

Detection and Identification of Species with Bacterial Cells Using a Plastic DNA Array

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The rapid identification of bacteria in many kinds of samples, *i.e.*, clinical, food, water, and material, is important from a hygienic standpoint. In this paper, we describe the development of a convenient bacterial identification system using bacterial cells by a plastic DNA array. The small plastic base, which was cut from an S-BIO[®] PrimeSurface[®] plastic base developed for the covalent immobilization of amino-modified DNA, was used as a substitute for a glass base. The species-specific primers were immobilized on the small plastic base and the spots specific to each bacterial species were observed after 2 types of thermal cycles in a single PCR tube using bacterial culture broth as a sample. The results obtained using the culture broth were the same as those obtained with total DNA extracted from bacterial cells. The detection limits of *Staphylococcus aureus* (*S. aureus*) ATCC25923 and *Escherichia coli* (*E. coli*) ATCC25922 were 8.7×10^3 cells/ μ l and 2.1×10^2 cells/ μ l, respectively. This system is useful and convenient for the identification of bacteria in many types of samples. Moreover, further improvements in conditions, such as the ingredients in the reaction mixture, thermal cycles, and the steps of visualization would result in a more efficient system of identification.

Key words — DNA chip, DNA microarray, identification of bacteria, plastic base

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INTRODUCTION

The rapid and accurate detection and identification of bacteria in many types of samples, *i.e.*, clinical, food, water, and material, is useful and important from the standpoint of hygiene. Most conventional assays for bacterial detection and identification depend on the culturing of bacteria and require several days for completion. Moreover, certain bacteria may or may not form visible colonies on plates after several days.

DNA microarrays based on hybridization between fluorescent-labeled single-strand nucleotide chains and presynthesized oligonucleotides immobilized on a solid phase have emerged as powerful and promising revolutionary tools for large-scale parallel genetic analysis.¹⁻³⁾ Currently, an oligonucleotide-based microarray offers several advantages over cDNA microarrays and has a global application in gene expression profiling studies, genotypes such as single nucleotide polymorphisms (SNPs), and resequencing. A DNA microarray was utilized for the detection and identification of bacteria in food samples using fluorescence-labeled 16S rDNA fragments amplified by the polymerase chain reaction (PCR).⁴⁾ Moreover, an on-chip PCR was used to identify bacteria in clinical samples; this involves the amplification of a partial 23S rDNA fragment and extension of a species-specific primer immobilized on a glass slide.⁵⁾ In both bacterial identification systems, DNA extracts were used as a templates for the amplification of partial 16S or 23S rDNA fragments.

Recently, a new plastic base termed S-BIO[®] PrimeSurface[®] was developed for the DNA microarray, and it was applied to SNPs using multiple primers extension (MPEX) by DNA polymerase.^{6,7)} In this paper, we described the use of the plastic base in the development of a convenient bacterial identification system using bacterial cells by an on-chip PCR method. As seen in the case of total DNA samples extracted from bacterial cells, specific spots for each bacterial strain were observed on the chip when a bacterial culture broth was used for the PCR. This identification system may be used to detect and identify bacteria in many types of samples.

MATERIALS AND METHODS

Bacterial Strains and Culture conditions — *Staphylococcus aureus* (*S. aureus*) ATCC25923,

Escherichia coli (*E. coli*) ATCC25922, *Salmonella enterica* (*S. enterica*) serovar Typhimurium ATCC14028, and *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC27853 were purchased from the American Type Culture Collection, U.S.A. *S. enterica* serovar Enteritidis IID604 was kindly provided by the Institute of Medical Science, University of Tokyo, Japan. These strains were cultured on nutrient agar plates or in nutrient broth (Eiken, Tokyo, Japan) at 37°C. *S. aureus* ATCC25923 was grown in nutrient broth supplemented with 0.5% glycine for the isolation of total DNA. The colony forming unit (CFU) of bacterial liquid culture was determined using the serial dilution method with plates of nutrient agar (Eiken).

Oligonucleotide Immobilization on the S-BIO[®] PrimeSurface[®] Plastic Base— The small plastic base (2 × 15 × 1 mm), which was cut from the S-BIO[®] PrimeSurface[®] plastic base (25 × 75 × 1 mm; Sumitomo Bakelite, Tokyo, Japan) developed for covalent immobilization of amino-modified DNA, was used in this study. The sequences of the species-specific primers immobilized on base plates are shown in Table 1. The immobilization of amino-modified oligonucleotides on the small plastic base was carried out following the manufacturer's instructions. A solution of 2 μM oligonucleotides in 1 × S-BIO spotting solution (Sumitomo Bakelite) was prepared. Drops of approximately 0.2 μl were spotted on the plastic base using a micropipette. The spotted base was heated at 80°C for 1 hr to fix the oligonucleotides and was then soaked in 1 × S-BIO blocking solution (Sumitomo Bakelite) to inactivate the unreacted surface of the base. After washing the chip with water and drying using an air spray, it was stored in a desiccator.

On-chip PCR Using the One-step or Two-step Method— On-chip PCR was performed by 2 different methods, namely, the one-step method or the two-step method. On-chip PCR using the one-

step method was described in a previous paper.⁵⁾ The 74 μl PCR mixture was prepared in a 0.2 ml PCR tube containing 1 × Ex *Taq* buffer (Takara, Otsu, Japan); 20 μM each of dATP, dCTP, and dGTP; 6.7 μM, dTTP; 13.3 μM, biotin-11-dUTP (PerkinElmer, Waltham, MA, U.S.A.); 67 μM, 23S rDNA specific primers (43a2, 69ar2, and 69arrh); and 2.5 U of Ex *Taq* DNA polymerase (Takara). One microliter of DNA solution, bacterial culture broth, or its diluted bacterial broth was added, and a piece of oligonucleotide-immobilized chip was placed in the tube prior to thermal cycling. Thermal cycling was carried out as follows: initial denaturation at 98°C for 3 min followed by 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 65°C, and extension for 2 min at 72°C, using a GeneAmp[®] PCR System 9700 (Applied Biosystems, FosterCity, CA, U.S.A.).

A single PCR tube was used in the two-step method, but 2 thermal cycles were used; the first PCR was to amplify the 23S rDNA partial fragment and the second was for the extension of the immobilized oligonucleotides. The 24 μl PCR mixture used for the first PCR was prepared in a 0.2 ml PCR tube and contained 1 × Ex *Taq* buffer; 100 μM of each dATP (2'-deoxyadenosine 5'-triphosphate), dCTP (2'-deoxycytidine 5'-triphosphate), dGTP (2'-deoxyguanosine 5'-triphosphate), and dTTP (2'-deoxythymidine 5'-triphosphate); 20 μM, 23S rDNA specific primers (43a2, 69ar2, and 69arrh); and 2.5 U of Ex *Taq* DNA polymerase. One microliter of extracted DNA solution or a diluted solution of bacterial culture broth was added to the tube prior to thermal cycling. The thermal cycles were as follows: 7 min of denaturation at 95°C, followed by 30 cycles of denaturation for 1 min at 95°C, annealing and extension for 2 min at 70°C, and a final extension step of 5 min at 72°C. Before the second thermal cycling, 50 μl of the solution containing 1 × Ex *Taq* buffer; 20 μM of each

Table 1. Sequences of Primers Used in This Study

Primer	Sequence (5' to 3')	Selectivity	5' modification
43a2	GACAGCCAGGATGTTGGCTTAGAAGCAGC	Universal	none
69ar2	GGAATTCGCTACCTTAGGACCGTTATAGTTACG	Universal	none
69arrh	GGAATTCGCTACCTTAGGATGGTTATAGTTACC	Universal	none
SA-1	CTAAGGGCGTTGAAGCATGATCGTA	<i>S. aureus</i>	NH ₂ -
ECO-2	GCACGCTGATATGTAGGTGAAGCGACT	<i>E. coli</i>	NH ₂ -
SAL-1	TGTGTGTTCCAGGTAAATCCGGTTC	<i>S. enterica</i>	NH ₂ -
PA-1	GTTAATCGACGCAGGGTTAGTCGGTT	<i>P. aeruginosa</i>	NH ₂ -

Sequences of these primers have been previously described by Mitterer, G. *et al.*⁵⁾

dATP, dCTP, dGTP, and biotin-11-dUTP (Biotin-11-2'-deoxyuridine-5'-triphosphate); and 2.5 U of Ex *Taq* DNA polymerase was added to the tube and a piece of oligonucleotide immobilized chip was put in the tube. The second thermal cycling was carried out as follows: initial denaturation at 95°C for 5 min followed by 15 cycles of denaturation for 30 s at 95°C; annealing for 30 s at 65°C; and extension for 2 min at 72°C. The chip with the extended primer was washed with S-BIO washing buffer (Sumitomo Bakelite) including 0.1% sodium dodecyl sulfate (SDS), 1/10 × washing buffer and 1/100 × washing buffer before the detecting the signals.

Detection of Signals on the Chip and the Partial Fragment of 23S rDNA — The washed chip was exposed to alkaline phosphatase-conjugated streptavidin (1/1000 dilution; PerkinElmer) in 1 × S-BIO dilution solution (Sumitomo Bakelite). It was incubated at 37°C for 30 min, then washed in the washing buffer, including 0.1% SDS, 1/10 washing buffer and 1/100 washing buffer. The washed chip was put in 5-bromo-4-chloro-3'-indolylphosphatease (BCIP)/nitro-blue tetrazolium chloride (NTB) (PerkinElmer) and incubated at 37°C for 15 min, then washed in distilled water. After drying with an air spray, the image on the chip was scanned using a GT-9400UF scanner (Epson, Suwa, Japan). Agarose gel electrophoresis was per-

formed to examine the amplified partial 23S rDNA fragments in the residual reaction solution.

RESULTS AND DISCUSSIONS

On-chip PCR with Purified DNA

In the Japanese Pharmacopoeia, Fifteenth Edition, 4 bacterial species, namely, *S. aureus*, *E. coli*, *S. enterica*, and *P. aeruginosa*, were the target species for specific bacterial examination in a microorganism limiting test.⁸⁾ We, therefore, selected the 5 strains as described in Materials and Methods. DNA extracts, which were isolated from the bacterial culture, were used as samples for on-chip PCR in a previous report.⁵⁾ We also verified that the total DNA extracted from bacterial cells could be used as a sample for the both of on-chip PCR protocols using the small plastic base. Total DNA was extracted from an overnight culture of bacterial cells. A specific spot was observed for each bacterial strain on the chip using both the on-chip PCR methods (Fig. 1). All spots appeared on the chip in case of the control sample comprising a mix of 5 types of DNA. The amplified 1.0 kb DNA fragments of the partial 23S rDNA were observed in all the reaction mixtures by agarose gel electrophoresis (data not shown). On the chips for

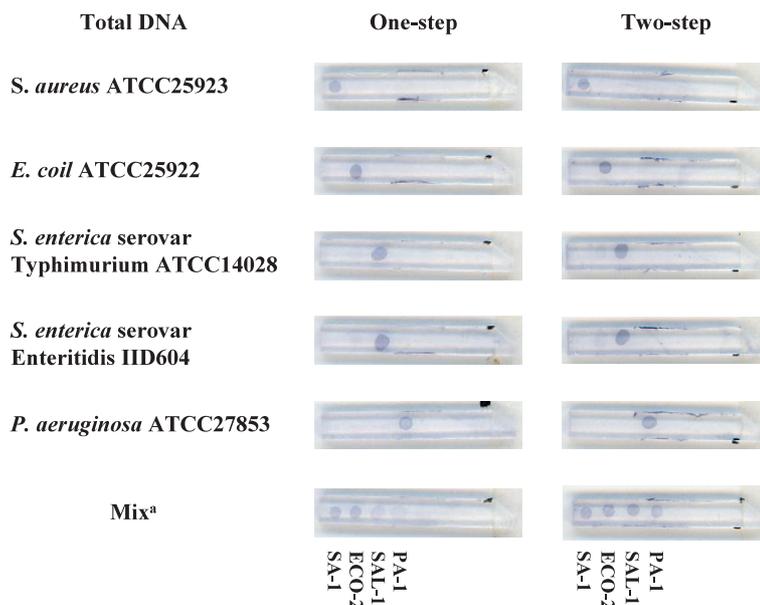


Fig. 1. Detection of Total Bacterial DNA Using the One-step and Two-step Methods

The SA-1, ECO-2, SAL-1, and PA-1 spots are positioned at the region of the selective primer for each species. The concentrations of total DNA extracted from *S. aureus* ATCC25923, *E. coli* ATCC25922, *S. enterica* serovar Typhimurium ATCC14028, *S. enterica* serovar Enteritidis IID604, and *P. aeruginosa* ATCC27853 were 8.0 µg/ml, 0.25 µg/ml, 0.25 µg/ml, 0.31 µg/ml, and 0.26 µg/ml, respectively. a: An equal volume of each DNA solution extracted from 5 strains was mixed.

S. aureus ATCC25923, *E. coli* ATCC25922, and *P. aeruginosa* ATCC27853, specific spots of only SA-1, ECO-2, and PA-1 respectively, were observed. However, weak spots on the chips of *S. enterica* serovar Typhimurium ATCC14028 and *S. enterica* serovar Enteritidis IID604 appeared in the ECO-2 position, and a specific strong spot appeared in the SAL-1 position. The taxonomical relationships between the *S. enterica* and *E. coli*, both of which belong to the family *Enterobacteriaceae*, are closer than those of *S. aureus* or *P. aeruginosa*.⁹⁾ The complete sequences of the 23S rRNA gene of *S. enterica* serovar Typhimurium, *E. coli*, *S. aureus*,

and *P. aeruginosa* can be obtained from the DNA database under accession numbers AE006468, V00348, X68425, and AE004091, respectively. The sequence similarity of the amplified 23S rDNA region between *S. enterica* serovar Typhimurium and *E. coli* was 91.0%. On the other hand, the similarities of *S. enterica* serovar Typhimurium with *S. aureus* and *P. aeruginosa* were 64.4% and 79.4%, respectively. Therefore, nonspecific weak spots of ECO-2 appeared on the chips of *S. enterica* serovar Typhimurium ATCC14028 and *S. enterica* serovar Enteritidis IID604.

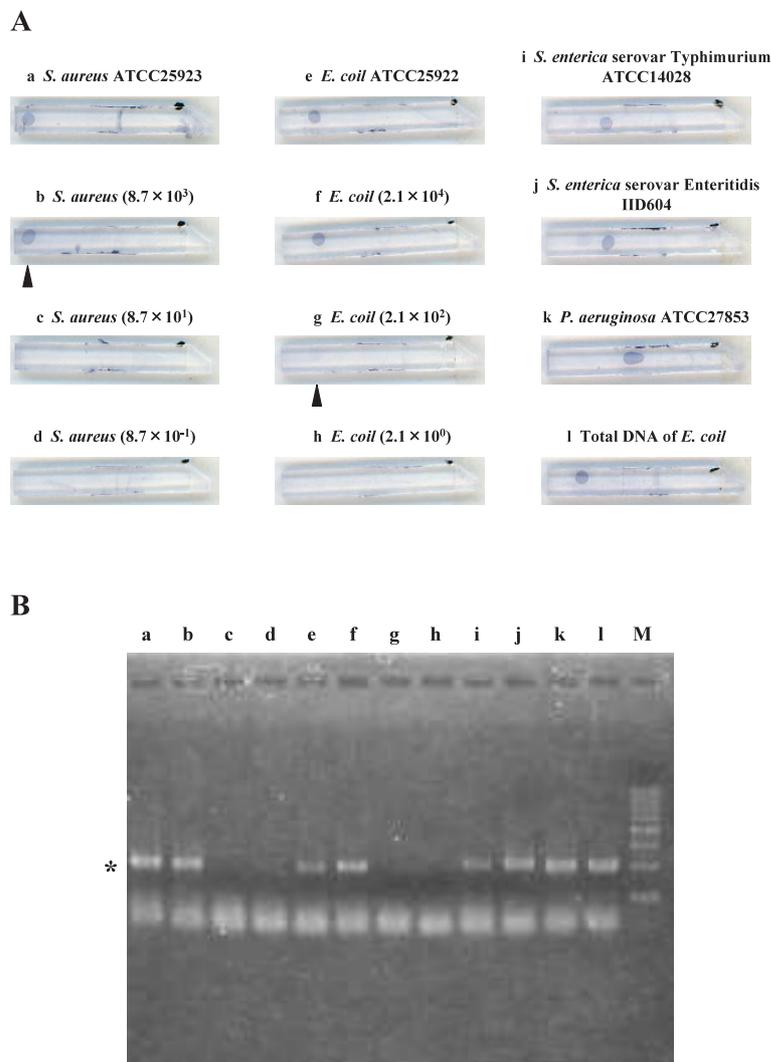


Fig. 2. Detection of Bacteria in Bacterial Culture Broth Using Two-step Method and Agarose Gel Electrophoresis of the Amplified Partial 23S rDNA Fragments in Residual Reaction Solutions.

A: Detection of bacteria in bacterial culture broth and its dilutions of the broth using the two-step method. The concentration of cells in each diluted sample is shown in parentheses. The positions of the selective primers immobilized on the chip are similar to those in Fig. 1. The specific spots of *S. aureus* ATCC25923 and *E. coli* ATCC25922 on the detection limit are shown with arrows. The concentration of total DNA extracted from *E. coli* ATCC25922 was 0.25 $\mu\text{g}/\text{ml}$ (chip l). B: Observation of partial 23S rDNA fragments in residual reaction solutions using the two-step method with agarose gel electrophoresis. Ten microliters of the reaction solution residue after the second PCR was applied to the agarose gel. An asterisk shows the position of the partial 23S rDNA fragment (1.0 kb). Lanes a–l correspond to the chips soaked in the residual reaction solution in Fig. 2A. M: 500 to 5000 kb DNA ladder marker (Takara).

On-chip PCR with Bacterial Cells

The on-chip PCR using the small plastic base was performed using 2 different PCR protocols, and specific spots appeared on the chips in case of both protocols using the total DNA. However, if the samples could be analyzed directly without DNA extraction, it would be very convenient to identify the bacterial species in water, food, and the environment. Next, we attempted to use the bacterial culture broth directly for the on-chip PCR using the small plastic base. Five kinds of bacteria were cultured overnight in nutrient broth, and the culture broths of *E. coli* and *S. aureus* were serially diluted with 0.9% saline. All the culture broth and the dilutions were applied to the reaction solutions instead of the total DNA. Specific spots for each bacterial strain were observed on the chip with the bacterial culture broth, using the two-step method (Fig. 2A). Moreover, weak spots on the chips of *S. enterica* appeared in the ECO-2 position in the same as weak spots derived from the total DNA of *S. enterica*. The amplified 1.0 kb DNA fragments of the partial 23S rDNA fragment were recognized in all the residual reaction solutions by agarose gel electrophoresis (Fig. 2B). On the other hand, we could not obtain clear results with the bacterial culture broth using the one-step method (data not shown). When the diluted culture broth of *S. aureus* was applied as a sample, the 10^{-2} dilution (8.7×10^3 cells/ μ l) displayed the specific *S. aureus* spot on the chip by the two-step method, but no spot appeared on the chips in case of the 10^{-4} and 10^{-6} dilutions. A clear specific spot was observed at the ECO-2 position with the 10^{-2} dilution of the *E. coli* broth (2.1×10^4 cells/ μ l); moreover, the specific spot weakly appeared at the ECO-2 position with the 10^{-4} dilution of the *E. coli* broth (2.1×10^2 cells/ μ l). Agarose gel electrophoresis demonstrated the amplified 1.0 kb DNA fragments of partial 23S rDNA in the residual reaction solution in case of the 10^{-2} dilutions of the *S. aureus* and *E. coli* broth, but there were no PCR fragments in the case of the 10^{-4} and 10^{-6} dilutions of both strains (Fig. 2B). It was presumed that the partial 23S rDNA fragments of *E. coli* ATCC25922 were amplified in the reaction mixture containing the 10^{-4} dilution of *E. coli* broth, but most of the fragments were attached to the *E. coli* specific primer ECO-2 immobilized on the chip. Therefore, the concentration of the amplified fragments in the reaction solution residues was too low to be observed on the agarose gel electrophoresis.

We could detect the specific spot on each chip with bacterial culture broth as well as total DNA extracted from bacterial cells by the two-step method. Moreover, 10^2 to 10^3 of bacterial cells were also detected with the plastic chip. Ideally, 1 cell in 1 μ l of sample should be detected in a microorganism limiting test. The clear specific spot was observed at the ECO-2 position with 10 ng of total DNA purified from *E. coli* cells; moreover, the specific spot weakly appeared with 1.0 ng of the total DNA (data not shown). *E. coli* K-12 has 4.6 Mbp chromosomal DNA, and 7 complete sets of bacterial 16S, 23S, and 5S rRNA genes lie on the chromosomal DNA.¹⁰⁾ Since the mass of *E. coli* chromosomal DNA in a cell is approximately 50 fg, 140 to 1400 genes encoding 23S rRNA were included in 1.0 to 10 ng of the total DNA. In this study, 2.1×10^2 cells of *E. coli* ATCC25922 could be detected using the two-step method. According to calculations, 1470 copies of the 23S rRNA gene were included in the reaction mixture, and the copy number was almost the same as that in 10 ng of the total DNA. Therefore, the limit of detection in the reaction mixture might be 10^2 to 10^3 bacterial cells using the two-step method. In this study, 4 types of specific primers were used to identify and detect the 4 bacterial species. However, many kinds of bacteria besides these 4 bacterial species used in this study exist in clinical, food, water, and material samples. These unspecified bacteria can be detected with a universal primer that anneals with the 23S rRNA gene of almost bacterial strains. Results using the two-step method were obtained within 5 to 6 hr as in the previous report using DNA extracts.⁵⁾ Further improvements in the conditions of this system, *i.e.*, composition of the reaction mixture, thermal cycle conditions, and visualization, will definitely result in a more efficient identification system and a convenient method for the detection and identification of bacteria in various samples. Our basic examinations demonstrated that the specific spots derived from 10 to 100 copies of template DNA could be observed on the plastic base when the Cy5-labeled extended nucleic acid chains on the plastic base were captured with a laser scanner (data not shown). Therefore, the limit of detection can be improved using the Cy5 detection system.

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