(Figure 2A, right panels). Consistent with this observation, a substantial amount of EGFP-Fn42k was purified from the culture supernatants as described above, indicating improved protein production by the secretion signal.

Binding activity of EGFP-Fn42k to gelatin was analyzed by gelatin affinity chromatography (5). Recombinant EGFP-Fn42k and FITC-labeled native human Fn42k prepared as described previously (4) were loaded onto a gelatin-Sepharose column, and bound fractions were eluted using a urea gradient (0–6 M) and detected by a fluorescence HPLC monitor (model RF535; Shimadzu, Kyoto, Japan). Figure 2B shows the elution profile of recombinant and native Fn42k, indicating that recombinant Fn42k retains the full activity of native Fn42k.

The set of vectors described here can be used to obtain secreted forms of recombinant proteins from insect cell culture with N-terminal $(His)_6$- and EGFP-tags (1). Use of $(His)_6$ tag allows for fast and easy purification of proteins from supernatants of Sf9 cell culture, and the EGFP tag permits early and simple detection of recombinant viruses during the preparation. The presence of a cleavage site between EGFP and the protein of interest enables the isolation of the protein. Because of its ability to produce heterologous proteins rapidly in an eukaryotic environment, the baculovirus gene expression system has been used to produce a number of complex proteins (9). However, a large fraction of recombinant protein produced in insect cells can sometimes be poorly processed and accumulate as aggregates (3). Our results also show that the secretory pathway in insect cells facilitates the appropriate folding and disulfide bond formation necessary for the biological activity of eukaryotic proteins.

REFERENCES

1. **Cha, H.J., N.G. Dalal, V.N. Vakharia, and W.E. Bentley.** 1999. Expression and purification of human interleukin-2 simplified as a fusion with green fluorescent protein in suspended Sf-9 insect cells. J. Biotechnol. *69*:9-17.
2. **Cha, H.J., T. Gotoh, and W.E. Bentley.** 1997. Simplification of titer determination for recombinant baculovirus by green fluorescent protein marker. BioTechniques *23*:782-786.
3. **Hsu, T.A. and M.J. Betenbaugh.** 1997. Coexpression of molecular chaperone BiP improves immunoglobulin solubility and IgG secretion from *Trichoplusia ni* insect cells. Biotechnol. Prog. *13*:96-104.
4. **Ingham, K.C., S.A. Brew, and M.M. Migliorini.** 1989. Further localization of the gelatin-binding determinants within fibronectin. Active fragments devoid of type II homologous repeat modules. J. Biol. Chem. *264*:16977-16980.
5. **Isaacs, B.S., S.A. Brew, and K.C. Ingham.** 1989. Reversible unfolding of the gelatin-binding domain of fibronectin: structural stability in relation to function. Biochemistry *28*:842-850.
6. **Lippincott-Schwartz, J., E. Snapp, and A. Kenworthy.** 2001. Studying protein dynamics in living cells. Nat. Rev. Mol. Cell Biol. *2*:444-456.
7. **O'Reilly D.R., L.K. Miller, and V.A. Luckow.** 1992. Post-translational modification, p. 216-236. *In* Baculovirus Expression Vectors: A Laboratory Manual. W.H. Freeman and Company, New York.
8. **Patel, R.S., E. Odermatt, J.E. Schwarzbauer, and R.O. Hynes.** 1987. Organization of the fibronectin gene provides evidence for exon shuffling during evolution. EMBO J. *6*:2565-2572.
9. **Possee, R.D.** 1997. Baculoviruses as expression vectors. Curr. Opin. Biotechnol. *8*:569-572.
10. **Wilson, L.E., N. Wilkinson, S.A. Marlow, R.D. Possee, and L.A. King.** 1997. Identification of recombinant baculoviruses using green fluorescent protein as a selectable marker. BioTechniques *22*:674-681.

We would like to thank Shelesa Brew for preparation of FITC-labeled Fn42k fragment. This work was supported by National Institutes of Health grant no. HL21791 to K.C.I. Address correspondence to Dr. Yasuhiro Katagiri, National Heart, Lung, and Blood Institute, National Institutes of Health, Building 10, Room 8C106, 9000 Rockville Pike, Bethesda, MD 20892, USA. e-mail: katagir@helix.nih.gov

Received 4 December 2001; accepted 28 March 2002.

Yasuhiro Katagiri and Kenneth C. Ingham
American Red Cross Holland Laboratory Rockville, MD, USA

Optimization of Fusion PCR for In Vitro Construction of Gene Knockout Fragments

BioTechniques 33:26-32 (July 2002)

Some eukaryotic and prokaryotic organisms such as *Saccharomyces cerevisiae* and *Synechocystis* can spontaneously take up foreign DNA and integrate it into their genomes via homologous recombination (5). This property provides the opportunity to knock out genes in these model organisms by transformation with linear DNA, in which a selectable marker is flanked by a chromosomal sequence that surrounds the targeted gene. For this reason, fusion PCR was developed to rapidly construct this kind of fragment in vitro (1). Here we describe our efforts to optimize fusion PCR for the construction of gene knockout fragments in the widely used cyanobacterial model organism, *Synechocystis* sp. PCC 6803. In this study, the genomic sequences of *Synechocystis* were obtained through CyanoBase (http://www.kazusa.or.jp/cyano/cyano.html). All primers were designed to amplify the gene-flanking segments through a Web-based program (http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer) (Table 1). Each primer was 23–26 bases in length, with a T_m of 58°C–62°C. The plasmids serving as a source of template used to amplify the selectable cassettes of chloramphenicol, kanamycin, and streptomycin/spectinomycin, respectively, were members of the set of plasmids produced by the Wolk group (2).

For the amplification of flanking regions of a target gene, the primers distal from the selectable marker insertion site were simple primers complementary to the target sequence. However, the primers directly adjacent to the insertion site were chimeric: their 5′-ends were complementary to the primers used to amplify the selectable gene and their 3′-ends were complementary to the target sequence. The products of this primary amplification were purified by electrophoresis using 1% low-melting agarose. Target bands were excised and stored in 1.5 mL Eppendorf® tubes at

Table 1. Sequences of Primer Pairs Used to Amplify Flanking Regions of Target Genes in *Synechocystis* sp. PCC 6803 and Selectable Markers

Primer	Sequence (5′→3′)[a]
sll0247[b]	
upstream	ATCAATTACTTCCAGCACCACGTC
	GTTTGTTCGCCCAGCTTCTGTATGATAGGTTTGCACAGA-ATTGCCTC
slr1595	
upstream	AATACCATCACCGACCCATTTAAGG
	GAAGACGAAAGGGCCTCGTGATACTATTGGTGTCCATC-GTTGTGATGC
downstream	**GATGAATGGCAGAAATTCGAAAGC**CCCCAGCCCATTAA-AATCCATTA
	CATTACCCTGGCGACTATTTACCAA
streptomycin/	CATACAGAAGCTGGGCGAACAAAC
spectinomycin	TTGTGTAGGGCTTATTATGCACGC
chloramphenicol	GTATCACGAGGCCCTTTCGTCTTC
	GCTTTCGAATTTCTGCCATTCATC

[a]Boldface indicates homology to selectable marker.

[b]Gene identification numbers as in CyanoBase (http://www.kazusa.or.jp/cyano/cyano.html).

-20°C before usage. The frozen gel slices were centrifuged at 12 000× *g* for 3 min, and the resulting supernatants were used as templates in fusion PCR experiments that exhibit differential performance, depending on the combinations of templates (Figure 1).

To assure the fidelity of the amplified sequence, a proofreading DNA polymerase was generally employed to amplify the flanking regions of the target gene and selectable cassette (1). As mentioned above, the additional sequences mediating the follow-up fusion PCR are added to the 5′-end of one primer of each primer pair for the amplification of the flanking regions. This additional sequence may cause PCR difficulty. In some cases, the desired products are not abundant enough to be visible on the gel, despite extreme care in the design of the primers and execution of the reaction. One solution is to use PCR enhancers such as betaine, DMSO, formamide, or glycerol to improve the match of primer and template during the annealing process. Therefore, we evaluated the effects of these PCR enhancers on the production of the chimeric products. We identified that Platinum® *Pfx* DNA polymerase in combination with its enhancer (both from Invitrogen, Carlsbad, CA, USA) were ideal candidates for this application. The enzyme is supplied with an enhancer, a co-solvent purported to have no negative effects on the proofreading capacity of the *Pfx* DNA polymerase, according to the manufacturer's tests (3). The additional but important advantage is that the enzyme produces a high yield of products compared to other proofreading DNA polymerases such as *PfuTurbo*™ (Stratagene, La Jolla, CA, USA). Little enhancer (1–3×) was often enough to achieve the success of difficult amplifications (Figure 2A). The high yield of the products was obtained with an optimized concentration of the enhancer. As a result, enriched templates after gel purification greatly improved the following fusion PCR. Individual amplification reactions contained 1× *Pfx* amplification buffer, 0.2 mM each dNTP, 1 mM $MgSO_4$, 1 µM each primer, 500 ng template of chromosomal DNA, 1–3× enhancer, and 2.5 U Platinum *Pfx* DNA polymerase in a final volume of 100 µL. The cycling conditions started with a denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, and 68°C for 1 min/kb. A final extension step at 68°C for 10 min was performed. Fewer cycles (e.g., 25 versus 35) can also be used to minimize the error frequency but often at

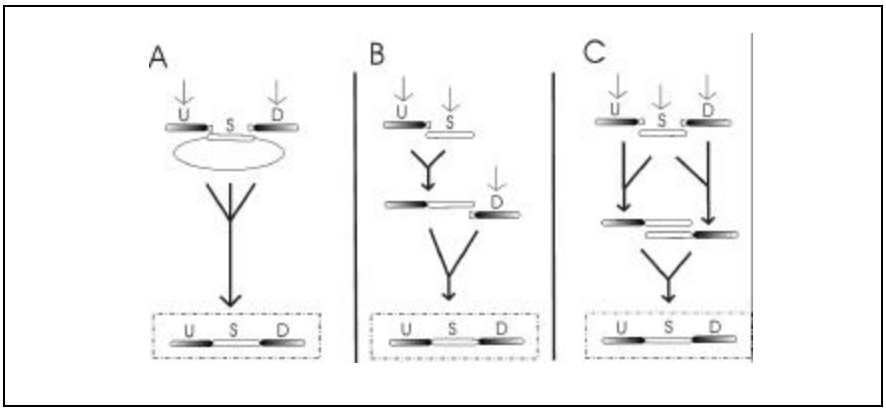

Figure 1. Diagram of fusion PCR methods for in vitro construction of gene knockout fragments developed in this study and elsewhere. The gene knockout fragment consists of three parts: two PCR-amplified products with sequences homologous to the flanking regions of the targeted gene to be replaced, flanking a selectable marker gene such as an antibiotic resistance cassette (U and D, up- and downstream flanks of the target gene, respectively. S, selectable marker.) The flanking regions include approximately 24 bases of sequence introduced through amplification with bipartite primers having homology to the selectable marker gene at their 5′-end. Method (A) uses two standard primary reactions (thin arrows) to produce the flanking regions upstream and downstream from the chromosomal DNA. These fragments are then mixed with a plasmid carrying the selectable marker gene and, in one fusion PCR (thick arrows), are fused to the selectable marker. The second method (B) employs the same modified flanks and a PCR-amplified selectable marker gene but uses them in two separate fusion reactions. Using the product of the fusion of the upstream flank to the selectable marker as template, the downstream flank is introduced through a second fusion event. The final method described here (C) uses the same primary amplified products as in (B) and three fusion events. The first two are the separate fusions of each flank to the selectable marker. The products of these two reactions are then combined for a final fusion event, again depending on the homologous sequence, which includes the entire selectable marker gene.

some sacrifice in yield. The orange band of products from the 100-µL reaction was often visible on the gel, containing 0.5 µg/mL ethidium bromide after electrophoresis. Therefore, the target fragment could be readily excised without the exposure of UV light.

For the fusion PCR step, *Taq* DNA polymerase (Promega, Madison, WI, USA) is generally employed because proofreading DNA polymerase is sensitive to template sources containing agarose and electrophoresis buffer (1). We compromised by using mixed DNA polymerases that keep 3′–5′ proofreading activity and improve the yield at the same time (Figure 2B). The optimized fusion PCR mixture contained 1× *Taq* DNA amplification buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, and 1% Triton® X-100), 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 1 µM each primer, 5 µL each purified template, 1.25 U *Taq* DNA polymerase, and 1.25 U Platinum *Pfx* DNA polymerase in a final volume of 100 µL. The cycling conditions started with a denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min/kb. Fewer cycles can be used to minimize the error frequency. A final extension step at 72°C for 10 min was performed. The amplified fragments were collected by ethanol precipitation and dissolved in sterile water for use in transformation. It is worthwhile to mention that, according to our tests, high-yield *Pfx* DNA polymerase alone could produce a reasonable number of fusion fragments, even in the presence of the template sources containing agarose and electrophoresis buffer. Therefore, the proofreading enzyme alone could be employed to complete all steps of fusion PCR for specific applications.

In the primary amplification, one flanking sequence is fused to a selectable marker, and a second one is subsequently fused to the purified intermediate fragment (1). However, this is not the optimal solution because the yield of the final fused fragment is still quite low, often as a result of an unstable match between the two templates through approximately 24 bases of the introduced primer sequence (Figures 1B and 2B). We found that the yield of the final fused fragment was much higher when both intermediate fusion fragments were used as the templates for a final fusion PCR [i.e., the two flanking sequences were separately fused to a selectable marker, and then both purified intermediate fragments served as templates for the final fusion PCR (Figures 1C and 2B)]. The entire sequence of the antibiotic cassettes can be annealed when two intermediate fragments serve as templates in the final fusion PCR. Transformation with linear DNA generally demands a large number of DNA fragments. Moreover, the fragments produced in high-yield PCR can be divided into aliquots for later use. Therefore, it is necessary to improve the yield of desired fragments in the final fusion PCR.

Recently, another method was developed to perform fusion PCR (4,6). This alternative utilizes two purified flanking sequences combined with a plasmid carrying the selectable marker to serve as the templates for one-step fusion PCR (Figure 1A). It seems to be a simple strategy because only one round of fusion PCR is required. We tested the method with three plasmids carrying chloramphenicol, kanamycin, and streptomycin/spectinomycin resistance genes, respectively. However, none of the desired fragments was visible on the gel for two of the three plasmid templates. Even when PCR was successful, the yield of desired fragments was much lower for this combination of templates (Figure 2B). Moreover, there were more nonspecific products. This kind of fusion PCR could be partially improved by increasing the concentrations of plasmid templates. Apparently,

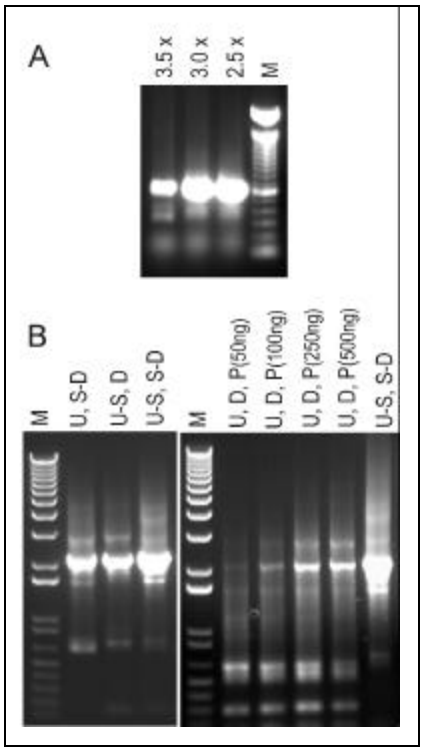

Figure 2. Optimization of fusion PCR steps for in vitro construction of gene knockout fragments. An equal volume of each 100-µL PCR was separated by size on an agarose gel and stained with ethidium bromide. (A) The amplification of the upstream flanking region of *sll0247* in *Synechocystis* with Platinum *Pfx* DNA polymerase and 2.5, 3.0, and 3.5× PCR enhancer supplied (Invitrogen). M, 100-bp DNA ladder (Invitrogen). (B) Final fusion PCR for the construction of *slr1595* knockout fragment with different combinations of templates. Mixed *Taq* and *Pfx* DNA polymerases were used. M, 1 Kb Plus DNA Ladder™ (Invitrogen); U and D, up- and downstream flanking regions of *slr1595*; S, chloramphenicol resistance cassette; P, plasmid providing chloramphenicol cassette with the amount of plasmid indicated in parentheses; and U-S and S-D, fused intermediate fragments.

the poor PCR performance is due to the unstable matching of the templates through only 24 bases of introduced primer sequence. The other drawback to this method is that it requires two rare fusion events to occur simultaneously for success. Whether this fusion PCR method works may largely depend on the sequences of gene-flanking regions and the plasmid providing the selectable marker.

Colony PCR has become a popular method to monitor the segregation of gene disruption after transformation. Unfortunately, some eukaryotic and prokaryotic organisms such as *S. cerevisiae* and *Synechocystis* possess cell walls, causing increased difficulty in releasing genomic DNA during colony PCR. It is necessary to optimize colony PCR to assure the constant performance and increase its sensitivity to confirm complete segregation. For this reason, we tested several conditions to improve colony PCR in *Synechocystis*. Figure 3 shows that even when *Synechocystis* colonies were pretreated with Lyse-N-Go™ PCR reagent (Pierce Chemical, Rockford, IL, USA), the yields of products remained low. In contrast, we found that the colony PCR of *Synechocystis* was markedly improved with the use of Herculase® DNA polymerase buffer (Stratagene) (Figure 3). The addition of DMSO could further increase the yield of products (Figure 3). The Herculase amplification buffer greatly enhances the colony PCR of the bacterium. The combination of Herculase buffer with other mixed enzymes such as *Taq* and *Pfx* DNA polymerases can also produce high yields of colony PCR products in *Synechocystis*; however, we have not yet found any other suitable amplification buffer as effective as Herculase buffer. We cannot define which substance in the buffer eases difficult colony PCR because the components of the buffer are not available from the supplier. We also tested the YieldAce™ DNA polymerase system (Stratagene) but found no improvement. Individual amplification reactions contained 1× Herculase amplification buffer, 0.2 mM each dNTP, 1 µM each primer, 3–6 colonies of bacterium, 2.5 U Herculase or the same units of other mixed enzymes such as *Taq* and Platinum *Pfx* DNA polymerases with a ratio of 1:1, and 0%–3% DMSO in a final volume of 50 µL. The cycling conditions started with a denaturation step at 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, 80°C for 15 s, 65°C for 15 s, 55°C for 1 min, 65°C for 15 s, 72°C for 1 min/kb, and 85°C 15 s. We performed a final cycle of 94°C for 1 min, 55°C for 1 min, and 72°C for 10 min.

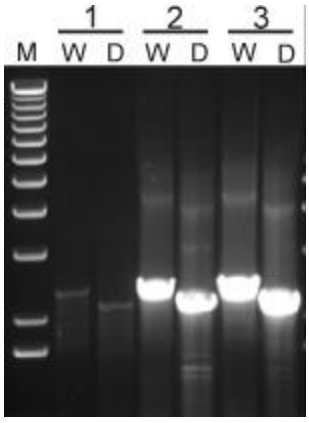

Figure 3. Colony PCRs of *Synechocystis* with *Taq* or Herculase DNA polymerases. An equal volume of each 100-µL PCR was separated by size on an agarose gel and stained with ethidium bromide. (1) *Taq* DNA polymerase. Colonies were treated with Lyse-N-Go PCR reagent before PCR. (2) Herculase DNA polymerase. (3) Herculase DNA polymerase and 3% DMSO. M, 1 Kb Plus DNA Ladder; W, wild-type; and D, deletion mutant in which *slr1595* was substituted with a chloramphenicol resistance cassette.

In summary, mixing *Taq* and proofreading DNA polymerases can improve yield and sequence fidelity in the application of fusion PCR. The match of templates is much more stable when two intermediate products with long stretches of overlapping homology serve as the templates for final fusion PCR, thereby specifically enhancing the synthesis of desired fragments. For difficult colony PCR of bacteria that possess cell walls, combining the Herculase amplification buffer and mixed DNA polymerases can greatly enhance colony PCR performance.

REFERENCES

1. **Amberg, D.C., D. Botstein, and E.M. Beasley.** 1995. Precise gene disruption in *Saccharomyces cerevisiae* by double fusion polymerase chain reaction. Yeast *11*:1275-1280.
2. **Elhai, J. and C.P. Wolk.** 1988. A versatile class of positive-selection vectors based on the nonviability of palindrome-containing plasmids that allows cloning into long polylinkers. Gene *68*:119-138.
3. **Lackovich, J., J.E. Lee, P. Chang, and A. Rashtchian.** 2001. Measuring fidelity of Platinum *Pfx* DNA polymerase. Focus *23*:6-7.
4. **Taroncher-Oldenburg, G. and G. Stephanopoulos.** 2000. Targeted, PCR-based gene disruption in cyanobacteria: inactivation of the polyhydroxyalkanoic acid synthase genes in *Synechocystis* sp. PCC6803. Appl. Microbiol. Biotechnol. *54*:677-680.
5. **Vermaas, W.F.** 1998. Gene modifications and mutation mapping to study the function of photosystem II. Methods Enzymol. *297*:293-310.
6. **Wach, A.** 1996. PCR synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. Yeast *12*:259 265.

This work was funded by the National Science Foundation. *Address correspondence to: Robert L. Burnap, Department of Microbiology & Molecular Genetics, 307 Life Sciences East, Oklahoma State University, OK 74075, USA. e-mail: burnap@biochem.okstate.edu*

Received 16 January 2002; accepted March 2002.

Hong-Liang Wang, Bradley L. Postier, and Robert L. Burnap
Oklahoma State University
Stillwater, OK, USA

Cyclosporin A Improves the Selection of Cells Transfected with the Puromycin Acetyltransferase Gene

BioTechniques 33:32-36 (July 2002)

The genetic manipulation of eukaryotic cells often relies on selection of the transfected or infected cells with a gene that confers resistance to cytotoxic drugs. An increasingly commonly used cytotoxic drug for this purpose is puromycin, an inhibitor of protein biosynthesis that is inactivated by puromycin