Adhesion and invasion-related genes of Edwardsiella tarda ETSJ54

Genes relacionados con la adhesión e invasión de Edwardsiella tarda ETSJ54

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Resumen

Edwardsiella tarda es una bacteria Gram-negativa que causa una enfermedad supurativa sistémica en peces y humanos. Recientemente, el secuenciamiento del genoma de una cepa de E. tarda altamente virulenta y resistente a múltiples antibióticos aislada en China demostró que este microorganismo posee un número de genes asociados con virulencia y sistemas secretores de toxinas que explican, en cierto grado, su capacidad de sobrevivir dentro de células fagocíticas y de infectar a diversos hospederos. Sin embargo, esta cepa de E. tarda carece de motilidad y tiene una estructura genética codificadora del flagelo y un sistema secretor tipo IV incompletos. En este estudio, a través de un secuenciamiento genómico parcial de librerías de ADN de E. tarda ETSJ54 construidas en vectores cósmido y plásmido, se identificaron un número de genes asociados a estructuras de superficie como invasinas, pili, sistema secretor tipo IV, fimbria y otros genes relacionados con virulencia. Es de notar que la secuencia de nucleótidos de la gran mayoría de dichos genes presentó identidad con genes previamente reportados en el genoma de E. tarda EIB202; sin embargo, algunos genes no tuvieron identidad alguna con aquellos reportados para este microorganismo. Los resultados indican que E. tarda ETSJ54 posee varios elementos genéticos asociados con adhesión, invasión y colonización de tejidos de peces y diferencias en el contenido genético entre cepas de origen geográfico distinto constituye un estímulo para el desarrollo de nuevos proyectos de secuenciamiento que permitan identificar los genes asociados a este sistema secretor que aún no han sido descritos en E. tarda.

Palabras clave: Adhesinas, fimbria, patogénesis, pili/sistema secretor tipo IV, virulencia.

Abstract

Edwardsiella tarda is a Gram-negative bacterium that causes a systemic suppurative disease in fish and humans. Recently, the complete genome sequence of a highly virulent and multidrug resistant E. tarda strain isolated in China indicated that this microorganism harbor a number of virulence- and toxin secretion system related genes that explain in some extent it capacity to survive within phagocytic cells and to infect a variety of hosts. However, this particular E. tarda strain was found to be no motile and appear to have a truncated flagellum structure and several type IV secretion system (T4SS) genes were missing. In this study, through a partial genome sequencing of cosmid and plasmid DNA libraries generated from a virulent E. tarda strain (ETSJ54), we identified several genes associated with Invasins, Pili, type IV secretion system, Fimbria and other genes related to its virulence. Of note, the majority of ETSJ54 genes sequences had nucleotide and deduced amino acid sequence identity to those gene sequences published in E. tarda EIB202 strain, however, some T4SS genes did not have identity to any previously reported E. tarda genes. The results indicate that E. tarda ETSJ54 possess various genetic elements associated with adhesion, invasion and colonization of fish tissues and differences in gene content between distinct geographical E. tarda isolates may encourage new genome sequencing projects to identify the missing genes associated with this particular secretion system.

Keywords: Adhesins, fimbria, pathogenesis, pili/T4SS genes, virulence.

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Edwardsiella tarda is a member of the enterobacteriaceae family that causes edwardsiellosis, a systemic suppurative disease of marine and freshwater fishes around the world (Miyazaki and Kaige, 1985), such as Japanese flounder (Paralychthys olivaceus) cultured in Japan and hybrid tilapia (Oreochromis spp.) cultured in Colombia (Iregui et al., 2012), and most region of the world. The bacterium may also cause sporadic infections in mammals including humans (Verjan et al., 2012). The infection in man often occurs accidentally during manipulation of aquatic animals or their products and varies from self-limited gastrointestinal and extraintestinal infections with systemic abscesses up to lethal septicemia (Spencer et al., 2008; Wang et al., 2005).

The pathogenesis of edwardsiellosis is complex and associated to multiple virulence factors that might be encoded in the chromosome or in conjugative plasmids (Wang et al., 2009), some of which were early described such as hemolysins (Hirono et al., 1997) that enable the bacteria to have access to essential elements such as phosphate, iron and other nutrients; siderophores that mediate iron uptake and motility conferred by the flagellum that may enable the bacteria to reach an appropriate niche for colonization (Mathew et al., 2001). In addition, E. tarda possesses several virulence-associated secretion systems that range from type I (TISS) to VI (TVISS). The type III secretion system (TTSS) is an important mechanism of virulence in E. tarda (Rao et al., 2004; Zheng et al., 2005), that appear to confer survival and replication within macrophages (Okuda et al., 2006). E. tarda as a typical intracellular pathogen resists to reactive oxygen species (ROS) and survives within phagocytic cells (Ishibe et al., 2008), a feature that is partially due to the presence of various enzymes including an iron-cofactored superoxide dismutase (FeSOD) (Cheng et al., 2010), and heat shock proteins HtpG and DnaJ (Hsp90 and Hsp40, respectively) that mediate resistance to various stress conditions including ROS (Dang et al., 2011). The type IV secretion system TIVSS is a membrane transporter 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). A number of genes coding for Pili and TIVSS were reported recently in E. tarda EIB202, however, they appear to be incomplete (Wang et al., 2009).

Previous studies by our group reported the identification of seven antigenic protein coding genes of E. tarda ETSJ54 strain (Verjan et al., 2005). Subsequently, the usefulness and protective effects of some of those genes and the recombinant proteins in vaccinated fish were reported by others (Hou et al., 2009). Recently, we described a number of genes associated with the virulence of ETSJ54 obtained throughout a random genome analysis (Verjan et al., 2013), where chemotaxis, flagellum, endotoxin (LPS) and capsular polysaccharide, iron uptake, proteases, intra-macrophage survival and toxin secretion by a type I and a type III secretion systems were the most relevant findings. In this study, we identify and annotate gene sequences coding for major structural components of the Pili/TIVSS, Fimbria and invasins, some of them appear to be absent in the previously sequenced genome of E. tarda EIB202 strain isolated from an outbreak of disease in turbot (Scophthalmus maximus) cultured in China (Wang et al., 2009). The genes were deposited in the GeneBank database in 2005, and a discussion of the putative roles of those genes of E. tarda is presented. The results show that E. tarda ETSJ54 possess various genetic elements associated with adhesion, invasion and colonization of fish tissues.

Material and methods

Bacterial strains and culture conditions

E. tarda ETSJ54 was isolated from an outbreak of disease in Japanese flounder (Paralichthys olivaceus) in Shizuoka, Japan and was described previously (Verjan, Hirono and Aoki, 2005). The bacterium was grown on heart infusion medium (Difco Laboratories, Detroit, MI, USA) at 30 °C. Bacterial strains and plasmids used in this study are described in Table 1. Escherichia coli strains XL1-Blue MR was used as host for recombinant cosmid and JM109 was used as host for recombinant plasmid. Both E. coli strains were grown in Luria-Bertani (LB) or 2× YT medium at 37 °C and when required, ampicillin at concentrations of 50 µg/ml and chloramphenicol at 20 µg/ml were added (Sambrook and Russell, 2001).

Generation of ETSJ54 genomic DNA libraries

Genomic DNA from ETSJ54 was isolated by the method of Ausubel (Ausubel et al., 1994), and used to construct Cosmid and plasmid DNA libraries as previously described (Verjan et al., 2013). Briefly, the genomic DNA was partially digested with Sau3A1 enzyme and the DNA fragments in the 20-40 Kbp range were dephosphorylated with calf intestinal alkaline phosphatase (Promega, Madison, WI, USA) and ligated into the BamHI site of Supercos I vector (Stratagene, La Jolla, CA, U.S.A). The recombinant molecules were packaged into lambda (λ) phage particles (Epicentre Technologies, Madison, WI, USA) and used to infect E. coli XL1-Blue MR.

Plasmid libraries were constructed in puC118 plasmid vector (Takara, Ohtsu, Japan). All DNA, cosmid and plasmid preparations were carried out using standard procedures (Sambrook and Russell, 2001).

Table 1. Bacterial strains, cosmid and plasmids used in this work

Bacterial strains and plasmids	Genotype, phenotype or characteristics	Source or reference
Edwardsiella tarda ETSJ54	Wild type	Verjan et al., 2005
Escherichia coli		
DH5α	F-, *Φ80*d *lacZ* ΔM15, Δ(*lacZYA-argF),* U169, *deoR, rec*A1, *endA*1, *hsdR17* (r_K-,m_K+), phoA, *sup*E44, λ-, thi-1, *gyrA96*, *relA*1. Recipient for recombinant plasmids.	BRL (USA)
XL1BlueMR	Δ(*mcrA*) 183 Δ (*mcr*CB-*hsd*SMR-*mrr*) 173 *end*A1 *sup*E44 thi-1 *rec*A1 *gyr*A96 *rel*A1 *lac*. Recipient for recombinant cosmid.	Stratagene, La Jolla, California
JM109	*recA*1, *supE*44, *endA*1, *hsdR*17, *gyrA*96, *relA*1 thi Δ(*lac-proAB*) F' [*tra*D36, *pro*AB+, *lacI*^{q} *lacZ*1ΔM15]. Recipient for recombinant plasmids.	Takara, Tokyo Japan
Cosmid and plasmids vectors		
SuperCos I	Ampicillin resistant (Ap^r) cosmid vector	Stratagene, La Jolla, CA
pGEM-T Easy Vector	Ampicillin resistant (Ap^r) *lacZ* cloning vector	Promega, Madison, WI
pUC118	Ampicillin resistant (Ap^r) *lacZ* cloning vector	Pharmacia
pHSG398	Chloramphenicol resistant (Cm^r) cloning vector	Takara, Tokyo Japan
pBluescriptII SK+	Ampicillin resistant (Ap^r), lacZ α-complementing cloning vector	Stratagene, La Jolla, California
Cosmid (KEC) and plasmid (KES) clones encoding T4SS		
KEC07_B04_T7, KEC09_E10_T7	Encoding ETSJ54 *traB*	This study
KEC04_F09_T3, KEC04_F09_T3	Encoding ETSJ54 *traF*	This study
KEC01_A07_T7	Encoding ETSJ54 *traH*	This study
KES02_E05_T3	Encoding prepilin peptidase dependent protein D	This study
KEC09_D07_T7	Encoding typeIV pilus biogenesis protein PilM	This study

Subcloning and DNA sequencing

Cosmid and plasmid libraries were plated on LB agar plates with ampicillin and single colonies were randomly isolated and grown in 2 × YT 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, whereas Plasmid DNA was sequenced with M13F (5′-GTAAAACGACGGCCAGTACG-3′) and M13R (5′-ACTATCTAGAGCGGCCGCTT-3′) primer sets. Cosmid DNA was digested with several restriction enzymes (i.e, BamHI, EcoRI, EcoRV, HincII, HindIII, PstI, SacI, or SacII) and the DNA fragments ligated into plasmid vectors (pUC118, pBluescript, and pHSG399) for sequencing. 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). Details of the Subcloning strategy were reported recently (Verjan et al., 2013).

Gene analysis

The DNA sequence data of ETSJ54 were compared with those in the GenBank database using the BLASTX (Version 2.2.28+) software (Zhang et al., 2000) of the National Center for Biotechnology Information, to identify DNA sequences that resemble our query sequence based on similarity of the nucleotide sequence. The identified closest homologous gene sequence 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 putative adhesion-invasion-related genes of E. tarda ETSJ54 were submitted to the GenBank database and the accession numbers are showed in Table 2.

Table 2. Adhesion and invasion-related genes of Edwardsiella tarda ETSJ54

Category	Gene	Putative name/function	Accession No.	Close related sp. (June 2013)	Query Cover	E-value	Amino acid Identity
Pili/T4SS							
1	ppdD	Prepilin peptidase dependent protein D	AB231503	*E. tarda* EIB202	100%	7,00E-89	98%
2	traB	type IV conjugative transfer system protein	AB231507	*Escherichia coli* 3.4880	93%	0.0	93%
3	traF	type IV conjugative transfer system protein	AB231506	*Photobacterium damselae* subsp. Piscicida	98%	3,00E-54	80%
4	traH	type IV conjugative transfer system protein	AB231505	*Vibrio mimicus* VM603	100%	3,00E-99	95%
5	pilM	type IV pilus biogenesis protein	AB831782	*E. tarda* C07-087	99%	3,00E-138	99%
Fimbria							
6	HofB	Transport protein, biogenesis of fimbria	AB231504	*E. tarda* EIB202	95%	2,00E-67	100%
7		Fimbrial subunit CS5	AB231508	*Edwardsiella tarda* C07-087	100%	2,00E-20	90%
8	lpfB,	Long polar fimbrial chaperone	AB231509	*E. tarda* EIB202	95%	1,00E-77	98%
9	bcfC	Fimbrial outer membrane usher protein	AB231510	*E. tarda* EIB202	100%	9,00E-147	99%
10	ghfA	Pilin protein, major fimbrial protein	AB231511	*E. tarda* EIB202	91%	1,00E-124	100%
11	afaC	Outer membrane usher protein	AB231512	*E. tarda* FL6-60	100%	0.0	99%
Invasin							
12		invasin family protein	AB831786	*E. tarda*	79%	3,00E-109	96%
13	invasin	Invasin	AB231513	*E. tarda* EIB202	100%	0.0	97%
Others putative virulence-related genes							
14	ttrA	Tetrathionate reductase, subunit A	AB231566	*E. tarda* EIB202	100%	0.0	99%
15	ttrB	Tetrathionate reductase, subunit B	AB231567	*E. tarda* EIB202	97%	2,00E-80	68%
16	ttrR	Tetrathionate reductase complex:response regulator	AB231568	*E. tarda* EIB202	100%	5,00E-147	99%
17	vacJ	VacJ lipoprotein	AB231569	*E. tarda* EIB202	98%	2,00E-38	95%
18	tolC	Outer membrane protein tolC precursor	AB231570	*E. tarda* EIB202	97%	7,00E-87	98%
19	ompS1	OmpK37, outer membrane porin	AB231571	*E. tarda* C07-087	100%	0.0	99%
20	DsbA	Putative disulfide isomerase	AB231572	*E. tarda* EIB202	100%	5,00E-102	100%
21	asnC	Regulatory protein AsnC	AB231573	*E. tarda* EIB202	100%	2,00E-107	99%
22	dvsB	tRNA-dihydrouridine synthase B	AB231574	*E. ictaluri*	100%	6,00E-57	94%
23	mrcA	Peptidoglycan synthetase; penicillin-binding protein 1A	AB231575	*Yersinia pestis* biovar Orientalis	100%	2,00E-28	82%
24	hemY	Putative protoheme IX biogenesis protein	AB231576	*E. tarda* EIB202	94%	5,00E-80	97%
25	glnD	Protein-PII, uridylyltransferase	AB231577	*E. tarda* EIB202	100%	3,00E-168	96%
26	gpi	Glucose-6-phosphate isomerase	AB231578	*E. tarda* EIB202	100%	1,00E-159	100%
27	aroC	Chorismate synthase	AB231579	*E. tarda* EIB202	100%	4,00E-123	98%
28	purM	Phosphoribosylformylglycinamidine cyclo-ligase	AB231580	*E. tarda* EIB202	96%	2,00E-178	100%
29	pyrC	dihydroorotase	AB231581	*E. tarda* EIB202	86%	2,00E-54	95%
30	spoT	guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase	AB231582	*E. tarda* EIB202	100%	3,00E-162	99%

Results

A total of 30 protein-coding genes associated with adhesion, invasion and virulence of E. tarda ETSJ54 are presented in Table 2. The annotated E. tarda gene sequences were classified into Pili/TIVSS- and fimbria-related genes, invasin and other putative virulence-related genes. The genes related to fimbria, invasin and other putative virulence-related genes were deposited in the GenBank database in 2005, when no genome from E. tarda was available. An updated comparison of their nucleotide sequence by using the BLASTX software of NCBI, indicate that a number of DNA sequences have nucleotide and deduced amino acid identity to genes and proteins reported in the E. tarda EIB202 genome (Wang et al., 2009), E. tarda C07-087 (Tekedar et al., 2013) or E. tarda FL6-60 (van Soest et al., 2011). Interestingly, the gene sequences coding for the type IV conjugative transfer system proteins traB, traF and traH in E. tarda ETSJ54 did not have any nucleotide or 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 TIVSS-related genes of E. tarda ETSJ54 showed nucleotide and deduced amino acid sequence identity to traB gene of Escherichia coli, traF of Photobacterium damselae (Formally Pasteurella piscicida) and Vibrio mimicus VM603, whereas the fimbria, invasin and other virulence-related genes had nucleotide and deduced amino acid identity to those in the previously reported E. tarda genome, indicating that although the TIVSS is highly conserved within enterobacteriaceae members, there could be E. tarda strains lacking those genetic elements or that they may be missing in the sequenced genomes. Another possibility is that a number of non-characterized proteins might be associated with those functions.

The E. tarda ETSJ54 type IV conjugative transfer system genes showed 80-95 % nucleotide and deduced amino acid sequence identity to the genes

and proteins of other enterobacteriaceae members with a nucleotide sequence coverage ranging from 93 to 100% (Table 2). A partial nucleotide and deduced amino acid sequence of the E. tarda ETSJ54 type IV conjugative transfer system protein TraB is presented in Figure 1. The provided TraB DNA sequence is 1130 bp in length and encode a protein of 360 amino acids, possesses one BamHI restriction site at position 1056, three HincII restriction sites at positions 816, 834 and 1036 and one SacI restriction site at position 304. The stop codon (TAA) is indicated at position 1083, whereas the start codon and a range of amino acids at the N-terminal region are missing (Figure 1).

positions 74 and 75 that correspond to the positions 144 and 145 of E. coli TraB protein. Additionally, ETSJ54 TraB protein showed an extended C-terminal region with 5 amino acids additions regarding to all aligned protein sequences.

To understand the phylogenetic relationships of E. tarda ETSJ54 Tra proteins with other members of the enterobacteriaceae family, we used both the Molecular Evolutionary Genetics Analysis (MEGA) version 5.2 software and the BLAST pairwise alignment with the Neighbor Joining method to estimate the phylogenetic distances between the E. tarda ETSJ54 TraB protein sequence and the

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TGGTAGAGCAAGAGAGAGCTGGAGTTACAAAGGATACTGCCGGTAAATCCAGTGATGTTGGTAGAGAGATTTCTCGCCTACGCCAAGACC    90
  V  E  Q  E  R  A  G  V  T  K  D  T  A  G  K  S  S  D  V  G  R  E  I  S  R  L  R  Q  D  L
TTGATCGCCTGACTCAGAAAAATATGGAGTTGGCTAAGAAGGTTGAAACTGGTGAGGCTGGTGGAAAAACGGCCTCTTCAACAGGTGATG   180
  D  R  L  T  Q  K  N  M  E  L  A  K  K  V  E  T  G  E  A  G  G  K  T  A  S  S  T  G  D  A
CCAGAGCAGATGTTAATGGTGCATCAGACGGTGAAGGTCAAGGTCAGTTCATGGAGAAAAAGCTGGACTACAAAGATCCGGCATCTTTTT   270
  R  A  D  V  N  G  A  S  D  G  E  G  Q  G  Q  F  M  E  K  K  L  D  Y  K  D  P  A  S  F  F
                                  SacI
TCCGTGATGCACCACTCCCGGACTCGAAGGGTGGAGCTCCAGCAACAGGTAAAGGTGATGGCCGTGATGCCACTAAGCCAGGCATTCAGA   360
  R  D  A  P  L  P  D  S  K  G  G  A  P  A  T  G  K  G  D  G  R  D  A  T  K  P  G  I  Q  I
TTGTGAGCTACTCACAGAAGGCCCCAGAAGTTGATGTGAAAGACGACAAAGATGATGAGTCCCTCTATCTGCCGTCAGGTTCCATCCTGA   450
  V  S  Y  S  Q  K  A  P  E  V  D  V  K  D  D  K  D  D  E  S  L  Y  L  P  S  G  S  I  L  T
CAGGTGTACTTATCAACGGTATGGATGCGCCTACTTCCCAGGGTGCCCGACGAGATCCATTCCCTTCAACTCTTCGGATTCAGAAAGAAG   540
  G  V  L  I  N  G  M  D  A  P  T  S  Q  G  A  R  R  D  P  F  P  S  T  L  R  I  Q  K  E  A
CAATATTGCCTAATCGTTTCCGTGCGGATGTCAGAGAGTGTTTCCTGATTGTTTCAGGCTATGGCGACCTTAGTTCTGAACGAGCCTACC   630
  I  L  P  N  R  F  R  A  D  V  R  E  C  F  L  I  V  S  G  Y  G  D  L  S  S  E  R  A  Y  L
TACGCGGTGAAACATTCTCTTGTGTCAGGGAAGACGGTGGGGTGATTGAAGCGAGGCTTGATTCTTATGCGGTTGGTGAGGACGGAAAGG   720
  R  G  E  T  F  S  C  V  R  E  D  G  G  V  I  E  A  R  L  D  S  Y  A  V  G  E  D  G  K  A
CTGGTGTTCGAGGCCGCGTTGTATCGAAGCAAGGGCAGATTATTGCCAAGAGCTTGATGGCTGGGTTCCTTGGTGGCGTGTCTGAGGCCT   810
  G  V  R  G  R  V  V  S  K  Q  G  Q  I  I  A  K  S  L  M  A  G  F  L  G  G  V  S  E  A  F
     HincII           HincII
TTGATGTCAACCCTGTACCAGTTGTTAACACTAACCCTGGCTCAAATACCCAGTATCAGTCAGTTTTCTCTGATCAGATGTTTCAGGGGG   900
  D  V  N  P  V  P  V  V  N  T  N  P  G  S  N  T  Q  Y  Q  S  V  F  S  D  Q  M  F  Q  G  A
CTGCTGCTAAGGGAGCGAGCAAGGCGCTGGATCGTATCGCTCAATTCTACATCGACATGGCCGAAAGTATCTTCCCGGTCATCGAGGTCG   990
  A  A  K  G  A  S  K  A  L  D  R  I  A  Q  F  Y  I  D  M  A  E  S  I  F  P  V  I  E  V  D
                                        HincII              BamHI
ATGCTGGGCGCCAAGTAGACATCATTGTGACAAAATGCACCAAGTGTCAACGACCGGGTAAAAAGGGATCCACTTACTCTCCACCGACGG  1080
  A  G  R  Q  V  D  I  I  V  T  K  C  T  K  C  Q  R  P  G  K  K  G  S  T  Y  S  P  P  T  V
TTTAATATTGATCCACCGTTTTACCTAGGATTAGCTTCCGC                                                    1130
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Figure 1. Partial nucleotide and deduced amino acid sequence of ETSJ54 type IV conjugative transfer system protein TraB. The nucleotide sequence is 1130 bp in length, encoding a protein of 360 amino acids and possesses restriction sites SacI, HincII and BamHI (underlined) enzymes and a stop codon a position 1083. The start codon and a range of amino acids at the N-terminal region are missing.

A multi-alignment of E. tarda ETSJ54 TraB deduced amino acid sequence was constructed with the closest homologous protein sequences and presented in Figure 2. Despite the missing N-terminal region of TraB protein, the deduced amino acid sequence of ETSJ54 TraB showed 93% identity to E. coli TraB protein (315/338, 93%) and also high identity to the homologous proteins of Photobacterium damsealae subsp. Piscicida (315/338, 93%), Vibrio mimicus VM603 (315/338, 93%) and Xenorhabdus nematophila (318/338, 94%) and Aeromonas hydrophila (306/340, 90%). Of note, ETSJ54 TraB protein showed two amino acid additions (Glycine and Glutamine) at the homologous protein in other bacteria. Figure 3 shows that E. tarda ETSJ54 TraB protein cluster more closely with Aeromonas hydrophila and Xenorhabdus nematophila ATCC 19061 TraB proteins and that it may have evolved or acquired those genes from the fish pathogen Aeromonas salmonicida.

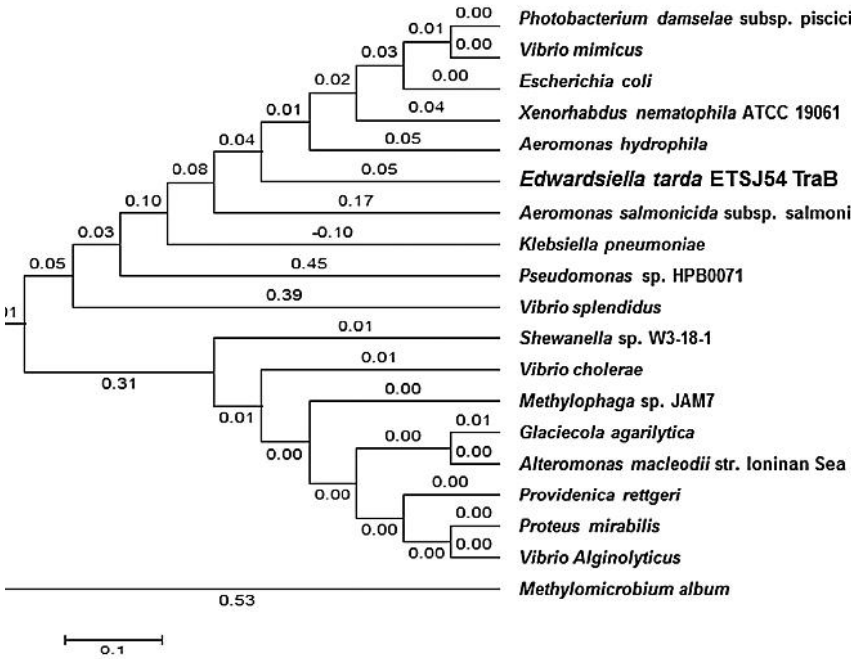

Figure 3. Phylogenetic distances between various type IV conjugative transfer system TraB proteins. The tree image was constructed by using the Molecular Evolutionary Genetics Analysis (MEGA) version 5.2, using the Neighbor Joining method. The accession numbers are the following: Photobacterium damselae subsp. piscicida, YP_908651; Vibrio mimicus, WP_000595748; Escherichia coli, WP_001694498; Xenorhabdus nematophila ATCC19061, YP_003662308; Aeromonas hydrophila, YP_002995568; Aeromonas salmonicida subsp. salmonicida A449, YP_001144206; Klebsiella pneumoniae, YP_002302231; Pseudomonas sp. HPB0071, WP_010799361;Vibrio splendidus, WP_017085225; Shewanella sp. W3-18-1, YP_962511; Vibrio cholera, WP_001896332; Methylophaga sp. JAM7, YP_006292395; Glaciecola agarilytica, WP_008305516; Alteromonas macleodii str. ¨Ioninan sea UM4b¨, YP_008183174; Providencia rettgeri, AAM08009; Proteus mirabilis, WP_004249357; Vibrio alginolyticus, WP_005396819; Methylomicrobium album, WP_005369499; and the E. tarda ETSJ54 TraB is shown in bold.

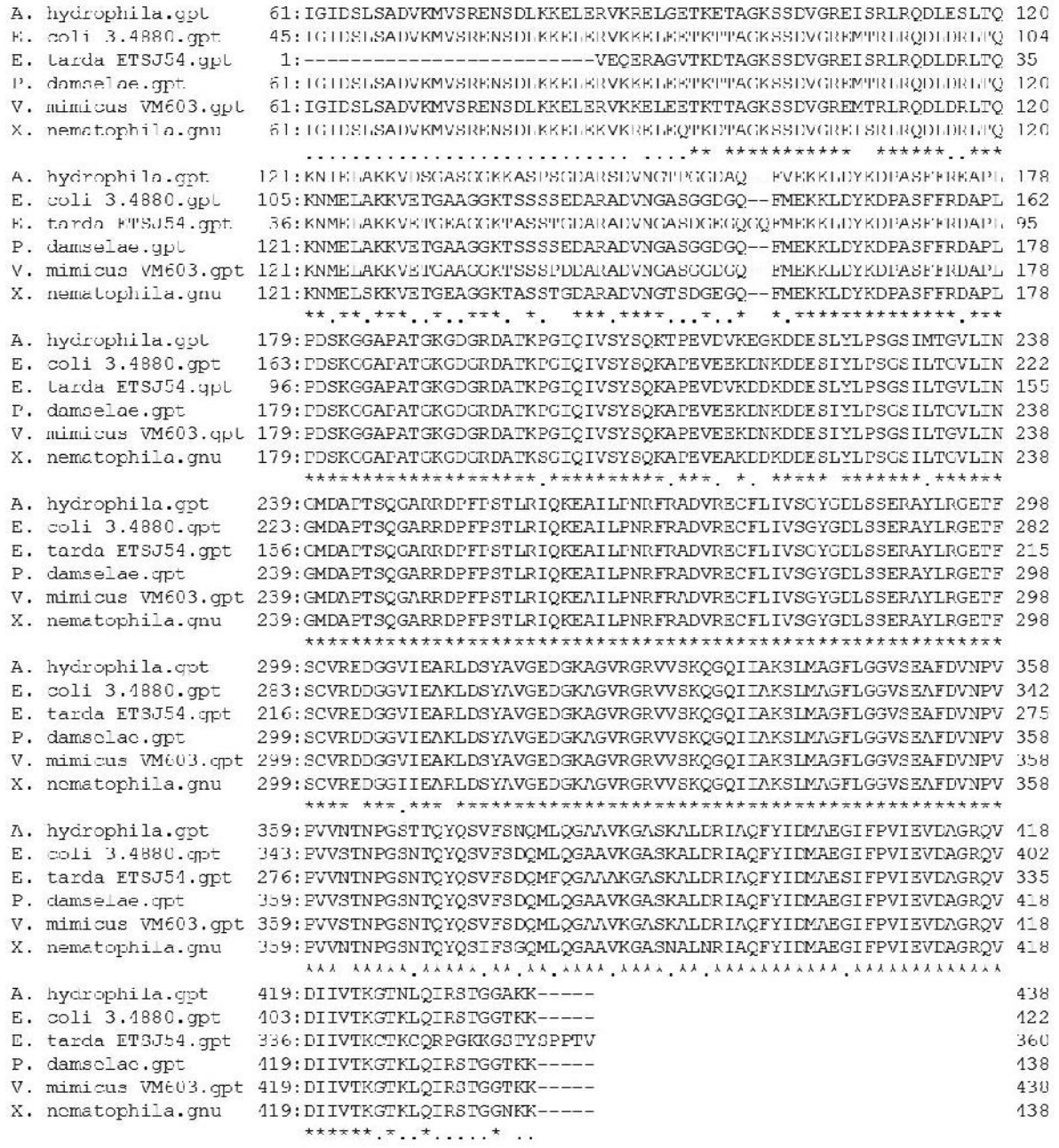

Figure 2. Multialignment of ETSJ54 TraB deduced amino acid sequence with homologous proteins from other bacteria. ETSJ54 TraB shows two amino acid additions (Glycine and Glutamine) at the positions 144 and 145 of E. coli TraB protein. The asterisks at the bottom indicate identical amino acids whereas the dots indicate different amino acids regarding to the aligned amino acid sequences from other bacteria.

Discussion

Bacterial adhesion and invasion in E. tarda ETSJ54

Surface structures in E. tarda might be involved in adhesion, invasion and colonization of host tissues, thus contributing to its pathogenicity, among them are adhesins, flagellum, fimbria, pilus and the T3SS apparatus (Ehrbar and Hardt, 2005). In addition, enzymes such as the sialidase NanA has also been involved in the ability of E. tarda to disseminate and to colonize fish tissues (Jin et al., 2012). Virulence-related genes are usually located in mobile genetic elements such as pathogenicity islands and plasmids, and those plasmids coding for antibiotic resistance genes, transposases and conjugal transfer genes have also been associated with E. tarda virulence (Yu et al., 2012). Bacterial adhesins are classified into two major classes, fimbrial adhesins that includes fimbria, pili, curli and type IV pili,

and nonfimbrial adhesins such as outer membrane proteins (OMPs) and flagella (Amano, 2010). Pili, fimbria, invasins, flagella (Friedlander et al., 2013) and OMPs (Confer and Ayalew, 2013) are associated with adhesion, invasion and internalization of many invasive pathogens, including invasive non-typhoidal strains of Salmonella, which possess a number of fimbrial operons, colonization factors and typhoid-associated virulence genes (Suez et al., 2013). Uropathogenic E. coli strains take advantage of those structures to mediated adhesion and invasion of the bladder and kidney epithelial cells (Niemann et al., 2004), and in Bordetella pertussis for adhesion to mucosal surfaces of the respiratory tract (Finlay and Falkow, 1997). E. tarda ETSJ54 appear to express those surface structures (Table 2), that may allow stable contacts with the host cells before initiating the invasion process, and may allow the bacteria to sense what kind of host cell is at the front, and probably what kind of pathogenic mechanism is needed to be activated, to carry on the infectious process. Upon bacterial internalization by epithelial cells, the pathogen can induce apoptosis or cell lysis to obtain nutrients and replicate. The identified Pili- and TIVSS-related genes, some of them flanked by transposases or associated with plasmid-encoded genes may also indicate the capability of horizontal acquisition and transfer of genetic material between E. tarda and other bacteria. However, it remains to be determined the precise origin of this transport system, since at present we have not isolated any plasmid from this strain.

Transfer of genetic material between bacteria can occurs through bacterial conjugation, a process that involves the formation of a mating bridge and a close contact between donor and recipient cells. The most studied mechanism is the plasmid-encoded extracellular filament or F pilus (Frost et al., 1994) that allows intergeneric and interkingdom F plasmid transfer. Among the E. tarda ETSJ54 TIVSS-related genes, we identified genes such as traB, traF, and traH involved in F pilus assembly during DNA transfer (Table 2). The E. tarda ETSJ54 tra genes were found in close proximity to transposase genes IS21, IS100 and IstB of Escherichia coli, suggesting that E. tarda ETSJ54 TIVSS-related genes might have been acquired horizontally and encoded in an uncharacterized plasmid, however, and the presence of mobile genetic elements suggest they could be integrated in the genome of E. tarda. A preliminary phylogenetic analysis based on the E. tarda ETSJ54 TraB protein sequence indicated that the E. tarda type IV conjugative transfer system might have been acquired from common fish pathogens such as Aeromonas salmonicida (Figure 3), suggesting a most probably inter-genus transfer from this microorganism.

Invasin binds tightly to a family of β_1 integrins and induces formation of pseudopods that mediate the bacterial uptake by epithelial cells and macrophages (Isberg et al., 2000; Niemann, Schubert and Heinz, 2004). Invasin also mediates activation of mucosal immune responses by induction of signaling events in epithelial cells that give rise to cytokines (TNFα, IL-1α, IL-1β) and chemokines (IL-8, MCP-1) production, and granulocyte-macrophage colony stimulating factor (GM-CSF) expression, and subsequent recruitment of leukocytes, in order to establish an infection (Grassl et al., 2003). The attachment of invasin to β_1 integrins is the key step in the contact-dependent secretion of virulence factors by the type III secretion system. An invasin-deficient E. tarda showed reduced bacterial virulence and impaired adhesion and invasion capabilities to host cells (Li et al., 2012). Those surface structures in E. tarda may be involved in a variety of functions and their potential roles in the pathogenesis of the disease, are yet to be recognized.

Nonfimbrial adhesins facilitates the bacterial attachment and invasion to polarized cells from the intestinal epithelium (Griessl et al., 2013). In E. tarda, nonfimbrial adhesins were initially associated with hemagglutination properties (Wong et al., 1989), later, a fimbrial gene cluster of E. tarda was associated with hemagglutination activities in erythrocytes from various animals' species including fish (Sakai et al., 2003). The fimbrial genes identified in this study matched well with those recently reported in E. tarda EIB202 (Wang et al., 2009), however, the precise function/role in disease and pathogenicity is currently unknown and the expression of recombinant proteins might be an useful strategy to evaluate their utility in disease prevention.

Other virulence-related genes in E. tarda

There are a number of virulence-related genes described in other Gram-negative pathogens that need to be characterized in the fish animal model of edwardsiellosis. We grouped here other putative virulence-related genes in the E. tarda genome considered important virulence factors in other bacteria. For example, outer membrane proteins such as tolC, and ompS1 are associated with multi-drug efflux systems (Lin et al., 2002). DsbA, asnC, yhdG, mrcA, and the putative glycosylase or exported protein of Y. pestis and hemY, contribute to P. mirabilis pathogenesis of urinary tract infections (Burall et al., 2004). The gene products of glnD, pgi, and aroC were associated with intracellular survival of Brucella suis in human macrophages (Foulongne et al., 2000), and purM, pyrC, and spoT are essential genes for B. abortus intracellular growth in HeLa cells (Kim et al., 2003). The vacJ lipoprotein, a virulence

factor associated with the intercellular spreading of S. flexneri (Suzuki et al., 1994) was also found in E. tarda. However, its sequence showed several internal stop codons and need to be re-sequenced to confirm this finding and to provide a better evidence of the involvement of this mechanism in the induction of disease by E. tarda ETSJ54. A functional characterization of these proteins will also clarify their roles in edwardsiellosis.

Conclusions

E. tarda is a pathogenic Gram-negative bacterium that causes edwardsiellosis, a suppurative disease of fish and mammals. Recently we performed a random genome analysis of a virulent strain of E. tarda (ETSJ54) and here we identified a series of virulence-related genes coding for surface structures involved in adhesion, invasion and colonization of fish tissues. Of note, we annotated genes coding for a TIVSS that were absent in the previously reported genome of a virulent and multi-drug resistant E. tarda EIB202 isolated in China. The identified genes in E. tarda ETSJ54 may be useful tools to explore their roles adhesion and invasion during infection of multiple hosts and encourage the study of other type IV secretion system genes no identified so far in this bacterium.

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