Characterization of *Edwardsiella ictaluri* Isolated from Wild Ayu *Plecoglossus altivelis* in Japan

Takahiro Nagai^{1, 2*}, Emi Iwamoto², Takamitsu Sakai³, Taeko Arima⁴,
Kozue Tensha⁵, Yoshisuke Iida¹, Takaji Iida³ and Toshihiro Nakai²

¹Fisheries and Marine Technology Center, Hiroshima Prefectural Technology Research Institute,
Kure, Hiroshima 737-1207, Japan

²Graduate School of Biosphere Science, Hiroshima University,
Higashi-Hiroshima, 739-8528, Japan

³National Research Institute of Aquaculture, Fisheries Research Agency, Minami-Ise,
Mie 516-0193, Japan

⁴Tokyo Metropolitan Islands Area Research and Development Center of Agriculture,
Forestry and Fisheries, Minato, Tokyo 105-0022, Japan

⁵Inland Sea Division, Yamaguchi Prefectural Fisheries Research Center,
Aio-Futashima, Yamaguchi 754-0893, Japan

(Received August 30, 2008)

ABSTRACT—A Gram-negative bacterium was isolated from diseased wild ayu *Plecoglossus altivelis*, which were caught in rivers in Japan from August to October in 2007. All four isolates examined showed the same morphological, physiological and biochemical characteristics and were classified into the genus *Edwardsiella*. The isolates were differentiated from *E. tarda* in respect of its negative production of indole and no growth at 37°C, and different from a reference strain of *E. ictaluri* (JCM1680) in its positive production of hydrogen sulfide. All isolates were completely identical in the partial nucleotide sequences of 16S rDNA, a type 1 fimbrial gene (*etfA*) and a heat shock protein gene (*dnaJ*), and these sequences showed high similarity (100%, 99.7% and 100%, respectively) with *E. ictaluri* but low similarity (99.7%, 92.5% and 87.3%, respectively) with *E. tarda*. Based on these phenotypic and genetic characteristics, the present isolates from ayu were identified as *E. ictaluri*.

Key words: *Edwardsiella ictaluri*, characterization, *Plecoglossus altivelis*, ayu

The genus *Edwardsiella* includes two fish pathogens, *E. tarda* and *E. ictaluri*, both of which have caused serious disease problems in fish culture industries. *E. tarda* (Ewing *et al.*, 1965), which was earlier called *Paracolobactrum anguillimortiferum* (Hoshina, 1962; Wakabayashi and Egusa, 1973; Sakazaki and Tamura, 1975), is the causative agent of edwardsiellosis in many cultured freshwater and marine fish species worldwide (Plumb, 1999; Muroga, 2001). In Japan, edwardsiellosis was first reported in cultured Japanese eel *Anguilla japonica* (Wakabayashi and Egusa, 1973), and then the disease has caused a serious disease problem not only in cultured eel but also in cultured marine fishes, particularly Japanese flounder *Paralichthys olivaceus* and red

sea bream *Pagrus major* (Kusuda *et al.*, 1977; Yasunaga *et al.*, 1982; Nakatsugawa, 1983). *E. ictaluri* (Hawke *et al.*, 1981) is the causative agent of enteric septicemia of catfish (ESC), the most important disease for economic loss in cultured channel catfish *Ictalurus punctatus* in the United States (Plumb, 1999). *E. ictaluri* was also isolated from some cultured freshwater catfish species in Thailand (Kasornchandra *et al.*, 1987), Vietnam (Crumlish *et al.*, 2002) and Indonesia (Yuasa *et al.*, 2003). However, there have been no reports on isolation of the bacterium in Japan.

In 2007, a Gram-negative bacterium was isolated from diseased wild ayu *Plecoglossus altivelis*, which were caught in rivers of three locations, Tokyo Metropolitan, Yamaguchi Prefecture and Hiroshima Prefecture, Japan (Sakai *et al.*, 2008), and the disease was diagnosed as *E. ictaluri* infection. This paper describes the

* Corresponding author
E-mail: t-nagai86032@pref.hiroshima.lg.jp

detailed phenotypic and genetic characteristics of the *E. ictaluri* strains isolated from diseased wild ayu.

Materials and Methods

Bacteria

Four strains, FPC1091, FPC1092, FPC1093 and PH-0744, used in this study are shown in Table 1. These were isolated from the kidney of diseased ayu, which were caught in rivers in Tokyo Metropolis, Yamaguchi Prefecture and Hiroshima Prefecture from August to October 2007 (Sakai *et al.*, 2008). Bacteria were cultured on Trypto-Soya agar (TSA; Nissui, Japan), and stored in heart infusion broth (HIB; Eiken, Japan) containing 10% glycerol at -80°C until used.

Table 1. Four bacterial strains used in this study.

Strain	Isolation		
	Organ	Location (Prefecture)	Date
FPC1091	Kidney	Yamaguchi	Sep. 12, 2007
FPC1092	Kidney	Tokyo	Aug. 14, 2007
FPC1093	Kidney	Tokyo	Aug. 14, 2007
PH-0744	Kidney	Hiroshima	Oct. 5, 2007

All strains were isolated from diseased ayu caught in rivers.

Bacteriological examinations

Bacterial cell morphology was examined by a light microscope with the Gram staining and by a scanning electron microscope (S-4300, Hitachi). Biochemical and physiological characterization tests were carried out by standard methods. In all the tests, the results were recorded after incubation at 25°C for a required period, unless cited otherwise. Motility was examined by two methods, the wet mount method with light microscopy and culture method with SIM medium (Nissui). *E. ictaluri* JCM1680 (= ATCC33202), *E. tarda* FK1051 (motile strain) isolated from diseased Japanese flounder and *E. tarda* E01-14 (non-motile strain) isolated from diseased red sea bream (Okuda *et al.*, 2007) were used as reference strains in motility, hydrogen sulfide (H_2S) production and indole production tests.

The optimum growth temperature of the strains was examined at various temperatures ranging from 10°C to 37°C . Bacteria were inoculated in HIB and incubated under shaking condition at 60 rpm, and the growth was monitored photometrically for 60 h.

Genetic analysis

Genomic DNA of the strains was extracted by a boiling method. One colony was suspended in $300\ \mu\text{L}$ TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and boiled for 5 min. After centrifugation ($10,000 \times g$, 10 min, 4°C), supernatant was used for polymerase chain reaction (PCR). Fragment of 16S rDNA was amplified using the

universal primer pair, 20F and 1500R (Weisburg *et al.*, 1991). Cycling conditions of the PCR were 94°C (30 s), 51°C (1 min), 72°C (1 min) for 30 cycles, which included an initial denaturation step (94°C , 5 min) and a final elongation step (72°C , 5 min). The *eftA* gene, one of the type 1 fimbrial gene clusters of *E. tarda*, was amplified using the primer set A (Sakai *et al.*, 2007). Cycling conditions were 94°C (20 s), 55°C (20 s), 72°C (1 min) for 30 cycles, which included an initial denaturation step (94°C , 5 min) and a final elongation step (72°C , 5 min). The *dnaJ* gene, one of the heat shock protein genes, was amplified using the primer pair, DN1-1F and DN1-2R (Nhung *et al.*, 2007). Cycling conditions were 94°C (30 s), 50°C (30 s), 72°C (1 min) for 35 cycles, which included an initial denaturation step (94°C , 3 min) and a final elongation step (72°C , 7 min). The *dnaJ* sequences of two *E. ictaluri* strains JF0208 (Yuasa *et al.*, 2003) and JCM1680 (= ATCC33202), nine *E. tarda* strains NE8003, E22, E381, TC165, TC166, NB8030, TC223, SU138, ATCC15947, one *E. hoshinae* strain ATCC33379 and *Erwinia aphidicola* IAM14479 as the out group were also determined to compare with those of the present strains.

The PCR products of 16S rDNA and the *eftA* were purified by the MinElute PCR purification kit (Quiagen, USA), and the PCR products of the *dnaJ* were cloned into a pGEM-T Easy vector (Promega, USA) using *Escherichia coli* JM109. Nucleotide sequences were determined with the BigDye terminator cycle sequencing kit (v.1.1, Applied Biosystems) and the 310 genetic analyzer or 3730 DNA analyzer (Applied Biosystems, USA). The sequence data obtained from the present strains were compared with the sequence data in the DDBJ databases using the BLAST algorithm, and the phylogenetic analyses based on the *dnaJ* sequences were carried out by the neighbor-joining (NJ) method using the MEGA4 software (<http://www.megasoftware.net/>).

Results and Discussion

All the four strains from diseased wild ayu showed the same characteristics in the morphological, physiological and biochemical tests (Table 2). They were Gram-negative, facultatively anaerobic, cytochrome oxidase-negative, catalase-positive short rods (Fig. 1). These and other characteristics indicated that the strains were classified into the genus *Edwardsiella*. All isolates grew at temperatures ranging from 10.0°C to 34.2°C but not at 37°C , with the optimum growth temperature at approximately 30°C . The representative result with PH-0744 is shown in Fig. 2.

Two species in the genus *Edwardsiella*, *E. ictaluri* and *E. tarda*, are known as fish pathogens, which can be differentiated from each other biochemically by the production of indole and H_2S , where both were positive for

Table 2. Morphological, physiological and biochemical characteristics of the present strains, *Edwardsiella ictaluri* and *E. tarda*.

Characteristic	Present strains	<i>E. ictaluri</i> ^{*1}	<i>E. tarda</i> ^{*1}
Gram stain	—	—	—
Form	Short rod	Short rod	Short rod
Motility at 25°C	+ ^{*2}	+	+
Growth at 37°C	—	±	+
NaCl tolerance			
1.0%	+	+	+
1.5%	+	+	+
4.0%	—	—	+
Cytochrome oxidase	—	—	—
Catalase	+	+	+
OF test	F	F	F
H ₂ S	+ ^{*3}	—	+
Indole	—	—	+
Methyl red test	+	—	+
Voges-Proskauer test	—	—	—
Citrate	— ^{*4}	— ^{*5}	— ^{*5}
Arginine dihydrolase	—	—	—
Lysine decarboxylase	+	+	+
Ornithine decarboxylase	+	+	+
Gas from glucose	+	+	+
Acid production from			
D-mannose	+	+	+
Maltose	+	+	+
D-mannitol	—	—	—
Sucrose	—	—	—
Trehalose	—	—	—
L-arabinose	—	—	—

^{*1} Plumb (1999) partly modified.

^{*2} Weak motility was observed in SIM medium at 25°C, but not by the wet mount method under light microscopy.

^{*3} Weak H₂S production was observed in SIM medium at 25°C.

^{*4} Simmon's.

^{*5} Christensen's.

Fig. 1. Gram-negative short rod (PH-0744 strain) isolated from diseased wild ayu. (A) Light microscopy (Gram staining), (B) Scanning electron microscopy.

E. tarda and both negative for *E. ictaluri* (Plumb, 1999). *E. tarda* grows well at 37°C, whereas *E. ictaluri* shows poor or no growth at 37°C (Plumb, 1999). *E. ictaluri* is biochemically less active than other *Edwardsiella* species, and it appears to be homogeneous (Waltman *et al.*, 1986). The characteristics of

the present strains were clearly different from those of *E. tarda* in points of negative production of indole and no growth at 37°C, and were different from *E. ictaluri* in its positive but weak production of H₂S and positive reaction in methyl red (MR) test (Table 2). Neither motility by the wet mount method under light microscopy nor flagel-

lation by electron microscopy was observed (Fig. 1B). However, when the cultures of SIM at 25°C were compared with the non-motile *E. tarda* strain, slightly spreading growth from inoculated area was observed in the present strains and *E. ictaluri* JCM1680 strain (Fig.

Fig. 2. Effect of temperature on the growth of the strain PH-0744. Bacteria were cultured in heart infusion broth by shaking (60 rpm) at 10–37°C, and the growth after 14 h- (●) and 60 h- incubation (○) was shown as optical density (OD) at 660 nm.

Fig. 3. Motility, H₂S and indole tests in SIM medium of *E. tarda* and *E. ictaluri* strains. Bacteria were cultured at 25°C for 48 h. (A) for motility, (B) for H₂S and indole production (Ehrlich-Böhne reagent-I), (C) for indole production 10 min after addition of Ehrlich-Böhne reagent-II. 1: *E. tarda* FK1051, 2: *E. tarda* E01-14, 3: *E. ictaluri* JCM1680, 4-7: the present strains, PH-0744, FPC1091, FPC1092, FPC1093.

3A), suggesting weak motility. A slight black coloration indicating production of H₂S was found in the center of the inoculated SIM medium after 48 h or longer inoculation at 25°C, but not in *E. ictaluri* JCM1680 strain (Fig. 3A and B). The coloration around the limited area was quite similar to that of the non-motile *E. tarda* strain. Waltman *et al.* (1986) reported that *E. ictaluri* exhibited no H₂S production in SIM medium at any incubation temperatures of 20°C, 30°C and 37°C, but H₂S production was observed in some isolates using lead acetate paper. These results suggest that H₂S productivity is not a key character to differentiate between *E. tarda* and *E. ictaluri*. Both the present strains and *E. ictaluri* JCM1680 strain gave negative result in the indole production test with SIM medium (Fig. 3B and C). The present strains were positive in the MR test, but there is also discrepancy in the MR test between Waltman *et al.* (1986) and Plumb (1999). Although the present strains were closely similar to the reported *E. ictaluri* strains on the biochemical characteristics, further comparative examinations with reference strains of *E. ictaluri* are required on motility, H₂S production, MR test and other characteristics in order to understand diversity among *E. ictaluri* strains.

The similarities of nucleotide sequences between the present strains and *Edwardsiella* species are shown in Table 3. All the strains were completely identical in the partial nucleotide sequences of 16S rDNA, the *etfA* and *dnaJ* genes. The 16S rDNA (1426 bp) of the isolates showed 100% similarity to that of *E. ictaluri* (DDBJ accession no. AB050826) and 99.7% similarity to that of *E. tarda* (DDBJ accession no. AB050832). Because the sequences of 16S rDNA were very close between *E. ictaluri* and *E. tarda*, the *etfA*, one of type 1 fimbrial gene

Table 3. Similarities in the partial sequences of 16S rDNA, *etfA* and *dnaJ* among the present strains, *E. ictaluri* and *E. tarda*.

Gene (target size)	Bacteria	Percent similarity to the present strains (FPC1091, FPC1092, FPC1093, PH-0744)
16S rDNA (1426 bp)	<i>E. ictaluri</i> (AB050826 ^{*1})	100
	<i>E. tarda</i> (AB050832 ^{*1})	99.7
<i>etfA</i> (372 bp)	<i>E. ictaluri</i> (AY626368 ^{*1})	99.7
	<i>E. tarda</i> (AF491964 ^{*1})	92.5
<i>dnaJ</i> (719 bp)	<i>E. ictaluri</i> JF0208 ^{*2}	100
	<i>E. tarda</i> ATCC15947	87.3

^{*1} Accession no. in DDBJ.

^{*2} Catfish strain (Yuasa *et al.*, 2003).

Fig. 4. Phylogenetic relationships of the genus *Edwardsiella* and present strains. The phylogenetic tree was inferred from the *dnaJ* sequences by the neighbor-joining (NJ) method. Bootstrap probabilities (%) are shown on the internal branches. The origins of the strains are shown in parentheses following strain name.

clusters of *E. tarda*, which were involved in the pathogenesis (Sakai *et al.*, 2007), and the *dnaJ*, heat shock protein gene (Nhung *et al.*, 2007), were compared for further genetic analysis. The nucleotide sequences of the *etfA* (372 bp) of the strains showed 99.7% similarity to that of *E. ictaluri* (DDBJ accession no. AY626368), while the similarity was lower (92.5%) to that of *E. tarda* (DDBJ accession no. AF491964). The nucleotide sequences of the *dnaJ* (719 bp) of the strains showed 100% similarity to that of *E. ictaluri* JF0208, while the similarity was lower (87.3%) to that of *E. tarda* ATCC15947. Phylogenetic tree inferred from the *dnaJ* sequences is shown in Fig. 4. The present strains were included in the cluster of *E. ictaluri*, which was clearly separated from the clusters of *E. tarda*. Therefore, the strains are genetically closely related to *E. ictaluri*. The nucleotide sequence data obtained in this study appear in the DDBJ database under accession numbers AB453281, AB453282 and AB454422–AB454435. From these phenotypic and genetic characterization tests, it was concluded that the present ayu isolates can be identified as *E. ictaluri*.

As cited before, ESC has a fairly limited geographical distribution, the United States and Southeast Asian countries, and furthermore *E. ictaluri* has a considerably narrow host range than other warm-water fish pathogenic bacteria (Plumb, 1999). *E. ictaluri* has been mostly isolated from affected catfish species; channel catfish *Ictalurus punctatus*, blue catfish *I. furcatus*, white catfish *Ameiurus catus*, brown bullhead *A. nebulosus*,

walking catfish *Clarias batrachus* and striped catfish *Pangasius hypophthalmus* (Plumb, 1999; Crumlish *et al.*, 2002), except for a few ornamental fishes (Humphrey *et al.*, 1986; Plumb, 1999) and one salmonid fish, rainbow trout *Oncorhynchus mykiss* (Keskin *et al.*, 2004). Therefore, ayu in the present study is a new host species of *E. ictaluri*.

E. ictaluri infection of fish in Japan was first found in wild populations of ayu in 2007, as described in Sakai *et al.* (2008). No mass mortalities by *E. ictaluri* infection have been recorded in cultured populations of ayu before and after the above occurrence. Considering that new diseases are usually noticed first in cultured fishes and *E. ictaluri* has a narrow host range, the present *E. ictaluri* infection in wild ayu seems to be strange. The routes, from where *E. ictaluri* was introduced into Japanese rivers, are remained unknown, though there are some speculative ideas. Further detailed epidemiological and pathological studies on the infection and the agent are required to know the transmission route and a possibility that *E. ictaluri* infection spreads to other domestic freshwater fishes living in Japanese rivers, particularly catfish species.

Acknowledgements

We thank Dr. S. Tanaka and Dr. K. Yuasa for providing *E. tarda* strain (E01-14) and *E. ictaluri* strain (JF0208), respectively, used in this study. We also thank Dr. H. Takayama for his help on the observation of

bacterial strain by SEM. This study was supported in part by a special grant from the Japan Fisheries Resource Conservation Association.

References

- Crumlish, M., T. T. Dung, J. F. Turnbull, N. T. N. Ngoc and H. W. Ferguson (2002): Identification of *Edwardsiella ictaluri* from diseased freshwater catfish, *Pangasius hypophthalmus* (Sauvage), cultured in the Mekong Delta, Vietnam. *J. Fish Dis.*, **25**, 733–736.
- Ewing, W. H., A. C. McWhorter, M. R. Escobar and A. H. Lubin (1965): *Edwardsiella*, a new genus of *Enterobacteriaceae* based on a new species, *Edwardsiella tarda*. *Int. Bull. Bact. Nom. Taxon.*, **15**, 33–38.
- Hawke, J. P., A. C. McWhorter, A. C. Steigerwalt and D. J. Brenner (1981): *Edwardsiella ictaluri* sp. nov., the causative agent of enteric septicemia of catfish. *Int. J. Syst. Bacteriol.*, **31**, 396–400.
- Hoshina, T. (1962): On a new bacterium, *Paracolobactrum anguillimortiferum* n. sp. *Bull. Japan. Soc. Sci. Fish.*, **28**, 162–164.
- Humphrey, J. D., C. Lancaster, N. Gudkovs and W. McDonald (1986): Exotic bacterial pathogens *Edwardsiella tarda* and *Edwardsiella ictaluri* from imported ornamental fish *Beta splendens* and *Puntius conchoni*, respectively: isolation and quarantine significance. *Aust. Vet. J.*, **63**, 369–371.
- Kasornchandra, J., W. A. Rogers and J. A. Plumb (1987): *Edwardsiella ictaluri* from walking catfish, *Clarias batrachus* L., in Thailand. *J. Fish Dis.*, **10**, 137–138.
- Keskin, O., S. Seğer, M. İzgür, S. Türkyilmaz and R.S. Mkakosya (2004): *Edwardsiella ictaluri* infection in rainbow trout (*Oncorhynchus mykiss*). *Turk. J. Vet. Anim. Sci.*, **28**, 649–653.
- Kusuda, R., T. Itami, M. Munekiyo and H. Nakajima (1977): Characteristics of a *Edwardsiella* sp. from an epizootic of cultured crimson sea breams. *Bull. Japan. Soc. Sci. Fish.*, **43**, 129–134. (In Japanese with English abstract)
- Muroga, K. (2001): Viral and bacterial diseases of marine fish and shellfish in Japanese hatcheries. *Aquaculture*, **202**, 23–44.
- Nakatsugawa, T. (1983): *Edwardsiella tarda* isolated from cultured flounder. *Fish Pathol.*, **18**, 99–101. (In Japanese with English abstract)
- Nhung, P. H., K. Ohkusu, N. Mishima, M. Noda, M. M. Shah, X. Sun, M. Hayashi and T. Ezaki (2007): Phylogeny and species identification of the family *Enterobacteriaceae* based on *dnaJ* sequences. *Diagn. Microbiol. Infect. Dis.*, **58**, 153–161.
- Okuda, J., F. Murayama, E. Yamanoi, E. Iwamoto, S. Matsuoka, M. Nishibuchi and T. Nakai (2007): Base changes in the *fliC* gene of *Edwardsiella tarda*, possible effects on flagellation and motility. *Dis. Aquat. Org.*, **76**, 113–121.
- Plumb, J. A. (1999): Catfish bacterial diseases. In "Health maintenance and principal microbial diseases of cultured fish". Iowa State University Press, Iowa, pp. 181–209.
- Sakai, T., T. Iida, K. Osatomi and K. Kanai (2007): Detection of type 1 fimbrial genes in fish pathogenic and non-pathogenic *Edwardsiella tarda* strains by PCR. *Fish Pathol.*, **42**, 115–117.
- Sakai, T., T. Kamaishi, M. Sano, K. Tensha, T. Arima, Y. Iida, T. Nagai, T. Nakai and T. Iida (2008): Outbreaks of *Edwardsiella ictaluri* infection in Ayu *Plecoglossus altivelis* in Japanese Rivers. *Fish Pathol.*, **43**, 152–157.
- Sakazaki, R. and K. Tamura (1975): Priority of the specific epithet *anguillimortiferum* over the specific epithet *tarda* in the name of the organism presently known as *Edwardsiella tarda*. *Int. J. Syst. Bacteriol.*, **25**, 219–220.
- Wakabayashi, H. and S. Egusa (1973): *Edwardsiella tarda* (*Paracolobactrum anguillimortiferum*) associated with pond-cultured eel diseases. *Bull. Japan. Soc. Sci. Fish.*, **39**, 931–939.
- Waltman, W. D., E. B. Shotts and T. C. Hsu (1986): Biochemical characteristics of *Edwardsiella ictaluri*. *Appl. Environ. Microbiol.*, **51**, 101–104.
- Weisburg, W. G., S. M. Barns, D. A. Pelletier and D. J. Lane (1991): 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.*, **173**, 697–703.
- Yasunaga, N., S. Ogawa and K. Hatai (1982): Characteristics of the fish pathogen *Edwardsiella* isolated from several species of cultured marine fishes. *Bull. Nagasaki Pref. Inst. Fish.*, **8**, 57–65. (In Japanese with English abstract)
- Yuasa, K., E. B. Kholidin, N. Panigoro and K. Hatai (2003): First isolation of *Edwardsiella ictaluri* from cultured striped catfish *Pangasius hypophthalmus* in Indonesia. *Fish Pathol.*, **38**, 181–183.