Gene 434 (2009) 43–49

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Methods paper

Reliable fusion PCR mediated by GC-rich overlap sequences

Kamonchai Cha-aim [a], Tomoaki Fukunaga [c], Hisashi Hoshida [b], Rinji Akada [c,*]

[a] Department of Applied Chemistry, Graduate School of Science and Engineering, Yamaguchi University, Tokiwadai, Ube 755-8611, Japan
[b] Innovation Center, Yamaguchi University, Tokiwadai, Ube 755-8611, Japan
[c] Department of Applied Molecular Bioscience, Graduate School of Medicine, Yamaguchi University, Tokiwadai, Ube 755-8611, Japan

ARTICLE INFO

Article history:
Received 27 June 2008
Received in revised form 7 October 2008
Accepted 9 December 2008
Available online 29 December 2008

Received by M. Di Giulio

Keywords:
Overlap extension
Annealing
Recombinant DNA
Gene targeting
Saccharomyces cerevisiae

ABSTRACT

Recombinant DNA technology largely depends upon *Escherichia coli* plasmid construction via restriction enzyme digestion and DNA ligation. Overlap extension PCR is another simple technique for constructing recombinant DNA but is not commonly used. This is likely due to the inefficiency of fusion after the annealing of overlaps that are generally designed from authentic sequences in the DNA fragments. In our current study, we describe the development of novel overlap sequences that can be used for the construction of fusion DNA fragments, including the one-step fusion of three fragments in a single PCR and also for in-frame fusions. Novel poly G or C stretches showed strong and also specific annealing to the complementary sequences in the fusion PCR. This DNA fusion method is thus both a simple and versatile recombinant DNA technique.

1. Introduction

Recombinant DNA technology has developed in parallel with the construction of *Escherichia coli* plasmid vectors containing DNA sequences that are desired for a range of molecular biology applications. These sequences include drug-resistance genes, *E. coli* replication origins, promoters, terminators, expression markers, and multicloning sites (Wu et al., 1989; Ausubel et al., 1999; Sambrook and Russell, 2001). Restriction digestion of DNA and subsequent ligation to desired sites within vectors is a well established recombinant DNA procedure. Recombination-targeted sequences have also recently been used for the construction of recombinant plasmids, known as Gateway™ technology (Alberti et al., 2007; Freuler et al., 2008). Alternatively, DNA fragments can be fused by overlap extension PCR (Heckman and Pease, 2007).

Overlap extension was developed in the late 1980s (Ho et al., 1989; Horton et al., 1989) and used not only for constructing recombinant DNAs but also for site-directed mutagenesis and the cloning of spliced segments. Theoretically, PCR-driven overlap extension is a simple procedure but it has not been commonly used when compared with recombinant plasmid construction. This may be due to the technical requirements for the adjustment of PCR conditions, the cleanliness of the initial DNA template, and the accurate design of primers (Heckman and Pease, 2007). However, if recombinant DNA constructs could be readily generated by PCR, this would provide a very useful tool for genome-wide analyses using large numbers of DNA fragments (Winzeler et al., 1999; Ghaemmaghami et al., 2003; Huh et al., 2003).

To more easily apply overlap extension PCR to the generation of conventional DNA vectors, we expected that intense annealing of overlapping sequences of target fragments should occur under simple PCR conditions. In general, the primer length is known to be an important factor in annealing efficiency but we present our findings herein that short G/C stretches can effectively produce specific annealing in a fusion PCR. The aims of our current study were to show 1) sequences that would be effective for overlap extension PCR, 2) sequences that would enable a successful one-step fusion PCR of multiple DNA fragments, and 3) overlapping sequences that could be used for the in-frame fusion of two functional genes. Significantly, the annealing sequences we describe in this present study can be applied to any molecular methodology that requires the joining of two or more DNA sequences.

2. Materials and methods

2.1. Yeast strains, primers, and plasmids

Saccharomyces cerevisiae strains BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), BY4704 (*MAT***a** *ade2Δ::hisG his3Δ200 leu2Δ0 met15Δ0 trp1Δ63*) and BY4743 (*MAT***a**/*MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0*

Abbreviations: *E. coli*, *Escherichia coli*; kb, 1000 bp; nt, nucleotides; RNase, ribonuclease; SDS, sodium dodecyl sulfate; GFP, green fluorescence protein.

* Corresponding author. Department of Applied Molecular Bioscience, Yamaguchi University Graduate School of Medicine, Tokiwadai, Ube 755-8611, Japan. Tel.: +81 836 85 9292; +81 836 85 9201.
E-mail address: rinji@yamaguchi-u.ac.jp (R. Akada).

0378-1119/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.gene.2008.12.014

met15Δ0/+ lys2Δ0/+) were used in this study (Brachmann et al., 1998). The primers used are listed in Supplementary Table 1.

The plasmids used were p316TDHTAAter, p316TDHTAA, p316TDH3p, pST106, pBH1-TAA (Nagashima et al., 1992), and pRS316 (Sikorski and Hieter, 1989). The construction of these plasmids is described in the Supplementary materials and methods. p316TDHTAAter and p316TDHTAA contain the *TAA* (*Aspergillus oryzae* α-amylase) gene amplified from pBH1-TAA.

Yeast chromosomal DNAs were also used as PCR templates. The construction of the yeast strains is described in the Supplementary materials and methods. Chromosomal DNA from the RAK3600 strain (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0::PpHIS3*) was used as the template for *Pichia pastoris HIS3* (*PpHIS3*) amplification. Chromosomal DNA from the RAK3656 strain (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0::* $_{CAx10}$*TDH3pTAA*$_{C15}$*PGKterPpHIS3*) was used as the template for amplification of the *PGK* terminator-*PpHIS3* (*terPpHIS3*) fragment. Chromosomal DNA from RAK4700 strain (*ura3Δ0::URA3-GAL10p-yEGFP-Bax-PpHIS3*) was used for amplification of *URA3-GAL10p-yEGFP* fragment using the URA3-300 primer and each primer in the yEGFPc set. BY4704 was used for *BOI1* amplification using the URA3-3′40c-BOI1c primer and each primer in the BOIp701 set. The fusion overlap sequences comprise the authentic sequence, C_{12}, CA_{x9}, $C_3G_3C_3G_3C_3$, or $C_5G_5C_5$ (Table 1).

2.2. Media

Yeast cells were grown in YPD (1% yeast extract, 2% polypeptone, 2% glucose, and 2% agar if necessary) YPGal contained 2% galactose instead of 2% glucose. Synthetic drop out media (0.17% yeast nitrogen base without amino acids and without ammonium sulfate, 0.5% ammonium sulfate, 2% glucose, and required nutrients) were prepared as described previously (Ausubel et al., 1999).

2.3. Chromosomal DNA preparation

Yeast cells were grown in 2 ml YPD, collected by centrifugation, and suspended in 0.3 ml SET buffer (1.2 M Sorbitol, 20 mM Tris–HCl, and 10 mM EDTA, pH 7.5). The suspension was then mixed with 20 μl of zymolyase solution containing 3 mg/ml Zymolyase 100 T (Seikagaku Kogyo, Tokyo, Japan) and 10% β-mercaptoethanol in SET buffer, and incubated at 37 °C for 30 min. The cells were lysed by the addition of 50 μl of 10% SDS, and DNA was extracted with 0.2 ml of phenol/chloroform. DNA was precipitated with ethanol and treated with RNase, followed by extraction again with phenol/chloroform and ethanol precipitation. DNA samples were eventually dissolved in sterile water and stored at −20 °C.

Table 1
Fusion sequence abbreviations

Name	Sequence
C_{15}	ccccccccccccccc
G_{15}	gggggggggggggggg
C_9	ccccccccc
G_9	ggggggggg
C_{12}	cccccccccccc
G_{12}	gggggggggggg
$CG_{x7.5}$	cgcgcgcgcgcgcgc
$GC_{x7.5}$	gcgcgcgcgcgcgcg
$C_5TC_4TC_3TC_2TCT$	ccccctccccctcctcctct
$AGAG_2AG_3AG_4AG_5$	agaggagggagggagggggg
$TC_{15}T$	tccccccccccccct
$AG_{15}A$	agggggggggggggga
$T_3C_{15}T_3$	tttccccccccccccctttt
$A_3G_{15}A_3$	aaagggggggggggggggaaa
$C_5T_3C_5T_3C_5T_3$	ccccctttccccctttccccttt
$A_3G_5A_3G_5A_3G_5$	aaaggggaaaggggaaaggggg
A_{15}	aaaaaaaaaaaaaaa
T_{15}	ttttttttttttttt
CT_{x15}	ctctctctctctctctctctctctctct
AG_{x15}	agagagagagagagagagagagagagag
CT_{x10}	ctctctctctctctctct
AG_{x10}	agagagagagagagagag
CA_{x10}	cacacacacacacacaca
TG_{x10}	tgtgtgtgtgtgtgtgtg
$G_3C_3G_3C_3G_3$	gggcccgggcccggg
$C_3G_3C_3G_3C_3$	cccgggcccgggccc
$G_5C_5G_5$	gggggccccggggg
$C_5G_5C_5$	cccccgggggccccc

2.4. PCR

All PCR amplifications were carried out using KOD Plus DNA polymerase according to the manufacturer's instructions (Toyobo, Osaka, Japan) in a final volume of 10 μl. The PCR cycles comprised an initial denaturation step at 94 °C for 1 min, followed by 30 cycles of 94 °C for 20 s, 50–68 °C for 30 s, and 68 °C for indicated times. DNA fragments for fusion were amplified using a 60 °C annealing temperature and between 1.5–6 min of extension time. For fusion PCR, the annealing temperatures and extension times were as indicated in the figures. PCR products were excised from agarose gels using filter tips as described previously (Dean and Greenwald, 1995). DNA solutions used for fusion PCR were quantified using a Qubit™ fluorometer (Invitrogen, Carlsbad, CA) and adjusted to 0.5 ng/μl with sterile water. For fusion PCR, 0.4 μl aliquots of DNA solution were added to a 10 μl final reaction volume. Amplified fragments were analyzed on 0.7% agarose gels using 1 kb DNA ladder molecular size markers (New England Biolabs, Ipswich, MA). One μl of the final 10 μl PCR mixture was resolved by agarose gel electrophoresis unless otherwise indicated. DNA sequencing was performed using the BigDye terminator V3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA).

2.5. Yeast transformation

Yeast cells were transformed using the conventional lithium acetate method (Ausubel et al., 1999). The cells were initially grown in YPD overnight, from which 1 ml of the culture was mixed with 9 ml of fresh YPD, and incubated for a further 5 h at 28 °C with shaking. The yeast cells were then collected by centrifugation, washed once with 1 ml of sterile water, and suspended in approximately 150 μl of water. The cells were next mixed with transformation solutions as follows; 120 μl of 60% polyethyleneglycol 3350 (Sigma-Aldrich, Tokyo, Japan), 10 μl of 10 mg/ml carrier DNA, 5 μl of 4 M lithium acetate, 65 μl of yeast suspension, and 50 ng of DNA amplified by PCR. This mixture was incubated at 42 °C for 1 h.

3. Results

3.1. Comparison of fusion PCR approaches using overlaps of authentic sequences or a designed C_{15}/G_{15} sequence

To demonstrate the successful construction of fusion DNA molecules using PCR, the α-amylase (*TDH3p-TAA*) and yeast transformation marker (*PpHIS3*) genes were used as test cases (Fig. 1A). A standard overlap extension PCR using primers that were designed based on the *PGK1* terminator sequence was first performed. Overlaps of different lengths, such as 20, 30, and 40 nucleotides (nt), were compared (Fig. 1A). *TDH3p-TAA* fragments were amplified with primer pairs for TDH3-572 and either PGK1ter1-20c-TAAc, PGK1ter1-30c-TAAc, or PGK1ter1-40c-TAAc from the p316TDHTAA plasmid. *PpHIS3* marker fragments were amplified with URA3-200c and a counter primer (either PGKter1-20-PpHIS3, PGKter1-30-PpHIS3 or PGKter1-40-PpHIS3) using RAK3600 as the chromosomal DNA template. The *TDH3p-TAA* and *PpHIS3* DNA fragments were purified from an agarose gel (Dean and Greenwald, 1995), mixed, and then PCR amplified using TDH3-572 and URA3-200c primers. As shown in Fig. 1C, fusion DNA fragments of 3.3 kb were produced using a 68 °C annealing