APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1993, p. 3117–3119 Vol. 59, No. 9
0099-2240/93/093117-03$02.00/0

NOTES

Development of a Random Amplification of Polymorphic DNA Typing Method for *Listeria monocytogenes*

L. M. LAWRENCE,[1,2]* J. HARVEY,[2] AND A. GILMOUR[1,2]

Department of Food Science (Food Microbiology), The Queen's University of Belfast,[1] and Department of Agriculture for Northern Ireland, Agriculture and Food Science Centre,[2] Newforge Lane, Belfast BT9 5PX, Northern Ireland, United Kingdom

Received 19 March 1993/Accepted 14 June 1993

The 10-mer primer OPM-01 (5′-GTT GGT GGC T-3′) was used to generate random amplification of polymorphic DNA (RAPD) profiles by polymerase chain reaction for 91 strains of *Listeria monocytogenes* from raw milk, food, and veterinary, medical, and food-environmental sources. The profiles obtained contained 1 to 10 bands within the molecular size range of 0.5 to 5.0 kbp. Reproducibility was enhanced by annealing at low stringency and introducing a 1-min ramp time between annealing and extension temperatures. Thirty-three RAPD profiles were observed, with specific profiles being observed for strains from each source. RAPD profiles allowed discrimination within serogroups, although five RAPD profiles which were not confined to one serotype were found. Within food strains, one RAPD profile was more common than others, suggesting this to be a common type among strains from this source.

Polymerase chain reaction (PCR) amplification of polymorphic DNA sequences using short arbitrary oligonucleotide primers was first described in 1990, when DNA polymorphisms amplified by means of 9- or 10-nucleotide primers were used as genetic markers (12). The use of the method to generate fingerprints for DNA from bacteria and rice (11) and from viral, bacterial, fungal, plant, and animal origins by means of an arbitrary primer as short as 5 nucleotides (3) has also been reported.

The amplification of short arbitrary stretches of total bacterial DNA with an 8-nucleotide primer has been used to produce highly characteristic and complex DNA fingerprints which have been used to distinguish among clinical isolates of *Streptococcus uberis*, *Klebsiella pneumoniae*, and *Escherichia coli* (1). Also, by use of 9-mer primers, *Bacillus thuringiensis* has been identified and differentiated from *Bacillus cereus*, a closely related species (2), thus highlighting the potential of this diagnostic tool for bacterial identification and taxonomy.

Arbitrary products produced by PCR have been shown to detect polymorphisms in DNA from bacterial isolates of the same species, in a study in which a range of *Campylobacter jejuni* isolates were typed by using a randomly designed 10-mer primer (8). Profiles obtained by the random amplification of polymorphic DNA (RAPD) allowed discrimination of strains within given Penner and Lior serotypes. Typing of *Listeria* spp. by this method has also been reported (4, 9). By using a 10-mer primer for a total of 60 strains of *Listeria* which included all seven *Listeria* species and all known serovars, 29 different banding patterns were observed (9). Again, analysis allowed discrimination of strains with the same serotype, indicating the potential use of RAPD as a typing system for *Listeria monocytogenes*. In a comparative study of RAPD analysis and the more conventional method, phage typing, for *L. monocytogenes* (7), it was found that for strains isolated during six different outbreaks of listeriosis, analysis of RAPD profiles by the single 10-mer primer HLWL74 (5′-ACG TAT CTG C-3′) could advantageously replace phage typing.

Czajka et al. (4) also demonstrated the ability to discriminate among *L. monocytogenes* strains of different serotypes with the same 16S rRNA sequences. However, when a group of strains of the same serotype (4b) were examined, they all gave identical RAPD patterns.

In this study, we have developed an alternative protocol for typing *L. monocytogenes*, in which the RAPD profiles obtained by use of the 10-mer oligonucleotide OPM-01 produced simpler and better-resolved patterns than have previously been reported (9) and also allowed discrimination among serotype 4b strains. The feasibility of OPM-01 for RAPD analysis of 91 strains of *L. monocytogenes* is also reported.

The bacterial strains came from a range of sources, which included raw milk (29 strains), food (44 strains), and veterinary (5 strains), medical (6 strains), and food-environmental (5 strains) sources. Two strains (CRA 198 and CRA 433) which were isolated from outbreaks of listeriosis were also included. Serotyping was carried out by the methods of Seeliger and Höhne (10) for some of the isolates, while the remainder were tested against bacteriological listeria O antiserum types 1 and 4 according to the manufacturer's instructions (Difco).

Preliminary studies were carried out with whole-cell lysates as a source of DNA; a series of 10-mer primers (60% G and/or C residues) from commercially available kits (Operon Technology, Alameda, Calif.) were screened for their suitability in generating reproducible fingerprints which allowed discrimination among *L. monocytogenes* strains. When a suitable primer was identified, optimization with respect to the concentrations of primer, target DNA, DNA *Taq* polymerase, $MgCl_2$, and deoxynucleotide triphosphates (dNTPs)

* Corresponding author.

was carried out. Subsequently, optimization of PCR and electrophoresis conditions was carried out. The following protocol has been used successfully to subtype strains of *L. monocytogenes*.

Cells were cultured overnight at 30°C in brain heart infusion (Oxoid; 10 ml), washed in saline (0.9% NaCl [wt/vol]), and pelleted by centrifugation (15 min at 4,000 × *g*) in an MSE Centaur 2 centrifuge, and the supernatant was discarded. Cells were resuspended in sterile distilled water (100 μl) and lysed by heating in a microprocessor-controlled thermal cycler (Perkin-Elmer Cetus) at 100°C for 10 min followed by cooling to 4°C. From this lysate, a 100-fold dilution (in distilled water) was prepared by adding 10 μl of lysate to sterile distilled water (990 μl) and used as a source of DNA in the amplification reaction. An estimated 2.5×10^4 cells were added to the amplification mixture. The 10-mer primer OPM-01 (5′-GTT GGT GGC T-3′) was selected after screening kits OPA and OPM, each containing 20 random-sequence 10-mer primers (Operon Technology), in preliminary experiments and was used throughout the remainder of this study. Fifty-microliter reaction mixtures were prepared, each containing 1.25 U of DNA *Taq* polymerase (Perkin-Elmer Cetus), 10 mM Tris-HCl (pH 8.3) at 25°C, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP, 1.38 μM primer OPM-01, and 2.5 μl of the diluted cell lysate. The reaction mixture was overlaid with liquid paraffin and cycled through the following temperature profile 44 times: 94°C for 1 min, 30°C for 2 min, and 72°C for 2 min, with a 1-min ramp time between the annealing and extension temperatures. This was followed by a final cycle of 94°C for 1 min, 30°C for 2 min, and 72°C for 10 min. Samples were held at 4°C until electrophoresis. Incubation was performed in a DNA Thermocycler (Perkin-Elmer Cetus).

Amplified DNA product (10 μl) was mixed with 5 μl of loading buffer (40% [wt/vol] sucrose in distilled water, 0.25% bromophenol blue) and resolved by electrophoresis on 2.0% agarose gels (agarose NA; Pharmacia Biotech, Ltd.) in Tris-borate buffer (TBE buffer) (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA [pH 8.0]) at 70 V for 4 h with a 200/2.0 power pack (Bio-Rad). Gels were stained with ethidium bromide (1.0 μg ml^{-1}) for 30 min, rinsed in tap water, and photographed under UV transillumination. *Eco*RI- and *Hin*dIII-digested λ DNA was included on the gels as a molecular size standard. Controls which contained no cell lysate were made for each incubation.

The method used to produce cell lysate as a source of DNA in the amplification protocol was standardized, and this allowed reproducible patterns to be obtained (results not shown). The screening of various primers in preliminary experiments was carried out to obtain RAPD profiles containing between 1 and 10 bands within the molecular size range of 0.5 to 5.0 kbp. This was achieved by primer annealing at low stringency (30°C) for 2 min and the introduction of a 1-min ramp time between the annealing and extension temperatures. This resulted in increased efficiency of PCR amplification due to stabilization of the primer during annealing, and therefore the hybrid formed between the primer and template DNA and has been found to overcome variation in the relative intensities of bands larger than 1.5 kbp, which has been reported to be a problem (2). Ramping therefore was found to allow resolution of DNA fragments across a broader molecular weight range with enhanced reproducibility.

With the oligonucleotide OPM-01 used as a primer, 91 strains of *L. monocytogenes* were analyzed. Thirty-three different RAPD profiles, arbitrarily designated I through XXXIII, were obtained for this group of strains (Table 1), and an example of each profile is shown in Fig. 1. Control assays in which no cell lysate was added yielded no detectable amplified product.

TABLE 1. Serotypes and RAPD types of *L. monocytogenes* determined with primer OPM-01

Strain source and no.	Serotype	RAPD type
Raw milk		
787, 945	1/2a	I
44, 554, 1035, 472, 1632, 1757	1/2a	II
1496, 1612	1/2a	III
792, 950	1/2b	II
60, 1351	1/2b	III
1750	1/2b	IV
1371	1/2c	II
876, 955, 1240	1/2c	III
1041, 2068	1/2	II
1802	1/2	V
2034, 1807, 2071	1/2	III
242, 481	4b	VI
1040	4b	V
566	4b	VII
Food		
2576, 2745	1/2a	VIII
2582	1/2a	IX
2433	1/2a	X
2708, 2922, 4645, 4714, 4136, 4823, 2940	1/2	VIII
3023	1/2	XI
3677	1/2	XII
2876, 4826, 4832, 4616, 3952, 3303, 4746, 4396, 4777, 2620, 2996, 3371	1/2	X
3536	1/2	XIII
4279	1/2	XIV
3275, 3571, 3848	1/2	XV
5093	1/2	XVI
3884	1/2	XVII
4894	1/2	XVIII
4168	1/2	XIX
5083	1/2	XX
4055	1/2	XXI
2723	4b	XXII
3736	4b	XXIII
CRA 198	4b	XXIV
CRA 433	4b	XXV
3141, 3456, 4580, 4880, 5156	4	XXVI
3780	4	VIII
Veterinary sources		
4426	1/2a	IX
4973	1/2a	XXVII
4678	1/2a	X
4979	4b	XXII
4974	4b	XXVIII
Medical sources		
21	1/2a	XVII
20	1/2b	XXIX
22	1/2c	VIII
23	4b	XXX
24	4b	XXXI
25	4b	XXII
Food-environmental sources		
26, 29	1/2a	X
28, 30	1/2a	XXXII
27	1/2c	XXXIII

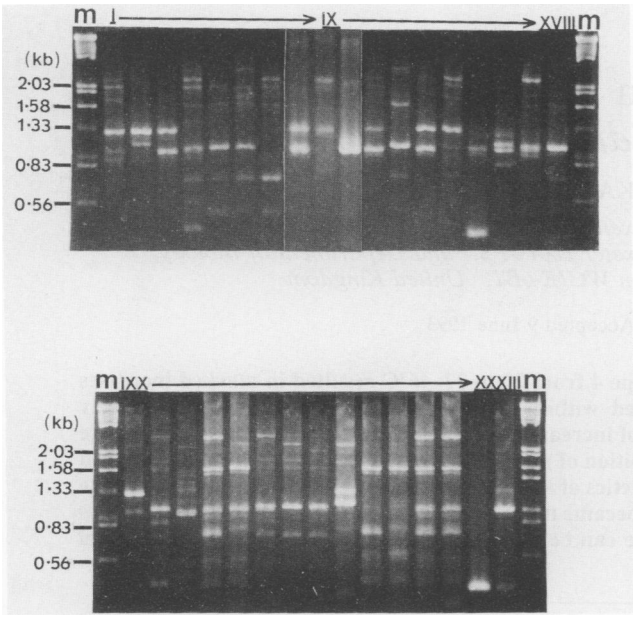

FIG. 1. RAPD profiles determined with primer OPM-01 for 91 strains of *L. monocytogenes*. Lanes I through XXXIII, RAPD types I through XXXIII, respectively; lanes m, molecular size standards (in kilobase pairs).

Within the group of 29 *L. monocytogenes* strains isolated from raw milk (5), seven RAPD profiles were observed (I through VII), and these profiles were specific to isolates from this source. Within the group of 44 *L. monocytogenes* strains isolated from various foods (6), 19 RAPD profiles were observed (VIII through XXVI), with 15 of these profiles specific to isolates from this group. Also within the food group, RAPD profile X (lane X in Fig. 1) was observed for 13 of the strains, suggesting that this is a common type among food strains. *L. monocytogenes* isolates from veterinary (five strains), medical (six strains), and food-environmental (five strains) sources yielded five, six, and three RAPD profiles, respectively, with two profiles specific to veterinary strains (XXVII and XXVIII), three to medical strains (XXIX through XXXI), and two to food-environmental strains (XXXII and XXXIII).

RAPD analysis allowed discrimination among isolates of the same serotype and also among isolates from a common source. However, identical RAPD profiles were also observed for some isolates distinguishable by serotyping; e.g., RAPD type II was produced by strains of serotypes 1/2a, 1/2b, and 1/2c, as was RAPD type III. This supports earlier studies (9) in which identical RAPD profiles for *Listeria* isolates of different serotypes were also found. Within serogroup 1/2 (72 strains), 23 RAPD profiles were observed; 12 RAPD profiles were obtained for serogroup 4 (19 strains). Two RAPD types (V and VIII), however, were found in both serogroups 1/2 and 4. The profiles obtained for serogroup 4 strains contained a number of bands of similar sizes, e.g., profiles XXII, XXIII, XXX, and XXXI, and this kind of relationship between RAPD profile and serotype has also been reported in the analysis of *B. thuringiensis* (2), of which strains belonging to the same serovar also produced closely related banding patterns. In *L. monocytogenes*, this may be due to the homogenous nature of serogroup 4 strains and may help to explain the findings of Czajka et al. (4), who found a lack of discrimination among serotype 4b strains by RAPD. Since in the present study there was discrimination among 4b strains isolated from the same source, it appears that the primer OPM-01 and the conditions outlined above offer a typing method which is more discriminatory than that previously described (4). There were, however, five RAPD profiles produced by strains of more than one serotype (profiles II, III, V, VIII, and XXII), and this would suggest that the regions of DNA amplified in the PCR are not serotype specific.

RAPD using primer OPM-01 has been found to be a rapid and valuable technique for typing *L. monocytogenes*. It has certain advantages over more-traditional typing techniques such as serotyping, because RAPD offers greater discrimination of strains. However, in some cases serotyping allowed discrimination within a particular RAPD type, so, used in combination, serotyping and RAPD offer a higher level of differentiation than either method used alone. In comparison with other molecular techniques for typing *L. monocytogenes*, such as restriction endonuclease analysis and restriction fragment length polymorphism, RAPD is more rapid and less labor-intensive, eliminates the need for pure DNA, and allows better discrimination within serogroup 4 strains.

L. M. Lawrence is currently in receipt of a Department of Agriculture for Northern Ireland postgraduate studentship.

REFERENCES

1. **Bassam, B. J., G. Caetano-Anollés, and P. M. Gresshoff.** 1992. DNA amplification fingerprinting of bacteria. Appl. Microbiol. Biotechnol. **38:**70–76.
2. **Brousseau, R., A. Saint-Onge, G. Préfontaine, L. Masson, and J. Cabana.** 1993. Arbitrary primer polymerase chain reaction, a powerful method to identify *Bacillus thuringiensis* serovars and strains. Appl. Environ. Microbiol. **59:**114–119.
3. **Caetano-Anollés, G., B. J. Bassam, and P. M. Gresshoff.** 1991. DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. Bio/Technology **9:**553–557.
4. **Czajka, J., N. Bsat, M. Piani, W. Russ, K. Sultana, M. Wiedmann, R. Whitaker, and C. A. Batt.** 1993. Differentiation of *Listeria monocytogenes* and *Listeria innocua* by 16S rRNA genes and intraspecies discrimination of *Listeria monocytogenes* strains by random amplified polymorphic DNA polymorphisms. Appl. Environ. Microbiol. **59:**304–308.
5. **Harvey, J., and A. Gilmour.** 1992. Occurrence of *Listeria* species in raw milk and dairy products produced in Northern Ireland. J. Appl. Bacteriol. **72:**119–125.
6. **Harvey, J., and A. Gilmour.** Occurrence and characteristics of *Listeria* in foods produced in Northern Ireland. Int. J. Food Microbiol., in press.
7. **Mazurier, S., A. Audurier, N. Marquet-van der Mee, S. Notermans, and K. Wernars.** 1992. A comparative study of randomly amplified polymorphic DNA analysis and conventional phage typing for epidemiological studies of *Listeria monocytogenes* isolates. Res. Microbiol. **143:**507–512.
8. **Mazurier, S., A. Van de Giessen, K. Heuvelman, and K. Wernars.** 1992. RAPD analysis of *Campylobacter* isolates: DNA fingerprinting without the need to purify DNA. Lett. Appl. Microbiol. **14:**260–262.
9. **Mazurier, S., and K. Wernars.** 1992. Typing of *Listeria* strains by random amplification of polymorphic DNA. Res. Microbiol. **143:**499–505.
10. **Seeliger, H. P. R., and K. Höhne.** 1979. Serogrouping of *Listeria monocytogenes*. Methods microbiol. **13:**31–49.
11. **Welsh, J., and M. McClelland.** 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. **18:**7213–7218.
12. **Williams, J. G. K., A. R. Kubelik, K. J. Livak, J. A. Rafalski, and S. V. Tingey.** 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. **18:**6531–6535.