PCR-Based Random Amplified Polymorphic DNA Fingerprinting of *Yersinia pseudotuberculosis* and Its Practical Applications

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The random amplified polymorphic DNA (RAPD) fingerprinting method was used to distinguish between various strains of *Yersinia pseudotuberculosis*, a causative agent of gastroenteritis in humans. The RAPD test uses arbitrarily chosen DNA oligomers of 10 nucleotides to prime DNA synthesis from genomic sites to which they are accidentally matched or almost matched. Most 10-nucleotide primers yielded strain-specific arrays. Ten *Y. pseudotuberculosis* type strains were distinguishable from each other by analyzing the RAPD arrays produced by using primers with a 50% G+C content. The RAPD patterns of *Y. pseudotuberculosis* strains were found to be constant regardless of the presence or absence of the large plasmid. RAPD tests were subsequently used to identify 30 clinical isolates of *Y. pseudotuberculosis* that were collected as the causative agent of an outbreak of Izumi fever, a disease showing clinical symptoms characteristic of atypical scarlet fever, in Japan. The RAPD arrays from all of the isolates yielded common patterns that were unique to each primer used. Since those 30 isolates belonged to serotype 5a and the restriction digest patterns of their large plasmids were all the same, the results of the RAPD tests confirmed the view that those isolates were from a single source and indicated that the RAPD test can be practically applied to survey transmission of the bacterium in humans.

*Yersinia pseudotuberculosis* causes various human diseases with symptoms ranging from gastroenteritis to septicemia. Recently, it was demonstrated that a large plasmid of about 70 kb is necessary for full expression of virulence in *yersiniae* (6, 26), and various virulence-associated genes have been identified on this plasmid (2, 3, 12). *Y. pseudotuberculosis* sources are frequently isolated from water and soil and from numerous animal species, for example, rodents and birds (5, 24). Wild animals, which are often asymptomatic carriers, are considered the natural reservoir for the bacterium. Humans and animals are contaminated orally either by direct contact with sick or asymptomatic animals or by ingestion of food or drinking water contaminated with excretions from these animals (4). Thus, identification of the origin of an outbreak or tracing transmission patterns in human populations is not an easy task. Although serotyping and biochemical characterization are routinely used to identify *Y. pseudotuberculosis* strains, a variety of DNA-based methods has recently been developed for *yersiniae*, including analysis of restriction fragment polymorphisms in chromosomal DNA (9, 11, 14) or the 70-kb plasmid DNA (10), Southern hybridization with various gene probes (11), and production of specific PCR products (7, 18, 27).

In this context, efficient, sensitive, and reliable methods are needed in order to identify possible sources of transmission to humans. We report here the use of a PCR-based DNA fingerprinting method using random amplified polymorphic DNA (RAPD) to detect DNA sequence diversity among *Y. pseudotuberculosis* isolates (23, 25). This method uses an oligonucleotide of arbitrarily chosen sequence to prime DNA synthesis from pairs of sites to which it is matched or partially matched and results in strain-specific arrays of DNA products. Using the RAPD method, we found that each type strain of *Y. pseudotuberculosis* tested was distinguishable by using one, or at the most two, primer. The data presented also indicate that the RAPD method is feasible for investigating the source of an outbreak associated with *Y. pseudotuberculosis* infection.

**MATERIALS AND METHODS**

Strains used in this study. Ten *Y. pseudotuberculosis* type strains for serotyping are listed in Table 1. Although 30 clinical *Y. pseudotuberculosis* isolates from an outbreak in Aomori Prefecture, Japan, in 1991 (19) were used in this study, only one representative isolate, NO3, is shown in Table 1, because all of them had the same phenotypes. All of these clinical isolates were from feces of individual patients, except for two strains which were isolated from feces of cooks and one strain from a waterway in a feeding facility.

Total and plasmid DNA preparation. Bacterial cells were grown in L broth for 20 h at 25°C with shaking. Total DNAs from *Y. pseudotuberculosis* were isolated according to previously described methods (15). Plasmid DNAs were isolated as previously described (21), with the following modifications. Briefly, bacterial cell pellets obtained from 1.5 ml of overnight culture were suspended in 100 µl of solution 1 (40 mM Tris-acetate, 2 mM EDTA, pH 7.4) and mixed with 200 µl of solution 2 (5 mM Tris, 3% sodium dodecyl sulfate, pH 12.6) and the mixture was incubated at 60°C for 60 min before phenol-chloroform (1:1 [vol/vol]) extraction. After centrifugation, 15 µl of the supernatants was used for agarose gel electrophoresis to determine the presence or
TABLE 1. Y. pseudotuberculosis strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Plasmid</th>
<th>Characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H655</td>
<td>1a</td>
<td>-</td>
<td>+</td>
<td>TMRL/</td>
</tr>
<tr>
<td>Pa5806</td>
<td>1b</td>
<td>+</td>
<td>+</td>
<td>NT + TMRL</td>
</tr>
<tr>
<td>H148</td>
<td>2a</td>
<td>+</td>
<td>+</td>
<td>NT + TMRL</td>
</tr>
<tr>
<td>H110</td>
<td>2b</td>
<td>+</td>
<td>+</td>
<td>NT + TMRL</td>
</tr>
<tr>
<td>H113</td>
<td>2c</td>
<td>+</td>
<td>+</td>
<td>NT + TMRL</td>
</tr>
<tr>
<td>Pa4333</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>NT + TMRL</td>
</tr>
<tr>
<td>H112</td>
<td>4a</td>
<td>+</td>
<td>+</td>
<td>NT + TMRL</td>
</tr>
<tr>
<td>CYP86-1</td>
<td>4b</td>
<td>+</td>
<td>+</td>
<td>+ TMRL</td>
</tr>
<tr>
<td>H123</td>
<td>5a</td>
<td>+</td>
<td>+</td>
<td>+ TMRL</td>
</tr>
<tr>
<td>H113</td>
<td>5b</td>
<td>+</td>
<td>+</td>
<td>+ NT + TMRL</td>
</tr>
<tr>
<td>N03</td>
<td>5a</td>
<td>+</td>
<td>+</td>
<td>+ + 19</td>
</tr>
<tr>
<td>H153</td>
<td>5a</td>
<td>+</td>
<td>+</td>
<td>NT + TMRL</td>
</tr>
</tbody>
</table>

* Ten strains (all except N03 and H153) are standard strains for serotyping. N03 is a strain representative of 30 clinical isolates from an outbreak in Aomori, Japan (19).
  a + and − indicate the presence and absence, respectively, of the large plasmid.
  b Crb, Congo red binding ability.
  c Invasiveness (Inv) of the epithelial cells. NT, not tested.
  d Positive (+) or negative (−) autoagglutination (Agg) in broth at 37°C after 24 h of incubation.
  / TMRL, Tokyo Metropolitan Research Laboratory of Public Health.

absence of the large virulence plasmid and 200 μl was further purified by ethanol precipitation in order to obtain DNA suitable for BamHI restriction analysis, as previously described (21).

Characterization of Y. pseudotuberculosis virulence-associated factors. Congo red binding ability was tested on tryptic soy agar plates containing 0.05% Congo red (Congo red plates) as previously described (21). Tests for autoagglutination were carried out according to the methods described by Laird and Cavanaugh (13). Invasiveness into the eukaryotic LLC-MK2 epithelial cells was tested by gentamicin killing assays and Giemsa staining techniques as previously described (16, 17).

Isolation of Crb− derivatives. Since the large plasmid codes for the Congo red-binding phenotype (Crb) on Congo red plates and is not maintained stably, it was possible to detect Crb− variants derived from Crb+ Y. pseudotuberculosis strains and thus isolate plasmid-cured derivatives as described previously (20, 21).

RAPD fingerprinting. PCR was carried out in a volume of 25 μl containing 20 ng of Y. pseudotuberculosis total DNA, 3 mM MgCl2, 20 pmol of primer, 1 U of Taq DNA polymerase (Promega), and 250 μM (each) dCTP, dGTP, dATP, and dTTP (Pharmacia) in 10 mM Tris-Cl (pH 8.3)–50 mM KCl–0.1% Triton X-100 under a drop of mineral oil, essentially as described previously (1, 25). An Astec program temperature control system PC-700 (Astec Co., Ltd., Tokyo, Japan) was used for amplification. The cycling program was 4 cycles of 94°C for 5 min, 36°C for 5 min, and 72°C for 5 min; 30 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min; and a final incubation at 72°C for 10 min. After PCR, 10- to 20-μl aliquots of the product were subjected to electrophoresis in 2% agarose gels, followed by ethidium bromide staining and photography under UV light.

RESULTS

Identification of useful primers. To identify primers that generate informative arrays of PCR products, we first under-

Table 2. Primers used in RAPD tests with Y. pseudotuberculosis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>G+C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP40</td>
<td>GCGACGCAA</td>
<td>70</td>
</tr>
<tr>
<td>AP41</td>
<td>GCCATCCCCA</td>
<td>70</td>
</tr>
<tr>
<td>AP42</td>
<td>AACGCGAAC</td>
<td>100</td>
</tr>
<tr>
<td>AP43</td>
<td>GTGGATGGGA</td>
<td>60</td>
</tr>
<tr>
<td>AP44</td>
<td>AGCAGATTTC</td>
<td>50</td>
</tr>
<tr>
<td>AP45</td>
<td>GTCAACGAAG</td>
<td>50</td>
</tr>
<tr>
<td>AP46</td>
<td>GAGCACAAGA</td>
<td>50</td>
</tr>
<tr>
<td>AP47</td>
<td>CCGGAAAATAG</td>
<td>50</td>
</tr>
</tbody>
</table>

* All primers have been used previously for RAPD analysis in H. pylori (1).

took RAPD tests on total DNA preparations from three type strains, H655, Pa4333, and H123, of Y. pseudotuberculosis belonging to serotypes 1a, 3, and 5a, respectively (Table 1). Eight different 10-nucleotide oligonucleotides that had been used for RAPD fingerprinting of Helicobacter pylori (1) (Table 2) were chosen as the arbitrary primers and examined for the ability to give rise to informative RAPD arrays. The results indicated that all arbitrary primers gave rise to arrays of up to about 15 bands, ranging from 0.5 to 3 kb (Fig. 1). Since the concentration of MgCl2 in the PCR mixture for the RAPD tests is a critical factor in producing informative arrays (1), the optimal concentration of MgCl2 for Yersinia DNA was determined. Since a heavy background of nonspecific bands appeared at higher concentrations of MgCl2 but numbers of informative bands were reduced at lower concentrations (data not shown), an intermediate concentration, 3 mM, gave rise to the most useful arrays of bands. On the other hand, changes in the concentration of primers or genomic DNA did not substantially affect the RAPD patterns under the PCR conditions described in Materials and Methods (data not shown).

Since primers with a 50 or 60% G+C content (AP42 through AP47) produced fewer bands than those with 70% (Fig. 1), their RAPD patterns were used to distinguish strains from each other. On the basis of these results, the RAPD patterns appearing from 10 type strains of Y. pseudotuberculosis were subsequently investigated by using four primers with a 50% G+C content, AP44, AP45, AP46, and AP47 (Table 2). Although many common bands appeared among those 10 strains in the RAPD arrays with each primer, most of the primers yielded their own RAPD pattern, unique to each strain (Fig. 2), suggesting that the RAPD arrays arising from the chosen arbitrary primers can be used to identify strains and that the RAPD tests could be applied to the epidemiology of Y. pseudotuberculosis.

Effect of the presence of the large plasmid in Y. pseudotuberculosis on RAPD patterns. Since fully virulent Y. pseudotuberculosis strains possess a large plasmid but frequently lose it during subculturing at 37°C or during long storage at 4°C (6, 26), we checked to see whether the presence or absence of the large plasmid affected the pattern of RAPD arrays. Hence, we undertook to cure the large plasmid from three Y. pseudotuberculosis strains, CYP86-1, H123, and N03 (Table 1), by subculturing for 200 generations at 37°C (see Materials and Methods). By isolating Crb− colonies from those three strains, we obtained plasmid-cured derivatives (data not shown). Those plasmid-cured derivatives had lost the ability to autoagglutinate but still possessed the ability to invade the epithelial cells (8). Using total DNA preparations from the three pairs of strains, we investigated the effect of the presence or absence of the large plasmid on...
the RAPD patterns with each of the three primers, AP40, AP42, and AP44, representing primers with 70, 60, and 50% G+C contents, respectively (Table 2). The results showed that there was no detectable difference in the RAPD patterns between the plasmid-cured and the plasmid-harboring strains (Fig. 3), indicating that all RAPD patterns obtained from the Y. pseudotuberculosis strains result from diversity in the DNA sequence of the chromosome but not in that of the large plasmid. Moreover, the results showed that the RAPD patterns from Y. pseudotuberculosis after subculturing for 200 generations are identical to those of the starting strains, indicating that its chromosome is constant in laboratory culture.

**Application of RAPD test to an epidemiological study of Y. pseudotuberculosis.** An epidemic fever associated with Y. pseudotuberculosis infection broke out in an area in Aomori Prefecture in Japan in 1991 (19). Thirty independent isolates of Y. pseudotuberculosis were sampled during June and July 1991 and partly characterized to elucidate the source of the outbreak. Because all the isolates were of serotype 5a and possessed large plasmids showing the same BamHI digestion

![FIG. 1. Representative results of tests to identify suitable primers for RAPD analysis. Eight kinds of arbitrary primers shown in Table 2 and three representative Y. pseudotuberculosis type strains, H655 (lanes 1), Pa3433 (lanes 2), and H123 (lanes 3), were used for the RAPD tests. Arbitrary primer code numbers are shown over this photograph and in all of the other figures. HindIII-digested bacteriophage lambda DNA was used as a size marker for all of the figures in this study.](image1)

![FIG. 2. Comparison of the RAPD patterns of 10 type strains. Four kinds of informative primers, AP44, AP45, AP46, and AP47, with a 50% G+C content were used. The 10 Y. pseudotuberculosis type strains used were H655 (lanes 1), Pa5806 (lanes 2), H148 (lanes 3), H110 (lanes 4), H133 (lanes 5), Pa3433 (lanes 6), H112 (lanes 7), CYP86-1 (lanes 8), H123 (lanes 9), and H113 (lanes 10).](image2)

![FIG. 3. Effect of the presence of the large plasmid in Y. pseudotuberculosis on RAPD patterns. Profiles of RAPD patterns obtained with arbitrary primers AP40, AP42, and AP44. Lanes 1, 3, and 5 contain plasmid-harboring strains, CYP86-1, H123, and NO3, respectively. Lanes 2, 4, and 6 contain plasmid-cured derivatives of CYP86-1, H123, and NO3, respectively.](image3)
pattern, it was suspected that the outbreak was due to a single *Y. pseudotuberculosis* strain, probably from a single unknown source (19). Hence, in order to determine whether the outbreak was due to a single clone of the bacteria and to demonstrate the versatility of the RAPD test, we used all 30 isolates and two type strains, H123 and H153 (Table 1), to investigate the RAPD patterns produced with primers AP44, AP45, AP46, and AP47.

Representative results of RAPD analysis from 4 of the 30 isolates associated with the outbreak are shown in Fig. 4. The RAPD patterns of all 30 strains were indistinguishable from each other (Fig. 4, lanes 1 to 4 for AP44, AP45, AP46, and AP47), whereas those of the two type strains H123 and H153 were clearly different (Fig. 4, lanes 5 and 6 for AP44, AP45, AP46, and AP47). These results indicate that a single clone of *Y. pseudotuberculosis* was associated with the outbreak and indicate that the RAPD tests performed in this study will be useful for epidemiological studies of *Y. pseudotuberculosis*.

**DISCUSSION**

PCR-based DNA fingerprinting methods, termed RAPD (23, 24), have recently been used to distinguish between strains of *H. pylori* to trace patterns of transmission in human populations and were found to be a more sensitive way of detecting diversity than restriction fragment length polymorphism analysis (1). Hence, we performed RAPD tests to investigate their usefulness in distinguishing between *Y. pseudotuberculosis* strains and showed that this method is applicable to epidemiological studies.

The RAPD tests in this study were carried out by using eight 10-nucleotide primers, consisting of three classes with respect to different G+C contents, 50, 60, and 70% (1). Those primers were originally prepared for RAPD tests on higher plant genomes (25) and subsequently used for identification of clinical isolates of *H. pylori* (1). On the basis of the profiles of informative RAPD arrays from the 3 type strains of *Y. pseudotuberculosis* with each of the eight primers (Fig. 1), we further investigated RAPD arrays from 10 type strains of *Y. pseudotuberculosis* with primers AP44 through AP47 (Fig. 2). The RAPD arrays obtained with each primer were specific for each strain, except for those from strains H148 and H110 with AP44 (Fig. 2, lanes 3 and 4). However, the RAPD arrays produced from strains H148 and H110 were different when other primers were used (Fig. 2, AP45, AP46, and AP47, lanes 3 and 4), suggesting that at least two different primers must be used to distinguish strains by RAPD analysis.

*Yersinia* harbor a large, 70-kb plasmid, which is essential for expressing the full virulence phenotype of *Y. pseudotuberculosis* (6, 26). However, this large plasmid is often lost during subculturing at 37°C or storage for long periods at 4°C (6, 26). We thus investigated whether the presence or absence of the large-plasmid DNA affected the patterns of the RAPD arrays by constructing three sets of *Y. pseudotuberculosis* strains, each consisting of plasmid-cured and plasmid-harboring strains. Our data showed that the RAPD patterns in each set were the same regardless of the presence or absence of the large plasmid, indicating that RAPD arrays reflected DNA diversity in the chromosome but not in the large plasmid.

It has been shown that the pattern of BamHI digests of the large plasmid of *Y. pseudotuberculosis* strains in combination with serotyping serves as a useful means for strain identity (10, 19). Although profiles of the electrophoresed BamHI restriction fragments from strains H148, H110, Pa3433, and H112 were different from each other (data not shown), those of the large plasmids from strains CYP86-1, H123, and H153 were indistinguishable (data not shown). Thus, analysis of BamHI digests of the large plasmid alone is not necessarily useful for strain identification, as further restriction analysis is needed to distinguish strains. In contrast to this, the results of the RAPD tests performed on the three strains CYP86-1, H123, and H153 showed that this method was capable of clearly distinguishing each strain (Fig. 2).

The results of RAPD testing on the type strains of *Y. pseudotuberculosis* with four different primers (Fig. 2) prompted us to evaluate its feasibility in an epidemiological study. To do this, we used 30 clinical isolates of *Y. pseudotuberculosis* from an outbreak. The illness was associated with symptoms characteristic of atypical scarlet fever, called Izumi fever in Japan, and has sporadically broken out in Japan (22). All of the isolates were shown to belong to serotype 5a and had uniform large-plasmid BamHI digestion patterns, indicating that the outbreak originated from a single source (19). Analysis of the RAPD arrays from the 30 isolates with each of the four primers (AP44, AP45, AP46, and AP47) showed that the same patterns, unique to each
primer used, appeared in every case (Fig. 4). These results, taken together, strongly support the view that the 30 isolates associated with the outbreak were derived from a common source and demonstrate that the RAPD tests performed in this study will be useful in epidemiological surveys of Y. pseudotuberculosis.

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