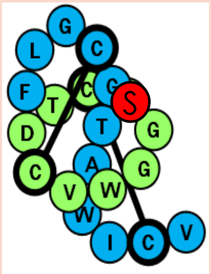


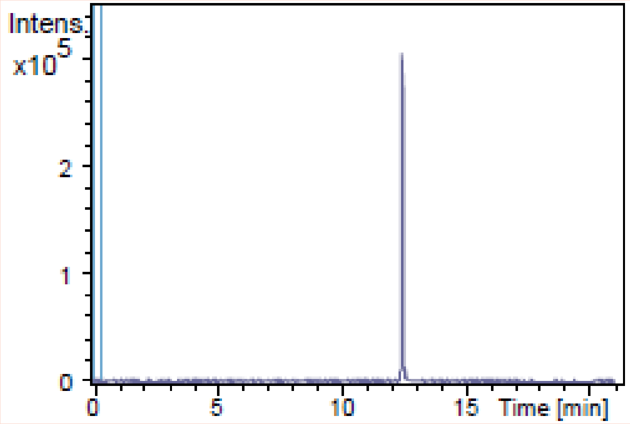
Integration in *S. coelicolor* genome



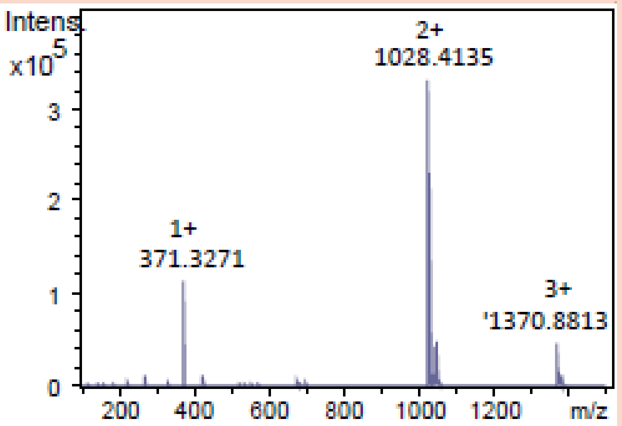
Small scale production

Mass spectrometry analysis (LCMS)



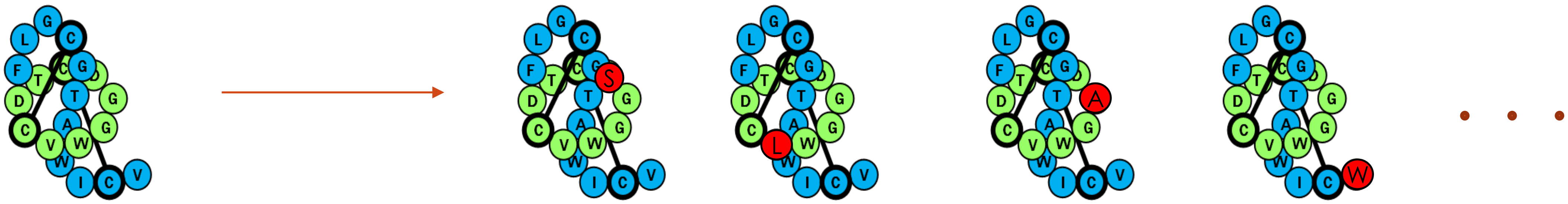


EIC 1028,41



Mass spectra

FURTHER MODIFICATIONS

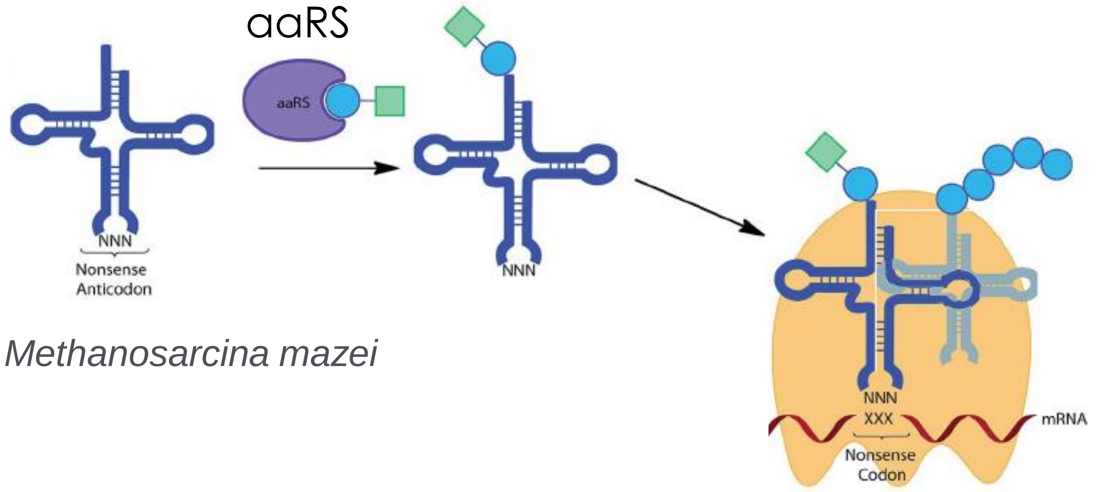


svi var1 var2 var3 var4

Can we obtain a variant that would contain non natural amino acids ?

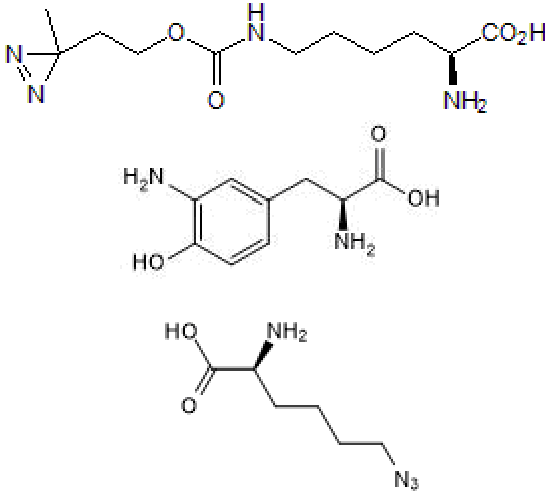
INCORPORATION OF NON NATURAL AMINO ACIDS THROUGH EXTENSION OF THE GENETIC CODE OF *STREPTOMYCES*

Orthogonal tRNA/aa-tRNA synthetase

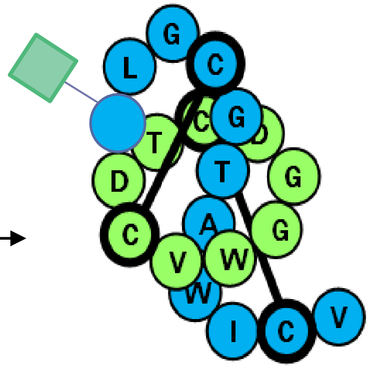


Methanosarcina mazei

TAG



Peptide with non natural amino acid



Reactive group:
Searching for the target protein,
Enrichment, Imaging *in situ*

Dumas et al., Chem. Sci., 2015

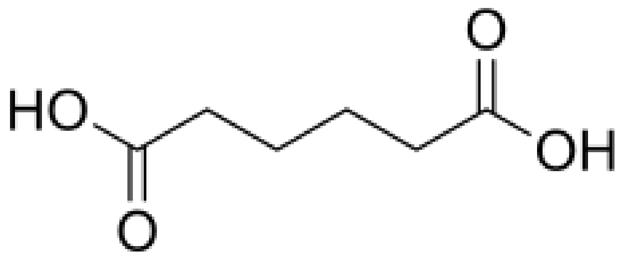
SUMMARY CONCERNING THE SPECIALIZED METABOLISM

- Presentation of some DNA assembly methods
- Explanation on NRPS and RiPP biosynthesis processes
- Through the two examples on congocidine refactoring, and sviceucin analog production

… but there is much more out there !

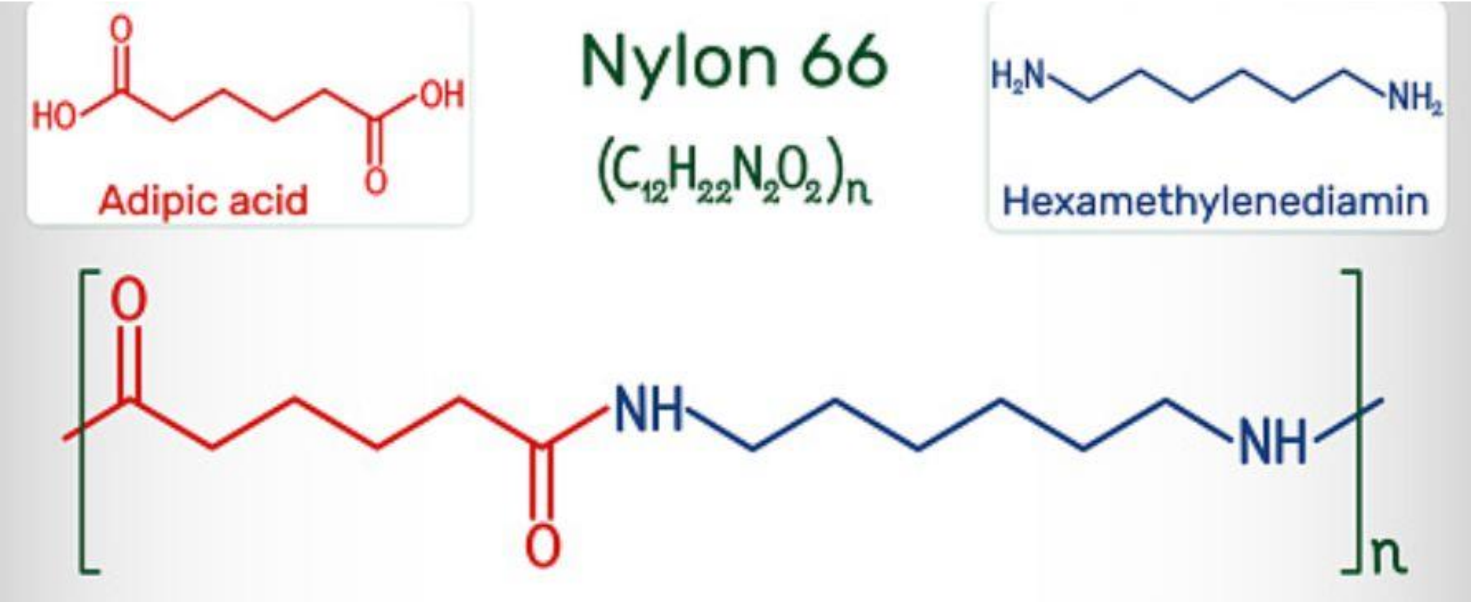
One growing field is to produce interesting molecules from biology,

instead of chemistry



PRODUCING ADIPIC ACID

A COMPONENT OF NYLON (1938)

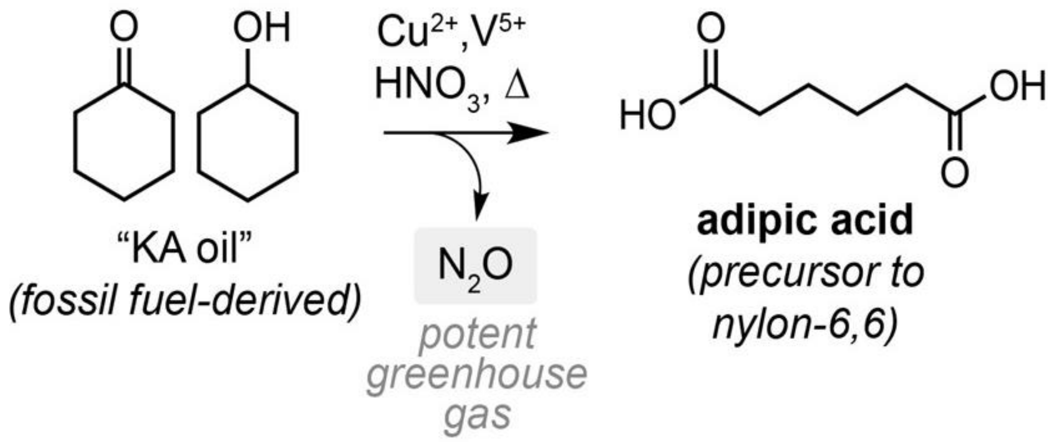


Condensation occurs spontaneously under solvent-free conditions, at elevated temperature and pressure and in high yield

https://europlas.com.vn/en-US/blog-1/nylon-66-what-is-it-properties-benefits-and-applications

CHEMICAL SYNTHESIS OF ADIPIC ACID

cyclohexanone and cyclohexanol



Suitor et al. *ACS synthetic biology* vol. 9,9 (2020): 2472-2476.

CHEMICAL SYNTHESIS OF ADIPIC ACID

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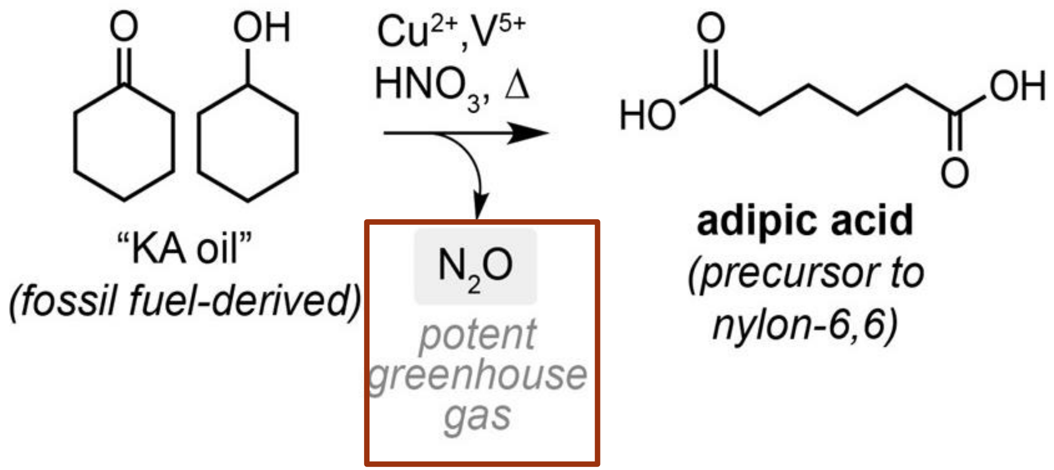


annual production of 2.6 million tons

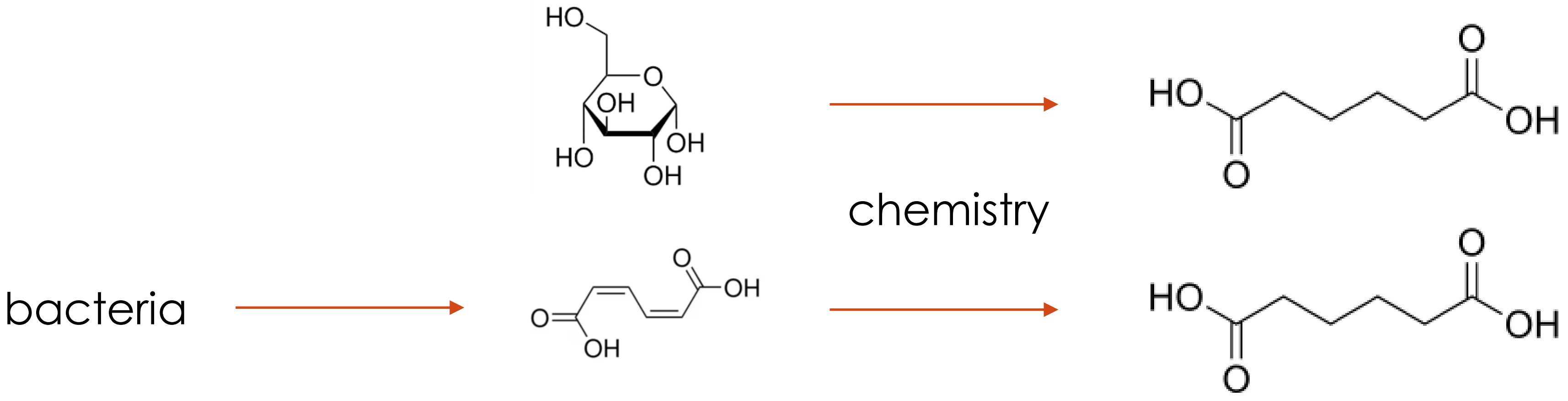
1 kg of N_2O equates to 298 kg CO_2 equivalents

10% of anthropogenic nitrous oxide emissions are emitted from this single industrial reaction to produce nylon

Steele and Wallace *Nature chemistry* vol. 16,5 (2024)
Suitor et al. *ACS synthetic biology* vol. 9,9 (2020): 2472-2476.

CAN WE PRODUCE ADIPIC ACID DIFFERENTLY?

- Semi synthetic approaches from glucose or cis-cis-muconic acid



Still relies on metal catalysts, petrochemical reagents, elevated reaction temperatures and pressures

Steele and Wallace *Nature chemistry* vol. 16,5 (2024)

CAN WE PRODUCE ADIPIC ACID DIFFERENTLY?

- Semi synthetic approaches from glucose or cis-cis-muconic acid
- Biological routes: which chassis??

Escherichia coli, Corynebacterium glutamicum, Saccharomyces cerevisiae, Pseudomonas aeruginosa…

- Which pathway?? Which carbon source??

Glucose, glycerol, cellulose, lignin, plastics…

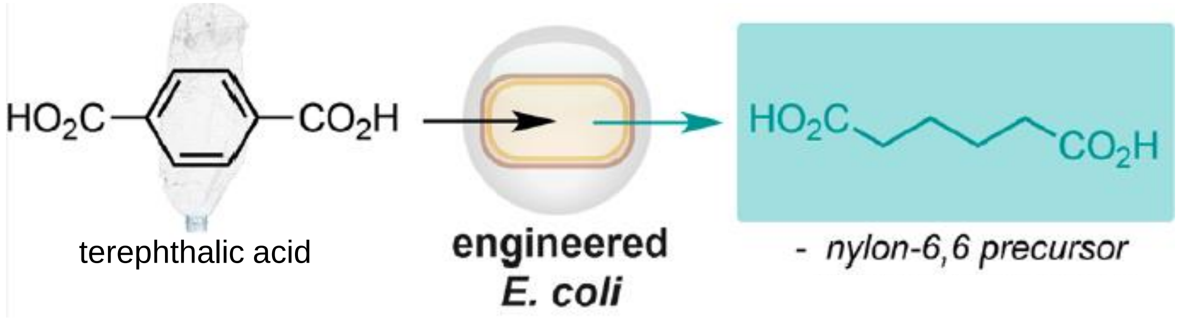
Steele and Wallace *Nature chemistry* vol. 16,5 (2024)

CAN WE PRODUCE ADIPIC ACID DIFFERENTLY?

ACS central science
http://pubs.acs.org/journal/acscii
Article

Microbial Upcycling of Waste PET to Adipic Acid

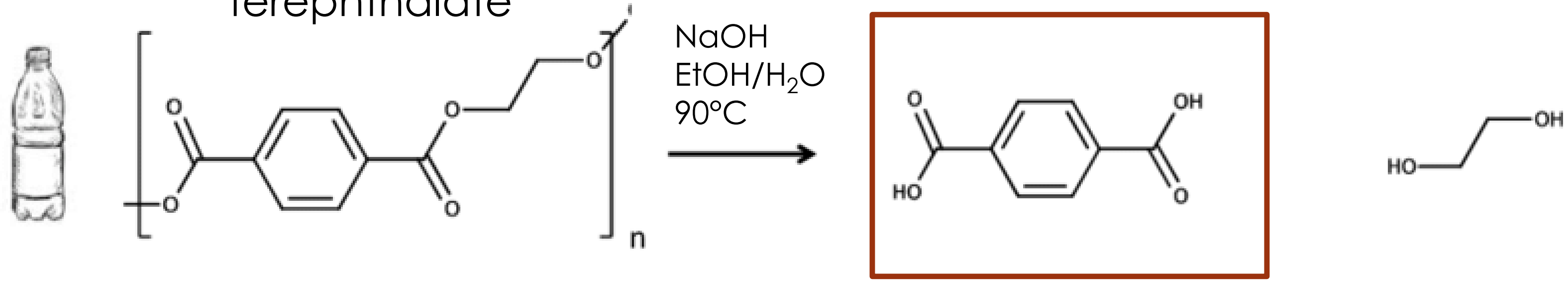
Marcos Valenzuela-Ortega,[§] Jack T. Suitor,[§] Mirren F. M. White, Trevor Hinchcliffe, and Stephen Wallace*



Valenzuela-Ortega, et al. *ACS central science* vol. 9,11 2057-2063. 1 Nov. 2023

PET WASTE

polyethylene terephthalate



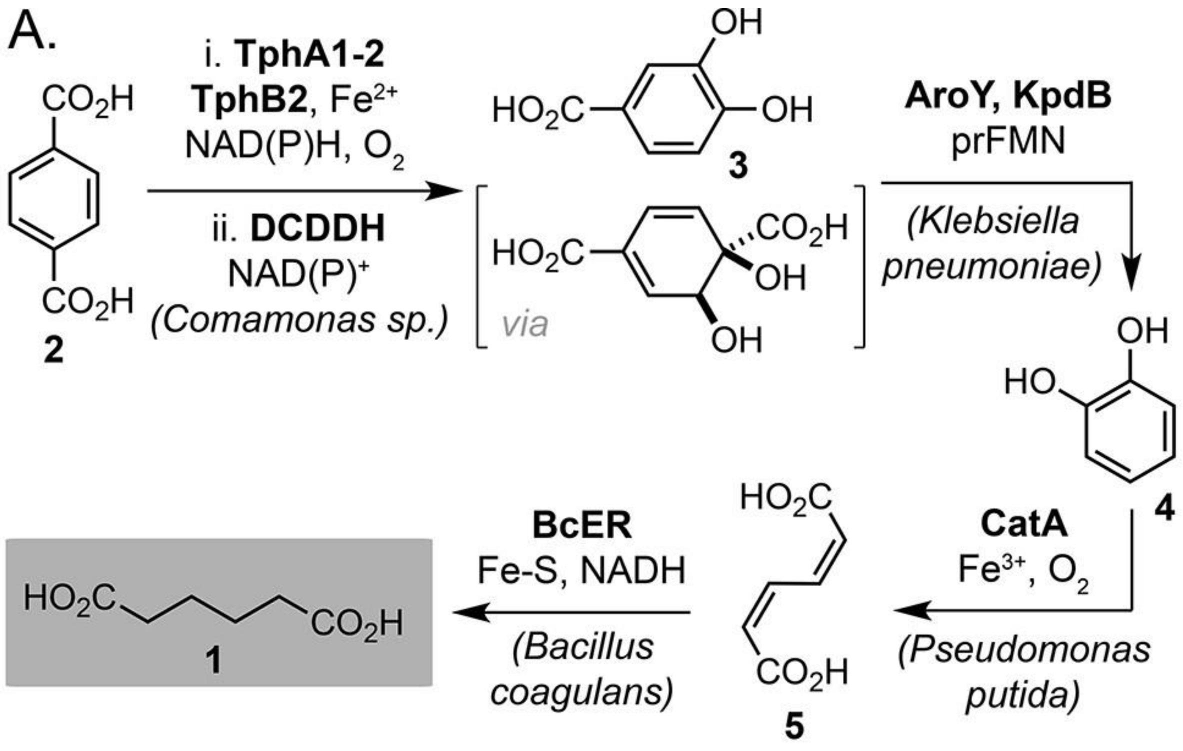
terephthalic acid (TA) and ethylene glycol

About 25 M ton/year of post-consumer PET waste

Salvador de Laraet al. (2019). Genes. 10. 373.

THE PROPOSED PATHWAY FROM TA TO ADIPIC ACID (AA)

terephthalic acid (TA)



Adipic acid (AA)

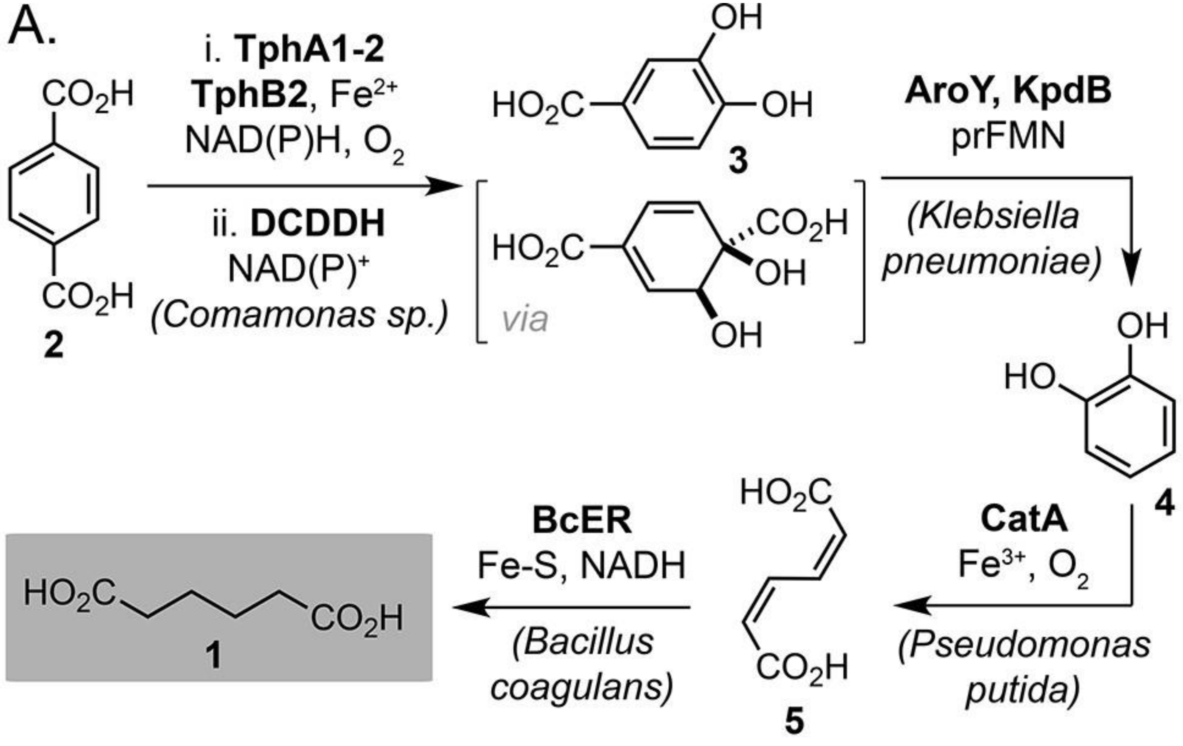
TPADO, a heterotrimeric O2-dependent dioxygenase
DCDDH, a NAD+-dependent dehydrogenase

AroY, a protocatechuate decarboxylase, **KpdB** is the Bsubunit of 4-hydroxybenzoate decarboxylase

CatA is a non-heme Fe(III)-dependent dioxygenase
BcER is a [4Fe−4S]-dependent oxidoreductase

THE PROPOSED PATHWAY FROM TA TO ADIPIC ACID (AA)

terephthalic acid (TA)



Adipic acid (AA)

TPADO, a heterotrimeric O2-dependent dioxygenase
DCDDH, a NAD+-dependent dehydrogenase

AroY, a protocatechuate decarboxylase, **KpdB** is the Bsubunit of 4-hydroxybenzoate decarboxylase

CatA is a non-heme Fe(III)-dependent dioxygenase
BcER is a [4Fe−4S]-dependent oxidoreductase

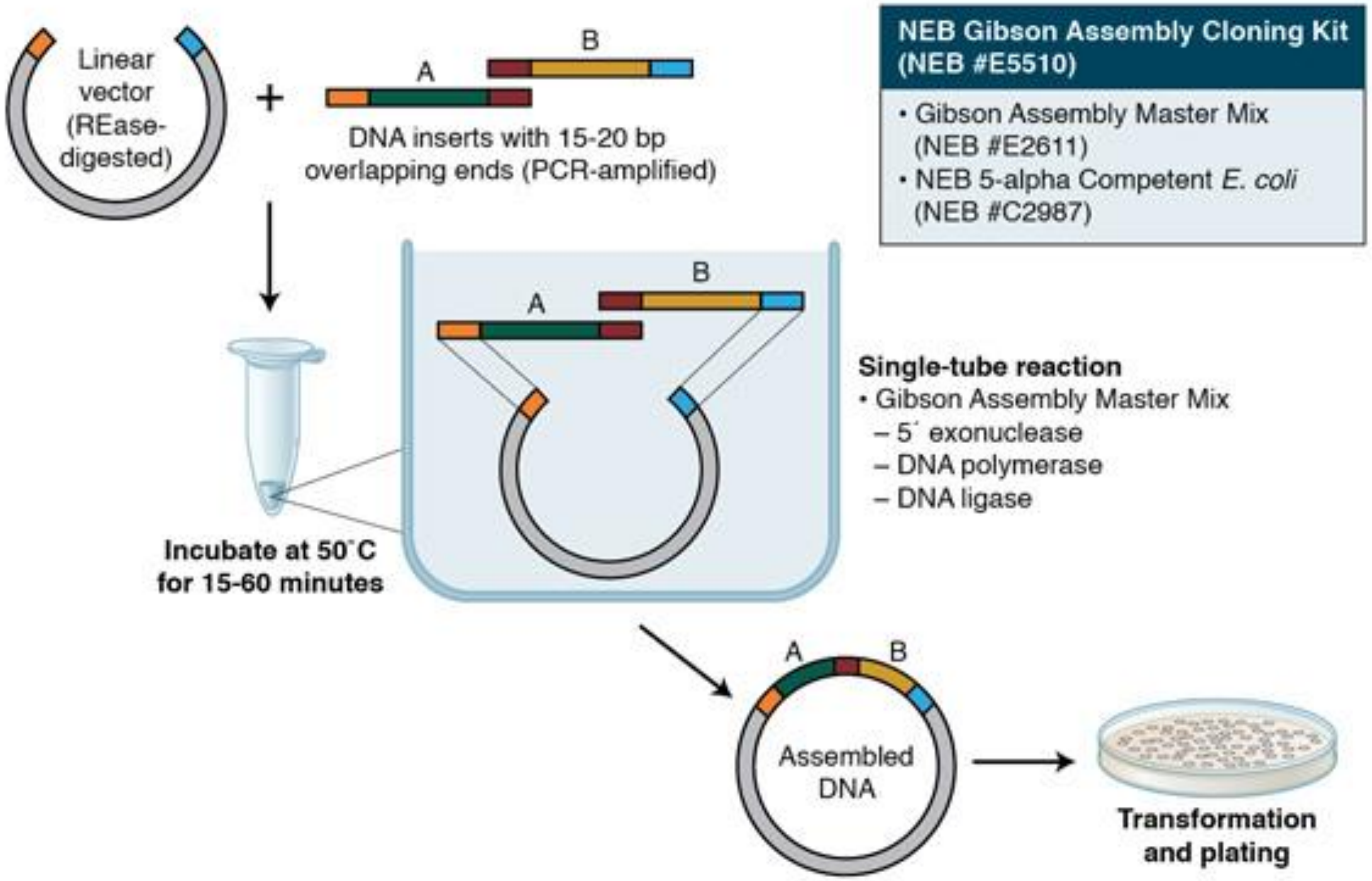
Construction of one vector with *aroY*, *kpdB*, *catA* and *bcER*

How do they proceed??

METHODOLOGY TO CONSTRUCT THE VECTORS

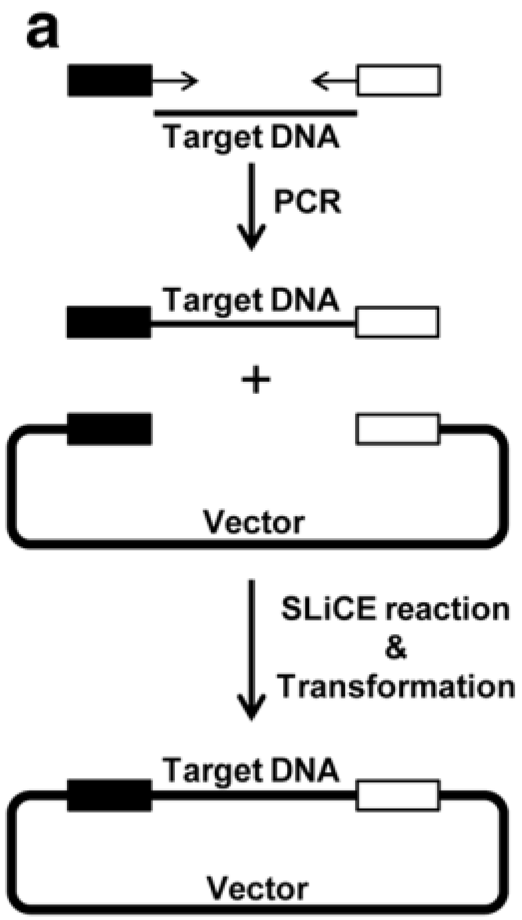
- All genes are codon-optimized and synthetized
- Genes are introduced in vectors using restriction enzymes
- Genes are combined using SLiCE cloning: SEAMLESS LIGATION CLONING EXTRACT

GIBSON ASSEMBLY



- https://www.neb.com/en/applications/cloning-and-synthetic-biology/dna-assembly-and-cloning/gibson-assembly

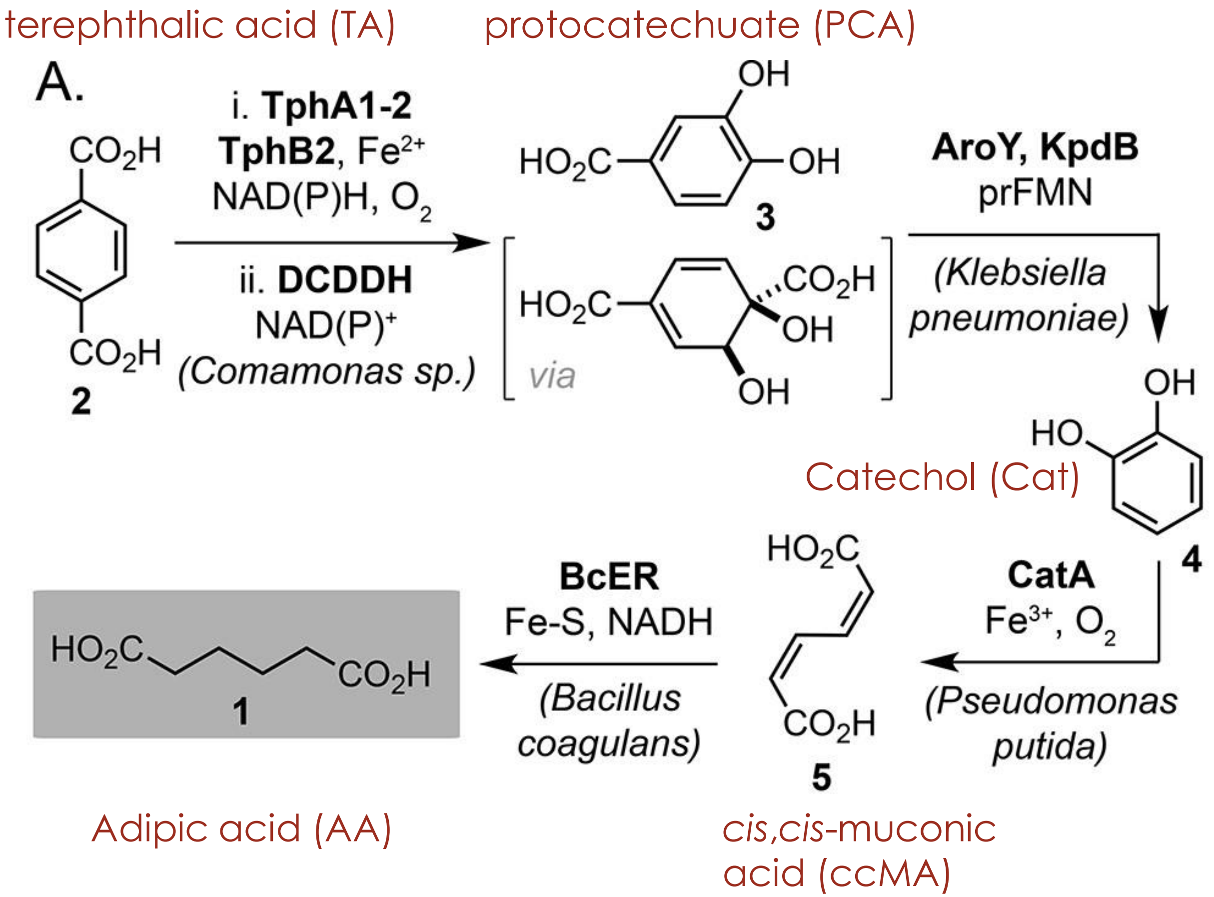
SEAMLESS LIGATION CLONING EXTRACT (SLICE) CLONING METHOD



- in vitro recombination (1h, 37°C) between short regions of homologies (15–52 bp) in bacterial cell extracts
- does not require the use of enzymes for the modification of vector and insert end sequences

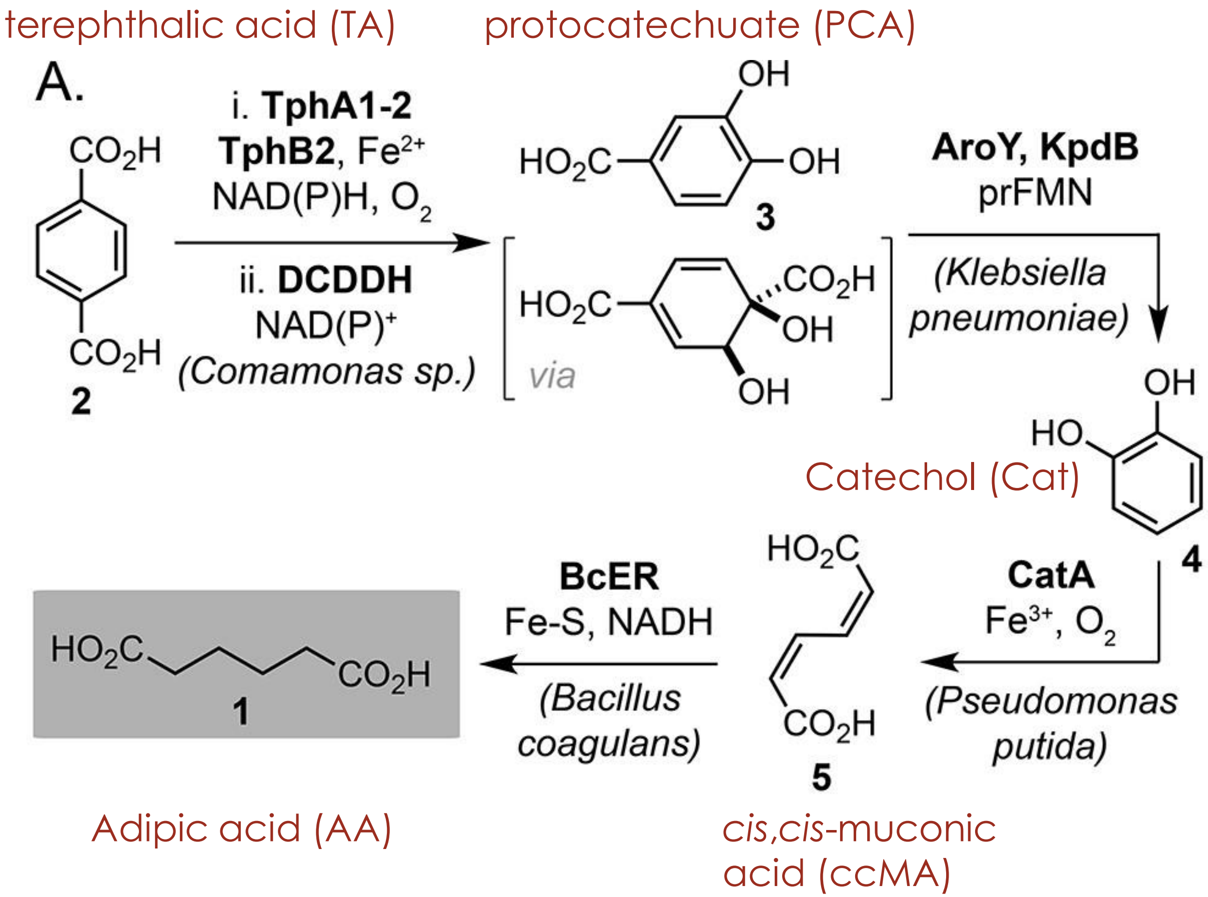
Motohashi K. *BMC biotechnology* vol. 15 47. 3 Jun. 2015, Zhang Y, et al. Methods Mol Biol. 2014;1116:235-44.

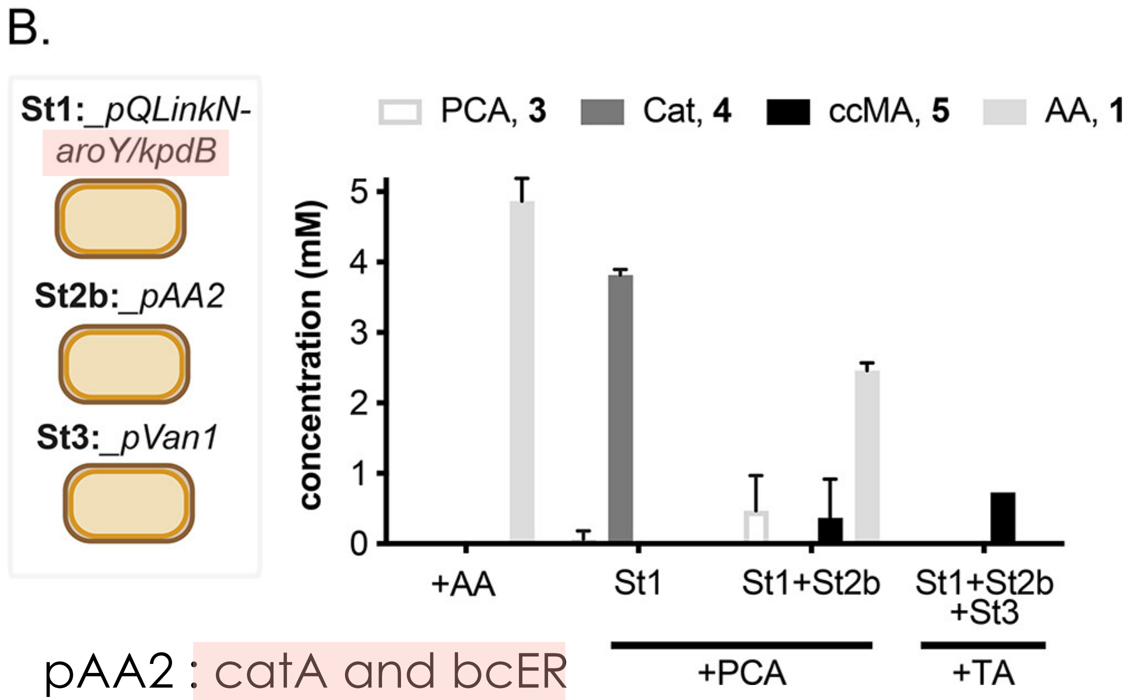
THE PROPOSED PATHWAY FROM TA TO ADIPIC ACID (AA)



pAA2 : catA and bcER
pVAN1 : TPADO and DCDDH

THE PROPOSED PATHWAY FROM TA TO ADIPIC ACID (AA)





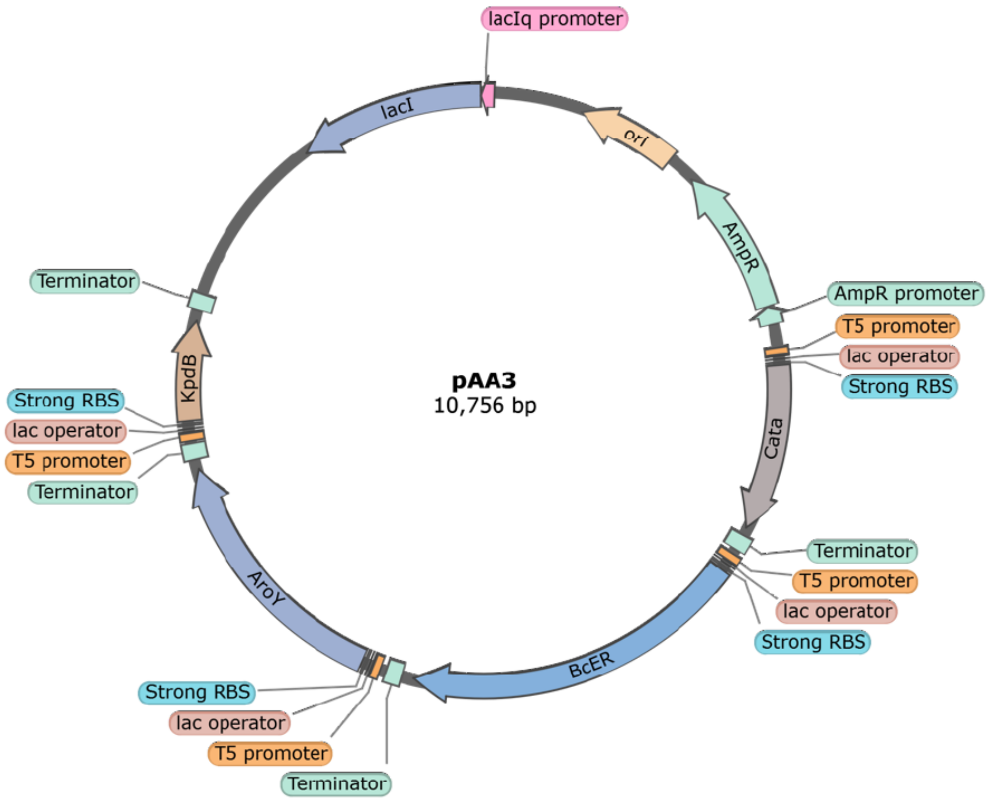
pAA2 : catA and bcER
pVAN1 : TPADO and DCDDH

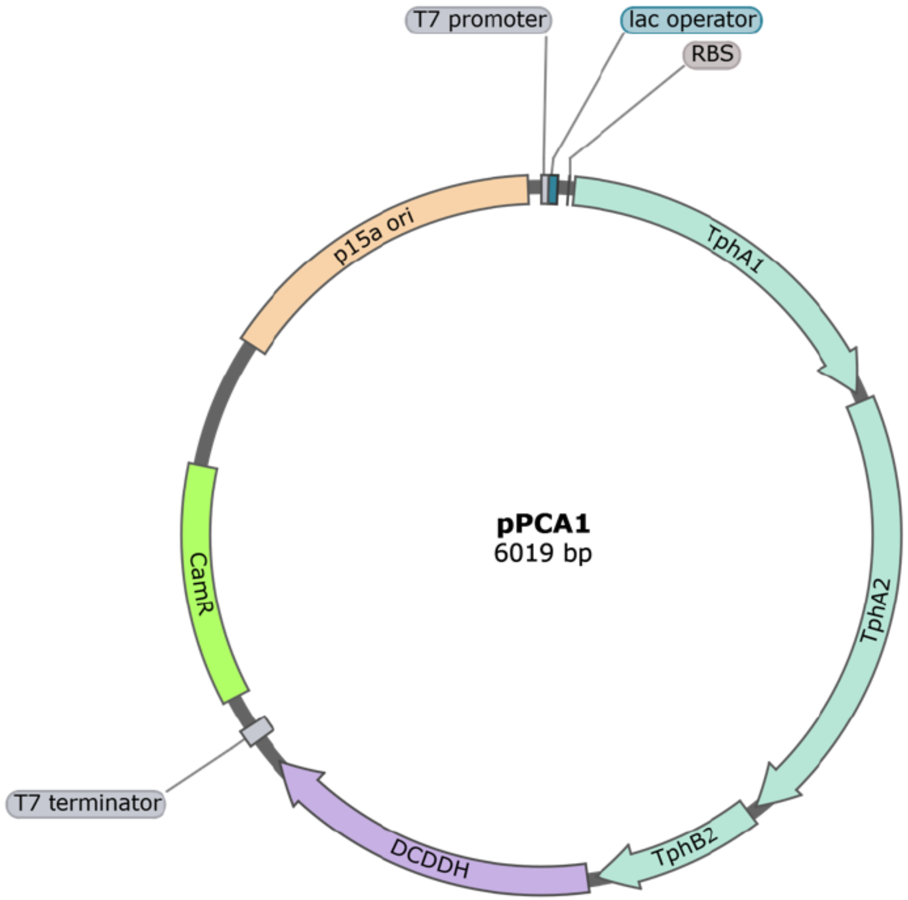
From PCA, they manage to produce AA, but not from TA
TPADO and bcER are evaluated as bottlenecks

HOW TO IMPROVE A YIELD?

- Localize all the pathway enzymes in a single cell, on two plasmids

Technique : SLiCE cloning: seamless ligation cloning extract



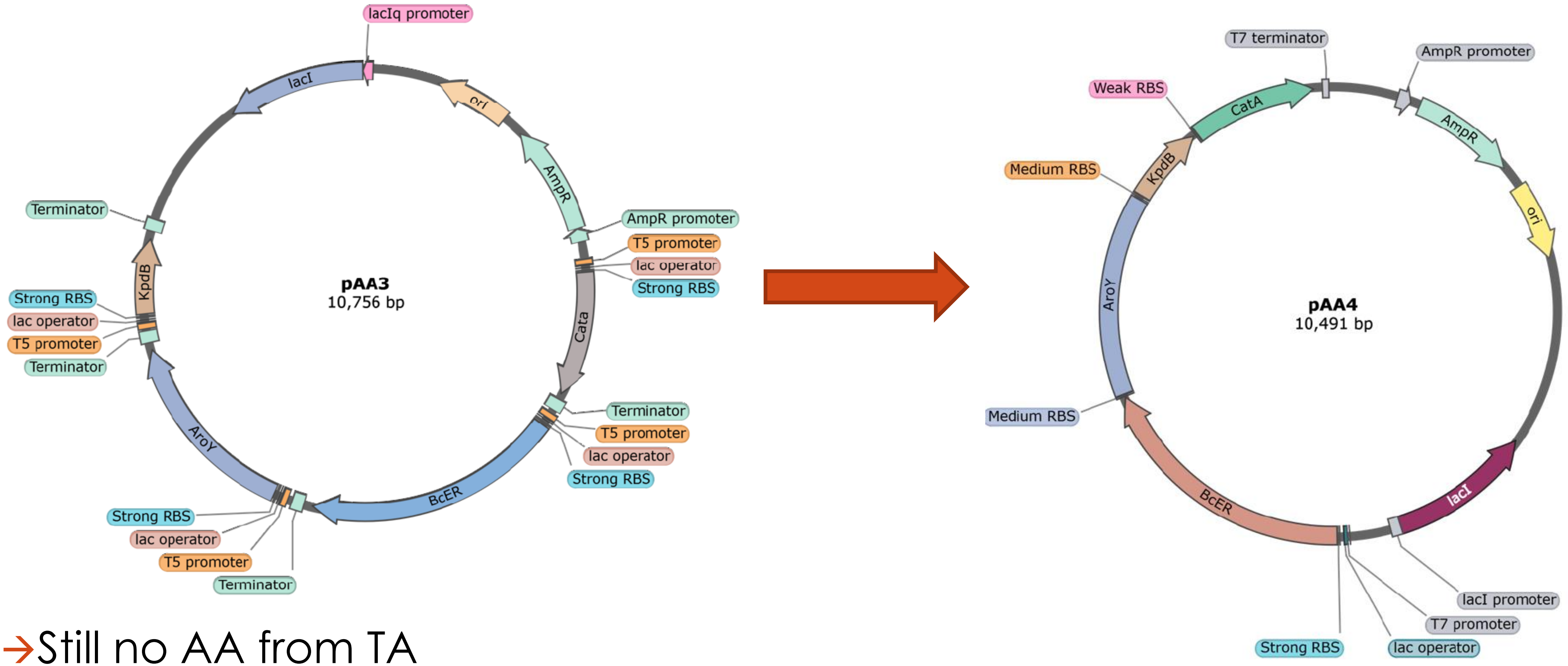


→Plenty of ccMA (better product flux), but no AA

→BcER is a limiting step

HOW TO IMPROVE A YIELD?

- Modify the plasmid to make a polycistronic mRNA



→Still no AA from TA

→19% yield AA from ccMA

HOW TO IMPROVE A YIELD?

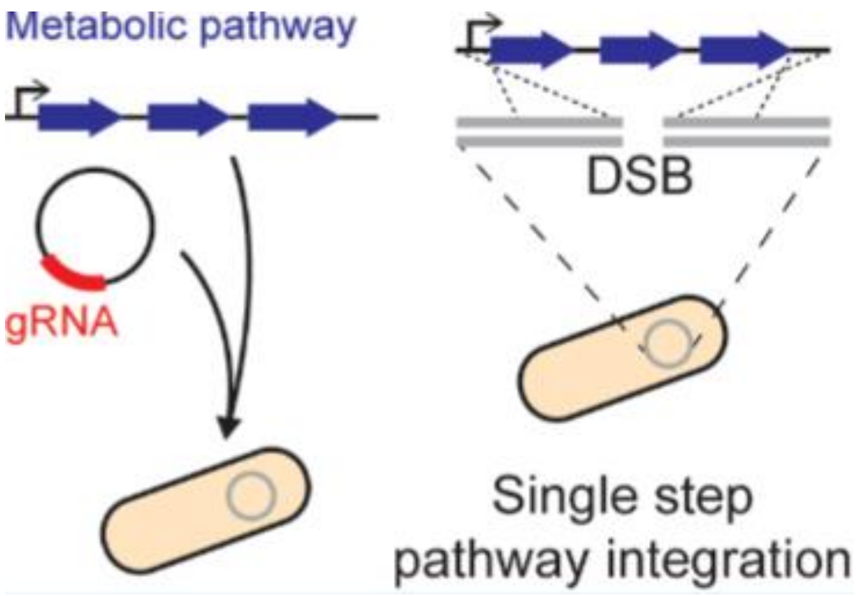
- Modify the plasmid to make a polycistronic mRNA

→Still no AA from TA

- Genome integration of genes TPADO and DCDDH

CRISPR-Cas9/λ-red method

CRISPR-Cas9/λ-red method



- Integration of the cassette from pPCA1 coding for *tphA1, tphA2, tphB2, dcddh*
- Donor plasmid with locus-specific 600-bp homology arms amplified from *E. coli* BL21(DE3) genomic DNA and the cassette amplified from pPCA1
- gRNA plasmid was generated by PCR and SLiCE assembly
- co-transforming the locus-specific gRNA, donor plasmid and pX2-Cas9
- Integration was confirmed by colony PCR
- CRISPR plasmids were cured by growing cells at 42 °C overnight without antibiotic selection

Bassalo, Marcelo C et al. ACS synthetic biology vol. 5,7 (2016): 561-8.

HOW TO IMPROVE A YIELD?

- Modify the plasmid to make a polycistronic mRNA

→Still no AA

- Genome integration of genes TPADO and DCDDH

→No AA, even less ccMA

HOW TO IMPROVE A YIELD?

- Modify the plasmid to make a polycistronic mRNA

→Still no AA

- Genome integration of genes TPADO and DCDDH

→No AA, even less ccMA

- Chaperone co-expression

→ also makes it worse (no AA, less ccMA)

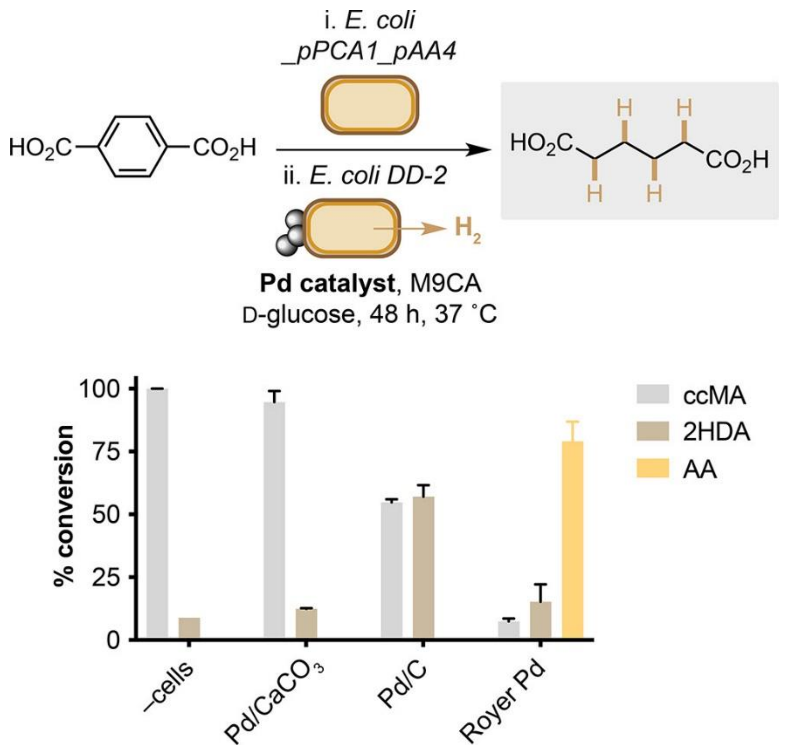
- Swapping the genes from the two plasmids

→ Faster production of PCA, but less yield

Back to pPCA1 and pAA4, the two plasmid system, focus on optimization of the whole-cell biotransformation

CHEMICAL APPROACH TO OVERCOME BcER LIMITATIONS

Biocompatible chemistry to replace the activity of BcER by converting ccMA to AA using a H2-generating strain of *E. coli* (DD-2) and a membrane-bound Pd catalyst.



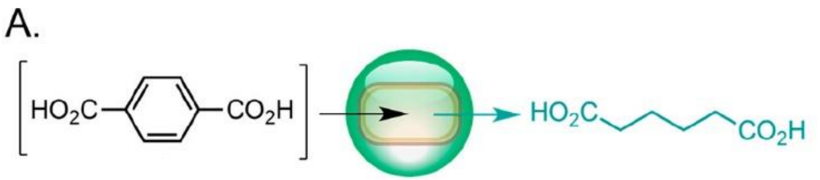
ccMA produced by the *E. coli* pPCA1_pAA4

Removal of cells by centrifugation

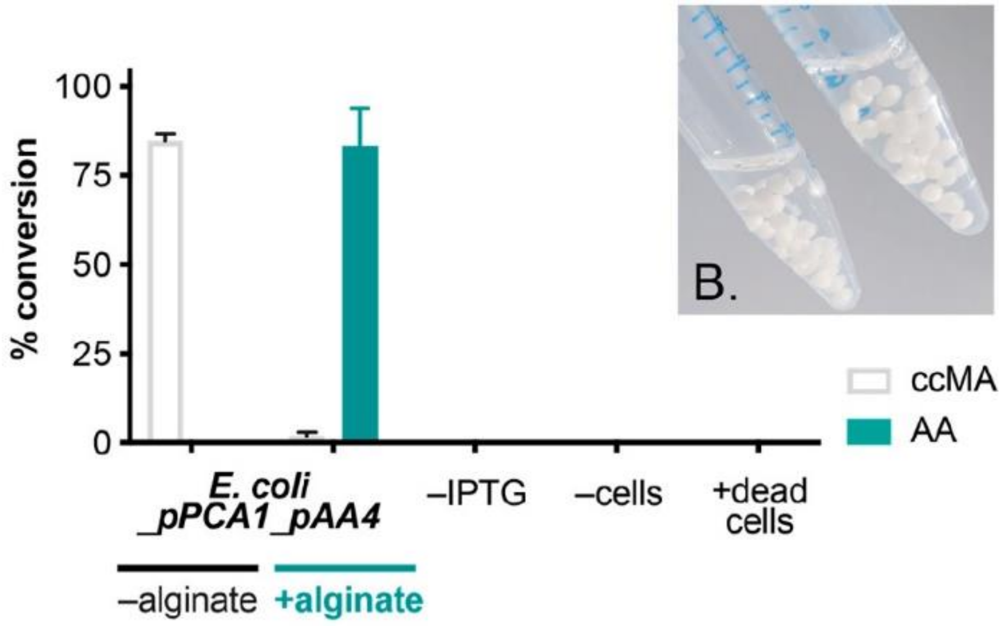
Supernatant introduced to a culture of *E. coli* DD-2

E. coli DD2 has a pathway consisting of a **pyruvate ferredoxin oxidoreductase** (PFOR) from *Desulfovibrio africanus*, **hydrogenase maturation factors** from *Chlamydomonas reinhardtii*, and a **ferredoxin and [Fe−Fe] hydrogenase** from *Clostridium acetobutylicum*, which together enable the anaerobic production of H2(g) from D-glucose

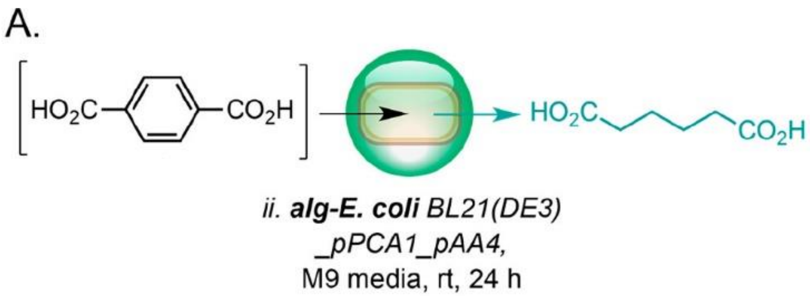
USE OF CELLS SUPPORTED IN ALGINATE HYDROGEL



Alginate hydrogel is known to increase the stability of enzymes *in vitro*



USE OF CELLS SUPPORTED IN ALGINATE HYDROGEL



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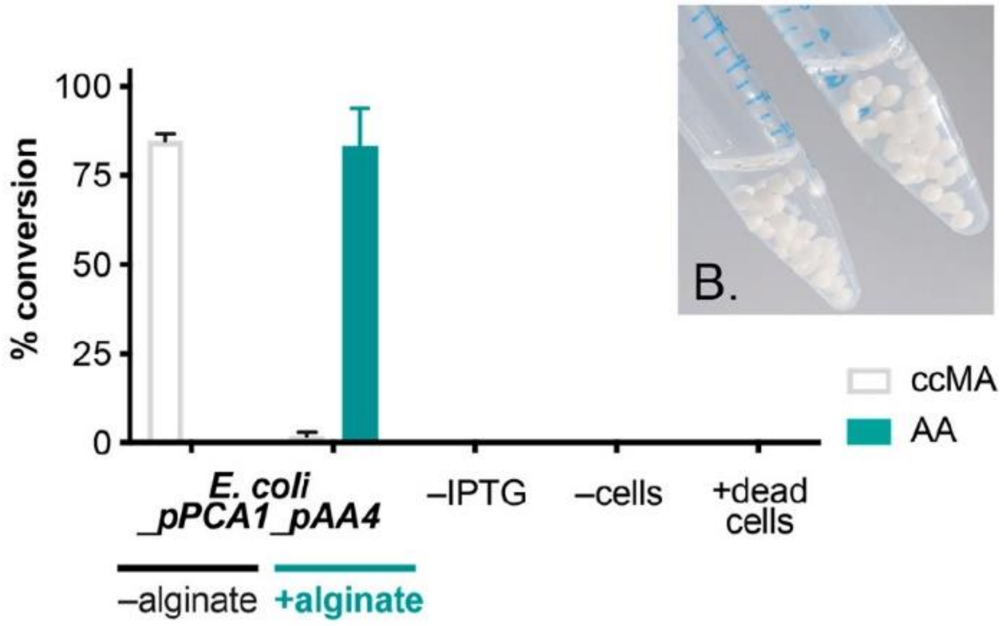
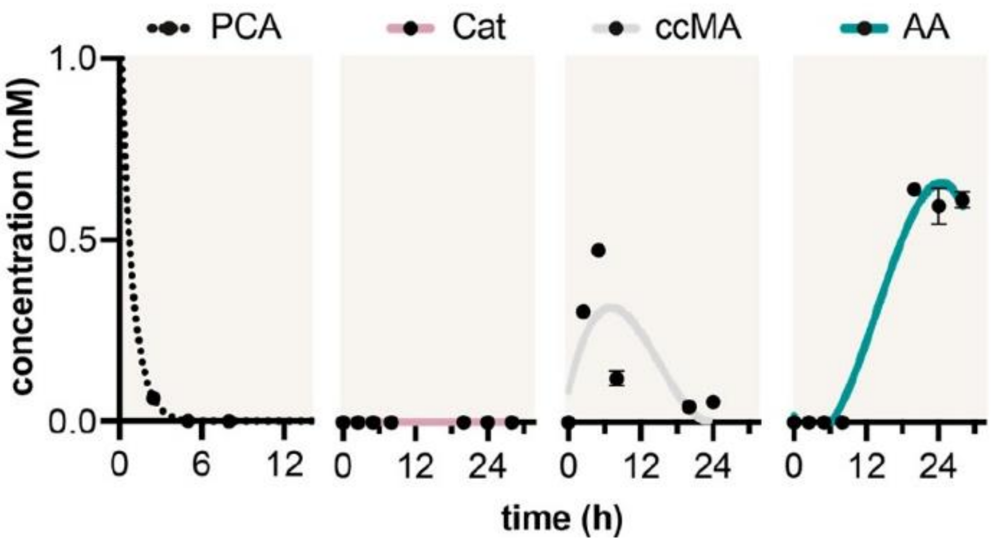




Alginate support likely improves the oxygen tolerance and/or stability of the [4Fe−4S]-containing BcER enzyme.

MORE THINGS THEY TRIED

- Increase NADH availability

Coexpression of NAD+-dependent formate dehydrogenase

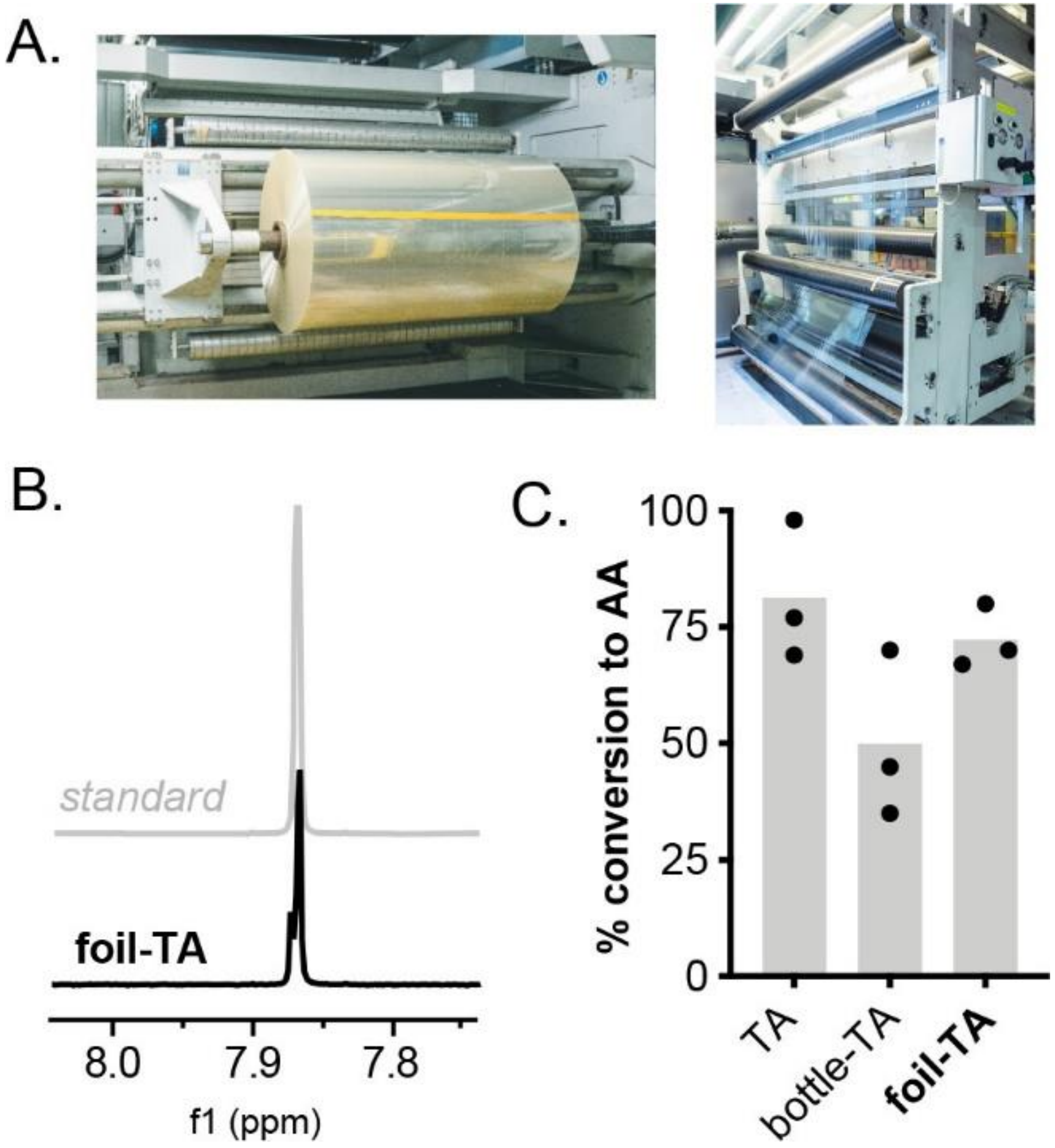
Switching the carbon source from D-glucose to D-mannitol or D-sorbitol

Co-addition of glucose and sorbitol at 1:1 mol equivalent or increasing the concentration of glucose 2-fold

Things they say they will study in more details:

- Examine the inhibition of BcER by TA
- Study pH-dependent TA diffusion and flux at physiological pH

THEIR PROOF OF CONCEPT IN "REAL CONDITIONS"



stamping foil

alkaline hydrolysis conditions (aq. NaOH, EtOH, 90°C, 1 h)

Their conclusion:

Product conversion is high (79%, 115 mg/L) and occurs in aqueous media under ambient conditions (room temperature, pH 7.4) in 24 h.

Conclusions to keep in mind

- Do not underestimate the versatility of microorganisms!
- Keep an open mind: what is specific to your project?

Their is no perfect host, no perfect assembly method, but to take into account their specifities is an opportunity

- Do not limit yourself to one field, combining methods/tools from different fields is also an advantage
- And of course… reality remains challenging, so be prepared for failure…

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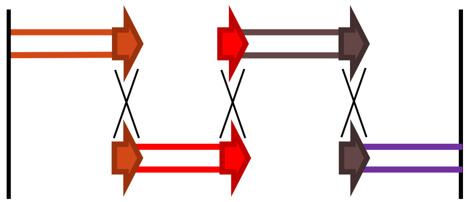
Pascal Belin
Carine Tellier-Lebègue
Matthieu Fonvielle
All the team

DNA ASSEMBLY METHODS

<u>Site-specific recombination</u>

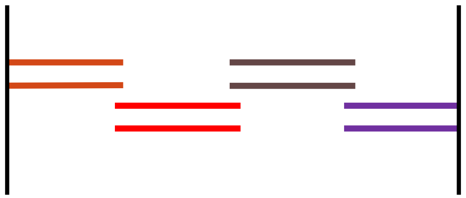


ΦBT1 Site-specific integrase
In vitro

ΦC31 Site-specific integrase
In vitro

<u>Homologous recombination</u>

in vitro



in vivo

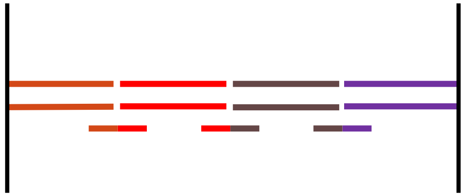
Gibson assembly
One step
3’ overhang, T5 exonuclease

SLIC
Sequence and ligation-independant cloning
5’ overhang, T4, RecA

DNA assembler
Saccharomyces cerevisiae

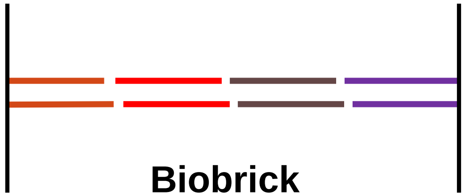
LLHR
Linear to linear homologous recombination
RedET-mediated in *E. coli*
In vivo assembly

<u>Bridging oligos</u>



LCR
Ligase cycling reaction
One step, scarless

<u>Restriction digestion/ligation</u>



Biobrick
*Eco*RI, *Xba*I, *Spe*I, *Pst*I

Golden Gate
*Bsa*I, one step

DIVERSITY OF DNA ASSEMBLY METHODS:

In vivo Assembly

SLIC, SliCE, USER

iBrick

CPEC

linear-linear homologous recombination

Biobrick, BglBrick

TAR cloning

Modular Overlap-Directed Assembly with Linkers (MODAL)

modular cloning (MoClo)

BASIC

Restriction enzymes

Gibson assembly

LCR

Golden Gate

PaperClip

MASTER

Phage φBT1 integrase-mediated site-specific recombination

Site-specific recombination-based tandem assembly (SSRTA)

GoldenBraid

DNA assembler

PIPE cloning

Direct Pathway cloning

AQUA Cloning

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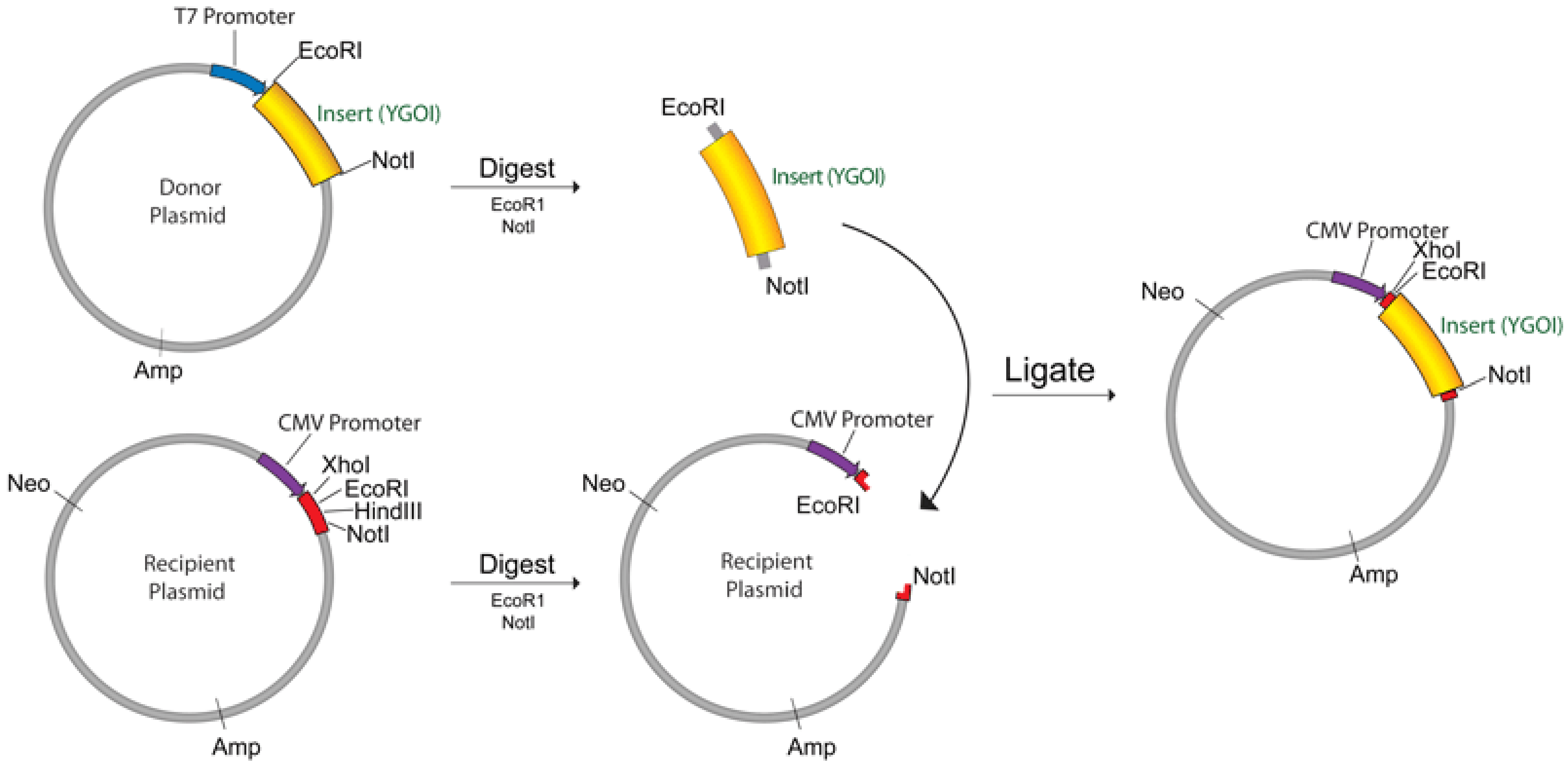
Cloning by restriction - ligation



https://www.addgene.org/protocols/subcloning/

The biobricks system