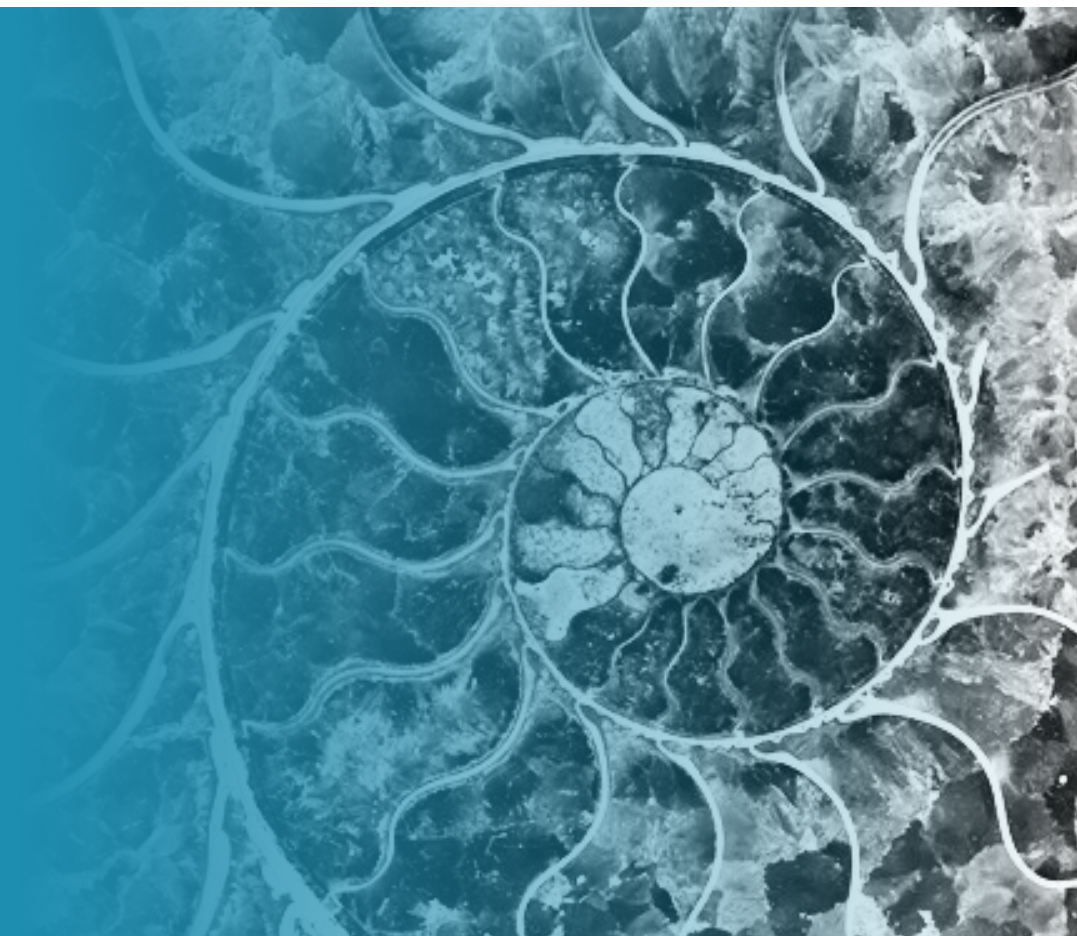


Directed evolution of compatible plasmid origins of replication

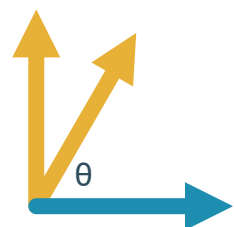
Towards a model of orthogonality



Prof. Vitor B. Pinheiro
Associate Professor

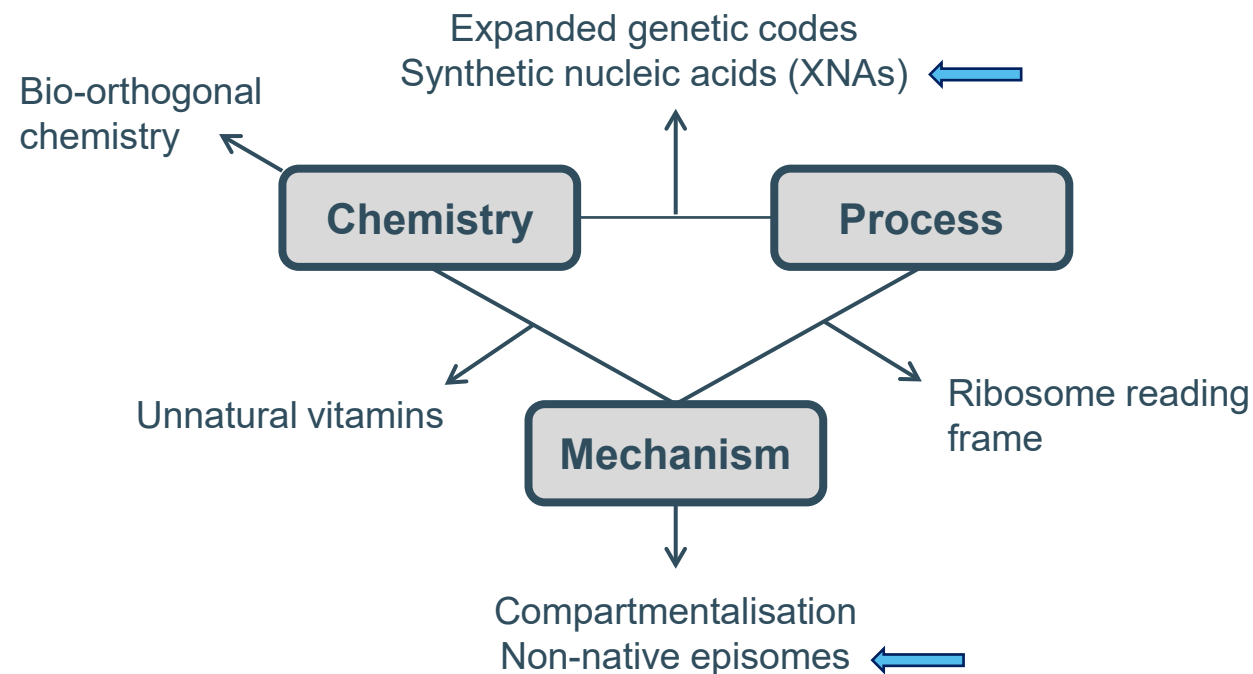
Orthogonality

- Borrowed concept from Mathematics
- There's no free ride in Biology
- There are also different routes towards orthogonality



$$\mathbf{a} \cdot \mathbf{b} = \|\mathbf{a}\| \|\mathbf{b}\| \cos\theta$$

If $\mathbf{a} \perp \mathbf{b}$, then $\mathbf{a} \cdot \mathbf{b} = 0$



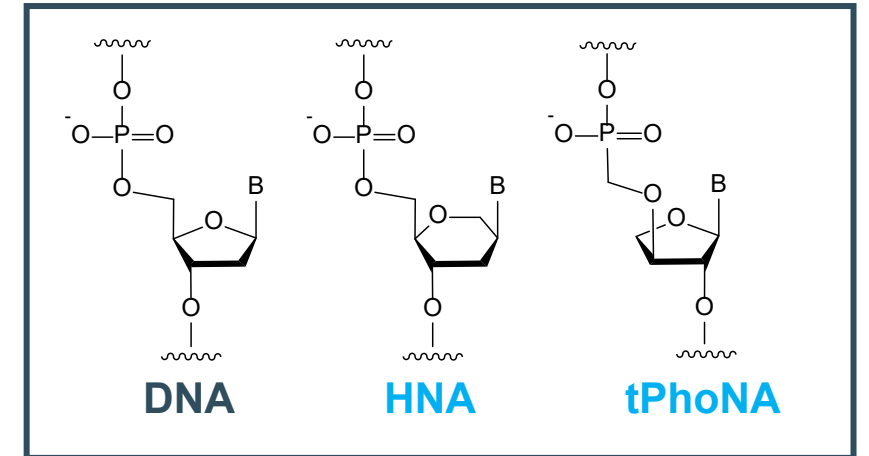
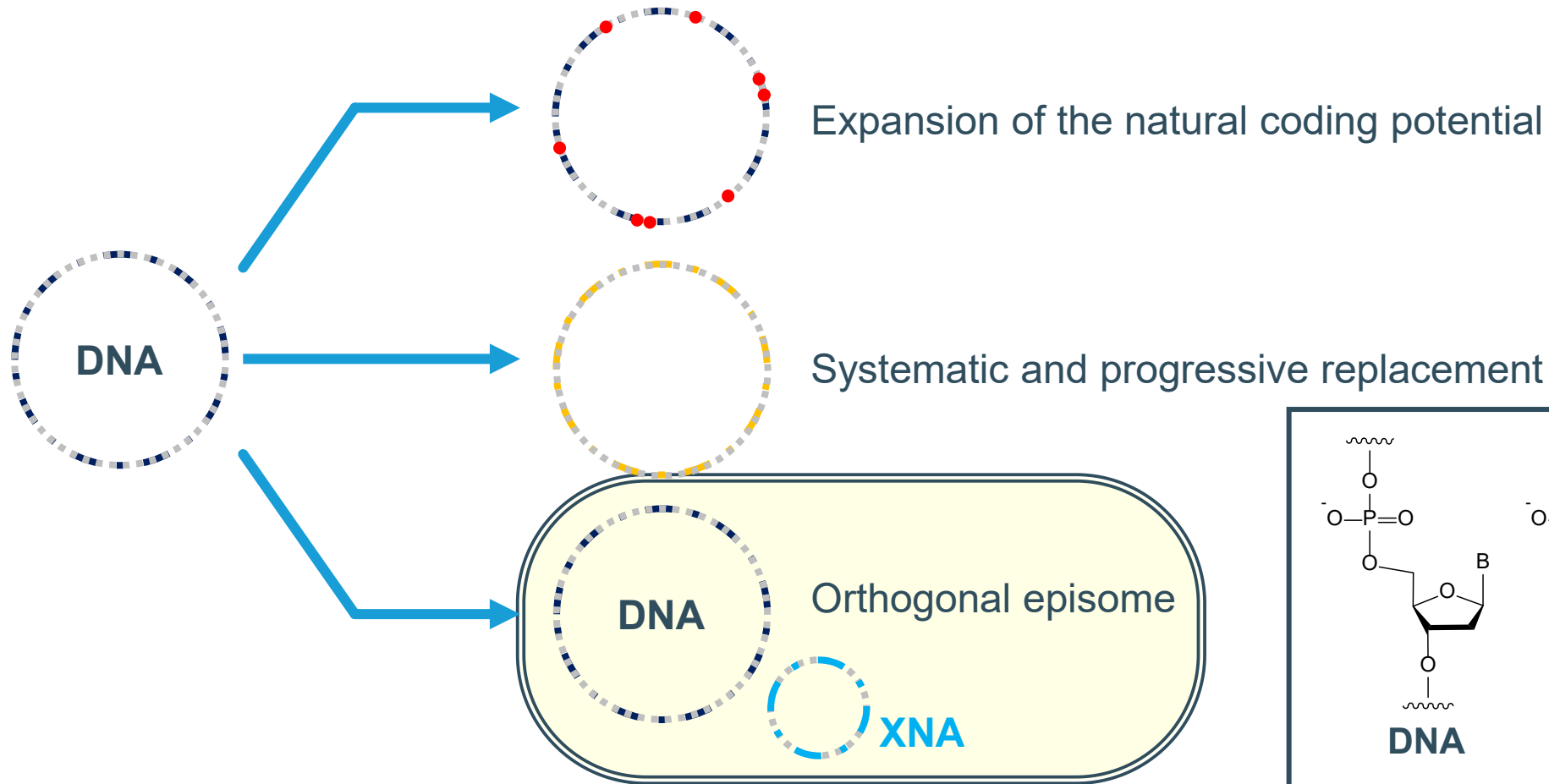
De Lorenzo (2011) **Bioengineered Bugs**
10.4161/bbug.2.1.13388

Gyorgy *et al.* (2015) **Biophysical Journal**
10.1016/j.bpj.2015.06.034

Torres *et al* (2016) **Essays in Biochemistry**
10.1042/EBC20160013

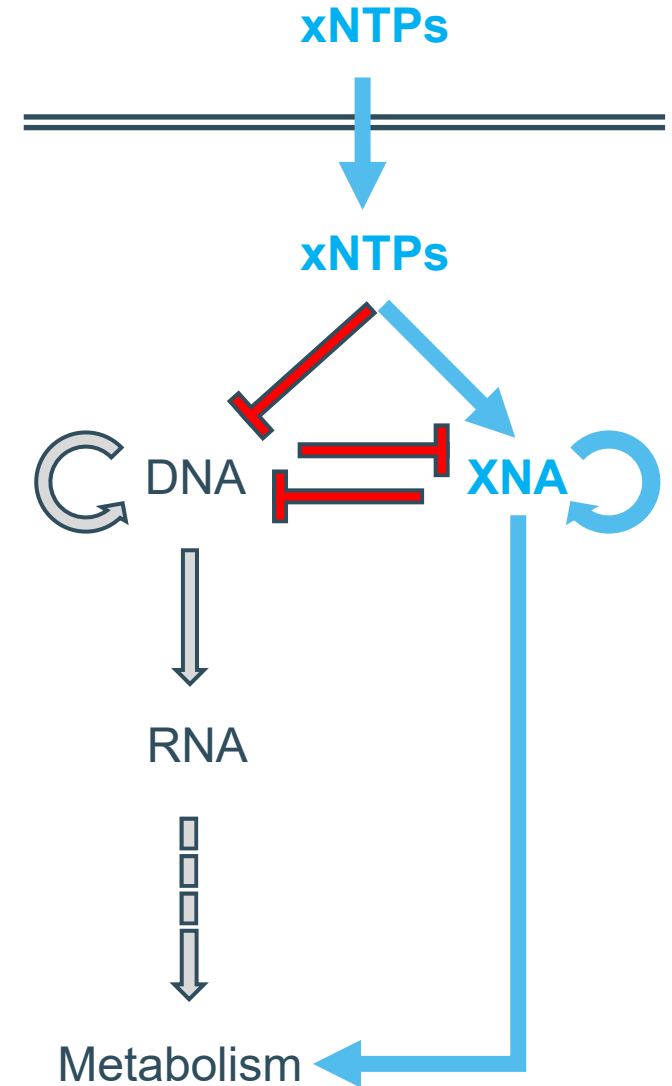
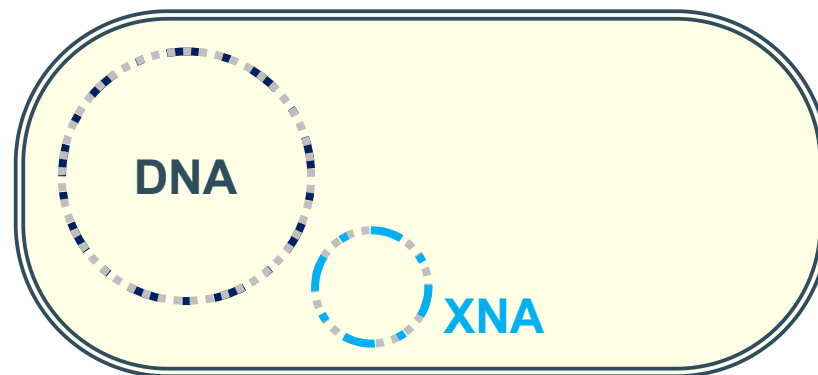
XNA biology

- Broadly 3 strategies to change the information storage medium in biology:



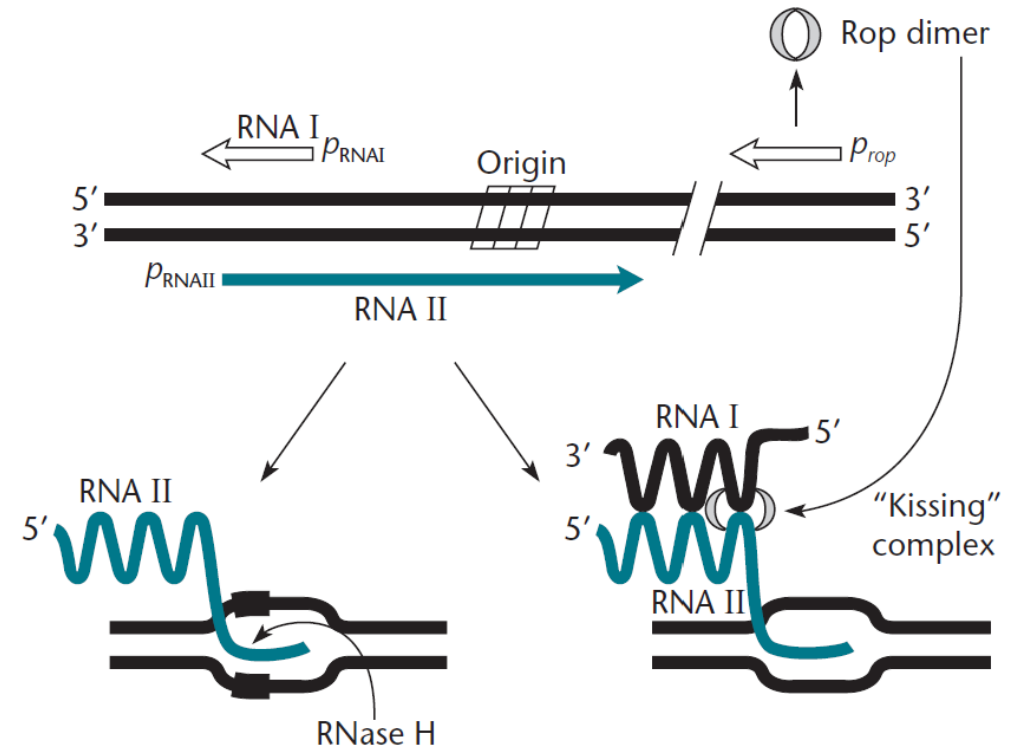
Life orthogonal

- Requires multiple orthogonal steps
- Orthogonality can be engineered but...
 - What's the best *orthogonality* to engineer?
 - How to quantify different *orthogonalities*?
 - Can the concept of *orthogonality* itself be improved?
- We need a **fast tractable tunable model** for orthogonality.



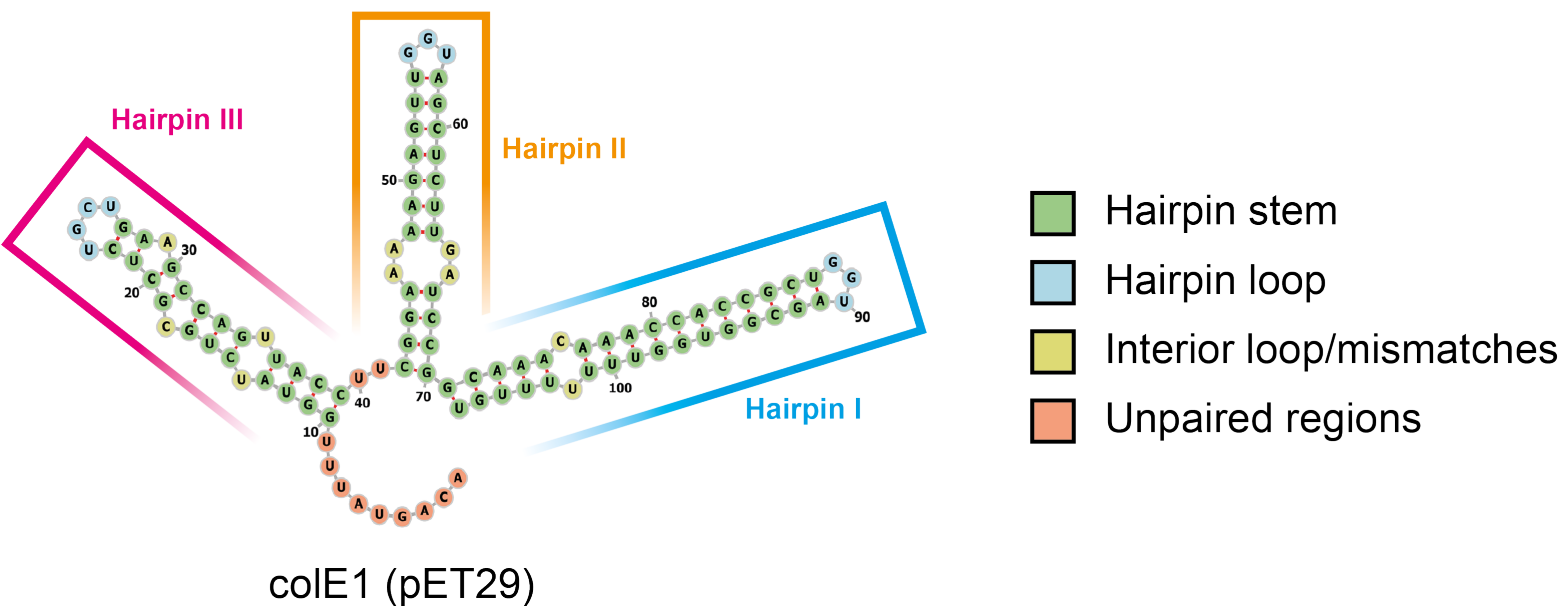
ColE1-family plasmids and plasmid compatibility

- colE1-family plasmids have a common replication mechanism
 - **Sense-antisense** overlapping gene pair
 - Interaction between two transcripts controls rate of replication initiation
- Two plasmids with the **same colE1 origin cannot stably co-exist** in a cell
 - Shared mechanism drives cell to lose one plasmid






Snyder *et al* (2013) **Mol. Gen. of Bacteria**
10.1128/9781555817169.ch4.f4.6

Plasmid compatibility

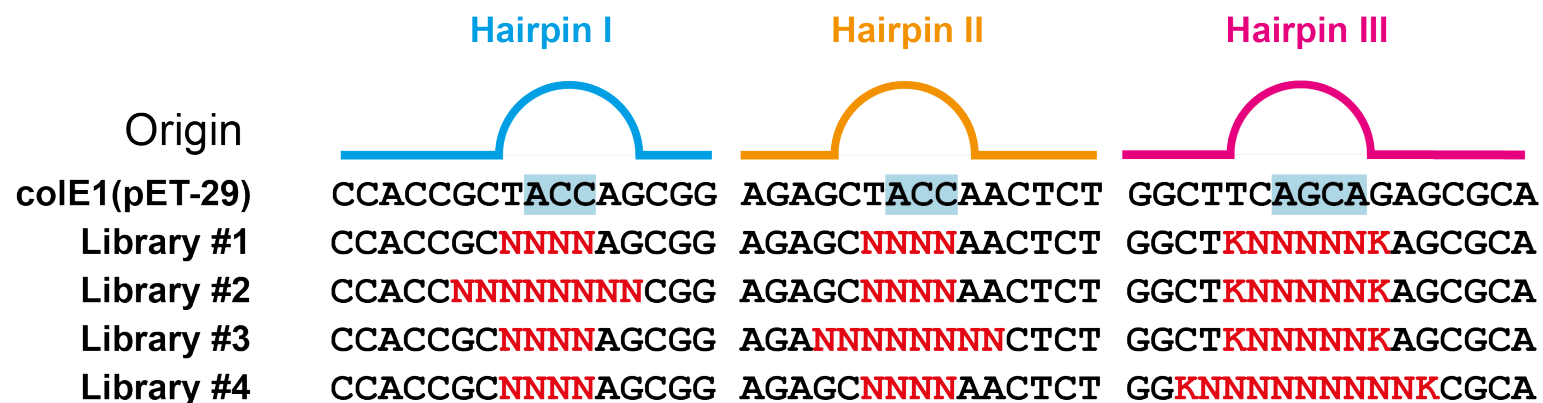


- Differences between compatibility groups is small
- Loop mutations are known to affect compatibility

	Hairpin I	Hairpin II	Hairpin III
Origin			
colE1(pET-29)	CCACCGCTACCAGCGG	AGAGCTACCAACTCT	GGCTTCAGCAGAGCGCA
p15A(pBAD30)	AA.....CTTGCAG..	T.....-G.AG.....
CDF(pCDF-1b)	AA...A.CTGGG.A..	TC..T.GGGG.ACTGTC.....A..
RSF1030(pRSFDuet-1)A...	T.....	GG...-G.AG..C....

Selecting novel plasmid origins

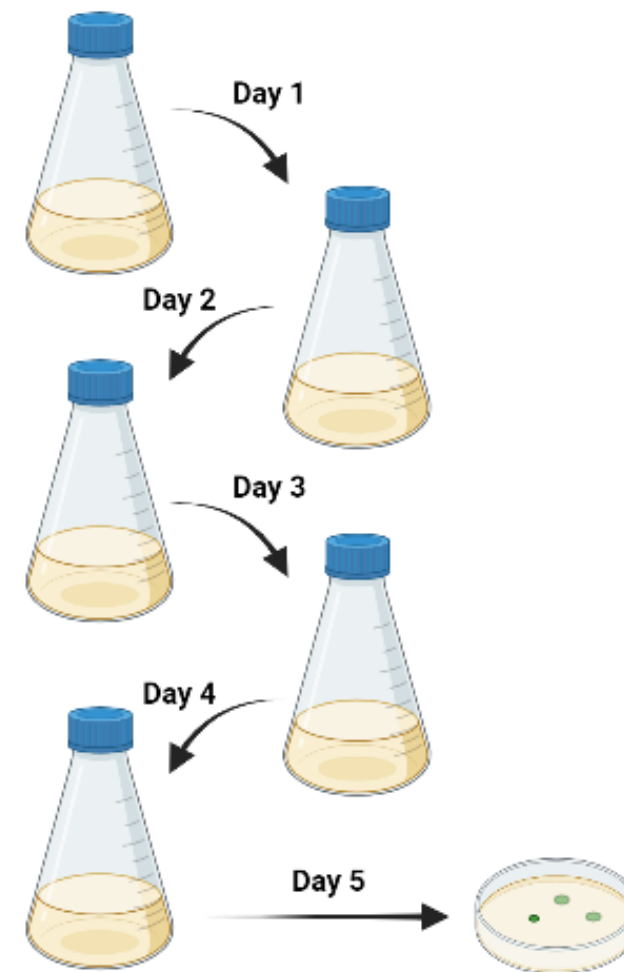
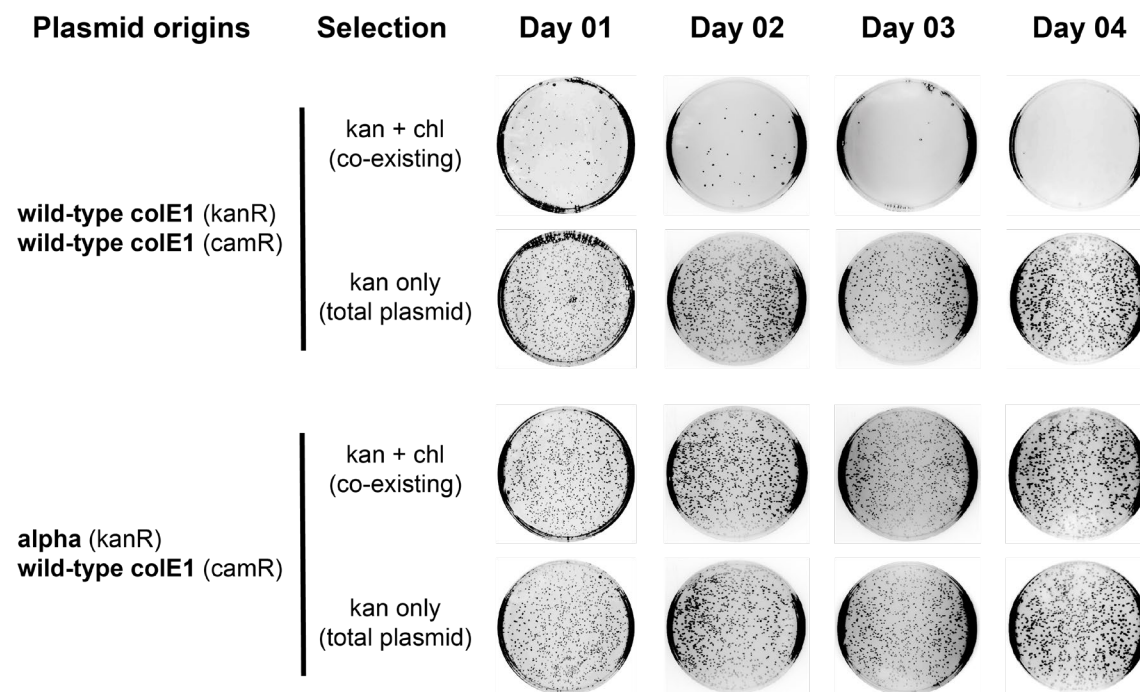
- Chemically synthesised diversity
- Viable plasmid origins selected by plating
 - Functional space not densely populated ($< 10^5$ from over 10^{10} variants)



Selecting novel compatible plasmid origins

- **Classic approach:**

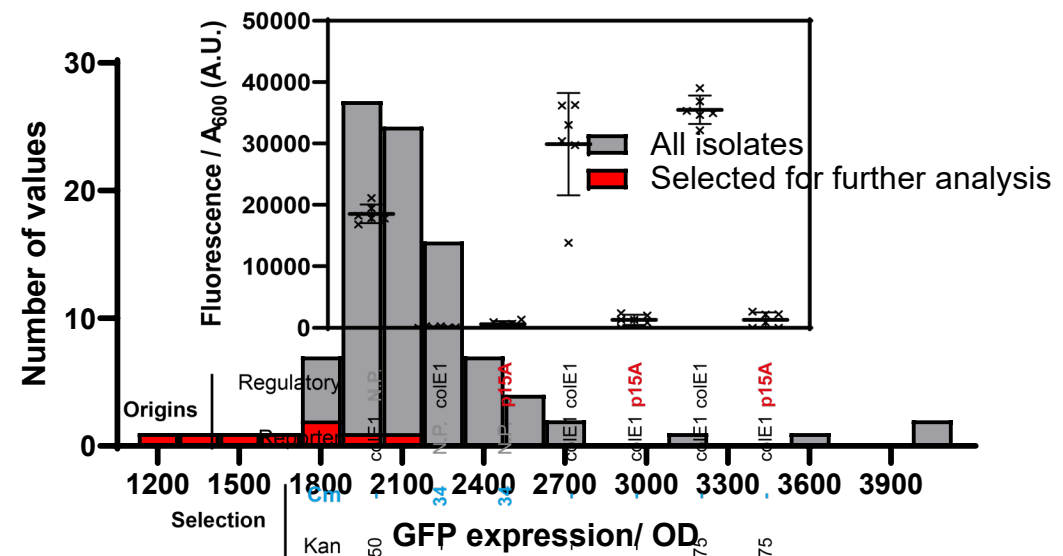
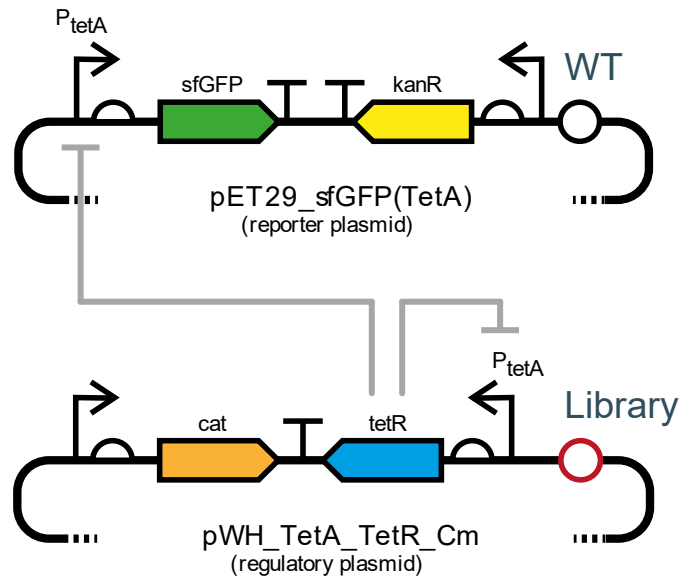
- serial passaging with (or without) selection
- plating under double selection



Created in BioRender.com

Selecting novel compatible plasmid origins

- **Scaled down assay:**
 - Serial passaging with (or without) reporter selection
 - One rather than five days - **fast**
 - Fluorescent readout
 - Selection and screening
 - Compatible with dynamic measurements



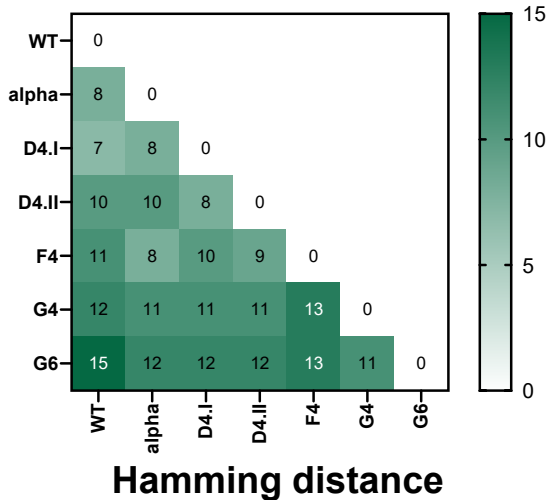
Characterising novel compatible origins

- Five origins selected for further characterisation
 - Based on sequence diversity
- Pairwise compatibility
 - Incompatibility taken from fluorescence outliers
 - **Not symmetric**

3rd dilution							
M9		pGFP(TetA)					
		WT	D4 I	G6	D4 II	F4	G4
pTetR	WT		21699	21105	22897	15320	16763
	D4 I	20591	574962	17190	17078	17176	18047
	G6	27180	213569	796289	22905	14470	21593
	D4 II	22785	64041	11803	339829	21765	23394
	F4	22291	45029	11608	24051	249330	17521
	G4	25794	70443	1119908	31474	149623	286265

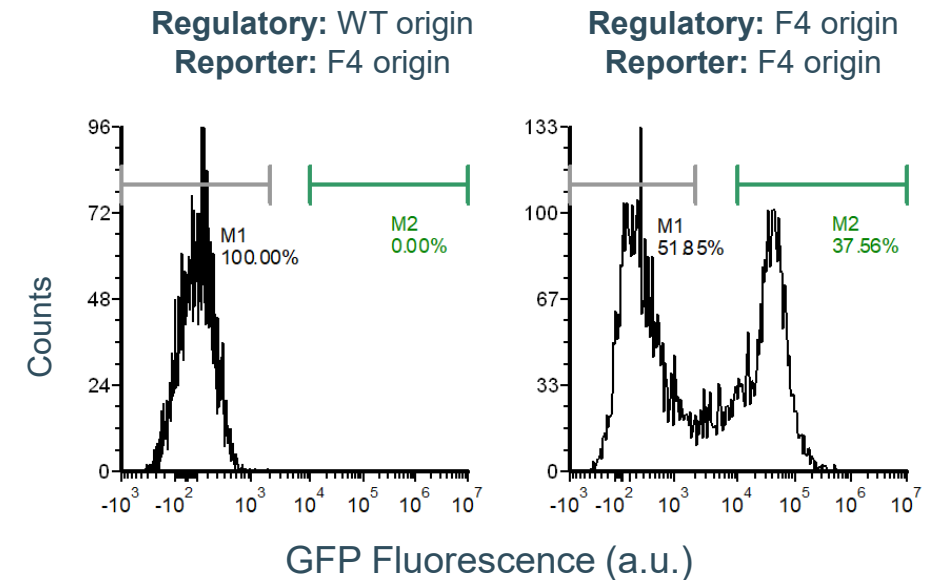
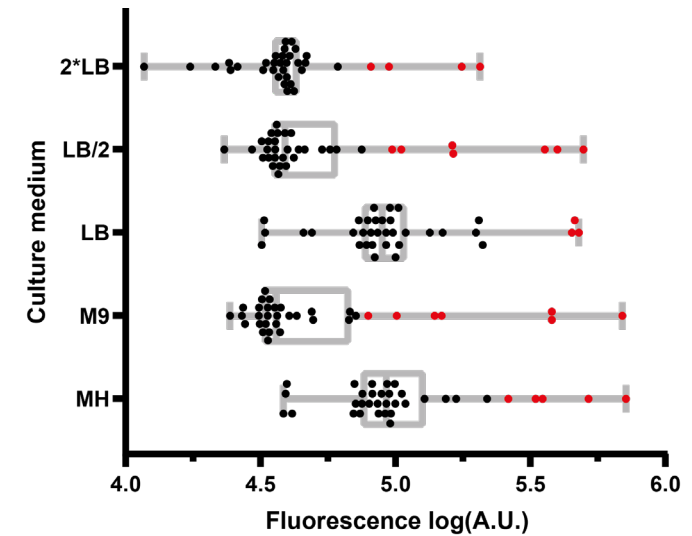
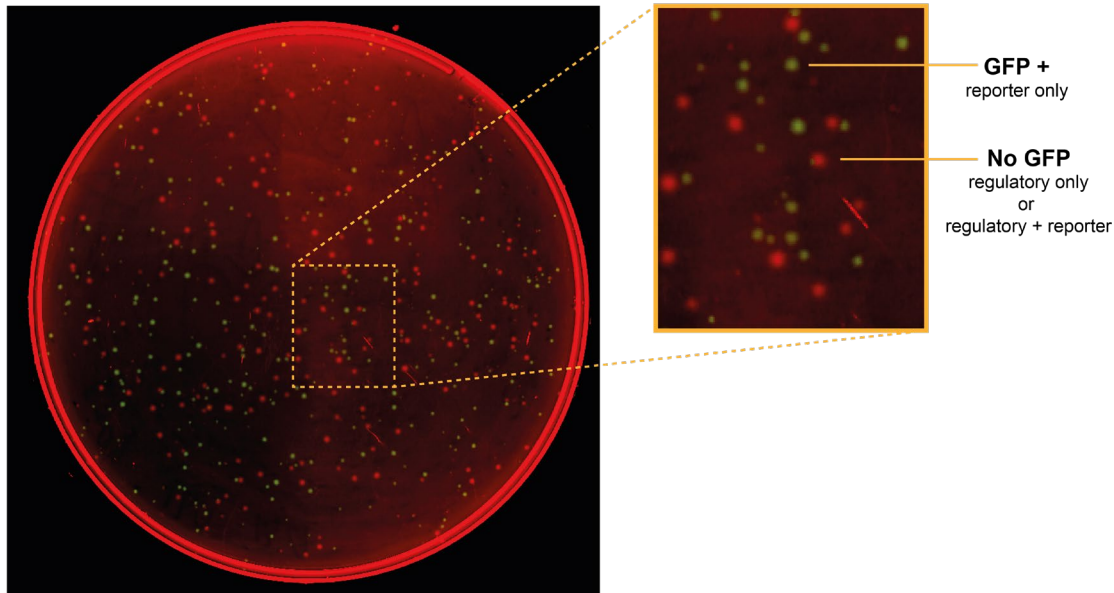
pGFP(TetA) positive control	738296
pTetR negative control	22328

Variant ID	Hairpin 1	Hairpin 2	Hairpin 3
Library #1	CCA-CCG CNNNN AGCGG	AGAG CNNNN AACTCT	GGCT KNNNNN KAGCGCAGATACC-AAATACTG
colE1-pET-29a(wt)	CCA-CCG CTACC AGCGG	AGAG CTACCA ACTCT	GGCT TCAGCAG AGCGCAGATACC-AAATACTG
D4.I	CCA-CCGC CTAG AGCGG	AGAG CGAC AACTCT	GGCT TTC GCAGAGCGCAGATACC-AAATACTG
D4.II	CCA-CCGCT TTGA -CGG	AGAG CGGT AACTCT	GGCT GAC GCAGAGCGCAGATACC-AAATACTG
F4.I	CCA-CCGC GCGT AGCGG	AGAG CAGAGA ACTCT	GGCT GATG CAGAGCGCAGATACC-AAATACTG
G4.I	CCA-CCG TGTCGAT CGG	AGAG ACCTA ACTCT	GGCT TTGGTG GAGCGCAGATACC-AAATACTG
G6.I	CCA-CCGC GGTG AGCGG	AGAG GGTA AACTCT	GG GCTGGTG GAGCGCAGATACC-AAATACTG



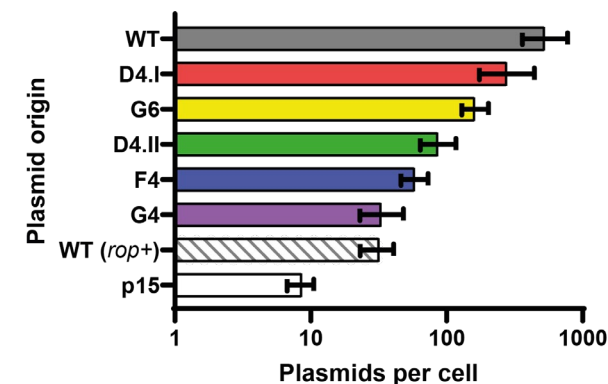
Directional orthogonality

- Rule out assay (or conditions) as source
 - Culture medium has an impact on the determination of outliers – **dynamic and static** components.
 - Assay is a cellular response
 - Classic approach comparison



Directional orthogonality

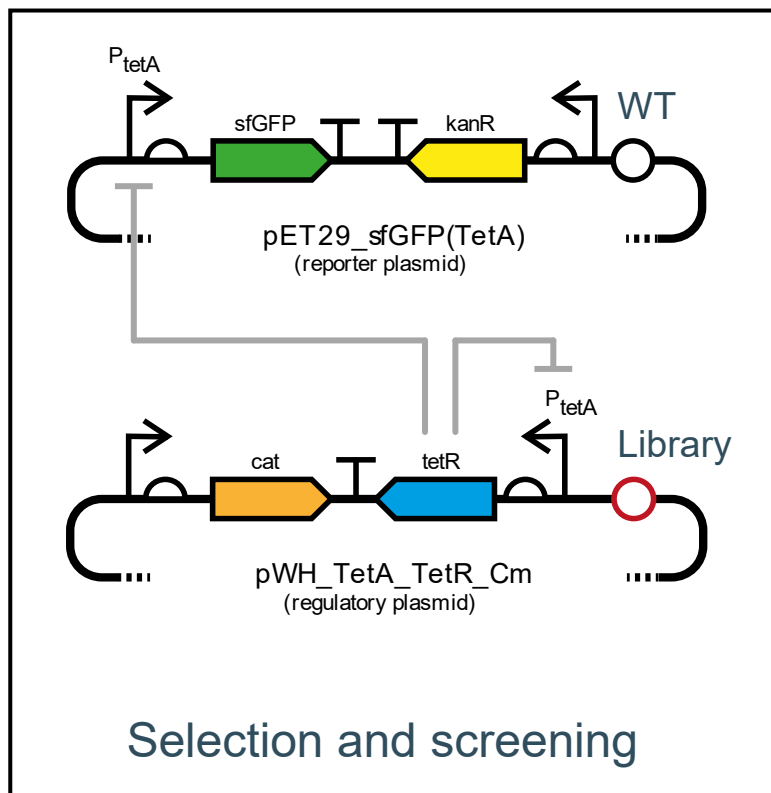
- Circuit-based assay is robust – **tractable**
- Plasmid copy number
 - Significant differences could explain symmetry break
 - **Not sufficient** – plasmid compatibility captures more complex behaviour
 - Complex behaviour is **circuit dependent**
- **Compatible with orthogonality being a continuous measure**



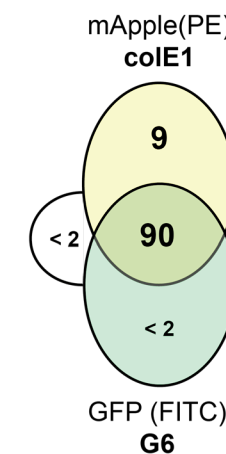
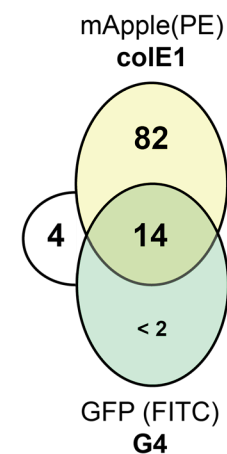
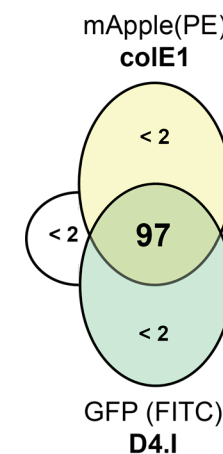
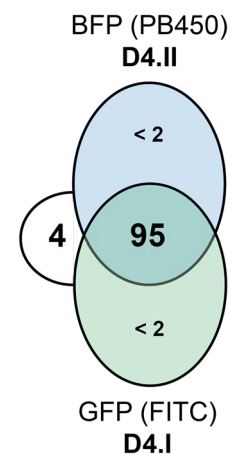
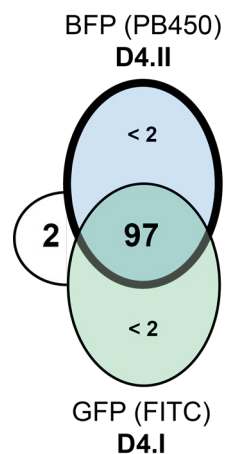
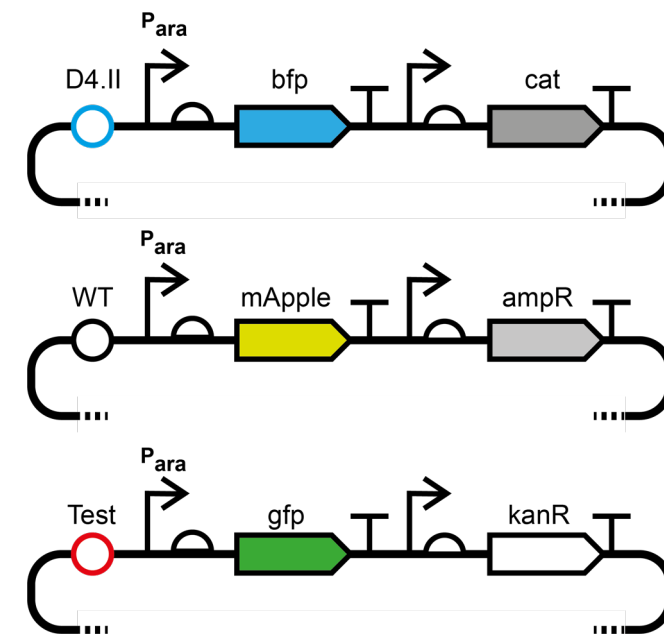
3rd dilution							
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pGFP(TetA) positive control	738296
pTetR negative control	22328

Circuit-dependent plasmid compatibility

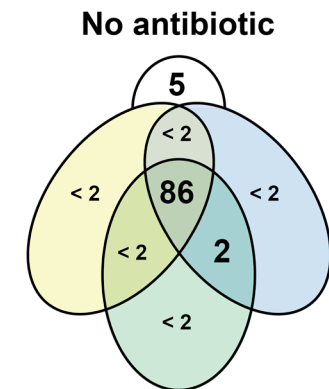
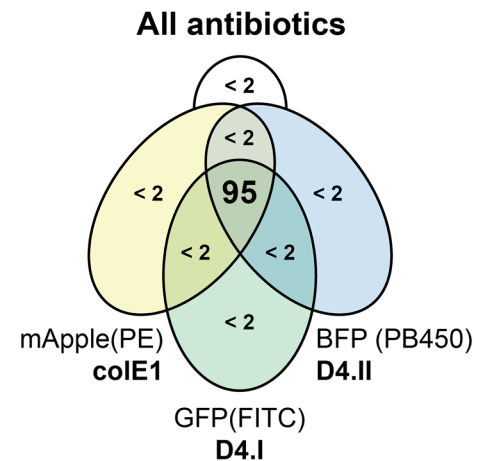
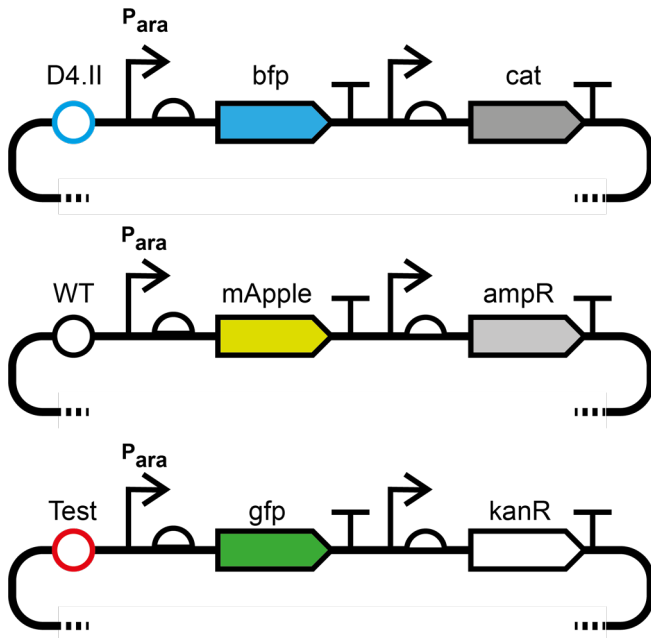


M9		WT	D4 I
		WT	D4 I
pTetR	WT		21699
	D4 I	20591	574962
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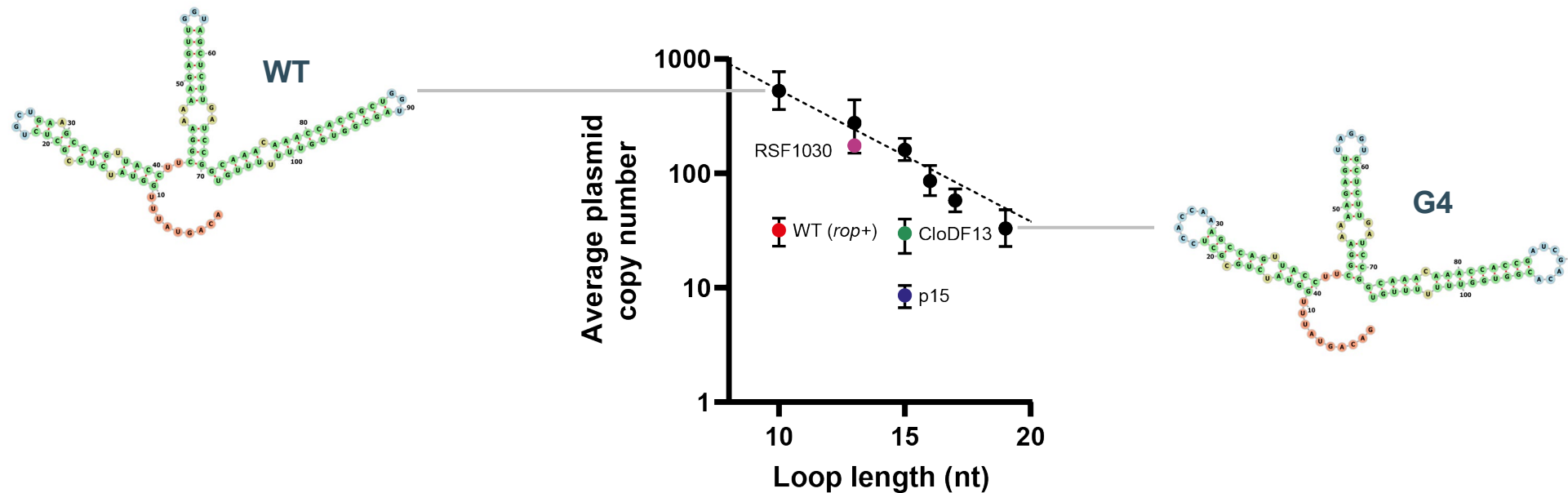
Plasmid cross-compatibility

- Pairwise compatibility is a good guide of cross-compatibility
- Compatibility affected by antibiotic selection - **tunable**



Potential engineering insight into plasmid copy number control

- Good correlation between total RNAI loop length and plasmid copy number
- This in addition to:
 - Regulation of RNAI and RNAII promoters
 - Rop regulation



Key points and acknowledgements

- Orthogonality is a continuum
 - Not necessarily linked to metabolic burden
- Plasmid compatibility is a **fast tractable tunable** model for orthogonality
 - Well suited to improve the biological definition
 - If complexity is tractable
- Analysis tools are well established and can benefit from further developments
- Novel origins can be harnessed for applications
 - Multiple compatible high copy number origins
 - Programmed plasmid loss
 - Optimization of copy number



- Eleftheria Stamou
- Santiago Chaillou
- Leticia Torres
- Ana B. Riesco
- Waren Hazelton
- Pinheiro Group

