The simple and rapid detection of specific PCR products from bacterial genomes using Zn finger proteins

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ABSTRACT

A novel method of rapid and specific detection of polymerase chain reaction (PCR) products from bacterial genomes using Zn finger proteins was developed. Zn finger proteins are DNA-binding proteins that can sequence specifically recognize PCR products. Since Zn finger proteins can directly detect PCR products without undergoing dehybridization, unlike probe DNA, and can double check the specific PCR amplification and sequence specificity of the PCR products, this novel method would be quick and highly accurate. In this study, we tried to detect *Legionella pneumophila* using Sp1. It was found that a 49 bp *L. pneumophila*-specific region containing the Sp1 recognition site is located on the *flh*A gene of the *L. pneumophila* genome. We succeeded in specifically detecting PCR products amplified from *L. pneumophila* in the presence of other bacterial genomes by ELISA, and demonstrated that Sp1 enables the discrimination of *L. pneumophila*-specific PCR products from others. By fluorescence depolarization measurement, these specific PCR products could be detected within 1 min. These results indicate that the rapid and simple detection of PCR products specific to *L. pneumophila* using a Zn finger protein was achieved. This methodology can be applied to the detection of other bacteria using various Zn finger proteins that have already been reported.

INTRODUCTION

The detection of pathogenic bacteria is important for our health and safety. The development of rapid and specific methods of detecting pathogenic bacteria in fields such as the food industry, clinical diagnosis and environmental control is required (1). Traditional methods, including culturing and immunological assays, remain the standard detection methods even now because of their high accuracy and sensitivity. However, it takes much time to detect bacteria using these methods, which require long culturing times. Other detection techniques that allow rapid and easy detection are also necessary.

In recent years, polymerase chain reaction (PCR) technology has been widely used to detect pathogenic bacteria (2,3). Bacterial genome DNA can be amplified by PCR in a short time, in contrast to culturing. Detection using PCR takes much less time than traditional detection methods. Thus, PCR technology has the potential to enable the rapid and specific detection of pathogenic bacteria via specific amplification and detection.

In PCR-based bacterial detection, PCR-amplified DNA must also be quickly and conveniently detected. Generally, the presence of amplified products can be confirmed by gel electrophoresis after PCR amplification. Several detection systems for pathogenic bacteria such as *Salmonella* based on the combination of PCR and gel electrophoresis have already been developed and commercialized. Gel electrophoresis is an easy method of detecting PCR products, but it cannot distinguish between specific amplified products and non-specific ones. Thus, gel electrophoresis is not sufficiently accurate to specifically detect PCR-amplified products.

To detect a target sequence specifically, DNA probe hybridization is generally performed (4,5). Although DNA probe hybridization provides more sequence specificity, the procedures to dehybridize the ssDNA from the amplified original dsDNA and to hybridize the DNA probe with the target sequence in the ssDNA are complicated. In addition, DNA probe hybridization is less efficient, since rehybridization of the separated ssDNA with the original complementary ssDNA occurs dominantly (6). We have

previously reported a PCR product detection method based on probe DNA hybridization with unilateral protruding DNA, but this procedure also requires several steps (7,8); recognition elements that can directly and specifically detect dsDNA are required for the rapid and specific detection of pathogenic bacteria.

Zn finger proteins are the most popular DNA-binding proteins in mammals. The most common Zn finger proteins are the C_2H_2 Zn finger proteins, whose structure is stabilized by a zinc ion bound to the Cys and His residues of each finger containing two β-strands and one α-helix (9–13). The C_2H_2 fingers can bind to DNA sequences with high affinity and specificity. Furthermore, it has been reported that different C_2H_2 Zn finger proteins can bind to different target sequences depending on the amino acid sequence of the fingers, the number of fingers and the combination of fingers (12). Various screening procedures and artificial design strategies have also been attempted to make Zn finger proteins bind to desired sequences (14–20). Such artificial Zn finger proteins are expected to be artificial transcriptional factors and artificial nucleases (20–23). A dsDNA detection system using a Zn finger protein, called 'Sequence-Enabled Reassembly' (SEER), has been reported (24–26). Although this system can distinguish target DNA from non-target DNA, only the binding ability of the Zn finger protein against short target sequences (<31 bp) has been investigated, and PCR product detection has not been reported to date. Thus, bacterial detection using Zn finger proteins has never been reported.

In this work, we describe the development of a novel methodology for the specific detection of amplified products from the genomes of pathogenic bacteria using a Zn finger protein. Our detection principle based on Zn finger proteins is schematically illustrated in Figure 1. In this system, a specific sequence from the bacterial genome is amplified, and the obtained PCR products are directly detected using the Zn finger protein. Thus, we expect to be able to double check PCR amplification and sequence specificity via direct detection using Zn finger proteins. This system of double checking and direct detection without dehybridization of the PCR products has the advantage of accurate and rapid detection, which is
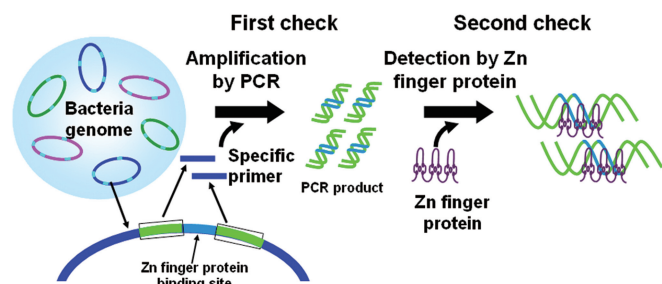
important in the detection of pathogenic bacteria. In principle, this system can also perform detection with DNA-binding proteins other than Zn finger proteins. We have already succeeded in constructing a *Salmonella* detection system using an engineered dsDNA-binding protein, DnaA IV (27). Zn finger proteins might be better suited than other DNA-binding proteins, since Zn finger proteins have high affinity and specificity for dsDNA as a monomer, and their binding mode has already been well studied.

To construct our system, the part of the bacterial genome containing the Zn finger protein recognition site should be amplified. It is highly possible that there are several Zn finger-binding sites in the genome of the target bacterium, and even in those of other bacteria, because some Zn finger proteins recognize short sequences, for example, 9 bp sequences in the case of well-characterized Zn finger proteins such as Zif268 and Sp1 (28–30). However, we need primers (e.g. 20-bp long) for PCR amplification, and the resulting 49 bp target sequence might be sufficiently specific to enable the detection of the target bacterial genome. To select the target sequence, we first searched for the Zn finger-binding sites and identified both ends of the genome sequence as primer regions for PCR, as shown in Figure 1.

To demonstrate the viability of this Zn finger protein-based detection system, we chose human transcription factor Sp1 as the Zn finger protein for dsDNA detection. Sp1 is a well-characterized C_2H_2 Zn finger protein that has three C_2H_2 fingers. Mutagenesis and NMR studies of the Zn finger domain of Sp1 have predicted the DNA-binding mode of Sp1 against 5′-GGG GCG GGG-3′ (29,30). This protein has a high binding affinity of ~3.5 nM against the GC box containing this 9 bp sequence (29).

We also chose *Legionella pneumophila* as the target pathogenic bacterium. *L. pneumophila* is the major causative agent of Legionnaires' disease. In recent years, *L. pneumophila* has often been found in man-made water systems such as cooling towers, hot springs and circulation type baths (31,32). The detection of *L. pneumophila* in man-made water systems is essential for preventing the spread of *Legionella* infection. The standard method of detecting *L. pneumophila* is culturing in selective media (33). However, it is difficult to detect *L. pneumophila* rapidly using the culture method since these bacteria grow slowly and culturing is therefore a time-consuming procedure (~3–6 days). Therefore, PCR-based detection of *L. pneumophila* is required. It has already been reported that *L. pneumophila* can be detected by the PCR amplification of conserved genes in *Legionella* such as the 16S rRNA gene (34,35), the macrophage infectivity potentiator (*mip*) gene (35–37) and others.

In this work, we tried to detect specific PCR products amplified from the *L. pneumophila* genome using Sp1 to demonstrate our novel methodology described above.

Figure 1. Scheme of the double-check detection system for the detection of pathogenic bacteria using a Zn finger protein. In the presence of the target bacterium, the target bacterium-specific region containing the Zn finger protein-binding site is amplified by PCR as the first check. The obtained PCR products are detected by the Zn finger protein as the second check.

MATERIALS AND METHODS

Materials

All biotinylated and fluorescein isothiocyanate (FITC)-labeled oligonucleotides were synthesized by Invitrogen

(California, USA). Genomic DNA from *L. pneumophila* subspp. *pneumophila* str. Philadelphia 1 (ATCC 33152D) was purchased from the American Type Culture Collection (Virginia, USA). The genomic DNA of the other organisms was prepared by us. The *L. pneumophila* serogroup 1 strain was grown on GVPC plates (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan) at 37°C for 5 days and the colonies were counted. Bath water and shower water were sampled from the laboratory staffs' houses. All other chemical reagents used were of analytical grade.

BLAST search of the target sequence from the *L. pneumophila* genome

We searched the Sp1-binding site, 5′-GGG GCG GGG-3′ (29,30), on the *L. pneumophila* genome using NCBI Nucleotide BLAST for short nearly exact matches (http://www.ncbi.nlm.nih.gov/BLAST/) limited by the Entrez query 'bacteria and *Legionella*'. From among the obtained data, we selected the target gene containing the Sp1-binding site in *L. pneumophila*. We also checked the specificity of the 49 bp target sequence, the selected 9 bp Sp1-binding site and the 20 bp primer regions at both ends among all the genomes using NCBI Nucleotide BLAST.

Expression and purification of GST fusion Sp1

The Zn finger domain from the human Sp1 gene was cloned from the human lymph node cDNA library (Takara Bio Inc., Otsu, Japan) into pGEX-2T vector (Promega, WI, USA), an *Escherichia coli* expression vector that produces GST fusion proteins (38). The plasmid was introduced into *E. coli* BL21 (DE3) cells. The clones were cultured at 37°C to an OD_{660} of 0.7. Then, the expression of GST fusion Sp1 was induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 30°C for 4 h. The cell pellet was collected by centrifugation at 3000 g for 10 min and resuspended in cell lysis buffer (PBS, 1% (v/v^1) Triton X-100, 5 mM DTT, 4 mM Pefabloc SC, pH 7.3). It was then homogenized using a French press and centrifuged at 20 000 g for 30 min at 4°C. Next, the GST fusion Sp1 was affinity purified using a GSTrap HF column (GE Healthcare UK Ltd., Bucks, England) after filtration with a 0.45 μm nitrocellulose filter. The purity of the collected GST-Sp1 was confirmed by SDS–PAGE using PhastGel Gradient 8–25 gels (GE Healthcare UK Ltd.). The activity of GST was measured colorimetrically at 340 nm in measurement solution (0.1 M PPB, 1 mM reduced glutathione, 1 mM 1-chloro-2,4-dinitrobenzene, pH 6.5).

dsDNA preparation

The reaction mixture (100 μl) contained 600 pmol ssDNA, 750 pmol complementary ssDNA and 50 mM NaCl. The mixtures were preheated to 95°C for 5 min and then gradually cooled down to 25°C for 90 min to prepare the dsDNA solution. We also used FITC-labeled or biotinylated ssDNA when necessary.

PCR amplification

Amplification reactions were performed in a final volume of 100 μl containing any template oligonucleotide or genome or the bacterium itself, 1 μM FITC-labeled 5′ primer, 1 μM biotinylated 3′ primer, 10 × PCR buffer (Applied Biosystems, CA, USA), 150 μM dNTP mixture (Applied Biosystems) and 2.5 U of AmpliTaq Gold DNA polymerase that can be hot started with low DNA contamination (Applied Biosystems). PCR amplification was performed on a Program Temp Control System PC-801 (Astec, Fukuoka, Japan). The temperature cycling was as follows: 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 48°C for 1 min and extension at 74°C for 1 min. When the template genome was below 1×10^4 copies, we used the other polymerase, Herculase II Fusion DNA polymerase (Stratagene, CA, USA), which is formulated for PCR with high yield and reliability. The amplification reactions for Herculase II Fusion DNA polymerase were performed in a final volume of 50 μl containing any template oligonucleotide or genome, 0.5 μM FITC-labeled 5′ primer, 0.5 μM biotinylated 3′ primer, 5× PCR buffer (Stratagene), 250 μM dNTP mixture (Stratagene) and 2.5 U of Herculase II Fusion DNA polymerase (Stratagene). PCR amplification was performed on the same machine as described above, and the temperature cycling was as follows: 98°C for 4 min, followed by 35 cycles of denaturation at 98°C for 20 s, annealing at 48°C for 20 s and extension at 72°C for 30 s. The PCR products were confirmed by gel electrophoresis in 3% agarose gels and visualized with ethidium bromide staining. For the PCR products from *L. pneumophila* serogroup 1 cells, the amplification reactions were performed under the same conditions as for Herculase II Fusion DNA polymerase, except for the use of bath water or shower water and the addition of 1×10^4 CFU of *L. pneumophila* serogroup 1 cells.

ELISA

We investigated the binding ability of Sp1 against the target dsDNA or PCR products by ELISA. The prepared dsDNA or PCR products, which were biotinylated, were diluted to a concentration of 100 μM with PBS (0.01 M phosphate buffer, pH 7.3, 0.15 M NaCl) containing 90 μM $ZnCl_2$, and 100 μl of the diluted dsDNA solution were added to the wells of a streptavidin-coated 96-well plate (Nunc, Roskilde, Denmark). The plates were incubated at room temperature for 1 h and then washed with PBS containing 90 μM $ZnCl_2$. One-hundred microliter of 2% skim milk in PBST (0.01 M phosphate buffer, pH 7.3, 0.15 M NaCl, 0.1% Triton) containing 90 μM $ZnCl_2$ were added to the wells, which were incubated for 1 h at room temperature and then washed as described above. GST-Sp1 solution was diluted to a concentration of 0.5 μM with 2% skim milk in PBST containing 90 μM $ZnCl_2$, and 100 μl of the mixture were added to each well. The plates were incubated at room temperature for 1 h and then washed using PBST containing 90 μM $ZnCl_2$. Horseradish peroxidase (HRP)-conjugated anti-GST antibody (GE Healthcare UK Ltd.) was diluted to a concentration

of 1 in 10 000 with 2% skim milk in PBST containing 90 μM ZnCl$_2$, and 100 μl of the mixture were added to each well. After incubation at room temperature for 1 h, the plates were washed as described above. Finally, 100 μl of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate solution in 50 mM citric acid, pH 7.3, containing 0.2% (w/w) hydrogen peroxidase were added to each well. The absorbance at 405 nm was measured using a microplate reader (Model 550, Bio-Rad Laboratories Inc., CA, USA) after 1 h.

Fluorescence depolarization measurement

The binding of Sp1 to the PCR products was also investigated by measuring the fluorescence depolarization using an automatic fluorescence polarimeter (FP-715, Jasco, Tokyo, Japan). FITC-labeled PCR products were prepared as described above. GST-Sp1 solution (2 μM) was added to a PCR product solution diluted 20-fold in PBS (0.01 M phosphate buffer, pH 7.3, 0.15 M NaCl) containing 90 μM ZnCl$_2$. The fluorescence depolarization was measured at an excitation of 485 nm and an emission of 530 nm.

RESULTS

BLAST search of the target sequence from the *L. pneumophila* genome

We first searched for the 9 bp recognition sequence of Sp1 (5′-GGG GCG GGG-3′) in the complete *L. pneumophila* genome by BLAST. As a result, there were two hits in the BLAST against the *L. pneumophila* subspp. *pneumophila* str. Philadelphia 1 genome that belonged to *L. pneumophila* serogroup 1. Among these hits, we chose *flh*A, which codes for the flagellar biosynthetic protein FlhA related to chemotaxis, motility and cell division (39), as the candidate for the detection of *L. pneumophila* subspp. *pneumophila* str. Philadelphia 1, because the other hit corresponding to a gene coding for methoxymalonyl CoA synthase has not been identified in most *L. pneumophila* species. There were 9 bp recognition sequences of Sp1 on the minus strand of the *flh*A gene in *L. pneumophila* subspp. *pneumophila* str. Philadelphia 1.

Since the estimated incidence rate of the 9 bp sequence of the Sp1 recognition site is once per 4^9 bp ($\sim 26 \times 10^4$ bp), we next identified the 49 bp PCR-amplified sequence containing the 9 bp of the Sp1 recognition site. The 20 bp genomic sequences at both ends of the 9 bp Sp1 recognition site on the *flh*A gene were used as the primer regions for PCR amplification. We also confirmed by BLAST whether or not the identified 49 bp sequence was specific to *L. pneumophila*. As shown in the Table 1, our identified 49 bp sequence was identical among *L. pneumophila* subspp. *pneumophila* str. Philadelphia 1, *L. pneumophila* str. Corby and *L. pneumophila* str. Paris, with a single base difference in *L. pneumophila* str. Lens. We also found that our selected 49 bp segment had little homology with other bacterial genomes (Table 1). These results indicate that our selected 49 bp target sequence would be the best genome region specifically detecting *L. pneumophila* using PCR amplification and Sp1.

Specific detection of *L. pneumophila*-specific PCR products by ELISA using Sp1

To demonstrate that Sp1 recognizes the sequence specific to *L. pneumophila*, first we tried to confirm the binding ability of Sp1 against a synthesized 49 bp target oligonucleotide by ELISA. Target dsDNA corresponding to *L. pneumophila* subspp. *pneumophila* str. Philadelphia 1, *L. pneumophila* str. Corby and *L. pneumophila* str. Paris, single-nucleotide-mutated dsDNA corresponding to *L. pneumophila* str. Lens and non-target dsDNA without the Sp1 recognition sequence were used. The results show that Sp1 could bind to both the target sequence (5′-GGG GCG GGG-3′) and the single-nucleotide-mutated sequence (5′-GG<u>A</u> GCG GGG-3′) corresponding to *L. pneumophila*, although the affinity against the single-nucleotide-mutated sequence was lower than that against the target sequence (data not shown). Thus, Sp1 might detect various strains of *L. pneumophila* even if a single-nucleotide mutation is introduced in the Sp1 recognition site.

Next, using the designed primers described above, we tried to specifically amplify the target sequence from the *L. pneumophila* genome. The *L. pneumophila* subspp.

Table 1. Homology between the Sp1 target sequences on the minus strand of *L. pneumophila* subspp. *pneumophila* str. Philadelphia 1, Corby, Lens, Paris and other organisms

| Species	Primer-binding region	Sp1-binding site	Primer-binding region
L. pneumophila str. Philadelphia1 *flh*A gene (minus strand)	A C T T T G T T G T A G T G A C A A A A	G G G G C G G G G	A G A G T A T C T G A A G T A A G T G C
L. pneumophila str. Paris *flh*A gene (minus strand)	A C T T T G T T G T A G T G A C A A A A	G G G G C G G G G	A G A G T A T C T G A A G T A A G T G C
L. pneumophila str. Corby *flh*A gene (minus strand)	A C T T T G T T G T A G T G A C A A A A	G G G G C G G G G	A G A G T A T C T G A A G T A A G T G C
L. pneumophila str. Lens *flh*A gene (minus strand)	A C T T T G T T G T A G T G A C A A A A	G G A G C G G G G	A G A G T A T C T G A A G T A A G T G C
Mus musculus BAC clone RP24-391P1 from chromosome 5	G A A C A T A A A T T T C T T T T T T C	G G G G C G G G G	A G A G T A T C T G G A G A G A C T T G
Geobacillus thermodenitrificans NG80-2 *rec*F	T A C T T G T T G T A G T G A C A A A A	G T T T G C A C T T T T T T C C G G A T C G C A T C A A G
Mus musculus chromosome 5 clone RP24-147H20	C A T T T G T T G T A G T G A C A A A A	A T T T A C T G C T T C T T T C T A A T G G A A G A A G C
Aeromonas hydrophila subspp. hydrophila ATCC 7966	C A C A G C A G A C G C T G G C A A A A	G G G G C G G G G	A G A G G G C A T C T C G A A C A C C G
Methanosarcina acetivorans str. C2A	A A A G A A A A A G C T T T C G T G T G A	G G G G C G G G G	A G A G T A T C T G G A A A T A T T G A
Corynebacterium efficiens YS-314	C G G C C C G A G G G G T G A C A A A A	G G G G C G G G G T G G G G A T T C C C A T C A T G G A A
Zebrafish DNA sequence from clone CH211-202N8	A C T T T G T T G T A G T G A C A A G A	T A A A G C A G G T A A A G C T G A T A A A G C T G G T A
Pan troglodytes BAC clone CH251-278I15	T T G G G G G G G G A T G G A A A A A	G G G G G G G A G A A G A A G A G G T T A G G A A G G A
Schistosoma japonicum SJCHGC08968 protein gene	C C A C T T G T T T A A A T G G C A C A C G T T C T A C A T G A G T A C T G A A G T A A G T A T
Homo sapiens BAC clone RP11-211J15	A C C T C C G C A T T T T T A A C T C T G C T T C T G G G A G A G T A T C T G A A G T G C A T C C
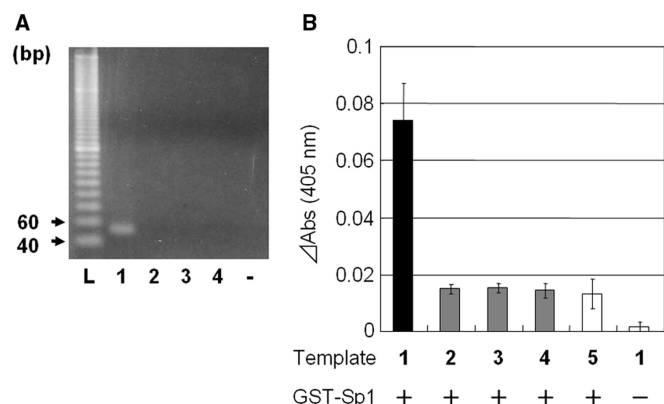
Figure 2. (**A**) Gel electrophoresis of the PCR products amplified from the bacterial genome using our designed primer set for Sp1. 'L' (lane 1) stands for the 20-bp DNA ladder. No. 1 (lane 2) indicates the *L. pneumophila* subspp. *pneumophila* str. Philadelphia 1 genome as the template. No. 2–4 (lanes 3–5) indicates the *E. coli* DH5α genome, *L. plantarum* and *P. vulgaris*, respectively. 'Minus' (−: lane 6) stands for no template. (**B**) Binding ability of Sp1 against the PCR products amplified from the bacterial genome by ELISA. Templates 1–4 indicate the *L. pneumophila* subspp. *pneumophila* str. Philadelphia 1 genome, *E. coli* DH5α genome, *L. plantarum* genome and *P. vulgaris* genome, respectively. No. 5 corresponds to no template. The absorbance at 405 nm was measured at room temperature after 60 min ($n = 3$).

pneumophila str. Philadelphia 1 genome, *E. coli* genome, *Lactobacillus plantarum* IAM1216 genome and *Proteus vulgaris* genome were used as templates for PCR. The PCR amplicon from each genome were examined by gel electrophoresis. In the presence of the *L. pneumophila* genome in the PCR solution, an amplified band of ∼49 bp was observed (Figure 2A, lane 2, sample 1). In contrast, in the presence of the other bacterial genomes in the PCR solution, no band was observed (Figure 2A, lanes 3–5, samples 2–4). These results indicate that our designed primer set for *L. pneumophila* enables us to specifically amplify the targeted region from the *L. pneumophila* genome.

To investigate the binding ability of Sp1, ELISA was carried out using Sp1 against the obtained PCR products. The absorbance at 405 nm increased in the presence of PCR products amplified from the *L. pneumophila* genome (Figure 2B, black bars). In contrast, low absorbance was observed in the solution containing PCR products from other bacterial genomes, as well as in the solution containing no template (Figure 2B, gray and white bars). These data suggest that Sp1 can specifically detect PCR products amplified from the *L. pneumophila* genome. Therefore, we succeeded in specifically detecting PCR products of the *L. pneumophila* genome using a Zn finger protein.

Bacterial genomes other than that of *L. pneumophila* are also contained in environmental samples. For example, the usual number of bacteria in bath water, which should be checked for *L. pneumophila*, seems to be 1×10^4–10^5 copies per milliliter. Therefore, to demonstrate the potential of our novel method for practical application, we should confirm that Sp1 can detect the PCR products of *L. pneumophila* in the presence of other bacterial genomes.
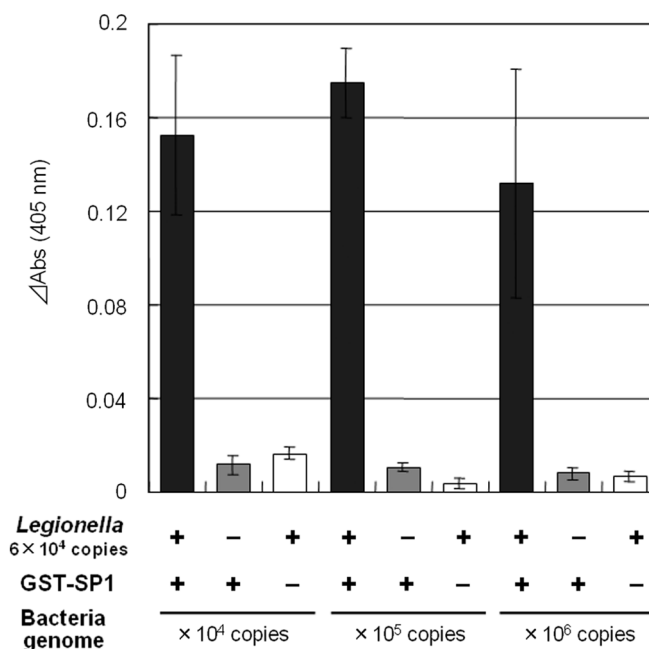

Figure 3. Binding ability of Sp1 against PCR products amplified from the *L. pneumophila* genome in the presence of other bacterial genomes by ELISA. In the PCR solution, there were 6×10^4 copies of the *L. pneumophila* subspp. *pneumophila* str. Philadelphia 1 genome as the template, $6 \times 10^8 \sim 10^{10}$ copies of other bacterial genomes, including the *E. coli* DH5α genome, *L. plantarum* genome and *P. vulgaris* genome, were present. The absorbance at 405 nm was measured at room temperature after 60 min ($n = 3$).

We tried to amplify the target sequence from *L. pneumophila* in the presence of other bacterial genomes, including the *E. coli* DH5α genome, *L. plantarum* genome and *P. vulgaris* genome. In samples containing 1×10^4–10^6 times more bacteria other than *L. pneumophila*, we were able to observe the amplified band of the 49 bp target sequence from the *L. pneumophila* genome by gel electrophoresis (data not shown). These data indicate that other bacterial genomes did not inhibit or affect the amplification of the target sequence from the *L. pneumophila* genome.

We confirmed the binding ability of Sp1 against the PCR solution obtained from other bacterial genomes by ELISA. The results show that Sp1 could specifically bind to PCR products amplified from *L. pneumophila* in the presence of a large number of other bacterial genomes (Figure 3). The other bacterial genomes had an insignificant effect on the binding ability of Sp1 against dsDNA, since the absorbance of each genome at 405 nm against *L. pneumophila*-specific PCR products was almost the same.

Since *L. pneumophila* is often detected in bath water or shower water, we also investigated the influence of the human genome on the detection of PCR products from *L. pneumophila*. Specific PCR amplification was observed by gel electrophoresis, and Sp1 could specifically bind to these PCR products in the presence of *L. pneumophila* and human genomic DNA. The absorbance at 405 nm in the presence of *L. pneumophila* was ∼25 ± 5 fold higher than that in the absence of *L. pneumophila* but with human
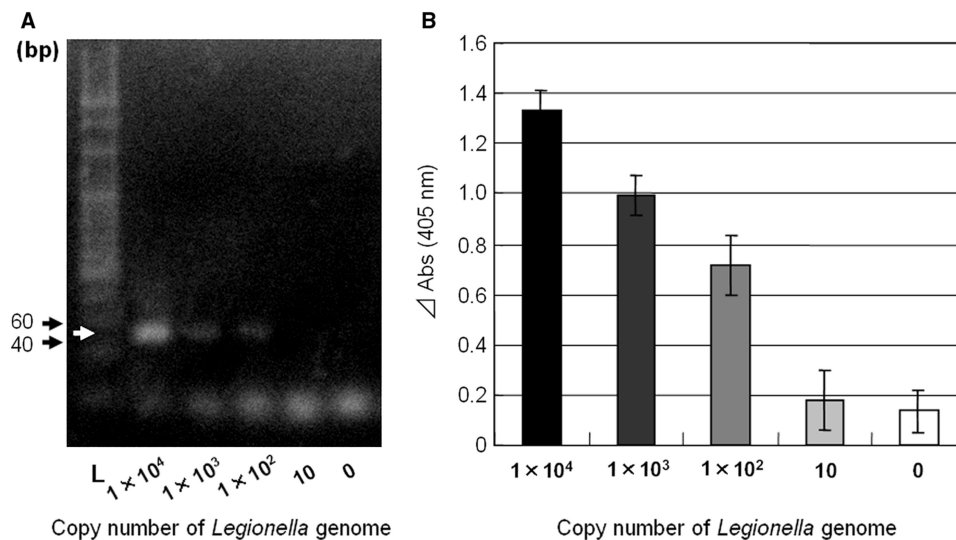
Figure 4. (**A**) Gel electrophoresis of the PCR products amplified from 0 to 1×10^4 copies of the *L. pneumophila* genome in the presence of other bacterial genomes. 'L' (lane 1) stands for the 20-bp DNA ladder. 1×10^4 times more other bacterial genomes were included for each copy of the *L. pneumophila* genome. (**B**) Binding ability of Sp1 against the PCR products amplified from 0 to 1×10^4 copies of the *L. pneumophila* genome by ELISA. The absorbance at 405 nm was measured at room temperature after 60 min ($n = 3$).

genomic DNA (data not shown). Thus, the human genomic DNA had no significant effect on the specific PCR amplification and detection using Sp1.

Using PCR products amplified from various copies of the *L. pneumophila* genome as templates, we examined the detection limit of Sp1 in the same experimental conditions. The gel electrophoresis results showed a clear amplified band in the presence of 1×10^2 copies of the *L. pneumophila* genome in the PCR mixture (Figure 4A). Similarly, we were able to detect PCR products from over 1×10^2 copies of the *L. pneumophila* genome with Sp1 (Figure 4B). Thus, the detection limit of Sp1 for the *L. pneumophila* genome by ELISA was 1×10^2 copies in the presence of other bacterial genomes.

Then, we applied this method to *L. pneumophila* detection in bath water samples. We checked the PCR amplification from *L. pneumophila* serogroup 1 cells in the samples and tried to detect this bacterium using Sp1. In the gel electrophoresis, amplified bands of ~49 bp were observed in three samples in the presence of 1×10^4 CFU of *L. pneumophila* serogroup 1 in the 77 μl of bath water or shower water per 100 μl of PCR solution. These data indicated that specific amplification from *L. pneumophila* cells in bath water and shower water could be achieved, even though there have been other some bacteria in the samples. ELISA was also carried out using Sp1 against the PCR products obtained from the *L. pneumophila* serogroup 1 cells. The absorbance at 405 nm in the presence of *L. pneumophila* serogroup 1 cells in the bath and shower water was significantly higher than that in the absence of *L. pneumophila* (Table 2). These results suggest that our detection system using Sp1 may work for real samples of *L. pneumophila*, especially serogroup 1, under environmental conditions such as bath water. Therefore, this method might be useful for the detection of pathogenic bacteria in environmental samples.

Table 2. The binding ability of Sp1 against PCR products amplified from *L. pneumophila* serogroup 1 cell in bath or shower water using ELISA

Sample	The ratio of ΔAbs (%) at 405 nm	
	$+$ *L. pneumophila* serogroup 1	$-$ *L. pneumophila* serogroup 1
Shower	2400 ± 6	100 ± 9
Bath water A	328 ± 20	100 ± 59
Bath water B	3500 ± 55	100 ± 458
Bath water C	Failure of PCR amplification	

Distinction between target PCR products and other products using Sp1

In bacterial detection, false positives and false negatives should be considered. One possibility of obtaining false positives is derived from non-specific amplification from other bacterial genomes in the PCR reaction. Thus, accurate detection requires a distinction between the target-specific amplification and the amplification of non-target sequences.

To demonstrate the high accuracy of our *Legionella* detection method, we confirmed whether or not Sp1 can distinguish between specific PCR products and others corresponding to simulated false positives. As specific PCR products, the target sequence containing the 9 bp Sp1-binding site (5′-GGG GCG GGG-3′) and the primer regions at both ends were used; as non-specific PCR products, the control sequence containing another 9 bp sequence (5′-GCG TGG GCG-3′) and the primer regions at both ends in the gene coding 2-deoxy-D-gluconate-3-dehydrogenase that is related with Carbohydrate Metabolism of *L. pneumophila* genome (Accession No. AE017354, region of 235777-236475)
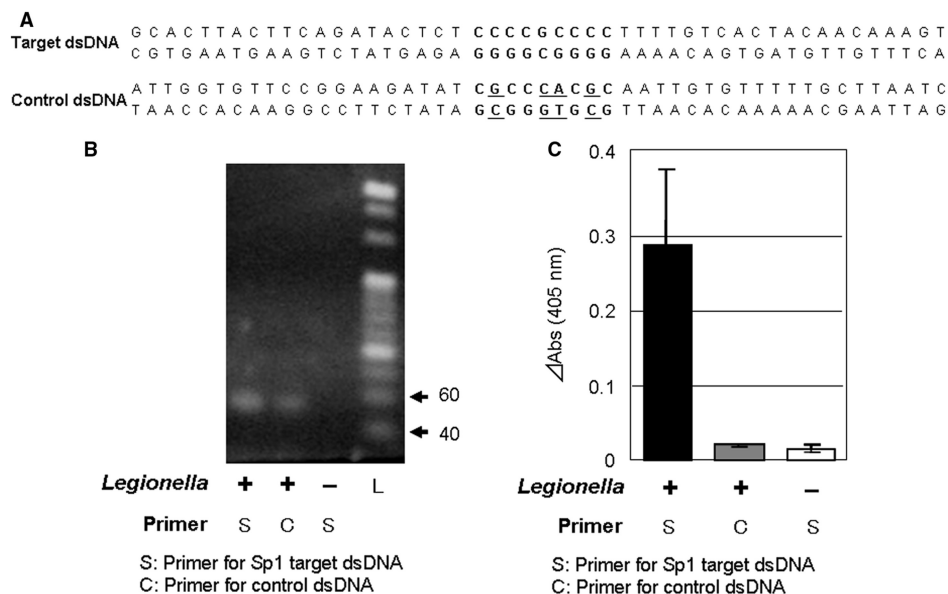
A
Target dsDNA

G C A C T T A C T T C A G A T A C T C T **C C C C G C C C C** T T T T G T C A C T A C A A C A A A G T
C G T G A A T G A A G T C T A T G A G A **G G G G C G G G G** A A A A C A G T G A T G T T G T T T C A

Control dsDNA

A T T G G T G T T C C G G A A G A T A T <u>C G C C C A C G C</u> A A T T G T G T T T T T G C T T A A T C
T A A C C A C A A G G C C T T C T A T A <u>G C G G G T G C G</u> T T A A C A C A A A A A C G A A T T A G

B

← 60
← 40

Legionella   +   +   −   L

Primer   S   C   S

S: Primer for Sp1 target dsDNA
C: Primer for control dsDNA

C

Legionella   +   +   −

Primer   S   C   S

S: Primer for Sp1 target dsDNA
C: Primer for control dsDNA

Figure 5. Distinction between the target PCR products and non-target ones using Sp1. (**A**) Sequence of the Sp1 target dsDNA and the control dsDNA. (**B**) Gel electrophoresis of the target and the control PCR products amplified from the *L. pneumophila* genome. Primers S (lanes 1 and 3) and Primer C (lane 2) indicate the primer for the Sp1 target sequence and the primer for the control sequence, respectively. 'L' (lane 4) stands for the 20-bp DNA ladder. (**C**) Binding ability of Sp1 against the target and the control PCR products amplified from the *L. pneumophila* genome by ELISA. The absorbance at 405 nm was measured at room temperature after 60 min ($n = 3$). Primers S and C indicate the primer for the Sp1 target sequence and the primer for the control sequence, respectively.

were identified (Figure 5A). Specific PCR products and the other PCR products were amplified from the *L. pneumophila* genome using 20 bp of each of the primer sequences, respectively. Although the amplified 49 bp bands were detected in the presence of the *L. pneumophila* genome by gel electrophoresis using each primer set (Figure 5B), Sp1 could specifically bind only to the specific PCR products, which contained the 9 bp Sp1-binding site, and not to others (Figure 5C). These results indicate that it is possible to discriminate specific PCR amplicon corresponding to true positives from others corresponding to false positives using the Sp1 Zn finger protein.

On the other hand, PCR inhibition by compounds present in certain environmental samples may induce the false negatives. Thus, whether the PCR reaction occurred or not should be confirmed. To confirm the PCR reaction, we designed a control template containing the Zif268 recognition sequence (5'-GCG TGG GCG-3') and the primer regions at both ends, as usually done in conventional PCR to discriminate false negatives. After the control template and the control primers for this sequence were added to the PCR solution in the presence of the *L. pneumophila* genome, multiplex PCR was performed. The target products containing the Sp1 recognition site and the control products containing the Zif268 recognition site in the obtained PCR amplicon were detected by Sp1 and Zif268, respectively, and the discrimination of false negative was achieved in this way (data not shown).

The rapid detection of *L. pneumophila*-specific PCR products using Sp1

Although Sp1 was able to specifically detect the PCR products from the *L. pneumophila* genome by ELISA, the detection procedure is somewhat time consuming and complicated. We tried to construct a more rapid detection method using Sp1 in conjunction with fluorescence depolarization measurement, which is a useful tool for detecting the interaction of fluorescence-labeled DNA–DNA, DNA–protein and protein–protein. Thus, the binding ability of Sp1 against fluorescence-labeled PCR products from the *L. pneumophila* genome in the presence of 1×10^4 times more other bacteria was checked by fluorescence depolarization measurement.

We observed that the ΔP ratio in the presence of PCR products amplified from the *L. pneumophila* genome as the template was significantly higher than the ratio in the absence of *L. pneumophila* genome (Figure 6). Although the ΔP values were only slightly different depending on the quantity of the PCR products, a significant difference in the ΔP values between the presence and the absence of *L. pneumophila* was clearly observed. Furthermore, the fluorescence depolarization measurement does not need B/F (bound/free) separation, unlike ELISA, and therefore allows quick detection. In fact, by fluorescence depolarization measurement, we were able to detect the PCR products of *L. pneumophila* within only 1 min. This means that our detection system for *L. pneumophila* is more rapid than the DNA–DNA hybridization method.

DISCUSSION

This is the first report of a detection system for PCR products from pathogenic bacteria using a Zn finger protein. Our concept enables the direct and selective detection of PCR products using Zn finger proteins that can bind to dsDNA; in contrast, the conventional method
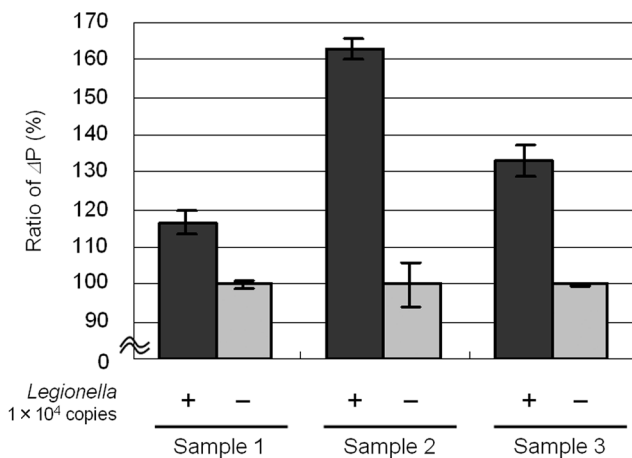
Figure 6. Measurement of the binding ability of Sp1 against PCR products amplified from 1×10^4 copies of the *L. pneumophila* genome by fluorescence depolarization ($n = 3$). Nearly 1×10^8 copies of other bacterial genomes were also included for each copy of the *L. pneumophila* genome. The fluorescence depolarization values were measured after 1 min using a PCR solution with no *L. pneumophila* genome (gray bar), and with a PCR solution with PCR products amplified from 1×10^4 copies of *L. pneumophila* (black bar). The PCR products were diluted 20-fold and GST-Sp1 was added to a final concentration of $2 \mu M$. The excitation and emission wavelengths were 495 and 530 nm, respectively. The fluorescence depolarization value (ΔP) was obtained by subtracting the initial fluorescence depolarization (P_0) from the fluorescence depolarization after mixing Sp1 and the diluted PCR products for 1 min (P_1). The ratio of ΔP for 1×10^4 copies of *L. pneumophila* was calculated by taking the ΔP of no *L. pneumophila* as 100%.

is based on the dehybridization of dsDNA and the hybridization of the DNA probe to ssDNA. In our novel system, only the measurement of the binding ability of Zn finger proteins against PCR products is necessary. Thus, the direct detection of dsDNA using Zn finger proteins speeds up and simplifies the detection of PCR amplicons from the bacterial genome. Moreover, this system allows the detection of the target bacterial DNA by double checking the PCR amplification and the detection using Zn finger proteins. Therefore, our novel bacterial detection methodology is superior to gel electrophoresis and the DNA–DNA probe hybridization method, since the efficiency of the DNA probe hybridization is low and the direct and specific double checking using the Zn finger protein makes our method both quick and accurate. To demonstrate the principle behind our novel detection system, we tried to detect *L. pneumophila* using Sp1.

We first selected a 49 bp sequence of the *L. pneumophila*-specific region containing a 9 bp Sp1-binding site and two 20 bp primer regions at both ends; this 49 bp sequence is located on the *flh*A gene of the *L. pneumophila* genome. In our methodology, the identification of the most suitable sequence for the detection of the target genome is the key point. The target sequence containing only the recognition site of the Zn finger protein is too short to be used for detecting specific bacteria, since its incidence rate would be high. Thus, the target sequence should be longer, to enable the specific detection of PCR products from the target genome. In this study, the estimated incidence rate of the 9 bp Sp1 recognition site is about once every 3×10^5 bp, whereas the estimated incidence rate of our 49 bp target region is about once every 3×10^{29} bp. Therefore, in principle, it is highly unlikely that our 49 bp target sequence would be found in non-target bacterial genomes. In fact, the homology analysis by BLAST showed that the 49 bp target region located on the *Legionella* genome has little homology with the genomes of other organisms. The target sequence should also not be too long because it takes a long time to amplify long sequences. For quick detection, shorter PCR products are preferable. We found that the length of 49 bp would be most suitable for specific and rapid amplification.

As expected, the 49 bp target sequence could be amplified from the *L. pneumophila* genome in the presence of a large number of unrelated bacterial genomes, as would be the case in environmental samples. We succeeded in specifically detecting PCR products amplified from *L. pneumophila* (over 100 copies) in the presence of other bacterial genomes by ELISA. Sp1 enables us to distinguish between specific PCR products and others. Thus, this system using a Zn finger protein can discriminate true positive results from false positive results, which is the main stumbling block in bacterial detection. In addition, we could also detect the specific PCR products of *L. pneumophila* in the presence of other bacterial genomes within only 1 min by fluorescence depolarization measurement.

In principle, this method can be applied to the detection of most bacterium. If there is a recognition site for a specific Zn finger protein in the genome of the target bacterium, the target sequence containing the binding site of the Zn finger protein and the primer regions can be easily identified. The Zn finger protein can also detect the PCR products from the identified sequence. Various Zn finger proteins, including Sp1, have already been reported, and each specifically binds to a certain recognition sequence, as mentioned above. It is highly probable that there is at least one Zn finger protein-binding site in most bacterial genome. Therefore, our novel methodology is expected to be generally applicable to the detection of most bacterium using a combination of various Zn finger proteins.

Of course, our detection method has some limitations. One of these limitations is that there is a limited number of genome sequences in the BLAST database, so we may not find the recognition site of certain bacterial sequences and confirm their specificity. In addition, there might be no recognition site in the targeted bacterium if the bacterial genome has already been sequenced. There is theoretically a 9 bp recognition site sequence at every 4^9 bps, however, in fact, specific sequences in most bacterial genomes might be conserved at some level. Another limitation is that there are recognition sites in the conserved region of some bacteria. However, we have already succeeded in detecting *Salmonella* using Zif268 (the report would be published elsewhere), *L. pneumophila* Philadelphia 1 using Sp2 (the report would be published elsewhere) and the Influenza A

virus using Sp1 (the report would be published elsewhere), which proves the versatility of our novel detection method.

We demonstrated the rapid and specific detection of PCR products amplified from a bacterial genome using a Zn finger protein. This methodology might be applied to the detection of other bacteria using various Zn finger proteins that have already been reported. We believe that our strategy would allow the rapid and simple detection of bacteria, and that it represents the significant application of Zn finger proteins to date.

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