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(54) Title: DETECTION ASSAY FOR *LISTERIA* AND *ERWINIA* MICROORGANISMS

(57) Abstract

A polymerase chain reaction-coupled ligase chain reaction is described for the identification of microorganisms based upon single-base-pair differences in the genetic information of each organism.

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Detection assay for Listeria and Erwinia microorganisms.

At the present time, many microorganisms can be assayed and identified only by time-consuming culture procedures. In many instances these microorganisms require a two-step protocol: an initial isolation and identification of the microorganism based upon characteristics of the genera, and a second isolation based upon the characteristics of the species within the genera. Only then can it be determined whether the species isolated is pathogenic.

Various techniques for detecting the presence of microorganisms in samples have been developed prior to the making of the present invention. The latest of these techniques have used either monoclonal antibodies or nucleic acid probes, or nucleic acid probes in conjunction with the polymerase chain reaction (PCR). The assay, according to the present invention, provides an alternative to these techniques.

In one aspect of the present invention, there is thus described an assay for determining the presence of *Listeria monocytogenes* in a clinical sample suspected of containing this organism. *L. monocytogenes* is a Gram-positive facultative anaerobe bacteria. It is ubiquitous in nature and has been linked to food-borne illness outbreaks involving a wide range of foods. The presence of *Listeria monocytogenes* in a prepared food products can result from contamination originating in food ingredients as well as during the processing or post-processing period in the food plant. It is known that once *Listeria monocytogenes* contaminates a food-processing plant, it can survive for a long time, if the temperature is low and the organism is protected by food components. The control of *Listeria monocytogenes* contamination involves the use of basic quality assurance systems such as the hazard analysis and critical control point (HACCP). Environmental and in-line samplings can play an important role in identifying problem areas and revealing plant conditions that may contribute to hygienic problems . An added emphasis on determining new and practical methods of pathogen control and detection in food plants is necessary in order to ensure safe food processing conditions .

Specific detection of *Listeria monocytogenes* is very important with regards to food hygiene and safety, since all other species within the genus *Listeria* appear to be non-pathogenic to humans. Conventional microbiological detection methods for *Listeria monocytogenes* are very time consuming and can take up to four weeks until final results are obtained. A variety of detection methods for *Listeria monocytogenes* based on nucleic acid probes or monoclonal antibodies have been described [see "Rapid methods for the detection of Listeria" in Molecular Approaches To Improving Food Quality and Safety, Bhatnagar and Cleveland (ed.) pgs 189-204, Van Norstand Reinhold (New York) (1992)]. Furthermore, various detection systems using the polymerase chain reaction (PCR) for specific detection of *Listeria monocytogenes* have been reported [see Appl. Environ. Microbiol. 56:2930 (1990); Appl. Environ. Microbiol. 58:2625 (1992); Appl. Environ. Microbiol. 57:606 (1991); J. Appl. Bacteriol. 73:53 (1992); J. Appl. Bacteriol. 70:372 (1991); Appl. Environ. Microbiol. 57:2576 (1991); and Appl. Environ. Microbiol 58:1564 (1992)]. Most of these methods have been applied to either pure bacterial cultures or food samples, to our knowledge there has only been one report describing the.use of a PCR assay for the detection of *Listeria monocytogenes* from surface samples. In the following description of the assay according to the present invention, it will be shown that discrimination between the closely related *L. innocua* (and a large number of other *Listeria* species) and *L. monocytogenes* may be readily accomplished

The selective detection of *Listeria monocytogenes* is of great importance, as the ecology of *Listeria* spp. is not well understood and the detection of *Listeria* spp. other than *Listeria monocytogenes* may not be an accurate indicator of a foods safety [see Food Microbiol. 6:49 (1989)].

The use of 16S rRNA as a distinct signature for bacteria has become the method of choice for identifying and differentiating microorganisms when no other nucleic acid sequence constitutes a unique desired target [see Microbiol. Rev. 51:221 (1987)]. A DNA probe based upon a 16S rRNA sequence (rDNA), which detects all *Listeria* spp., has been developed, although the sequences that discriminate it from other genera were not reported [see J. Assoc.. Off. Anal. Chem. 7:669 (1988)] Also, a 16S rRNA-based oligonucleotide probe specific for *L. monocytogenes* has been designed and was used in a hybridization assay [see Appl. Environ. Microbiol. 57:3666 (1991)].

The 16S rRNA nucleotide sequence from a number of *Listeria* spp. has been determined either after reverse transcription or from PCR-amplified genomic rDNA, and differences in single base pairs between *Listeria monocytogenes* and *Listeria innocua* have been described for the V2 and V9 regions [see Int. J. Syst. Bacteriol. 41:240 (1991)].

The ligase chain reaction (LCR) has been shown to be a highly sensitive and specific method for discriminating between DNA sequences differing in only a single base pair [see Proc. Natl. Acad. Sci. USA 88:189 (1991), the disclosure of which is incorporated, *in toto,* herein]. LCR is based on the principle of ligation of two adjacent synthetic oligonucleotide primers which hybridize uniquely to one strand of the target DNA. The junction of the two primers is positioned so that the nucleotide at the 3' end of the upstream primer coincides with a potential single-base-pair difference in the targeted sequence, which defines different alleles or species. That is, two pairs of primers, each pair adjacent to each other on one strand each of target DNA, are designed so that the 3' end of each upstream primer terminates at a nucleotide that is complementary in the targeted DNA but is mismatched in the DNA of closely related targets. If the base pair at that site matches the nucleotides at the 3' end of the upstream primer, the two adjoining primers can be covalently joined by the ligase. In the LCR, a second pair of primers complementary to the first pair are present, again with the nucleotide at the 3' end of the upstream primer denoting the sequence difference. In a cycling reaction with a thermostable *Thermus aquaticus* DNA ligase, the ligated product can then serve as a template for the next and subsequent reaction cycles leading to an exponential amplification process analogous to PCR amplification. If there is a mismatch at the primer junction, it will be discriminated by the thermostable ligase, and the primers will not be ligated. The absence of the ligated product therefore indicates at least a single-base-pair change in the target sequence. This was demonstrated by using LCR to discriminate between normal b^A and sickle b^S-globin genotypes in humans.

In brief, the first aspect of the present invention provides for an assay utilizing PCR primers that specifically amplify the 16S rDNA of all *Listeria* species present in the sample for the first step of the assay procedure, whereas the use of the LCR as the second step of the assay procedure is used to specifically confirm the presence of *L. monocytogenes.*

In still another aspect of the present invention there is described the use of a polymerase chain reaction-coupled ligase chain reaction assay to differentiate the plant pathogenic bacterium *Erwinia stewartii* from other *Erwiniae* species. This second aspect is essentially the same protocol as used in the first, but with different genera of bacteria.

Stewart's bacterial wilt is a disease of corn (*Zea mays* L.) caused by the bacterium *Erwinia stewartii* (Smith) Dye. On three to five leaf stage corn seedlings, the disease is characterized by water soaked lesions on leaves leading to stunted plants, with severe yield reductions in susceptible and moderately susceptible corn hybrids. The Corn flea beetle, *Chaetocnema pulicaria* Melsh., is the overwintering host and vector of *E. stewartii*, and the abundance of primary inoculum is related to the population of this beetle.

Because of its sporadic nature, the disease has not been thoroughly researched, and little is known about the distribution and prevalence of *E. stewartii* strains, the role of symptomless carriers, and prevalence of seed transmission of the bacteria. The potential risk of seek transmission is so important in international shipment of seed, that more than 50 countries ban import of seed corn unless certified to be free of *E. stewartii.* Usually seed is certified by field inspection or by laboratory isolation of the pathogen on general or selective media.

Since neither of these methods is very sensitive, a double sandwich enzyme linked immunosorbent assay (ELISA) was recently developed to detect low levels of *E. stewartii* in seed. This assay uses polyclonal antibodies for capture and monoclonal antibodies for detection, and has a detection limit of 10^5 cells per sample [see Phytopathology 81:839 (1991)].

Accordingly, to facilitate the understanding of the epidemiology of Stewart's wilt and to improve methods for detecting the pathogen, a species-specific nucleic acid probe (which in an enzymatic amplification format was believed to be able to decrease detection limits while retaining specificity and utility engendered in ELISA) was developed in accordance with the present invention to allow for the detection of *E. stewartii* directly from plant and vector material.

The rationale for this aspect of the present invention is the same as given before, specifically that the 16S rDNA is generally phylogenetically conserved, although limited variable regions, within rDNA constitute a distinct signature for bacterial species, and because of this the 16S rDNA has become the preferred method for identifying microorganisms when no other unique nucleic acid sequences are known to serve as potential targets.

In this aspect of the present invention, the 16S rDNA of *Erwinia stewartii* and *Erwinia herbicola* was amplified, coned and sequenced. Differences found between these two closely related species were used to design LCR primers that were successful in differentiating the corm pathogen from the other members of the genera.

These and other aspects of the present invention can be more fully understood by reference to the following examples and detailed discussion of the findings made with the assay according to the present invention.

EXAMPLE I

This example describes the preparation of genomic DNA and PCR of Listeria 16S rDNA according to one aspect of the present invention.

Listeriae were grown in 5 ml of *Listeria* enrichment broth (BBL, Becton Dickinson Microbiology Systems) at 37° C on a shaker at 150 rpm. Cells from an 8-h culture (approx. 10^9 cells) of *Listeria* spp. were pelleted, resuspended in 100 µl of distilled water, and then placed in a boiling-water bath for 10 min. a 1-µl aliquot of this lysate was used for PCR amplification of the V2 region with two primers for the 16S rDNA [see J. Bacteriol. 173:697 (1991)], 16S-5 (5'-CCGAATTCGTCGACAACAGAGTTTGATCATGG-3') and mp19-INT (5'-CGGAAACCCCCTAACACTTA-3'), or for amplification of the V9 region with two universal primers for the 16S rDNA [see J. Bacteriol. 173:697 (1991)], 16-3 (5'-CCCGGGATCCAAGCTTTAACCTTGTTACGACTT-3') AND MP-18 INT (5'-ATTAGATACCCTGGTAGTCC-3'). The amplified DNA was phenol-chloroform extracted, ethanol precipitated, and dissolved in TE buffer (10 mM Tris-HCL, 1 mM EDTA [pH 8.0]) by conventional procedures [see Maniatis et al, Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor (1989)]. Alternatively, the amplified DNA was used directly in the LCR. These preparations were analyzed for the presence of the expected 16S rDNA fragment of 840 bp for the V2 region or of 720 bp for the V9 region by electrophoresis on a 1.5% agarose gel in TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.2). The DNA concentration was estimated by visual comparison with a standard DNA preparation.

Specific nucleotide primers for use in both the LCR and LDR Primers for LCR and LDR were prepared following conventional solid state procedures. For the LCR, two sets (Lm 1, Lm 2R, Lm 3, and Lm 4R for the V2 region, and Lm 1a, Lm 2Ra, Lm 3a, and Lm 4Ra for the V9 region) of four different primers were used. The primer sequences are shown in the following Table 1:

TABLE 1.

Sequences and melting temperatures of primers used in the LCR

Primer	Sequences (5'-> 3')	Tm (° C)
V2 region-specific pairs:		
Lm 1 (labeled)	AGTGTGGCGC ATGCCAGCTC TTTTGA 26	78
Lm 3	GGACCGGGGC TAATACCGAA TGATAA 26	70
Lm 2R (labeled)	TATCATTCGG TATTAGCCCC GGTTT 25	72
Lm 4R	GGAAAAGCGT GGCATGCGCC ACACTT 26	76
V9 region-specific pairs:		
Lm 1a (labeled)	GTACAAAGGG TCGCGAAGCC G 21	68
Lm 3a	AAGCTACACA CGTGCTACAA TGGATA 26	70
Lm 2Ra (labeled)	ATCCATTGTA GCACGTGTGT AGC 23	68
LM 4Ra	AACGGCTTCG CGACCCTTTG TACT 24	70

The nucleotide sequence of the V2 region site in *L. monocytogenes* and in *L. innocua* used for LCR and locations of primers Lm 1a, Lm 3, and Lm 4R are depicted in the following Table 2. In this sequence, oligonucleotides Lm 3 and Lm 4R contained 2-nucleotide tails to prevent ligation of the 3'-end. The designation "X" in this sequence listing mark differences between L. monocytogenes and *L. innocua*; nucleotides that are identical in *L. monocytogenes* and *L. innocua* are shown as colons.

TABLE 2

L. monocytogenes:

```
              Lm 3
    GG________________________A             Lm 1
                               A_________________________
5' GGAAACCGGGGCTAATACCGAATGATAAAGTGTGGCGCATGCCACGCTTTTGAA
3' CCTTTGGCCCCGATTATGGCTTACTATTTCACACCGCGTACGGTGCGAAAACTT
    _________________________T
          Lm 2R                 T______________________GG
                                          Lm 4R
```

L. innocua:

```
   :::::::::::::::::::::::::::X::::::::::::::::::::X:::::
5' GGAAACCGGGGCTAATACCGAATGATAGAGTGTGGCGCATGCCACGCTCTTGAA
                              -------------V2-------------
```

The nucleotide sequence of the V9 region site in *L. monocytogenes* and in *L. innocua* used for LCR and locations of primers Lm 1a, Lm 2Ra, Lm 3a, and Lm 4Ra are depicted in the following table 3. Oligonucleotides Lm 3a and Lm 4Ra contained 2-nucleotide tails to prevent ligation of the 3' end.

TABLE 3

L. monocytogenes:

```
              Lm 3a
    AA________________________A             Lm 1a
                               G_____________________
5' CTGGGCTACACACGTGCTACAATGGATAGTACAAAGGGTCGCGAAGCCGC
3' GACCCGATGTGTGCACGATGTTACCTATCATGTTTCCCAGCGCTTCGGCG
    _________________________A
          Lm 2Ra                T___________________AA
                                          Lm 4Ra
```

L. innocua:

```
   :::::::::::::::::::::::::::X::::::::::::::::::::::
5' CTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGTCGCGAAGCCGC
                              -------------V9---------
```

For the ligase detection reaction (LDR) of the V9 region, either the primer pair Lm 1a plus Lm 3a or the pair Lm 2Ra plus Lm 4Ra was used.

Primers Lm 1, 1a, 2R, and 2Ra were 5'-end labeled with [g-^{32}P]ATP (Amersham) as described previously [see Proc. Natl. Acad. Sci. USA 88:189 (1991)].

The LCR and LDR were performed under conditions as described previously [see Proc. Natl. Acad. Sci. USA 88:189 (1991) and Clin. Chem. 37:1522 (1991)] with minor modifications.

The Listeria strains used in the LCR assay according to the present invention are described in the following Table 4 in which information about the serotypes was provided from the laboratories that provided strains and in which ND indicates that serotype was not determined and NT indicates serotype was not typeable. In the table, USDA represents the United States Department of Agriculture, FDA represents the United States Food and Drug Administration, ERRC represents the Eastern Regional Research Center,

TABLE 4

Species and Strain	Serotype	Source/Reference	LCR Product
L. monocytogenes:			
A/F2380	ND	USDA-ERRC	+
C/ATCC 15313	NT	USDA-ERRC	+
D/LCDC91-329	1/2a	USDA-ERRC	+
E	ND	USDA-ERRC	+
SLCC 53	1/2a	Univ. Vermont	+
SLCC2371	1/2a	Univ. Vermont	+
SLC 5779	1/2a	Univ. Vermont	+
ATCC 19112	1/2c	Univ. Vermont	+
Scott A	4b	Univ. Minnesota	+
L. innocua:			
SA 3 VT	ND	USDA	-
SH3V	ND	Univ. Minnesota	-
P5V5	ND	Univ. Minnesota	-
L. ivanovii:			
KC 1714	ND	USDA	-
L 29/R1-7	ND	Univ. Vermont/FDA	-
L 30/R1-8	ND	Univ. Vermont/FDA	-
L. seeligeri:			
	ND	Univ. Minnesota	-
L. welshimeri:			
H2.V.G.	ND	USDA	-
CCK9LG	ND	Univ. Minnesota	-
L. grayi:			
ATCC 19120/KC 1773	ND	USDA	-

A positive LCR product reaction indicates an LCR product on a denaturing polyacrylamide gel after 25 LCR cycles, as detected by autoradiograph exposure of 12 hours.

EXAMPLE II

One set of primers (50 fmol of each primer) were incubated in the presence of target 16S rDNA (5 fmol) in 50 µl of reaction buffer (50 mM Tris-HCL, 100 mM KCL, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM dithiothreitol, 1 mM NAD^+, 0.01% Triton X-100, 20 µg of salmon sperm DNA) with 75 nick-closing units of *T. aquaticus* DNA ligase (purified as described in Gene 109:1 (1991)), and overlaid with 50 µl of mineral oil. The reaction cycle was 1 min at 94°C followed by 4 min at 65°C, and this cycle was repeated 25 times. Annealing temperatures of 70 and 74°C were also tested in the LCR performed with the primers for the V2 region. The reaction was stopped by adding 40 µl of formamide containing 10 mM EDTA, 0.2% bromophenol blue, and 0.2% xylene cyanole. Alternatively, bacterial lysates (prepared as described above) were also used directly in the LCR.

As described above, only a single pair of primers was used in conducting the LDR, whereas two pair of primers were used in conducting the LCR.

To evaluate the overall sensitivity of the PCR-coupled LCR, serial dilutions of an 8-h *L. monocytogenes* culture were used to prepare boiled lysates and tested with the primers for the V9 region. These samples were analyzed for the PCR product after PCR amplification, and then 5 µl of the PCR product was used for the second LCR step.

EXAMPLE III

Electrophoresis was conducted as follows:

LCR samples were heated to 90° C for 5 min, and 10 µl was loaded onto a 16% polyacrylamide minigel (8 by 7.3 cm in Mini-PROTEAN II Electrophoresis Cell; Bio-Rad) containing 7 M urea. Electrophoresis was carried out in TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.2) at 175 V constant voltage for 1 hour. Gels were autoradiographed on Kodak X-Omat AR film at -20°C for 12 hours.

Results for one aspect of the present invention, that showing the ability of an LCR assay according to the present invention to discriminate different species of Listeria utilizing *L. monocytogenes* and *L. innocua* having previously reported single-base-pair differences at nucleotide 193 (V2 region) and at nucleotide 1259 (V9 region) in the 16S rDNA [see Appl. Environ. Microbiol 57:3666 (19910 and Int. J. Syst. Bacteriol. 41:240 (1991)] are reported below. All other *Listeria* spp. are far more divergent in that region. These two sites, specifically those depicted in Tables 2 and 3, were used to design primers for an LCR assay according to the present invention.

As depicted above, the LCR assay based on those single-base-pair differences according to the present invention was developed which is successful in distinguishing *L. monocytogenes* from other *Listeria* spp. This method was tested on 19 different *L.monocytogenes* strains and other *Listeria* spp. (Table 4), and even species closely related to *L. monocytogenes*, such as *L. innocua*, could be reliably distinguished.

When crude bacterial lysates were used without prior PCR in the LCR assay with the V9 primers, a ligation product was detectable with a 24 hour autoradiogram exposure. PCR amplification of either the V2 or the V9 region of the 16S rDNA was successful in generating DNA fragments which were suitable for use in the LCR. There was no significant difference in sensitivity or specificity whether phenol-chloroform-extracted or unpurified DNA from the PCR step was used for the LCR.

The two complementary pairs of primers designed for the V9 region had a low variation in melting temperature (68 to 70° C). When this set of primers was used in the LCR assay (as well as in LDR), clear differentiation between *L. monocytogenes* and other *Listeria* spp. was possible. For example, examination of representative autoradiographic results of the LCR for 13 *Listeria* spp. with the primers for the V9 region (*L. monocytogenes* strains Scott A; SICC 53; SLCC 5779; ATCC 19112; E; A; D; SLCC 2371; and C; *L. ivanovii* L 30; *L. innocua* SA 3 VT; *L. welshmerii* and *L. seeligeri*) clearly indicated two labeled primers, and upper and lower band, visible on the gel; the lower band being primer Lm 1a (21 nucleotides), and the higher one being primer Lm 2Ra (23 nucleotides). When autoradiographs of the ligated products (primer pairs Lm 1a plus 3a and Lm 2Ra plus 4Ra) only a single band appeared on the autoradiogram.

The addition of 0.01% Triton X-100 to the reaction mixture gave higher yields of ligation product with both LDR and LCR. This was quantified by excising the bands from gels after a 25-cycle LCR and measuring the specific activities of the ligation product and the unligated primers by liquid scintillation counting. Using 5 fmol of target DNA, a slightly higher amount of false-positive ligation was detected when 0.01% Triton X-100 was used than in LCR reactions without Triton X-100.

When 0.01% Triton X-100 was used, approximately 80% of the starting primers were ligated after 25 LCR cycles at a concentration of 5 fmol of target DNA. Since only one pair of primers was used in the LDR, only one band for the unligated labeled primer was seen (primer Lm 2Ra, 23 nucleotides), while the autoradiogram of the LCR, two bands of unligated primers were seen.

When *L. innocua* served as a target in the LCR with the V9 primer set, a very small amount of ligation product was detected only after the gel had been autoradiographed for more than 20 hours. In species distantly related to *L. monocytogenes* (e.g. *L. seeligeri* and *L. grayi*), no ligation product was observed even when the gel was autoradiographed for more than 36 hours.

When different dilutions of target cells were used for the PCR-coupled LCR, small numbers of target cells (150 to 15,000 CFU) gave no detectable PCR product in ethidium bromide-stained gels, but the second LCR step revealed the presence of *L. monocytogenes.*

The LCR primers for the V2 region exhibited a high degree of ligation in the absence of target DNA. Since the melting temperature of those primers ranged from 70 to 78° C, cycles with higher annealing temperatures (70 or 74° C) then originally described were tested. At an annealing temperature of 74° C, the amount of ligated product decreased drastically, not only in the absence but also in the presence of target; however, target-independent ligation products were still observed.

When LCR was first described for the detection of the β^A-globin gene [see Proc. Natl. Acad. Sci. USA 88:189 (1991)], sensitivity was reported to be limited to about 200 target molecules. PCR has been shown to have a much greater sensitivity, although it cannot always discriminate between two targets differing in only a single base pair. For example Bej and co-workers [see Appl. Environ. Microbiol. 56:307 (1990)] were able to detect fewer than 5 coliform bacteria in 100 ml of water by using PCR amplification. The PCR primers for the V2 region allowed the amplification of an 840-bp fragment, whereas the universal PCR primers for the V9 region amplified a 720-bp fragment. In the PCR amplification, no difference was observed in the results obtained with crude bacterial cell lysates and purified DNA.

When using the PCR to amplify the target region for the LCR, it is possible to combine the high sensitivity of the PCR with the high specificity of the LCR. Furthermore, the results of the assay according to the present invention clearly showed that a second LCR step can increase the sensitivity of the PCR step for the detection of *L. monocytogenes* when ethidium bromide staining of the the agarose gels is used to detect the PCR products. One primer of an LCR primer pair can easily be radioactively labeled following conventional protocols, and the ligation product can be detected by gel electrophoresis and subsequent autoradiography of the gel. This approach was shown to be highly sensitive {see Proc. Natl. Acad. Sci. USA 88:189 (1991)], so that even minimal amounts of ligation products could be detected; primers containing a mismatch with the target DNA yielded from <0.2 to 1.3% of the ligation product obtained with perfect complementarity, depending on the target nucleotides involved. As indicated above in Table 3, *L. monocytogenes* has an A-T base pair while *L. innocua* has a G-C base pair at nucleotide 1259 in the V9 target region. Based upon initial findings with G-T and C-A mismatches, a 1.3% background signal with *L. innocua* would be expected.

The inclusion of 0.01% Triton X-100 in the LCR reaction mixture increased LCR efficiency, but also intensified false-positive ligation when 5 fmol of target DNA was used and the gel was exposed for longer than 12 hours, a problem that should be eliminated by using either shorter exposure times or smaller amounts of target DNA.

The use of a PCR-coupled LCR for the specific detection of *L. monocytogenes* may be simplified by using a nonradioactive reporter. For example, the use of biotin, as discussed below for assaying for different species of *Erwinia*, for labeling one primer and of a suitable nonisotopic reporter group for the second primer following conventional protocols would allow product capture and detection in a manner amenable to automation [see for example Science 241:1077 (1988) and Proc. Natl. Acad. Sci. USA 87:8923 (1990)]. The following two examples and discussion describes such nonisotopic procedures for the detection of *L. monocytogenes.*

EXAMPLE IV

The ligase chain reaction was performed with the LCR primers described in Table 1. To facilitate the nonradioactive detection of the LCR products, primers Lm 3a and Lm 4Ra were synthesized with a biotin group at the 5' end by the Biotin-On (Clontech) phosphoramidite method with an Applied Biosystems 392 DNA synthesizer. Primers Lm 1a and Lm 2Ra were phosphorylated at the 5' end with 300 pmol of primer, 20 nmol of ATP, and 6 U of polynucleotide kinase (New England Biolabs) in 30 µl of 1 X polynucleotide kinase buffer at 37° C for 45 min, followed by 68° C for 10 min to inactivate the enzyme. The phosphorylated primers were then 3' labeled with a single 11-ddUPT digoxigen molecule (Boehringer Mannheim). The labeling reaction was performed as recommended by the manufacturer with 85 U of terminal deoxynucleotidyl transferase (US Biochemical).

LCR reactions were performed as described above with minor modifications. Primers Lm 1a, 2Ra, 3a, and 4Ra (100 fmol of each) were incubated with 1µl of PCR-amplified 16S genes coding for rRNA in 25 µl of LCR buffer containing 37.5 nick closing units of *Thermus aquaticus* DNA ligase [see Gene 109:1 (1991)].

EXAMPLE V

For the detection of the LCR products in the microtiter plate format, a modified procedure of Nickerson et al [see Proc. Natl. Acad. Sci. USA 87:8923 (1990)] was used with the following modifications. Five µl of the LCR reaction mixture diluted in 40 µl of buffer 1 (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) together with 10 µl of plate binding buffer (1 M NaCl, 0.75 M NaOH) was used per well. Substrates tested in the microtiter plate detection included Lumi-Phos 530 (Boehringer Mannheim) and p-nitrophenyl phosphate (p-NPP, Sigma). All detection reactions were run in duplicate. For the detection with p-NPP, a 12 hour incubation at ambient temperature was used. The optical density at 450 nm was calculated as the average of the two wells representing the same LCR reaction. In the detection mode with Lumi-Phos 530, a positive LCR result translates into a chemiluminescent reaction that can be seen as a bright dot on Polaroid type 612 film after a 5 min exposure.

Results are considered positive for *L. monocytogenes* when the signal intensity for a wall is clearly higher than the one obtained from the LCR with *L. innocua* PCR-amplified 16S genes coding for rRNA. In the detection with p-NPP, and LCR is considered positive for *L. monocytogenes* when the optical density at 450 nm calculated for the average of two replicates is 2 standard deviations higher than the optical density at 450 nm of *L. innocua* PCR-amplified 16S genes coding for rRNA.

Detection of the LCR products with the luminogenic substrate Lumi-Phos 530 and the chromogenic substrate p-NPP gave equivalent results when an incubation time of 12 hours was chosen for the latter substrate. When compared, however, the detection method with Lumi-Phos 530 is better with respect to the rapidity with which the results of the LCR are obtained; this assay can be finished in 3 hours, whereas both the radioactive assay described in Example III and the nonradioactive detection with p-NPP take more than 12 hours. On the other hand, the detection mode with p-NPP allows a noninstrumental semiquantitative readout of the yellow color.

The PCR-coupled LCR assay according to the present invention as described in Example V was tested on different *Listeria* strains as well as on other bacterial and showed highly specific results. The PCR-coupled LCR consistently allowed the detection of 10 CFU of *L. monocytogenes.*

The PCR-coupled LCR assay according to the present invention as described in the preceding examples can detect the presence of *Listeria* spp in the first PCR step while specifically recognizing *L. monocytogenes* in the second LCR step. This assay will therefor provide information equivalent to that provided by classical microbiological methods. The luminogenic nonisotopic detection method allows one to perform the assay in approximately 10 hours compared with the previously used isotopic detection method which needed almost 24 hours for completion. Since the detection of the LCR products in a microtiter plate with luminogenic or chromogenic substrates is amenable to automation, the present invention will prove useful for screening large amounts of samples, and will facilitate the use of this assay in a routine setting.

EXAMPLE VI

The growth conditions by which Erwinia species used in confirmation of the assay according to the present invention are given below.

Bacteria were stored frozen at -70° C, and cultured at 28° C on tryptone yeast extract phosphate medium consisting of 5 g yeast extract, 10 g Bacto-tryptone, 2.2 g $K_2HPO_4 \cdot 3H_20$, 0.75 g KH_2PO_4, and 18 g bacto-agar per liter of distilled water. Luria-Bertani medium (LB) was used to grow Erwinia for DNA preparations [see Current protocols in molecular biology, Ausubel et al eds., Greene Publishing Associates and Wiley Intersciences (1987)]. Identities of *Erwinia stewartii* strains were confirmed by colonial morphology on tryptone yeast extract phosphate media, oxidative/fermentative reaction, lack of motility, and pathogenicity tests on the susceptible sweet corn cultivar, Jubilee. Other *Erwinia* species used in this study were obtained from stocks kept in the Department of Plant Pathology, Cornell University. All *Erwinia* species and strains used in the making of this invention, their sources, along with the results of the assay according to the present invention are shown in the following table in which ATCC designates American Type Culture Collection, NCPPB designates National Collection of Plant Pathogenic Bacteria.

TABLE 5

Strain	Origin	LCR product
Erwinia stewartii:		
E-s-2	Corn, Cayuga Co., New York	+
E-s-4	Corn, Genesee Co., New York	+
E-s-13	Corn, Genesee Co., New York	+
E-s-20	Corn, Onondaga Co., New York	+
E-s-36b	Corn, Ontario Co., New York	+
E-s-37a	Corn, Yates Co., New York	+
E-s-50b	Corn, Suffolk Co., New York	+
E-s-62	Corn, Wye, Maryland	+
E-s-6	Corn, Illinois	+
Erwinia herbicola:		
E-h-107	Rhododendron, Netherlands	-
E-h-112y	Apple, United Kingdom	-
E-h-252	Apple, New York	-
E-h-262	Tomato, New York	-
E-h-306	unknown	-
E-h-312	New York	-
E-h-331	Canada, ATCC 33243	-
E-h-332	Pineapple, Brazil, ATCC 33244	-
E-h-342	Chevron	-
E-h-345	Chevron	-
E-h-351	Apple, New York	-
Erwinia amylovora:		
E-a-302	Apple, New Zealand, ATCC 15357	-
E-a-304	ATCC 19381	-
E-a-321	Hawthorne, France	-
Erwinia cartovora pv. betavasculorum:		
E-cb	NCPPB 2793	-

A positive LCR product reaction indicates an LCR product on a denaturing polyacrylamide gel after 25 LCR cycles, as detected by autoradiograph exposure of 12 hours.

A nucleotide sequence and alignment of a 120 nucleotide segment of 16S rDNA from E. stewartii 20 and E. herbicola 252 is depicted in the following Table 6. In Table 6, differences between the two bacterial sequences are depicted by "X", matches by ":", and a base that is missing relative to the *E. coli* sequences is indicated by ".". The numbers at the right hand end of the sequence are from alignment with *E. coli* 16S rDNA [see Proc. Natl. Acad. Sci. USA 75:4801 (1978)].

TABLE 6

E. stewartii	CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAA	968
	::	
E. herbicola	CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAA	
E. stewartii	CGCGAAGAACCTTACC.ATCCTTGACATCCAGCGAACTTG	1008
	:::::::::::::::: :::::::::::::::X::::::X	
E. herbicola	CGCGAAGAACCTTACC.ATCCTTGACATCCAGAGAACTTA	
E. stewartii	GCAGAGATGCCTTGGTGCCTTCGGGAACCGTGAGACAGGTG	1049
	::::::::::X::::::::::::::::::::::::::::::	
	GCAGAGATGCTTTGGTGCCTTCGGGAACCGTGAGACAGGTG	

EXAMPLE VII

The amplification, cloning, and sequencing of the 16S rDNA of *Erwinia* was conducted as follows:

Genomic DNA was isolated from *Erwinia stewartii* 20 and *Erwinia herbicola* 252 by the method of Ausubel *supra*, except that the lysates were extracted two times with chloroform to remove residual phenol. PCR amplification and sequencing of the 16S rDNA were carried out utilizing known protocols [see Appl. Environ. Microbiol. 59:304 (1993)], and primers 16S-P5 and 16S-P3, as shown in Table 6, were used to amplify the complete 16S rDNA [see Appl. Environ. Microbiol. 59:304 (1993) and J. Bacteriol. 173:697 (1991)]. Amplification reactions were performed on a Hybaid TRI thermocycler with 2.5 U Taq polymerase (Promega) and 100 ng of genomic DNA for one cycle of 10 minutes at 90° C, 25 cycles of 30 sec. at 94° C, 30 sec at 42° C, and 1 min at 72° C, followed by 1 cycle for 5 min at 72° C,. The fastest transitions between temperatures were used. The resulting PCR product was purified from residual primers and Taq DNA polymerase by phenol/chloroform extraction followed by ethanol precipitation using conventional protocols according to Maniatis, *supra*, and dissolved in distilled water. The resulting 1.5 kb purified PCR product was restricted with SalI and BamHI, and the restricted products were ligated into plasmid M13mp18/19 [see Gene 19:259 (1982)]. Sequences were determined using single-stranded M13mp18/19 template and conventional -40 primer [see Proc. Nat. Acad. Sci. USA 74:5463 (1977)]. A Sequenase kit with 7-deazaGTP (US Biochemicals) was employed for all sequencing reactions in order to resolve compressions that made the sequence difficult to read.

In conducting the amplification procedure, the 1.5 kb amplification product was first restricted with SalI and BamHI for ligation into M13 as described above. However, two fragments of approximately 0.9 and 0.6 kb were observed, and thus the amplification products were digested with each enzyme separately to reveal an unexpected SalI site, rarely found internally within the 16S sequence [see J. Bacteriol. 173:697 (1991)]. When sequenced and aligned to *Escherichia coli* 16S rDNA [see Proc. Natl. Acad. Sci. USA 75:4801 (1978)], the SalI restriction site was found to be 873 base pairs into the 16S rDNA gene of both *E. stewartii* and *E. herbicola* (see Table 6). The 624 bp SalI/BamHII 3' terminal fragment was therefore used in the ligation to M13mp18/19 described above.

Several single-stranded M13 templates were next sequenced for both *E. stewartii* and *E. herbicola*, and sequences were compared between species for differences and within the same species for discrepancies. When approximately 240 base pairs of the 16S rDNA was sequenced, comparisons revealed three base-pair differences in the V3 [see Nucleic Acids Res. 12:5837 (1984)] region, one at nucleotide 1001 (C->A), one at nucleotide 1008 (G->A), and one at nucleotide 1019 (C->T).

TABLE 7

Sequences and Melting Temperatures of LCR and PCR Primers

Primer	Sequence	T(° C)
PCR		
16S-P5	CCGAATTCGTCGACAACAGAGTTTGATCATGG	42
16S-P3	CCCGGGATCCAAGCTTTACCTTGTTACGACTT	46
LCR		
Es-1	GGCAGCGAACTTGGCAGAGATGCC	70
Es-2	TTGGTGCCTTCGGGAACCGTGA	70
Es-3	GCATCTCTGCCAAGTTCGCTGG	70
Es-4	GGCACGGTTCCCGAAGGCACCAAG	72

In the above table, the dissociation temperature (T) was calculated by the method of Suggs et al. [see Developmental Biology Using Purified Genes, Brown and Fox eds., pg 683, Academic Press (1981)].

In the above table it should become readily apparent that LCR primers Es-1 and Es-3 contain the base pair differences at positions 1001 and 1008 noted above, while primers Es-1 and Es-4 contain the single base pair difference at nucleotide 1019, so that, when primers annealed to the target DNA, only primers exactly complementary to the targeted *E. stewartii* DNA would be efficiently bound, ligated and amplified to yield a ligation product.

Two pair of LCR primers (Es-1 and Es-2, and Es-3 and Es-4) were designed to flank a single base pair difference determined by 16S rDNA sequence comparisons of *Erwinia stewartii* and *Erwinia herbicola* according to the following Table 8. More specifically, the LCR primers utilized according to the present invention are depicted with corresponding *E. stewartii* target sequences. The "*" indicates a ^{32}P ATP label (Amersham) produced using polynucleotide kinase [see Proc. Natl. Acad. Sci. USA 88:189 (1991)], and for each primer, only the two end bases are depicted. The dashed line represents the corresponding sequence on the *E. stewartii* target DNA; and primers Es1 and Es4 have a GG tail on their 5' ends.

TABLE 8

```
                                         Es2
                Es1         *TT------------------GA
     GGCA-------------------CC
5' CATCCAGCGAACTTGGCAGAGATGCCTTGGTGCCTTCGGGAACCGTGAGA 3'
3' GTAGGTCGCTTGAACCGTCTCTACGGAACCACGGAAGCCCTTGGCACTCT 5'
      GG-------------------CG*
            Es3              GA------------------ACGG
                                         Es4
```

In the primers depicted in Table 8, on the 5' end of Es1 and Es4, two nucleotide tails (GG) have been added to prevent ligation of the 3' ends [see PCR Methods Applications 1:5 (1991)]. Primer size was selected so that the primers would have a melting temperature of 70±2° C.

EXAMPLE VIII

Minipreparations of Genomic DNA from *Erwinia* isolates were prepared according to the methods of Ausubel *supra.* DNA concentration was determined by measuring the absorbance at 260 nm and 100 ng of DNA was used in the PCR. Alternatively, crude lysis of the bacterial cells could be (and was) performed to recover DNA. *Erwiniae* were grown overnight in 5 ml of LB medium at 28°C on a shaker at 150 rpm. Cells from the overnight cultures were pelleted, resuspended in 95 µl of IX PCR buffer (Promega), and lysed by incubation with 4 µl of lysozyme (50 mg/ml) for 15 min at ambient temperature. One µl proteinase K (20 mg/ml) was then added and the lysate was incubated for 1 hour at 60° C. The lysate was divided into two 50 ml aliquots and boiled for 20 minutes. A total of 5 µl of the boiled lysate was used for PCR.

EXAMPLE IX

The PCR/LCR condition and PCR amplifications conditions for the PCR-coupled LCR were: 1 cycle of 4 min at 92° C, followed by 25 cycles of 1 min at 94° C, 1.5 min at 50° C, 1.5 min at 72° C, and a final extension of 6 min at 72° C. Any residual Taq DNA polymerase activity was eliminated by one cycle of 25 min at 97° C. PCR product reactions were observed on 1.5% agarose gels after electrophoresis ant ethidium bromide staining as described above. LCR was performed as described in the literature [see Appl. Environ. Microbiol. 58:3443 (1992)]. Optimal reaction conditions were 25 cycles of 1 min at 94° C, followed by 4 min at 65° C. Electrophoresis of the resulting LCR products was carried out as described in Example III.

The two stage assay (an initial PCR amplification followed by LCR) according to the present invention was successful in distinguishing E. stewartii from other Erwinia species using bacterial genomic DNA. Whether the genomic DNA was from a minipreparation (either purified or unpurified following PCR) or from a crude lysate made no significant difference in the LCR assay. In a typical autoradiograph after LCR and gel electrophoresis, two bands were seen in each lane: an upper band corresponding to the 46 base pair ligation product, and a lower band corresponding to unligated radiolabelled primers, allowing for a clear distinction between *E. stewartii* and *E. herbicola* to be seen. Other *Erwinia* spp. when tested produced similar results to *E. herbicola*, ie, no, or only a trace background, LCR product was observed.

Thus, PCR-coupled LCR has been shown to be a highly sensitive and specific method to detect and identify bacteria, and once detected to distinguish one species or strain from another. PCR enhances sensitivity by amplifying the target DNA region, and LCR provides the specificity since a positive reaction is only observed upon ligation of primer pairs with the correct 3' and 5' ends. The sequences of the LCR primers for detection of bacteria are preferably derived from the 16S rDNA region (which as a general rule is phylogenetically conserved among bacteria) of the bacteria genome. While variable segments in the 16S rDNA are highly but not completely homologous among closely related bacteria, they diverge sufficiently among distantly related bacteria so that discrimination of closely related bacterial virtually ensures discrimination of less closely related bacteria. Thus, the present invention is applicable for all bacterial assays, not just for assays regarding the specific *Listeria* and *Erwinia* species described herein.

As described herein, an advantages of the use of PCR-coupled LCR according to the present invention is that even if the target sequence for the LCR can only be amplified to the attomolar range (because of competition from other bacteria in the original sample, whose 16 rDNA is also amplified by the universal PCR primers), this amount of DNA is still sufficient to be detected by LCR. Thus, the assay system described herein provides a highly sensitive and specific means to identify and distinguish bacteria species and strains, one from the other.

A complete listing of the nucleotide sequences described herein is provided as follows:

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Carl A. Batt, Martin Wiedmann and Francis Barany

(ii) TITLE OF INVENTION: Assay For microorganisms By Polymerase Chain Reaction-Coupled Ligase Chain Reaction

(iii) NUMBER OF SEQUENCES: 28

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCGAATTCGT CGACAACAGA GTTTGATCAT GG 32

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGGAAACCCC CTAACACTTA 20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCCGGGATCC AAGCTTTAAC CTTGTTACGA CTT 33

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATTAGATACC CTGGTAGTCC 20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5

AGTGTGGCGC ATGCCAGCTC TTTTGA 26

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGACCGGGGC TAATACCGAA TGATAA 26

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TATCATTCGG TATTAGCCCC GGTTT 25

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGAAAAGCGT GGCATGCGCC ACACTT 26

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9

GTACAAAGGG TCGCGAAGCC G 21

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AAGCTACACA CGTGCTACAA TGGATA 26

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATCCATTGTA GCACGTGTGT AGC 23

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AACGGCTTCG CGACCCTTTG TACT 24

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGAAACCGGG GCTAATACCG AATGATAAAG TGTGGCGCAT .40
GCCACGCTTT TGAA 54

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TTCAAAAGCG TGGCATGCGC CACACTTTAT CATTCGGTAT 40
TAGCCCCGGT TTCC 54

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGAAACCGGG GCTAATACCG AATGATAGAG TGTGGCGCAT 40
GCCACGCTCT TGAA 54

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CTGGGCTACA CACGTGCTAC AATGGATAGT ACAAAGGGTC 40
GCGAAGCCGC 50

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCGGCTTCGC GACCCTTTGT ACTATCCATT GTAGCACGTG 40
TGTAGCCCAG 50

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:
CTGGGCTACA CACGTGCTAC AATGGATGGT ACAAAGGGTC 40
GCGAAGCCGC 50

(2) INFORMATION FOR SEQ ID NO:19:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 120 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:
CCCGCACAAG CGGTGGAGCA TGTGGTTTAA TTCGATGCAA 40
CGCGAAGAAC CTTACCATCC TTGACATCCA GCGAACTTGG 80
CAGAGATGCC TTGGTGCCTT CGGGAACCGT GAGACAGGTG 120

(2) INFORMATION FOR SEQ ID NO:20:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 120 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20
CCCGCACAAG CGGTGGAGCA TGTGGTTTAA TTCGATGCAA 40
CGCGAAGAAC CTTACCATCC TTGACATCCA GAGAACTTAG 80
CAGAGATGCT TTGGTGCCTT CGGGAACCGT GAGACAGGTG 120

(2) INFORMATION FOR SEQ ID NO:21:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:
CCGAATTCGT CGACAACAGA GTTTGATCAT GG 32

(2) INFORMATION FOR SEQ ID NO:22:
(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CCCGGGATCC AAGCTTTACC TTGTTACGAC TT 32

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GGCAGCGAAC TTGGCAGAGA TGCC 24

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TTGGTGCCTT CGGGAACCGT GA 22

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2111 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GCATCTCTGC CAAGTTCGCT GG 22

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:
GGCACGGTTC CCGAAGGCAC CAAG 24

(2) INFORMATION FOR SEQ ID NO:27:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:
CATCCAGCGA ACTTGGCAGA GATGCCTTGG TGCCTTCGGG 40
AACCGTGAGA 50

(2) INFORMATION FOR SEQ ID NO:28:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:
TCTCACGGTT CCCGAAGGCA CCAAGGCATC TCTGCCAAGT 40
TCGCTGGATG 50

While we have illustrated and described preferred embodiments of our invention, it is to be understood that this invention is capable of variation and modification, and we therefore do not wish to be limited to the precise terms set forth, but desire to avail ourselves of such changes and alterations which may be made for adapting the invention to various usages and conditions. For example, it is well within the skill of others to identify other primers which may be used in the present invention; and in optimizing the individual PCR and LCR reactions, the LCR and PCR cycles and temperatures may be adjusted according to optimal protocols for each test system. Accordingly, such changes and alterations are properly intended to be within the full range of equivalents, and therefore within the purview of the following claims.

Having thus described our invention and the manner and a process of making and using it in such full, clear, concise and exact terms so as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same;

WE CLAIM:

1. A method to discriminate one bacterial species or strain from another bacterial species or strain which comprises

identifying a single base pair mismatch between the 16S rDNA nucleotide sequences of each bacterial species,

designing and utilizing a compatible nucleotide PCR primer in a polymerase chain reaction to amplify the 16S rDNA of each bacterial species containing the single base pair mismatch,

designing and utilizing a compatible LCR nucleotide primer in a ligase chain reaction to determine whether the LCR primer will ligate the PCR-amplified 16S rDNA; and

determining whether ligation has occurred in the ligase chain reaction.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12508

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C12P 19/34; C07H 21/04
US CL :435/6, 91.2, 91.52; 536/24.32
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.2, 91.52; 536/24.32

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CA, MEDLINE, DNA DATA BASES: GENBANK, EMBL AND GENESEQ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	International Journal of Systematic Bacteriology, Volume 24, Number 2, issued April 1991, Collins et al., "Phylogenetic Analysis of the Genus *Listeria* Based on Reverse Transcriptase Sequencing of 16S rRNA", pages 240-246, see entire document.	1

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 25 JANUARY 1995	Date of mailing of the international search report 02 FEB 1995
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