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Quantification of the Detrimental Effect of a Single Primer-Template Mismatch by Real-Time PCR Using the 16S rRNA Gene as an Example[▽]

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We investigated the effects of internal primer-template mismatches on the efficiency of PCR amplification using the 16S rRNA gene as the model template DNA. We observed that the presence of a single mismatch in the second half of the primer extension sequence can result in an underestimation of up to 1,000-fold of the gene copy number, depending on the primer and position of the mismatch.

DNA polymerases catalyze the addition of nucleotides to the primer 3′-OH, as specified by complementarity to the template DNA. The principal primer characteristics that determine the efficiency and accuracy of PCR are melting temperature, secondary structures, and the complementarity between primers and targeted DNA. Mismatches between primers and targeted DNA can affect duplex stability, which might then hamper the ability of a system to amplify the template DNA. The effects of mismatches depend on numerous factors, such as oligonucleotide length and the nature and position of the mismatches. Several studies have investigated the effects of primer-template mismatches at the 3′ end of the primer sequence, and it has been demonstrated that PCR was prevented by a single mismatched base at the 3′ end (1, 3, 6, 8). In contrast, studies on the effect of internal mismatches are rarer and it is believed that such mismatches can be tolerated (8). However, to what extent such mismatches can affect the efficiency of the PCR is still unknown.

Since in many applications of PCR the template DNA is a mixture of homologous genes, it is important to find out how a primer-template mismatch can affect the accurate interpretation of the results. In microbiology, the most common example of amplification of homologous genes is the amplification of the 16S rRNA gene, which is used as a phylogenetic marker to investigate bacterial diversity in natural ecosystems (11, 15). Over the last 20 years, 16S rRNA has proved to be a powerful marker and has permitted the extension of the known bacterial diversity (5). However, within the 16S rRNA gene, the longest string of totally conserved bases is between positions 788 and 798, and most of the absolutely conserved regions are found in strings of less than 4 bases (2). Therefore, no existing primer targeting the prokaryotic 16S rRNA gene is universal, which can result in differential amplification in a mixture of template DNA from a complex microbiota, leading to a distorted view of microbial diversity (14).

The aim of this study was to use real-time PCR to quantify the effects of primer-template mismatches on PCR efficiency. For this purpose, the 16S rRNA gene was used as a model and the effects of mismatches at different positions either in the sequence of the 341F-534R universal primers (10, 16) or in the template DNA itself were compared. The 16S rRNA gene copy numbers were quantified by real-time PCR, which permits a clear comparison of amplification efficiencies between reactions.

The detrimental effect of primer-template mismatch was comprehensively studied by introducing base alterations either into the primer sequence or in the template sequence into which the primer extension occurs, thus testing a total of 21 primers and 19 DNA templates. Since the percent G+C is considered for melting temperature calculation and in order to avoid modification of the annealing temperature, mismatches were introduced only by base conversion. Forward 341F and reverse 534R primers with various single-base alterations were synthesized as shown in Table 1. DNA from three bacterial strains for which the complete genome has been sequenced, i.e., *Pseudomonas aeruginosa* PAO1, *Agrobacterium tumefaciens* C58, and *Sinorhizobium meliloti* 1021, were used as templates. The theoretical 16S rRNA gene copy number per ng of template genomic DNA was calculated using the following formula (Quanti Tect Sybr green PCR handbook; Qiagen, France): (16S rRNA copy number per ng) = $(6.023 \times 10^{23})/$ (genome size in base pairs $\times$ 660 $\times 10^9$) $\times$ (number of 16S rRNA copies per genome). We generated the 16S rRNA templates with the desired base alterations by amplifying the 16S rRNA from *P. aeruginosa* with the mispaired primers from Table 1. The resulting PCR products were cloned by using a pGEM-T Easy kit (Promega, France), and the presence of base alterations was verified by sequencing using a dye terminator cycle sequencing-1 kit (Beckman Coulter, France) and a Ceq 8000 sequencer (Beckman Coulter, France). The linearized plasmids containing the cloned PCR products were then used as template DNA for real-time PCR amplification with the 341F and 534R primers.

The quantification of 16S rRNA copies per ng of template DNA was based on the fluorescence intensity of Sybr green dye, which binds to double-stranded DNA. Real-time PCRs were carried out on an ABI Prism 7900 HT sequence detection system (Applied Biosystems, France). The 20-μl PCR mixture contained

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TABLE 1. Primers tested in our study[a]

Forward primer	Sequence 5′→3′	Reverse primer	Sequence 5′→3′
341F	CCTACGGGAGGCAGCAG	534R	ATTACCGCGGCTGCTGGCA
F2G	C**G**TACGGGAGGCAGCAG	R2A	A**A**TACCGCGGCTGCTGGCA
F5G	CCTA**G**GGGAGGCAGCAG	R6G	ATTAC**G**GCGGCTGCTGGCA
F8C	CCTACGG**C**AGGCAGCAG	R11G	ATTACCGCGG**G**TGCTGGCA
F11C	CCTACGGGAG**C**CAGCAG	R12A	ATTACCGCGGC**A**GCTGGCA
F12G	CCTACGGGAGG**G**AGCAG	R13C	ATTACCGCGGCT**C**CTGGCA
F13T	CCTACGGGAGGC**T**GCAG	R14G	ATTACCGCGGCTG**G**TGGCA
F14C	CCTACGGGAGGCA**C**CAG	R15A	ATTACCGCGGCTGC**A**GGCA
F15G	CCTACGGGAGGCAG**G**AG	R16C	ATTACCGCGGCTGCT**C**GCA
F16T	CCTACGGGAGGCAGC**T**G	R17C	ATTACCGCGGCTGCTG**C**CA
		R18G	ATTACCGCGGCTGCTGG**G**A

[a] Base conversions are indicated in bold.

10 μl of Absolute Sybr green ROX PCR master mix (ABgene, France), 1 μM of each primer, and 1.5 ng of genomic DNA or 0.05 ng of plasmidic DNA quantified by spectrophotometry at 260 nm using a Bio-Photometer (Eppendorf, Germany). Thermal cycling was performed as previously described (9). No-template controls were also included in all the assays. Melting curves were generated after amplification by increasing the temperature from 80°C to 95°C. Standard curves were obtained using 10-fold serial dilutions of a linearized plasmid containing cloned 16S rRNA genes from *Pseudomonas aeruginosa* PAO1. Three independent quantitative PCRs were performed for all primers, and an average value was calculated. The real-time PCR products were electrophoresed on 2% agarose gels to verify the presence of a single band of the expected size of 174 base pairs.

In a first assay, the same amount of genomic DNA was amplified with various sets of primers exhibiting single mismatches at different positions. DNAs from three different strains were used as templates to address the question of contextual effects on primer extension by varying the template sequence. There was no significant difference between the theoretical and observed gene copy numbers for any strain when the universal 341F-534R primers were used to quantify the 16S rRNA gene, which validates our assay (data not shown).

The mispaired forward and reverse primers shown in Table 1 were used in combination with the 534R and 341F primers, respectively. The impact of internal mismatches differed between the forward and reverse primers. The number of 16S rRNA copies per ng of DNA decreased from 10^6 to as low as 10^3 when estimated using mispaired forward primers (Fig. 1). A trend of increasing detrimental effects on PCR efficiency was observed when mismatches were moved toward the 3′ end of the primer, which indicates the importance of the position of the mismatch within the primer sequence for the stability of primer annealing. Thus, mismatches located closer to the 3′ end of the primers were more critical for PCR efficiency. The same trend was observed for all strains, suggesting that the genetic context had no effect. For the forward primers, a single mismatch at position −5, −6, or −8 of the 3′ end was sufficient to lead to an underestimation of 1 log of the gene copy number. In contrast, no negative effect was observed for the mispaired reverse primers when mismatches were located more than 4 bases away from the 3′ end, except for primer R13C (Fig. 1). The F11C, F14C, and R13C primers had a detrimental effect on PCR efficiency that was greater than the general trend, which was probably due to the generation of a CC-GG hairpin loop by the base conversion. In contrast to our results, Kwok et al. (8) demonstrated that single internal mismatches in one of the last 4 bases at the 3′ terminus had no significant effect on PCR product yield. This observed discrepancy between results is likely to be due to the fact that the final effect of mismatches is determined by numerous factors, such as the primer length, the nature and position of mismatches, and the annealing temperature of the primers. For instance, the primer used by Kwok et al. (8) was a 30-base oligonucleotide, whereas the lengths of the 341F and 534R primers were 17 and 19 bases, respectively. In addition, low-stringency PCR conditions were used by Kwok et al. (8), while Ishii and Fukui (7) showed that the bias due to the presence of mismatches was reduced at lower temperatures. In contrast to single mismatches, the effect of several internal mismatches on PCR has been more widely described in the literature. Thus, Guy et al. (4) reported that a few mismatches reduced the sensitivity of detection of *Giardia*, as seen by an increase in the cycle threshold values. Recently, Sipos et al. (12), using an equal mixture of two strains as template, showed a preferential amplification of the strain that perfectly matched the primer sequence compared to the amplification of the strain exhibiting three mismatches close to the 5′ end of the primer sequence. A more-pronounced reduction in PCR efficiency with an increasing number of mismatches was shown by Smith et al. (13). However, the results of the present study demonstrate that a single internal primer-template mismatch can also have a strong negative effect on PCR, depending on the position of the mismatch and on the nature of the primers used.

To test if the effects of mismatches on PCR efficiency were symmetrical, i.e., whether a base alteration on the DNA template was equivalent to a base alteration on the primer, the same amount of the linearized plasmids, containing 16S rRNA exhibiting single mismatches at different positions in the primer extension sequence, was amplified with the 341F-534R primers (Fig. 2). This approach was also used to check that the negative effect of a base conversion in the primer sequence observed in this study did not result from subsequent modifications of the primer secondary structure. A similar trend was observed when base alterations were located in the template sequence corresponding to the primer extension regions and when they were located in the primer sequence. Thus, a greater decrease in PCR efficiency with mismatches in the 3′ region

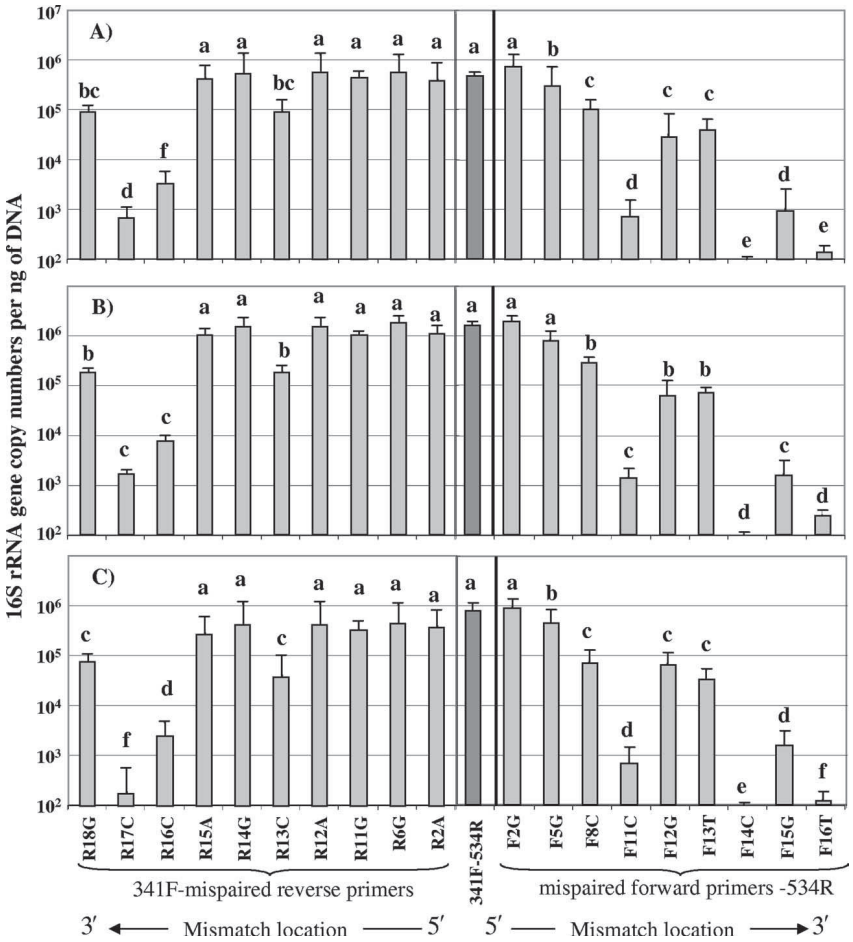

FIG. 1. Comparison of the 16S rRNA gene copy numbers in *Pseudomonas aeruginosa* PAO1 (A), *Agrobacterium tumefaciens* C58 (B), and *Sinorhizobium meliloti* 1021 (C), estimated by real-time PCR using the 341F-534R or the mispaired forward and reverse primers in combination with the 534R or 341F primers, respectively. Error bars show standard errors ($n = 3$). Mean values were compared by using a t test. Significantly different gene copy numbers ($P < 0.05$) are indicated by different letters.

was observed in both cases (Fig. 2). As an example, a base conversion at position 16 (position −1 of the 3′ end) on the forward primer or on the template DNA led in both cases to an underestimation of around 3 logs of the gene copy number (Fig. 1 and 2). The higher negative effects of base conversions at positions 11 and 14 on the forward primer or at position 13 on the reverse primer were not observed when the same base conversions were located in the template sequence, thus con-

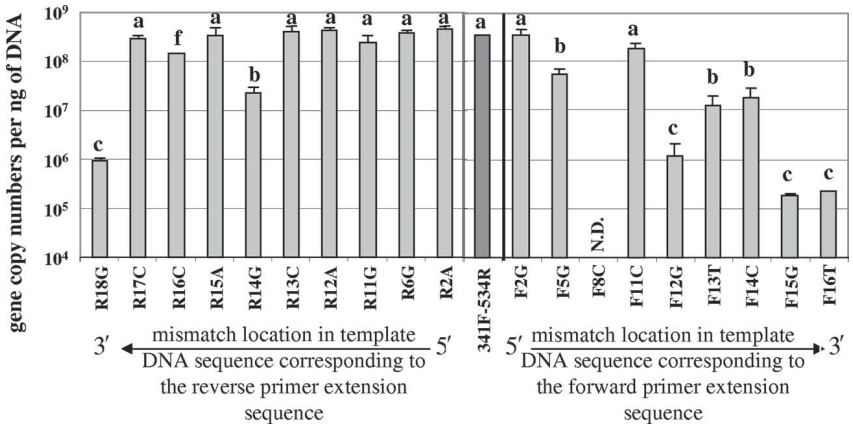

FIG. 2. Quantification of 16S rRNA gene copy number with primers 341F-534R in plasmids containing mutagenic 16S rRNA having a base conversion in the forward or reverse primer extension sequences. Error bars show standard errors ($n = 3$). Mean values were compared by using a t test. Significantly different gene copy numbers ($P < 0.05$) are indicated by different letters. N.D.: not determined.

firming that the presence of a hairpin loop in the primer sequence can also reduce PCR efficiency (Fig. 2). On the other hand, some base conversions in the template DNA (e.g., F12G and R14G) had a greater negative effect than when located in the primer sequences. Further experimentation is therefore required to clarify this issue.

In conclusion, this study demonstrates that the effect of a single internal primer-template mismatch is variable but can greatly decrease PCR efficiency, depending on its position and on the primer used. Such an effect of primer-template mismatches can distort the data used in drawing conclusions on the structure of a microbial community in a PCR-based approach, due to bias in the estimation of the relative proportions between groups possessing and not possessing mismatches with the primers. As a result, taxonomic groups that do not perfectly match the 16S rRNA universal primers are probably underrepresented in databases or in fingerprinting analysis. The presence of mismatches could also bias real-time PCR assays by leading to an underestimation of the actual gene copy number if measured against a perfectly matched standard. Instead of allowing some mismatches between primer and template rather than increasing the degeneracy of the primer, this study suggests that the use of multiple sets of primers targeting different subgroups may be preferable when the sequence polymorphism of the targeted gene family is high.

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