

Novel type 4 secretion system (T4SS)-related genes of *Edwardsiella tarda*

Nuevos genes relacionados con el sistema secretor tipo 4 de *Edwardsiella tarda*

Novo genes associados com o sistema secreção tipo 4 (T4SS) de *Edwardsiella tarda*

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Recibido: noviembre 02 de 2013 **Aceptado:** agosto 16 de 2015

Summary

Edwardsiella tarda is a Gram-negative bacterium that causes edwardsiellosis, a disease of fish and mammals including humans and characterized by multiple abscesses. Different strains of *E. tarda* possess a number of virulence, antibiotic-resistance, and toxin secretion system-related genes that explain in some extent its capacity to survive within phagocytic cells and to infect a variety of hosts. Previously we introduced a virulent *E. tarda* strain (ETSJ54) isolated from Japanese flounder (*Paralichthys olivaceus*) with edwardsiellosis and reported a number of virulence-related genes. In this study we have re-analyzed the sequencing data of ETSJ54 and identified novel type IV secretion system-related genes, most of them were flanked by transposase and plasmid encoding genes. Interestingly, their nucleotide sequence had no identity to those of the genes published in the *E. tarda* EIB202 genome, a virulent strain isolated from turbot (*Scophthalmus maximus*) in China. The results suggest differences in gene content between geographically distinct *E. tarda* strains that may encourage additional *E. tarda* genome sequencing projects.

Keywords: Pathogenesis, T4SS genes, Virulence.

Resumen

Edwardsiella tarda es una bacteria Gram-negativa responsable de edwardsiellosis, una enfermedad de peces y mamíferos incluido el humano, la cual se caracteriza por la formación de múltiples abscesos. Distintas cepas de *E. tarda* poseen un número de genes asociados con virulencia, resistencia a múltiples antibióticos y sistemas secretores de toxinas que explican en cierto grado su capacidad de sobrevivir dentro de células fagocíticas y de infectar a diversos hospederos. En estudios previos, nuestro grupo secuenció parcialmente el genoma de una cepa virulenta de *E. tarda* (ETSJ54) aislada de lenguado japonés (*Paralichthys olivaceus*) con edwardsiellosis y reportó un número de genes asociados a su virulencia. En este estudio

se ha re-analizado los datos de secuenciación y en este proceso se identificaron varios genes que codifican para la estructura de superficie Pili y el sistema secretor tipo IV, la mayoría de los cuales estuvieron rodeados por genes codificadores de transposasas y otros genes de origen plasmídico. La secuencia de nucleótidos de dichos genes no tuvieron identidad con la de los genes previamente reportados en *E. tarda* EIB202, una cepa virulenta aislada de turbot (*Scophthalmus maximus*) en China. Los resultados sugieren diferencias en el contenido genético de cepas de *E. tarda* de distinto origen geográfico y la necesidad de desarrollar nuevos proyectos de secuenciación de genomas de *E. tarda*.

Palabras clave: Patogenesis, Sistema secretor tipo IV, Virulencia.

Resumo

Edwardsiella tarda é uma bactéria Gram-negativas responsáveis pela edwardsiellosis, uma doença de peixes e mamíferos, incluindo os seres humanos, que é caracterizada pela formação de múltiplos abscessos. Diferentes cepas de *E. Tarda* possui um número de genes associados com a virulência e resistência a múltiplos antibióticos, sistemas secretores de toxinas, para explicar em certa medida a sua capacidade de sobreviver no interior das células fagocíticas e infectar diferentes hospedeiros. Em estudos anteriores o nosso grupo sequenciou parcialmente o genoma de uma estirpe virulenta de *E. tarda* (ETSJ54) isolado do linguado japonês *Paralichthys olivaceus* com edwardsiellosis e relatou uma série de genes associados à virulência. Neste estudo foram analisados de novo os dados de sequenciamento e, neste processo foram identificados vários genes que codificam para a estrutura de superfície Pili e sistema de secreção do tipo IV, a maioria dos quais foram cercados por transposase e outros genes de origem plasmídico. A sequência de nucleótidos dos genes não tinham identidade com os genes previamente relatados em *E. Tarda* EIB202, uma cepa virulenta isolado do turbot (*Scophthalmus maximus*) na China. Os resultados sugerem diferenças no conteúdo genético de cepas de *E. tarda* de origem geográfica diferente e a necessidade de desenvolver novos projetos de sequenciamento do genoma *E. tarda*.

Palavras-chave: Patogênese, tipo de sistema de secretorIV, virulência.

Introduction

Edwardsiella tarda is a member of the enterobacteriaceae family that causes edwardsiellosis, a systemic suppurative disease of marine and fresh-water fishes around the world (Miyazaki and Kaige, 1985), such as Japanese flounder (*Paralichthys olivaceus*) and turbot (*Scophthalmus maximus*) cultured in Japan and China respectively, and red tilapia (*Oreochromis* spp.) cultured in Colombia (Iregui et al., 2012). The bacterium may cause sporadic infections in humans that varies from self-limited gastrointestinal and extra-intestinal infections with systemic abscesses up to lethal septicemia (Wang et al., 2005; Spencer et al., 2008; Verjan et al., 2012).

The virulence of *E. tarda* strains is associated with multiple factors including siderophores and hemolysins (Hirono et al., 1997), motility conferred by the flagellum (Mathew et al., 2001), and a type three secretion system (TTSS) that confers survival and replication advantages within macrophages (Rao et al., 2004; Zheng et al., 2005; Okuda et al., 2006). *E. tarda* as a typical facultative intracellular pathogen resists reactive oxygen species (ROS) and survives within phagocytic cells (Ishibe et al., 2008), a feature that is partially due to the production of enzymes including an iron-cofactored superoxide dismutase (FeSOD) (Cheng et al., 2010), and heat shock proteins (Dang et al., 2011). *E. tarda* also possesses a type four secretion system (T4SS) involved in horizontal DNA transfer to other bacteria and eukaryotic cells, toxin secretion and injection of

virulence factors into host cells (Backert and Meyer, 2006). The genome of *E. tarda* EIB202, a virulent strain isolated from diseased turbot in China was recently reported, however, this particular strain appeared to be non motile and also possess an incomplete T4SS (Wang et al., 2009).

In previous studies, our group identified a number of antigenic and virulence-related genes in a motile *E. tarda* (ETSJ54) strain isolated from diseased Japanese flounder (Verjan et al., 2005; Verjan, 2005b; Verjan et al., 2013). In this study, we have re-analyzed the sequencing data of *E. tarda* ETSJ54 to identify and annotate gene sequences coding for major structural components of the Pili and T4SS. The results indicate that some of those genes were absent in the previously sequenced genome of *E. tarda* EIB202 (Wang et al., 2009). The novel type IV conjugative transfer system-related genes were deposited in the GenBank database, and their putative roles are discussed. The results suggest important differences in gene content between geographically distinct *E. tarda* strains that may support the need for new genome sequencing projects.

Material and methods

Bacterial strains and Genomic DNA libraries of ETSJ54

E. tarda (ETSJ54) and *Escherichia coli* strains XL1-Blue MR and JM109 used as host for recombinant cosmid

and generation of cosmid DNA libraries were described previously (Verjan *et al.*, 2005; Verjan *et al.*, 2013). Cosmid and plasmid preparation were performed following standard procedures (Sambrook and Russell, 2001). Table 1 shows a list of cosmid and plasmid clones encoding the identified ETSJ54 T4SS genes.

The nucleotide sequences were determined by the cycle sequencing method using Thermo sequenase fluorescent-labeled primer cycle sequencing kit (Amersham Pharmacia Biotech, Little Chalfont Buckinghamshire, UK). Briefly, cosmid and plasmid clones were cultured in LB agar plates with ampicillin and single colonies were randomly isolated and grown in 2YT broth for cosmid or plasmid DNA isolation. Sequencing of the terminal ends of cosmid DNA was performed with T3, 5´-(ATTAACCCTCACTAAAGGGA)-3´ and T7,5´-TAATACGACTCACTATAGGG-3´ primers sets to identify putative ORFs flanking the *E. tarda* DNA fragments. Detailed methods for genomic DNA library construction, subcloning and nucleotide sequence determination were reported recently (Verjan *et al.*, 2013). The DNA sequence data of ETSJ54 were compared with those in the GenBank (www.ncbi.nlm.nih.gov) database using the BLASTX (Version 2.2.28+) software (Zhang *et al.*, 2000) of the National Center for Biotechnology Information. The closest homologous gene sequences in other bacterial species allowed predicting its putative function or the potential origin of the DNA

sequence. Multialignment of protein sequences was carried out with BioEdit and Genetyx version 7 programs and phylogenetic analysis was performed with the Molecular Evolutionary Genetics Analysis (MEGA) version 5.2 (Tamura *et al.*, 2011), using the Neighbor Joining method. The novel T4SS-related genes of *E. tarda* ETSJ54 were submitted to the Gen-Bank database.

Results

A total of 9 protein-coding genes associated with the Pili and T4SS of *E. tarda* ETSJ54 were annotated, deposited in the GenBank database and the corresponding accession numbers are showed in Table 2. Comparison of their nucleotide sequence using the BLASTX software of NCBI, indicated that only two genes, those coding for the prepilin peptidase dependent protein D (ppdD) and the type IV pilus biogenesis protein (PilM) had identity to previously reported genes in *E. tarda* EIB202 genome (Wang *et al.*, 2009) and *E. tarda* C07-087 (Tekedar *et al.*, 2013). Interestingly, the remaining gene sequences coding for the T4SS in *E. tarda* ETSJ54 did not have any nucleotide or deduced amino acid sequence identity to genes or protein sequences in the published *E. tarda* genomes (Table 2).

Using the BLASTX software at the NCBI website, the T4SS-related genes of *E. tarda* ETSJ54 showed nucleotide and deduced amino acid sequence identity to ge-

Table 1. Cosmid and plasmids encoding *E. tarda* ETSJ54 T4SS genes

Cosmid and Plasmids	Gene	Source or Reference
Cosmid and plasmids vectors		
SuperCos I	Ampicillin resistant (Ap ^r) cosmis vector	Stratagene, La Jolla, CA
pUC118	Ampicillin resistant (Apr) lacZ cloning vector	Pharmacia
pHSG398	Chloramphenicol resistant (Cm ^r) cloning vector	Takara, Tokyo Japan
Cosmid (KEC) and plasmid (KES) clones encoding T4SS		
KEC07_B04_T7,KEC09_E10_T7	Encoding ETSJ54 <i>traB</i>	This study
KEC04_F09_T3,KEC04_F09_T7	Encoding ETSJ54 <i>traF</i>	This study
KEC01_A07_T7	Encoding ETSJ54 <i>traH</i>	This study
KES02_E05_T3	Encoding prepilin peptidase dependent protein D	This study
KEC09_D07_T7	Encoding type IV pilus biogenesis protein PilM	This study
KEC01_A10_T3,KEC07_H04_T7	Encoding ETSJ54 <i>traI</i>	This study
KEC01_E03_T7,KEC01_H04_T3	Encoding ETSJ54 <i>traD</i>	This study
KEC10_A04_T3	Encoding ETSJ54 <i>traE</i>	This study
KEC10_A04_T3	Encoding ETSJ54 <i>traK</i>	This study

DNA sequencing and analysis

Table 2. T4SS-related genes of *Edwardsiella tarda* ETSJ54

Category	Gene	Putative name/Function	Accession No.	Close related SP (Sep 2013)	Query Cover %	E-Value	Aminoacid Identity %
Pili/T4SS							
1	ppdD	Prepilin petidase dependent protein D	AB231503	<i>E. tarda</i> EIB202	100	7,00e-89	98
2	traB	Type IV conjugative transfer system protein	AB231507	<i>Escherichia coli</i> 3.4880	93	0.0	93
3	traD	Type IV conjugative transfer system protein	AB831781	<i>Salmonella enterica</i>	87	0.0	97
4	traE	Type IV conjugative transfer system protein	AB831783	<i>Xenorhabdus nematophila</i> ATCC 19061	98	1,00E-16	100
5	traF	Type IV conjugative transfer system protein	AB231506	<i>Photobacterium damsela</i>	98	3,00E-54	80
6	traH	Type IV conjugative transfer system protein	AB231505	<i>Vibrio mimicus</i> VM603	100	3,00E-99	95
7	traI	Type IV conjugative transfer system protein	AB831785	<i>Vibrio mimicus</i>	98	0.0	73
8	traK	Type IV conjugative transfer system protein	AB831784	<i>Vibrio mimicus</i>	61	1,00E-87	87
9	piM	Type IV pilus biogenesis protein	AB831782	<i>E. tarda</i> C07-087	99	3,00E-138	99

nes of well-known mammalian enteropathogens such as *E. coli* and *Salmonella enterica*, to fish pathogens such as *Photobacterium damsela* (*Formally Pasteurella piscicida*) and *Vibrio mimicus*, and to entomopathogens such as *Xenorhabdus nematophila*, indicating that although the T4SS is highly conserved within enterobacteriaceae members, there could be *E. tarda* strains such as the *E. tarda* EIB202 lacking those genetic elements or that they may be missing in the sequenced genomes. Another possibility could be that uncharacterized proteins grouped as hypothetical proteins in those *E. tarda* strains might be associated with the missing T4SS proteins.

The *E. tarda* ETSJ54 type IV conjugative transfer system genes showed 73-100 % nucleotide and deduced amino acid sequence identity to the genes and proteins of other enterobacteriaceae members with a nucleotide sequence coverage ranging from 61 to 100% (Table 2). A partial nucleotide and deduced amino acid sequence of the *E. tarda* ETSJ54 type IV conjugative transfer system protein TraI is presented in Figure 1. The provided TraI DNA sequence is 2004 bp in length and encodes a protein of 667 amino acids, possesses one HincII restriction site at position 280, one SacI restriction site at position 757 and one Pst I restriction site at position 805 (sequence underlined). The stop codon (TGA) is indicated at position 2002, whereas the start

codon and a range of amino acids at the N-terminal region are missing in this sequence (Figure 1).

A multi-alignment of *E. tarda* ETSJ54 TraI deduced amino acid sequence was constructed with the closest homologous protein sequences and a summary of this alignment is presented in Figure 2. Despite the missing N-terminal region of TraI protein, the deduced amino acid sequence of ETSJ54 TraI showed 75% identity to *Photobacterium damsela* subsp. *Piscicida* (502/670), *Vibrio mimicus* VM603 (503/674) and *Yersinia ruckeri* (503/670) TraI protein sequences. In addition, ETSJ54 TraI showed 74% identity to *E.coli* TraI (496/668) and 72% identity to *Aeromonas hydrophila* (480/667) and *Salmonella enterica* (477/667) TraI proteins, whereas it has 90% identity to a *Xenorhabdus nematophila* (341/377) TraI protein fragment of 377 amino acids. Of note, ETSJ54 TraI protein showed several amino acid deletions and additions when compared to *Photobacterium damsela* subsp. *Piscicida* TraI deduced amino acid sequence (Figure 2).

The novel *E. tarda* genes were compared to those in the GenBank database and similar phylogenetic distances between the *E. tarda* ETSJ54 TraI protein sequence and those proteins from other enterobacteriaceae were found. Figure 3 shows that *E. tarda* ETSJ54 TraI protein cluster more closely with *Xenorhabdus nema-*

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TCAGTCAGACAAAATCCCCGGCATCCAAGAGATCCAGATACGCTGGCCGACATCTCATCGAGCGGGTTTGTGTGCCAAACACTGTG 90
S V R Q N P R H P R D P D T L A D I L I E R G F A V P N T V
CAGGAAAAGGTGAGCCGAGCTACTCCGATATTGGGAAGTGTCTGCCGGAGATGCTCCAGGAAGCGACAGGCTCTTTGAAGATTTTGATG 180
Q E K G E R A Y Y R Y W E V L P E M L Q E A T G S L K I L M
CTCCGCCTCGAATCAAACGACTTGGTGTTCACCAATGAGCCACCATCAGCCGTTGCAGCAGAAAGTTGTTGGAGACGTTGAAGATGCTGAG 270
L R L E S N D L V F T N E P P S A V A A E V V G D V E D A E
HincII
ATTGAGTTCGTTGACCCCTGATGAGAGTGTGATGACGATCAAGATGAAGCGGAGTGTCTCTGAACGATGACATGTTGGCCGAGAGCAGGAA 360
I E F V D P D E S D D D Q D E G G V S L N D D M L A A E Q E
GCAGAGAAAAGCTCTAGCCGGTCTCGGTTTCGGTGTGCGATGGAATGCTGAAAAGCACTTCAACAGAACCCGAAAGACACCCGCCCAA 450
A E K A L A G L G F G D A M E M L K S T S T E P E D T P A Q
GGCCGGCTGAAAACAAAGGACTCGCCTAAAGCACCATCAGCCACAACCAAGCAAGGGAAAACAGGTTAAGGCTAAACCCAAAAAAGAC 540
G A A E T K D S P K A P S A T T P S K G K Q V K A K P K K D
ACAGAAGAACAAAACAGAGAAACCTGAGGCAAAAGAAGAGTTGTACCTCAGGACATCGCCAAAACCGCCACCTTTGGCGAACGATAAC 630
T E E Q T T E K P E A K E E L S P Q D I A K N A P P L A N D N
CCTCTGCAAGCTCAAGGATGTTGGGGTGGACTGGGTGACATCGACTTCCATTTGACGCTTTAACGCATCGACTGACGCTGCATT 720
P L Q A L K D V G G G L G D I D F P F D A F N A S T D A C I
SacI
GCTGACGAGAGAGCCCTTAAAACTCCAACGAGGCAGCTCGAACAGCCCAAGCCTGACTTCGTTCCAGAAGAACAAAACCTCCCTGCGAG 810
A D E E S L K N S N E A E L E Q P K P D F V P E E Q N S L Q
GACGACTTCCCCATGTTTGTAGTGGTTCAGATGAGCCTCCATCATGGGCTATTGAACCATGCCCTATGTTGGCCGTTGAGTCAGAAAG 900
D D D F P M F S G S D E P P S W A I E P L P M L A V E S E E
GCTTCCGCAACAGAAATGTACAAGCGAACATACCTAATCAGCAGGAAAAGGACTCTAAGACATTGCTGTCTGAGATGCTTGTCTGGC 990
A S A T T E M S Q A N I P N Q Q E K D S K T L L S E M L A G
TATGGCAAGCATCTGCTCTGCTTGTGAGCAGGCGATCATGCCTGTTCTGGAAGAAAAGAAGCGCTGGGAGAAGTTCTGTCTGTATGAG 1080
Y G E A S A L L E Q A I M P V L E G K K T L G E V L C L M K
GGCCAGGCGCTCATCTATACCCAGAAGGTCTCGGTTCATTAGTGTGCGCGTCAAGATTTCTCTCAAAGCTATCCCATGCCAATGCGATT 1170
G Q A V I L Y P E G A R S L G A P S E V L S K L S H A N A I
GTTCCAGACCTTATTGCTGGTTCGTAAGTGCAGATTTTCAGCGGTGTGAAGCAATTGTGCTGGCCCAACAACCTTTCAGATGCGGTC 1260
V P D P I M P G R K V R D F S G V K A I V L A Q Q L S D A V
GTAGCGCCATCAAGGAAGTAGAAGCGTCAATGGGTGGATACCAGGATGCTTTGAGCTTGTGTCTCTCTCTGATTCATGATGCAGAAAG 1350
V A A I K E V E A S M G G Y Q D V F E L V S P P D S D V R K
AGCAAAGTCTGCTCCGAAGCAACAAAGCCCAACCGATGCAGAAAAAACAACCAACAGAAAGTCTGATGCTGGATCAGGTCAAGCTGCACCG 1440
S K S A P K Q Q S Q P M Q K K N Q Q Q K S D A G S G Q A A P
GCAAAAGACACGAAAGGAAAGCCGCTCAGAAACCTCAAAGGAGGAAAAAGGTGACACCTCCTCTGTGGTTCGGGGCATGAAAGCAAG 1530
A K D T K G K A A Q K P Q K E K G D T S S V V A G H E S K
CCGATTGAAGAAAACAGAACGCTGGCTCGCCTTCCCAAGAGAGAGGCTCAAACCCGAAAGCTGTTGTAGTTTACCAAGTTGAGCGTGAAG 1620
P I E E K Q N V A R L P K R E A Q P E A V V V T K V E R E K
GGATTTGGTCACATCGAGTCCGAGAAAGGGAAGAGCAAGAGGTGAGGAGTTTGAACCCAAAGGCTAAAACAAAACCCGAAAGACCTC 1710
G L G H I E V R E R E E Q E V R E F E P P K A K T N P K D L
CTTTTTACTCAAAAAGAAAGACTTTTTTGGCCGCTGGGGTTCCCCCTTGAAAAAGCAATTTTTTATCCTCAAGGGAAATGAATCCC 1800
L F T T Q K E R L F C P P G G S P L K K A I F Y P S R E M N P
AAACGTTCCGGCAGGTGTTCCCTTACACCTGTCTTGAAGAAGACGGTGTCTTAGTAACCAAGCGATAAAGCGTTCGACATGTTGCCGGG 1890
K R S G R W F L T P V L E E D G C L V T S D K A F D M I A G
GAAAACATCGGCATCAACAACACATCCTCTGTGGGATGTTGAGCCGGGCACAAAACCGCCCTTGTCTCAAAAACGTCGAAGGAAATG 1980
E N I G I N K H I L C G M L S R A Q K R P L L K K R Q G K L
TATTTAGAGGTAATGAACATGA 2004
Y L E V N E T *

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Figure 1. Partial nucleotide and deduced amino acid sequence of *E. tarda* (ETSJ54) type IV conjugative transfer system protein Tral. The nucleotide sequence is 2004 bp in length, encoding a protein of 667 amino acids and possesses *SacI*, *HincII* and *PstI* (underlined) restriction enzyme sites and a stop codon a position 2002. The start codon and a range of amino acids at the N-terminal region are missing.

tophilia and *E. coli* Tral proteins and that it may have evolved from fish pathogens such as *Aeromonas hydrophila* or *Aeromonas salmonicida* Tral proteins.

Discussion

The bacterial Pili is classified as a fimbrial type adhesin that is involved in adhesion, invasion and colonization of host tissues. These processes are then improved by other non fimbrial adhesins such as the flagellum (Amano, 2010; Friedlander *et al.*, 2013), and outer membrane proteins (Confer and Ayalew, 2013). Enzymes such as sialidase NanA, may also contribute to the

adhesion, colonization and dissemination events of *E. tarda* in fish tissues (Jin *et al.*, 2012). The virulence and pathogenicity of *E. tarda* may also involve various surface structures including a Pili-associated type IV conjugative transfer system, a subset of the type IV secretion system (T4SS), that is usually located in mobile genetic elements such as pathogenicity islands or plasmids encoding antibiotic resistance genes and transposases (Yu *et al.*, 2012). The bacterial T4SS and particularly their effector proteins have been involved in many pathogenic mechanisms of bacteria including the interference of the actin cytoskeleton rearrangements by *Helicobacter pylori*, the exotoxin (pertussis toxin,

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E.coli.gpt      271:DVDEFSYGVVPERYVFDAIRRLVKTGKKNVNEPGAKVWHLNQGQVFIANKQLGDLYDLISHDKIPGIPRDPDTLADILIERGFAVPNTVQE 360
E.tarda.gpt    1:-----SVRQNPFRHPRDPDTLADILIERGFAVPNTVQE 32
P.damselae.gpt 271:DVDEFSYGVVPERYVFDAIRRLVKTGKKNVNEPGAKVWHLNQGQVFIANKQLGDLYDLISHDKIPGIPRDPDTLADILIERGFAVPNTVQE 360
S.enterica.gpt 271:DVNEFSYGVVPERYVFDAIRRLVKTGKKNVNEPGAKVWHLNQGQVFIANKQLGDLYDLISHDKIPGIPRDPDTLADILIERGFAVPNTVQE 360
V.mimicus.gpt 271:DVDEFSYGVVPERYVFDAIRRLVKTGKKNVNEPGAKVWHLNQGQVFIANKQLGDLYDLISHDKIPGIPRDPDTLADILIERGFAVPNTVQE 360
*****
E.coli.gpt      361:KGERAYRYWEVLPPEMLQEAAGSVKILMLRLESNDLVFTTEPPAAVAEEVVGVDAAEIEFVDPEEADDDGDDQEEGEAAINDMMLAAEQE 450
E.tarda.gpt    33:KGERAYRYWEVLPPEMLQEAATGSLKILMLRLESNDLVFTNEPPSAVAEEVVGVDAAEIEFVDPEESDD--DQDEGGVSNDDMMLAAEQE 120
P.damselae.gpt 361:KGERAYRYWEVLPPEMLQEAAGSVKILMLRLESNDLVFTTEPPAAVAEEVVGVDAAEIEFVDPEEADDDGDDQEEGEAAINDMMLAAEQE 450
S.enterica.gpt 361:KGERAYRYWEVLPPEMLQEAAGPVKILMLRLESNDLVFTTEPPAAVTGEVVGVDAAEIEFVDPEEAD--DQAEAGDAGINNDMLAAELE 448
V.mimicus.gpt 361:KGERAYRYWEVLPPEMLQEAAGSVKILMLRLESNDLVFTTEPPAAVAEEVVGVDAAEIEFVDPEEADDDGDDQEEGEAAINDMMLAAEQE 450
*****
E.coli.gpt      451:AEKALAGLPGDAMEMLKSTSDAVEEKPEQKIDAGPTESKPDAGKKGKPKQKPKGKAKPKSDTEKQPHKPEAKEDLSQDDIAKNAPPLAND 540
E.tarda.gpt    121:AEKALAGLPGDAMEMLKSTSTEPEDTPAQGAETKDSFKAPSATTPS--KGFQVKAKPKKDETEQTEKPEAKEELSQQDIAKNAPPLAND 209
P.damselae.gpt 451:AEKALAGLPGDAMEMLKSTSDAVEEKPEQKIDAGPTESKPDAGKKGKPKQKPKGKAKPKSDTEKQPHKPEAKEDLSQDDIAKNAPPLAND 540
S.enterica.gpt 449:AEKALAGLPGDAMEMLKSTSDIAEEKPEQEDTETELPKPDASKKGGKQKPKGKARA---EKQPQKPEVKEEDLSQDDIAKNAPPLAND 534
V.mimicus.gpt 451:AEKALAGLPGDAMEMLKSTSNTEVEETSEQEDVGSSTESSKPDAGKKGKPKQKPKGKAKPKSDTEKQPHKPEAKEDLSQDDIAKNAPPLAND 540
*****
E.coli.gpt      541:NPLQALKDVGGLGSDIDFPDAPNASETTSTDATNSEIPDVAMPKQEEQPKQDFVFPQNSLQGGDDPFMFGGSDPEPPSWAIEPLPMLT 630
E.tarda.gpt    210:NPLQALKDVGGLGSDIDFPDAPNASTDACLAEDESLKNSN---EAELEQKFPDFVPEEQNSLQGGDDPFMFGGSDPEPPSWAIEPLPMLA 295
P.damselae.gpt 541:NPLQALKDVGGLGSDIDFPDAPNASETTSTDATNSEIPDVAMPKQEEQPKQDFVFPQNSLQGGDDPFMFGGSDPEPPSWAIEPLPMLT 630
S.enterica.gpt 535:DPQLQALKDVGGLGSDIDFPDAPNASETTSTDATNSEIPDVEPEKQEEQPKQDFVFPQNSLQGGDDPFMFGGSDPEPPSWAIEPLPMLT 624
V.mimicus.gpt 541:NPLQALKDVGGLGSDIDFPDAPNASETTSTDATNSEIPDVAMPKQEEQAKQNFVFPQNSLQGGDDPFMFGGSDPEPPSWAIEPLPMLT 630
*****
E.coli.gpt      631:DAPEQPTHTPEMPHTDNVNHQEKDAKTLLVEMLSGYGEASALLEQAIMPVLEGGKTLGEVLCIMKGGAVILYPDARSLSGAPSEVLSKLS 720
E.tarda.gpt    296:VESEASATTEMQANIPNQEKDRTLLSEMLAGYGEASALLEQAIMPVLEGGKTLGEVLCIMKGGAVILYPDARSLSGAPSEVLSKLS 385
P.damselae.gpt 631:DAPEQPTHTPEMPHTDNVNHQEKDAKTLLVEMLSGYGEASALLEQAIMPVLEGGKTLGEVLCIMKGGAVILYPDARSLSGAPSEVLSKLS 720
S.enterica.gpt 625:DTPEQTIAPDPMQHAGKFKLEKDKATLLSEMLAGYGEASTLLEQAIMPVLEGGKTLGEVLCIMKGGAVILYPDARSLSGAPSEVLSKLS 714
V.mimicus.gpt 631:DVPEQPTHTPEMPHTDNVNHQEKDAKTLLVEMLAGYGEASALLEQAIMPVLEGGKTLGEVLCIMKGGAVILYPDARSLSGAPSEVLSKLS 720
*****
E.coli.gpt      721:HANAIVPDPIMPGRKVRDFSGVKAIVLAELQSDAVVAIKDAEASMGYQDAFELVSPGSDASKNKSAKPKQQR---KKAQQQKPEVNA 807
E.tarda.gpt    386:HANAIVPDPIMPGRKVRDFSGVKAIVLAELQSDAVVAIKVEASMGYQDFELVSPGSDVVRKKSAPKQQRQPMKKNQQRSDAGS 475
P.damselae.gpt 721:HANAIVPDPIMPGRKVRDFSGVKAIVLAELQSDAVVAIKDAEASMGYQDAFELVSPGSDASKNKSAKPKQQR---KKAQQQKPEVNA 807
S.enterica.gpt 715:HANAIVPDPIMPGRKVRDFSGVKAIVLDQSDAVVAIKDAEASMGYQDAFELVSPGSDASKNKSAKPKQQR---KKTQQQKPEVDA 801
V.mimicus.gpt 721:HANAIVPDPIMPGRKVRDFSGVKAIVLAELQSDAVVAIKVEASMGYQDAFELVSPGSDASKNKSAKPKQQR---KKAQQQKSEINA 807
*****
E.coli.gpt      808:GKASPEQKAKGKDSQPQKKEKVDV---TSPVEEQQRKPVQ--EKQNVARLPKREAQPVAPF--PKVEREKELGHVEVREDEPVEVRE 890
E.tarda.gpt    476:GQAAPAKDTKGAQAQKPKQKKGDT---SIVVAGHESKPIE--EKQNVARLPKREAQPEAVVTVKVEREKELGHIEVREDEPVEVRE 559
P.damselae.gpt 808:GKASPEQKAKGKDSQPQKKEKVDV---TSPVEEQQRKPVQ--EKQNVARLPKREAQPVAPF--PKVEREKELGHVEVREDEPVEVRE 890
S.enterica.gpt 802:G-AGSEQKTRKDSLQQQPKKQGNV---ASLVEEQKRRKPVQDQEKQNVARLPKREAQPVAPF--PKVEREKELGHVEVREDEPVEVRE 885
V.mimicus.gpt 808:GKAAPKAKGKDSQPQKKEKRDVGRDVAASVEEQKRRKPVQ--EKQNVARLPKREAQPVAPF--PKVEREKELGHVEVREDEPVEVRE 894
*****
E.coli.gpt      891:PPKARTNPKDI---NAEDFL-PSGVTP-QKALQML-KDMIQKRSGRWLVTPVLEEDGCLVTSDFKAFPMIAGENIGISKHILCGMLSPAQR 974
E.tarda.gpt    560:PPKARTNPKDLLEFKERLFCPPGGSPKKAIFYPSREMPKRSGRWLVTPVLEEDGCLVTSDFKAFPMIAGENIGINKHILCGMLSPAQR 649
P.damselae.gpt 891:PPKARTNPKDI---NAEDFL-PSGVTP-QKALQML-KDMIQKRSGRWLVTPVLEEDGCLVTSDFKAFPMIAGENIGISKHILCGMLSPAQR 974
S.enterica.gpt 886:PPKARTNPKDI---NEEDFL-PSGVTP-EKALLML-KDMIQKRSGRWLVTPVLEEDGCLVTSDFKAFPMIAGENIGISKHILYGLSPAQR 969
V.mimicus.gpt 895:PPKARTNPKDI---NAEDFL-PSGVTP-QKALQML-KDMIQKRSGRWLVTPVLEEDGCLVTSDFKAFPMIAGENIGISKHILCGMLSPAQR 978
*****
E.coli.gpt      975:RPLLKRRGKLYLEVNET 992
E.tarda.gpt    650:RPLLKRRGKLYLEVNET 667
P.damselae.gpt 975:RPLLKRRGKLYLEVNET 992
S.enterica.gpt 970:RPLLKRRGKLYLEVNET 987
V.mimicus.gpt 979:RPLLKRRGKLYLEVNET 996
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Figure 2. Multialignment of *E. tarda* (ETSJ54) Tral deduced amino acid sequence with homologous proteins from other bacteria. ETSJ54 Tral shows several amino acid additions and deletion when compared to the Tral proteins of other enterobacteriaceae members. The asterisks at the bottom indicate identical amino acids whereas the dots indicate different amino acids regarding to the aligned amino acid sequences.

Ptx) secretion by *Bordetella pertussis*, the prevention of phagosome-lysosome fusion that allows intracellular replication of *Legionella pneumophila* and *Brucella* species, and the modulation of vacuole biogenesis by *Coxiella* species (Backert and Meyer, 2006; Bruggemann *et al.*, 2006), and based on those properties, bacterial T4SS are beginning to be considered as an important DNA delivery tool for human cells that may allow the development of cell and tissue-specific gene therapies (Llosa *et al.*, 2012). At present, the exact function of *E. tarda* T4SS in the virulence and pathogenesis in fish and mammals remains unknown.

The T4SS are classified into four groups based on phylogenetic relationships and despite the lack of complete nomenclature and standardization, they include (1) F-T4SS (Tra/Trb), (2) P-T4SS (VirB/D4), (3) I-T4SS (Dot/Icm) that resembles the incompatibility (Inc) IncF, IncP and IncI plasmid conjugation systems, and (4) the GI-T4SS, associated with genomic islands (Juhas *et al.*, 2008). An alternative classification of T4SS was proposed based on their function which includes conjugation machines, effector translocators and DNA release/uptake systems (Alvarez-Martinez and Christie, 2009). Recently, a database called Atlas T4SS, holding a collection of 1,617 predicted proteins encoding the T4SS

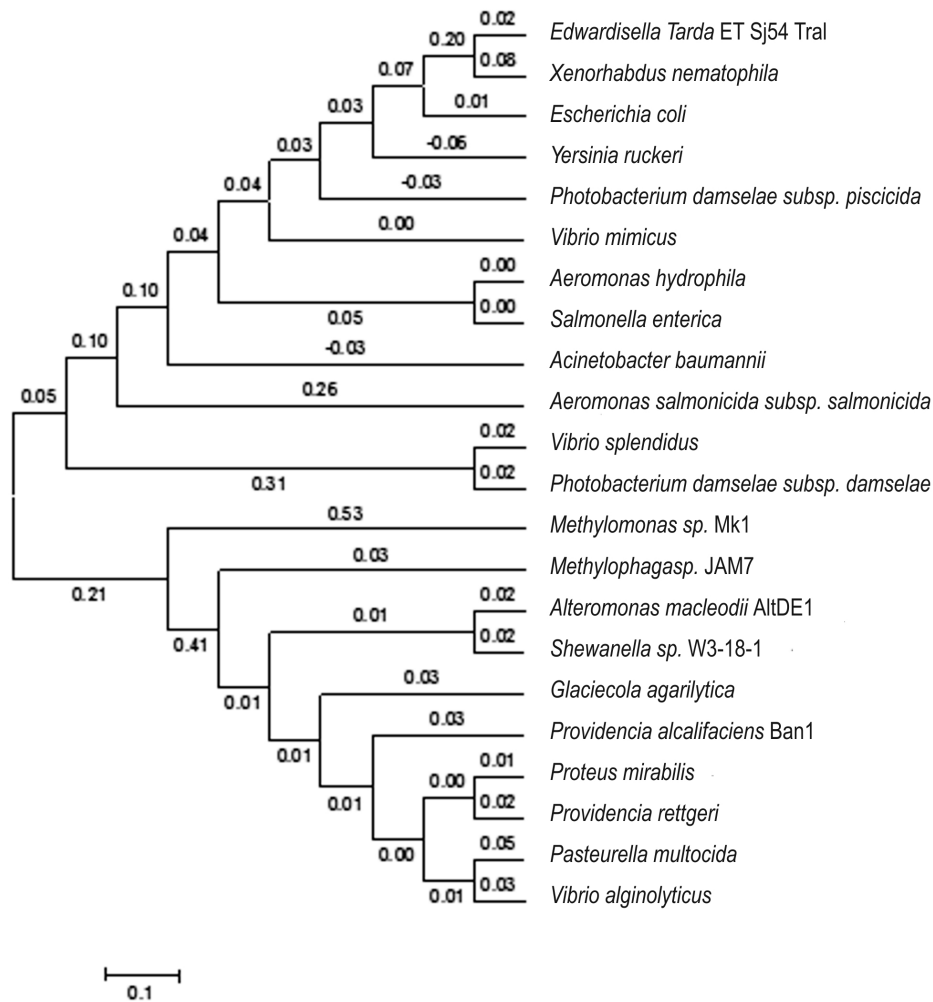


Figure 3. Phylogenetic distances between various type IV conjugative transfer system Tral proteins. Amino acid sequences from various strains were aligned and the tree image was constructed by using the Molecular Evolutionary Genetics Analysis (MEGA) version 5.2, using the Neighbor-Joining method. The accession numbers are the following: *Xenorhabdus nematophila*, WP_010845924; *Escherichia coli*, WP_001447845; *Yersinia ruckeri*, YP_001101767; *Photobacterium damsela subsp. piscicida*, YP_908640; *Vibrio mimicus*, WP_005507685; *Aeromonas hydrophila*, YP_002995557; *Salmonella enterica*, WP_000909947; *Acinetobacter baumannii*, WP_002093818; *Aeromonas salmonicida subsp. salmonicida*, YP_001144215; *Vibrio splendidus*, WP_017085217; *Photobacterium damsela subsp. damsela*, YP_005352470; *Methylomonas sp. MK1*, WP_020481731; *Methylophagasp. JAM7*, YP_006292404; *Alteromonas macleodii AltDE1*, YP_006976049; *Shewanella sp. W3-18-1*, YP_962502; *Glaciecola agarilytica*, WP_008305530; *Providencia alcalifaciens Ban1*, ACV96076; *Proteus mirabilis*, WP_004249348; *Providencia rettgeri*, AAM08003; *Pasteurella multocida* WP_005737977; *Vibrio alginolyticus*, WP_005396791; and the *E. tarda* ETSJ54 Tral is shown in bold.

was developed to help the assignment of given coding sequence (Souza *et al.*, 2012). The identified genes in *E. tarda* ETSJ54 indicate that this bacterium possess a T4SS that maybe involved in protein-ss DNA complex transfer but functions in adhesion and invasion of fish tissues cannot be excluded. The *E. tarda* ETSJ54 T4SS may belong to the type I group or F-T4SS described in *E. coli* (Lawley *et al.*, 2003), and by using the Atlas T4SS database, we found that four out of seven (TraD,

TraB, TraF and TraH) *E. tarda* T4SS protein sequences had identity to previously reported *Haemophilus influenzae* conjugal transfer protein TraD (Plasmid ICE-hin1056), *Salmonella typhi* plasmid transfer protein TraB, *Salmonella typhi* conjugal transfer protein TraF-F, and *Legionella pneumophila subsp. pneumophila* F-type conjugal transfer protein TraH, respectively, whereas the *E. tarda* Tral, TraK and TraE did not have any significant hit when compared to the proteins in this

database. Of note, these proteins and microorganisms were different to the significant hits obtained by using the BLAST tool at the NCBI website (Table 2). These results also support the possibility of highly diverse T4SS protein sequences between enterobacteriaceae families and that the current available tools still have limitations to properly classify T4SS proteins.

The process of bacterial conjugation involves the formation of a mating bridge and a close contact between donor and recipient cells that allows the transfer of genetic material between bacteria. One of the most studied mechanisms is the plasmid-encoded extracellular filament or F pilus (Tra/Trb) in *E. coli* (Frost *et al.*, 1994) that allows intergeneric and interkingdom F plasmid transfer. Among the *E. tarda* ETSJ54 T4SS-related genes, we identified genes involved in binding and pumping of DNA into the recipient cell, traD that may also have a role in pilus assembly; DNA transfer (tra) genes such as traB, traE, traF, traK and traH involved in F pilus assembly; and tral that encodes a relaxase/helicase I, involved in two functions, oriT nicking and unwinding in the 5'-to-3' direction (Table 2), and finally a sequence encoding TraN-like protein associated with mating-pair stabilization (Not shown). The *E. tarda* ETSJ54 tra genes were found in close proximity to genes coding for multidrug efflux proteins of *Salmonella enterica*, sulfonamide resistance protein (Sul1) of *Pseudomonas putida* HB3267, and Streptomycin resistance protein (AadA) of *Aeromonas salmonicida subsp. salmonicida* A449, and particularly those genes were flanked by transposase genes IS21, IS100 and IstB of *Escherichia coli*, suggesting that *E. tarda* ETSJ54 T4SS-related genes might have been acquired horizontally and encoded in an uncharacterized plasmid, nevertheless, the presence of mobile genetic elements, currently known as "integrative and conjugative elements" (ICEs) suggests they could also be integrated in the genome of *E. tarda* ETSJ54. Integrated conjugative plasmids and ICE are known to mediate the unidirectional transfer of single strand large fragments of chromosomal DNA and shape the architecture of bacterial genomes (Alvarez-Martinez and Christie, 2009). Pathogens with extensive genetic diversity such as *Helicobacter pylori*, have also chromosomally encoded TraG-like proteins and relaxase (rlx) proteins (Backert *et al.*, 2005).

To obtain a better understanding of the evolutionary relationships of *E. tarda* T4SS genes, we performed a preliminary phylogenetic analysis and found that although the deduced amino acid sequence of *E. tarda* relaxase Tral has been acquired from common fish pathogens such as *Aeromonas* sp., and *Photobacterium damselae*, it appeared to be more closely related to a Tral fragment of the plant pathogen *X. nematophila* and to

E. coli Tral protein, suggesting a most probably inter-genus transfer from *E. coli*. The relationship of ETSJ54 Tral with the plant pathogen *X. nematophila* is currently unknown. Additional studies are required to define the precise origin and function of *E. tarda* T4SS-related genes and detection/identification of other potential tra genes such as the regulatory genes of the F transfer operon (traJ), pilus synthesis and assembly (traA, traQ, traX), mating-aggregate stabilization (traG) and surface exclusion (traT and traS) genes (Frost *et al.*, 1994) that are currently missing or have not been identified.

Conclusions

E. tarda virulence is complex and involves multiple factors including diverse toxin secretion systems such as the T4SS that may allow genetic material transfer between bacteria and bacteria-host cells, but also adhesion and colonization properties. A partial genome analysis of a virulent strain of *E. tarda* (ETSJ54) allowed the identification of a series of virulence-related genes coding for T4SS components that were absent in previously reported complete genome of a virulent and multi-drug resistant *E. tarda* EIB202 isolated in China. The T4SS-related genes in ETSJ54 were associated with various transposases genes and other mobile genetic elements, indicating that the process of DNA exchange and acquisition between *E. tarda* and other bacteria might be an active process of genetic material transfer and evolution. Further studies are needed to clarify the exact origin and role of this surface structure during infection of fish and mammal hosts.

Acknowledgements

This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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