Antibiotic-Free Fermentation

Plasmid maintenance without antibiotics or antibiotic resistance genes

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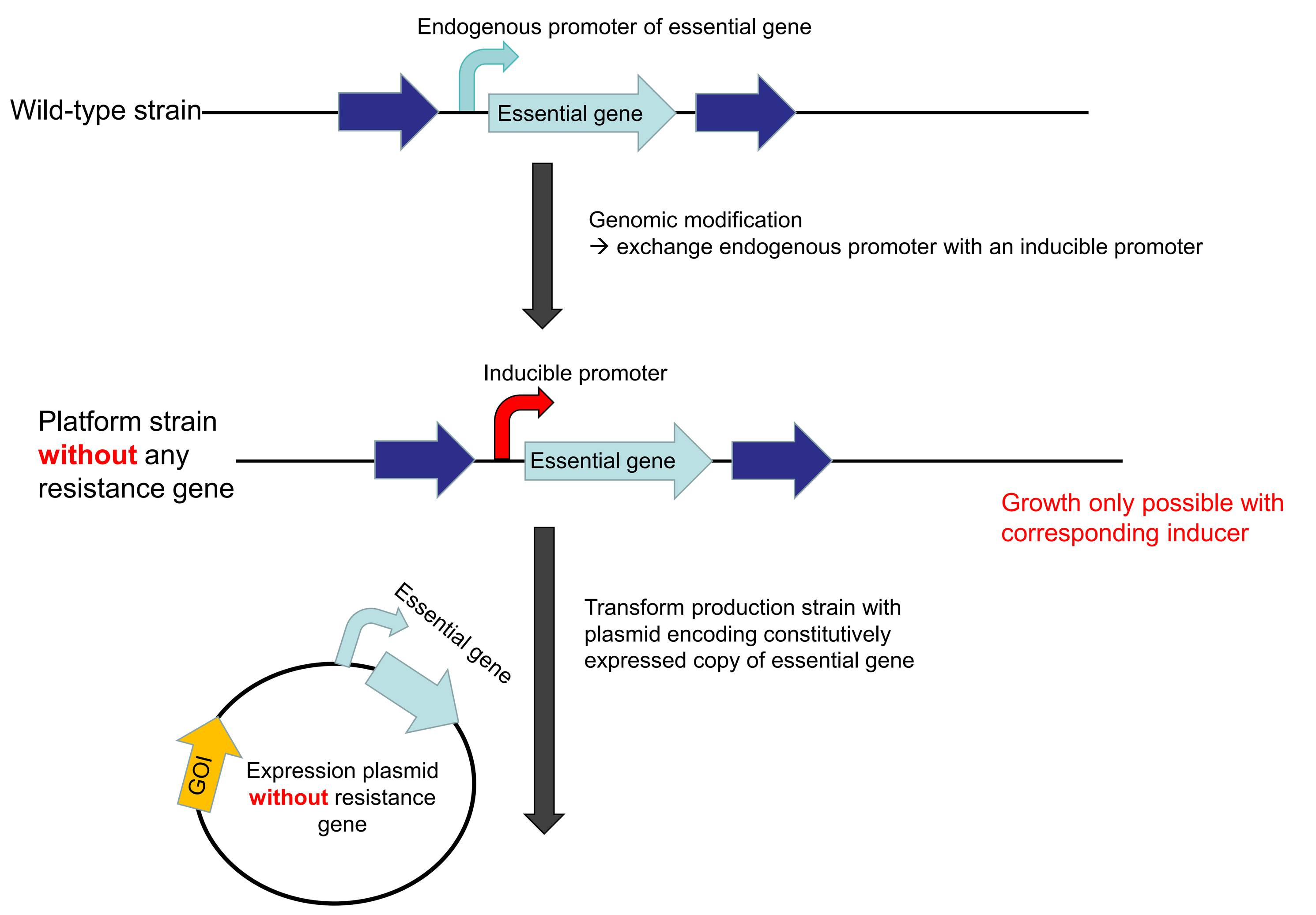
Antibiotic resistance genes used on plasmids for biomanufacturing raise safety concerns and are viewed critically by regulatory authorities. Residual antibiotics in the product could provoke allergic reactions in sensitive individuals [1] and the use of antibiotics could contribute to the development of antimicrobial resistance (AMR) through environmental contamination or horizontal gene transfer. In November 2021, the WHO declared antibiotic resistance to be one of the top 10 global public health threats facing humanity [2]. Eliminating antibiotic use in biomanufacturing would also have economic advantages. Antibiotic use is expensive and necessitates additional analytical and purification steps. Furthermore, antibiotic resistance genes impose a metabolic burden on host cells, decreasing the microbial fermentation capacity. **Gen-H's antibiotic-independent plasmid maintenance system overcomes these disadvantages, providing a simple strategy for antibiotic-free protein expression without any antibiotic resistance genes [3, 4].**

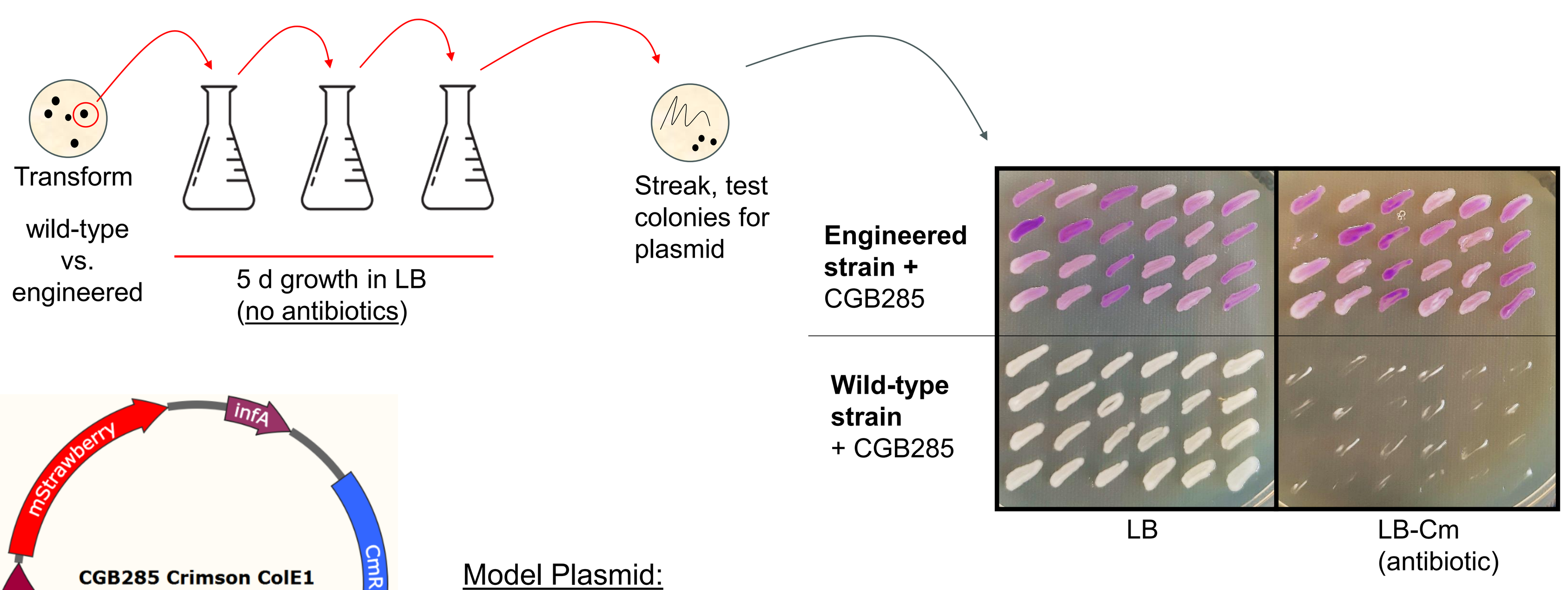
Introduction: Mechanism

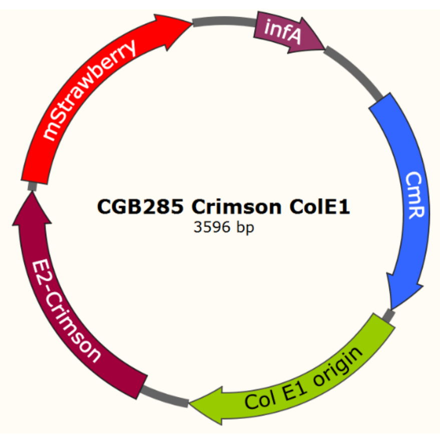
An essential endogenous gene in the genome of the production strain is replaced by an inducible copy of the essential gene. The resulting platform strain only grows with the corresponding inducer. This strain can be transformed with a plasmid that encodes a constitutively expressed copy of the essential gene and the gene(s) of interest (GOI). In the absence of the inducer, the plasmid is essential for growth.



Stable plasmid maintenance: the plasmid is essential for growth in the absence of inducer
No antibiotics or antibiotic resistance genes are needed

Results: Long term antibiotic-free plasmid maintenance

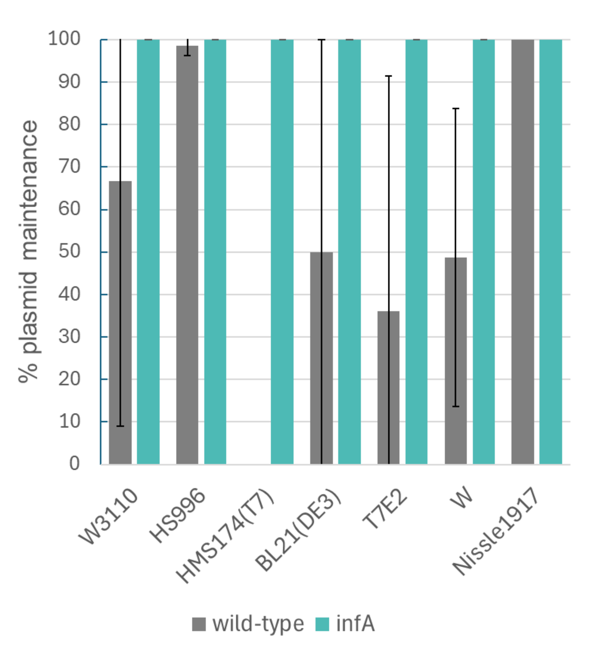




Model Plasmid:
- *inf*A marker
- Cm resistance gene
- chromoprotein

After 5 days growth in non-selective conditions, the wild-type strain showed complete plasmid loss, while **all tested colonies from the engineered strain maintained the plasmid**.

Strict plasmid maintenance in a wide variety of *E. coli* strain backgrounds

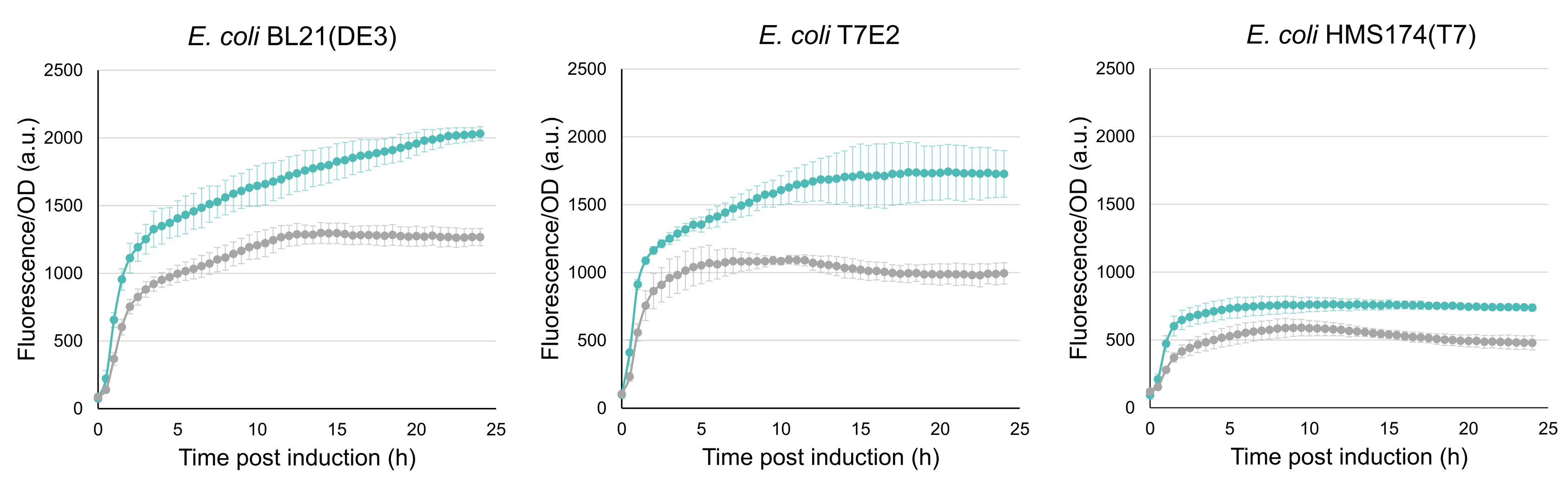


Plasmid maintenance was examined in 7 strain pairs after 24 h growth in LB using the strategy shown above. The wild-type strains showed variable plasmid loss. **All engineered strains maintained the plasmid.**
(3 independent cultures, each with 18 – 24 colonies tested)

Strain	Notes
HS996-*inf*A	K-12 strain, DH10β-derivative, *inf*A-selection
BL21(DE3)-*inf*A	B strain, T7-expression competent, *inf*A-selection
T7E2-*inf*A	B strain, BL21(DE3) with DE3 prophage removed, T7-expression competent, *inf*A-selection [5]
HMS174(T7)-*inf*A	K-12 strain, HMS174(DE3) with DE3 prophage removed, T7-expression competent, *inf*A-selection
W-*inf*A	W strain, sucrose utilizing, *inf*A-selection
W3110-*inf*A	K-12 strain, *inf*A-selection
Nissle1917-*inf*A	Probiotic strain with GRAS status, *inf*A-selection

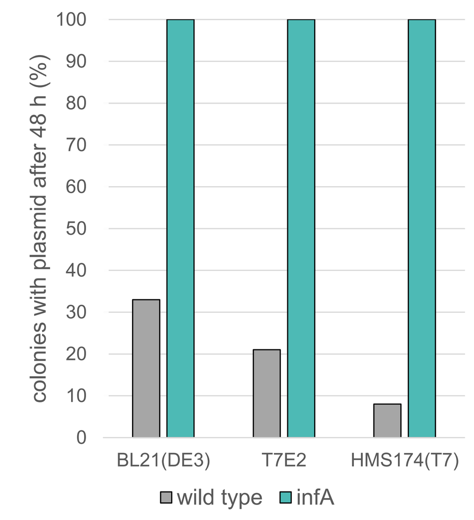
Results: Protein Expression

Protein expression levels were examined using a plasmid expressing GFP from a T7 promoter. A direct comparison was performed with three wild-type and engineered (antibiotic-free) strain pairs (n = 3 biological replicates). Wild-type strains were grown with antibiotic selection pressure (Cm, 30 µg/ml).



The *inf*A-based selection yielded higher expression than the wild-type strains due to plasmid loss in the wild-type strains despite antibiotic-based plasmid selection.

antibiotic-free *infA* selection
wild-type strains with antibiotics



Colonies were isolated after expression and 24 single colonies from each culture were patched on LB and LB-Cm to check for plasmid presence. Significant plasmid loss was observed in the wild-type strains despite antibiotic supplementation.

***infA*-based selection showed higher stability than antibiotic selection**

Protein expression of an enzyme was scaled up to 10,000 L for a commercial fermentation using the *infA* engineered version of BL21(DE3). The scale-up steps were performed in parallel using wild-type BL21(DE3) with antibiotic supplementation (Kan 30 µg/ml) to compare yields and specific activity.

Construct	Geometric Volume	U/mg of total protein *
ARG-free	3 L	13
Standard	3 L	12 ± 3
ARG-free	750 L	20 ± 3
Standard	750 L	14 ± 3
ARG-free	10 m^3	14 ± 3

*The results show data from triplicate fermentations, apart from the 3 L ARG-free fermentation, which was performed only once.

The antibiotic resistance gene (ARG)-free cultures delivered a commercial product with similar yield and specific activity, offering an antibiotic-free alternative.

Conclusions

Plasmid maintenance using *infA*-based selection is compatible with different *E. coli* strain backgrounds and plasmid copy numbers. The engineered strains do not differ significantly in their growth rate or productivity. Furthermore, the high flexibility of this selection method makes it widely applicable; previously established plasmid-based processes can be easily made antibiotic-free using this method.

Summary: Gen-H Antibiotic-free Fermentation System

(EP22160310 and EP21174990)

- **No antibiotic resistance genes** in the strain or the plasmid
- **Flexible:** one strain can be used for various plasmids without helper plasmids or strain modifications
- **Temperature independent**
- **Robust & reliable:** does not rely on a negative selection, essential gene product cannot be scavenged from media
- **No media requirements**
- **Adaptable:** existing strains and processes can be easily converted to be made antibiotic-free

References

[1] Vandermeulen *et al.*, 2011, New Generation of Plasmid Backbones Devoid of Antibiotic Resistance Marker for Gene Therapy Trials; The American Society of Gene & Cell Therapy, 19, 11, 1942
[2] https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance
[3] Brechun, *et al.*, 2024, Method for plasmid-based antibiotic-free fermentation; Microbial Cell Factories, 23(18), doi: 10.1186/s12934-023-02291-z
[4] Brechun, *et al.*, 2026 Inducible complementation for antibiotic-free plasmid-based biomanufacturing in industrially relevant strains; Frontiers in Industrial Microbiology, 3(1725733), doi: 10.3389/finmi.2025.1725733
[5] Noll *et al.*, 2013, Gezielte Optimierung von *Escherichia coli* BL21(DE3); Biospektrum, 19, 211