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Adaptor long-range PCR procedure for clone-specific characterization and chromosomal localization

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An efficient adaptor long-range PCR (ALR-PCR) procedure was developed to detect genomic rearrangements in high-plasticity genomic regions between closely related strains of bacteria. The method was precisely optimized using a combination of high-speed experimental steps for the chromosomal localization and elucidation of deletions, inversions, duplications, or inserted sequences within a clone-specific flanking region. The advantages of this strategy are: (i) ready-to-use polymerase mixtures and Master mix (ready-to-use reaction mixtures with polymerase MasterAmp™ and buffer 2× Premix 4); (ii) a 5-min ligation procedure; (iii) rapid purification of DNA digests; (iv) optimized DNA template concentration protocol to avoid nonspecific amplification and high backgrounds; (v) long-range PCR protocol to obtain at least 9.6 kb single PCR products; (vi) two-step PCR cycling with the same annealing and extension temperature at 68°C; (vii) simple design of the adaptors according to the preferred restriction endonuclease enzyme; and (viii) simple technology and equipment required. The application of this method for a tester-specific suppressive subtractive hybridization (SSH) clone of Brucella melitensis 16M revealed an 837-bp deletion and a 7255-bp DNA transfer from one chromosomal location to another for Brucella abortus 2308 used as a driver.

INTRODUCTION

Comparative in silico analyses of completed bacterial genomes have elucidated genetic and biological similarities and differences between closely and distantly related species. However, because there are still many voids in the list of completed genomes, suppressive subtractive hybridization (SSH; References 1 and 2) has proven to be highly effective in identifying genetic differences between closely related strains and species of bacteria. The genome of interest is termed the “tester” DNA and the comparison is referred to as the “driver” DNA. As useful as SSH has been in defining genomic differences, it is limited in detecting chromosomal rearrangements such as inversions, duplications, and translocations. The analysis and chromosomal localization of SSH clones provide additional information on DNA sequence inversions and translocations by using adaptor-mediated PCR technology.

Several PCR methods have been described to amplify unknown DNA fragments using specific DNA polymerases (3,4), different types

of oligonucleotide primers such as degenerate oligonucleotide-primed PCR (DOP-PCR; Reference 5), linker adaptor-mediated PCR (LAM-PCR; Reference 6), rapid amplification of cDNA ends (RACE-PCR; Reference 7), adaptor-tagged competitive PCR (ATAC-PCR; Reference 8), serial analysis of gene expression (SAGE; Reference 9), and long and accurate PCR (LA-PCR; Reference 10). Comparison of these methods revealed no simplicity, low reproducibility, high backgrounds of nonspecific PCR products, inefficient homopolymer tailing, small PCR product amplification, and laborious time-consuming procedures (5–8,11,12).

As a consequence, we decided to develop adaptor long-range PCR (ALR-PCR; Figure 1) to examine each SSH clone when *Brucella melitensis* 16M was the tester and *Brucella abortus* 2308 was the driver (13). The terms 16M and 2308 will be used to signify these species throughout this publication. Specifically, ALR-PCR was employed to identify genomic rearrangements in large unknown DNA sequences adjacent to a particular

tester-specific SSH clone. This approach was derived from the modification of protocols determining the 5′ and 3′ of cDNA ends (10), representational difference analysis (RDA) of cDNA (14), and was optimized with reagents for high reproducibility and velocity.

MATERIALS AND METHODS

Detailed descriptions of the bacterial strains, chromosomal genomic DNA isolation, and application of SSH technique to obtain the tester-specific clones have been reported elsewhere (13).

Oligonucleotide Primers

The oligonucleotide primers synthesized for this research were purchased from MWG-Biotech (High Point, NC, USA). The 12-mer primer PAOK-*SphI*-12 (5′-GTACTAGCGAGC-3′; underlined nucleotides were designed according to *SphI*-specific recognition site) was designed to complement the universal 24-mer primer PAOKARA-24 (5′-TCGGTAGAGTCGATTC-GCTCGCTA-3′) to ensure the correct polarity of ligation with the cohesive-ended genomic DNA fragments. The recessed ends of the ligated DNA fragments were automatically filled in during the first round of PCR in the presence of dNTPs and DNA polymerase. The forward primer PAOK-1999F (5′-GATGATTCTTCCTGTGCCGGAT-3′) was designed upstream of the SSH clone f44 using the 16M genome (14), and the reverse primer PAOK-*SphI*-R (5′-ATCTCAGC-TAAGCGAGCGATCATG-3′) was designed on the adaptor, including the *SphI*-specific CATG recognition site at the 3′ end for enhanced PCR specificity. The primers for PCR amplification were designed to anneal at 68°C according to Wallace et al. (15).

Generation of DNA Adaptor-Linked Digests

The cohesive-ended restriction endonuclease *SphI* (New England Biolabs, Beverly, MA, USA) was used to digest 2308 genomic DNA in a reaction mixture of 50 µL containing 5 µL of 10× restriction endonuclease enzyme buffer (NEB 2; New England Biolabs), 1100 ng DNA, and 5 U restriction endonuclease enzyme for 4 h at 37°C and then purified using

the QIAquick® Nucleotide Removal Kit (Qiagen, Valencia, CA, USA). *SphI* DNA digests were ligated to the adaptors at room temperature for 5 min in a reaction mixture of 100 µL containing 50 µL 2× Quick Ligation™ Buffer (New England Biolabs), 3.5 µL of primer PAOK-*SphI*-12 (20 pM/µL), 3.5 µL of primer PAOKARA-24 (20 pM/µL), and 5 µL Quick T4 DNA Ligase (New England Biolabs). Prior to the addition of the Quick T4 DNA Ligase, annealing of the 12- and 24-mer oligonucleotide primers to form the adaptor was achieved by heating at 50°C for 1 min, and then

cooling to 10°C at 1°C per min using a DNA thermal cycler (Perkin Elmer GeneAmp® PCR System Model 9700; Applied Biosystems, Foster City, CA, USA). Quick T4 DNA Ligase was inactivated by heating at 65°C for 10 min.

PCR Amplification

PCR amplification was carried out using the GeneAmp DNA thermal cycler programmed to denature DNA at 95°C for 15 s, and 35 cycles of annealing and extension at 68°C for 15 min in a 50-µL mixture containing

2.5 U MasterAmp™ Extra-Long DNA Polymerase (EPICENTRE, Madison, WI, USA), 25 µL MasterAmp Extra-Long PCR 2× Premix 4 (EPICENTRE), 1 µL (50 pM/µL) of each oligonucleotide primer, and 10 ng *SphI* DNA adaptor-linked digests. An initial 2-min adaptor extension at 68°C, 2-min denaturation at 95°C, and a final 10-min extension at 68°C were included in the program.

Sequence Analysis

The DNA sequencing of PCR products was conducted by MWG-Biotech. Sequence data were analyzed using the MapDraw, EditSeq, SeqMan, and MegAlign programs from the DNASTAR Nucleotide Sequence Analysis Package (www.dnastar.com) and were searched using the 16M genome available at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) and Integrated Genomics ERGO™ (www.integratedgenomics.com).

RESULTS AND DISCUSSION

Although several approaches have been developed using adaptors for PCR amplification of unknown DNA fragments flanking a known sequence (3–10), those techniques have been limited by a relative short walking distance, low specificity, low efficiency, and/or extensive cycling, which contributed to a higher error rate in PCR products (3). Therefore, several improved adaptor-mediated PCR methods needed to be developed to utilize new biotechnological systems and reagents for simplifying and minimizing complex experimental steps. The strategy described here is displayed in Figure 1, using cleaved genomic DNA fragments ligated onto site-specific oligonucleotide adaptors to characterize the flanking regions of differential SSH clones generated by the subtraction of closely related *Brucella* genomes. As an example, the application of this ALR-PCR strategy using a tester-specific SSH clone (f44; Reference 13) elucidated an 837-bp deletion and the exact location of a 640-kb inversion.

The cohesive-end *SphI* restriction endonuclease was selected to cleave genomic 2308 DNA, generating average DNA fragments of 7–10 kb, which were then ligated onto *SphI*-

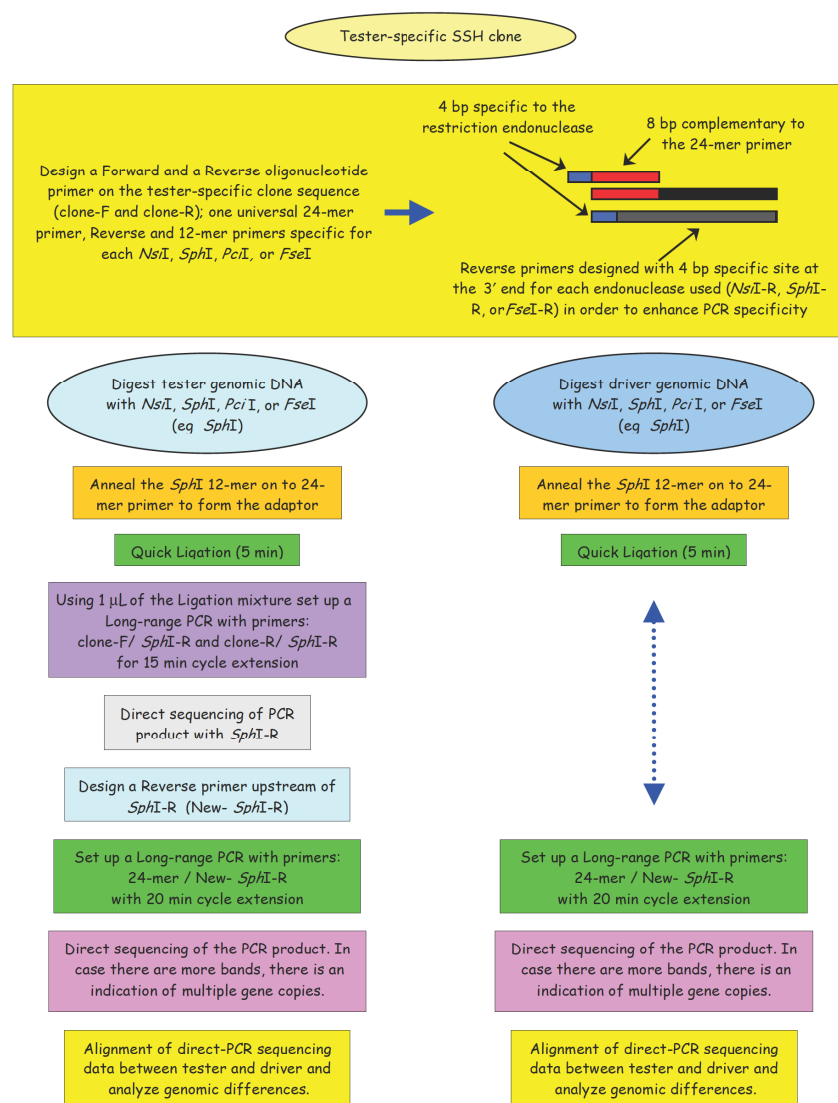


Figure 1. Diagram of efficient adaptor long-range PCR (ALR-PCR). SSH, suppressive subtractive hybridization.

specific adaptors to isolate large genomic regions by long-range PCR. The advantage of ALR-PCR is that by using short oligonucleotide primers with no chemical modifications at the 3' end and synthesizing the adaptors prior to ligation, one can apply stringent PCR conditions for better amplification specificity. Modification at the 3' end of the 12-mer primer of the adaptor (Figure 1) allows this method to utilize DNA template digests from various restriction endonuclease enzymes, depending on the genome of the organism under investigation. This can be achieved by creating new specific adaptors, replacing the four nucleotides at the 5' end of the 12-mer primer; that is, the site 5'-ACGT-3'

for *NsiI* digests, 5'-GGCC-3' for *FseI* digests, etc. (Figure 1).

High backgrounds yielding nonspecific PCR products are a frequent problem in adaptor-linked PCR procedures (3,7,10). Long-range PCRs were sensitive to DNA template concentration using optimum 5–15 ng amounts of 2308 *SphI* digests in this study for the efficient amplification of large PCR products and low background. This drastically reduced the generation of nonspecific PCR product production obtained with high DNA template concentrations (Figure 2).

The forward oligonucleotide primer PAOK-1999F was designed to anneal upstream of the location of clone f44, and the reverse PAOK-*SphI*-R on the

adaptor included the CATG site at the 3' end for enhanced specificity (Figure 3). Although the ALR-PCR amplification product was expected to be smaller than 4192 bp [based on the genome of 16M chromosome II, chr. 303770–307961 (16) and the deletion identified from the analysis of clone f44], a 9619-bp DNA fragment was generated. The difference in the expected and observed size of the PCR products indicated a large genomic rearrangement at this chromosomal region of 2308. Indeed, direct sequencing of the 9619-bp PCR product confirmed a total of 837 bp deletion at the flanking region of f44, disrupting the BMEII0291 and BMEII0292 genes as well as a 7255-bp fragment (Figure 3) located approximately 740 kb downstream from clone f44 in the annotated 16M genome (16M chromosome II, chr. 1047279–1039998). Thus, the application of ALR-PCR confirmed the disruption of the BMEII0291 and BMEII0292 genes and a 7255-bp DNA transfer from one chromosomal location to another.

In the current study, ALR-PCR is used as a powerful tool to compare genomic sequence data among the different *Brucella* strains, providing insights into their host range, virulence factors, evolutionary origins, and phylogenetic relationships. ALR-PCR was also successfully used to identify various deletions in adjacent genomic regions of 45 other SSH clones, leading to the development of strain-specific PCR-based diagnostic tools for *Brucellae* (experiments in progress).

In summary, the powerfulness of ALR-PCR is anticipated to be a combination of the following parameters: (i) ready-to-use polymerase mixtures and a master mixture, which reduce time, experimental steps, and the requirements of PCR optimization for each polymerase mixture (3,4); (ii) a 5-min ligation procedure; (iii) rapid purification of DNA digests; (iv) optimized DNA template concentration protocol to avoid nonspecific amplification and high backgrounds; (v) long-range PCR protocol to obtain at least 9.6 kb single PCR products; similar work but with different conditions (17) has emphasized the problems associated with PCR-amplifying large PCR products; (vi) two-step PCR cycling with the same annealing and extension temperature at 68°C, resulting in high-stringency amplification as previously

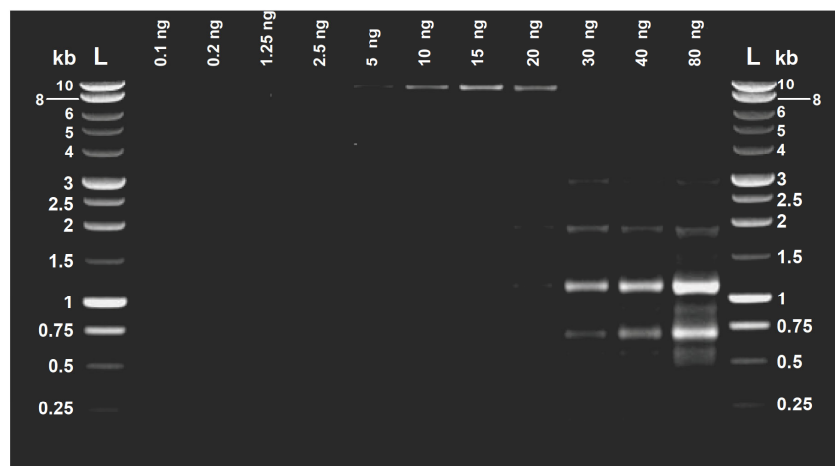


Figure 2. Optimization of DNA template concentration of the adaptor long-range PCR (ALR-PCR).

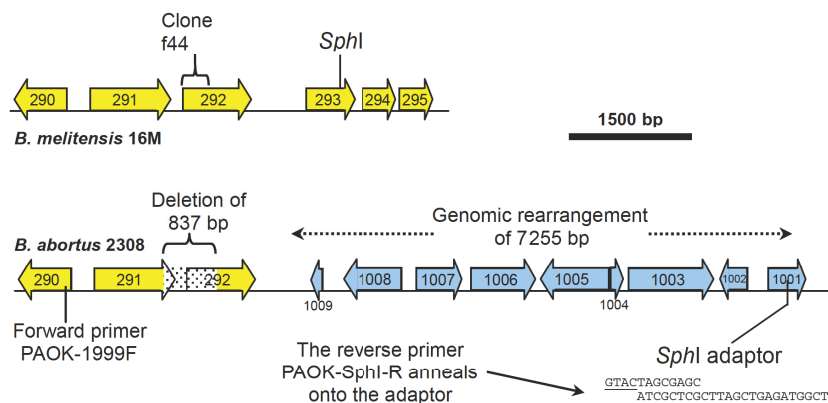


Figure 3. Genome analysis in a scaled diagram after the application of adaptor long-range PCR (ALR-PCR) for the f44 suppressive subtractive hybridization (SSH) clone. *B. melitensis*, *Brucella melitensis*; *B. abortus*, *Brucella abortus*.

described (18,19) and also applied in our work; (vii) simple design of the adaptors according to the preferred restriction endonuclease enzyme; and (viii) simple technology and equipment required. ALR-PCR therefore offers a more simple, flexible, and user-friendly approach over other adaptor-PCR procedures.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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