In-frame deletion of *Escherichia coli* essential genes in complex regulon

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A conditional knockout-rescue system was developed to construct an in-frame deletion strain of *Escherichia coli* essential genes. The target was flanked with marker genes and FRT (FLP recognition target) sites, and a plasmid containing arabinose-induced FLP recombinase was transformed. After arabinose induction, cells could survive only when target protein activity was provided in trans. We selected three essential genes as targets, *yaeT*, *fabZ*, and *dnaE*, which are components of the complex eight-gene regulon *yaeT-hlpA-lpxD-fabZ-lpxA-lpxB-rnhB-dnaE*. Deletion of these three genes exhibit no polar effects on their adjacent genes in terms of cell viability, meaning that this system not only allows for the simplified study of protein interactions and homolog screening in other organisms, but also facilitates the null mutant construction of essential genes.

INTRODUCTION

Because of their core functions and evolutionary conservation, essential genes are of particular interest and serve as starting points for minimal genome designs (1). However, the difficulties in essential gene mutation have impeded their study. Many approaches have been developed to study gene function, such as temperature-sensitive (TS) mutants (2,3,4), controlled gene expression (5,6), RNA interference (7), and tRNA suppression (8). However, leaky gene expression (9), design difficulties (2,3,4), laborious work (8), and unregulated expression (5,6) have hindered their application.

The eight-gene regulon *yaeT-hlpA-lpxD-fabZ-lpxA-lpxB-rnhB-dnaE* at 4.30 min on the *Escherichia coli* genome, is complex, containing six essential genes that participate in DNA replication, repair, immune reaction, and signal transduction. The *fabZ* gene encodes a (3R)-hydroxymyristol acyl carrier protein dehydratase, and the *lpxD-fabZ-lpxA* gene cluster is involved in lipid A biosynthesis in many bacteria (10,11). *YaeT*, a highly conserved protein (12), is required for gram-negative outer membrane protein assembly (13,14,15,16). In cells lacking *YaeT*, insertion, folding, and oligomerization of outer membrane

proteins are defective (17,18). The *dnaE* gene encodes the alpha-catalytic subunit of the DNA polymerase III holoenzyme. The TS *dnaE* allele blocks DNA synthesis at the initiation stage of DNA replication and elongation

(19,20). Although many studies about this operon have been reported, null mutants have not been obtained.

In this work, a conditional knockout-rescue system for the in-frame deletion of essential genes was established. Using this system, three deletion strains of *yaeT*, *fabZ*, or *dnaE* genes were constructed and the polarity effects of deletion were investigated.

MATERIALS AND METHODS

Strains, Plasmids, Media, and Culture Conditions

Strains and plasmids are listed in Table 1. M63 minimal medium (21) contained 18 amino acids (no methionine or cysteine), thiamine (1 µg/ml), and 0.2% of a specified carbon source (22). For agar plates, 10 g agar were added per liter and both 100 µg/ml ampicillin and 10 µg/ml tetracycline were used for antibiotic resistance selection. Sucrose was added at a concentration of 5%. *E. coli* DH5α

Figure 1. System strategy and structure of eight-gene operon. (A) Strategy. A, chromosomal target essential gene; *tet*, positive marker gene; *sacB*, negative marker gene; X, sample gene. (B) The *yaeT-hlpA-lpxD-fabZ-lpxA-lpxB-rnhB-dnaE* operon structure and the three target genes marked with an asterisk.

Table 1. Strains and Plasmids

Name	Description	Source/Reference
Strains		
DY329	Lam ^r , IN (<i>rrnD-rrnE</i>) 1, <i>rph-1</i> Δ ^{acU169} <i>nadA::Tn10</i> <i>gal490</i> λ <i>cl857</i> Δ(<i>cro-bioA</i>)	(25)
DH5α	F' <i>endA1 hsdR17</i> (<i>r_K-m_K</i>) <i>glnV44 thi-1 recA1 gyrA</i> (<i>Nal^R</i>) <i>relA1</i> Δ (<i>lacIZYA-argF</i>)U169 <i>deoR</i> (Φ80 <i>dlac</i> Δ (<i>lacZ</i>)M15)	(23)
DY1	DY329, Δ <i>yecl:: (PBAD-Flp-sacB-bla)</i> , Suc ^s , Ap ^r	This work
DY2	DY1, Δ (<i>sacB-bla</i>), Suc ^r , Ap ^s	This work
DYSB	DY2, (<i>FRT-sacB-tet-FRT</i>), Suc ^s , Tc ^r	This work
DYAY	DY329, Δ <i>yecl:: (FRT-sacB-tet-FRT)</i> , Suc ^s , Tc ^r	This work
FSYTF	DY329, Δ <i>yaeT:: (FRT-sacB-yaeT-tet-FRT)</i> , Suc ^s , Tc ^r	This work
FSFTF	DY329, Δ <i>fabZ:: (FRT-sacB-fabZ-tet-FRT)</i> , Suc ^s , Tc ^r	This work
FSDTF	DY329, Δ <i>dnaE:: (FRT-sacB-dnaE-tet-FRT)</i> , Suc ^s , Tc ^r	This work
Plasmids		
pEX18Tc	<i>oriT⁺</i> , <i>sacB⁺</i> , gene replacement vector with MCS from pUC18, Suc ^s , Tc ^r	(24)
pPS854	Vector containing two <i>FRT</i> sites and MCS from pUC18, Ap ^r	(24)
pFLP2	Vector containing <i>cl857</i> -controlled FLP encoding sequence, Suc ^s , Ap ^r	(24)
pBAD18	Vector containing PBAD operon, Ap ^r	(5)
pPST	pPS854 with <i>sacB</i> and <i>tet</i> inserted into <i>FRT</i> sites, Tc ^r , Ap ^r	This work
pPST1	pPST, <i>yaeT</i>	This work
pPST2	pPST, <i>fabZ</i>	This work
pPST3	pPST, <i>dnaE</i>	This work
pBFLP	pBAD18, <i>Flp</i>	This work
pBFLPE	pBFLP, <i>gapAp</i>	This work
pBFLPE1	pBFLPE, <i>yaeT</i>	This work
pBFLPE2	pBFLPE, <i>yaeT</i> , <i>hlpA</i> and <i>lpxD</i>	This work
pBFLPE3	pBFLPE, <i>fabZ</i>	This work
pBFLPE4	pBFLPE, <i>lpxD</i> and <i>fabZ</i>	This work
pBFLPE5	pBFLPE, <i>fabZ</i> and <i>lpxAB</i>	This work
pBFLPE6	pBFLPE, <i>dnaE</i>	This work
pBFLPE7	pBFLPE, <i>rnhB</i> and <i>dnaE</i>	This work

cells were grown at 37°C and strains derived from DY329 cells were grown at 32°C.

PCR and Oligonucleotides

PCR (polymerase chain reaction) was performed with *LA Taq* DNA polymerase according to the manufacturer's protocol (TaKaRa Biocompany, Dalian, China). DNA sequences of the PCR primers are listed in Table 2. Lyophilized primers were synthesized by Invitrogen Ltd. (Shanghai, China), dissolved in H₂O, and stored at -20°C. General techniques for plasmid DNA preparation, restriction enzyme manip-

ulation, molecular cloning, and agarose gel electrophoresis were carried out per standard protocols. Restriction endonucleases and ligase were purchased from TaKaRa Biocompany. Plasmid transformations and preparations were done in *E. coli* DH5α cells (23).

Plasmid Construction

The *Flp-sacB* fragment amplified from pFLP2 (24) was inserted into the *EcoRI/HindIII* site of pBAD18 (5) to construct pBFLP, containing the arabinose-controlled *Flp* and *sacB* genes. After *BamHI/HindIII* digestion and ligation, the *gapAp* fragment

replaced the *sacB* gene of pBFLP to produce pBFLPE containing the *gapA* promoter, *RBS* (ribosome binding sequence), and several restriction sites (*SphI*, *SacI*, *SalI*, *KpnI*, *XhoI*). Plasmid pBFLPE1-7 was generated by ligation of *XhoI/SphI*-digested pBFLPE with different cassettes, *yaeT*, *yaeT-hlpA-lpxD*, *fabZ*, *lpxD-fabZ*, *fabZ-lpxAB*, *dnaE*, and *rnhB-dnaE*, which could express these gene(s) constitutively.

pPST was generated by inserting the *EcoRI/EcoRV*-digested *sacB-tet* fragment from pEX18Tc (24) into pPS854, which had been treated identically (24). pPST1-3 containing a *FRT-sacB-target-tet-FRT* cassette was constructed by the ligation of *SspI*-treated *yaeT*, *fabZ*, and *dnaE* fragments into the pre-digested pPST.

Strain Construction

Strains were constructed by homologous recombination as described previously (25). DY1 was generated by replacing the *yecl* gene of DY329 with the *PBAD-Flp-sacB-bla* cassette amplified from pBFLP. DY2 was engineered from DY1 by deleting the marker genes and DY2 was modified to produce DYSB by inserting the *FRT-sacB-tet-FRT* (sb) cassette into the downstream *Flp* gene. DYAY was generated by replacing the *yecl* gene of DY329 with the *FRT-sacB-tet-FRT* (ay) cassette. FSYTF, FSFTF, and FSDTF were constructed separately by substituting the *yaeT*, *fabZ*, and *dnaE* genes of DY329 for the *FRT-sacB-fabZ-tet-FRT* (FSYTF), *FRT-sacB-fabZ-tet-FRT* (FSFTF), and *FRT-sacB-dnaE-tet-FRT* (FSDTF) fragments.

System Efficiency Detection

pPST and pBFLPE were transformed into DY2 and DYAY, respectively. The two transformants and DYSB were inoculated into 1 ml of M63 medium and cultured at 32°C until the OD₆₀₀ reached approximately 0.3. Cells were harvested and spread on M63 plates containing ampicillin and arabinose of different concentrations (from 0.05% to 0.5%). Control experiments were performed with glycerol as the inducer. The following day, 100 colonies were picked from each plate and screened in

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Table 2. Sequences of PCR Primers

Name	Sequence	Description
1	AAGGTCGAATTCactatTTTactctggcgg	Amplification of <i>Flp-sacB</i> cassette
2	TTGCATAAGCTTcggcattttctttgctgt	
3	GGGGGGGATCCgctcacatctcactttaatcg	Amplification of <i>gapAp</i> fragment
4	GGGGGAAGCTTGCATGCGAGCTCGTCGACGGTACCTCGAGCCACCAgctattttagtg	
5	AAGGTCGAATTCggagacatgaacgatgaacatc	Amplification of <i>sacB-tet</i> cassette
6	TTGCATGATATCccattcaggtcgaggtgg	
7	CAGACCAATGGCTGCCAGGGCTTTAGTAAATTTGTGCA Tttatgacaactgacggctac	Amplification of <i>PBAD-Flp-sacB-bla</i> cassette
8	TCCGAAATGTGATCGTGGAGTCAATTCTGACGATGATcagttaccaatgcttaacagtg	
9	AGACTACCTTTTCATCCTACATAAATAGACGCATATAAatcatcgctcag aattgactccac	Amplification of <i>sacB-bla</i> deletion cassette
10	cgctgaatgccgatcaggaa	
11	GTACTAGACTACCTTTCATCCTACATAAATAGACGCATATgagctcgaattggggatctt	Amplification of <i>FRT-sacB-tet-FRT</i> (sb) cassette
12	GTCCGAAATGTGATCGTGGAGTCAATTCTGACGATGATgaattagcttcaaaagcgctct	
13	CCAGACCAATGGCTGCCAGGGCTTTAGTAAATTTGTGATgagctcgaattggggatctt	Amplification of <i>FRT-sacB-tet-FRT</i> (ay) cassette
14	GTCCGAAATGTGATCGTGGAGTCAATTCTGACGATGATgaattagcttcaaaagcgctct	
15	GGGGGGAATATTAGGAGGCTCGAGatgtctgaaccacgttctgt	Amplification of <i>dnaE</i> fragment
16	GGGGGGAATATTGCATGCTtagtcaaactccagttccacc	
17	CTTTGGCCTGTCAAACGCGCACTGGGACTTGC GTCCTGgagctcgaattggggatctt	Amplification of <i>FRT-sacB-dnaE-tet-FRT (FSDTF)</i> cassette
18	TCAAATCAAGGAAATTCAGACTCATAGTATTCTGTGagaattagcttcaaaagcgctct	
19	actgggacttgcgtcctga	Detection primer of chromosomal <i>dnaE</i>
20	ctcatcctgacggctaacc	
21	GGGGGGAATATTAGGAGGCTCGAGatggcgatgaaaaagttgtcta	Amplification of <i>yaeT</i> fragment
22	GGGGGGAATATTGCATGCTtaccaggtttaccgatgttaa	
23	CTCGTTATGAGAGTTAGTTAGGAAGAACGCATAATAACGgagctcgaattggggatctt	Amplification of <i>FRT-sacB-yaeT-tet-FRT (FSYTF)</i> cassette
24	TCATCGCTACACTACCACTACATTCCTTTGTGGAGAACACgaattagcttcaaaagcgctct	
25	aggaagaacgcataataacg	Detection primer of chromosomal <i>yaeT</i>
26	cattcctttgtggagaacac	
27	GGGGGGAATATTAGGAGGCTCGAGttgactactaacactcatactctgc	Amplification of <i>fabZ</i> fragment
28	GGGGGGAATATTGCATGCTcaggcctcccggctacga	
29	TTATTATTGTCGTTTCTTATATTTTGACAGGAAGAGTATCgagctcgaattggggatctt	Amplification of <i>FRT-sacB-fabZ-tet-FRT (FSFTF)</i> cassette
30	TGGCGGTTGGATGCACAAAGGCGGATTATCAATCACGTGagaattagcttcaaaagcgctct	
31	cttatattttgacaggaagagtac	Detection primer of chromosomal <i>fabZ</i>
32	gcggatttatcaatcacgta	
33	GGGGGGCTCGAGatggcgatgaaaaagttgtcta	Amplification of <i>yaeT-hlpA-lpxD</i> fragment
34	GGGGGGCATGCTtagtctgttgattaaccttgcg	
35	GGGGGGCTCGAGatgcctcaattcgactggc	Amplification of <i>lpxD-fabZ</i> fragment
36	GGGGGGCATGCTcaggcctcccggctacga	
37	GGGGGGCTCGAGttgactactaacactcatactctgc	Amplification of <i>fabZ-lpxAB</i> fragment
38	GGGGGGCATGCTcattgtgttaactccagaac	
39	GGGGGGCTCGAGatgatcgaattgtttatccgc	Amplification of <i>rnhB-dnaE</i> fragment
40	GGGGGGCATGCTtagtcaaactccagttccacc	
41	ccgcttgacgctgcgtaag	Detection primer of plasmid DNA
42	ctcatccgcaaaacagcc	

All primers are listed from 5' to 3'. The odd and even primers represent forward and reverse primers, respectively. The underlined bases are introduced to create restriction sites for DNA cloning. The lowercase bases match the template, but the capitalized bases are added for either DNA cloning or homologous recombination.

Table 3. System Excision Efficiency under Different Conditions

Conditions	DY2 with pPST	DYAY with pBFLPE	DYSB
0.05% arabinose/glycerol	41%/0%	46%/0%	37%/0%
0.1% arabinose/glycerol	82%/0%	88%/0%	79%/0%
0.2% arabinose/glycerol	98%/0%	100%/0%	94%/0%
0.3% arabinose/glycerol	97%/0%	100%/0%	92%/0%
0.4% arabinose/glycerol	97%/0%	100%/0%	90%/0%
0.5% arabinose/glycerol	98%/0%	99%/0%	93%/0%

One hundred colonies were tested in each experiment and the percentages indicate the number of Suc^r/Tet^s colonies/100, followed by the calculated excision efficiency.

Table 4. Deletion-complementation Efficiency of *yaeT*, *fabZ*, and *dnaE* Genes

Plasmids	Transformants	Colony Number	Frequency
(A) FSYTF			
pBFLPE	(-)	0	/
pBFLPE1	(+)	354	20/20 (100%)
pBFLPE2	(+)	339	20/20 (100%)
pBFLPE3	(-)	0	/
(B) FSFTF			
pBFLPE	(-)	0	/
pBFLPE2	(-)	0	/
pBFLPE3	(+)	398	20/20 (100%)
pBFLPE4	(+)	392	20/20 (100%)
pBFLPE5	(+)	383	20/20 (100%)
(C) FSDTF			
pBFLPE	(-)	0	/
pBFLPE5	(-)	0	/
pBFLPE6	(+)	417	20/20 (100%)
pBFLPE7	(+)	403	20/20 (100%)

Transformants were selected for Amp^r, Suc^r, and Tet^s. (-), no colonies, (+), positive colonies. The next column shows the positive colony number with 100 ng plasmid DNA transformed. In general, 20 colonies were selected in each experiment for PCR and the fractions indicate the number of colonies in which the chromosomal target genes were deleted/20, followed by the calculated frequency in %.

parallel on plates containing sucrose and tetracycline, respectively.

Construction of *yaeT*, *fabZ*, or *dnaE* In-frame Deletion Strains

FSYTF was transformed with pBFLPE, pBFLPE1, pBFLPE2, and pBFLPE3, respectively. A single colony was picked and cultured to OD₆₀₀ = 0.3; the cells were then harvested, spread on M63 plates containing ampicillin and 0.2% arabinose, and incubated overnight. Control experiments were performed with 0.2% glycerol. Deletion efficiency of the chromosomal *yaeT* gene and compensatory frequency were detected by PCR. FSFTF was transformed with pBFLPE, pBFLPE2, pBFLPE3, pBFLPE4, and pBFLPE5, while FSDTF was transformed with

pBFLPE, pBFLPE5, pBFLPE6, and pBFLPE7 separately. These constructs were induced and detected as described above.

RESULTS AND DISCUSSION

In this work, a conditional knockout-rescue system was established to construct an in-frame deletion strain of certain essential genes (Figure 1A). Two *FRT* sites and positive/negative marker genes were integrated into the *E. coli* DNA, flanking the essential target genes. A plasmid containing arabinose-induced FLP and a constitutively expressed sample gene was transformed into the modified strain. After arabinose induction, cells could survive only when target protein activity was

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provided from a plasmid in trans.

To make this system efficient and suitable for widespread use, the deletion efficiency and the stability of gene expression were considered. We used positive and negative marker genes, *tet* and *sacB*, to detect the excision efficiency of FLP recombinase under different conditions; DY2 with pPST, DYAY with pBFLPE, and DYSB were all used to study position effects. After induction with arabinose of different concentrations, the *Suc^r/Tet^s* colonies were numbered to calculate excision efficiency. Results showed that no excision was detected with glycerol. Excision efficiency increased gradually with increasing arabinose concentrations and reached a maximum at 0.2%. Among the three strains, DYAY with pBFLPE exhibited the highest excision efficiency of nearly 100% (Table 3). This is consistent with previous work stating that the excision reaction rate exhibited strong dependence on FLP concentration when FLP was present in excess relative to available recombination sites (26). These experiments were performed in M63 medium as we found that the excision efficiency in M63 medium was higher than that in LB medium (data not shown), perhaps due to the more tightly regulated PBAD expression in LB medium than in minimal medium (22). All these results indicate that arabinose-controlled FLP is tightly regulated and its excision efficiency is sufficiently high for the study of essential genes.

We further selected three essential genes, *yaeT*, *fabZ*, and *dnaE*, as targets (Figure 1B), to analyze the null mutant construction feasibility of this system. Three strains, FSYTF, FSFTF, and FSDTF, were obtained by flanking the target with both *FRT* sites and marker genes without changing the promoter(s).

To construct a null mutant of the *yaeT* gene, several plasmids were transformed into FSYTF. After arabinose induction, positive colonies (*Suc^r/Tet^s*, +) were only observed in strains containing a second-copy *yaeT* gene, but no colonies were found in other transformants under any tested conditions (*Suc^r/Tet^s*, -). Additionally, PCR results showed that the chromosomal *yaeT* gene was absent and cells could

only grow when a copy was provided in trans. Other genes failed to complement this deletion (Table 4A). When *fabZ* and *dnaE* genes were used as targets, similar results were found (Table 4, B and C). As expected, no positive transformants were observed in any strains when glycerol was used as an inducer (data not shown). These results indicated that this system could be used for the deletion of essential genes with high efficiency and accuracy. Note that the number of positive transformants (+) could vary from a few to several thousand depending on the quality and amount of plasmid DNA used, the quality of the electrocompetent strains, as well as the electroporation chambers used. But no transformants could be observed in cases indicated by (-).

When we study an operon containing essential genes, one notable question is the polar effects of essential gene deletion on the neighboring genes. To detect whether polar effects exist, we transformed each of the three strains not only with a plasmid harboring the appropriate target essential gene, but also with a plasmid carrying the target and the upstream or downstream gene(s). (Table 1). No differences on cell viability were found (Table 4), which demonstrates that the deletion of the three essential genes (*yaeT*, *fabZ*, or *dnaE*) did not exert significant polarity effects on neighboring genes.

In summary, this conditional knockout-rescue system completely removed the essential targets from the *E. coli* genome without affecting the function of other genes in the same operon. True null mutants can both eliminate leaky expression and be used in functional studies of genes expressed at low levels. Additionally, the interactions of remaining protein fragments with complementing proteins or other proteins are not possible. Moreover, the target genes expressed under their own promoters before deletion can avoid improper gene expression and undetectable compensatory mutations. Therefore, this system provides a new way for the functional study of essential genes in a complex operon and for screening homologs (e.g., DNA library screening) in other organisms.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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