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Direct and Rapid Detection of *Erysipelothrix rhusiopathiae* DNA in Animals by PCR

SOU-ICHI MAKINO,[1]* YUMIKO OKADA,[1] TSUTOMU MARUYAMA,[1] KIYOYASU ISHIKAWA,[2] TOSHIO TAKAHASHI,[2] MASAYUKI NAKAMURA,[2] TAKAYUKI EZAKI,[3] AND HIROSHI MORITA[4]

Department of Veterinary Public Health, The Institute of Public Health, Minato-ku, Tokyo 108,[1] National Veterinary Assay Laboratory, Tokyo 185,[2] Department of Bacteriology, Gifu University School of Medicine, Gifu 500,[3] and Yokohama Research Center, Chisso Corporation, Yokohama, Kanagawa 236,[4] Japan

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***Erysipelothrix rhusiopathiae* is a gram-positive rod capable of causing erysipelas in swine. To establish a method for specifically detecting *E. rhusiopathiae* for practical applications, such as for the inspection of slaughterhouses, the feasibility of using primers derived from the DNA sequence coding for 16S rRNA in a PCR-specific detection system was investigated. Oligonucleotide primers were designed to amplify a 407-bp DNA fragment by PCR. The amplification was specific to the *Erysipelothrix* DNA but not to that of other bacterial genera tested. This PCR-based method efficiently and specifically detected the *Erysipelothrix* DNA sequence in joint and spleen samples from mice within 6 h, and application of the 407-bp DNA segment from samples containing very low numbers of bacteria (<20 bacteria per spleen from mice) was possible. Although this PCR amplification is specific for the *Erysipelothrix* genus, which contains at least two species, *E. rhusiopathiae* and *E. tonsillarum*, it can be concluded that all *Erysipelothrix* strains detected by this PCR system in diseased pigs are *E. rhusiopathiae* because only *E. rhusiopathiae* is virulent for pigs. These results show that this PCR amplification system using the DNA sequence coding for 16S rRNA is very rapid and reliable and avoids cumbersome and lengthy cultivation steps, demonstrating that this system could be used for practical applications.**

Erysipelothrix rhusiopathiae is a small gram-positive rod that causes erysipelas in swine and a variety of diseases in other animals and birds, as well as erysipeloid, a human skin disease (23). Erysipelas can occur as an acute septicemia or chronic disease with development of arthritic lesions and endocarditis, which causes great economic loss and continues to be a major problem in swine-producing areas of the world.

In Japan, erysipelas is generally found in several thousand slaughter-bound swine every year, all parts of which must be wasted. Therefore, the detection of *E. rhusiopathiae* in slaughter-bound swine is very important, but it is difficult to distinguish *E. rhusiopathiae* from gram-positive bacterial species such as *Listeria monocytogenes*, *Corynebacterium pyogenes*, and *Streptococcus suis*, which have similar biochemical characteristics and are usually causative agents of arthritis and endocarditis in swine. The routine detection method for *E. rhusiopathiae* involves direct culturing from preparations derived from the joints, heart, and other organs of infected swine on selective media and subsequent enrichment culturing in liquid medium to obtain heat-stable antigen by hot aqueous extraction for serotyping (2). However, this method is time-consuming, since enrichment schemes may require up to 4 days for the identification of *E. rhusiopathiae*. Hence, it is highly desirable from an economic and health viewpoint to establish a highly sensitive, reliable, rapid, simple, and widely applicable method for detecting *E. rhusiopathiae* in livestock.

Recently, several methods have been proposed to replace the time-consuming classical techniques for detecting bacterial pathogens, for example, DNA-DNA hybridization with a bacterium-specific probe and PCR. PCR techniques especially (10) could be an attractive tool for detection of pathogens directly from food, meat, biopsy samples, and other sources (1, 6, 7, 13, 19, 20, 22). However, the complex composition of those products might compel extensive purification procedures in order to obtain DNA of a purity suitable for PCR amplification.

In this study, we have evaluated the use of PCR amplification in detecting *E. rhusiopathiae*, which could be widely applicable for slaughterhouses. The experiments described here are based on the amplification and subsequent detection of fragments of *Erysipelothrix* DNA coding for 16S rRNA, which can be amplified specifically for *Erysipelothrix* species.

MATERIALS AND METHODS

Animals and bacterial strains used. Specific-pathogen-free mice (BALB/c) 4 to 8 weeks old were supplied by the Animal Medicine Section in the Institute of Public Health. All bacterial strains used in this study are listed in Tables 1 and 2.

DNA preparation. To isolate total DNA from the bacteria shown in Table 2, bacterial cells from 1.5 ml of bacterial culture grown for 24 h were suspended in 200 μl of TES buffer (50 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl [pH 8.0]) containing 10 μl of lysozyme (10 mg/ml) and were incubated for 30 min at room temperature or at 37°C before the addition of 10 μl of 10% sodium dodecyl sulfate (SDS) and 10 μl of proteinase K (20 mg/ml). After further incubation at 55°C for 60 min, the crude DNA preparation was treated with RNase, extracted three times with phenol-chloroform, precipitated with ethanol, and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). To isolate total DNA from *E. rhusiopathiae* strains, TES buffer containing 10 μl of lysozyme (10 mg/ml) and 10 μl of *N*-acetylmuramidase SG (1 mg/ml;

* Corresponding author. Mailing address: Department of Veterinary Public Health, The Institute of Public Health, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan. Phone: 81-3-3441-7111. Fax: 81-3-3446-4314.

TABLE 1. *Erysipelothrix* strains used in this study[a]

Erysipelothrix strain	Serovar or type
E. rhusiopathiae	
ME-7	1a
Fujisawa	1a
422/1E1	1b
ATCC 19414	2
Koganei[b]	2
Nagasaki	2
R32E11	2
Doggerscharbe	4
Pécs 67	5
Tuzok	6
Dolphin E-1	6
Goda	8
Kaparek	9
IV12/8	11
Pécs 9	12
Pécs 3597	15
Tanzania	16
545	17
2017	19
Bāno 36	21
MEW 22	N
E. tonsillarum	
Witlling	3
ATCC 43339	7
ATCC 43338	7
P-43	7
L1-3	7
Lengyel-P	10
Iszap-4	14
2553	20
Bāno 107	22
KS20A	23
Erysipelothrix spp.	
Pécs 56	13
Shiribeshi-17	13
Shiribeshi-19	13
715	18

[a] All strains except for Nagasaki are preserved in National Veterinary Assay Laboratory, Tokyo, Japan. Strain Nagasaki was a kind gift from T. Sawada, Nippon Veterinary and Animal Science University, Tokyo, Japan. Fine designations of all of the strains are described in reference 15.

[b] Strain Koganei is used as a vaccine strain in Japan.

Seikagaku Kogyou Co. Ltd., Tokyo, Japan) was used before SDS and proteinase K treatment.

DNA amplification and gel electrophoresis. The *Erysipelothrix* primers were derived from the DNA sequence coding for 16S rRNA (21), EMBL accession no. M23728. Primers, MO101 (5′AGATGCCATAGAAACTGGTA3′) and MO102 (5′CTGTATCCGCCATAACTA3′) amplified a 407-bp DNA sequence (nucleotides 634 to 1040) by the PCR method in a reaction mixture (100 μl) containing 10 mM Tris-HCl (pH 8.3); 60 mM KCl; 1.5 mM $MgCl_2$; 0.1% Triton X-100; 200 mM (each) dATP, dTTP, dCTP, and dGTP; 50 pmol of oligonucleotide primers; 2.5 U of *Taq* DNA polymerase (Promega Co., Madison, Wis.); and 0.1 μg of various DNAs. A Perkin-Elmer PJ2000 thermal cycler was used for amplification. Denaturation was carried out at 94°C for 1.0 min, annealing was at 54°C for 2 min, and extension was at 72°C for 2 min; amplification was repeated for 30 cycles. Prior to cycling, the samples were heated at 94°C for 2 min. Finally, an additional extension step was performed at 72°C for 7 min. The amplified products were separated on 1% agarose gels and stained with ethidium bromide.

Infection experiments with mice and preparation of samples for PCR. Mice were inoculated subcutaneously with appropriate dilutions of bacterial cell suspensions of *E. rhusiopathiae* Nagasaki or Koganei grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.1% Tween 80 at 37°C for 24 h. After 3 or 4 days, stifle joints and spleens were isolated. Those isolates were finely chopped with a sharp knife and vortexed with 400 μl of phosphate-buffered saline (PBS). Twenty microliters of the supernatants was spread on brain heart infusion agar plates supplemented with 0.1% Tween 80 to calculate the number of CFU. One hundred microliters of the supernatants was mixed with 200 μl of TES buffer containing 10 μl of lysozyme (10 mg/ml) and 10 μl of *N*-acetylmuramidase SG (1 mg/ml) and incubated at 37°C for 30 min, and 10 μl of 10% SDS, 10 μl of proteinase K (20 mg/ml), and 1 μl of RNase (10 mg/ml) were added. After incubation at 55°C for 60 min, the DNAs were extracted three times with phenol-chloroform, precipitated with ethanol, and dissolved in 100 μl of sterilized water. One and 10 μl of the suspensions of the samples prepared from joints and spleen, respectively, were directly used for the PCR.

Other techniques. Southern hybridization was performed according to the method described by Maniatis et al. (9) with the DIG DNA Labeling kit and DIG Luminescent Detection kit (Boehringer Mannheim, Mannheim, Germany). DNA was sequenced by the dideoxy chain termination method of Sanger et al. (11) with the Bca Best sequencing kit (Takara Shuzo Co. Ltd., Kyoto, Japan). PCR products were treated by DNA polymerase to produce flush ends and were cloned into the *Sma*I site of pUC119 and pUC118 (Takara Shuzo Co., Ltd., Kyoto, Japan).

RESULTS

PCR with total DNA from various bacteria. Although bacterial DNA sequences coding for 16S rRNA generally contain many universal short DNA regions, two short DNA segments within the DNA sequence coding for *Erysipelothrix* 16S rRNA (21), which seem to be specific to *Erysipelothrix* species, were selected to synthesize oligonucleotide primers for PCR and were designated MO101 and MO102. PCR was performed with purified total DNA from *E. rhusiopathiae* ATCC 19414 with various concentrations of $MgCl_2$ in the reaction mixture and at various annealing temperatures. Optimum conditions for PCR were determined as described in Materials and Methods. Under those conditions, only a single band corresponding to a 407-bp DNA segment derived from the DNA sequence coding for 16S rRNA was amplified (Fig. 1, lane 1). To test whether this PCR system would be useful for detecting *Erysipelothrix* DNA, PCR was performed with 35 *Erysipelothrix* strains, including all serotypes, as shown in Table 1. A single band corresponding to a 407-bp DNA segment was amplified in the DNA samples from all *Erysipelothrix* strains, representative results of which are shown in Fig. 1. All of the bands strongly hybridized with a 407-bp DNA fragment amplified from *E. rhusiopathiae* ATCC 19414 DNA by PCR (Fig. 1B). We then determined the DNA sequences of both of the amplified 407-bp DNA fragments from ATCC 19414 and ATCC 43339 and found them to be identical to the published sequence (21) (data not shown), suggesting that the nucleotide sequence of a 407-bp region in 16S rRNA is highly conserved in all serotypes of *Erysipelothrix* strains. Those results demonstrated that PCR with MO101 and MO102 primers could give rise to DNA amplification from all *Erysipelothrix* strains.

TABLE 2. Bacterial strains used in this study

Organism type	Group	Strain	Source or reference
Gram positive	A	*Bacillus anthracis* Davis	5
		Bacillus anthracis Pasteur II	8, 18
		Bacillus cereus IID872	5
		Bacillus licheniformis NIAH227	This laboratory
		Bacillus megaterium NIAH368	5
		Bacillus subtilis NIAH801	5
		Bacillus thuringiensis	This laboratory
		Clostridium botulinum 003-9	S. Kozaki[a]
		Clostridium difficile 7626	Gifu[b]
		Clostridium perfringens 5256	Gifu
	B	*Corynebacterium diphtheriae* 3182	Gifu
		Corynebacterium pyogenes	This laboratory
		Enterococcus faecalis 8357	Gifu
		Streptococcus suis	T. Maeda[c]
		Lactococcus lactis 8591	Gifu
		Listeria monocytogenes EGD	This laboratory
		Mycobacterium tuberculosis 27874	Gifu
		Staphylococcus aureus	This laboratory
		Streptococcus pneumoniae	This laboratory
Gram negative	C	*Actinobacillus pleuropneumoniae* Ngl	This laboratory
		Aeromonas sp. strain ATCC 9071	This laboratory
		Campylobacter fetus 8746	Gifu
		Campylobacter jejuni 91-569	S. Kaneko
		Enterohemorrhagic *Escherichia coli*	This laboratory
		Enteropathogenic *Escherichia coli*	This laboratory
		Enterotoxigenic *Escherichia coli*	This laboratory
		Klebsiella pneumoniae IID5209	This laboratory
	D	*Legionella pneumophila* 10260	Gifu
		Pasteurella multocida 87-37	S. Kaneko[d]
		Proteus vulgaris IID874	This laboratory
		Pseudomonas acidovorans 11501	Gifu
		Pseudomonas aeruginosa P13	This laboratory
		Salmonella choleraesuis SB242	This laboratory
		Salmonella enteritidis	This laboratory
	E	*Salmonella typhimurium* LT2	This laboratory
		Serratia marcescens IID5218	This laboratory
		Shigella flexneri YSH6000	3
		Vibrio cholerae IID936	This laboratory
		Vibrio parahaemolyticus 91-572	S. Kaneko
		Yersinia enterocolitica	This laboratory
		Yersinia pseudotuberculosis	This laboratory

[a] S. Kozaki, University of Osaka, Osaka, Japan.
[b] Department of Bacteriology, Gifu University School of Medicine, Gifu, Japan.
[c] T. Maeda, The Inspection Center of the Kennan Slaughterhouse, Ibaraki, Japan.
[d] S. Kaneko, The Metropolitan Institute of Hygiene, Tokyo, Japan.

To test whether this PCR amplification was specific to *Erysipelothrix* species, PCR was performed for all of the strains shown in Table 2. No amplified DNA was seen in any of the mixtures without *E. rhusiopathiae* ATCC 19414 DNA (Fig. 2, lanes 1, 3, 5, 7, and 9), whereas a single 407-bp band that hybridized with DNA derived from *E. rhusiopathiae* ATCC 19414 DNA samples (Fig. 1, lane 1) was amplified in DNA mixtures containing *E. rhusiopathiae* ATCC 19414 DNA (Fig. 2, lanes 2, 4, 6, 8, and 10). This clearly demonstrates that PCR with the MO101 and MO102 primers gives rise to DNA amplification specific to *Erysipelothrix* species.

Animal model experiments. Since *E. rhusiopathiae* is virulent for mice and similar clinical signs such as arthritis and systemic disease are observed in both mice and swine, mice are generally used for the official approval of live-organism erysipelas vaccine and to examine the pathogenicity of *Erysipelothrix* species. Thus, we used mice to test whether the PCR detection system with MO101 and MO102 primers would allow detection of *Erysipelothrix* species in an animal model. BALB/c mice were inoculated subcutaneously into the right inner thigh with 10-fold dilutions (inoculum sizes of 4.6×10^7 to 4.6×10^3 CFU) of overnight cultures of *E. rhusiopathiae* Nagasaki or Koganei in brain heart infusion broth with 0.1% Tween 80. At 3 or 4 days after being challenged, all mice survived but displayed arthritic symptoms regardless of the inoculum size present, and then stifle joints and spleen were taken to calculate the number of bacteria and were subjected to PCR as described in Materials and Methods. The joint samples contained 1.2×10^4 to 6.2×10^4 CFU, which corresponds to the number of bacterial cells in the joint samples from cases of

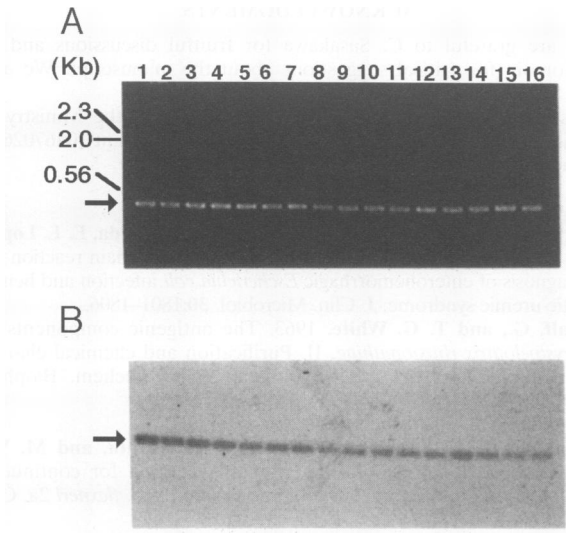

FIG. 1. PCR amplification of a 407-bp DNA sequence from total DNAs of various *Erysipelothrix* strains and Southern hybridization to an *E. rhusiopathiae* probe. *Erysipelothrix* strains used are representative of all serotypes listed in Table 1. Lanes (with serovar or serotype in parentheses): 1, ATCC 19414 (1a; a type strain); 2, 422/1E1 (1b); 3, R32E11 (2); 4, Witlling (3); 5, Pécs 67 (5); 6, ATCC 43339 (7); 7, Kaparek (9); 8, IV12/8 (11); 9, Pécs 9 (12); 10, Pécs 56 (13); 11, Pécs 3597 (15); 12, 545 (17); 13, 715 (18); 14, 2017 (19); 15, 2553 (20); 16, MEW 22 (N). (A) Ethidium bromide-stained 1.0% agarose gel. (B) Southern hybridization autoradiogram. A 407-bp DNA fragment amplified with *E. rhusiopathiae* ATCC 19414 DNA was used as the probe. Arrowheads indicate the 407-bp DNA fragment. Size markers are shown on the left.

swine erysipelas (13a), and the spleen samples contained less than 20 CFU regardless of the inoculum size.

To prepare the samples for PCR, the boiling method described in reference 6 and the lysis method described in Materials and Methods were performed. In the boiling method, the samples were prepared for PCR by boiling at 100°C for 15 min followed by centrifugation, and an aliquot of

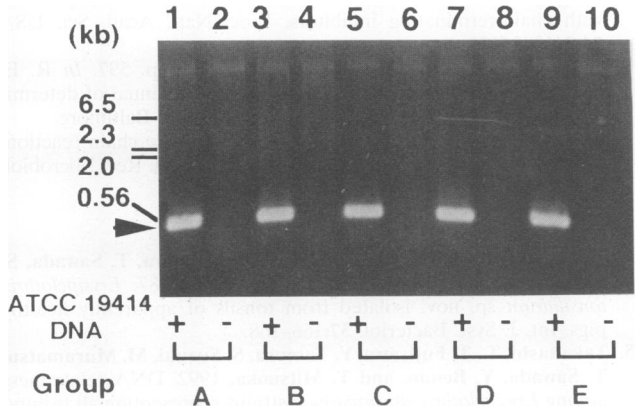

FIG. 2. PCR amplification of the 407-bp DNA fragment from total DNAs of various bacterial strains. Mixtures A, B, C, D, and E indicate pooled DNA samples containing total DNAs isolated from all strains in groups A, B, C, D, and E as shown in Table 2, respectively, with (+) and without (−) *E. rhusiopathiae* ATCC 19414 DNA. For each PCR, 0.25 μg of DNA from each sample was used. An arrowhead indicates the 407-bp DNA fragment. Size markers are shown on the left.

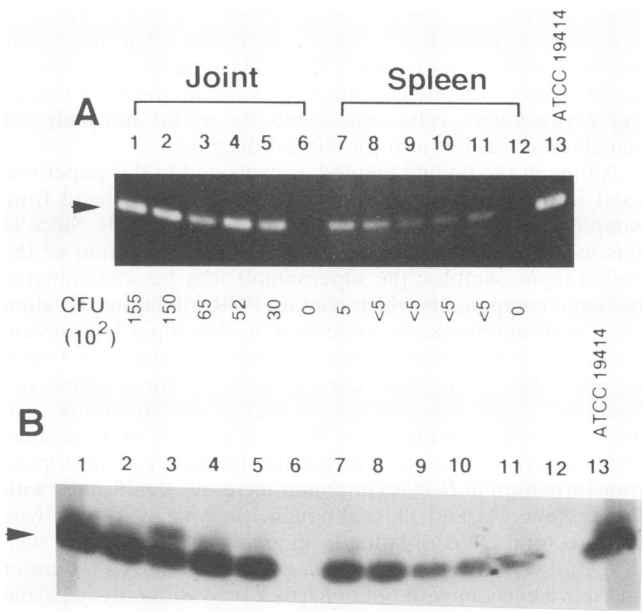

FIG. 3. PCR amplification of the 407-bp DNA fragment in samples from mice infected with *E. rhusiopathiae* and Southern hybridization to an *E. rhusiopathiae* probe. Lanes 1 to 5 and 7 to 11 show PCR amplifications from mice infected with *E. rhusiopathiae*. Lanes 6 and 12 show PCR amplifications from mice not infected with *E. rhusiopathiae*. Lane 13 shows PCR amplification with *E. rhusiopathiae* ATCC 19414 purified DNA. PCR samples were prepared from joints (lanes 1 to 6) and spleen (lanes 7 to 12). The arrowheads indicate the 407-bp DNA band. (A) Ethidium bromide-stained 1.0% agarose gel. The values given under panel A indicate the bacterial numbers (CFU) isolated from samples before preparation for PCR. (B) Southern hybridization autoradiogram. A 407-bp DNA fragment amplified with *E. rhusiopathiae* ATCC 19414 DNA was used as the probe.

the supernatant was directly used for PCR. However, samples prepared by the boiling method were not suitable for PCR, since no visible DNA bands appeared (data not shown), while a 407-bp band was amplified from all samples prepared by the lysis method established in this study (Fig. 3A, lanes 1 to 5 and 7 to 11). The differences in intensity of the 407-bp bands amplified from joint and spleen samples are thought to reflect the number of bacterial cells contained in each sample (Fig. 3A). Although we could recover very few bacterial cells from the spleen samples, PCR was able to efficiently amplify a 407-bp band. Those amplified 407-bp bands specifically hybridized with a 407-bp band amplified from *E. rhusiopathiae* ATCC 19414 DNA (Fig. 3B, lanes 1 to 5 and 7 to 11), demonstrating that this PCR system is useful in detecting *Erysipelothrix* DNA directly from live samples.

DISCUSSION

In this study, we have established a PCR system that detects *Erysipelothrix* DNA from animal samples without cultivation by using oligonucleotide primers complementary to the DNA sequence coding for 16S rRNA. This PCR system was found to be highly specific to *Erysipelothrix* species, because the oligonucleotide primers used hybridized uniquely to *Erysipelothrix* total DNA, giving rise to an amplified 407-bp DNA sequence (Fig. 1). This PCR system is also highly sensitive because a 407-bp DNA sequence was amplified from the mouse spleen containing only a few bacterial cells. Moreover, since about 10 to 10^4 *Erysipelothrix* cells could be generally recovered from the

tissue samples in cases of swine erysipelas (13b), with this system, it is possible to make a diagnosis from very small numbers of bacteria within a day, indicating that this system could replace the time-consuming classical methods for detecting *Erysipelothrix* cells and would be useful not only for slaughterhouses but also for clinical diagnosis.

Although the boiling method as explained in this paper was used in an attempt to amplify the 407-bp DNA band from samples, no DNA bands could be amplified by PCR. Since in this method PCR was done by using the supernatant of the boiled tissue samples, the supernatants may be contaminated by tissue components which contain PCR inhibitors. To eliminate such inhibitors, we undertook to develop a lysis system for sample preparation. In this system, we utilized *N*-acetylmuramidase SG as the lytic enzyme, because this enzyme can hydrolyze a glycoside linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine in the cell wall of gram-positive bacteria. We have observed that the frequency of protoplast transformation in *Bacillus anthracis* increases significantly with this enzyme (4) and that, although lysozyme is a good lytic enzyme, total DNA isolation from gram-positive bacteria such as *L. monocytogenes*, some *Bacillus* strains, and *S. suis* is easier and more efficient with both enzymes than with only lysozyme (data not shown). Thus, this enzyme could be widely applicable for genetic studies of gram-positive bacteria. Moreover, when the samples were prepared by the boiling method with bacteria grown in liquid medium or on agar medium, a 407-bp DNA band specific for *Erysipelothrix* species could efficiently be amplified (data not shown). Since the boiling method is faster and simpler than the lysis method for preparing samples, the conclusion is that, although the lysis methods described here are better for tissue samples, the boiling methods are better for in vitro studies.

It has long been thought that the *Erysipelothrix* genus consists of a single species, *E. rhusiopathiae* (12, 17). According to recent studies with DNA-DNA hybridization, however, this genus now seems to consist of at least two species, *E. rhusiopathiae* and *E. tonsillarum* (15). In this study, PCR with MO101 and MO102 primers amplified a 407-bp DNA band from *E. tonsillarum*, which strongly hybridized to a 407-bp band amplified from *E. rhusiopathiae* total DNA (Fig. 1). Moreover, the nucleotide sequences of both amplified DNA fragments were almost identical (2a), implying that both species are indistinguishable from each other by this PCR method and that this PCR system will thus detect all *Erysipelothrix* species. However, *E. tonsillarum* is usually found in the tonsils of apparently healthy pigs and is avirulent for pigs, but *E. rhusiopathiae* is fully virulent (14–16). If an *Erysipelothrix* species is detected in joints or/and other organs by the PCR system developed in this study, it is thus most unlikely to be *E. tonsillarum*.

PCR with MO101 and MO102 primers has been shown to be highly specific for *Erysipelothrix* species (Fig. 2). However, when DNA-DNA hybridization with a 407-bp fragment amplified with *E. rhusiopathiae* ATCC 19414 DNA as a probe was performed against all strains listed in Table 2, some DNA bands hybridized to various extents with those of some other gram-positive bacterial species (data not shown). This suggests that the 407-bp region in the DNA sequence encoding the 16S rRNA is partially conserved among bacterial species. When we constructed two inner primers located between the MO101 and MO102 primers in the DNA sequence coding for 16S rRNA, a single strong band was amplified not only in *E. rhusiopathiae* but also in *S. suis* total DNA (data not shown). We therefore concluded that MO101 and MO102 primers are the best combination for the PCR amplification-specific detection of *Erysipelothrix* species.

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