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Research paper

Development of a loop-mediated isothermal amplification method for the rapid detection of the dioxin-degrading bacterium *Ochrobactrum anthropi* in soil

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ABSTRACT

In this study, loop-mediated isothermal amplification (LAMP) and real-time LAMP assays were developed to detect the dioxin-degrading bacterium *Ochrobactrum anthropi* strain BD-1 in soil. Four primers were designed to use ITS gene amplification for the strain *O. anthropi* BD-1. The real-time LAMP assay was found to accomplish the reaction by 1 pg of genomic DNA load when used for nucleic acid amplification. This assay was then applied to detect *O. anthropi* BD-1 in eight soil samples collected from a dioxin-contaminated site. The results demonstrated that these newly developed LAMP and real-time LAMP assays will not only be useful and efficient tools for detecting the target gene, but also be used as molecular tools for monitoring the growth of dioxin-degrading *O. anthropi* in the soil. This is the first report to demonstrate the use of LAMP assays to monitor the presence of *O. anthropi* in dioxin-contaminated soil. The application of this method should improve the biomonitoring of dioxin contamination.

1. Introduction

Dioxins compounds are the products/by-products of many human activities, including incineration (Altwicker and Milligan, 1993). Once dioxins are formed, they tend to accumulate in the environment and cause enormous damage to living systems (Bonn, 1998). Dioxins can persist in the soil environment for a long period of time (Lahl et al., 1991). The pollution with dioxins occurring at Seveso, Italy, has shown that these compounds have a half-life time of 10 years (Eskenazi et al., 2002). In a human body, the half-life time of dioxins could last 7 years (Pirkle et al., 1989). The 2,3,7,8-TCDD used in this study belongs to one kind of dioxin group. Previous animal testing demonstrated that the LD_{50} of 2,3,7,8-TCDD could vary widely. A previous study shows that 2,3,7,8-TCDD could be thousand times more toxic than nicotine, hundred times more toxic than pufferfish toxin, and five times more toxic than ricin (Kociba and Schwetz, 1982). Without any doubt, this chemical is one of the most toxic artificial chemicals in the world (McCrady et al., 1990). The USEPA and WHO have classified dioxins as a probable carcinogen. The IARC (International Agency for Research on Cancer) announced in 1997 that 2,3,7,8-TCDD is a first grade certain carcinogen.

The level of environmental pollution can be determined by physical, chemical and bio-indication methods. However, the physical or chemical detection of toxicants usually depends on high cost of the equipment and complexity of the processes in samples' pretreatment.

In recent years, there has been growing interests in using bio-indicators for monitoring environmental pollution (Achazi and Van Gestel, 2003; Cenci and Jones, 2009; Fontanetti et al., 2010; Ronald et al., 2013). Bioindicators are species or chemicals used to monitor pollutants in some environments or ecosystems. They can be any kind of biological species or a group of species whose function, population are used to determine the integrity of ecosystems. The reasons why bioindicators may become popular in monitoring pollution are many. The most significant one is that they are very cost-effective with high accuracy. For example, instead of examining the entire biota, it is possible to assess the pollution based on a small amount of biota, if a bioindicator is used (Paoletti, 1999). In some cases, it may be extremely useful for monitoring species that could provide early warning of change (Spellerberg, 1993). Bio-indicators are also a good way to monitor the effects of toxic materials on organisms (Bridgham, 1988).

Recently, bio-indication was proposed as a better way to

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monitor dioxin contamination problems (Focant, 2006; Chang, 2008). From mid-1980s, even though many dioxin biodegradation microorganisms were found to be able to convert dioxin into carbon source (Matsumura and Quensen, 1983; Armengaud et al., 1998; Wittich, 1998; Nojiri and Omori, 2002; Hiraishi, 2003), dioxin bio-indication still remains as a time-consuming approach in the laboratory.

DNA-based methods for bacterial identification are well established and have been widely and successful applied to bacterial identification. Such molecular-based methods include DNA sequence analysis, PCR, RT-PCR and oligonucleotide probe hybridization. These approaches adopted for bacterial identification purposes have several advantages that include greater sensitivity, better specificity, simplicity, rapid turnover and good reproducibility. Moreover, some of these approaches not only determine bacteria identity, but also measure the bacteria load in the process, for example, the real-time nucleic acid amplification (Yang et al., 2006; Singh et al., 2007; Miyauchi et al., 2008; Sahni et al., 2011).

The loop-mediated isothermal amplification (LAMP) assay is a potential method for the rapid amplification of DNA, with time saving and high sensitivity. This approach has developed significantly in the past years, with isothermal DNA amplification using four to six specifically designed primers (Notomi et al., 2000). The assay uses Bst DNA polymerase rather than Taq DNA polymerase in the test, which means that a LAMP reaction can be carried out without the denaturation step like PCR (Notomi et al., 2000; Nagamine et al., 2001). Recently, this LAMP assay has been improved and applied to detecting a wide range of different microbes and pathogens including protozoa, bacteria, and viruses (Aoi et al., 2006; Qiao et al., 2008; Salih et al., 2008; Shivappa et al., 2008; Yamazaki et al., 2009; Lee et al., 2011). Consequently, LAMP has become a powerful tool for carrying out nucleic acid-based diagnosis and detection since it is time-saving and requires relatively simple equipment.

Okamura et al. conducted a LAMP reaction in 2008. In this test, a positive reaction was observed for *Salmonella* (Okamura et al., 2008). In addition, LAMP also offers a means of reducing genotyping costs as well as simplifying testing procedures (Lee et al., 2009).

This study was aimed at using LAMP to assess quickly the microorganism present at a dioxin contaminated site. It was hoped that this approach could overcome the limitation of bioindicators and move forward the environmental biotechnology. To achieve the goals, a LAMP assay that used the 16S to 23S rRNA internal transcribed spacer (ITS) region of *Ochrobacterum anthropic* as the target DNA for rapid identification of dioxin contamination in the soil was displayed. This paper presents the first study describing the application of LAMP for the detection of dioxin.

2. Materials and methods

2.1. Bacterial strains

The dioxin-degrading strain *O. anthropi* BD-1 was originally isolated from the An-shan plant, in southern Taiwan. The plant was built to manufacture pesticides, including pentachlorophenol (PCP). The An-shun plant was identified by the Taiwanese government as a major site of contamination with both PCP and dioxin. The details of the procedure used to isolate the BD-1 strain have been previously described (Chen et al., 2010). The BD-1 strain was cultured by inoculating the organism into Minimal Salts Basal (MSB) medium supplemented with dioxin (10 μg/L of 2,3,7,8-TCDD) and 1 g/L of glucose, followed by incubation for 72 h at 30 °C with shaking at 160 rpm. Six other strains including *Ochrobactrum anthropi* BCRC 14348, *O. anthropi* BCRC 17142, *O. anthropi* BCRC 17248, *O. intermedium* BCRC 17247, *O. grignonense* BCRC 17249, and *O. tritici* BCRC 17250, were purchased from FIRDI (The Food Industry Research and Development Institute, Hsichu, Taiwan), and used to evaluate the ITS gene specificity of the LAMP. These six *Ochrobactrum* strains were grown for 48 h in nutrient broth (NB) at 30 °C. Genomic DNA extracted from *Brucella melitensis* was kindly provided by the Department of Medical Laboratory Science of the China Medical University Hospital (CMUH), Taiwan.

2.2. 2,3,7,8-TCDD detection

The dioxins were extracted from soil samples and purified for gas chromatography/high-resolution mass spectrometry (GC-HR-MS) analysis. The primary process consisted of pretreatment by freeze-drying, grinding, and screening with 150 meshes. The 1613-^{13}C was then added as an isotopic internal standard. This was then followed by Soxhlet extraction, concentration, and purification. Each sample was analyzed by the $^{13}C^{12}$-isotope dilution method, using GC/HRMS. Dioxins present in the soil were extracted by the solid phase micro-extraction (SPME) method. Detection of dioxins and various metabolites was performed by GC-HR-MS on a National Institute of Environmental Analysis (NIEA) M801.11B, 2005 method (Chen et al., 2010).

2.3. DNA extraction

Genomic DNA was extracted from pure cultures of the various strains using a Geneaid bacterial genomic DNA extraction kit (Presto™ Mini gDNA Bacteria Kit, Geneaid, Taipei, Taiwan), according to the manufacturer's instructions. Bacterial genomic DNA was extracted from the soil samples using a PowerSoil® DNA isolation kit purchased from MO BIO Laboratories, Inc. (Carlsbad, California, USA). All genomic DNA samples were stored at −20 °C until further use for LAMP and real-time LAMP assays. The concentration of the pure bacterial genomic DNA was measured with a spectrophotometer at 260 nm (NanoVue Plus™, GE Healthcare, Ltd. (Pollards Wood, UK).

2.4. Cloning and sequencing of the 16S–23S rRNA ITS region from O. anthropi BD-1

The 16S–23S rRNA intergenic spacer (ITS) sequence of *O. anthropi* BD-1 was cloned into a pGEM-T Easy vector (Promega, USA) and sequenced; cloning procedures were performed according to the manufacturer's instructions. DNA from the ITS region of *O. anthropi* BD-1 was amplified by PCR using the following forward and reverse primers, OA-ITS1-F (5′-AGTTGGTTTTACCCGAAGGC-3′) and OA-ITS-R (5′-TCAAAGGGTATTCGCACCTC-3′). The primers were designed based on various published 16S–23S rRNA sequences (GenBank accession Nos. CP000758, AJ867297, AJ867296, AJ867294, AJ867331, AJ867323, AJ867347, AJ867326). The PCR was performed in a total volume of 50 μL containing 1× PCR buffer [10 mM Tris–HCl pH 8.3, 50 mM KCl], 10 mM of each dNTP, 2 mM $MgCl_2$, 100 nM of each OA-ITS1-F and OA-ITS-R primer, 1 unit of Taq DNA polymerase, and 100 ng of genomic template DNA. The PCR conditions were as follows: 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 60 °C for 30 s, and 72 °C for 90 s, with a final extension at 72 °C for 10 min. Finally, 3 μL of the PCR product was analyzed by electrophoresis on a 1.5% agarose gel. DNA sequencing was performed to confirm the presence of the ITS sequence in the pGEM-T Easy vector.

2.5. Design of LAMP primers

Three sets of primers, Spec1, Spec2, and Spec3 were used. The

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primers (F3, B3 FIP, and BIP) used for the detection of *O. anthropi* BD-1 were designed based on the 16S–23S rRNA ITS sequence obtained in this study, and the published ITS sequences of other *Ochrobactrum* species (GenBank accession Nos. CP000758, AJ867297, AJ867296, AJ867294, AJ867331, AJ867323, AJ867347, AJ867326), which were used as reference sequences. The LAMP primers were designed by using the Primer Explorer ver. 3 software (http://primerexplorer.jp; Eiken Chemical Co. Ltd., Japan). The primer sequences and their target positions are outlined in Table 1.

2.6. LAMP reaction and product detection

The LAMP reaction was performed in 25 μL of a previously described reaction mixture (Huang et al., 2010). For each LAMP reaction, a different amount of genomic DNA was added to the reaction tube as a template and the samples were then incubated at 65 °C for 1 h on a heating device (PCR machine, BioRad). After the reaction was completed, 3 μL of the LAMP product was analyzed by electrophoresis on a 1.5% agarose gel.

2.7. Specificity and sensitivity of the LAMP assay

The specificity of the LAMP assay for detecting *O. anthropi* BD-1 was determined using genomic DNA from *O. anthropi* BCRC 14348, *O. anthropi* BCRC 17142, *O. anthropi* BCRC 17248, *O. intermedium* BCRC 17247, *O. grignonense* BCRC 17249, *O. tritici* BCRC 17250, and *B. melitensis*, which were used as reference strains. The sensitivity of the LAMP reaction for detecting *O. anthropi* BD-1 was determined using varying amounts of the *O. anthropi* BD-1 genomic DNA as templates to determine the detection limit of the assay.

2.8. Real-time LAMP

Real-time LAMP was performed by measuring the increase in fluorescence intensity during the LAMP reaction by using a real-time thermal cycler (StepOne™ Real-time System, Applied Biosystems®, USA). The LAMP reaction was performed in eight linked optical tubes on a thermal cycler using SYBR Green I (Invitrogen) as the intercalation dye for the LAMP product (Yang et al., 2011). The real-time LAMP reaction was performed using the Spec3 LAMP primers and the same reaction mixture and conditions as those used for amplifying the ITS region by LAMP extracted from strain BD1 was used. A serial dilution of the purified bacterial genomic DNA solution (100 ng, 10 ng, 1 ng, 100 pg and 10 pg) was used to establish a standard curve. To obtain this standard curve, the amount of bacterial genomic DNA used in each amplification was plotted against the time at which the fluorescence signal (ΔRn) increased to the threshold level (ΔRn = 1). This approach was based on a previous study by Aoi et al. (2006). The fluorescence signal was collected at 10-s intervals by the tube scanner while the real-time LAMP was being performed.

3. Results

3.1. Cloning and sequencing of the 16SA–23S rRNA region for use as target DNA for the LAMP primers

As a result, the ITS gene from five selected *Ochrobactrum* strains obtained the similarity lower than strain BD1 (higher than 95%). It indicated the ITS gene from BD1 ensures enough specificity for the LAMP assays. To design the LAMP primers, the genomic DNA was used to amplify the 16S–23S rRNA ITS region and then, sequenced and compared with the regions of *Ochrobactrum* species for evaluating the genetic variation. The related *Ochrobactrum* strains were *O. anthropic* BCRC, *O. intermedium*, *O. grignonense,* O. *lupine*, and *O. tritici*. The *O. anthropi* BCRC and *O. anthropi* BD-1 strains showed the highest similarity (94%), and *O. oryzae* showed the least similarity (85%) to *O. anthropi* BD-1. The other strains show the similarity of 89%, 88%, 92% to strain of *O. intermedium*, *O. oryzae, O. lupine,* respectively. In general, if the similarity is greater than 96%, we say that is high similarity ITS gene. In this study, we found the other five strains have similarity below 95% when compare to BD1. So, it indicates that strain BD1's ITS gene obtains the specificity among the strains. The high level of variation (over 15%) we observed in 16S–23S rRNA ITS region helped us to design LAMP primers that could potentially distinguish the BD-1 strain from related species of *Ochrobactrum*.

3.2. Establishment of a LAMP method for the specific detection of the dioxin-degrading strain O. anthropi

Based on the above findings, three primer sets, Spec1, Spec2, and Spec3 each with four primers were designed for LAMP amplification, and four specific primers were F3, B3, FIP, and BIP. All of which were used for amplify the 16S–23S rRNA ITS DNA of BD-1 under isothermal conditions. The design sequences of the primers are shown in Table 1. As illustrated in Fig. 1, the LAMP products formed ladder-like patterns after separation by agarose gel electrophoresis. When Spec1 and Spec2 primer sets were used to perform the LAMP reaction, they amplified the BD-1 strain as well as other species of *Ochrobactrum*. In contrast, when Spec3 primer set was used to perform the LAMP, the typical pattern of ladder-like fragments was only detected in the sample containing BD-1 genomic DNA (Fig. 1). Moreover, restriction digestion of the LAMP products yielded a single DNA fragment with a low molecular weight (Fig. 2). These findings indicated that LAMP product was indeed specific and was produced only by the amplification of 16S–23S rRNA ITS DNA from *O. anthropi* BD-1 by the Spec3 primer

Table 1
Loop-mediated isothermal amplification primers used in this study.

Sets	Primers	Sequences
Spec1	ITS-Spec1-F3	5′-CGCTTGATAAGCGTGGGGTC-3′
	ITS-Spec1-B3	5′-AGTCCGCAAACTTGGTTCTT-3′
	ITS-Spec1-FIP	5′-CTCTCCCAGCTGAGCTATGGCGGTTCAAGTCCTCCCAGGCC-3′
	ITS-Spec1-BIP	5′-GCTTTGCAAGCAGGGGGTCGTCTGCCGTCCTACTCTACACC-3′
Spec2	ITS-Spec1-F3	5′-GGGTCGGAGGTTCAAGTC-3′
	ITS-Spec1-B3	5′-ACTTCGTAAAGTCCGCAAAC-3′
	ITS-Spec1-FIP	5′-TGCAAAGCAGGTGCTCTCCCACTCCCAGGCCCACCAAA-3′
	ITS-Spec1-BIP	5′-GGTCGTCGGTTCGATCCCGTCTTCCTCGATGATTGACTGCCG-3′
Spec3	ITS-Spec3-F3	5′-GGGTCGGAGGTTCAAGTC-3′
	ITS-Spec3-B3	5′-TACAGAACACGCAGACTTCG-3′
	ITS-Spec3-FIP	5′-CTTGCAAAGCAGGTGCTCTCCTCCCAGGCCCACCAAATAT-3′
	ITS-Spec3-BIP	5′-GTTTGGTGTAGAGTAGGACGGCCTTGGTTCTTTCCTCGATGA-3′

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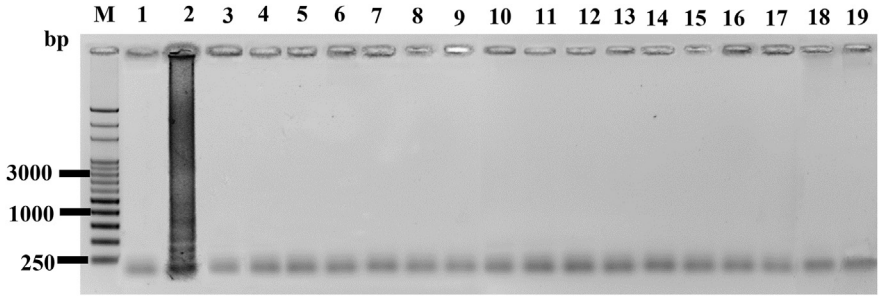

Fig. 1. Specificity of various LAMP primers sets evaluated by agarose gel electrophoresis for the detection of BD-1strain.The spec3 agarose gel is shown here: Lane1, negative control; lane 2, genomic DNA of BD-1; lane 3, genomic DNA of *O. anthropi* (BCRC14348); lane 4, genomic DNA of *O. anthropi* (BCRC17142); lane 5, genomic DNA of *O. intermedium*; lane 6, genomic DNA of *O. anthropi* (BCRC17248); lane 7, *O. grignonense*; lane 8, genomic DNA of *O. tritici*; lane 9, genomic DNA of *Brucella* sp., lane10, genomic DNA of *Arthrobacter* sp. (IL01); lane11, genomic DNA of *Arthrobacter* sp. (IL02); lane 13, genomic DNA of *Arthrobacter* sp. (IL03); lane 14, genomic DNA of *E. coli*; lane 15, genomic DNA of *Staphylococcus* sp.; lane 16, genomic DNA of *B. subtilis*; lane 17, genomic DNA of *A. xylosoxidans*; lane 18, genomic DNA of *R. mannitolilytica*; lane 19, genomic DNA of *Agromyces* sp.

set. Thus, a LAMP method for the detection of *O. anthropi* BD-1 has been successfully established using the Spec3 primer set.

3.3. Sensitivity of the LAMP method for detecting the dioxin-degrading strain O. anthropi BD-1

To determine the sensitivity of the LAMP assay, different amounts of purified bacterial genomic DNA were used as templates. As illustrated in Fig. 3A, the genomic DNA added to each LAMP reaction ranged from 100 ng to 1 pg, at 10-fold dilutions. By using Spec3 primer set, the LAMP products were obtained at concentrations as low as 1 ng. It gives a detection limit of 1 ng for the LAMP reaction. To improve the sensitivity of LAMP, FLP, a primer with an extra loop was designed and used. As shown in Fig. 3B, when the FLP primer was added to Spec3 primer set, the detection limit was reduced to less than 1 pg. It indicates that FLP primer improved the sensitivity by at least 1000-folds. In addition, the presence of FLP primer also improved the speed of ITS amplification (see Fig. 4B). It requires only about 30 min for a positive amplification when the FLP loop was used. These results were compared with those without the FLP loop primer (Fig. 4A). In which, at least 1 h was needed to accumulate sufficient LAMP product for a positive result. Thus, both the sensitivity and speed of the LAMP assay for detection of strain BD-1 were significantly improved by the adding of the FLP primer.

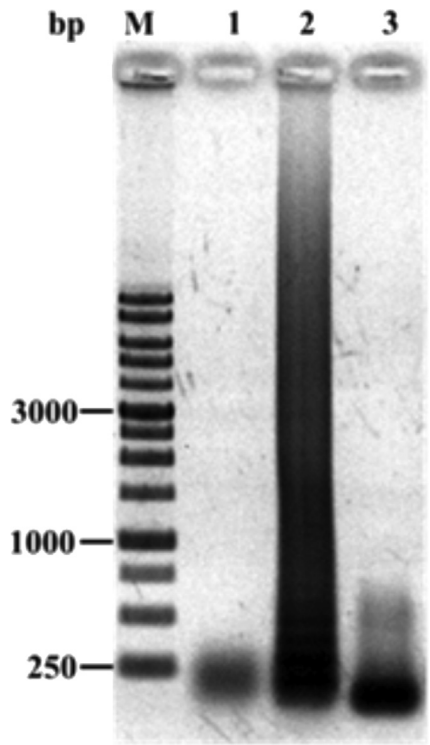

Fig. 2. Restriction digestion of LAMP products. In the gel: Lane 1, controls; lane 2, the LAMP product without addition of *Pvu*II; lane 3, the LAMP product with addition of *Pvu*II.

3.4. Development of a real-time LAMP assay for quantification of the dioxin-degrading strain O. anthropi BD-1

Real-time LAMP was performed isothermally using a real-time thermal cycler that occurs during the LAMP reaction. As seen in Fig. 5A, the real-time LAMP reaction was performed using varying amounts of 10-fold diluted genomic DNA as the template. While the genomic DNA added to the LAMP reaction increased, the time required to detect the fluorescence signal decreased. When 100 ng of DNA was loaded, the initiation time of the LAMP reaction was 13 min 17 s, and it was 18 min 45 s and 25 min 23 s when 1 ng and 10 pg of DNA was loaded, respectively. These results were used to plot a standard curve of DNA concentration against initiation time of the LAMP reaction which as shown in Fig. 5B. Regression analysis of this curve showed a negative and highly linear correlation with R^2 value of 0.93 ($p = 0.02$). To calculate the corresponding amount of BD1 bacteria present in cfu/mL (Fig. 5C), the relationship: $Y = 1 \times 10^{11} X + 4.6 \times 10^{-17}$ (Equation (1) in Fig. 5 (C)), with R^2 value of 0.99 ($p = 0.01$). These findings were then validated by separately inoculating two pure colonies of BD1 into liquid culture, followed by incubation for 18.5 min and 20 min. DNA was extracted and the concentrations were found to be 1.23 ng and 348 pg. Using Equation (1), the bacterial counts were determined to be 123 and 35 cfu/mL. The number of bacteria present was then estimated by using a spectrophotometer and the spread plate method. The bacteria were present at DNA concentrations of 1.07 ng and 329 pg, and the number counts were 107 cfu/mL and 33 cfu/mL per 1 g soil. The spectrophotometric and spread plate methods had 85.1% and 93.9% accuracy, respectively. These findings indicate that real-time LAMP assays can be used to estimate DNA concentrations and bacterial counts of samples with acceptable accuracy and reliability.

3.5. Application of real-time LAMP for the detection of dioxin-degrading O. anthropi BD-1 in environmental soil samples

After the development and validation of the LAMP and real-time LAMP assays for detecting *O. anthropi* BD-1, we assayed eight soil

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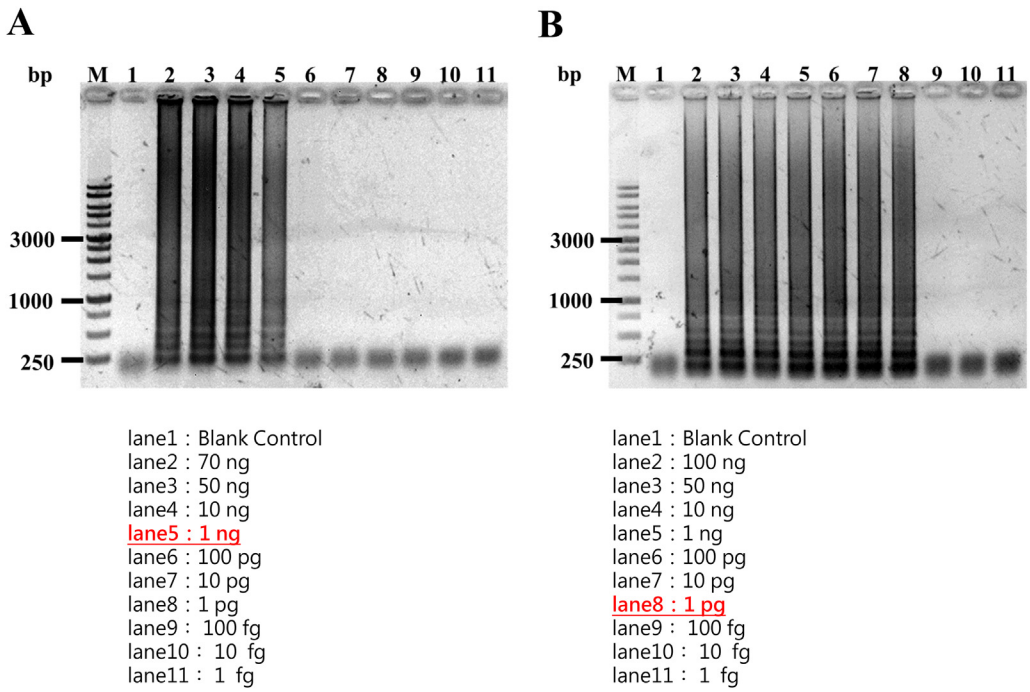

Fig. 3. The sensitivity of the LAMP reaction for detection of *O. anthropi* BD-1. In the gel: Lane1, negative control; lane 2, 100 ng (of genomic DNA); lane 3, 50 ng; lane 4, 10 ng; lane 5, 1 ng; lane 6, 100 pg; lane 7, 10 pg; lane 8, 1 pg; lane 9, 100 fg; lane 10, 10 fg; lane 11, 1 fg.

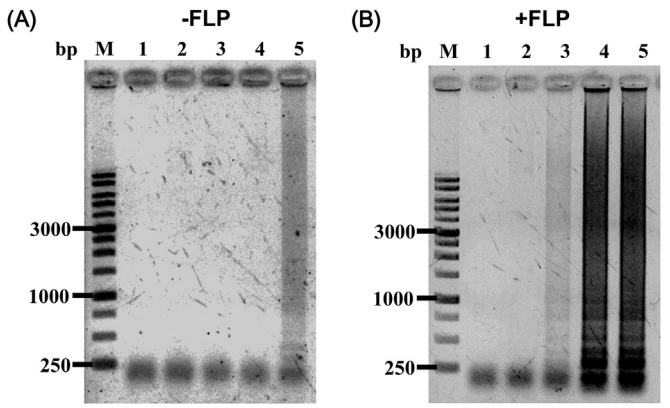

Fig. 4. The reactivity of the LAMP reaction, it was improved by additional of a loop primer. In the gel: (A) without addition of FLP (B) reactivity evaluated in terms of the initiation time required: Lane 1, negative control; lane 2, 15 min (initiation time); lane 3, 30 min; lane 4, 45 min; lane 5, 60 min.

samples collected from a dioxin-contaminated site. The presence of *O. anthropi* BD-1 in these soil samples had been previously confirmed by traditional cultural identification (Gong, 2009). Bacterial genomic DNA was extracted from the soil samples and subjected to the real-time LAMP assay. The products obtained were resolved by agarose gel electrophoresis and are shown in Fig. 6. The intensity of the ladder-like DNA fragments varied between the different soil samples. When fluorescence intensity was measured by the LAMP reaction progressing, it was possible to establish the initiation time of LAMP reaction for each soil sample. The samples 4, 5, and 6 showed shorter initiation times of LAMP reaction of 20.3 min, 19.4 min, and 18.9 min, respectively (Fig. 6). In contrast, the initiation times of the remaining soil samples were all >24 min. These results were in agreement with the DNA electrophoresis patterns observed (Table 2). The three samples that showed short initiation times also showed high-intensity DNA fragment patterns. The BD-1 bacterial loads of samples 4, 5, and 6 were estimated using the standard curve (Equation (1)) and the bacterial counts of the three samples were determined to be 26, 55, and 86 cfu/mL, respectively. Collectively, these results show that LAMP and real-time LAMP can be used to detect the dioxin-degrading BD-1 strain in environmental soil samples and to determine the amount of *O. anthropi* BD-1 present in each sample.

4. Discussion

We found that the BD1 isolated using dioxin 2,3,7,8 TCDD as the sole carbon source in a enrich culture. The strain BD1 obtains the closer genetic relation to the genus of *Sphingomonas* species with the Bootstrap value of 95. Phylogenetic analysis shows that strain BD1 has similar genetic relationship with the strain of *Agrobacterium tumefaciens*, *Rhizobium etli*, *Agrobacterium radiobacter*, and *Sphingomonas* species (Gong, 2009).

In this study, we successful developed rapid, sensitive, and simple LAMP and real-time LAMP assays for detecting the dioxin-degrading strain *O. anthropi* BD-1 in real soil samples. We confirmed that these LAMP and real-time LAMP methods, allow the specific detection of BD-1 in soil samples. LAMP primer design is an important consideration when choosing a specific target DNA for identification of a bacterium. In a previous study, DNA encoding the 16S rRNA region, the *recA* gene, and the 16S–23S rRNA ITS sequence were used as the target sequences for bacterial identification (Lai, 2013). However, prior to conducting this study, we cloned and sequenced the DNAs encoding the 16S rRNA and the *recA* gene from the BD-1 strain and compared them with the sequences from *O. intermedium*, *O. grignonense*, *O. tritici,* and *B. melitensis.* We found that the DNA encoding the 16S rRNA of O. anthropi BD-1 was 100% identical to that of various *Ochrobactrum* strains, rendering this sequence unsuitable as a tool for strain differentiation (data not shown). A similar analysis of the *recA* gene sequences from the various Ochrobactrum strains showed that the sequence identity varied from 90% to 99% (data not shown).

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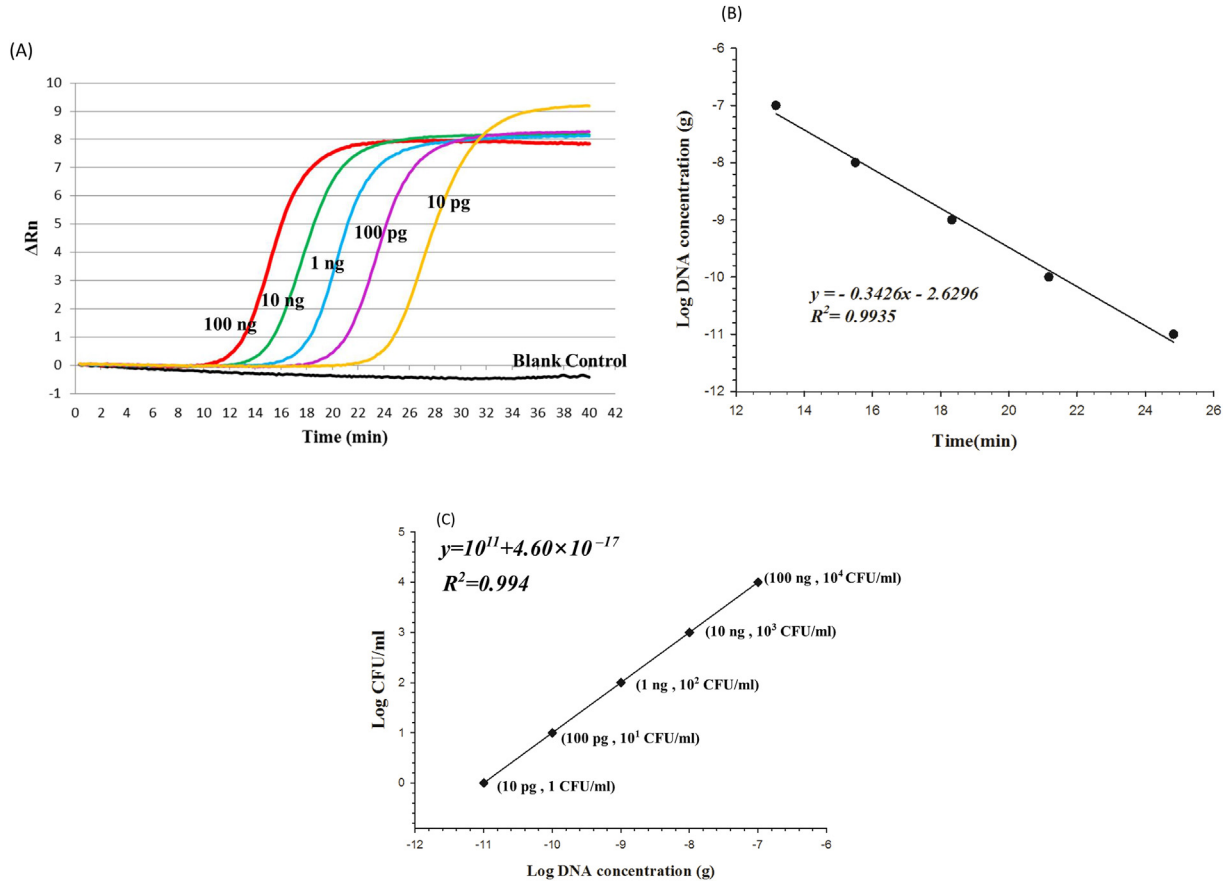

Fig. 5. Correlation between initiation time for real-time LAMP and the amount of *O. anthropi* BD-1 genomic DNA loaded. (A) The initiation time for real-time LAMP corresponded to the amount of genomic DNA loaded. (B) A correlation between the initiation time for real-time LAMP and the amount of genomic DNA added to the reaction. (C) A linear correlation between the amount of genomic DNA and the concentration of the BD-1 cells (CFU/mL).

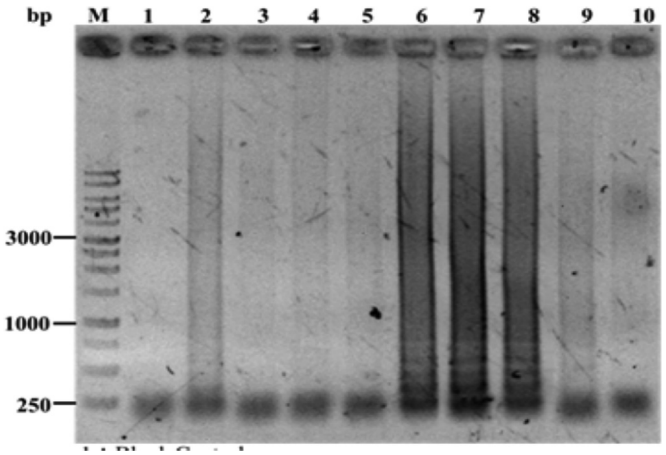

Fig. 6. Detection of *O. anthropi* BD-1 in dioxin-contaminated soil samples using LAMP. Eight soil samples containing strain BD-1 were collected from dioxin-contaminated areas. The presence of the BD-1 strain in the soil was confirmed by tradition cultural identification methods. The total genomic DNA as the templates for the LAMP was extracted from each soil sample. In the gel: Lane 1, negative control; lane 2, genomic DNA mixture of BD-1, and other bacterial strains including lanes 3–10 corresponding to soil samples No. 1–8, respectively.

O. anthropi BCRC 14348 and *O. anthropi* BD-1 showed the highest identity at 99%. This lack of variation would hamper the specificity of a LAMP assay using the *recA* gene sequences. In contrast, the 16S–23S rRNA ITS sequences were found to be 85%–94% identical. This level of genetic variation is sufficient to allow the design of specific LAMP primers, that even variation in a single nucleotide between sequences can be detected, allowing precise discrimination between strains. Our findings demonstrate that the 16S–23S rRNA ITS region of *O. anthropi* BD-1 with its high level of genetic variation relative to other *Ochrobactrum* strains is well suited for use as a target for the highly specific Spec3 LAMP primers used for distinguishing *O. anthropi* BD-1 from other species of genus *Ochrobactrum* (Fig. 1).

LAMP is a method for the rapid amplification of DNA with high specificity and efficiency under isothermal conditions, using four to six specifically designed primers. The LAMP reaction can also be conducted without the denaturation step essential to PCR since the DNA amplification occurs via the strand displacement activity of the *Bst* DNA polymerase. Additionally, the LAMP method can be performed directly on crude biological materials, such as blood, without the need for DNA purification (Njiru et al., 2008). Recently, methods for LAMP have been improved and applied to the detection of various pathogens and genetically modified organisms (GMOs) (Lee et al., 2009). However, the LAMP is seldom applied in environmental microbiology. LAMP has become a very powerful tool for detecting specific target DNAs because of its time-saving and simple equipment. Once the appropriate target DNA for *O. anthropi* BD-1 is established, LAMP could be used to identify DNA from this dioxin-degrading strain more efficiently than sequencing of other conventional identification methods. Since the LAMP method can only be used for one specific strain, we recognize the method limitation while many dioxin biodegradation bacteria

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Table 2
The microbial estimation and detection of 2,3,7,8-TCDD for the soil samples.

Samples number	Initial time of real-time LAMP (minute)	CFU/ml of 1 g soil	2,3,7,8-TCDD concentration in the soil (ng-TEQ/kg soil)
1	NR[a]	5.4 × 104	ND[b]
2	NR	3.2 × 103	ND
3	NR	1.1 × 103	ND
4	20.3	1.5 × 103	96.3
5	19.4	1.3 × 103	78.3
6	18.9	4.4 × 103	1260.2
7	NR	1.2 × 105	ND
8	NR	8.3 × 104	ND

[a] None reaction during the real-time LAMP thermal cycler. The reaction time delay will be invalid when it greater than 24 min and we identify it none reaction.
[b] None detected, the detection limit of the GC/HR/MS is 5 ng-TEQ/kg soil, the concentration below the detection limit will none detected.

strains exist simultaneously in one site. Nevertheless, this study is applicable at a particular contaminated site to detect the local dioxin degraders.

In general, four or six primers are used to perform LAMP assays. In our experience, LAMP reactions using four primers performed well. The sensitivity may be improved by adjusting a variety of different factors, including the primer sequences, the reaction buffer, and the purity of the nucleic acid template. Thus, future studies should address the optimization of this LAMP reaction, notwithstanding the increased sensitivity obtained when the FLP primer was added to the reaction (Fig. 3).

When the LAMP reaction was initialized by this study, two outer primers, F3 and B3, of the Spec3 target the first 20–229 nucleotides of ITS DNA sequence. The LAMP then amplifies a 210-bp DNA fragment under isothermal conditions, and this amplicon acts as the initiation substrate for the two inner primers, FIP and BIP, which leads to the formation of the ladder-like LAMP DNA fragments. The amplicon derived from the first 20–229 nucleotides of the 16S–23S rRNA ITS of the BD-1 strain contains one *Pvu*II restriction enzyme site, which allows the ladder-like LAMP DNA fragments to be digested by *Pvu*II, producing a single identifiable linear DNA band for the verification of the LAMP product.

Of the eight soil samples obtained from 2,3,7,8-TCDD-contaminated site, three samples produced strong signals for both the LAMP and real-time LAMP assays. Using real-time LAMP, it was possible to monitor as well as quantify the presence of the dioxin-degrading strain *O. anthropi* BD-1. Using GC-HR-MS, 2,3,7,8-TCDD could only be detected in 3 of the 8 soil samples; samples 4, 5, and 6 showed concentrations of 96.3, 78.3, and 1260.2 ng-TEQ/kg soil, respectively. The amounts of specific target DNA from *O. anthropi* BD-1 detected by real-time LAMP assays of these samples were 255.2, 554.3, and 864.2 pg, respectively. These findings indicate that it was possible to detect significant amounts of *O. anthropi* BD-1 genomic DNA in contaminated soil. Thus, there appears to be a direct correlation between level of 2,3,7,8-TCDD dioxin contamination, the ability to isolate bacteria capable of dioxin biotransformation, and the presence of *O. anthropi* BD-1 genomic DNA. In spite of the low level of *O. anthropi* BD-1 present in the soil samples, the presence of a specific strain of bacteria that can grow in the presence of toxic dioxins was confirmed by real-time LAMP. This method should help to quickly identify dioxin contamination by measuring the real-time LAMP initiation time for soil samples.

5. Conclusions

In this study, we successful developed rapid, sensitive, and simple LAMP and real-time LAMP assays for detecting the dioxin-degrading strain *O. anthropi* BD-1 in real soil samples. The 16S–23S rRNA ITS sequences were found to be 85%–94% identical. This level of genetic variation is sufficient to allow the design of specific LAMP primers and suitable for use as a target for distinguishing *O. anthropi* BD-1 from other species of genus *Ochrobactrum*. The amplicon derived from the first 20–229 nucleotides of the 16S–23S rRNA ITS of the BD-1 strain contains one *Pvu*II restriction enzyme site, which allows the ladder-like LAMP DNA fragments to be digested by *Pvu*II and producing a single identifiable linear DNA band for the verification of the LAMP product. When using real-time LAMP assays to the real samples, there appears to be a direct correlation between the level of 2,3,7,8-TCDD dioxin contamination, the ability to isolate bacteria capable of dioxin biotransformation, and the presence of *O. anthropi* BD-1 genomic DNA.

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