Recombineering and its application for tailor made production strains

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Gene Bridges GmbH

June 2016
Gene Bridges

- founded in 2000
- EMBL spin-off
- no venture capital
- located at Technology Park Heidelberg
- exclusive rights for commercialization of patents regarding recombination based on λ red genes or recE/recT genes filed by EMBL
Recombineering

Recombination-mediated genetic engineering

- mediated by a protein pair: Redα/β from λ phage or RecE/T from Rac prophage
- linear DNA molecule necessary
- short homology arms from 35 – 50 bp
- no limitation to restriction sites
- no specific recombination sites required
- nucleotide-precise at any desired position
- applicable to any DNA engineering tasks on very large constructs

→ efficient **genetic engineering of *E. coli*** by targeted gene knock-outs or knock-ins and other user defined modifications
Application of recombineering: efficient genetic engineering of *E. coli* → strain development
Production strain improvement: *E. coli* BL21(DE3)
deletion of *E. coli* chromosome region (knock-out)

removal of 89% (= 38 kb) DE3 prophage sequence from *E. coli* chromosome
→ improved safety regarding plaque formation (phage activity)

*E. coli* BL21(DE3) → modified strain

*E. coli* T7E1

Example 1
Production strain improvement: *E. coli* BL21(DE3)

deletion of *E. coli* chromosome region (knock-out)

consistent bacteria growth and heterologous protein expression pattern in comparison to “wildtype” *E. coli* BL21(DE3)

Example 1

![Graph showing protein expression pattern](image)

**E. coli T7E1**
Production strain improvement: \textit{E. coli} BL21(DE3)
gene knock-in in \textit{E. coli} T7E1

gluconoylation
\textit{E. coli} BL21(DE3) is \textit{pgl}-deficient
PGL catalyzes hydrolysis of 6-phosphogluconolactone to 6-phosphogluconate prevents accumulation and random gluconoylation of cellular proteins

Example 2
Production strain improvement: *E. coli* BL21(DE3) gene knock-in in *E. coli* T7E1

gluconoylation
Production strain improvement: *E. coli* BL21(DE3)
gene knock-in in *E. coli* T7E1

integration of *pgl* gene from *E. coli* MG1665 in the genome of *E. coli* T7E1, while simultaneous removing of cryptic Rac prophage

→ no gluconoylation of heterologous expressed protein at all in modified strain *E. coli* T7E2, in contrast to 6.4% gluconoylation of the target protein in wildtype

Noll et al., 2013, Gezielte Optimierung von *Escherichia coli* BL21(DE3), Biospektrum, 19, 211
Production strain improvements: *E. coli* T7E2
gene knock-in: genomic integration of expression cassette
→ genomic expression construct

circular map of the *E. coli* T7 E2 genome and potential expression cassette integration sites
Production strain improvements: *E. coli* T7E2
gene knock-in: genomic integration of expression cassette
→ genomic expression construct

batch fermentation of *E. coli* T7E2
genomic expression constructs

25 mL, 37°C, 225 rpm, + 1mM IPTG
without antibiotics!
Plasmid maintenance without antibiotics
collaboration with University of Stockholm/PlasEX AB
transfer of an essential gene to the plasmid

**conventional**

<table>
<thead>
<tr>
<th>aat</th>
<th>infA</th>
<th>serW</th>
</tr>
</thead>
</table>

**E. coli** BL21(DE3)

<table>
<thead>
<tr>
<th>lacI</th>
<th>ori</th>
<th>pQET7-TNFα</th>
<th>5746 bp</th>
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</thead>
<tbody>
<tr>
<td>pT7</td>
<td>km^R</td>
<td>TFα</td>
<td></td>
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**infA-based**

<table>
<thead>
<tr>
<th>aat</th>
<th>serW</th>
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**E. coli** BL21(DE3) ΔinfA

<table>
<thead>
<tr>
<th>lacI</th>
<th>ori</th>
<th>pQET7-infA-TNFα</th>
<th>5345 bp</th>
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</thead>
<tbody>
<tr>
<td>pT7</td>
<td>km^R</td>
<td>TFα</td>
<td></td>
</tr>
</tbody>
</table>

infA coding for translation factor 1, IF1

Plasmid maintenance without antibiotics

collaboration with University of Stockholm/PlasEX AB

favorable from cost and safety perspectives

applicable in defined and broth media; no risk of cross-feeding
Plasmid maintenance without antibiotics
collaboration with University of Stockholm/PlasEX AB

Example 4

WO2003000881 A3
Pathway engineering: multiple knock-outs

enhancing succinate production

→ improved succinate production up to 47%

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Pathway engineering: genomic knock-ins

promoter fine tuning

replacement of native genomic localized *pgi* promoter (fused to *lacZ*) by a synthetic promoter library:

\[ N_5TTGACAN_{17}TATAATN_5AAATCAGAAGAGTATTGCTAATG \]

→ promoter sequences with 25% – 570% activity of native promoter in blue white screening

Braatsch et al., 2008, *Escherichia coli* strains with promoter libraries constructed by Red/ET recombination pave the way for transcriptional fine-tuning, BioTechniques, 45, 335
Multiple knock-outs: eliminating prophage Mu: approx. 37 kDa

E. coli

E. coli ΔG::km^R

E. coli ΔMu::cm^R

E. coli ΔMu

Lösch et al., 2007, Red/ET Recombination. λ vs. Mu: base-precise modification of the E. coli genome, BIOspektrum
Genomic knock-ins: reporter fusion

in cooperation with Weizmann Institute of Science, Israel

Example 8

Arbel-Goren et al., 2013, Effects of post-transcriptional regulation on phenotypic noise in *Escherichia coli*, Nucl. Acid Res., 9, 4825

Gene Bridges GmbH – Heidelberg, Germany – www.genebridges.com
Other applications 1)

Boston Mountain Biotech, USA

- Lotus® platform, links downstream contaminating host cell proteins (HCPs) to potential upstream cell line modifications
- identifying cellular proteins that exert the greatest burden on downstream processing and then shutting off these genes in the production strain
- Lotus® *E. coli* manufacturing platform: “Separatome-based protein expression and purification platform” (US8927231), cataloged *E. coli*’s separatome collection of proteins that bind to the purification column, and subsequent knock-out of the corresponding genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>IS</th>
<th>E/N</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>hldD</td>
<td>0.07259</td>
<td>N</td>
<td>synthesis of ADP-heptose precursor of core LPS</td>
</tr>
<tr>
<td>usg</td>
<td>0.01034</td>
<td>N</td>
<td>unknown</td>
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<tr>
<td>rraA</td>
<td>0.00928</td>
<td>N</td>
<td>inhibits RNase E</td>
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<tr>
<td>rpoB</td>
<td>0.00876</td>
<td>E</td>
<td>RNA polymerase, β subunit</td>
</tr>
<tr>
<td>rpoC</td>
<td>0.00811</td>
<td>E</td>
<td>RNA polymerase, β' subunit</td>
</tr>
<tr>
<td>tufA</td>
<td>0.00738</td>
<td>E</td>
<td>elongation factor Tu</td>
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<tr>
<td>cubA</td>
<td>0.00736</td>
<td>N</td>
<td>copper binding protein</td>
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<tr>
<td>ptsI</td>
<td>0.00724</td>
<td>E</td>
<td>PTS enzyme I</td>
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<tr>
<td>nagD</td>
<td>0.00661</td>
<td>N</td>
<td>UMP phosphatase</td>
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<tr>
<td>ycfD</td>
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<td>E</td>
<td>ribosomal protein-arginine oxygenase</td>
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<td>speA</td>
<td>0.00589</td>
<td>N</td>
<td>arginine decarboxylase, biosynthetic</td>
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<tr>
<td>gltA</td>
<td>0.00550</td>
<td>N</td>
<td>L-1,2-propanediol dehydrogenase / glycerol dehydrogenase</td>
</tr>
</tbody>
</table>

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Other applications 2)

**BAC-based Expression System Technology** in mammalian cells (BESTcell™)

stable mammalian cells for bio-production (The Antibody Lab GmbH, Austria)

- reliable high yield expression
- shorten timeline to stable clones in comparison to classical methods
- utilizing BACs as carriers
  - expression independent of the integration site in the host chromatin
  - expression dependent on the copy numbers integrated
  - high levels of expression
  - expression should be stable over time

Gene Bridges recombineering technology and experience enables a defined and rapid access to any chromosomal and plasmid modification for your strain optimization.

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