Application of Randomly Amplified Polymorphic DNA Fingerprinting for Species Identification of Bacteria Isolated from Bovine Milk

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ABSTRACT

A polymerase chain reaction-based DNA fingerprinting system for species identification of bacteria in milk was developed using randomly amplified polymorphic DNA. A total of 108 organisms including 24 ATCC reference strains and 84 wild-type isolates belonging to gram-negative, Staphylococcus, Enterococcus, and Streptococcus species were used to develop the system. Organisms included in the study were those that are isolated frequently from milk. Forty primers from two commercially available primer kits were evaluated to determine the "ideal" primer that could be used for several bacterial species. Over 960 DNA fingerprint patterns were analyzed by laser densitometry. Seven of the 40 primers met criteria established for primer selection. However, only primers OPE-4 (5' GTGACATGCC-3') and OPE-20 (5'-AACGGTGACC-3') allowed differentiation between all 19 ATCC bacterial species included in the study. The other five primers were restricted to either gram-negative bacteria (OPA-7, OPA-14), Staphylococcus species (OPA-13, OPA-14, OPA-18), or Streptococcus species (OPA-3). Primers OPE-4 and OPE-20 were further evaluated using 84 wild-type isolates. A bacterial species identification scheme was developed based on characteristic polymorphic DNA fragments obtained with primers OPE-4 and OPE-20. Results of this study suggest that RAPD fingerprinting has the potential for being developed into a rapid and accurate method for species identification of bacteria in milk.

Key words: Bacterial identification, milk, PCR, randomly amplified polymorphic DNA (RAPD) fingerprinting

Identification of pathogenic microorganisms is important for surveillance, prevention and control of foodborne diseases (6). The search for reliable and rapid methods for detection of specific groups of organisms in foods has taken on an increased urgency with the steady rise of reported foodborne illnesses in the United States (2, 7, 19). Development of DNA-based techniques for identification of microbial pathogens has gained the attention of food microbiologists and researchers in the area of diagnostic microbiology. The greatest advantage of DNA-based identification techniques is that methods focus on the unique nucleic acid composition of the microorganism rather than on the phenotypic expression of products that nucleic acids encode. This approach eliminates the cumbersome process of phenotypic characterization of microbial pathogens, which tends to be time consuming.

The polymerase chain reaction (PCR) has become an important diagnostic tool in microbiology. Typing of bacteria employing PCR for species identification using 16S ribosomal DNA (13, 14, 18), arbitrary primers (12, 15, 20), species-specific DNA (4), transfer RNA intergenic spacers (22), repetitive extragenic palindromic elements and entero-bacterial repetitive intergenic consensus sequences (28) have been described. The technique of randomly amplified polymorphic DNA (RAPD) fingerprinting analysis using arbitrary primers has been evaluated critically for reproducibility, reliability, and validity (5, 9, 24, 25).

Although RAPD fingerprinting analysis has become an accepted and reliable tool for subtyping a wide variety of bacteria within a genus or species, its potential use for species identification has been limited to a few bacterial species belonging to the genera Streptococcus and Enterococcus (17) and Staphylococcus (20). The objectives of this study were: (i) to identify an ideal primer(s) that could be used for bacterial species identification, (ii) to define a systematic approach for evaluating primers and bacterial DNA fingerprint patterns, and (iii) to develop a scheme for bacterial species identification based on DNA fingerprint patterns.

MATERIALS AND METHODS

Bacterial strains

Twenty-four American Type Culture Collection (ATCC, Rockville, MD) reference strains including Staphylococcus aureus (10832, 13709), Staphylococcus chromogenes (43764), Staphylococcus xylosus (35663), Escherichia coli (4350, 31619, 12014, 23545), Enterobacter cloacae (961), Klebsiella pneumoniae (27736), Proteus mirabilis (35659), Salmonella typhimurium (14028), Serratia marcescens (13880), Pseudomonas aeruginosa...
DNA isolation

Bacterial species DNA was isolated using a modified InstaGene protocol (BioRad, Melville, NY) as described by Gillespie et al. (8). Modifications were as follows. Genomic DNA was isolated from 1.5 ml of overnight tryptic soy broth (Difco Laboratories, Detroit, MI) culture. The cells were washed once with 1 ml of 10 mM Tris, 5 mM EDTA, pH 7.8 (TE buffer). InstaGene purification matrix (0.2 ml) was added to the bacterial pellet. The mixture was vortexed and incubated at 56°C for 30 min followed by a 10 min incubation at 99°C. The cell lysate was then centrifuged for 5 min at 14,000 rpm. From the supernatant, 0.175 ml was withdrawn and the DNA was quantified using a Du 640 spectrophotometer (Beckman, Fullerton, CA). Absorbance of a 1:50 dilution of DNA in sterile water was measured at 260 and 280 nm.

Randomly amplified polymorphic DNA

Amplification of DNA was done essentially as described by Williams et al. (31) except that 30 ng of DNA, 10 pmoles of primer and 3.5 u of Taq DNA polymerase (Promega, San Diego, CA) were used. Following an initial denaturation step of 94.5°C for 120 s, parameters for the thermocycler (EasyCycling Series TwinBlock System; Ericomp Inc., San Diego, CA) were set at 94.5°C for 70 s, 33°C for 60 s and 72°C for 130 s. Thirty-five cycles of DNA amplification were performed. Ramping time from 94.5°C to 33°C was 150 to 165 s. Electrophoresis, visualization, and documentation of amplified products were done as described previously (17).

Evaluation of RAPD fingerprint patterns

The negative of the Polaroid film was scanned with a computer-integrated laser densitometer (Ultrascan XL; LKB Produkter AB, Bromma, Sweden). Scans were evaluated with Gelscan XL version 2.0 software package (Pharmacia LKB Biotechnology, Uppsala, Sweden) to determine the number and size of DNA fragments. Densitometric data were transferred to Alpha Four (Alpha Software Corporation, Burlington, MA) database management software to compare fingerprint patterns.

Selection of primers based on RAPD fingerprint patterns

RAPD fingerprint patterns of ATCC reference strains were examined for: (i) number of DNA fragments, (ii) optical density of each fragment, and (iii) size of fragments (kb). The first screening step categorized primers into two groups based on optical density and number of DNA fragments. Primers that generated DNA fragments between 0.2 kb and 2.0 kb with at least 2 but not more than 7 fragments in a fingerprint pattern with an optical density (OD) >0.25 for all species included within the group (staphylococci, gram-negative, or streptococci/enterococci) were identified. A ±2% variation in the size of the DNA fragment as compared to that of the reference strain was scored as a “similar” fragment. In the second screening step, primers were evaluated for reproducibility of DNA fingerprint patterns. Reproducibility of a DNA fingerprint pattern generated with a primer was determined based on size, number, and OD of the fragments. If similar DNA fingerprint patterns were obtained on at least three different occasions, then the primer was selected for evaluation with wild type isolates.

Species identification scheme

Wild type isolates (n = 84) belonging to 15 species were examined for RAPD fingerprint patterns using primers OPE-4 and OPE-20. The Clique-compatibility program in the software package Phylip version 3.3 developed by Felsenstein (University of Washington, Seattle, WA), was used to construct phenograms. The Clique program uses the compatibility method for unrooted two-state characters to obtain the largest cliques of characters expressed in the form of possible trees (phenograms). The molecular size (in kb) of amplified fragments ranging from 0.2 to 2.0 were used to define a data matrix by scoring the DNA fragment as either 0 (absent) or 1 (present). The DNA fragment that was common to the group was called the primary fragment, while other fragments characteristic of the species were called secondary fragments. Similar or dissimilar DNA fragments were identified on the basis of size and optical density of the fragment.

RESULTS

The ATCC reference strains, including three Staphylococcus species, seven gram-negative species, two Enterococcus, and six Streptococcus species, were used to evaluate 40 primers from commercially available primer kits A (OPA-01-20) and E (OPE-01-20). A total of 960 RAPD fingerprint patterns were evaluated using set criteria established to identify the “ideal” primer(s) for species identification of bacteria from milk. Fifteen primers including 10 from kit OPA (OPA-1, 3, 5-7, 9, 10, 11, 13, 14, 18) and 5 from kit OPE (OPE-2, 3, 4, 15, 20) met the established criteria. The 15 primers were then evaluated for reproducibility. Primers OPA-3, 7, 14, 13, and 18 and primers OPE-4 and -20 gave identical RAPD fingerprint patterns based on optical density, size, and number of DNA fragments on three separate occasions for a given group of ATCC strains. The other primers that gave reproducible fingerprint patterns were found suitable for either Staphylococcus species (OPA-13, OPA-14, OPA-18), gram-negative (OPA-7, OPA-14), or Streptococcus and Enterococcus (OPA-3) species. Primers OPE-4 and OPE-20 were the only primers suitable for all three groups of ATCC reference strains included in the study.

Primers OPE-4 and OPE-20 were evaluated further with 84 wild-type isolates comprising 15 bacterial species (Fig. 1 and 2). Phenogram analysis of RAPD fingerprint patterns resulted in development of a species identification scheme for 15 bacterial species using either primer OPE-4 (Fig. 3) or OPE-20 (Fig. 4). Differentiation of 15 bacterial species using PCR-based DNA fingerprinting with primer OPE-4 resulted in assigning the organisms to 5 groups, each group identified by a primary fragment (Fig. 3). Similarly, PCR-based DNA fingerprinting with primer OPE-20 resulted in assigning the organisms to 5 groups (Fig. 4). Determination of secondary fragments allowed identification of the bacteria (Fig. 3 and 4).
RAPD FINGERPRINTING FOR BACTERIA IN MILK

FIGURE 3. Differentiation of 15 bacterial species using PCR-based DNA fingerprinting with primer OPE-4. For a given DNA fingerprint pattern, the primary DNA fragment is located first, followed by secondary DNA fragments that match an organism which shares the same primary fragment within a group.

DISCUSSION

Scientific advances in diagnostic technology and instrumentation in the past decade have resulted in a wide variety of biochemical and enzymatic tests for species identification of bacteria (11, 27). Most of these tests allow identification of bacteria based on phenotypic characterization utilizing biochemical tests, serotyping, and enzymatic profiles. Identification methods based on phenotypic characterization have been evaluated extensively (1, 10, 18, 21, 26, 29).

In recent years, researchers have focused on developing alternate methods to overcome some of the difficulties associated with phenotypic characterization of bacteria. Studies have shown that nucleic acid analysis could provide an alternative approach to species identification using phenotypic characterization, especially for bacterial species that are phenotypically indistinguishable (13, 16). RAPD is produced by enzymatic amplification of genomic bacterial DNA using primers that are not directed or targeted to any known sequence of the bacterial genome. Amplification of template DNA is directed by one or more arbitrary oligonucleotide primers to produce a characteristic spectrum of products. Several variations of this technique have been developed each having differences in DNA amplification conditions, length of primer, and resolution of products obtained (3, 30, 31). Although RAPD has been utilized successfully for subtyping several species of bacteria, the lack of a simplified analytical approach to interpret DNA fingerprint patterns has hampered precise comparison of results from different laboratories and thus slowed universal adoption of the RAPD technique for subtyping bacteria. This has also led to underevaluating the potential of RAPD for bacterial species identification.

With the objective of establishing a simplified method to PCR-based fingerprinting, we undertook screening of 40 commercially available primers. Commercially available arbitrary 10-mer primers were used in our study to preclude differences observed with primer quality, quantity, and concentration used for RAPD analysis. Lack of consensus on which primer to use has lead to uncontrolled proliferation of arbitrary primers, making it difficult for researchers to determine the "ideal" primer that could be of value for RAPD analysis. The "ideal" primer(s) was hypothesized as a primer that resulted in at least 2 to 7 fragments with an OD of >0.25 that gave reproducible DNA fingerprint patterns.

Phenogram analysis was done to determine the largest cluster of shared DNA fragments across the several bacterial species included in the study. This resulted in development of a species identification scheme for two primers, OPE-4 and OPE-20, that gave suitable DNA fingerprint patterns for several different species including gram-negative, staphylococci, streptococci and enterococci, and representative wild-type species.

In conclusion, results of this study suggest that PCR-based DNA fingerprinting using RAPD can be used for...
identifying several bacterial species isolated frequently from milk. Further examination of additional wild-type isolates in a blind study needs to be conducted to critically evaluate this technique as a rapid and accurate method for species identification of bacteria.

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REFERENCES