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Characterization of the unusual non-thiosulfate-reducing *Edwardsiella tarda* isolated from eel (*Anguilla japonica*) farms

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ABSTRACT

Unusual *Edwardsiella tarda*, that do not produce hydrogen sulfide (H_2S) from thiosulfate, was isolated from diseased eels (*Anguilla japonica*) in varying farming sites. In this study, we examined the biochemical characteristics of H_2S production from the bacterium *E. tarda*. Diseased eels, along with pond water, were sampled from eight eel farms. All isolates were used for biochemical characterization and 16S rRNA and fimbrial gene sequencing. The *phs* gene of some isolates were also sequenced. H_2S production was analyzed from either thiosulfate ($S_2O_3^{2-}$)- or sulfite (SO_3^-)-containing media. DNA analysis identified all isolates as *E. tarda*. Of the 17 *E. tarda* isolates, 11 were unable to reduce thiosulfate to H_2S, while all isolates produced H_2S from sulfite. In addition, non-thiosulfate-reducing *E. tarda* were isolated from four of the eight sampled eel farms. In media containing sulfate (SO_4^{2-}) and in media without thiosulfate or sulfite, no H_2S production was observed. In addition, glucose and galactose appeared to repress thiosulfate reduction in all strains, whereas these different carbon sources did not affect sulfite reduction. This indicates that thiosulfate and sulfite reduction pathways do not overlap in *E. tarda*. For this reason, careful attention should be made when identifying or differentiating non-thiosulfate-reducing *E. tarda* using H_2S production characteristics.

1. Introduction

The genus *Edwardsiella* is within the family *Enterobacteriaceae* and is composed of three species: *Edwardsiella hoshinae*, *Edwardsiella ictaluri*, and *Edwardsiella tarda* (Sakazaki, 2001). The bacterium *E. 1ictaluri* is often associated with causing enteric septicemia in channel catfish (Hawke et al., 1981). *E. tarda* is the causative agent of edwardsiellosis in freshwater and marine fish (Thune et al., 1993) as well as a human pathogen (Janda and Abbott, 1993). A number of eel disease cases have been associated with *E. tarda* in Japan and Korea (e.g., Joh et al., 2010; Park et al., 1983). Although the first edwardsiellosis reported by Alcaide et al. (2006) originated from European eels (*Anguilla anguilla*) in Spain, the emergence of *E. tarda* still seems to be confined within the USA, Japan and Korea (Hah et al., 1984; Meyer and Bullock, 1973; Wakabayashi and Egusa, 1973).

In clinical laboratories, one of the most often used phenotypic traits for the differentiation of *Enterobacteriaceae* members is the production of sulfides, which, in many cases, includes the formation of bacterial colonies with black precipitates or centers. For instances, media using this phenotypic character are commercially available and widely used to isolate *E. tarda* in laboratories (e.g., Leotta et al., 2009; Xiao et al., 2009). These media include motility indole lysine sulfide medium, lysine iron agar, Kligler iron agar, peptone iron agar, sulfide-indole-motility (SIM) medium, Triple sugar iron agar, Hektoen enteric agar (HEA), and Salmonella Shigella (SS) agar, all of which contain sulfur and iron compounds.

E. tarda showed little phenotypic variability, although they are captured from two different continents, Asia and North America (Austin and Austin, 2007). Thus, *E. tarda* is easy to identify clinically based on the production of lysine, ornithine decarboxylase, and hydrogen sulfide (H_2S), and the inability to ferment most carbohydrates (Walton et al., 1993). H_2S may be generated through the degradation of sulfur-containing amino acids or reduction of inorganic sulfur compounds such as elemental sulfur, sulfite, thiosulfate and sulfate (Linderholm et al., 2008). Despite the importance of H_2S production as a criterion in terms of *E. tarda* identification, understanding of the biochemistry underlying H_2S production in this pathogen is limited. This study describes an unusual strain of *E. tarda* with the ability to produce H_2S from sulfite (SO_3^-), but not from thiosulfate ($S_2O_3^{2-}$).

2. Materials and methods

2.1. Samplings of diseased eels and eel pond water

Diseased eels (*Anguilla japonica*) or eel pond water, which were kept at 24–26 °C regardless of season, were sampled from a total of

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eight eel farms in different locations throughout the Republic of Korea at different sampling time points (Jan., Feb., Mar. and Jun.) in 2010. At each sampling site, including 2 or 3 different farms, 3–5 diseased eels and/or 1 l of pond water were obtained.

The most common clinical signs of a diseased eel observed in this study were hemorrhages around the anus and abdominal fin. Occasionally, some fish exhibited poorly pigmented convex swollen lesions filled with abscesses on the body surface posterior to the pectoral fin (Fig. 1). Internal signs of disease were ascitic fluid and liver and intestine hemorrhage. Some fish showed severely damaged livers, revealing hemorrhage and perforation (Fig. 1).

2.2. Bacterial isolation and strains used in this study

E. tarda were isolated from the kidney, spleen or liver of diseased fish using the brain heart infusion agar (BHIA; Difco, Sparks, MD, USA) containing 1% (w/v) sodium chloride and SS medium (Difco, Sparks, MD, USA). The plates were incubated at 25 °C for 48 h, after which representative colonies were selected and streaked out to obtain single pure cultures. For eel-containing tank water, three 10-fold serial dilutions of the water were spread on SS agar and incubated at 25 °C for 48 h. Different colonies, including hydrogen sulfide producing colonies determined by the colony morphology, were selected and purified by streaking and re-streaking on fresh media.

A total of 21 *E. tarda* isolates and reference strains were used in this study and are listed in Table 1. *E. tarda* KE1 and ET82015, originated from diseased olive flounder and eel, respectively, were used as reference strains. *E. ictaluri* ATCC 33202 was also used for comparison.

2.3. Genotypic and phenotypic characterization

DNA was extracted from all bacteria isolated from the eel tank water and from the internal organs of the diseased eels. Bacterial genomic DNA was isolated using the Genomic DNA extraction kit (Bioneer, Deajeon, Korea), following the manufacturer's protocol. The DNA templates were amplified using the polymerase chain reaction (PCR) method on a Bioneer MyGenie' 96 Gradient Thermal Block (Daejeon, Korea), using universal primers that amplified a region of the 16S rRNA gene (Table 1) obtained from Bioneer (Daejeon, Korea). The DNA templates were amplified by initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 90 s, and a final extension at 72 °C for 5 min. The upstream regions of the fimbrial gene cluster were also amplified and sequenced using the primers presented in Table 1. PCR amplification was conducted according to a previously described method (Sakai et al., 2009). Furthermore, phs-F and -R primers were used to amplify the *phs* operon (phcA, phcB, and phcC), which encodes the thiosulfate reductase, of bacteria (KE1, ETW11, ETW12, and ETW31) under the following conditions: 30 cycles of denaturation at 94 °C for 90 s, annealing at 58 °C for 30 s, extension at 72 °C for 3 min, and a final extension at 72 °C for 5 min. Amplicons of the *phs* gene (=3873 bp) of a reference strain (KE1) and an isolate (ETW11) were sequenced using the following primers (phsF1 = CGCCTGTCCGGCGCTAAACT; phsF2 = ACTCTGCACCTCCGCCGACT; phsF3 = GTCTCGCCGGCCAGCTTGTT; phsF4 = AAGAACGACGACTGATGCTC; phsR1 = ACGCCGGACTACTGGCCACA; phsR2 = AACAAGCTGGCCGGCGAGAC). Sequencing of the amplified DNA fragment was performed using the fD1 primer, and an automatic sequencer (Applied Biosystems 3730xl DNA Analyzer). All sequences were submitted to similarity searches using the BLAST program. Representative sequences were deposited in GenBank under accession numbers (HQ852206–HQ852222 for 16S rRNA gene; JQ625503 and JQ625504 for fimbrial gene). All the isolates were tested for biochemical characteristics using the API 20E kit (bioMérieux, Marcy I'Etoile, France). SIM medium was used for indole production and motility.

2.4. Production of hydrogen sulfide

H_2S production was detected using different media containing either thiosulfate or sulfite. Sodium thiosulfate ($Na_2S_2O_3$) and ferrous or ferric compounds are indicators of H_2S production in SS agar, triple sugar iron (TSI) agar (Difco, USA) and SIM medium (Difco, USA) and the API 20E kit. Nutrient agar supplemented with 1 mM $FeCl_2$ and either 6 mM $Na_2S_2O_3$, 12 mM Na_2SO_3, 12 mM Na_2SO_4 or 0.2% of L-cysteine (Sigma; St. Louise, MO, USA) was also used for the detection of H_2S production. Furthermore, we used nutrient agar (plus the ferrous and sulfur compounds) containing 5.6 mM glucose or galactose and 10 mM potassium nitrate (KNO_3) to assess the effects of the carbon source and electron acceptor on H_2S production (Table 3).

A pure culture of *E. tarda* isolates or strains were spread on petridishes of SS agar, and inoculated using an inoculating needle by

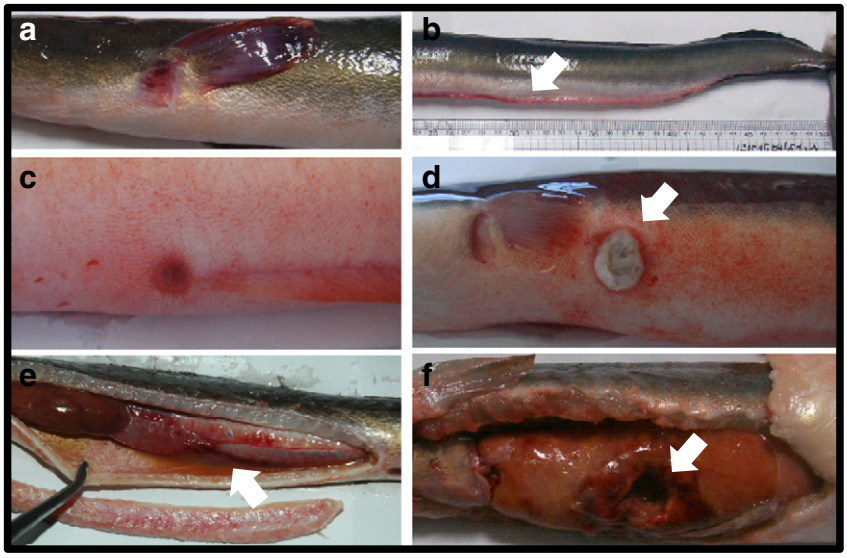

Fig. 1. Clinical symptoms in eel (*Anguilla japonica*), from which *Edwardsiella tarda* was isolated. (a) and (b), hemorrhage of the pectoral and anal fins; (c), hemorrhage around the anus and on the abdomen; (d), poorly pigmented convex swollen lesion (arrow) filled with abscesses on the body surface of the eel; (e), ascitic fluid (arrow) in the intraperitoneal cavity and congested liver; and (f), severely damaged (perforated) liver with hemorrhages.