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Technical Report

A simple and reliable method to conduct and monitor expression cassette integration into the *Escherichia coli* chromosome

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We report a method for the integration of expression cassettes into the *Escherichia coli* chromosome using rare and dispensable sugar degradation gene loci as sites for integration. Clones carrying successfully recombined DNA fragments in the chromosome are easily screened using a solid differential medium containing the respective sugar compound. As an example for the heterologous expression of a complex natural product biosynthesis pathway, we show the stepwise chromosomal integration of the zeaxanthin biosynthesis pathway from *Pantoea ananatis* into *E. coli*.

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1 Introduction

The introduction of new genes into a host organism or increasing activities of genes is an important assignment in metabolic engineering. The advantage of using plasmids to express recombinant genes lies in the increased copy number. Thus, plasmids may be the best choice for the cloning and short-term expression of recombinant genes, in particular for the maximum overproduction of a given protein. Especially in metabolic engineering applications, however, a too strong gene expression may be unfavorable for long-term productivity [1]. To overcome this problem, low copy number plasmids have been used. Still, these recombinant plasmids tend to structural instability, and selection markers (*e.g.*, antibiotic resistance genes or plasmid addiction systems) are required to maintain the vectors in the host cell [2]. This procedure leads to the growth inhibition or killing of plasmid-free cells, but it does not avoid segregational instability of plasmids. Stable and selection marker-free maintenance of recombinant genes may be accomplished by integration into the chromosome replicon of a host. For *E. coli*, various methods have been developed for the modification of bacterial chromosomal DNA based on recombination mediated by the λ-Red- or the *recET* recombinase system [3–5]. These methodologies have mainly been used for fast disruption (knockout) of chromosomal genes in *E. coli* and in other bacteria like *Salmonella, Pantoea,* or *Haemophilus* [6–8]. Beside the specific gene deletion, this recombinase system has further been used, among others, for the integration of heterologous genes (knockin) into *E. coli* vectors [9, 10], for the replacement of chromosomal promoters in *E. coli* [11], or in combination with a *rpsL* counter selection for the introduction of single point mutations into *E. coli* [12].

Here we describe a simple method for the stable chromosomal integration of PCR products carrying full expression cassettes for the moderate production of heterologous proteins in *E. coli*. As *E. coli* harbors a variety of rare and dispensable sugar degradation genes, which are not relevant for most industrial applications, these loci can be used for site-specific integration of recombinant genes. To highlight this, we consecutively integrated the carotenoid biosynthesis pathway genes *crtE*, *crtB*, *crtI*, *crtY*, and *crtZ* from *Pantoea ananatis* into an *E.*

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Abbreviation: FRT, FLP recognition target

Table 1. Strains and plasmids

Strains	Relevant genotype or sequence	Source
E. coli DH5α	F⁻, ϕ80d, *lacZ*ΔM15, *endA*1, *recA*1, *hsdR*17($r_K^-m_K^-$), *supE*44, *thi*-1, *gyrA*96, *relA*1, Δ(*lacZYA-argF*)U169	Laboratory strain
E. coli BW25113	*lacI*, $rrnB_{T14}$, $\Delta lacZ_{WJ16}$, *hsdR514*, $\Delta araBAD_{AH33}$, $\Delta rhaBAD_{LD78}$	[3]
E. coli BW25113 *Lac*⁺	$rrnB_{T14}$, *hsdR514*, $\Delta araBAD_{AH33}$, $\Delta rhaBAD_{LD78}$	This study
E. coli BW-E	*E. coli* BW25113 *lac*⁺, *malEFG::crtE-FRT-cat-FRT*	This study
E. coli BW-BE	*E. coli* BW25113 *lac*⁺, *malEFG::crtE, fucIK ::crtB*	This study
E. coli BW-BIE	*E. coli* BW25113 *lac*⁺, *malEFG::crtE, fucIK ::crtB, xylAB::crtI-FRT-cat-FRT*	This study
E. coli BW-CARO	*E. coli* BW25113, *malEFG::crtE, fucIK ::crtB, xylAB::crtI, lacZYA::crtY*	This study
E. coli BW-ZEA	*E. coli* BW25113, *malEFG::crtE, fucIK ::crtB, xylAB::crtI, lacZYA::crtY, melAB::crtZ- FRT-kan-FRT*	This study
Plasmids		
pKD46	P_{araB} γ β exo (red recombinase), Amp^R	[3]
pKD3	FRT-*cat*-FRT, PS1, PS2, oriR6K, Amp^R, Cm^R	[3]
pKD4	FRT-*kan*-FRT, PS1, PS2, oriR6K, Amp^R, Km^R	[3]
pCP20	FLP^+, λ $cI857^+$, λ p_R Rep^{ts}, Amp^R, Cm^R	[16]
pCAR25	pUC19, *P. ananatis crtEXYIBZ* gene cluster	[13]
pJF119ΔN	cloning vector, RBS, P_{tac}, Amp^R	[14]
pCAS30-FRT-*cat*-FRT	pJF119ΔN, *P. ananatis crtE* gene, FRT-sites, Amp^R, Cm^R	[15]
pJF119-*crtB*-FRT-*kan*-FRT	pJF119ΔN, *P. ananatis crtB* gene, FRT-sites, Amp^R, Km^R	This study
pJF119-*crtI*-FRT-*cat*-FRT	pJF119ΔN, *P. ananatis crtI* gene, FRT-sites, Amp^R, Cm^R	This study
pJF119-*crtY*-FRT-*kan*-FRT	pJF119ΔN, *P. ananatis crtY* gene, FRT-sites, Amp^R, Km^R	This study
pJF119-*crtZ*-FRT-*kan*-FRT	pJF119ΔN, *P. ananatis crtZ* gene, FRT-sites, Amp^R, Km^R	This study

coli host strain (BW25113 Lac⁺) to ultimately come up with a zeaxanthin producer. Formerly, this had been accomplished with recombinant plasmids [13].

2 Material and methods

2.1 Bacterial strains, growth conditions, and media

The bacterial strains used in this study were *Escherichia coli* BW25113, *E. coli* BW25113 lac⁺, and *E. coli* DH5α. If not stated otherwise, strains of *E. coli* were grown at 37°C in LB medium. Antibiotics were used at the following final concentrations: ampicillin 100 μg/mL, chloramphenicol 50 μg/mL, kanamycin 50 μg/mL. Difco MacConkey agar was purchased from Nordwald (Hamburg, Germany). Carotenoid standards were provided by the Institute of Technical Biochemistry, University of Stuttgart. All other chemical and reagents are from Sigma-Aldrich or Roth (Germany).

2.2 Cloning and amplification of *crt*-genes expression cassettes

The genes *crtB, crtI, crtY, crtZ* were PCR amplified from plasmid pCAR25 [13] using oligonucleotide primers listed in Table S1 (Supporting Information). The PCR products of *crtB, crtY, and crtZ* were treated each with *Nde*I and *Bam*HI restriction enzymes and were ligated into the *Nde*I/*Bam*HI-treated vector pJF119ΔN [14], respectively. The PCR product of *crtI* was hydrolyzed using *Eco*RI and *Bgl*II restriction enzymes and subsequently ligated into *Eco*RI/*Bam*HI-hydrolyzed pJF119ΔN. The products of ligation were transformed into *E. coli* DH5α and the resulting plasmids were verified by DNA sequencing. Each plasmid (pJF119ΔN::*crtB*, pJF119ΔN::*crtI*, pJF119ΔN::*crtY*, and pJF119ΔN::*crtZ*) was treated by *Hin*dIII and ligated to a likewise treated FLP recognition target (FRT)-*cat*-FRT PCR fragment (in case of pJF119ΔN::*crtI*) or FRT-*kan*-FRT PCR fragment in case of pJF119ΔN::*crtB,Y,or Z)*. The FRT-*cat*-FRT PCR product derived from pKD3 and the FRT-*kan*-FRT PCR product derived from pKD4 as template using primer PKD34rrn and PKD34oriR6 for amplification (see Supporting Information, Table S1).

The individual expression cassettes of each *crt*-gene from plasmid pCAS30-FRT-*cat*-FRT, [15], pJF119-*crtB*-FRT-*kan*-FRT, pJF119-*crtI*-FRT-*cat*-FRT, pJF119-*crtY*-FRT-*kan*-FRT, and pJF119-*crtZ*-FRT-*kan*-FRT (see Table 1), respectively, was amplified by PCR. The oligonucleotide primers are shown in Table S1. PCR conditions were: DNA denaturation (95°C, 40 s); primer annealing (50°C, 30 s); polymerization (72°C, 60 s/kb); 30 cycles; DNA-polymerase (proofreading PrimeStar-poly-

merase (Lonza, Verviers, Belgium). PCR products were column purified (PCR-purification-kit; Qiagen, Germany), treated with *Dpn*I restriction enzyme, and subsequently purified from agarose gel (0.8%) (gel extraction kit, Qiagen).

2.3 Chromosomal integration

For integration of the expression cassettes the purified PCR products were each transformed into *E. coli* BW25113 and its variant strains carrying plasmid pKD46. Expression of the λ-Red enzymes and the preparation of competent cells were carried out as described previously [3]. Competent cells were electroporated with 0.2–0.4 μg PCR product. After electroporation the cells were resuspended in 1 mL LB medium and incubated at 30°C over 12 h with shaking. Subsequently, the cell suspension was spread onto MacConkey agar plates containing antibiotic (kanamycin or chloramphenicol) and 1% sugar (maltose, L-fucose, D-xylose, lactose, or melibiose), corresponding to the targeted genes responsible for sugar degradation. MacConkey agar plates were incubated overnight at 37°C. Five to ten pale colonies per transformation were picked and checked regarding the correct recombination event by PCR (data not shown). The antibiotic resistance cassette was eliminated using the plasmid pCP20 as previously described [16].

2.4 Extraction and analysis of carotenoids from *E. coli* cultures

E. coli strains carrying chromosomal integrated *crt*-genes were cultivated in LB medium. Samples (1 mL) were withdrawn from cultures after 24 h. The cells were harvested by centrifugation, washed with cold water, and subsequently extracted with acetone (500 μL) for 15 min at 50°C. Insoluble components of the extract were removed by centrifugation (20 000 × *g*). HPLC analysis were performed on Dionex HPLC Instrument (Idstein, Germany), installed with a Chromeleon Software, Gina autosampler, P580 pumps, and a detector with UV lamp. Products were analyzed by loading 20 μL of the supernatant onto a C30-reverse-phase HPLC column (250 mm × 4.6 mm, 5 μm, YMC-Europa GmbH, Dinslaken, Germany) attached with a guard column containing matrix of same material as the column. Solvent flow rate of 1.0 mL/min was used. The solvents used were Solvent A consisting of MTBE/methanol/water (19:80:1) and Solvent B consisting of MTBE/methanol/water (90:9:1). Gradient conditions were equilibration in 70% B and then 0–30 min linear gradient from 70% B to 100% B.

3 Results

3.1 Construction of integrable DNA fragments

Each single *P. ananatis* gene needed for the heterologous biosynthesis of zeaxanthin in *E. coli* (*crtE, crtB, crtI, crtY, crtZ*) was first cloned into the expression vector pJF119ΔN. To allow transient selection for chromosomal integration, the cloned genes were hooked up to a downstream antibiotic resistance cassette (Kan^R or Cm^R). To ease the subcloning, *Hin*dIII restriction sites had been introduced into these resistance gene cassettes, which also carried flanking FRT sites. The gained plasmids (Table 1) each contained an expression cassette including P_{tac}-promoter, Shine-Dalgarno se-

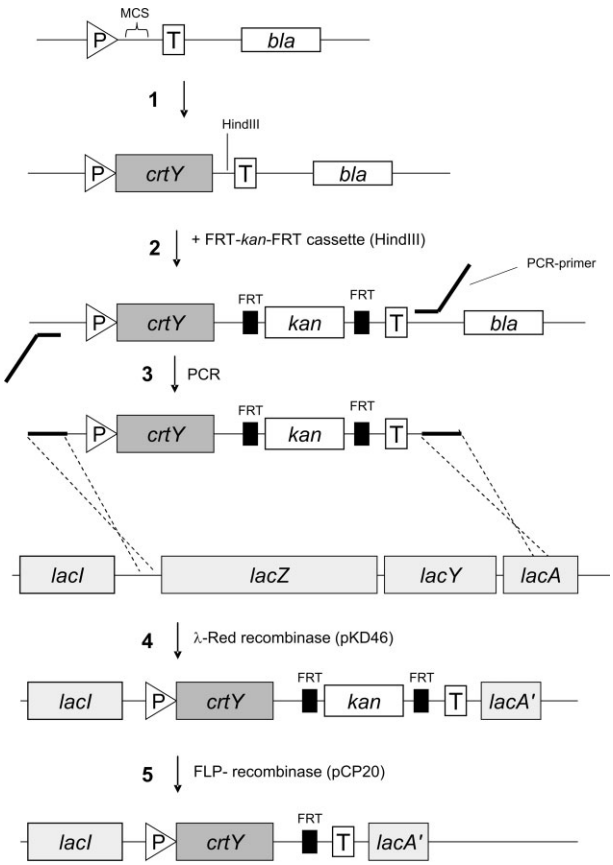

Figure 1. Integration strategy for the introduction of heterologous gene expression cassettes into the chromosome of *E. coli*. Step 1: Cloning of a given gene (e.g., *crtY*) into a standard *E. coli* expression vector (e.g., pJF119ΔN); step 2: cloning of a FRT-*kan*-FRT (*Hin*dIII) fragment downstream of the heterologous gene; step 3: PCR amplification of the expression cassette using extended primer with 50–60 bp homology arms; step 4: transformation of the PCR product in *E. coli* expressing λ-red recombination enzymes (pKD46) and replacement of sugar degradation genes (e.g., *lacZYA*) by homologous recombination, screening of pale colonies on MacConkey agar plates; step 5: elimination of the resistance marker using Flip recombinase (pCP20). (P, promoter; T, transcription terminator; MCS, multiple cloning site).

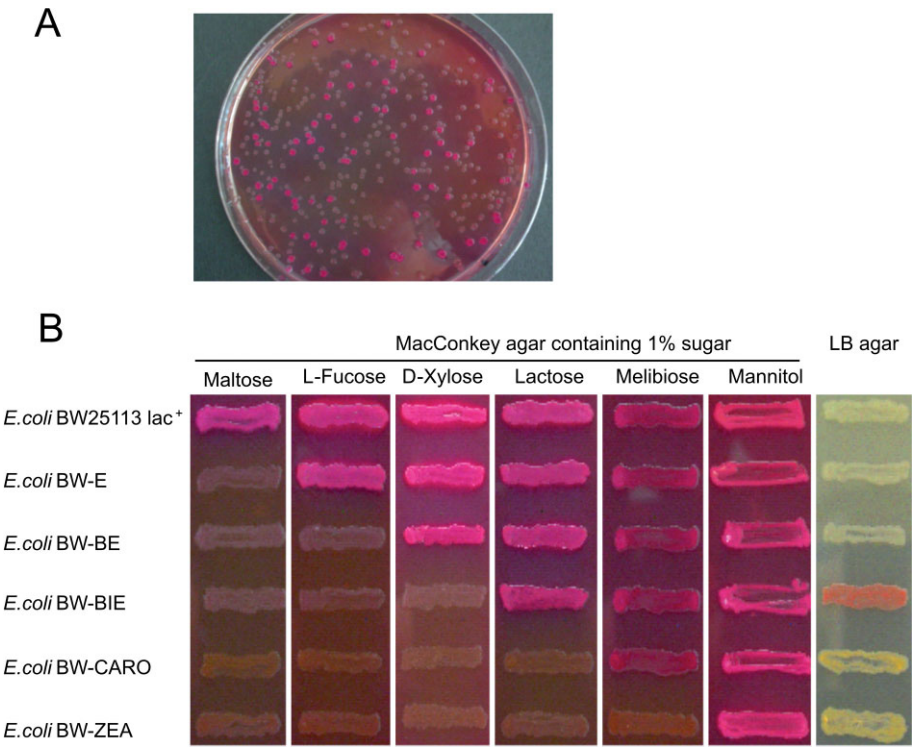

Figure 2. Cultivation of recombinant strains on differential indicator agar plates. (A) MacConkey agar transformation plate (1% D-xylose, 50 μg/mL chloramphenicol) after electroporation of a DNA fragment carrying expression cassette P_{tac}-*crtI*-FRT-*cat*-FRT into *E. coli* BW-BE/ pKD46. (B) Tracing of the chromosomal integration by cultivating engineered *E. coli* strains on pH indicator plates and for carotenoid formation on LB-agar plate (*E. coli* BW-BIE, lycopene; *E. coli* BW-CARO, β-carotene; *E. coli* BW-ZEA, zeaxanthin). Cultivation of recombinant strains on MacConkey agar plates (37°C for 12 h) and on LB agar plate (37°C for 24 h) (as indicated).

quence (SD), the *crt*-gene of interest, FRT-*cat*-FRT or FRT-*kan*-FRT, and a transcription terminator sequence (*rrnB*). The expression cassettes were individually amplified by PCR (Fig. 1) using a 70–80-base oligonucleotide primer, resulting in linear DNA molecules that were flanked by 50–60-nucleotide (nt) terminal extensions with sequence homology to the targeted chromosomal sequence [3].

3.2 Chromosomal integration of cassettes for the expression of heterologous genes

As recipient strain we used a derivative of *E. coli* BW25113 that had been made *lacZ* positive by transduction with a P1-phage lysate grown on wild-type *E. coli* LJ110 [17]. The five PCR products containing the expression cassettes (P_{tac}-SD-*crt* gene-AB^R-*rrnB-term*) were transformed by electroporation into *E. coli* BW25113 Lac^+/pKD46 expressing the λ-Red genes (γ, β, exo). After transformation and phenotypic expression the cell suspension was spread on MacConkey agar plates containing the respective antibiotic (kanamycin or chloramphenicol) and 1% sugar, corresponding to the targeted genes responsible for sugar degradation. After incubation, pale colonies (Fig. 2A) were picked and the correct chromosomal insertion of the respective heterologous expression cassette was verified by PCR analysis. As integration sites, we chose the dispensable sugar degradation markers for maltose, L-fucose, D-xylose, lactose, and melibiose. Thus, we stepwise replaced the genes *malEFG*, *fucPIK*, *xylAB*, *lacZYA*, and *melAB* by an expression cassette containing the respective *crt* gene and the FRT-flanked antibiotic-resistance gene. A visual summary is given in Fig. 2B. Once integration was verified, the FRT-flanked helper gene cassettes *cat* and *kan* were removed using the transiently expressed FLP recombinase from pCP20. The loss of the antibiotic resistance markers was verified by antibiotic sensitivity and by PCR verification. Thus, by knockout and knockin, we first inserted the *crtE*-FRT-*cat*-FRT expression cassette into the maltose locus (*malEFG*) followed by the replacement of *fucPIK* by the *crtB*-FRT-*kan*-FRT cassette. After displacement of the *cat* and *kan* genes, the cleaned antibiotic resistance gene-free strain (*E. coli* BW-BE) was used for integration of further *crt*-expression cassettes by the recombineering method. Insertion of the next expression cassette in line of the zeaxanthin biosynthesis pathway (*crtI*-FRT-*cat*-FRT) into the xylose locus (*xylAB*) yielded strain *E. coli* BW-BIE. In the next round integration of *crtY*-FRT-*kan*-FRT followed by replacing *lacZYA* and subsequent elimination of the antibiotic resistance cassette yielded strain *E. coli* BW-CARO (Table 1). It should be noted that elimination of the resistance cassettes (*kan* or *cat*) with the pCP20 plasmid can be done either at each

step, or by removing two different antibiotic resistance genes at one time. Finally, gene *crtZ* (*crtZ*-FRT-*kan*-FRT) was integrated into the melibiose degradation genes (*melAB*) resulting in strain *E. coli* BW-ZEA (Table 1).

3.3 Extraction and HPLC analysis of carotenoids

The functional expression of the integrated heterologous genes was verified by detecting the respective isoprenoid products. The product of CrtE, geranylgeranyl-pyrophosphate, was shown after extraction from *E. coli* crude extracts as described recently [15]. The recombinant *E. coli* strains BW-BE, BW-BIE, BW-CARO, and BW-ZEA were cultivated in complex medium and carotenoid formation was determined by HPLC analysis (Fig. 3). The recombinant strains (*E. coli* BW-BE, *E. coli* BW-BIE, *E. coli* BW-CARO, and *E. coli* BW-ZEA) showed product formation as expected from their respective genotype [13]. No pathway intermediates were detected underlining that only end products were accumulated and enzyme functions were sufficient. Moreover, the formation of carotenoids by the recombinant strains, especially the red lycopene compound did not negatively interfere with the visual screening on the MacConkey agar plates. The red dye formation of the toluylene red, caused by the acid formation of the cells, was much faster and the dye's color intensity was significantly higher than that of lycopene (Fig. 2).

To check the stability of the recombinant *crt* genes, the strains *E. coli* BW-BIE, *E. coli* BW-CARO, and *E. coli* BW-ZEA, respectively, were repeatedly cultivated on LB-agar medium (>100 generations) as well as grown in large-scale fed-batch cultivation (15 L) with mineral medium (not shown). Successive generations were analyzed concerning the functionality and localization of recombinant genes. In no case did we observed a loss or migration of any of the inserted genes, as verified by PCR and by analysis of the carotenoid product pattern.

4 Discussion

We show a facilitated method by which heterologous genes can be inserted into the chromosome of *E. coli* to create stable production strains, especially for metabolic engineering of multistep heterologous biosynthesis pathways. In theory the recombineering technique allows the insertion of homologous or heterologous genes at any position on the chromosome, unless it would be lethal for the organism [4]. Both the replacement of genes and the insertion in intercistronic regions are possible. Inserted genes should be integrated in a direct way with a negligible physiological effect on the host strain. For *E. coli*, we propose using dispensable genes such as the genes for rare sugar degradation of *E. coli* (>20 operons available [18, 19]), as ideal sites for gene replacements. Their disruption has no primary adverse effects for biotechnological applications. Most remarkably, the loss of these sugar degradation genes and the concomitant insertion of a recombinant DNA fragment can be screened in a precise way using MacConkey agar pH indicator plates supplemented with the respective sugar compound. Screening for pale colonies on the MacConkey plates facilitates the detection of correct inserts, as we noticed that a significant amount of clones (from 20 to 70%) showed a Cm^R or Km^R phenotype that turned red on MacConkey sugar plates (Fig. 2). Such high rates of false-positive clones by

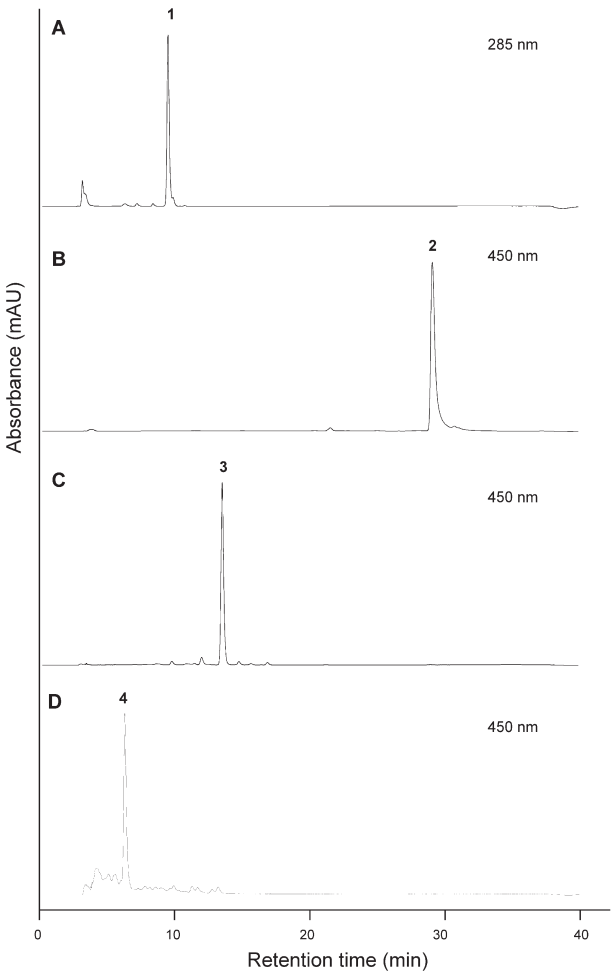

Figure 3. HPLC-analysis of carotenoids in extracts from recombinant E. coli strains. (A) *E. coli* BW-BE; (B) *E. coli* BW-BIE; (C) *E. coli* BW-CARO; (D) *E. coli* BW-ZEA; 1, phytoene; 2, lycopene; 3, β-carotene; 4, zeaxanthin (detection wavelength as indicated). Peaks were identified by co-chromatography with authentic standard compounds.

the λ-Red recombineering system were not observed in approaches such as gene knockout or promoter replacement [3, 11]. These false-positive clones could stem from undesired recombination events mediated by illegitimate cross-overs or the cross-over of homologous DNA segments, *e.g.*, *rrnB*. In our hands, all analyzed pale transformants showed an accurate "knockin by knockout" event in the respective sugar loci.

The zeaxanthin biosynthesis genes were used as proof of principle for this method because the functional gene expression was easily detectable by visual inspection of colony color [lycopene, β-carotene, zeaxanthin (Fig. 2B)], or formation of carotenoids was detected after extraction and HPLC examination (Fig. 3). Low-level expression of *crt* genes from plasmids or after chromosomal integration can lead to a product formation that is at least equal or even increased if compared to high-level overexpression [15, 20–22]. This makes it an interesting example for the advantages of chromosomal integration of heterologous genes. In principle, genes of the same bacterial strain can also be used for defined copy number increases of a gene (our unpublished observations).

Other methods used for the integration of heterologous biosynthesis genes into the *E. coli* chromosome relied on the use of plasmid transformation or utilized the insertion into phage attachment sites (*e.g.*, [20, 23, 24]). While the insertion *via* phage attachment sites is efficient [23], cloning and screening is time consuming compared to the targeting and integration of a linear PCR product into an easily screenable chromosomal locus. Limitations of our method lie, of course, in the reliability and efficiency of the used DNA polymerase that constrict the size of DNA that can be integrated. In our lab, linear DNA fragments up to 6 kb have been successfully integrated so far (data not shown). The abundance of screenable sugar degradation loci in the *E. coli* chromosome enables the integration of multistep biosynthetic pathways, as shown in this work. A further expression cassette, for example of *crtX* (zeaxanthin glucosyltransferase gene [13]), could be integrated, *e.g.*, in the mannitol degradation locus (*mtlAD*) (Fig. 3). Further sugar degradation loci that can be used in the same way of integration, like the series used in this study, are, for example, L-rhamnose (*rhaBAD*), D-galactose (*galK*), D-ribose (*rbsD-K*), L-arabinose (*araBAD*), *N*-acetylglucosamine (*nagAB*), D-allose (*alsB-K*), trehalose (*treBC*), dulcitol (*gatA-D*), sorbitol (*srlA-D*), mannonate (*uxuAB*) and others [18]. So, a stepwise engineering of complex natural product biosynthesis pathways (*e.g.*, antibiotics) in *E. coli* could be achieved. The assembly of a heterologous pathway can either be carried out by a stepwise direct insertion of linear DNA fragments into the same host, as shown in this work, or by a parallel integration approach. In this case *E. coli* strains with only one expression cassette insertion are constructed and the pathway assembly into one host can be achieved by P1 transductions from a sibling strain, whereas the same MacConkey agar screening assays can be used.

If necessary, the expression level of single genes can be adjusted by the use of different promoters by the use of different expression plasmids (promoter exchanges at the chromosomal level have been described recently [11]) or *via* a stepwise gene amplification of the same expression cassette, which could be inserted at two or more chromosomal loci.

In summary, the combination of the λ-Red recombineering system [4] and the precise localization of the recombination event by screening on differential medium demonstrates an efficient methodology of construction of markerless and stable recombinant *E. coli* strains useful for microbial biotechnology applications.

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5 References

[1] Jones, K. L., Kim, S. W., Keasling, J. D., Low-copy plasmids can perform as well as or better than high-copy plasmids for metabolic engineering of bacteria. *Metab. Eng.* 2000, *2*, 328–338.

[2] Friehs, K. Plasmid copy number and plasmid stability. *Adv. Biochem. Eng. Biotechnol.* 2004, *86*, 47–82.

[3] Datsenko, K. A., Wanner, B. L., One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* 2000, 97, 6640–6645.

[4] Sharan, S. K., Thomason, L. C., Kuznetsov S. G., Court, D. L., Recombineering: A homologous recombination-based method of genetic engineering. *Nat. Protoc.* 2009, *4*, 206–223.

[5] Zhang, Y., Buchholz, F., Muyrers, J. P., Stewart, A. F., A new logic for DNA engineering using recombination in *Escherichia coli*. *Nat. Genet.* 1998, *20*, 123–128.

[6] Gerlach, R. G., Jäckel, D., Hölzer, S. U., Hensel, M., Rapid oligonucleotide-based recombineering of the chromosome of *Salmonella enterica*. *Appl. Environ. Microbiol.* 2009, *75*, 1575–1580.

[7] Tracy, E., Ye, F., Baker, B. D., Munson, R. S. Jr., Construction of non-polar mutants in *Haemophilus influenzae* using FLP recombinase technology. *BMC Mol. Biol.* 2008, *9*, 101.

[8] Katashkina, J. I., Hara, Y., Golubeva, L. I., Andreeva, I. G. *et al.,* Use of the n Red-recombineering method for genetic engineering of *Pantoea ananatis*. *BMC Mol. Biol.* 2009, *10*, 34.

[9] Rivero-Müller, A., Lajić, S., Huhtaniemi, I., Assisted large fragment insertion by Red/ET-recombination (ALFIRE)-an alternative and enhanced method for large fragment recombineering. *Nucleic Acids Res.* 2007, *35*, e78.

[10] Wenzel, S. C., Gross, F., Zhang, Y., Fu, J. *et al*., Heterologous expression of a myxobacterial natural products assembly line in pseudomonads via red/ET recombineering. *Chem. Biol.* 2005, *12*, 349–356.

[11] Yuan, L. Z., Rouvière, P. E., Larossa, R. A., Suh, W., Chromosomal promoter replacement of the isoprenoid pathway for enhancing carotenoid production in *E. coli*. *Metab. Eng*. 2006, *8*, 79–90.

[12] Heermann, R., Zeppenfeld, T., Jung, K., Simple generation of site-directed point mutations in *Escherichia coli* chromosome using Red/ET recombination. *Microb. Cell Fact.* 2008, *7*, 14.

[13] Misawa, N., Nakagawa, M., Kobayashi, K., Yamano, S. *et al*., Elucidation of the *Erwinia uredovora* carotenoid biosynthetic pathway by functional analysis of gene products expressed in *Escherichia coli*. *J. Bacteriol*. 1990, *172*, 6704–6712.

[14] Albermann, C., Ghanegaonkar, S., Lemuth, K., Vallon, T. *et al.*, Biosynthesis of the vitamin E compound δ-tocotrienol in recombinant *Escherichia coli* cells. *ChemBioChem* 2008, *9*, 2524–2533.

[15] Vallon, T., Ghanegaonkar, S., Vielhauer, O., Müller, A. *et al.,* Quantitative analysis of isoprenoid diphosphate intermediates in recombinant and wild-type *Escherichia coli* strains. *Appl. Microbiol. Biotechnol*. 2008, *81*, 175–182.

[16] Cherepanov, P. P., Wackernagel, W., Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* 1995, *158*, 9–14.

[17] Zeppenfeld, T., Larisch, C., Lengeler, J. W., Jahreis, K., Glucose transporter mutants of *Escherichia coli* K-12 with changes in substrate recognition of IICB(Glc) and induction behavior of the *ptsG* gene. *J. Bacteriol*. 2000, *182*, 4443–4452.

[18] Lin, E. C. C., Dissimilatory pathways for sugar, polyols, and carboxylates, in: Neidhardt, F. C. (Ed.), *Escherichia coli* and *Salmonella: Cellular and Molecular Biology,* 2nd edn. ASM Press, Washington, D.C. 1996, pp. 307–342.

[19] Herring, C. D., Glasner, J. D., Blattner, F. R., Gene replacement without selection: Regulated suppression of amber mutations in *Escherichia coli*. *Gene* 2003, *311,* 153–163.

[20] Chiang, C. J., Chen, P. T., Chao, Y. P., Replicon-free and markerless methods for genomic insertion of DNAs in phage attachment sites and controlled expression of chromosomal genes in *Escherichia coli*. *Biotechnol. Bioeng*. 2008, *101*, 985–995.

[21] Tao, L., Jackson, R. E., Rouviere, P. E., Cheng, Q., Isolation of chromosomal mutations that affect carotenoid production in *Escherichia coli*: Mutations alter copy number of ColE1-type plasmids. *FEMS Microbiol. Lett.* 2005, *243*, 227–233.

[22] Yoon, S. H., Kim, J. E., Lee, S. H., Park, H. M. *et al.,* Engineering the lycopene synthetic pathway in *E. coli* by comparison of the carotenoid genes of *Pantoea agglomerans* and *Pantoea ananatis*. *Appl. Microbiol. Biotechnol*. 2007, *74*, 131–139.

[23] Minaeva, N. I., Gak, E. R., Zimenkov, D. V., Skorokhodova A. Y. *et al*., Dual-in/out strategy for genes integration into bacterial chromosome: A novel approach to step-by-step construction of plasmid-less marker-less recombinant *E. coli* strains with predesigned genome structure. *BMC Biotechnol*. 2008, *8*, 63.

[24] Snell, K. D., Draths, K. M., Frost, J. W., Synthetic modification of the *Escherichia coli* chromosome: Enhancing the biocatalytic conversion of glucose into aromatic chemicals. *J. Am. Chem. Soc.* 1996, *118*, 5605–5614.