Strategy for chromosomal gene targeting in RecA-deficient *Escherichia coli* strains

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Reengineering DNA by homologous recombination in Escherichia coli often depends on helper functions provided on a temporarily introduced replicon that is subsequently cured from the cells. The suicide vector pKSS offers a new curing strategy. pKSS specifies a variant of phenylalanyl-transfer RNA (tRNA) synthetase conferring relaxed substrate specificity towards phenylalanine analogs that results in their lethal incorporation into cellular proteins. Consequently, the presence of p-chlorophenylalanine selects for strains that have lost pKSS. This principle, in conjunction with a plasmid-borne recA gene, was exploited for targeted chromosomal mutagenesis by double homologous recombination in RecA-negative E. coli strains. Gene replacement with a kanamycin-resistance cassette was possible in a single step by plating on kanamycin and p-chlorophenylalanine agar plates and incubating at 37°C. The presence of the correct chromosomal mutation and the absence of the plasmid were established by several control experiments. A simple screen confirmed the desired resistance phenotype in 44% of the initially selected clones, and 75% of these had the correct genotype.

INTRODUCTION

DNA engineering in *Escherichia coli* by double homologous recombination is a key technology in functional genomics (1). To precisely swap genetic information between two DNA molecules in vivo, scientists have the choice between two fundamentally distinct pathways. The first pathway, termed Red/ET recombination, is mediated either by the RecE/RecT protein pair from phage *Rac* or the Red α /Red β protein pair from phage λ (1–3). The second pathway is based on the RecA function naturally occurring in wild-type *E. coli* cells (4,5).

Red/ET recombination involves transformation of linear DNA containing, for example, an antibiotic resistance marker and short (approximately 50 bp) DNA regions adjacent to the sequence to be targeted. Critical for its success is precise control of phage gene expression. For this reason, the phage genes reside either on a low-copy plasmid (6) or are integrated in the bacterial chromosome (7,8). To subsequently remove the phage genes, either the plasmid has to be cured, which is generally done by using a temperature-sensitive replication origin, or the

integrated genes have to be eliminated by another homologous recombination. While the latter and its verification are very time-consuming, the high temperature selection requirement (up to 43°C) limits plasmid curing to temperature-tolerant strains and precludes the introduction of temperature-sensitive chromosomal lesions.

RecA-dependent homologous recombination strategies also often rely on conditional plasmid replication (4,9,10). For instance, a desired mutation linked to an antibiotic resistance marker on such a plasmid can be chromosomally integrated by establishing conditions incompatible with plasmid replication and simultaneously selecting for the antibiotic resistance. Frequently, a temperature-sensitive derivative of plasmid pSC101 is used (4), which again necessitates selection at elevated temperatures. Suicide plasmids with an *ori*_{R6K}, which only replicate in strains carrying a *pir* gene, are also popular, but critical factors here are the copy number and the need for a Pir⁺ host for plasmid handling (9). Other strategies to select for plasmid loss require specific mutations in a chromosomal gene, for example in *polA* (11) or in *rpsL* (12), which are often undesirable

in a “designer” strain. Alternatively, linear DNA containing the reengineered chromosomal locus can be transformed into exonuclease-deficient strains in which integration into the chromosome can occur by RecA-dependent recombination (13). Drawbacks are low transformation yields (7) and the need for a time-consuming P1 transduction to transfer the chromosomal mutation into the desired host.

In this work, we present an alternative plasmid-curing method to introduce mutations into the *E. coli* chromosome by RecA-mediated homologous recombination, which requires neither selection at 43°C nor specific mutant strains. The method is based on the suicide vector pKSS (14) and allows for the straightforward one-step selection of correct clones. By temporarily providing the RecA function on the suicide replicon (15), chromosomal engineering can be accomplished even in RecA-deficient strains, the recommended choice for stable plasmid cloning.

MATERIALS AND METHODS

Escherichia coli Strains, Plasmids, and Oligonucleotides

The RecA-positive and kanamycin-resistant strain AD202 (16) and RecA-negative strains KA12 (17) and KA13 (18) were described previously. Strains XL1-Blue (Stratagene, La Jolla, CA, USA) and KA13 were used for standard cloning. KA35 (this work) is KA12 $\Delta(dsbaA)::neo$.

Oligonucleotides RECA-5' (5'-GCACCGTCTGGTTTGCTTTT-3') and RECA-3' (5'-CATCAAGCCG-GCTGTCATGGCATATCCTTACAA-3') were synthesized with a Gene Assembler[®] Plus (Amersham Biosciences, Piscataway, NJ, USA). All other oligonucleotides were from Microsynth (Balgach, Switzerland). Plasmids pKSS (14) and pKY102 (19) were described previously. pMG205 was constructed by a four-way ligation including a fragment of pKSS, the two DNA regions (*dsbA1* and *dsbA2*) flanking the gene to be knocked out and the aminoglycoside 3'-phosphotransferase gene (*neo*) responsible for kanamycin resistance

(Km^R). The 2029-bp *dsbA1* fragment was obtained by PCR amplification of chromosomal KA13 DNA with primers DSBA1-5' (5'-GATAATCTAGAG-TAACGGTATCATCGTTTTT-3') and DSBA1-3' (5'-GATAATCGGCC-GTCTTTTTCATGATCTACTCTCT-3'), followed by restriction digestion with *Xba*I and *Eag*I. For the 2071-bp *dsbA2* fragment, primers DSBA2-5' (5'-GATGAATTCGGCCGTAT-GCTGATACAGTAAAATATCT-3') and DSBA2-3' (5'-GATGATCCGCG-GCCTGCATCCCACCACCATT-3') were used followed by restriction digestion with *Sac*II and *Eco*RI. PCR with primers KAN-5' (5'-GATGATCGGCCGGAAGC-CACGTTGTGTCTCAA-3') and KAN-3' (5'-GATGATCGGCCGAATTC-GCCTCGTGAAGAAGGTGTT-3') and chromosomal AD202 DNA as template yielded the 1208-bp *neo* gene fragment after restriction digestion with *Eag*I and *Eco*RI. The three digested PCR products were ligated to the 4117-bp *Xba*I-*Sac*II fragment from pKSS giving pMG205. Insertion of the 1332-bp *Sac*II-*Ngo*MIV *recA*

gene fragment generated after PCR amplification of pKY102 with primers RECA-5' and RECA-3' into pMG205 cut with *Sac*II and *Ngo*MIV (9095 bp) resulted in plasmid pMG207 (Figure 1). To verify the chromosomal lesion, two primers hybridizing to chromosomal DNA outside of the segments cloned in pMG207 were used (DSBAKO-5', 5'-CGGTACCACTCCACGCGGCAAA-3' and DSBKO-3', 5'-CCGGAGC-GACGTGGTAAAGATGT-3').

Media and Growth Conditions

LB medium and LB agar medium were from Q-BIOgene (Montreal, Canada). YEG-CI medium (14) containing 25 µg/mL kanamycin sulfate was used for direct selection. YEG-CI Km₂₅ agar was prepared as follows: 5 g yeast extract (Oxoid, Basingstoke, UK), 10 g NaCl, 15 g agar (A-7002; Sigma, St. Louis, MO, USA), and 2 g D,L-*p*-chlorophenylalanine (13,071-0; Sigma) were autoclaved in 1 L ultrapurified water. Then, 20 mL 20% glucose (w/v) and 500 µL 50 mg/mL kanamycin sulfate were added.

General Molecular Biological Techniques

Restriction enzymes and T4-DNA-ligase were from New England BioLabs (Beverly, MA, USA), and JumpStart™ *Taq* DNA polymerase was obtained from Sigma. All enzymes were used as specified by the manufacturers. CaCl₂ transformation and agarose gel electrophoresis was done by standard techniques (20). Plasmid DNA was isolated by JETquick Plasmid Miniprep Spin Columns (GENOMED, Research Triangle Park, NC, USA). Chromosomal *E. coli* DNA was isolated from the pellet of 5-mL cultures by resuspension in 500 µL G1 buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/mL RNase A), cell lysis at 4°C for 5 min after adding 20 µL lysozyme (50 mg/mL) and 50 µL 10% sodium dodecyl sulfate, and extraction (three times) with 1 volume phenol/CHCl₃ after adding 12 µL 5 M NaCl. The DNA was precipitated with 2.5 volumes 100% ethanol, washed with 70% ethanol, and dissolved in 50 µL TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Analytical PCRs contained 40 ng chromosomal DNA, 25 pmol primers, 0.2 mM dNTPs, and 2.5 U JumpStart *Taq* DNA polymerase in the supplied buffer in a total volume of 50 µL. Forty-five PCR cycles were run with annealing temperatures between 53° and 65°C, depending on the primer pairs.

RecA Assay

Strains harboring a functional RecA protein are capable of repairing DNA damage introduced by low doses of UV irradiation while strains lacking RecA (*recA* mutants) cannot survive (21). To check the RecA phenotype, cells of a fresh, medium-sized colony were resuspended in 300 µL 1× A salts [10× A salts are 105 g K₂HPO₄, 45 g KH₂PO₄, 10 g (NH₄)₂SO₄, and 5 g sodium-citrate in 1 L ultrapurified water]. Three microliters of each suspension were spotted to corresponding grid positions on two identical LB plates. One of these plates was irradiated with UV light (for 3 min, 254 nm, 2 × 6 W lamp, distance 60 cm to an open Petri dish). The conditions for the appropriate UV

Figure 1. Double homologous recombination. The knockout plasmid pMG207 is shown with the two approximately 2.0-kb DNA regions (*dsbA1* and *dsbA2*) flanking *dsbA* on the chromosome and the *bla* (providing ampicillin resistance; Ap^R), *neo*, *recA*, and *pheS* Gly294 genes. Homologous recombination is symbolized by X. Relevant restriction sites are shown. The maps are not drawn to scale. Wt, wild-type.

dose may vary among *E. coli* strains and need to be established with control RecA⁺ and RecA⁻ strains. Both LB plates were incubated in the dark for 24 h at 30°C.

RESULTS AND DISCUSSION

Customized *E. coli* strain construction may require precise and stable chromosomal deletions in RecA-deficient host strains. To replace *dsbA* encoding a periplasmic disulfide bond oxidase (22) with a Km^R cassette (*neo*) in the Δ *recA* strain KA12, we have developed a new strategy that temporarily provides the RecA function necessary for targeted homologous recombination. This strategy was based on the suicide vector pKSS (14), which carries a modified *pheS* gene, whose Ala294Gly mutation affords an α subunit of phenylalanyl-transfer RNA (tRNA) synthetase with relaxed substrate specificity (23). On medium containing *p*-chlorophenylalanine, a strain harboring pKSS cannot survive because the amino acid analog is detrimentally incorporated into cellular proteins instead of phenylalanine (14,24). Consequently, selection with *p*-chlorophenylalanine and kanamycin should yield strains that have lost the plasmid (and concomitantly, the *pheS* and *recA* genes) and have integrated the *neo* gene into their chromosome.

Plasmid pMG207 was assembled for the chromosomal knockout of *dsbA* (Figure 1). Table 1 shows the timeline for the chromosomal mutagenesis. Transformants with plasmid pMG207 were grown in LB Km₂₅ liquid medium in the absence of ampicillin to allow for double homologous recombination (Figure 1) and spontaneous plasmid loss. Subsequent plating on YEG-Cl Km₂₅ [containing *p*-chlorophenylalanine, but only little competing phenylalanine (14)] and incubation at 37°C for 2 days yielded strains with an integrated *neo* gene but lacking plasmid. The 37°C incubation step resulted in enhanced selection pressure for plasmid loss since we observed that *E. coli* cells harboring high-copy number plasmids with the *recA* gene (such as pMG207) have difficulties growing at 37°C. As shown in Table 1, 14 of the 32 tested clones

Table 1. Protocol for Strain Construction by the Day^a

Day	Manipulation	Results
Day 1	Transform KA12 with pMG207; select on LB Ap ₁₅₀ Km ₂₅ plates, 30°C.	
Day 2	Streak-out transformants to single colonies on LB Ap ₁₅₀ Km ₂₅ plates; incubate overnight at 30°C.	
Day 3	Inoculate 5 mL LB Km ₂₅ liquid medium with a single colony; incubate overnight at 30°C.	Two cultures were started in parallel.
Day 4	10× and 100× dilution of the grown culture in 1× A salts. Plate 100 μ L of each dilution onto YEG-Cl Km ₂₅ selection plates; incubate at 37°C for 2 days.	Plating of 4 × 100 μ L of 10× dilution gave 47 colonies.
Day 6	Streak-out 32 colonies to obtain single colonies on YEG-Cl Km ₂₅ plates; incubate at 37°C for 2 days.	
Day 8	Spot 32 colonies (one from each purification) onto LB Ap ₁₅₀ , LB Km ₂₅ , and LB plates (in this order). Useful controls: KA12 (RecA ⁻ , Ap ^S , Km ^S), AD202 (RecA ⁺ , Ap ^S , Km ^R), and KA12/pMG207 (RecA ⁺ , Ap ^R , Km ^R); incubate for 24 h at 30°C.	14 out of 32 colonies were Ap ^S and Km ^R (desired phenotype).
Day 9	Repurify eight candidate KA35 colonies to single colonies on LB Km ₂₅ plates and incubate for 24 h at 30°C.	
Day 10	RecA assay of the eight candidate clones; incubate over night at 30°C. Inoculate 4 × 5 mL LB with KA35 candidates; incubate overnight at 30°C.	All tested Ap ^S and Km ^R clones were RecA ⁻ .
Day 11	Isolate chromosomal DNA of the four cultures and as a control of KA12. Chromosomal PCR.	
Day 12	Restriction digest of the chromosomal PCR fragments; agarose gel analysis.	Three of four clones had the correct genotype.

^aSubscripted numbers refer to final antibiotic concentrations in μ g/mL [sodium ampicillin (Ap) or kanamycin sulfate (Km)]. Superscripted ^R and ^S refer to resistance and sensitivity phenotypes, respectively.

(44%) exhibited the desired resistance phenotype on master plates [ampicillin-sensitive (Ap^S) and Km^R]. In fact, these 14 clones already grew better on the YEG-Cl Km₂₅ plates (day 6) than the 18 other ones. The false positives showed inappropriate combinations of antibiotic resistances; 10 clones were still ampicillin-resistant and Km^R, and some even had problems growing on LB without antibiotics. The RecA assay confirmed that all Ap^S clones were also RecA⁻ as expected, since the corresponding genes were linked on the plasmid. Chromosomal PCR (not shown) with primers annealing to *dsbA* outside of the segments present in pMG207 further ruled out plasmid cointegrates. Restriction analysis of the PCR products confirmed the correct location of the lesion on the chromosome for three of four investigated candidates for the new *dsbA* knockout strain KA35; the inappro-

priate clone had too large an insertion. With plasmid pMG207 at hand, the entire strain construction procedure was completed within 12 days, including all verification steps (Table 1). It takes just four additional days to assemble a new customized knockout plasmid starting from pMG207 (plus two more days for plasmid verification).

In conclusion, we present here a system for targeted chromosomal mutagenesis that is complementary to the previously described methods and overcomes some of their drawbacks and limitations: (i) By provision of a *recA* gene on the suicide plasmid, RecA-negative precursor strains can be reengineered, including most common cloning hosts and well-established “designer” strains for directed evolution experiments (17,25). (ii) Selection for *p*-chlorophenylalanine resistance directly identifies clones that have lost the suicide plasmid, making

a high-temperature (up to 43°C) incubation step, which is often harmful for *E. coli* or incompatible with the phenotype of a new chromosomal mutation, unnecessary. (iii) To cure the suicide vector, no additional chromosomal mutation is required in the host. (iv) The system is very efficient with one third of the selected clones having the correct genotype. (v) With the integration plasmid at hand, strain construction and verification takes less than 2 weeks. Furthermore, the method requires very little interaction time; except for the final PCR analysis, the daily tasks are accomplished within 1 h. (vi) Unlike other conditional plasmids in use, our knockout plasmid is a high-copy number replicon making DNA isolation and handling easier. (vii) The system is highly versatile; by exchanging the flanking regions of the targeted gene (*dsbA1* and *dsbA2*; Figure 1) in pMG207, we have constructed two other chromosomal mutations with yields similar to those presented in this work. The *neo* gene can easily be replaced with a different antibiotic resistance cassette. If required, the resistance gene can be provided with flanking *FRT* or *loxP* sites for subsequent excision from the chromosome by a site-specific recombination step based on the well-established *Flp/FRT* or *Cre/loxP* systems, respectively (6,10).

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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