Enzyme Electrophoresis, Catalase Test and PCR-RFLP Analysis for the Typing of *Edwardsiella tarda*

Yoshiyuki Yamada and Hisatsugu Wakabayashi

Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences,
The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan

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Enzyme electrophoresis for superoxide dismutase (SOD) and catalase divided 144 *Edwardsiella tarda* strains into two groups; group 1 contained all the strains isolated from diseased fish in Japan and group 2 included 25 strains from non-disease sources and reference strain ATCC15947^T. These groups could be similarly distinguished by a simple catalase test; group 1 showed an instantaneous bubbling, whereas in group 2 bubbling was delayed or weak. PCR-RFLP analysis using *Hha*I revealed that the two groups differed in their 16S rDNA genotypes. Furthermore, this analysis subdivided group 1 into two genotypes.

Keywords: *Edwardsiella tarda*, SOD, catalase, 16S rDNA, enzyme electrophoresis, PCR, RFLP

Edwardsiellosis, caused by *Edwardsiella tarda*, has long been recognized as a common bacterial disease in freshwater and marine fish in Japan (Wakabayashi and Egusa, 1973; Kusuda *et al.*, 1976; Kubota *et al.*, 1981; Nakatsugawa, 1983).

Epidemiological surveys of *E. tarda* in Japan revealed that isolates from diseased fish and their environments (eel ponds) were classified into several different serotypes (A, B, C, D and unclassified), with most of the strains belonging to type A being virulent against fish (Park *et al.*, 1983; Rashid *et al.*, 1994). Serotyping has been one of the popular methods to type natural populations of bacterial species, but the analysis of the genetic structure of populations has recently been shown to be of great value in the study of variation within species in bacterial population genetics and systematics.

There are several approaches to assaying genetic variation of bacterial populations. Multilocus enzyme electrophoresis has the ability to reveal many variations in enzymes, with regard to their shape, net charge, catalytic efficiency and stability. The differences in these characters result from many aspects of the genetic differences. Hence, in many bacterial species phenotyping reveals the presence of genetic variation (Selander *et al.*, 1986). Inversely in the analysis of DNA, restriction fragment length polymorphism (RFLP) analysis of PCR-amplified DNA is one of the useful technique to compare the DNA sequences based on the distribution of restriction sites. Small subunits ribosomal DNA (16S

rDNA) was the most investigated and valuable tool to discuss the phylogenetic diversities among bacteria and RFLP analysis of PCR-amplified 16S rDNA was used for identification or grouping of bacterial isolates (Schmidt, 1994).

The aim of the present study was the typing of natural populations of *E. tarda* using enzyme electrophoresis, catalase test and PCR-RFLP analysis, and phylogenetic diversities among the populations were also discussed using these typing methods.

Materials and Methods

Bacteria

E. tarda ATCC15947^T was purchased from the American Type Culture Collection. Another 143 strains were isolated from diseased fish (Japanese eel, *Anguilla japonica*; Japanese flounder, *Paralichthys olivaceus*; Nile tilapia, *Oreochromis niloticus*; red sea bream, *Chrysophrys major*; loach, *Misgurnus anguillicaudatus* and ayu, *Plecoglossus altivelis*), apparently healthy Japanese eels, or their environments (eel ponds) during the past three decades. Localities of the diseased fish were Aichi, Ehime, Kagoshima, Miyazaki, Nagasaki, Niigata, Oita, Saga, Shizuoka, and Tokushima, whereas isolates from eel ponds were collected in Nagasaki and Shizuoka. The material collected was stored at -80°C in tryptose-yea (TS) broth (Nissui co.) containing 10% (v/v) glycerol until use.

Enzyme Electrophoresis

Each strain was cultured on TS agar at 25°C. Bacterial cells in a stationary phase were collected and suspended in distilled water. Cells were disrupted by sonication and then centrifuged at $16,800 \times g$ for 20 min at 4°C. The supernatant was collected and stored at -80°C until required for electrophoresis. A non-denatured polyacrylamide gel electrophoresis (native-PAGE) was performed on 12.5% or 7.5% acrylamide gels.

Visualization of SOD and catalase activities

Superoxide dismutase (SOD) activities on the 12.5% gels were visualized using nitro blue tetrazolium and riboflavin (Beauchamp *et al.*, 1971), whereas catalase activities on the 7.5% gels were visualized using horseradish peroxidase, H_2O_2 and diaminobenzidine (Clare *et al.*, 1984). To estimate the amount of metal contained in each SOD band staining was conducted with the following inhibitors; 3.7 mM H_2O_2 , and 1.9 mM KCN (Crapo *et al.*, 1991).

Catalase activity

Each strain was cultured on TS agar at 25°C for 48 h. A single colony was used for a simple catalase test by adding a drop of 3% H_2O_2 to the cells on a glass slide. The time required for the appearance of oxygen bubble was used as an estimation of activity; instantaneous bubbling was recorded as "immediate" and any delayed or weak bubbling was recorded as "delayed" (Chester *et al.*, 1987).

DNA extraction

Chromosomal DNA of 144 *E. tarda* strains were prepared by the boiled-extraction method (Aoki *et al.*, 1995). Briefly, this technique involves the bacterial cells suspended in distilled water being boiled at 100°C for 10 min. The supernatant containing the template DNA was then obtained by centrifugation at $16,800 \times g$ for 10 min at 4°C.

PCR amplification of 16S rDNA

The oligonucleotide primers used were those designed to anneal to conserved regions of the eubacterial 16S rRNA: forward, 5'-AGAGTTTGA-TCCTGGCTCAG-3'; reverse, 5'-GGTTACCTTGTTACGACTT-3' (Weisburg *et al.*, 1991).

PCR amplification was performed with a GeneAmp® PCR System 2400 (Perkin-Elmer Co.) using AmpliTaq Gold™ polymerase and 10× PCR buffer (Perkin-Elmer Co.) in a total reaction volume of 50 µl. This reaction

mixture contained 0.1–0.2 µg of template DNA (in DW), 20 µM of each deoxynucleoside triphosphates, 20 pM of each primer, 1.5 mM $MgCl_2$, and 1.25U of AmpliTaq Gold™ DNA polymerase. The following temperature profile was used for the amplification: preheating at 94°C for 9 min; 40 cycles of denaturation (94°C, 30s), annealing (50°C, 30s), and extension (72°C, 90s); and a final extension at 72°C for 7 min.

Restriction fragment analysis

Thirty-two representative strains of each type (groups 1 or 2) which were classified according to the enzyme electrophoresis and catalase tests, were used for screening of the appropriate enzyme for the 16S rDNA genotyping of *E. tarda*.

Eight micro-liters of the PCR products were digested with the 2.5 units of restriction endonucleases at 37°C for 150 min according to the manufacturer's instructions. The following 4 and 5 base-cutter enzymes were used: *AluI*, *DdeI*, *HinfI*, *RsaI* (TOYOBO Co. Ltd.), *HhaI* (TAKARA Shuzo Co.), *HaeIII*, *MspI*, *Sau3A* (BOEHRINGER MANNHEIM Co.). Restricted DNAs were analyzed by horizontal electrophoresis in 4% NuSieve 3 : 1 agarose gels (FMC Bioproducts Co.). Electrophoresis was carried out at 50V for 180 min using Mupid mini-gel electrophoresis apparatus (Advance Co.) in TAE electrophoresis buffer on ice. DNA fragments were stained by ethidium bromide, and visualized under UV light.

In *HhaI* digestion, which exhibited several different fragment patterns, stability of the identifying fragments was investigated using 5 units of enzyme and extended reaction times (180, 240 min). Then, RFLP analysis using *HhaI* was performed to the 144 *E. tarda* strains.

Results

Enzyme electrophoresis

Electrophoretic patterns of SOD and catalase are shown in Fig. 1. Electrophoretic mobilities of SOD and catalase divided the 144 *E. tarda* strains into two groups. Group 1 produced SOD type 1 and catalase type 1, and consisted of 118 strains. On the other hand, group 2 produced SOD type 2 and catalase type 2, and consisted of 26 strains. The reference strain ATCC15947^T was classified into group 2 (Table 1). The SOD inhibitors revealed that the metal contained in all strains was iron and hence it was identified as an iron-containing SOD (FeSOD).

Fig. 1. 1A: Electrophoretic patterns of superoxide dismutase (SOD) on a 12.5% acrylamide gel. The electrophoretic mobilities of SOD isolated from the *E. tarda* strains were separated into two types; lanes 1 to 6 were of type 1, and lanes 7 to 12 were of type 2. 1B: Electrophoretic patterns of catalase isolated from the *E. tarda* strains on a 7.5% acrylamide gel. Catalase was also separated into two types; lanes 1 to 6 were of type 1, and lanes 7 to 12 were of type 2. (The same samples were applied to the same lanes on both gels.)

Catalase test

The catalase activity of each strain depended on the catalase type. All strains producing catalase type 1 were classified as "immediate", whereas all members producing catalase type 2 were typed as "delayed" (Table 1).

16SrDNA PCR-RFLP analysis

The DNA fragments of the representative 32 strains showed the same pattern following restriction with the enzymes; *AluI*, *DdeI*, *HaeIII*, *HinfI*, *MspI*, *RsaI* and *Sau3A* (Data not shown). On the other hand, the strains exhibited several different patterns when digested with *HhaI* (Fig. 2). The genetic variation was typed (genotypes A, B and C) according to the presence or absence of 190, 100 and 90 bp fragments. Several strains of genotype C had an additional band of approximately 470 bp. Due to the weak staining of this band compared to its fragment size, this group was tentatively typed as C'. The identifying fragments of the three genotype were stable under the digestion using 5 units of enzyme and extended reaction times.

The RFLP analysis with *HhaI* was performed to the 144 *E. tarda* strains. It split the strains identified as group 1 by the enzyme electrophoresis and catalase test into genotypes A or B, whereas group 2 strains exhibited genotype C (Tables 1 and 2).

Discussion

In the present study, the phenotypings made according to the enzyme electrophoresis and catalase test gave a rough understanding of the populations of *E. tarda* investigated. These tests revealed that the Japanese isolates were divided into two major populations, groups 1 and 2.

The electrophoretic mobilities of enzymes are determined by their structures; i.e., by their amino acid composition (primary structure) and by additional folding and assembly into their secondary and tertiary structures. The variations in electrophoretic migration of FeSOD and catalase of *E. tarda* strains suggested that structural differences exist between strains in these enzymes or that post-translational modifications to them differ, with the result that groups 1 and 2 were quite different populations.

In a simple catalase test, groups 1 and 2 differed in the time required for the appearance of oxygen bubbles.

Table 1. The phenotyping of *E. tarda* strains according to enzyme electrophoresis and catalase test, and the relationship of these typings with 16S rDNA genotypes.

Populations of <i>E. tarda</i>	Enzyme electrophoresis		Catalase test	16S rDNA genotype
	FeSOD	catalase		
Group 1 (118 strains)	type 1	type 1	immediate	A, B
Group 2 (26 strains)	type 2	type 2	delayed	C, C'

Fig. 2. Restriction fragment length polymorphism (RFLP) patterns of *Hha*I digested 16S rDNA of representative *E. tarda* strains on a 4% NuSieve 3 : 1 agarose gel. The genetic variation was typed by the presence or absence of the 190, 100 and 90 bp fragments (see arrows) : genotype A; lanes 1 to 4, genotype B; lanes 5 to 8, genotype C; lanes 9 to 15 (lanes 13 to 15 had an additional band of approximately 470bp: C'). M; 100 bp DNA ladder (Laboratories, Inc.)

Table 2. The relationship between 16S rDNA genotypes of *E. tarda* strains as determined by RFLP analysis and the sources of these strains.

Sources of the strains	16S rDNA genotypes of <i>E. tarda</i>			
	A	B	C	C'
ATCC15947 ^T	—	—	1	—
Diseased fish				
Japanese eel	54	0	0	0
Japanese flounder	37	0	0	0
Red sea bream	0	13	0	0
Tilapia	0	2	0	0
Ayu	2	0	0	0
Loach	2	0	0	0
Intestinal contents of apparently healthy eel	2	0	5	3
Eel culture pond				
Water	5	0	10	1
Sediment	1	0	3	3
Total 144 strains	103	15	19	7

Chester *et al.* (1987) used this semiquantitative method as a supplemental test for rapid identification of members of the family *Enterobacteriaceae*. According to their survey, the family *Enterobacteriaceae* was divided into two major groups: "immediate" catalase reactors, comprising *Yersinia*, *Serratia*, *Proteus*, *Morganella*, *Providencia*, *Cedecea*, and *Hafnia* spp.; and "delayed"

catalase reactors, comprising *Escherichia*, *Shigella*, *Klebsiella*, *Enterobacter*, *Salmonella*, *Citrobacter*, *Edwardsiella*, *Kluyvera*, and *Tatumella* spp.. In the present study, 144 *E. tarda* strains were classified either as "immediate" or "delayed" catalase reactors. This fact suggests that the groups 1 and 2 might be phylogenetically distant.

RFLP analysis of the 16S rDNA indicated genetic variation between *E. tarda* strains which had been assigned to groups 1 and 2. Furthermore, RFLP also suggested that the strains comprising of group 1 were subdivided into genotypes A and B. The strains isolated from diseased Japanese eel, Japanese flounder, loach, and ayu belonged to genotype A, whereas the strains isolated from red sea bream and tilapia belonged to genotype B. Because of the conservative nature of the 16S rDNA, its comparison in its structure have been an essential tool for understanding phylogenetic and evolutionary relationships among bacteria (Weisburg *et al.*, 1991). The differences in the 16S rDNA genotype recorded here suggest that *E. tarda* might be subdivided into several subspecies.

The typing methods used in the current study were able to distinguish the strains that were isolated from diseased fish (group 1). Group 1 contained all of the strains which had high virulence to fishes and the strains classified into serotype A, whereas group 2 contained the low virulent strains and the strains classified into serotype B and C (Park *et al.*, 1983, Iida and Wakabayashi, 1990). Hence, the occurrence of edwardsiellosis in Japan might be caused by the population identified as group 1. In other words the strains of group 2 might not be involved in the occurrence of this disease. Recently, Itou *et al.* (1997) reported that H₂O₂ played an important role in the bactericidal processes of Japanese eel neutrophils, and that catalase inhibited their bactericidal activities. Indeed, it was demonstrated that strong catalase activities provided pathogens with resistance against the bactericidal activities of neutrophils and macrophages. Because the catalase activities of group 1 strains were higher than those of group 2, catalase activities of *E. tarda* might contribute as one of the virulence factors against fish.

Further studies are in progress on the sequencing of the 16S rDNA and the FeSOD gene to clarify the genetic differences and the phylogenetic distance among *E. tarda* populations, and the role of catalase for the pathogenicity is also investigating.

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