of chromosomal transcriptional fusions in Gram-negative bacteria that are sensitive to trimethoprim. These include most members of the *Enterobacteriaceae*, such as *Escherichia*, *Klebsiella* and *Proteus*. However, it is particularly useful in studies on those Gram-negative bacteria that exhibit low-level resistance to trimethoprim but high resistance to other commonly used antibiotics, such as members of the genus *Burkholderia* (particularly the human pathogens *B. cepacia* and *B. pseudomallei*) and the opportunist pathogen *Stenotrophomonas maltophilia* (formerly *Xanthomonas maltophilia*). The resultant single-copy fusions are more desirable for studying gene regulation because they avoid problems associated with multicopy systems such as the titration of regulatory proteins. This vector can also be adapted for the identification of bacterial promoters that are specifically expressed in an animal host or within cultured cell lines. The unique *NotI* site downstream of the promoterless *cat* gene facilitates the insertion of an additional reporter gene or antibiotic resistance marker as required.

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3' Overhangs Influence PCR-SSCP Patterns

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Single strand conformation polymorphism (SSCP) analysis has become a popular method for detecting sequence variation in PCR-amplified DNA. It is a highly sensitive technique that is capable of detecting single-base substitutions under optimized conditions (3). DNA amplified by DNA polymerases, which lack 3'→5' exonuclease (proofreading) activity, often has an additional nontemplate-directed nucleotide added at the 3' ends (1). The addition of the extra nucleotide is variable, and the nucleotide added is dependent on the 3' base existing at the blunt end (Table 1). Therefore, PCR products amplified by a DNA polymerase that exhibits extendase activity often consist of a mixture of blunt-ended DNA fragments and those with 3' overhangs. The presence of these 3' overhangs may affect the single strand conformation of PCR products and hence change the migration of bands on an SSCP gel. Furthermore, any factor that changes the addition of the 3' extra nucleotides or causes degradation of the 3' overhangs may alter the PCR-SSCP pattern. In this study, we tested the effect of the 3' overhangs generated by *Taq* DNA polymerase (Qiagen GmbH, Hilden, Germany) on SSCP banding patterns.

A fimbrial subunit gene (*fimA*) of *Dichelobacter nodosus* was amplified using the blunt end DNA polymerase, *Pwo* (Roche Molecular Biochemicals, Mannheim, Germany). After purification, half of the PCR product was treated with *Taq* DNA polymerase and then subjected to SSCP analysis. The PCR product treated with *Taq* DNA polymerase generated a different SSCP pattern from the untreated product (Figure 1a) and caused a reduction in the mobility of the slowest moving band. This mobility shift can be most clearly seen in Figure 1a, lane 2, where PCR products treated with and without *Taq* DNA polymerase were mixed and run together in the same lane. As *Pwo* DNA polymerase produces blunt-ended PCR products, the difference in migration pattern observed is thought to be the di-

Benchmarks

Table 1. The 3' Overhang Produced by *Taq* DNA Polymerase In Vitro (Modified from Reference 1)

3' End of Blunt-Ended DNA	3' Overhang Added
T	A
G	G>A>G ^a
C	A>C ^a
A	(A) ^b

^a> Represents bases at the left side are preferentially added over bases at the right side.

^bThe base in brackets means it will be added at the 3' end at a very low efficiency.

rect result of a single 3' end extra nucleotide added by *Taq* DNA polymerase to a majority of the PCR products. Consistent with this result, the PCR-SSCP patterns of the *fimA*, amplified separately by *Taq* DNA polymerase and *Pwo* DNA polymerase, were also different. As expected, an extra SSCP band was observed with DNA fragment amplified by *Taq* DNA polymerase (Figure 1b). However, it is generally accepted that a proofreading polymerase such as *Pwo* DNA polymerase has a higher fidelity than a non-proofreading polymerase such as *Taq* DNA polymerase, and, therefore, the difference in banding pattern may simply be due to PCR errors made by *Taq* DNA polymerase.

The influence of the 3' overhang on ssDNA secondary structure was predicted by the *mfold* server (<http://www.ibc.wustl.edu/~zucker/dna/forml.cgi>). Both the sense and antisense strands of amplified fragments were analyzed. A minor change in the secondary structure of one strand was predicted with the addition of an extra A to the 3' end. This is consistent with the SSCP result because only one band showed a change in mobility.

Differences in fluorescence-based SSCP patterns have been observed when PCR products are treated with and without Klenow fragment (4). Klenow fragment has 3'→5' exonuclease activity for both ds and ssDNA. Treatment of PCR product with Klenow fragment not only removes 3' overhangs but also may re-

Figure 1. PCR-SSCP analysis. A 263-bp fragment of *D. nodosus fimA* sequence was amplified using primers (5'-GCTCTGTACAGAAGCTGG-3' and 5'-AGGCACAAATTGGGATCAATATC-3'). Amplification consisted of denaturation at 94°C for 60 s, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 50 s, with a final extension at 72°C for 7 min. PCR products were mixed with a loading dye containing 95% formamide, 10 mM EDTA, 0.025% bromophenol blue and 0.025% xylene cyanol. After denaturation at 95°C for 5 min, samples were applied to a 16 × 18 cm 8% polyacrylamide gel (49:1). Electrophoresis was performed using PROTEAN II® xi Cell (Bio-Rad Laboratories, Hercules, CA, USA) at 160 V for 18 h at 4°C in 0.5× TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.0), and the gels were silver stained according to Merril et al. (2). (a) PCR was amplified using *Pwo* DNA polymerase. An aliquot of purified PCR product, using a PCR Purification Kit (Roche Molecular Biochemicals) was incubated with or without *Taq* DNA polymerase in a 30-μL reaction containing 1× PCR buffer, 1.5 mM MgCl₂ and 200 μM dNTPs at 72°C for 30 min. (b) PCR was amplified using *Taq* DNA polymerase or *Pwo* DNA polymerase.

sult in recessed ends of the dsDNA fragments. Thus, the differences observed by Sugano et al. (4) may not only represent the effect of 3' overhangs on SSCP pattern. The production of recessed ends is time and enzyme activity dependent. In our experience, an excessive amount of enzyme or prolonged treatment can result in the complete degradation of the PCR product (data not shown).

This experiment has demonstrated that PCR-SSCP is able to detect a single-nucleotide addition to the 3' end of a small DNA fragment (200–300 bp). Sensitivity may be decreased for larger DNA fragments. Blunt-ended PCR

products are therefore more appropriate for SSCP analysis. The generation of blunt-ended products by a DNA polymerase that does not exhibit extendase activity is preferable to the treatment of PCR products that have 3' overhangs with Klenow because the latter is both time consuming and the result is dependent on the conditions (i.e., time and enzyme activity) used.

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Canterbury, New Zealand Sequencing of β_2 -Adrenoceptor Gene PCR Products Using *Taq* BigDye™ Terminator Chemistry Results in Inaccurate Base Calling

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Direct sequencing of PCR products is often used to identify point mutations in human genomic DNA and to genotype for studies aimed at evaluating the role of genetic polymorphisms in various diseases (2,4,6,7,9). The β_2 -adrennergic receptor (β_2 AR) gene has three polymorphic point mutations located within a 126-bp span (9,12). Two of these mutations occur at β_2 AR coding region nucleotide positions 46 (A or G) and 79 (C or G) resulting in amino acid substitutions at codons 16 (Arg or Gly) and 27 (Gln or Glu), respectively. The third mutation occurs upstream from the coding region at nucleotide -47 (C or T), resulting in either an Arg or Cys at codon 19 of the gene's 5' leader cistron (5'LC) region. Restriction fragment length polymorphism (RFLP) methods can be used to determine β_2 AR genotypes at nucleotide positions -47 and 79; however, no restriction enzyme is currently available that recognizes one but not the other polymorphism at nucleotide 46. Given the close proximity of these polymorphisms, direct sequencing of a single PCR product seemed an efficient method for simultaneously genotyping the three polymorphic sites. We report our experience using PCR and direct sequencing to determine human β_2 AR genotype at nucleotides -47, 46, and 79. Specifically, this paper describes the difficulty in accurately interpreting β_2 AR genotypes with direct PCR cycle sequencing using the energy-transfer dichlororhodamine dye terminator chemistry.

Studies including genetic analysis were approved by the Investigational Review Boards at the University of Tennessee and the University of Florida, and each participant provided written informed consent for genetic analy-

sis before blood sample collection. None of the participants were known to be related. Genomic DNA was extracted from 300 μ L whole blood using a commercially available DNA isolation kit (Gentra Systems, Minneapolis, MN, USA). PCR of genomic DNA was used to generate a 352-bp fragment spanning the three polymorphisms of interest. The primers were synthesized by Life Technologies (Rockville, MD, USA), and the sequences were 5'-GAG-GCTTCCAGGCGTCC-3' upstream and 5'-CCAGGCCCATGACCAGAT-3' downstream. Each 25- μ L reaction contained 50–200 ng genomic DNA, 13.5 μ L deionized water, 2.5 μ L 10 $\times$ PCR buffer (Promega, Madison, WI, USA), 1.5 μ L 25 mM $MgCl_2$ (Promega), 2.5 μ L solution containing 2.5 mM each dNTP (Applied Biosystems, Foster City, CA, USA), 1.25 μ L each 10 μ M primer, 0.25 μ L 5 mM trimethylammonium chloride and 1.25 U *Taq* DNA polymerase (Promega). PCR was performed on a PTC-200™ Peltier Thermal Cycler (MJ Research, Waltham, MA, USA). After an initial denaturation step at 94°C for 5 min, the PCR consisted of 35 cycles of 94°C for 30 s, 61°C for 30 s and 72°C for 30 s, followed by a 10-min final extension at 72°C. To confirm product length, 5 μ L PCR product were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining.

Following purification with the QIAquick® PCR Purification Kit (Qiagen, Valencia, CA, USA), the PCR products were sequenced using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). DNA (10 ng) was mixed with 3 pmol sequencing primer and 2 μ L premixture (containing dye terminators, dNTPs, AmpliTaq® DNA Polymerase FS (Applied Biosystems), *rTth* pyrophosphatase, $MgCl_2$ and buffer) in a total volume of 20 μ L and placed in a 96-well plate (MJ Research). The forward sequencing primer used was 5'-CCCAGCCAGTGC-GCTTACCT-3'. The sequencing reaction consisted of 25 cycles at 96°C for 10 s and 60°C for 4 min. Unincorporated dye terminators were removed from the sequencing products before electrophoresis by gel filtration using MultiScreen® 96-well filtration plates