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Detection of Pathogenic Bacteria by Using Zinc Finger Protein Fused with Firefly Luciferase

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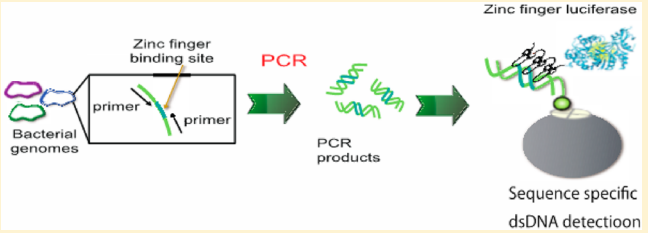
ABSTRACT: We constructed a novel bacterial genome detection system using zinc finger protein (ZF) fused with firefly luciferase (ZF-luciferase). Taking advantage of the direct recognition of double-stranded DNA (dsDNA) by ZF, we previously constructed bacterial genome detection systems that did not require dehybridization processes. To detect polymerase chain reaction (PCR) products rapidly and with a high sensitivity, we constructed two kinds of ZF-luciferase, Sp1-fused luciferase (Sp1-luciferase), and Zif268-fused luciferase (Zif268-luciferase). ZF-luciferase not only maintains luciferase activity but also shows dsDNA-binding ability and specificity. Furthermore, we succeeded in the detection of 10 copies of the genome of *Legionella pneumophila* and *Escherichia coli* O157. ZF-luciferase would be a useful tool for highly sensitive detection of pathogenic bacterial genome.



The species-specific detection of pathogenic bacteria is important for the prevention and identification of the problems related to foods, infectious disease, or biological warfare agents. Although conventional culture-based assays are sensitive, they are very time-consuming. To reduce the risk of those problems, assay results should be obtained within a few hours. Polymerase chain reaction (PCR) is often faster and more accurate than the culture-based method for the detection of bacterial genomes. PCR can amplify specific regions of the bacterial genome exponentially within a couple of hours.

Although PCR is a powerful method for the detection of bacterial genomes, specific amplification needs to be discriminated from nonspecific amplification. For specific detection of PCR products, analysis of internal sequences of PCR products by a probe DNA or a DNA-binding protein is required.

We have previously reported a system using zinc finger protein (ZF) for the detection of PCR products of pathogenic bacterial genomes (Figure 1a).[1,2] ZF is the most well-known DNA-binding protein in mammals. The most common ZF is the C_2H_2 ZF, whose structure is stabilized by a zinc ion bound to 2 cysteine and 2 histidine residues of each finger containing 2 β-strands and 1 α-helix. Design or screening of novel ZFs, in addition to natural ZF, that recognize any target sequences enables the development of the DNA-sensing system targeting any region of the genome.[3]

Previously, we used ZF-fused glutathione-S-transferase (GST-ZF) and a horseradish peroxidase (HRP)-conjugated anti-GST antibody for the detection of PCR products (Figure 1a). GST-ZF recognizes the internal sequence of PCR products, which are then detected by HRP-conjugated anti-GST antibody. Multistep operations are time-consuming and result in increased number of errors and decreased number of

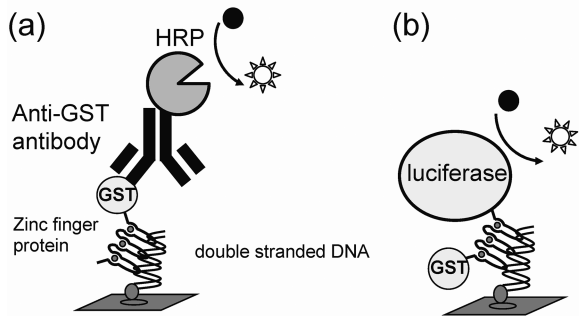


Figure 1. Comparison of pathogenic bacterial genome detection system using zinc finger protein-fused luciferase (ZF-luciferase) with glutathione-S-transferase-fused zinc finger protein (GST-ZF). Bacterial specific DNA was amplified by PCR using a suitable primer set. Amplified double stranded DNA (dsDNA) was detected by GST-ZF (a) or ZF-luciferase (b), which specifically recognize the central region of amplified dsDNA specifically. The interaction of GST-ZF with target dsDNA was detected by horseradish peroxidase-conjugated anti-GST antibody. The interaction of ZF-luciferase with target dsDNA was directly detected based on luciferase activity.

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signals because of the dissociation of ZF from DNA during incubation. Thus, we fused ZF with an enzyme whose activity can be easily measured for the direct detection of PCR products without the need for an enzyme-conjugated antibody.

Enzyme-linked immunosorbent assays (ELISA) ordinarily use HRP or alkaline phosphatase (ALP) as the labeling enzyme of an antibody. Commercialized HRP- or ALP-conjugated anti-antibody antibodies can also be used. However, because ZFs are small proteins, and lysine and cysteine residues that are ordinarily used for chemical conjugation are important for their scaffold formation and binding ability, chemical conjugation of enzymes with ZF would decrease the binding ability of ZF. Although enzyme fusion with ZF genetically is an attractive strategy, construction of HRP or ALP fusion protein is difficult because HRP cannot be easily expressed as a soluble enzyme in *Escherichia coli*[4] and ALP is a dimeric protein. Thus, we selected a firefly luciferase, which catalyzes D-luciferin oxidation accompanied by emission of high-intensity light signals; this enzyme is a monomeric protein that is expressed as a soluble enzyme in *E. coli*. Kobatake et al. successfully constructed firefly luciferase fusion protein with protein A without the loss of catalytic activity.[5] Because luminescence can be measured with high sensitivity, low noise, and over a wide dynamic range by employing photomultipliers or charge-coupled devices (CCD), firefly luciferase is employed for highly sensitive ATP detection and as a labeling enzyme for immunosensors.[6,7] Therefore, firefly luciferase is an attractive candidate enzyme for fusion with ZF.

In this study, we constructed two types of ZF-fused firefly luciferase (ZF-luciferase). Compared to previous detection systems using GST-fused ZF, our detection system using ZF-luciferase can eliminate some steps and be expected to increase sensitivity (Figure 1). Using our system, we attempted to detect pathogenic bacterial genomes and evaluate the effect of the luciferase on the specificity and sensitivity of PCR product detection.

EXPERIMENTAL SECTION

Cloning, Expression, and Purification of Fusion Proteins. The gene encoding firefly luciferase was amplified from pTrc99A-luciferase, which was donated by Kikkoman Corporation, using *Eco*RI-inserted forward primer 5′-CACA-GAATTCCTTAGCAACTGGTTTCTTCA-3′ and *Eco*RI-inserted reverse primer 5′-CTCTGGAATTCCTTAG-CAACTGGTTTCTTCA-3′. The amplified DNA was cloned into pGEX-Sp1[1] or pGEX-Zif268.[2] Firefly luciferase was expressed in *E. coli* BL21 (DE3) cells. Each clone was expressed by Overnight Express Autoinduction system at 20 °C for 24 h.[8] Those cells were harvested by centrifugation at 3 000*g* for 10 min and resuspended in cell lysis buffer (PBS [137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4], 1% [v/v] Triton X-100, 5 mM dithiothreitol, 4 mM Pefabloc SC [Roche Applied Science], pH 7.3). The cells were then homogenized using a French press and centrifuged at 20 000*g* for 30 min at 4 °C. The ZF-luciferase was purified using a GSTrap HF column (GE Healthcare Bioscience, Buckinghamshire, United Kingdom). The luciferase activity was measured after incubation for 1 min by mixing 20 μL of protein solution and 100 μL of PicaGene (Toyo Ink Co., Ltd., Tokyo, Japan) using ARVO MX 1420 (PerkinElmer Inc., MA).

Design of the Primer Set for Bacterial Detection. For *Legionella pneumophila*, the target sequence and primer set designed previously were used.[1] For *E. coli* O157, Sp1-binding site, 5′-GGGGCGGGG-3′, and Zif268-binding site, 5′-GCGTGGGCG-3′, were searched on the *E. coli* O157 genome by using NCBI Nucleotide BLAST for short, nearly exact matches (http://www.ncbi.nlm.nih.gov/BLAST/) limited by the Entrez query "bacteria and *Escherichia coli* O157". From among the data obtained, we selected the target gene containing the Zif268-binding site in *E. coli* O157. Using NCBI Nucleotide BLAST, we also checked the specificity of the 45-bp target sequence (5′- TTACCAATGAAGAA-TAACCGTGCGTGGGCGCTTATCAGTGGTCTG-3′), which included the selected 9-bp Zif268-binding site and the 21-bp primer region at the 5′ end and a 15-bp primer region at the 3′ end, among all the genomes.

Detection of DNA and PCR Products by Luciferase Assay. For the detection of synthetic DNA, double-stranded DNA (dsDNA) was prepared, as has been described elsewhere.[1,2] Biotinylated dsDNA was diluted to 100 nM with rinse buffer (90 μM $ZnCl_2$, 10 mM phosphate buffer, pH 7.3, 150 mM NaCl), and 100 μL of the diluted dsDNA was added to 15 μL of NeutrAvidin resin (Thermo Fisher Scientific Inc., MA), which was separated into a 500 μL tube. For the detection of PCR products, PCR amplification was carried out using Herculase II Fusion DNA polymerase (Agilent Technologies, Inc., CA). A volume of 50 μL of PCR products was added directly to 15 μL of NeutrAvidin resin. After 15-min incubation, NeutrAvidin resin was centrifuged and washed 2 times using rinse buffer. Thereafter, 100 μL of wash buffer (90 μM $ZnCl_2$, 10 mM phosphate buffer, pH 7.3, 0.15 M NaCl, 0.1% (v/v) Tween20) containing 2% skimmed milk and 1-mM d-biotin was added and incubated for 15 min. NeutrAvidin resin was then centrifuged and the supernatant was discarded. Purified fusion protein was diluted to 100 nM with wash buffer containing 2% skimmed milk and 1 mM d-biotin and added to the NeutrAvidin resin. After incubation for 15 min, NeutrAvidin resin was washed 3 times by wash buffer and once with wash buffer without Tween20. Finally, 100 μL of Picagene was added to each well and luminescence was measured after 1-min incubation by using ARVO MX 1420. All experiments were performed at room temperature. For detection of *E. coli* O157, streptavidin immobilized magnetic beads (Magnosphere MS300/Streptavidin, JSR Corporation, Tokyo, Japan) were used instead of NeutrAvidin.

RESULTS

Design and Characterization of ZF-luciferase. In this study, we constructed firefly luciferase fused with Sp1 or Zif268, which are well characterized 3-finger-type of ZF that can recognize 9-bp sequences, 5′-GGGGCGGGG-3′ and 5′-GCGTGGGCG-3′, respectively.[9,10] ZF-luciferase was expressed as a fusion protein with GST at the N-terminus to increase solubility and purification. After purification, we confirmed that luciferase activity of ZF-luciferase was sufficiently maintained although luminescent activity of the fusion protein decreased approximately 10 times compared to that of the nonfusion firefly luciferase.

Characterization of ZF-luciferase. First, Sp1-luciferase was evaluated for the binding ability and specificity against target DNA by using a 49-bp synthetic oligonucleotide that has an Sp1-recognition sequence and a primer region for PCR amplification. As negative controls, synthetic oligonucleotides with a mutation in the Sp1-recognition sequence, 5′-GGGACGGGG-3′, or a random DNA sequence other than Sp1-recognition sequence, 5′-TAAGCGATT-3′, were used.

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The binding ability of Sp1-luciferase was evaluated by a luciferase assay (Figure 2). Higher-intensity luminescent signal

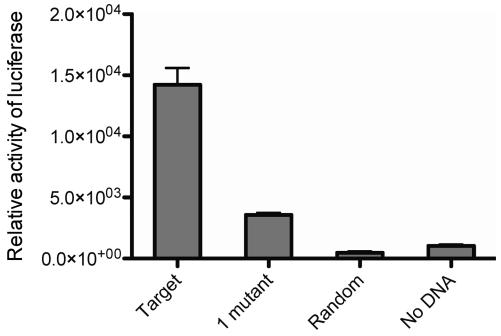

Figure 2. Binding ability and specificity of Sp1 fused firefly luciferase. Each biotinylated 10 pmol double-stranded DNA was immobilized on NeutrAvidin beads.

was observed from the bead-immobilized target DNA than from the control DNA. Zif268-luciferase also maintained the binding ability and specificity against target sequence (data not shown). Therefore, ZF-luciferase maintained not only the luciferase activity but also the DNA-binding ability and specificity.

Sp1-luciferase was then evaluated for a lower detection limit. A 22-bp synthetic oligonucleotide that contains the Sp1-recognition sequence without PCR amplification was used as a target DNA. A clear luminescent signal was observed in the presence of 10^{10} copies (~17 fmol) of DNA (S/N = 3) (Figure 3a). This detection limit is 100 times lower than the detection limit of our previous detection method using HRP-conjugated anti-GST antibody (10^{12} copies, ~1.7 pmol) (Figure 3b). For luminescence measurements, the substrate HRP has a higher background signal than luciferase does. Therefore, lower copies of DNA can be detected using luciferase than by ELISA using HRP-conjugated anti GST antibody.

Detection System Using ZF-luciferase for PCR Products of Pathogenic Bacteria. To demonstrate the sensitivity of Sp1-luciferase for the detection of pathogenic bacterial genomes, we used *L. pneumophila* whose genome has been previously detected by GST-ZF. For the specific detection of the *L. pneumophila* genome, we selected a 49-bp sequence within *flhA* that is a conserved region among *Legionella* species but is not found among other bacteria by BLAST search. We have previously detected 100 copies of the genome of *L. pneumophila* as the lower detection limit by using GST-fused Sp1 by ELISA with HRP-conjugated anti-GST antibody.

Figure 4a,b shows the results of *L. pneumophila* detection by Sp1-luciferase. After 35 cycles of PCR using 20-bp primers hybridized with upstream and downstream sequences of the Sp1-recognition sequence of the *flhA* gene, PCR products were detected using Sp1-luciferase. First, Sp1-luciferase was evaluated for specificity of bacterial genome detection. Sp1-luciferase showed luminescent signals in the presence of the *L. pneumophila* genome (12 000 copies) but not *E. coli* genome or *Proteus vulgaris* genome. Moreover, in the presence of a sample solution of *L. pneumophila*, *E. coli* genome, and *P. vulgaris* genome, we observed similar extent signals regardless of the *E. coli* genome or *P. vulgaris* genome presence in a sample solution of *L. pneumophila* (Figure 4a). These results indicate that firefly luciferase did not affect the specificity of the detection of the *L. pneumophila* genome by Sp1.

The lower detection limit of the *L. pneumophila* genome was evaluated using various copies of the genome. After amplification of $0-10^4$ copies of the genome by PCR, the products were detected by Sp1-luciferase. Higher-intensity luminescent signal was observed in the presence of 10 copies of the genome than in the absence of the bacterial genome (Figure 4b). These results indicate that PCR products of bacterial genomes can be detected with a higher sensitivity by using ZF-luciferase than HRP mediated detection using HRP conjugated anti-GST antibody and GST-ZF.

To illustrate the versatility of this system, another ZF-luciferase, Zif268-luciferase, was evaluated by detecting the genome of another bacterium, *E. coli* O157. Moreover, to construct an automatized detection system, we introduced magnetic beads instead of agarose beads into the luciferase assay. For *E. coli* O157 detection, we designed a new primer set using methods employed in other bacterial genome detection systems.[1,2] Although we found the Sp1-binding site on the *E. coli* O157 genome, the gene including Sp1-binding site was not specific to *E. coli* O157. On the other hand, the Zif268-binding site was also found on the *E. coli* O157 genome, and the sequence, including the *E. coli* O157-binding site and primer-binding regions, is specific for *E. coli* O157 in BLAST search. (We have recently found that this sequence is included in the *E. coli* O55 genome.)

Figure 4c,d shows the results of *E. coli* O157 detection. We observed a clear luminescent signal in the presence of *E. coli*

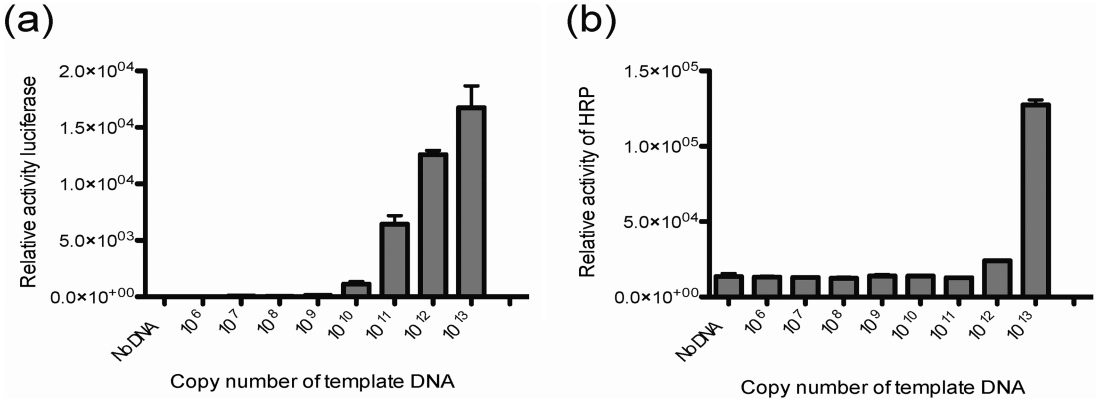

Figure 3. Detection limit of double-stranded DNA (dsDNA) by using Sp1-fused firefly luciferase (a) or GST-fused Sp1 (b). A total of 10^6-10^{13} copies of synthetic dsDNA were immobilized on NeutrAvidin beads.

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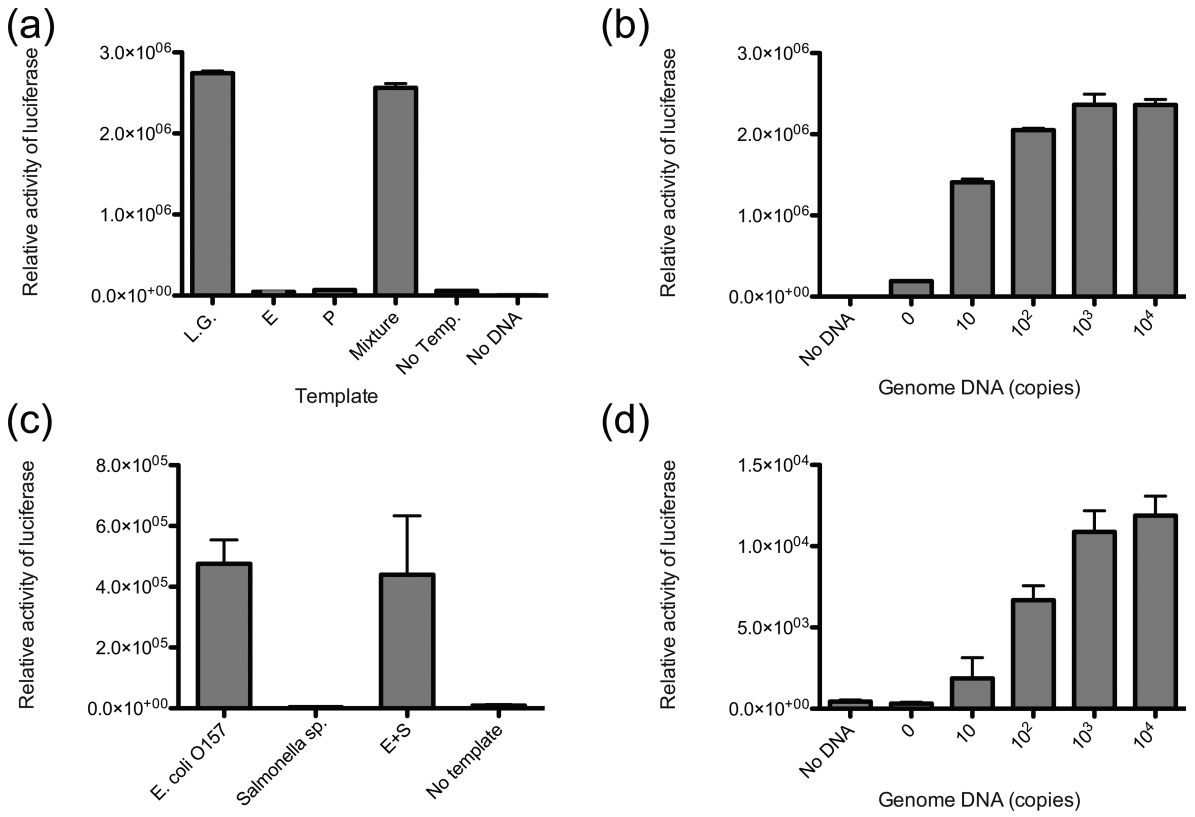

Figure 4. Detection of PCR products of *Legionella pneumophila* by using Sp1-luciferase (a) and (b) and that of *Escherichia coli* O157 by using Zif268-luciferase (c and d). (a) Specificity of Sp1-luciferase was evaluated using 1.2×10^4 copies of each bacterial genome. LG means genome of *L. pneumophila*. P means *Proteus vulgaris* genome. E means *E. coli* DH5α. Mixture means the sample that includes three bacterial genomes (*L. pneumophila, Proteus vulgaris, E. coli* DH5α genomes). (b) $0-10^4$ copies of each bacterial genome was added to each PCR solution. The sample named No DNA did not include a PCR solution and showed background signals resulting from a nonspecific interaction of ZF-luciferase with the NeutrAvidin beads. (c) Specificity of Zif268-luciferase was evaluated using 10^4 copies of each bacterial genome. (d) $0-10^4$ copies of each bacterial genome was added to each PCR solution. The sample named No DNAs did not include any PCR solutions and they show background signal resulting from nonspecific interaction of Zif268-luciferase with Magnosphere MS300/streptavidin beads.

O157 genome regardless of the presence of *Salmonella* sp. genome (Figure 4c). Furthermore we were able to distinguish *E. coli* O157 from *E. coli* DH5α (data not shown). The lower detection limit of *E. coli* O157 genome was evaluated using various copies of the genome. We observed a higher luminescent signal in the presence of 10 copies of *E. coli* O157 genome than in the absence of this genome. The signal intensity in the presence of 10 copies of *E. coli* O157 genome was lower than that in the presence of 10 copies of *L. pneumophila* genome (Figure 4d). We observed that the signal was almost completely saturated in the presence of 10^2 copies of the *L. pneumophila* genome but was not saturated in the presence of 10^2 copies of the *E. coli* O157 genome. This observation might be attributed to the difference in the binding ability between Sp1-luciferase and Zif268-luciferase.

DISCUSSION

ZF-based bacterial detection systems would be applicable to any bacterial genome by selection of a suitable ZF for each bacterial genome sequence. Several ZF sets need to be constructed for the application of this system to detect various bacterial genomes. We have already constructed two types of ZF-luciferase in addition to Sp1-luciferase and Zif268-luciferase: (1) Sp2-luciferase that recognizes 5′-GGGCGGGACT-3′[11] and (2) ZF-luciferase with ZF that recognizes 5′-GTAAATGAT-3′. All ZF-luciferases show similar binding abilities to their specific sequences and similar levels of luciferase activities. Therefore, this system would be able to detect any bacterium by using a suitable ZF-luciferase.

Furthermore our system includes simple processes: incubation, washing, and detection. Washing steps can be easily automated using magnetic beads. We would be able to construct the automatic detection system of bacterial genomes by ZF-luciferase using an automated pipetter.

Although ZFs show high sequence specificity, most ZFs also recognize dsDNAs with single base mutations in the target sequence. The recognition of dsDNAs by ZF-luciferase yields false-positive results. We think that a single base mutation would not cause serious problems, because the possibility that nonspecifically amplified PCR products will contain a single base mutation in the ZF recognition sequence is low; moreover, compared with a false-negative result, a false-positive result does not cause a serious problem. However, if a false-positive result does cause a serious problem in practice, a suitable ZF can be selected for the detection of a specific bacterial species.

This system relies on genomic databases to design a primer set for a specific detection. When we designed a primer set for *E. coli* O157, we did not find sequences that would be amplified using a designed primer by BLAST among whole genomic database, except *E. coli* O157. However, we have recently found complete match sequences by BLAST in *E. coli* O55. The strain *E. coli* O55 is similar to *E. coli* O157, and it is difficult to

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discriminate them by PCR although it should be discriminated.[12] Its genome has recently been analyzed.[13] This is a common problem for PCR-based detection. Because recent genomic DNA analyses using next-generation sequencers unveiled many types of bacterial genomes, designing of a more specific primer would be possible.

Stains et al. reported a ZF-based dsDNA detection system designated SEER (SEquence Enabled Reassembly).[14,15] They constructed a fusion protein of ZF with split green fluorescent protein (GFP) or β-lactamase (Lac). Binding of 2 ZF molecules that have a different recognition sequence enhances formation of the whole structure of GFP or Lac on dsDNA. SEER is an attractive methodology because dsDNA can be detected with a picomole detection limit (using Lac) and does not require bound-free separation. However, detection of a few copies of bacterial genomes corresponding to femtomoles of dsDNA after PCR amplification is required for food-risk management. ZF-luciferase would be an attractive dsDNA detection tool because it can detect subnanomolar levels of dsDNA after PCR amplification.

In conclusion, we developed a novel tool for the detection of dsDNA by using ZF-luciferase. ZF-luciferase was able to detect femtomole levels of dsDNA, indicating that it has the potential to detect minute quantities of the bacterial genome. In this study, we demonstrated the detection of two pathogenic bacteria by using ZF-luciferase with 10 copies as the lower detection limit. We have already constructed four types of ZF-luciferase, including the newly designed ZF. These fusion proteins also maintained the luciferase activity and the binding ability of each ZF. Therefore, we expect that this sensing system can be expanded for the detection of the genome of any bacteria.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Osawa, Y.; Ikebukuro, K.; Motoki, H.; Matsuo, T.; Horiuchi, M.; Sode, K. *Nucleic Acids Res.* **2008**, *36*, e68.

(2) Osawa, Y.; Ikebukuro, K.; Kumagai, T.; Motoki, H.; Matsuo, T.; Horiuchi, M.; Sode, K. *Biotechnol. Lett.* **2009**, *31*, 725−733.

(3) Pabo, C. O.; Peisach, E.; Grant, R. A. *Annu. Rev. Biochem.* **2001**, *70*, 313−340.

(4) Egorov, A. M.; Gazaryan, I. G.; Savelyev, S. V.; Fechina, V. A.; Veryovkin, A. N.; Kim, B. B. *Ann. N.Y. Acad. Sci.* **1991**, *646*, 35−40.

(5) Kobatake, E.; Iwai, T.; Ikariyama, Y.; Aizawa, M. *Anal. Biochem.* **1993**, *208*, 300−305.

(6) Seto, Y.; Iba, T.; Abe, K. *Luminescence* **2001**, *16*, 285−290.

(7) Seto, Y.; Ohkuma, H.; Takayasu, S.; Iba, T.; Umeda, A.; Abe, K. *Anal. Chim. Acta* **2001**, *429*, 19−26.

(8) Studier, F. W. *Protein Expr Purif* **2005**, *41*, 207−234.

(9) Yokono, M.; Saegusa, N.; Matsushita, K.; Sugiura, Y. *Biochemistry* **1998**, *37*, 6824−6832.

(10) Christy, B.; Nathans, D. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 8737−8741.

(11) Moorefield, K. S.; Fry, S. J.; Horowitz, J. M. *J. Biol. Chem.* **2004**, *279*, 13911−13924.

(12) Oberst, R. D.; Hays, M. P.; Bohra, L. K.; Phebus, R. K.; Yamashiro, C. T.; Paszko-Kolva, C.; Flood, S. J.; Sargeant, J. M.; Gillespie, J. R. *Appl. Environ. Microbiol.* **1998**, *64*, 3389−3396.

(13) Zhou, Z.; Li, X.; Liu, B.; Beutin, L.; Xu, J.; Ren, Y.; Feng, L.; Lan, R.; Reeves, P. R.; Wang, L. *PLoS One* **2010**, *5*, e8700.

(14) Ooi, A. T.; Stains, C. I.; Ghosh, I.; Segal, D. J. *Biochemistry* **2006**, *45*, 3620−3625.

(15) Stains, C. I.; Porter, J. R.; Ooi, A. T.; Segal, D. J.; Ghosh, I. *J. Am. Chem. Soc.* **2005**, *127*, 10782−10783.