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Rapid site-directed domain scanning mutagenesis of enteropathogenic *Escherichia coli espD*

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Abbreviations: bp, base pair; EPEC, enteropathogenic *Escherichia coli*; %G+C, percentage guanine plus cytosine DNA content; kb, kilobase; PAGE, polyacrylamide gel electrophoresis; Tm, melting temperature.

ABSTRACT

We developed a rapid mutagenesis method based on a modification of the QuikChange® system (Stratagene) to systemically replace endogenous gene sequences with a unique similar size sequence tag. The modifications are as follows: 1: the length of the anchoring homologous sequences of both mutagenesis primers were increased to 16 - 22 bp to achieve melting temperatures greater than 80°C. 2: the final concentrations of both primers were increased to 5-10 ng/μl and the final concentration of template to 1-2 ng/μl. 3: the annealing temperature was adjusted when necessary from 52°C to 58°C. We generated 25 sequential mutants in the cloned *espD* gene (1.2 kb), which encodes an essential component of the type III secretion translocon required for the pathogenesis of enteropathogenic *E. coli* (EPEC) infection. Each mutation consisted of the replacement of 15 codons (45 bp) with 8 codons representing a 24 bp sequence containing three unique restriction endonuclease sites (*Kpn*I/*Mfe*I/*Spe*I) starting from the second codon. The insertion of the restriction endonuclease sites provides a convenient method for further insertions of purification and/or epitope tags into permissive domains. This method is rapid, site-directed and allows for the systematic creation of mutants evenly distributed throughout the entire gene of interest.

INTRODUCTION

Domain scanning mutagenesis by inserting a signature tag in-frame of a gene of interest is a powerful way to study the domain functions of a gene of interest. Random mutagenesis methods based on transposons are often used for such purposes (1-3). However these methods have two major hindrances. One drawback is that they are time consuming and laborious. For example, we previously invested more than six months to mutate a 1-kb gene (4). Another drawback to random mutagenesis strategies is the uneven distribution of the mutations throughout the gene. As a result, an excess number of mutations must be generated to insure adequate coverage. Here, we report a new strategy to rapidly and systematically generate a large number of in-frame deletion-insertions throughout a gene of interest using a modification of the QuikChange® procedure (Stratagene). The method proved to be fast and economical.

MATERIALS AND METHODS

Plasmid construction

Mutagenesis was performed on the *espD* gene cloned into a minimal vector based on plasmid pACYC184 and

derived from pWSL-17 (4). Ten micrograms of pWSL-17 was digested first with *Ava*I and blunt-ended with mung bean nuclease at room temperature for 20 minutes, DNA was purified and digested with *Sac*II. The 2.2 kb band including the origin of replication and the gene encoding tetracycline resistance was gel purified. The *espD* gene from EPEC strain E2348/69 was PCR amplified for 35 cycles with sense primer 5′-AACCTATTAACGAGTGCACG-3′ and antisense primer 5′-TTAAACTCGACCGCTGAC-3′ (both at 0.4 μM), 1mM MgSO4, 0.2 μM dNTPs, 10 ng of template pJY26 (5), and 2.5 units of *Pfx* DNA polymerase (Invitrogen) at an annealing temperature of 51°C. A 1.2 kb PCR product was gel purified and cloned into pPCR-Script Amp (Stratagene) according to the manufacturer's instructions to generate pQWD1. The *espD* gene was confirmed by sequencing. pQWD1 was digested with *Eco*RI, blunt-ended with mung bean nuclease and purified. Following digestion with *Sac*II, the *espD* gene was gel purified, ligated to the pACYC184 fragment described above and transformed into DH5α to generate pQWD2. The sequences of the junction regions were verified. pQWD2 was transformed into EPEC *espD* mutant strain UMD870 to confirm its function by complementation (6).

Cell transformation

Competent *E. coli* DH5α cells were obtained from Invitrogen. EPEC *espD* mutant strain UMD870 was transformed by electroporation. A single colony was grown to OD_{600} = 0.6, and washed three times with ice-cold 10% glycerol. Plasmid pQWD2 (50-100 ng) was mixed with 45 μl of competent cells, transferred to a cold 1.5 cm electroporation cuvette and pulsed with 1.8 kilovolts using an *E. coli* gene pulser (Bio-Rad).

Modifications of the QuikChange® site-directed mutagenesis kit

The QuikChange® mutagenesis kit (Stratagene catalogue #200518) is commonly used for site-directed mutagenesis to change a single or a few amino acids. To adapt this system for larger deletions and insertions, we first increased the length of the homologous regions flanking the mutated sequence from 10-15 bps to 16-22 bps to obtain predicted melting temperatures (Tm) of 80°C or more. We used the following formula for calculating Tm (7):

Tm = 81.5 + 0.41(%GC) - 675/N, where N is the primer length and the value of %GC is expressed as a whole number. The terms %GC and N apply only to the homologous flanking sequences and not to the sequences that were inserted or deleted.

All mutagenic primers included the following inserted sequence: 5′-GGTACCGCGCAATTGGCGACTAGT-3′ (24 bps), which contains unique *Kpn*I/*Mfe*I/*Spe*I sites (Table 1). Primers were synthesized and PAGE-purified by Integrated DNA Technologies, Inc.

As a second modification of the procedure, the concentration of primers was increased from 2.5 ng/μl to 5-10 ng/μl. The concentration of plasmid template was kept at 1-2 ng/μl.

For convenience we used Maxi-efficient competent cells *E. coli* DH5α cells (Invitrogen) rather than XL-1 blue supercompetent *E. coli* cells and regular LB broth rather than NZY+ broth, as specified by Stratagene.

As a final modification, the PCR products of the mutagenesis reactions were digested with *Dpn*I for 2 hours instead of one hour to insure complete digestion of the parental plasmid.

We typically used the recommended annealing temperature (55°C) except when we did not obtain a product, in which case we varied the annealing temperature from 52°C to 58°C. The number of PCR cycles was kept at 18.

Verification of mutations

Plasmids were extracted from 1 to 6 colonies and digested with unique restriction enzymes (*Kpn*I, *Mfe*I or *Spe*I) and *Sac*II for analysis by 1% agarose electrophoresis to verify size and position. One or two plasmids with the correct restriction map were sent for sequencing (ABI Prism) to confirm the mutations.

Sequence data analysis: The DNAssist software program was used for sequence analysis and primer design.

Table 1: Properties of primers used to create deletion-insertion mutations in the *espD* gene.

Deleted codons	Primer properties: Sequence	Length (nucleotides)	(G+C)%	Tm (°C)	Concentration [ng/µl]
2-16	gtaaataaccggagataactatgGGTACCGCGCAATTGGCGACTAGTgctgctacggctacttcag	42	45%	83.96	5
17-31	cagttgtaaatccagcgcGGTACCGCGCAATTGGCGACTAGTactggctccagacctcac	36	56%	85.55	5
32-46	cagtctgaagtgacttccGGTACCGCGCAATTGGCGACTAGTtggacagaaagtaccgc	35	51%	83.12	5
47-61	gccccatcagcaagtGGTACCGCGCAATTGGCGACTAGTttagtgacgccctctgctg	34	59%	85.76	5
62-76	caccggcaggtcactcaGGTACCGCGCAATTGGCGACTAGTggtggtattagtggtgaag	36	57%	85.96	5
77-91	gaacagagggaacagaaGGTACCGCGCAATTGGCGACTAGTtctcaggtgaataacgttg	36	44%	80.95	5
92-106	gaacagagggaacagaaGGTACCGCGCAATTGGCGACTAGTtctcaggtgaataacgttg	36	44%	80.95	5
107-121	ctctggctatccttatcttGGTACCGCGCAATTGGCGACTAGTactctgttatccctgg	35	46%	80.95	5
122-136	ccatgatgatgatggccGGTACCGCGCAATTGGCGACTAGTaaaaactccaacgagatc	35	46%	80.95	5
137-151	cagagagttgcgagtatgGGTACCGCGCAATTGGCGACTAGTgataataaaacgctggag	36	44%	80.95	5
152-166	ggacaaaataaagcgctgGGTACCGCGCAATTGGCGACTAGTaaagccgaagagaaagc	35	46%	80.95	10[2]
167-181	gctcgaagaacaacaaGGTACCGCGCAATTGGCGACTAGTtttggttggttaggggttg	35	46%	80.95	5
182-196	gtaaaattgttggtcaggtcGGTACCGCGCAATTGGCGACTAGTaacccggcactctgggctg	39	54%	86.25	5
196-211	ctattgcagctatttttGGTACCGCGCAATTGGCGACTAGTgcactgcaaactgcagttg	36	42%	79.85	5
212-226	ctattagtgcaacagcaatgGGTACCGCGCAATTGGCGACTAGTgcgttaaaaacagcag	36	42%	79.85	5
227-241	gcgatgatgctccacagGGTACCGCGCAATTGGCGACTAGTgcaggtattctgacagc	34	56%	84.57	5
242-256	ggcggtctatctcttgccGGTACCGCGCAATTGGCGACTAGTtctaaagttggcgatgtc	36	53%	84.40	5
257-271	ggggtgtcttcacttataGGTACCGCGCAATTGGCGACTAGTaaagtagtcactacgctg	36	44%	80.95	5
272-286	gttggttcaaacatcgtaGGTACCGCGCAATTGGCGACTAGTtcgaaaatttcggcagtg	36	42%	79.85	5
287-301	gacacttttgttgataatgttgcaGGTACCGCGCAATTGGCGACTAGTtctattggtacaactgtg	42	36%	80.07	5
302-316	gtttaactacttcttcgcgcGGTACCGCGCAATTGGCGACTAGTgtgttatcacaggtttctg	39	44%	82.07	5
317-331	gacgcagcgtattataatGGTACCGCGCAATTGGCGACTAGTcaaagtgagtacttaagtc	37	38%	78.75	5
332-346	gctgtggaaaatttaactcgaGGTACCGCGCAATTGGCGACTAGTgctactctcgagctgcaaaac	42	45%	83.96	5
347-361	gcgaaggcagagctggaaaaaGGTACCGCGCAATTGGCGACTAGTtctcagctgatgtctgattc	41	49%	85.04	5
362-376	cgaattatatacagagtgctGGTACCGCGCAATTGGCGACTAGTagcggtcgagtttaag	36	42%	79.85	7.5

[1]Lower case letters represent *espD* sequences while upper case letters represent the inserted sequence.
[2]This primer was not PAGE-purified prior to use.

RESULTS AND DISCUSSION

A modified QuikChange® procedure can be used to engineer systematic insertions and deletions in a cloned gene

Previously, we analyzed the 1-kb *espB* gene of EPEC using the linker scanning mutagenesis kit from New England Biolabs, which is based on *in vitro* Tn*7* random insertions (4). Although we were able to generate 42 mutants using this system, we found the procedure to be laborious and the mutations to be unevenly distributed along the gene. The QuikChange® mutagenesis kit was originally designed for site-directed mutagenesis targeting single or few base pairs in a cloned gene. Having successfully adapted this procedure to insert a variety of purification and epitope tags into permissive sites of the *espB* gene (data not shown), we then applied these modifications to systemically mutate the *espD* gene for analysis. To reduce the cost of primers, we initially attempted to use unpurified primers, but were able to generate products for only one out of three primer pairs attempted. Therefore we subsequently used PAGE-purified primers to increase efficiency. Using this procedure, we systematically substituted a 24-bp linker sequence for sequential 45 bp of endogenous *espD* sequences. In less than two months, we successfully generated 25 insertion/deletion mutants that evenly covered the entire gene (Table 2), leaving no region un-mutated. All the mutants were verified by restriction enzyme digestion and sequencing.

Some regions are more difficult to mutate than others, but all desired mutants were obtained after altering the annealing temperature

We were able to obtain 80% (20/25) of the mutants with just one or two rounds of the procedure. For the other five mutants, we were able to obtain products after three to six attempts by either reducing (4) or increasing (1) the annealing temperature (Table 2). This apparent variation in required annealing temperature was unanticipated

based on primer analysis, but it may depend on physical properties of the particular region of the gene targeted or of the primers. Alternatively it may have been the result of stochastic forces. In other applications, we used an annealing temperature up to 60°C successfully.

Not all the transformants contain the desired mutation

Although the PCR mixture was digested with *Dpn*I at 37°C for two hours instead of one hour, we found that not all of the colonies contained the correct mutation. To analyze the plasmids we made use of one of the unique inserted restriction sites (*Kpn*I, *Mfe*I or *Spe*I and another unique site (*Sac*II) on the vector. Typically we obtained hundreds of transformants on each plate and the majority of those analyzed had the correct digestion pattern (Table 2). Without exception, plasmids with the correct digestion pattern were verified by sequencing to have the desired mutation. In some cases (pQWD21), the desired mutation was obtained from just one colony; while in other cases (pQWD9) up to 14 colonies were analyzed to find three that were correct (Table 2). Overall, 117/147 (80%) of the colonies analyzed were correct. The percentage of correct colonies per mutation varied from 21-100% (mean 85%, median 100%).

Other applications

We intentionally designed our primers with unique restriction endonuclease sites to permit efficient insertion of any desired sequence into sites subsequently found to be permissive. Using this strategy, we have inserted purification and epitope tags into some of these sites (data not shown). Rather than modifying genes for analysis on plasmids, the targeted gene could be cloned on a suicide vector and the resulting mutants could be introduced by homologous recombination for analysis in the native position. In addition to providing a more efficient method to scan domain functions through entire genes of interest, this method may also be used to target specific domains for deletion or swapping. This adaptation may be especially useful in structural biology when it is necessary to remove or replace domains to allow crystallization, or to replace particular domains to disrupt local structure for functional studies. In addition, this procedure may be useful for domain swapping between genes.

Summary and conclusions

We describe an adaptation of the QuikChange mutagenesis procedure that rapidly allowed us to create a set of systematic deletion-insertion mutants spanning the *espD* gene. This procedure has a number of advantages compared to the widely used transposon scanning linker insertion mutagenesis method. First, it is site-directed rather than random, allowing the entire gene to be covered equally. Second, the size and characteristics of the linkers can be engineered instead of fixed. For example, histidine, FLAG or other tags can be easily embedded in the linker and the size of linker or deleted sequence can be changed, making the method flexible and versatile. Lastly, the procedure is more rapid, efficient, and reliable. We intend to analyze the mutated genes by transforming the plasmids into an EPEC *espD* null mutant and assaying phenotypes that require EspD. One drawback of the procedure is the high cost of PAGE-purified primers. We spent $2875.50 for the 25 primer pairs used in this study. However, we feel that this cost is more than offset by labor savings incurred when using much more time-consuming methods.

Table 2: Results of mutagenesis reactions.

		Reaction Conditions		Results		
Plasmid name	Deleted codons	Template concentration [ng/µl]	Annealing temperature (°C)	Rounds required[1]	Clones verified	Clones tested
pQWD10	2-16	2	55	1	6	6
pQWD11	17-31	1	55	1	6	6
pQWD12	32-46	1	55	1	6	6
pQWD13	47-61	1	55	1	5	6
pQWD14	62-76	1	52	3	4	4
pQWD15	77-91	1	55	1	5	6
pQWD16	91-106	1	55	1	6	6
pQWD17	107-121	1	55	1	6	6
pQWD18	122-136	1	55	1	5	6
pQWD19	137-151	1	55	1	6	6
pQWD7	152-166	2	55	1	4	9
pQWD20	167-181	2	53	3	2	6
pQWD5	182-196	1	55	1	6	6
pQWD21	197-211	1	55	1	1	1
pQWD22	212-226	2	55	2	6	6
pQWD23	227-241	1	55	1	2	5
pQWD8	242-256	2	52	4	3	6
pQWD24	257-271	2	55	2	2	2
pQWD25	272-286	2	55	1	6	6
pQWD26	287-301	2	55	2	6	6
pQWD27	302-316	2	55	2	4	5
pQWD28	317-331	2	55	1	5	5
pQWD29	332-346	2	58	6	6	6
pQWD9	347-361	2	52	4	3	14
pQWD30	362-376	2	55	2	6	6

[1]Number of reactions required to obtain desired mutation. Some reactions yielded no clones.

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PROTOCOLS

1. Construction of a plasmid encoding the gene of interest

Clone the gene of interest into a plasmid by standard molecular cloning techniques and transform the plasmid into a Dam+ *E. coli* strain. Confirm the correct DNA sequence of the gene. Verify that the cloned gene retains known function(s). Purify the plasmid, dilute the concentration to 50 ng/μl and store at -20°C.

2. Primer design and preparation

The procedure follows the basic protocol of the QuikChange® kit (for details consult: http://www.stratagene.com/manuals/200518.pdf). Design the primers so that the desired mutation occurs at the center of the primer. The forward and reverse primers should be complementary to each other (i.e. anneal to the same location on opposite strands of the template) and both ends are either G or C. Adjust the length of the primers so that the estimated Tm is greater than or equal to 80°C. The homologous flanking regions on both sides of the inserted sequences should be 16 to 22 bps in length rather than the recommended 10 to 15 bps. The melting temperature is estimated based on the formula below (7):

Tm = 81.5 + 0.41(%GC) - 675/N, where N is the primer length and the %GC is entered as a whole number. Note, %GC and N do not include the insertion or deletion sequence.

The primers are purified by PAGE, dissolved in ultra-pure water to a concentration of 250 ng/μl, and stored at -20°C.

[Note: we purchased PAGE-purified primers. However, protocols for PAGE purification are available and may result in significant cost-savings (8).]

3. Thermal cycling conditions

Add the following components in order in 0.2 ml or 0.5 ml thin walled PCR tubes:
- 5 μl of 10X reaction buffer from the QuikChange kit
- 1 μl or 2μl of dsDNA plasmid template (to yield final concentration of 1-2 ng/μl)
- 1μl of primer 1 (to yield final concentration of 5 ng/μl)
- 1μl of primer 2 (to yield final concentration of 5 ng/μl)
- 1μl of dNTP from the kit

Add 40 μl of autoclaved MiliQ water
Add 1μl of pfuTurbo DNA polymerase (2.5U/μl)
Mix by gentle flicking

Place the tube in Thermal Cycler with a heated lid and program as follows:
- One cycle
- 95°C, 30 seconds
- Eighteen cycles
- 95°C for 30 seconds
- 55°C (or 52°C-58°C) for 60 seconds
- 68°C for 60 seconds/1 kb of plasmid length
- Cool down the reaction on ice for one minute

[Notes: we did not test other thermostable polymerases but found no sequence errors in any of our clones. Thermal cycling was performed using a Gene Engine PTC-200 cycler (MJ Research) which has a ramping speed of 3°C /second.]

4. Digestion of PCR mixture with *Dpn*I

Add 1 µl of DpnI (10 U/µl) to the PCR tube, gently mix and digest at 37°C for two hours. If subsequent plasmid analysis reveals that clones lack the desired mutation, increase the digestion time to three hours.

5. PCR product mixture check (optional)

Load 10 µl of digested mixture on a 1% agarose gel to check for the amplified product. In our experience the mutagenesis will be successful if a weak band is visible and may be successful even if no product is seen.

6. Transformation of competent bacteria

Add 1 µl to 5 µl of the *Dpn*I digested amplification product to 100 µl of Maxi-efficiency competent *E. coli* DH5α cells (Invitrogen cat #18258-012) for transformation (transformation efficiency $> 1 \times 10^9/\mu g$) according to the manufacturer's instructions (http://www.invitrogen.com/content/sfs/manuals/18258012.pdf). Save the rest of the digested mixture at -20°C for future use if needed.

After incubation in SOC media (2% Bacto Tryptone, 0.5% Bacto Yeast Extract, 10mM NaCl, 2.5mM KCl,10mM MgCl, 10mM $MgSO_4$, 20mM glucose) at 37°C for one hour, spread 100 µl on plates containing appropriate antibiotic selection. Centrifuge the remaining 900 µl for 15 seconds at maximum speed and resuspend in 100ul SOC. Spread on plates containing appropriate antibiotic selection. Incubate overnight at 37°C.

7. Analysis of transformants

Isolate plasmids from four to six colonies, digest about 200 ng of DNA with unique enzymes for one hour at 37°C and analyze on a 1% agarose gel. Select one or two plasmids with the correct restriction digestion pattern for sequencing to confirm the desired mutations.