Engineering live biotherapeutics: approaches, challenges and applications

Brandon Childers [1], Mary O'Connell-Motherway [2], Constantine Stavropoulos [1], Joshua Corkhill [1], Christina Sparre [1], Lorenzo Pavarini [1], Emma Raftis [1], Imke Mulder [1] and Delphine Lauté-Caly [1]

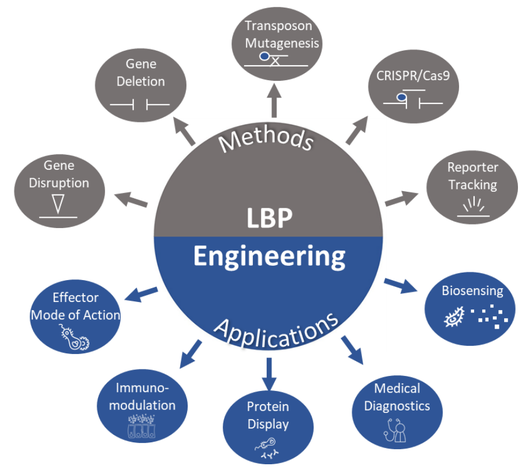
[1] 4D Pharma Research Ltd, Life Sciences Innovation Building, Cornhill Road, AB25 2SZ, Aberdeen, UK
[2] APC Microbiome Ireland, University College Cork, Western Road, Cork, Ireland

4D pharma PLC is a pharmaceutical company focussed on developing live biotherapeutic products (LBPs) from the human gut microbiome. LBPs represent a new class of drugs that contain live organisms for the prevention, treatment or cure of disease. 4D Pharma currently has clinical stage programmes in cancer, asthma, airway hyper-responsiveness in Covid-19 and a strong pipeline of pre-clinical programmes in autoimmunity, inflammation, immuno-oncology and CNS diseases.

Introduction

The ability to genetically manipulate LBPs has numerous crucial research applications, from comparative strain characterisation to in-depth dissection of bacterial-host interactions and elucidation of mechanisms of action. Bacterial engineering is therefore a powerful tool for functional characterisation of effector molecules in drug development platforms. Engineering LBPs isolated from the human gut microbiome requires specific customised approaches as selected strains often belong to novel or poorly characterised species. Human isolates are often fastidious and can be recalcitrant to genetic manipulation. Key considerations for engineering LBPs and developing tailored methods and tools include choice of transformation methods, plasmid elements and strain characteristics such as the presence of native plasmids or restriction-modification systems.

LBP engineering



Genetic tractability of LBPs

Strain-specific tool development

- Strain characteristics (ATB resistance, native plasmids, restriction modification systems, etc.) are considered to identify suitable methods and plasmids (Fig. 1).
- Custom shuttle vectors are generated, containing origin(s) of replication and a suitable antibiotic resistance marker. An origin of transfer is added for conjugation experiments (Fig. 1).
- The choice of replication origin can have a noticeable impact on transformation efficiency. As an example, *Lactobacillus* strains showed higher electroporation efficiencies using the *Lactococcus lactis* origin or replication pSH71 (Fig. 2).

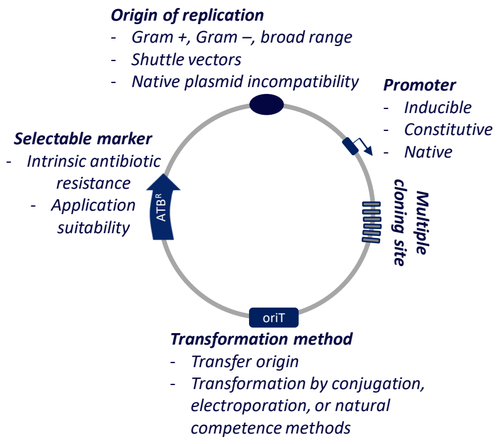


Figure 1. Plasmid schematic

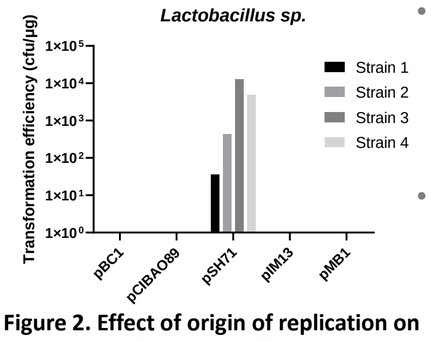


Figure 2. Effect of origin of replication on transformation efficiency

- Several transformation techniques can be tested to increase the chances of gaining genetic tractability.
- Conjugation was more successful to transform *Clostridium* strains than electroporation (Fig. 3).

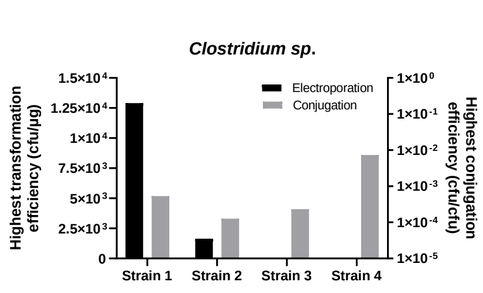


Figure 3. Effect of transformation method on transformation efficiency

Engineering of recalcitrant species

- To establish if a *Bifidobacterium breve* strain was genetically accessible, we performed transformation experiments with five plasmids with different replication origins and antibiotic resistance cassettes (Table 1).

Table 1. List of plasmids tested

Plasmid	Origin of replication	Antibiotic resistance
pNZ8048	SH71	Chloramphenicol
pNZEm	SH71	Erythromycin
pAM5	pBC1	Tetracycline
pPKCm	pCIBA089	Chloramphenicol
pSKEm	pCIBA089	Erythromycin

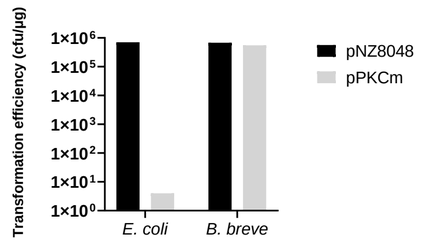


Figure 4. Transformation efficiencies obtained with plasmids propagated in *E. coli* and *B. breve*

- Transformation efficiency increased for pPKCm when the plasmid DNA was isolated from *B. breve* (Fig. 4). This suggested that restriction-modification (RM) systems were impairing the genetic accessibility of this *Bifidobacterium* strain.

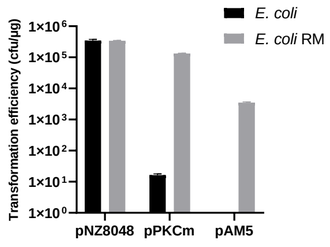


Figure 5. Improvement in transformation efficiencies obtained after propagation in *E. coli* harbouring *B. breve* RM system

- To circumvent this RM, the methylase and specificity genes of the RM system of this *B. breve* strain were cloned in *E. coli.* Methylation of plasmids in the RM-expressing *E. coli* strain allowed higher transformation efficiencies to be achieved (Fig. 5).
- The RM system of a *B. breve* strain was successfully used to propagate plasmids in *E. coli* and optimise transformation efficiency.

Engineering strategies to elucidate mode of action of LBPs

In vivo tracking

- Bioluminescent or fluorescent derivatives can be generated using plasmid-encoded *gfp* or *lux* operon.
- Strains expressing *lux* genes can be tracked by luminescence measurement (Fig. 6).

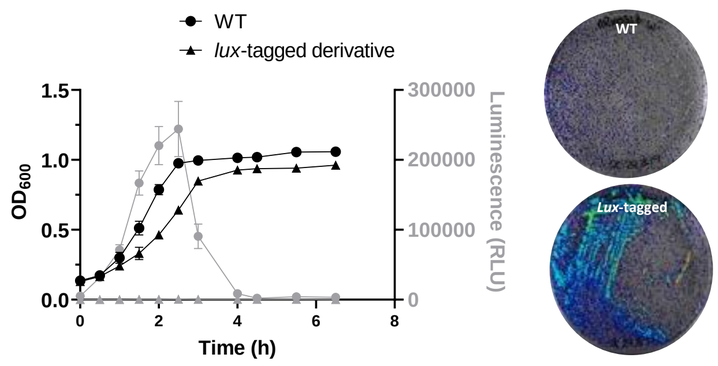


Figure 6. Light emission by a strain harbouring a plasmid-encoded lux operon

- Such derivatives allow the generation of valuable preclinical data through characterisation of LBPs *in vivo* localisation (*e.g.* GI tract compartment, translocation to peripheral organs) and behaviour (survival, colonisation) (Fig. 7).

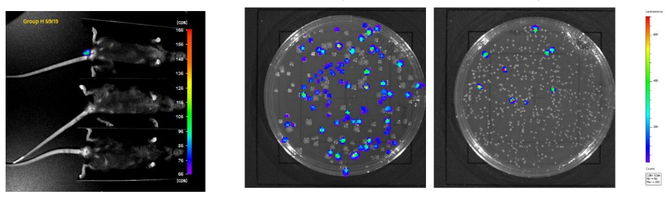


Figure 7. Survival of a *lux*-tagged LBP after transit through SPF mice

Loss of function analysis

- Gene inactivation is a useful technique to elucidate mode of action through loss of function analysis.
- Numerous methods have been developed for gene editing in bacteria, including gene disruption by plasmid insertion and clean deletion through homologous recombination (Fig. 8).

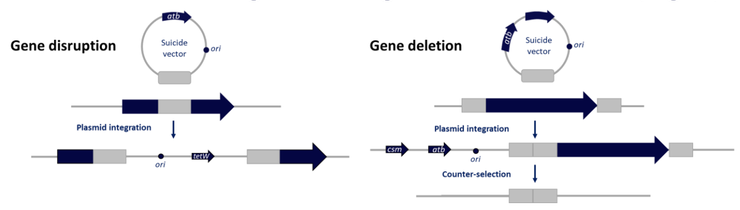
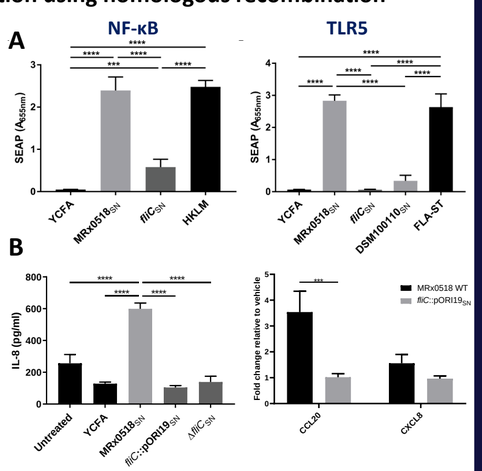


Figure 8. Gene inactivation using homologous recombination

- NFκB and TLR5 signalling (Fig. 9A) and immune stimulation in intestinal epithelial cells (Fig. 9B) were significantly reduced by culture supernatants of a flagellin insertion mutant *fliC*::pORI19 and a flagellin deletion mutant Δ*fliC* in comparison to the wild-type strain.



Lauté-Caly and Raftis *et al.*, 2019, Scientific Reports

Figure 9. Effect of inactivation of the flagellin gene in TLR signalling (A) and immune stimulation (B)

- Flagellin was identified as a key immuno-modulatory protein in *Enterococcus gallinarum* MRx0518.

Potency

- Once a proteinaceous effector has been identified, it can be overexpressed and produced for further characterisation and potency investigation.
- Flagellins from MRx0518 and another highly motile *E. gallinarum* DSM100110 (Fig. 10) were produced using heterologous protein expression in *E. coli* (Fig. 11).

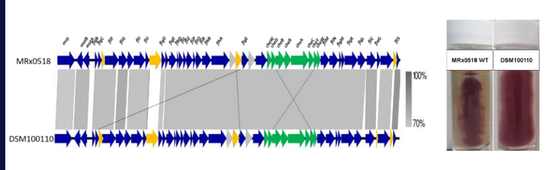


Figure 10. Both *E. gallinarum* strains harbour functional flagella

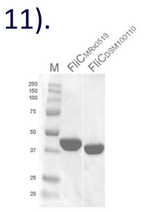
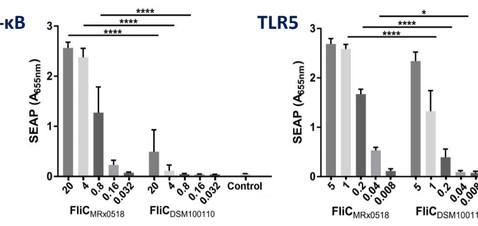


Figure 11. Purified recombinant flagellins

- MRx0518 flagellin was more potent than flagellin from DSM100110 when tested on TLR reporter cells (Fig. 12).



Lauté-Caly and Raftis *et al.*, 2019, Scientific Reports

Figure 12. NFκB and TLR5 response induced by recombinant flagellins

- A high level of diversity within the flagellin sequences of those two strains was demonstrated and prompted us to investigate their implication in the observed potency difference.
- Use of engineering and heterologous gene expression also provide options for further lead optimisation.

Conclusion

Establishing a genetic platform that incorporates tailored strain-specific tools for commensal bacteria is essential to facilitate the development of genetically tractable LBPs. As an example of the application of such methods to effective LBP development, we presented how gene inactivation and heterologous gene expression in *Enterococcus gallinarum* MRx0518, an LBP currently in clinical trials as an immuno-oncology therapeutic, has been successfully used to identify bacterial effector molecules involved in immune stimulation and establish their role in observed therapeutic effects. LBP engineering provides essential information for the development of therapeutic products, including strain characterisation, pharmacodynamics data and lead optimisation.

4D pharma PLC "Developing Science, Delivering Therapies"