A Novel Eukaryote-Made Thermstable DNA Polymerase Which Is Free from Bacterial DNA Contamination

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To achieve the production of a thermostable DNA polymerase free from bacterial DNA contamination, we developed eukaryote-made thermostable DNA (Tag) polymerase. The novel eukaryote-made thermostable DNA polymerase resolves the problem of contaminating bacterial DNA in conventional bacterially made thermostable DNA polymerase as a result of its manufacture and incomplete purification. Using eukaryote-made thermostable DNA polymerase, the sensitive and reliable detection of bacteria becomes feasible for large fields, thereby making the development of a wide range of powerful applications possible.

The PCR-based detection of a broad range of bacteria is widely applied for the screening of clinical, environmental, and manufacturing samples to diagnose infectious diseases or to detect a bacterial contamination. If bacterial species are unknown, a broad range of bacteria can be detected by using bacterial universal primers targeting highly conserved regions in the bacterial 16S rRNA gene (24). In cases where bacterial DNA is detected, it can be further characterized by DNA sequencing (16), sequence-specific hybridization (15), or other methods, including size-dependent electrophoretic separation (8), a restriction fragment length polymorphism analysis (13), pyrosequencing (10), a single-strand conformation polymorphism analysis (7), and mass spectrometry (14). As PCR is capable of 10^6- to 10^7-fold amplification of a single copy of template DNA, PCR may enable the identification of bacterial DNA even in some culture-negative samples and can provide more rapid diagnostic results than culture analysis in emergency situations, such as septic shock (11). However, the validity of bacterial universal PCRs is compromised by unclear results, especially when amplifying a small amount of bacterial DNA (26).

Concerning bacterial universal PCRs, the main problem is the achievement of sensitive and reliable detection of bacteria with a clear negative control. Over the years, this has been an unsolved problem because the reagents used in the PCR process are contaminated with trace amounts of bacterial DNA, especially the thermostable DNA polymerase (e.g., Taq polymerase) (2, 9, 16, 20). Whenever target DNA is present in very small quantities, contaminating DNA becomes a major problem, since even low-copy-number contamination will be amplified, thus leading to problems in obtaining low detection limits.

A number of studies have indicated the presence of contaminating bacterial DNA in commercial preparations of recombinant thermostable DNA polymerases as a result of its manufacture and incomplete purification (2, 9, 16, 20). Estimates of contamination in commercially available lots of thermostable DNA polymerases range from 10^2 to 10^5 genome equivalents of bacterial DNA per unit of enzyme (2, 16, 20). Although thermostable DNA polymerase is usually manufactured using Escherichia coli, the contaminating DNA is not always originated from host cells but from multiple bacterial sources (9, 20). Our experiments indicated that the contaminating bacterial DNA is traced mainly from bacterial host cells, with a small amount of multiple bacteria (data not shown).

No universally applicable method for completely removing bacterial DNA from thermostable DNA polymerase preparations has been established (2, 19). Removal of DNA contamination from thermostable DNA polymerase has been approached by physical, chemical, and enzymatic treatments using UV irradiation (18), psoralens with long-wave UV light (9, 16), ethidium monoazide treatment (22), restriction endonuclease digestion (1, 17), ultrafiltration (17), and digestion with DNase I (5, 21). These methods have been tested individually, and inconsistent decontamination results have been reported for all of them (2, 12, 19). In fact, Philipp et al. reported that they were unable to achieve an improvement in sensitivity lower than that obtained for a concentration of 2,500 bacterial CFU/ml sample material using restriction enzymes and microfiltration (19). Furthermore, most of the decontamination procedures lead to a decreased performance of the thermostable DNA polymerase (2, 12). Therefore, reliable and efficient decontamination methods need to be developed.

To resolve the problem, we propose a novel approach to make thermostable DNA polymerase free from bacterial DNA contamination.

MATERIALS AND METHODS

Manufacturing of Taq polymerase by using Saccharomyces cerevisiae (yeast) cells. Restriction enzymes and the ligation kit were purchased from Takara Bio (Shiga, Japan). The pYES2 vector and custom primers were purchased from...
Invitrogen (Carlsbad, CA). *S. cerevisiae* strain *× 2180-1A (ATCC 204594) was obtained from the ATCC (Manassas, VA). Yeast extract, peptone, yeast nitrogen base, and Casamino Acids were obtained from Difco (Detroit, MI). The transformation kit was purchased from ZYMOR Research (Orange, CA). SYBR Green I was purchased from Sigma-Aldrich (Milwaukee, WI). Dimethyl sulfoxide (DMSO) was purchased from Kanto Chemical (Tokyo, Japan). M13mp18 single-stranded DNA was purchased from Bayou Biosab (Harahan, LA). dATP, dGTP, dCTP, and dTTP were obtained from PerkinElmer (Norwalk, CT). Nonidet P-40 was purchased from MP Biomedicals (Aurora, OH). All other reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

The Taq polymerase gene (AB609596) was codon usage optimized for expression in *Saccharomyces cerevisiae* was chemically synthesized with an HindIII site at the 5’ end and an EcoRI site at the 3’ end and inserted into the pUC57 plasmid, pUC-TA01, from the GenScript Corporation (Piscataway, NJ). The pUC-TA01 vector was double digested with HindIII and EcoRI and subsequently inserted into the HindIII-EcoRI sites of the pYES2 vector, pYES-TA01, using a DNA ligation kit, version 2.1.

The pYES-TA01 vector was transformed into the *S. cerevisiae* strain *× 2180-1A (ara3-52) with a Frozen-EZ Yeast Transformation II kit. The colonies of the pYES-TA01 transformant were selected by SD medium containing 0.1% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 0.2% galactose, 0.2% dropout powder (2 g Casamino Acids, 20 mg adenine, 20 mg tryptophan), 100 g/l threitol (DTT), 0.1 mM EDTA, 0.5% NP-40, 0.5% Tween 20, and 50% glycerol, and incubated with 50 ml of 50 mM Tris-HCl buffer, pH 7.5. Elution of the Taq polymerase fraction was achieved with 50 ml of 50 mM Tris-HCl, pH 7.5. The eluted fraction was applied to a 5-ml column of HiTrap Q HP (GE Healthcare, Piscataway, NJ) equilibrated with 50 ml of 50 mM Tris-HCl, pH 7.5. Elution of the Taq polymerase fraction was achieved with 50 ml of 50 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl. The eluted fraction was applied to a 5-ml column of HiTrap heparin HP (GE Healthcare) equilibrated with 50 ml of 50 mM Tris-HCl, pH 7.5, and the Taq polymerase fraction was eluted with 25 ml of 50 mM Tris-HCl buffer, pH 7.5, containing 400 mM NaCl. The target fraction was concentrated and exchanged into the stock buffer solution containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.5% NP-40, 0.5% Tween 20, and 50% glycerol, by an Amicon Ultra PL-50 filter (Millipore, Milford, MA) in order to obtain the final Taq polymerase concentration of 0.1 to 1.0 mg/ml.

The activity of Taq polymerase was determined by a fluorescence-based DNA polymerase assay as previously described (21). The reaction mixture contained 14 pmol of M13mp18 DNA and 24 pmol of primer (5’TCCCCGATCGACGTTGAAACCGACCCGGCCAGTG-3’ and reverse, 5’GGACTACCAGGGTATCTAATCCT-3’), and to universally amplify 287 bp of bacterial 16S rRNA genes (forward, 5’TGCCATCCAGCCTGTTGGAATACGA; reverse, 5’TGCAGATGCTATGATTCTCTCT-3’) and to universally amplify 146 bp of fungal 18S rRNA genes (forward, 5’TATACCGTCGGTGCTACACCA-3’; reverse, 5’GCATTCTCTTATGTTCGACCGCT-3’). The mixture was subjected to a total of 30, 40, 60, or 100 cycles of PCR amplification. These samples were denatured at 39°C for 30 s at 55°C, and subjected to extension for 60 s at 72°C with a thermal cycler (Gene Amp 2400-R; PerkinElmer). Amplicons were purified from samples or negative controls (QIAquick PCR purification kit; Qiagen) and sequenced (310 base sequencing performed using the BLAST nucleotide database tool (http://www.ncbi.nlm.nih.gov/).

RESULTS AND DISCUSSION
At the beginning, we confirmed the presence of contaminating bacterial DNA in commercial thermostable DNA polymerases from eight companies (A to H) using bacterial universal primers (Fig. 1a). All these polymerases were rec-
sequences between bacteria and eukaryotes are quite different. According to the universal phylogenetic tree determined from rRNA sequence comparisons, there are large sequence distances between eubacteria and eukaryotes (25). Second, as eukaryotic host cells are used, antibacterial drugs can be used during the steps of manufacturing the recombinant thermostable DNA polymerases. Consequently, contaminating bacteria can be blocked. However, as antibacterial drugs inhibit or kill bacteria but do not eliminate their DNA, it is therefore necessary to use antibacterial drugs as soon as the eukaryotic host cells start to grow. No similar approach has been reported before.

To demonstrate the advantages of using this new method, we confirmed the nonexistence of contaminating bacterial DNA in yeast-made and plant-made Taq polymerase using bacterial universal primers (Fig. 1b). No contaminating bacterial DNA was detected, even after 100 cycles of PCR amplification, which indicates that the sensitive and reliable detection of bacteria without any unclear results is feasible using eukaryote-made thermostable DNA polymerase.

As a practical example, we performed a sensitive PCR assay (60 cycles) for bacterial contamination of household water using bacterial universal primers (Fig. 2a). Unlike conventional bacterially made Taq polymerase, eukaryote-made Taq polymerase does not detect any contaminating bacterial DNA, and so a very low abundance of bacteria in hot spring water could thus be reliably detected (Fig. 2a, sample C). The amplicon that appeared in hot spring water was subjected to a sequencing analysis and was identified as Pseudomonas alcaligenes (data not shown).

Pathogenic bacteria are the most frequent causes of septicemia, but fungi can also be isolated in a minority of patients (6). Indeed clinical laboratories do not usually perform PCR directly on blood to identify either bacterial or fungal pathogens, but we would like to propose the possibility of conducting the PCR-based detection of pathogens. To prevent the occurrence of unclear results in PCR-based assaying of clinical samples for both bacterial and fungal pathogens, a two-step usage of thermostable DNA polymerases is recommended. That is, to detect bacterial pathogens, eukaryote-made thermostable DNA polymerase can be used in combination with bacterial universal primers. In contrast, to detect fungal pathogens, conventional bacterially made thermostable DNA polymerase, which is usually free from fungal DNA contamination, can be used in combination with fungal universal primers (Fig. 2b). Although bacterially made thermostable DNA polymerase contains trace amounts of DNA from bacterial host cells, no fungal universal primers can bind to bacterial genomic DNA, because the base sequence distances between eubacteria and eukaryotes (25). According to the universal phylogenetic tree determined from rRNA sequence comparisons, there are large sequence distances between eubacteria and eukaryotes (25).
becomes feasible in practice for various fields, e.g., medicine, livestock raising, household water, purified water used in pharmaceutical manufacturing processes, food products, cell culture infection, and research in microbiology, etc. This achievement is therefore expected to enable the development of a wide range of powerful applications.

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