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Efficient and seamless DNA recombineering using a thymidylate synthase A selection system in *Escherichia coli*

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ABSTRACT

λ-Red system-based recombinogenic engineering is a powerful new method to engineer DNA without the need for restriction enzymes or ligases. Here, we report the use of a single selectable marker to enhance the usefulness of this approach. The strategy is to utilize the thymidylate synthase A (*thyA*) gene, which encodes an enzyme involved in the synthesis of thymidine 5′-triphosphate, for both positive and negative selection. With this approach, we successfully created point mutations in plasmid and bacterial artificial chromosome (BAC) DNA containing the mouse *Col10a1* gene. The results showed that the *thyA* selection system is highly efficient and accurate, giving an average of >90% selection efficiency. This selection system produces DNA that is free from permanent integration of unwanted sequences, thus allowing unlimited rounds of modifications if required.

INTRODUCTION

Recent advances in homologous recombination-based DNA engineering technology, termed recombinogenic engineering or recombineering, have led to the development of highly efficient procedures with which chromosomal or plasmid DNA can be modified in *Escherichia coli* by the introduction of mutations, such as single base substitutions, deletions and insertions. These methods are based upon the *rac*-encoded RecET system or the bacteriophage λ-Red recombination system (1–6), eliminating the need for *in vitro* manipulations using restriction enzymes or DNA ligases (7,8). The three proteins of the Red system, Exo, Beta and Gam, mediate recombination between a linear double-stranded DNA donor and its homologous target sequence by promoting double-strand break repair (9–15).

This system, while powerful, suffers from a relatively low rate of recombination events, so that screening of a large number of bacterial colonies is still required in most cases. This limitation can be overcome by incorporating a selectable marker to assist in selection of the desired recombinants. Selection protocols generally use antibiotic resistance genes flanked by loxP or frt sites, such that the selectable marker can later be removed by *cre*- or *flp*-mediated recombination (10). This method is highly efficient but unavoidably leaves at least one loxP or frt sequence behind. Alternatives include antibiotic-*sac*B cassettes, such as *Sac*B–neo, which have been used in a two-step modification procedure to avoid unwanted residual DNA (4,16,17). In the first round of recombination, cells are transformed with the *sac*B–neo fusion cassette and are selected for their resistance to kanamycin. Subsequently, an appropriate DNA cassette is introduced to replace the entire *sac*B–neo cassette to create the desired change in DNA sequence. Cells retaining the *sac*B marker gene will not survive in 7% sucrose. However, these methods can result in unwanted rearrangement (18) and require labor-intensive screening procedures to distinguish clones with the desired recombineered product from cells that survived owing to mutational events that prevented the expression of the counter-selectable gene.

Here, we describe a novel selection approach in *E.coli* utilizing the λ-Red recombineering system. It is a single selectable marker approach with selection criteria based on previous work performed in *Bacillus subtilis* (9,19). In brief, the selectable marker is the enzyme encoded by the

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thymidylate synthase A (*thyA*) gene, which is involved in the *de novo* synthesis of dTTP from dUMP. Without *thyA*, the cell is unable to synthesize DNA and, therefore, will not grow in minimum growth media. Thus, in a *thyA*-null *E.coli* mutant (Δ*thyA*), recombinants containing an exogenous *thyA* gene can be selected for (positive selection) by culture in growth medium in the absence of thymine. When thymine is provided, *de novo* dTTP synthesis can proceed without the need for ThyA function.

The activity of ThyA requires the cofactor tetrahydrofolate (THF), which is converted to dihydrofolate (DHF) in the process. THF is also an important cofactor for many other essential cellular reactions and is replenished from DHF by the action of dihydrofolate reductase, which can be inhibited by trimethoprim. Thus, recombination events in which the *thyA* gene is removed can then be selected for (negative selection) using growth media containing thymine and trimethoprim. Under these culture conditions, cells with functional *thyA* exhaust the supply of THF then growth is supressed because it cannot be replenished in the presence of trimethoprim. In cells lacking a functional *thyA* gene, sufficient THF is maintained for other cellular reactions and dTTP can be synthesized from thymine; thus, these cells will grow.

We have generated a *thyA*-null *E.coli* deletion strain harboring a defective lambda prophage, which contains the three Red genes with their expression tightly controlled by a temperature-sensitive repressor. Red induction occurs when the temperature is switched from the normal 32 to 42°C. At the higher temperature, the repressor comes off, allowing the expression of Exo, Bet and Gam. We have also established culture conditions for both positive and negative selection when an exogenous *thyA* gene is re-introduced and subsequently removed. The strategy is that using this strain of *E.coli* as a host, a *thyA* gene with its promoter can be introduced anywhere in a plasmid or bacterial artificial chromosomes (BACs) DNA sequence specifically by homologous recombination, and this event is selected for using positive selection. Subsequently, point mutations, deletions or insertions can be introduced precisely in the plasmid or BAC DNA through the removal of the *thyA* gene, again by homologous recombination, and this event is selected for using positive selection for growth in the absence of ThyA. In a proof of principle experiment, point mutations were introduced into the mouse collagen X gene (*Col10a1*) in plasmid and BAC DNA with selection efficiency >90%.

MATERIALS AND METHODS

Bacterial strains and growth media

Two bacterial strains, DY380 and QW1, were used in this study. DY380 is a gift from Dr Don Court (10) and is a modification of DH10B [F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*) 7649 *gal*U *gal*K *rps*L *nup*G] that includes the Red genes [λcI857 (*cro-bio*A) <> *tet*] as a prophage. QW1 is a further modification of DY380 created in the current study with the thyA gene deleted [Δ*thyA*].

Cells were grown in a modified M9 minimal medium (48 mM $Na_2HPO_4.7H_2O$, 22 mM KH_2PO_4, 8.6 mM NaCl and 18.7 mM NH_4Cl), containing 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, 0.4% (w/v) glucose, 0.6 mM leucine, valine and isoleucine, 30 nM vitamin B1, 1% (v/v) Luria–Bertani (LB) broth and 1× MM1 containing 1 nM each of $ZnSO_4$, $CuSO_4$, Na_2MoO_4, $CoCl_2$, $MnSO_4$, $CrCl_3$, $NiCl_2$. Specific growth selection medium was supplemented with either or combination of 50 μg/ml ampicillin, 12.5 μg/ml chloramphenicol, 0.79 mM thymine (Sigma) and 10 μg/ml (for plasmids) or 20 μg/ml (for BACs) trimethoprim (Sigma). For growth on agar plates, 2.5% (w/v) noble agar (Sigma) was included in the appropriate medium. All chemicals were of analytical grade.

Polymerase chain reaction

PCR was used for the amplification of DNA fragments with the GeneAmp PCR System 9700 (Applied Biosystems). The high-fidelity *Pfx* DNA polymerase (Invitrogen) was used in all the reactions, prepared as specified by the supplier. The general condition included an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 45 s to 1 min 30 s, depending on the length of the DNA to be amplified. PCR products were gel-purified and recovered using the gel purification kit from Qiagen. The quality and concentration of DNA were analyzed by gel electrophoresis and UV spectrophotometry, respectively.

Preparation of DNA fragment for recombineering procedures

All linear DNA fragments were generated by PCR amplification and the appropriate primers are shown in Table 1. Primers thyAF1 and thyAR1 were used to amplify a 1470 bp DNA fragment (Figure 2A) containing the full-length *thyA* gene (thyA-FL) using DY380 chromosomal DNA as template. The amplified DNA was cloned into pBluescript KS II (+) (Stratagene) to generate the plasmid, pThyA-FL.

A 744 bp DNA fragment (Figure 2A) containing a truncated 'inactive' version of the *thyA* gene (Δ*thyA*) was generated by overlapping PCR using pThyA-FL plasmid DNA as a template. The primers used to generate the two overlapping fragments were thyAF1 and thyAR2, and thyAF2 and thyAR1 (Table 1). The resultant amplification fragments were purified and used as templates in the second round of overlapping PCR with primers thyAF1 and thyAR1. In Table 1, the nucleotides in bold represent the overlapping regions where the two fragments will anneal to one another.

A 1124 bp DNA fragment (Figure 2A) containing an 'active' version of the *thyA* gene lacking a small region 3′ of the translation stop and terminator of the *thyA* gene (*thyA*Δ3′) was amplified using primers thyAF1 and thyAR3 (Table 1) with plasmid pThyA-FL as a template. This fragment was cloned into pBluescript KS II (+) to generate the plasmid, pThyA-Δ3′.

For site-directed mutagenesis in a plasmid containing the mouse collagen X gene (*Col10a1*), two PCR-amplified DNA fragments were generated, X-thyAΔ3′-X′ and ColX-G18D. The X-thyAΔ3′-X′ fragment was amplified using pThyA-Δ3′ plasmid DNA as template and primers ColX-thyAF and ColX-thyAR (Table 1). The highlighted nucleotides in the primers are nucleotide sequence of *Col10a1* flanking the site of mutagenesis. The ColX-G18D fragment was generated by overlapping PCR (Figure 2B) as described below and in

Table 1. Normal and mutant primers for PCR site-directed mutagenesis

Primers	Primer sequence (5′ to 3′)	Position	Orientation
thyA-F1	GCTGCTGCTGGAAGGTGTGGT	−277 to −257	Forward
thyA-R1	CCCTCCCTGATAACGCTGATA	+1193 to +1173	Reverse
thyA-F2	**GTTTTAACCTGCAAGATGG***CCGTTAAATTCTTCGAGACGC*	**+104 to +122**::*+849* to *+869*	Forward
thyA-R2	**GCGTCTCGAAGAATTTAACGG***CCATCTTGCAGGTTAAAAC*	**+869 to +849**::*+122* to *+104*	Reverse
thyA-R3	GGGTAAAAAAACCGACGCACAC	+847 to +826	Reverse
ColXF	CAAGGCAGTTTCCAAACACTAG	−223 to −202	Forward
ColXR	CCATGCATCATTCCGCTGTAC	+307 to +287	Reverse
ColX-thyAF	**TTCTCTTCTGCTTTTCAGAATCTATCTGAAAATATGCTG CCTCAAATACC***GCTGCTGCTGGAAGGTGTGGTCGTC*	**−33 to +17**::*−277 to −253*	Forward
ColX-thyAR	**ACCTTTACTCTTTATGGCGTATGGGATGAAGTATTGTGT CTTGGGGCTAG***GGGTAAAAAAACCGACGCACACTGGCG*	**+156 to +107**::*+847 to +820*	Reverse
ColX-mR	T***ATC***ATGAACCAGGGTCAAGAAC	+55 to +33	Reverse
ColX-mF	CAT***GAT***ATGTTTTATGCTGAACGG	+49 to +72	Forward

The positions of the primers are relative to the start codon of the mouse *Col10a1* or the *thyA* genes. The sequences in bold letters are overhang regions. In primers ColX-thyAF and ColX-thyAR, *Col10a1* sequences are in bold letters and *thyA* sequences are in italics. The sequences that are in both bold and italics in primers ColX-mF and ColX-mR represent the codon to be mutated in the mouse *Col10a1* gene. Sequence assignment numbers for the collagen X primers are taken from Kong *et al*. (27) and for thyA from Belfort *et al*. (28).

previous mutagenesis of the human *COL10A1* gene (20). In brief, two overlapping DNA fragments were generated using primers ColX-F and ColX-mR, ColX-mF and ColX-R. The overlap fragment contained a 2 bp substitution changing the codon GGG for glycine at residue 18 (relative to the start of translation) to GAT for aspartate in the precollagen X α-chain, analogous to a mutation in the signal peptide cleavage site (21).

For site-directed mutagenesis in a *Col10a1* containing BAC, primers (ColX-F and ColX-R) were used to amplify a 566 bp PCR fragment from a plasmid (pG18D-Flag) containing a different variant of the G18D mutation (GGG to GAC) and an in-frame insertion of the Flag® sequence (5′-GACGACGATGACAAGCTTGCGGGCCCA-3′) at the 3′ region of exon 2 (Figure 2C).

Electroporation of DNA into bacterial cells

Appropriate bacterial cells were prepared for electroporation as described previously. The Red gene functions were induced at 42°C for 15 min to allow the expression of Exo, Bet and Gam as the temperature-sensitive repressor falls off at this high temperature. Cells grown at 32°C without induction were used as a control (5). In brief, 50 µl of 'competent' cells were mixed with 2–8 µg of the PCR-amplified DNA in a pre-cooled 0.1 cm Gene Pulser cuvette (Bio-Rad Laboratories) and electroporated using the Bio-Rad Gene Pulser with conditions set at 1.8 kV, 25 µF and 200 ohms. Following electroporation, the cells were grown in 1 ml of LB broth at 32°C for 1–1.5 h, collected by centrifugation and plated at various dilutions onto M9 minimal medium agar plates with appropriate supplements for selective growth, and incubated at 32°C for 48–72 h.

Generation of a *thyA*-null bacterial strain (QW1)

The *thyA*-null strain (QW1) was constructed by electroporating the 744 bp 'inactive' ΔthyA DNA fragment (created by overlapping PCR as described above) into DY380 following activation of the λ-Red recombinase system. Clones with the appropriate homologous recombination event inactivating the endogenous *thyA* gene were selected for culturing electroporated cells on modified M9 minimal growth agar plates supplemented with 0.79 mM thymine and 10 µg/ml trimethoprim.

Mutagenesis of the mouse *Col10a1* gene

A plasmid, pKM2-ColX, containing 10 kb of the mouse *Col10a1* gene was first transformed into QW1. Next, 4 µg of X-*thyA*Δ3′-X′ DNA fragment (constructed as described above) was electroporated into 'competent' QW1 cells harboring pKM2-ColX. Appropriate clones with a functional *thyA* gene were selected by growth on modified M9 minimal growth media agar in the absence of thymine. Clones (QW1-pKM2-ColX-ThyA) that grew under these conditions had a functional *thyA* gene introduced into pKM2-ColX at the desired location by homologous recombination. Subsequently, 4 µg of ColX-G18D DNA (generated as described above) were electroporated into 'competent' cells derived from a QW1-pKM2-ColX-ThyA clone. Cells were then grown on modified M9 minimal agar plates supplemented with 0.79 mM thymine and 10 µg/ml trimethoprim to select for the absence of *thy*A gene function.

Similar procedures were carried out for mutagenesis in a 319 kb BAC (RP23-194I3, accession no. AC021709.12) containing the mouse *Col10a1* gene, except that in the final mutagenesis step, between 2 and 8 µg of a PCR product, FColX-G18D (Figure 2C) was electroporated into 'competent' cells derived from QW1 containing the RP23-194I3 BAC with *thyA* introduced at the appropriate site for subsequent mutagenesis.

RESULTS

Generation of a *thyA*-null bacterial strain (QW1)

Utilizing the λ-Red recombination function present in DY380, the *thyA* gene was inactivated through homologous recombination between DY380 chromosomal DNA and a PCR-amplified DNA fragment, ΔthyA. The overall strategy is outlined in Figure 1A. Growth selection for the absence of ThyA function resulted in >250 colonies when the λ-Red

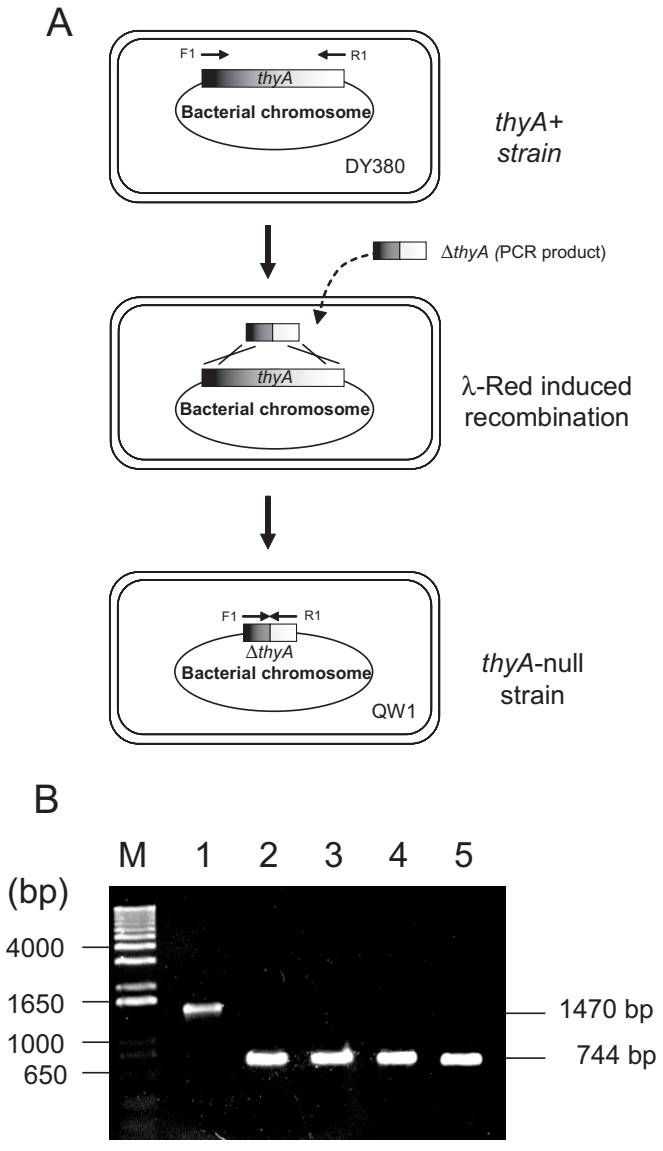

Figure 1. Generation of a *thyA*-null bacterial strain (QW1). (**A**) Schematic representation of the strategy to inactivate the endogenous *thyA* gene in DY380. The *thyA* gene is deleted through homologous recombination between the endogenous gene and a DNA fragment ΔthyA generated by PCR (see Materials and Methods for details). This process is assisted by the λ-Red recombination system present in DY380. (**B**) Genotype analysis of the resultant colonies following appropriate growth selection for the absence of ThyA function. The DNA products after PCR amplification of chromosomal DNA with primers F1 and R1 flanking the *thyA* gene were analyzed by 1% (w/v) agarose gel electrophoresis stained with ethidium bromide. Lanes 2–5 are PCR products (744 bp) from four representative *thyA*-null colonies; lane 1 is the 1470 bp PCR product from the parental strain DY380; M is a DNA molecular size marker (Invitrogen).

recombination system was activated (42°C) prior to electroporation as compared with only two colonies without prior activation (maintained at 32°C) (Table 2). Twelve colonies were selected for analysis; deletion of the *thyA* gene from the bacterial genome was confirmed by PCR amplification using primers flanking the *thyA* gene in eleven of the twelve colonies (Table 2 and Figure 1B). One colony with the correct recombination was selected as the newly generated *thyA*-null strain (QW1), where sequencing of the PCR product confirmed the expected deletion in the gene (data not shown).

Establishment and confirmation of selective growth conditions

To establish that QW1 can be utilized to facilitate the selection of homologous recombination events between exogenous DNAs, a number of 'rescue' assays were performed to test the newly designed thyA selection system. The introduction of a full-length functional *thyA* gene in a plasmid (pThyA-FL) restored 'normal' cell function in QW1. Under the appropriate growth selection for the presence of ThyA function, >1300 colonies were observed (Table 2). A similar result was obtained for the introduction of plasmid pThyA-Δ3′ (Table 2), indicating that DNA sequence encoded in *thyA* Δ3′ is also fully functional. The use of *thyA*Δ3′ is preferred for subsequent mutagenesis experiments as it lacks 347 bp 3′ to the terminator of the *thyA* gene, minimizing potential recombination events where the *thyA* gene may recombine back into the original position in the bacterial chromosome, introducing false positives. Furthermore, we have shown that transformation of linear PCR DNA fragments, *thyA*-FL or *thyA*Δ3′ (Figure 2A), did not result in ThyA function, indicating that during the selective growth period, the linear DNAs are likely to be degraded and will not contribute to false positives. This is supported by the very low frequency or absence of colonies in transformation of QW1 with *thyA*-FL or *thyA*Δ3′ PCR-amplified DNA fragments when the λ-Red recombination system is not active (Table 2).

Site-directed mutagenesis of the mouse *Col10a1* gene

As a proof of principle experiment, we utilized the ThyA selection system in QW1 to introduce a specific 2 bp mutation in a plasmid containing the mouse *Col10a1* gene relying solely on the λ-Red recombination system. The overall strategy is illustrated in Figure 3A. It involves two rounds of homologous recombination in QW1. The first round is between a plasmid containing the normal *Col10a1* sequence (pKM2-ColX) and a PCR-generated DNA fragment containing Δ*thyA* sequence flanked by 50 bp of *Col10a1* sequences, corresponding to the 5′ and 3′ regions of the intended site of mutagenesis, respectively. When the λ-Red recombination system is activated, the 50 bp flanking sequences of homology to normal *Col10a1* sequence are sufficient to allow homologous recombination in QW1 to promote the incorporation of the Δ*thyA* gene into pKM2-ColX, with an efficiency of 1.32×10^4 recombinants/μg DNA when growth was selected in conditional medium lacking thymine (Table 2). Of the 13 colonies selected, 1 colony showed only the parental plasmid, while 12 colonies showed a varying mixture (~1:2) of parental and correctly recombined plasmids by PCR amplification of the region corresponding to the site of Δ*thyA* integration. Colonies containing only the recombinant plasmid were obtained by retransformation in QW1 and selected for ThyA function. A representation of the respective PCR product with (1565 bp) and without (530 bp) Δ*thyA* integration is shown in Figure 3B.

With Δ*thyA* integrated, the strategy is to remove it in a second recombination event whereby the desired 2 bp substitution mutation is introduced into the plasmid. The base

Table 2. Summary of the *thyA*-null experiment, rescue assays, first and second stages of *Col10a1* mutagenesis

Experiment	Bacterial strain and DNA fragment/plasmid used	Induction of λ-Red system	Total number of colonies	No. of correctly targeted colonies relative to the number of colonies screened	Selection efficiency (%)
thyA-null strain (QW1)	DY380 + Δ*thyA* (PCR)	+	255	11 out of 12	92
	DY380 + Δ*thyA* (PCR)	–	2	0 out of 2	—
Rescue assays	QW1 + p*ThyA*-FL	–	1346	—	—
	QW1 + p*ThyA*Δ3′	–	1386	—	—
	QW1 + *thyA*-FL (PCR)	–	0	—	—
	QW1 + *thyA*-Δ3′ (PCR)	–	0	—	—
First stage of *Col10a1* mutagenesis	QW1-pKM2-ColX + X-*thyA*Δ3′-X′ (PCR)	+	2500	12 out of 13	96
		–	1	0 out of 1	—
Second stage of *Col10a1* mutagenesis	QW1-(X-*thyA*Δ3′-X′) + ColX-G10D (PCR)	+	1260	14 out of 16	94
		–	1	0 out of 1	—

The values given are the result from a typical experiment, while the selection efficiency percentage is the mean of three independent experiments. PCR denotes DNA fragments generated by PCR.

changes were first introduced into a PCR product (ColX-G18D) containing *Col10a1* sequence by overlapping PCR as shown schematically in Figure 2B. Through homologous recombination of this PCR with pKM2-ColX-Δ*thyA*, the Δ*thyA* integration was removed and the 2 bp substitution introduced precisely. When selected in culture conditions for cells without ThyA function, we obtained on average >1200 colonies (Table 2) when the λ-Red recombination system was activated. Of the 16 colonies selected, 14 showed the correct recombination event confirmed by PCR amplification of the region containing the 2 bp substitution with only trace amounts of parental plasmids. Thus, this indicates that the recombination can be very efficient as previously reported (8), and that the counter selection process is sufficient to differentiate growth of cells with significant differences in the level of ThyA function. Furthermore, there will be a selection pressure for the enrichment of progenies with reducing ThyA function through random segregation.

Because the base pair changes also introduced a new BspHI restriction enzyme site, the presence of the mutation was monitored by BspHI digestion of the PCR product (Figure 3C), and confirmed by DNA sequencing of the plasmids isolated from the appropriate clones (Figure 3D). Together with the data from the generation of the *thyA*-null strain (QW1), this shows that the selectivity of the system is extremely high with ~90% of the colonies exhibiting the expected phenotype with respect to ThyA function.

To demonstrate that the ThyA recombineering system can be applied to BACs, we performed mutagenesis in a 317 kb BAC (RP23-194I3) containing the mouse *Col10a1* gene. Compared with plasmid DNA, the total number of colonies was lower for BACs, in the order of 5–10, 10–20 and 30–40 colonies using 2, 4 and 8 μg of PCR product for transformation, respectively. However, the selection efficiency remained high with >90% of the colonies contained the appropriate recombination event for the insertion of *thyA* into the BAC (9 from 9 colonies selected), or its subsequent removal (9 from 10 colonies selected). Introduction of *thyA* into the BAC was monitored by PCR as described above (data not shown), and confirmed by Southern blot analysis (Figure 4A). The DNA sequence of a final modified BAC (FColX-BAC-G18D) containing the G18D mutation with the Flag® sequence in exon 2 is shown in Figure 4B.

DISCUSSION

With the completion of many genome projects, the next step is to understand the function and regulation of genes that are discovered. Functional genomics will play a major role. There is a need for the ability to manipulate DNA for expression studies, to create targeting vectors for making transgenic organisms and to investigate the regulation of gene expression and gene function through site-specific mutagenesis.

Recently, the development of recombineering procedures utilizing homologous recombination in bacteria has allowed DNA manipulation without the need for restriction enzymes, large plasmid DNA fragments or vectors carrying large DNA inserts, such as BACs, P1-derived or yeast artificial chromosomes. However, the efficiency is generally low and recovering the appropriate clones requires the screening of hundreds to thousands of colonies, or having to engineer a gene for growth selection, such as an antibiotic gene (22,23). Furthermore, some approaches retain unwanted residual DNA sequences that may interfere with gene function or are limited to a single manipulation. Here, we have developed a unique selection system in *E.coli* to enhance the usefulness and efficiency of the recombineering technique, utilizing a positive–negative selection system based on a single marker, the *thyA* gene, allowing seamless multiple manipulations of DNA.

With the *thyA* selection system, as a proof of principle experiment, we have successfully created a 2 bp mutation in exon 2 of the mouse collagen X gene, *Col10a1*. Overall, this system showed an average selection efficiency of >90%, requiring the screening of fewer than 10 colonies to ensure a successful outcome. This is a valuable improvement of the bacteriophage λ-Red recombination system, with advantages over existing systems. Its greatest advantage is that the selection of clones with or without ThyA function is based on growth of the bacteria concerned, with minimal background ranging from 0.01 to 0.08%.

Under optimal conditions, the efficiency of the bacteriophage λ-Red recombination system without selection has been reported at best to be only 0.1% (5) and 0.17% (10) of surviving cells. Therefore, although this is a single step without further manipulations, more than 1000 colonies need to be screened to obtain one or two recombinants. With appropriate selection using the ThyA system, we have obtained 14 recombinants from 16 colonies.

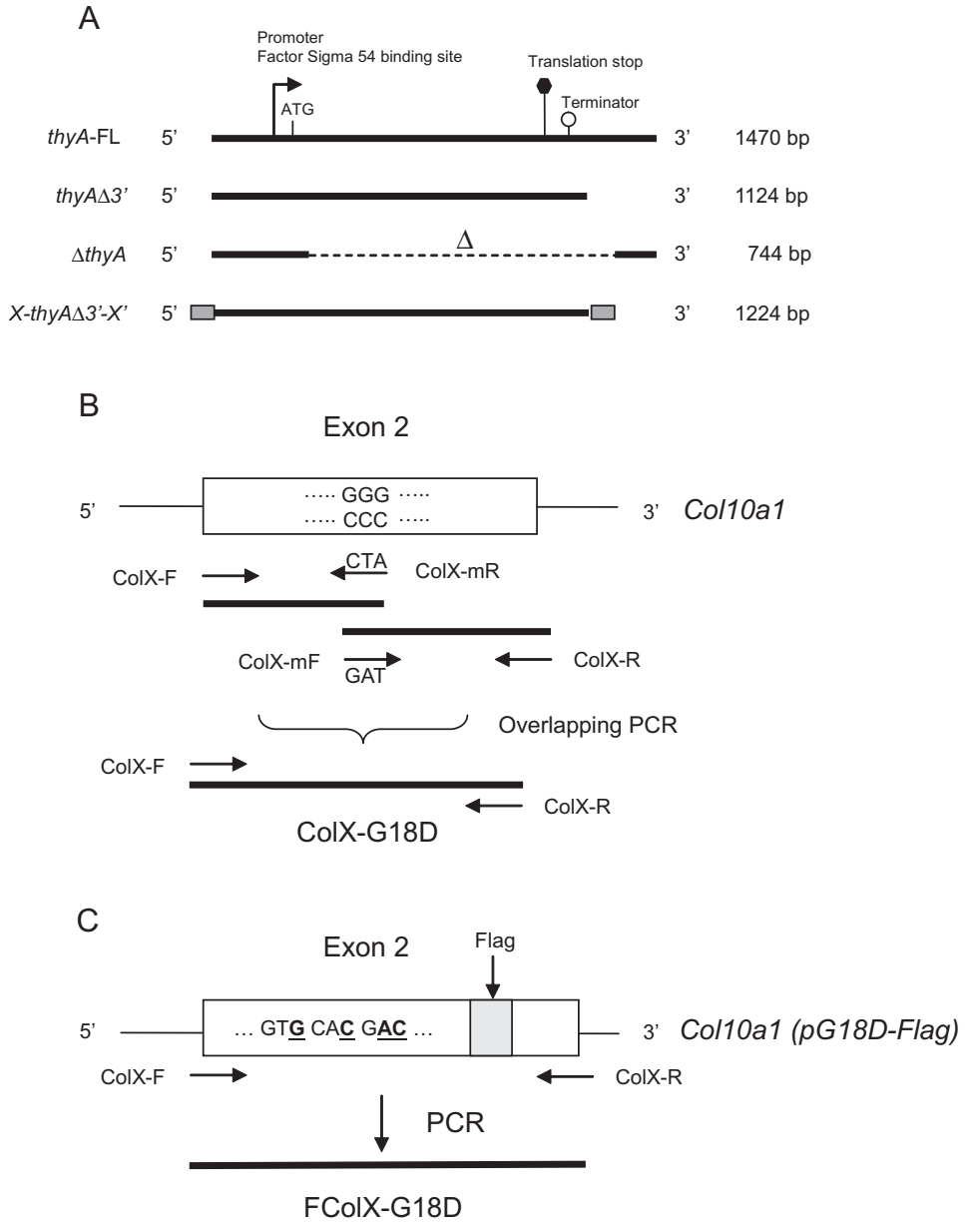

Figure 2. Schematic representation of the PCR DNA fragments ThyA-F, ThyA-Δ3′, ΔThyA and X-thyAΔ3′-X′. (**A**) PCR amplification using primers thyA-F1 and thyA-R1 (Table 1) amplifies a 1470 bp fragment (thyA-FL) of the *thyA* gene containing the promoter, Factor Sigma 54 binding site and the transcription termination flanking the start and stop codons for translation. The 1124 bp product, thyA-Δ3′, amplified using primers thyA-F1 and thyA-R3 (Table 1), lacks 347 bp 3′ of the transcription terminator. ΔthyA is a 744 bp product generated by overlapping PCR (see Materials and Methods for details) with most of the coding sequence deleted (+123 to +848; relative to the translational start site), and Δ in the diagram represents the deleted region. X-thyAΔ3′-X′ is a fusion product amplified using primers ColX-thyAF and ColX-thyAR with 5′ and 3′ overhang sequences for the regions of the *Col10a1* gene for homologous recombination. (**B**) Schematic representation of the strategy for the generation of the ColX-G18D fragment. Two PCR products were first generated using primer sets, ColX-F/ColX-mR and ColX-mF/ColX-R. These products, containing complementary sequences from primers ColX-mF and ColX-mR, were used in an overlapping PCR to produce ColX-G18G using primers ColX-F and ColX-R. (**C**) Schematic representation for the amplification of a PCR product from a plasmid, pG18D-Flag, using primers ColX-F and ColX-R. pG18D-Flag contains mutations (bold and underlined) around the signal peptide cleavage site of collagen X, and a Flag® sequence inserted at the 3′ region of exon 2 (shaded region).

Our system is superior to similar ones that positively select for growth of the desired recombinants, such as the use of a selection cassette containing the EcoRI endonuclease and an antibiotic gene that allows selection of the desired clone at an efficiency of only 0.35% (2 recombinants from 570 colonies) (24). In this system, expression of EcoRI endonuclease, which is toxic to cells in the absence of the corresponding methylase, is induced by the addition of isopropyl-β-d-thiogalactopyranoside to kill unwanted clones. Despite the high killing efficiency of EcoRI endonuclease, there is still a high background with >95% false-positive clones. The same group, wanting to improve the efficiency, altered the selection cassette to contain a rarer cutter, I-SceI endonuclease, and an antibiotic gene. However, the efficiency was

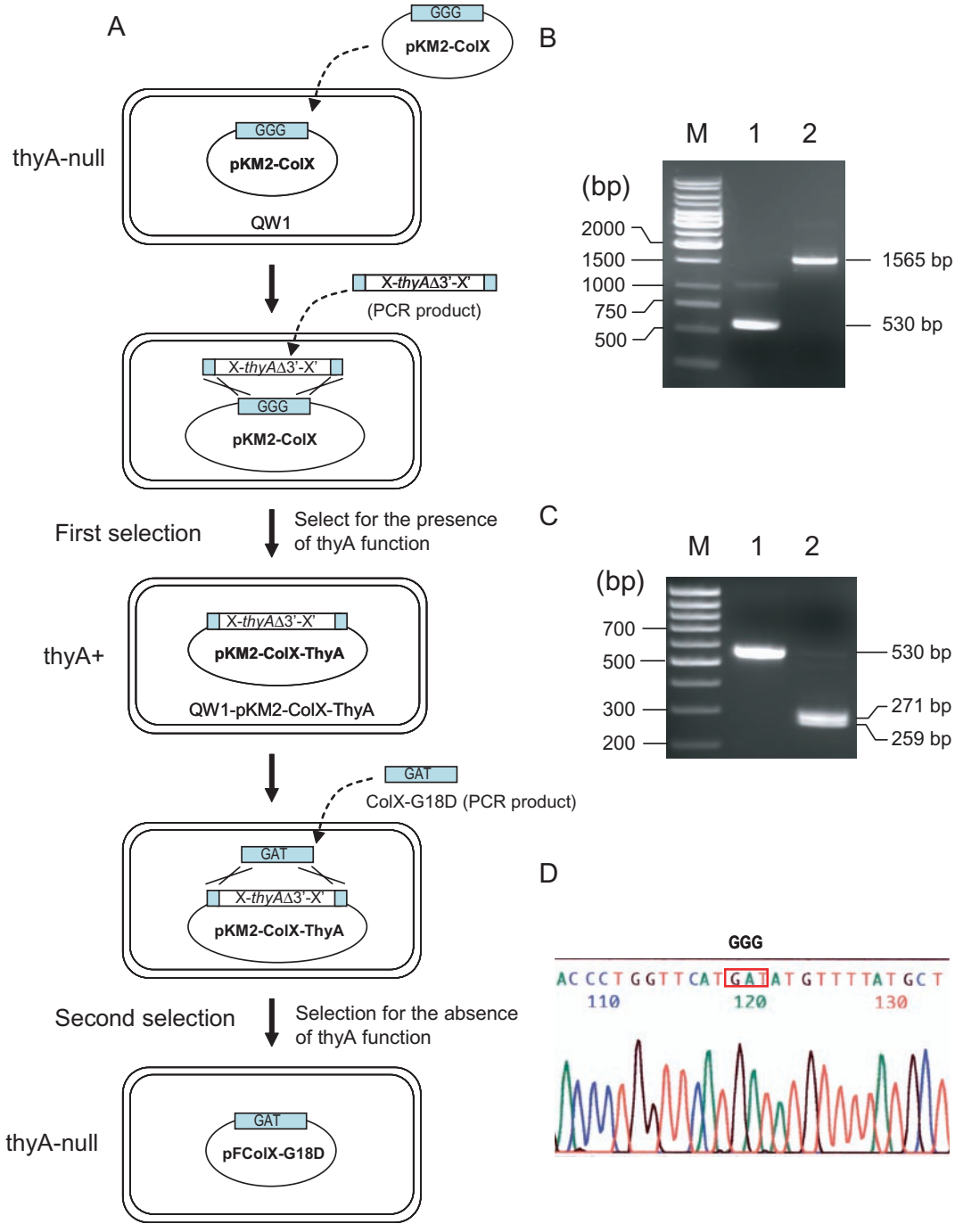

Figure 3. Mutagenesis of the mouse collagen X gene (*Col10a1*) using QW1. (**A**) A schematic presentation of selective growth for *thyA* gene replacement and removal through two rounds of homologous recombination events to introduce a 2 bp substitution in exon 2 of *Col10a1*. pKM2-ColX is a plasmid containing the normal *Col10a1* gene with GGG as the codon for Gly[18]. pKM2-ColX was first transformed into a *thyA*-null strain (QW1). This was followed by a second transformation with a PCR product (X-thyAΔ3′-X′) containing flanking sequences homologous to the *Col10a1* gene (shaded region). Through homologous recombination, a specific region of the *Col10a1* gene in pKM2-ColX is replaced with *thyA* and this event is selected for by growth in defined medium (see Materials and Methods for details) that requires ThyA function. A selected colony is then transformed with a PCR product containing *Col10a1* sequence with a 2 bp mutation at codon 18 (GGG to GAT), representing a G18D amino acid substitution. Activation of homologous recombination will remove the thyAΔ3′ sequence and introduce the G18D mutation to produce plasmid pFColX-G18D; growth conditions are used that select for the absence of ThyA function. (**B**) Confirmation of *thyA*Δ3′ integration in pKM2-ColX following the first round of homologous recombination. Colonies derived from growth selection for the reintroduction of ThyA function in QW1 were analyzed by PCR amplification of the resultant plasmid using primers ColX-F and ColX-R (Table 1). Lanes 1 and 2 show the amplification products from pKM2-ColX (530 bp) and pKM2-ColX-ThyA (1565 bp), respectively. M is a DNA molecular size marker (1 kb GeneRuler, MBI Fermentas). (**C**) Confirmation of *thyA*Δ3′ removal and introduction of a 2 bp substitution in pKM2-ColX following the second round of homologous recombination. The 2 bp substitution mutation introduces a new BspH1 restriction enzyme site in the 530 bp PCR product and will result in two fragments of 271 and 259 bp following digestion. M is a DNA molecular size marker (100 bp GeneRuler, MBI Fermentas)); lanes 1 and 2 are the PCR fragments from pKM2-ColX and pColX-G18D digested with BspH1, respectively. (**D**) DNA sequencing of pFColX-G18D showing that the codon GGG (glycine) has been specifically changed to GAT (aspartate) following the two rounds of homologous recombination using the *thyA* selection system.

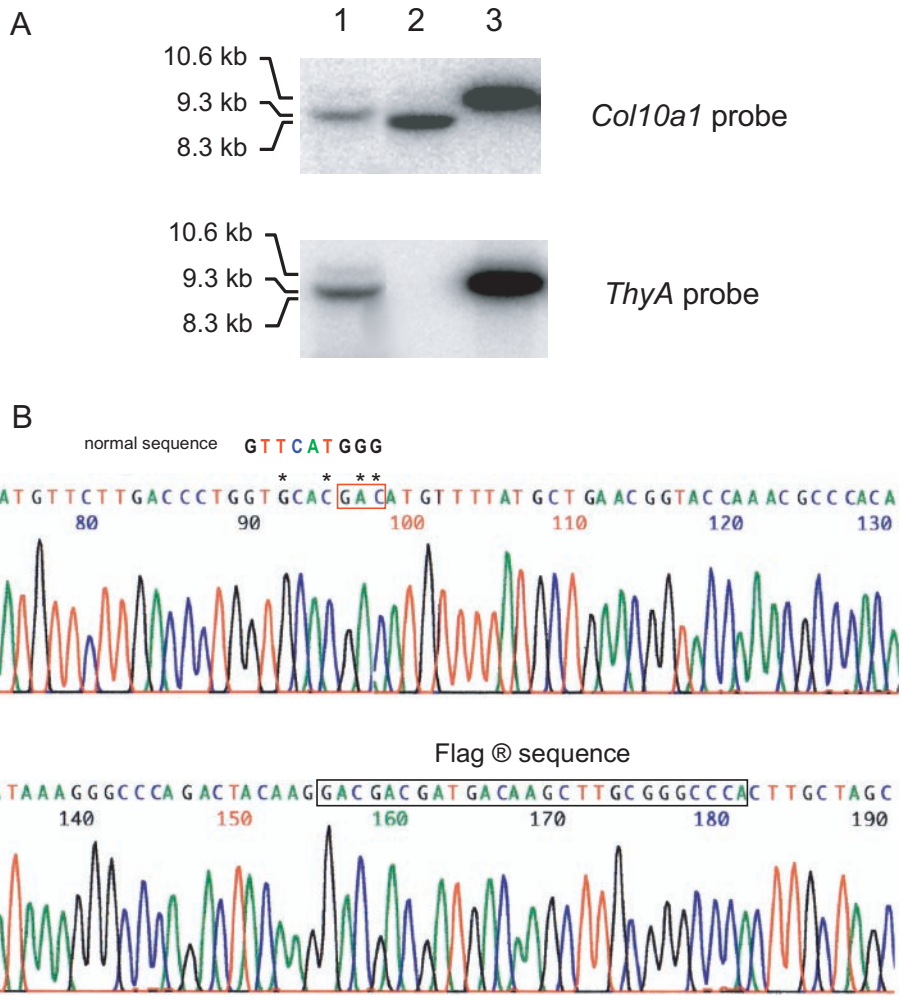

Figure 4. Southern blot analysis and DNA sequence of a modified BAC containing the mouse *Col10a1* gene. (**A**) Southern blot of the *Col10a1*-containing BAC modified with *thyA* (lane 1) and the parental BAC (lane 2), and the plasmid pKM2-ColX-ThyA (lane 3) digested with EcoRI. DNA transferred onto Hybond-N^+ membranes were hybridized consecutively with a 567 bp *Col10a1* (+2494 to +3060) probe, and a 656 bp *ThyA* (+191 to +846) probe with a stripping process in between. The respective 8.3 and 9.3 kb EcoRI fragments from the parental and *thyA*-containing BACs are indicated. EcoRI digest of pKM2-ColX-ThyA results in a 10.6 kb fragment containing both *Col10a1* and vector sequences. (**B**) DNA sequencing of the modified BAC (FColX-BAC-G18D) following the two rounds of homologous recombination using the *thyA* selection system. The engineered nucleic acid changes are highlighted with an asterisk, the codon GGG (Glycine) to GAC (aspartate) mutation is highlighted with a red box and the Flag® sequence with a black box.

improved to only 7.5% (7 recombinants from 93 colonies) upon counter selection (25).

Other recent counter-selection strategies for BAC modifications use the fused *sacB*/*Neo* gene (26) and the *rspL* gene (18). However, these are negative selection systems that suffer from a high background owing to mutational events affecting the SacB protein (26) or rearrangements (18).

Apart from counter-selection systems, selective procedures like the use of antibiotics and site-specific recombinases, such as *Cre*, and restriction enzyme digestion are also methods for eliminating selection markers (3,10,26). However, these protocols create an end product that is not 'clean' in that there remains a marker or a tag within the final product. These tags may be problematic, causing unpredicted splicing, altered binding of transcription factors or affecting regulation of gene expression at distant sites that cannot be predicted from sequence analysis alone.

By combining the advantages of the λ-Red recombination and the selection of ThyA function in *E.coli*, we have developed a system that has high and accurate efficiency in the selection of a well-proven homologous recombination event induced by specific genes of the λ-prophage. This system should be applicable to plasmids, P1-derived artificial chromosomes or bacterial artificial chromosomes. In theory, this system can allow unlimited seamless modifications in different regions of the vectors or chromosomes concerned.

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