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Large antibiotic-resistance plasmid of *Edwardsiella tarda* contributes to virulence in fish☆

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ABSTRACT

Edwardsiella tarda, an enteric gram negative bacterium, infects a wide range of fish and causes a systemic fish disease called edwardsiellosis. *E. tarda* CK41, isolated from Japanese flounder diagnosed with edwardsiellosis, has exhibited a high degree of resistance to multiple antibiotics, including kanamycin, tetracycline, streptomycin, among others. As the bacterial antibiotic-resistance genes are usually contained in plasmids, we hypothesized that *E. tarda* CK41 may harbor one or more plasmids for antibiotic resistance. We showed the existence of plasmids in *E. tarda* CK41, and the size of the plasmid, designated as pCK41, was estimated to be approximately 70 kb. *Escherichia coli* DH5α transformed by the pCK41 plasmid exhibited an antibiotic-resistance phenotype against kanamycin (30 μg/mL), tetracycline (30 μg/mL), and streptomycin (10 μg/mL), indicating the existence of at least 3 antibiotic-resistance genes in pCK41. Through a procedure for pCK41 plasmid curing, a plasmid-cured strain, designated as *E. tarda* CK108, was identified, which was unable to grow in the presence of either kanamycin or tetracycline. As virulence-associated genes are occasionally encoded in bacterial plasmids, we examined the virulence of *E. tarda* CK108 in Japanese flounder. The virulence of plasmid-cured *E. tarda* CK108 was lower (survival rate 80%) than that of CK41 (20%), indicating the existence of virulence-associated genes in pCK41. The strain also appeared to be attenuated in both goldfish and zebrafish pathogenesis models. To analyze genes for antibiotic resistance and virulence in pCK41, the entire nucleotide sequences of pCK41 were determined (GenBank accession number: HQ332785). A total of 84 open reading frames (ORFs) were annotated. The pCK41 plasmid consists of potential virulence genes, transposases, plasmid maintenance genes, antibiotic-resistance genes (including kanamycin, tetracycline, and streptomycin), conjugal transfer genes, and unknown ORFs. These results suggest that pCK41 is a virulence plasmid of substantial importance in the *E. tarda* pathogenesis to fish.

1. Introduction

Edwardsiella tarda is a gram negative bacterium in the family of Enterobacteriaceae and causes a hemorrhagic septicemia in fish, called edwardsiellosis [1]. Edwardsiellosis is often associated with poor water quality and stress [2] and causes high mortality and severe economic losses in farmed fish, such as flounders [3], eels [4], chinook salmon [5], and catfish [6]. It has a broad host range, causing disease in higher vertebrates such as reptiles, birds, and mammals including humans [7]. This broad host range suggests variation of serotypes in *E. tarda*. *E. tarda* has been differentiated according to the O-antigen in lipopolysaccharide, and there are 61 different *E. tarda* serotypes [8].

Natural infection in fish is thought to occur through waterborne contact with *E. tarda* [1]. Susceptible fish species may take up *E. tarda* through the skin or gills, or orally [9]. Disease signs may include extensive skin lesions that progress into necrotic abscesses, distended abdomen and swollen anus due to the accumulation of ascetic fluid, pigment loss, enlarged kidney, and abscesses on internal organs [1,6].

Although very little is known about the virulence mechanisms of *E. tarda*, the pathogenesis is known to be multifactorial [10]. Several potential virulence factors have been reported, including the ability to invade epithelial cells [11,12], resist serum [7], and produce substances such as hemolysins [13], catalases [14], and toxins [15] for disseminating infection. A type III secretion system

Abbreviations: DAP, diaminopimelic acid; IP, intraperitoneal; LB, Luria–Bertani broth; NFRDI, National Fisheries Research and Development Institute; ORF, open reading frame; QPCR, quantitative PCR.

☆ GenBank id: HQ332785.

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(TTSS) [16,17], type VI secretion system (T6SS) and a putative secretion system were discovered to play important roles in *E. tarda* pathogenesis [18].

To prevent or relieve edwardsiellosis, antibiotic agents have generally been used in aquaculture. The massive use of antibiotic agents in commercial fish escalates the selective pressure on microbial flora and promotes the natural increase in antibiotic resistance. Not only can resistant bacteria multiply after an antibiotic has suppressed sensitive bacteria, but they can also transfer their resistance genes to other bacteria that have never been exposed to the antibiotics [19]. Furthermore, antibiotic resistance has been shown to be an adaptive mechanism contributing to bacterial virulence [20]. Antibiotic resistance and virulence genes are being located in bacterial chromosomes as well as on plasmids, associated in gene clusters to form resistance or pathogenicity islands [21].

The majority of pathogenicity studies for *E. tarda* are antibiotic susceptibility studies and plasmid analyses [22]. Plasmids isolated from *E. tarda*, containing resistance genes to sulphonamide and tetracycline jointly, or to sulphonamide, tetracycline, streptomycin, kanamycin, and chloramphenicol together, have been reported [23,24]. In this study, we identified and characterized a large plasmid in *E. tarda* CK41. After curing of the plasmid, we were able to investigate the contribution of the plasmid to *E. tarda* virulence. We herein report the 72.8 kb sequence and annotation of the large plasmid, designated as pCK41, from *E. tarda* CK41. The plasmid contained putative genes for virulence and antibiotics resistance. The plasmid-cured *E. tarda* CK108 strain exhibited increased LD_{50} in fish infection models compared with the parental strain CK41, indicating that pCK41, a virulence plasmid, plays a role in the *E. tarda* infection in fish.

2. Materials and methods

2.1. Bacterial strains, primers, and media

The bacterial strains and primers used in this study are listed in Table 1. *E. tarda* CK41 is a pathogenic bacterium, isolated from diseased flounder, provided by the National Fisheries Research and Development Institute (NFRDI) in the Republic of Korea. The purified plasmid pCK41 was transferred to and maintained in *Escherichia coli* DH5α. All strains were grown in Luria–Bertani broth (LB) or LB agar at 37 °C (for *E. coli*) or 25 °C (for *E. tarda*). Antibiotics, when required, were added to the culture medium in the following concentrations; tetracycline, 15 μg/mL; kanamycin, 50 μg/mL. Diaminopimelic acid (DAP) was added (50 μg/mL) for the Asd^- strains. For the fish infection experiments, bacterial cells grown in fresh media were harvested by centrifugation (5000 × *g*, 10 min, 25 °C), and resuspended in PBS. Bacterial concentrations were adjusted by spectrophotometer and confirmed by viable count after serial dilution.

2.2. Plasmid curing

The modified method described by Guerry and Colwell was adopted [25] for the curing of plasmids using novobiocin (Sigma, St. Louis, MO, USA) [26]. To determine the optimum concentration of novobiocin for *E. tarda*, serially diluted novobiocin from 50 μg/mL to 1.6 μg/mL was added to *E. tarda* culture in LB broth. The highest concentration of novobiocin permitting bacterial growth was used in the plasmid curing procedures. *E. tarda* cells inoculated into LB broth containing 25 μg/mL novobiocin were cultured up to OD_{600} 0.6 at 37 °C with aeration. After this SDS was added into the culture to a final concentration of 1%, and the culture temperature was shifted to 42 °C for 2 h. During this process a few cells may lose their plasmid and switch to the antibiotic sensitive phenotype. The plasmid-cured strains were screened by comparison of bacterial growth on LB agar with and without antibiotics.

Table 1
Bacterial strains, plasmids and primers used in this study.

Strains or primers	Characteristics or sequences[a]	Reference
Bacterial strains		
Edwardsiella tarda CK41	Wild type (isolates from diseased flounder), Km^R, Tet^R, Sm^R, Cm^S	NFRDI[b]
Edwardsiella tarda CK108	CK41 derivative, pCK41 cured	This study
Edwardsiella tarda CK215	CK108 derivative, Δasd::km, Km^R	Lab collection
Escherichia coli DH5α	Transformation host, F^-, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-, mk^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1	Promega
Plasmids		
pYA3342	pBR322ori, Asd^+	[29]
pBP884	5771 bp DNA fragment harboring the BOX 2 region of pCK41 in pYA3342	This study
pBP945	5706 bp DNA fragment harboring the BOX 1 region of pCK41 in pYA3342	This study
Primers		
HY726	aacgggttagcgttcatcac	This study
HY727	cgggattatggttctgatgg	This study
HY728	acgttaagatcaccggttcg	This study
HY729	gaacgacagttccagggtgt	This study
HY783 (*Bam*HI)	gaattcctcattccccgcttgt	This study
HY784 (*Pst*I)	ctgcagcttcacgctggcagaa	This study
HY866 (*Bam*HI)	ggattcggtggcagagtttatagcc	This study
HY867 (*Pst*I)	ctgcagtgcagcggttttgatagta	This study

[a] Underlines indicate restriction enzyme site for the enzyme indicated in parentheses of the primer names.
[b] The National Fisheries Research and Development Institute (NFRDI) in the Republic of Korea.

2.3. Fish

Japanese flounder (*Paralichthys olivaceus*) were monitored at the NFRDI facilities in the Republic of Korea. Goldfish (*Carassius auratus*) and zebrafish (*Danio rerio*) were monitored at the Laboratory of Bacterial Pathogenesis facilities, Pusan National University. All fish were obtained from commercial fish farms and maintained in aerated tanks supplied with static susceptible water at 25 °C. All fish were anesthetized with tricaine (ethyl 3-aminobenzoate methanesulfonate salt, Sigma) prior to experiments involving injection or sacrifice. Commercially available feeds were used to feed fish. The antibiotic contamination of feeds was checked before use.

2.4. DNA manipulation and nucleotide sequence determination

DNA manipulation was carried out as described by Sambrook and William Russell [27]. To purify plasmids, *E. tarda* CK41 was cultivated overnight in LB containing kanamycin and tetracycline, and the plasmid DNA was prepared using the modified methods of Sambrook and William Russell with the treatment of alkaline solution and SDS [27]. Extracted plasmid fraction was subjected to CsCl gradient ultracentrifugation to get pure plasmid. Residual sheared chromosome DNA fragments were removed by treating BAL31 nuclease (New England Biolabs, Beverly, MA, USA). Some of the purified plasmids were used for the *E. coli* transformation. The complete nucleotide sequence of the plasmid was determined by Solgent™ (Daejeon, Korea) using the Roche GS-FLX system. A total of 7,630,563 bp (average read length 335.7 bp) was obtained resulting in 99-fold coverage of the plasmid. Assembly was performed and produced 25 contigs ranging from 318 bp to 14,015 bp.