Análisis genómico al azar de *Edwardsiella tarda* ETSJ54: anotación de genes relacionados con virulencia

A random genome analysis of *Edwardsiella tarda* ETSJ54: annotation of putative virulence- related genes

A análise do genoma aleatória de *Edwardsiella tarda* ETSJ54: anotação de genes de virulência relacionados ao putativos

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Resumen

Como un paso inicial para comprender los mecanismos de patogenicidad usados por *Edwardsiella tarda* durante la infección en peces, se llevo a cabo un secuenciamiento genómico parcial y al azar de librerias de ADN construidas en vectores cosmido y plasmido generadas a partir de una cepa (ETSJ54) virulenta de *E. tarda* para identificar genes presumiblemente relacionados con su virulencia. Los genes relacionados con virulencia de acuerdo a la semejanza en las secuencias de nucleotides con otras especies bacterianas fueron agrupados en nueve categorías que incluyeron quimiotaxis y motilidad, endotoxina (LPS), secreción de toxinas por los sistemas scretorios I y III, adquisición de hierro, proteasas y sobreviviencia dentro de macrófagos. Los resultados indican que *E. tarda* posee un amplio rango de genes involucrados en la virulencia y en la patogenicidad de generos bacterianos diversos y especies como *Salmonella, Yersinia* and *Vibrios.* Los resultados también indican que existe un alto flujo de genes en el genoma de *E. tarda* que podrían explicar en algún grado su potencial de infectar y causar enfermedad en varias especies animales.

Palabras clave: *Edwardsiellosis*, secuenciamiento genómico, virulencia, patogénesis.

Abstract

As an initial step to understand the pathogenic mechanisms displayed by *Edwardsiella tarda* during infection in fish, we conducted a random genome sequencing of cosmid and plasmid DNA libraries generated from a virulent *E. tarda* strain (ETSJ54) to identify putative virulence-related genes. The assumed virulence-related genes of *E. tarda* were grouped into nine categories including chemotaxis and motility, adhesion and invasion, endotoxin (LPS), toxin secretion by type I and type III secretion systems, iron uptake, proteases, and intra-macrophage survival. The results reveal that *E. tarda* is equipped with a wide range of genes involved in virulence and pathogenesis of diverse bacterial genera and species including *Salmo-*

nella, Yersinia and *Vibrios* species. The results also indicate a high genetic flux in the *E. tarda* genome that could explain in some extent its potential to infect and to cause disease in a number of animal species.

Key words: *Edwardsiellosis*, genome sequencing, virulence, pathogenesis.

Resumo

Como um passo inicial para entender os mecanismos patogenéticos expostos por *Edwardsiella tarda* durante a infecção no peixe, conduzimos uma genoma sequencing de cosmid e plasmad ADN bibliotecas geradas de um virulento *E. tarda* tensão (ETSJ54) para identificar genes putativos relacionados com virulência. Os genes relacionados com virulência assumidos de *E. tarda* foram agrupados em ocho categorias inclusive chemotaxis e motility, endotoxin (LPS), tipo I e tipo III sistemas de substância segreda, compreensão de ferro, procaçoadores, e intra-macrophage sobrevivência. Os resultados revelam que *E. tarda* é equipado com uma larga variedade de genes implicados na virulência e pathogenesis de gêneros bacterianos diversos e espécie inclusive Salmonella, Yersinia e espécie Vibrios. Os resultados também indicam um alto fluxo genético no *E. tarda* genoma que pode explicar em alguma extensão o seu potencial para infeccionar e causar a doença em um número de espécie dos animais.

Palavras-chave: Edwardsiellosis, genoma sequencing, virulência, pathogenesis.

Introduction

Edwardsiellosis is a systemic suppurative disease caused by the Gram-negative bacterium *Edwardsiella tarda,* a member of the family enterobacteriaceae (Ewing *et al.,* 1965). *E. tarda* is usually found in water-living animals, causing disease in cultured marine and fresh-water fishes around the world (Miyazaki and Kaige 1985). The bacterium may also cause sporadic infections in birds, frogs, reptiles, marine and terrestrial mammals including humans (Verjan *et al.,* 2012). The infection in man often occurs accidentally during manipulation of aquatic animals and range from self-limited gastrointestinal and extraintestinal infections up to lethal septicemia (Wang *et al.,* 2005; Spencer *et al.,* 2008).

Multiple proteins appear to be involved in the virulence and pathogenesis of *E. tarda* infections, some of them are hemolysins (Hirono *et al.,* 1997), siderophore production, resistance to serum killing, motility mediated by the flagella, and phosphate uptake (Mathew *et al.,* 2001), a sialidase Nan A that increase colonization of fish tissues (Jin *et al.,* 2012), a type III secretion system that allow survival and replication of *E. tarda* within macrophages (Okuda *et al.,* 2006), a DNA adenine methylase (Dam) that reduce UV radiation and H_2O_2 sensibility (Sun *et al.,* 2010), an iron-cofactored superoxide dismutase (FeSOD) that inhibits macrophage-mediated immune responses (Cheng *et al.,* 2010), and plasmids coding antibiotic resistance genes, transposases and conjugal transfer genes have also been associated with *E. tarda* virulence (Yu *et al.,* 2012).

The above studies have contributed substantially to understand the pathogenic mechanisms used by *E. tarda* during the infection process in fish, and the information gathered from the whole genome sequence of *E. tarda* EIB202 strain showed that a substantial proportion of the genome is devoted to the growth and survival under diverse conditions including intracellular niches (Wang *et al.,* 2009). We initially reported the identification of seven antigenic protein coding genes of *E. tarda* ETSJ54 strain (Verjan *et al.,* 2005), and subsequent studies by others reported the usefulness and protective effects of some of those proteins in vaccinated fish (Hou *et al.,* 2009). Our group also performed a partial genome sequencing of the *E. tarda* ETSJ54 genome and deposited in the Gene Bank database a number of virulent-related genes (Verjan, 2005). Here, we present the annotation and a discussion of the putative roles of those genes that were available since 2005, before the whole genome of *E. tarda* was published. By that time there were no many sequenced genes of *E. tarda* available and by using the basic local alignment search tool (BlastX, version 2.2.28+), the obtained nucleotide sequences resembled those from many Gram-negative enteropathogens, however, an up-to-date BlastP (BlastP, version 2.2.28+) results is presented here and indicate that almost all the coded proteins of the *E. tarda* ETSJ54 genome correspond to those of the *E. tarda* EIB202 strain (Wang *et al.,* 2009). The results shows that *E. tarda* ETSJ54, is equipped with the genes coding for major surface structures involved in motility, lipopolysaccharides and capsular polysaccharides, endo and exo-toxin secretion, iron uptake, intramacrophage survival and proteases among others. The presence of a variety of insertion sequence elements not only indicates a high genetic flux in the *E. tarda* genome but also suggests this bacterium has a highly dynamic and potentially rapidly evolving genome that could explain in some extent its potential to infect and to cause disease in a number of animal species.

Material and methods

Bacterial strains and culture conditions

E. tarda SJ54 (ETSJ54) was isolated from an outbreak of disease in Japanese flounder (*Paralichthys olivaceus*) in Shizuoka, Japan. The bacterium was grown on heart infusion medium (Difco Laboratories, Detroit, MI, USA) at 30 °C. All bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* strains XL1-Blue MR and JM109 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).

Construction of genomic DNA libraries

Genomic DNA from ETSJ54 was isolated by the method of Ausubel (Ausubel *et al.,* 1994), and partially digested with a fixed concentration of *Sau*3A1 enzyme at the indicated time-lapses (30s, 60s, 90s, 2′, 3′, 5′, 7′, 10′ and 15′). Genomic DNA fragments obtained at each digestion period were separated in 1% agarose by pulsed field gel electrophoresis (PFGE), with pulse times of 5s to 20s at 6 volts for 8 hr. Genomic DNA fragments in the 20-40 Kbp range (Figure 1) were dephosphorylated with calf intestinal alkaline phosphatase (Promega, Madison, WI, USA) and ligated into the *Bam*HI 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. Genomic DNA from ETSJ54 was also subjected to random mechanical shearing by using an ultrasonic disrupter UD-21 (Tomy Digital Biology Co, Tokyo Japan), coupled with a micro tip to produce small DNA fragments (0.5-2 kbp) by ultrasounds. The DNA fragments were ligated into the plasmid vector puC118 (Takara, Ohtsu, Japan) to generate a plasmid DNA library. *E. coli* JM109 was transformed with recombinant plasmids by the heat shock method and all DNA, cosmid and plasmid preparations were carried out using standard

Table 1. Strains and plasmids used in this work

Bacterial strains and plasmids	Genotype, phenotype or characteristics	Source or reference
Edwardsiella tarda ETSJ54	Wild type	This study
Escherichia coli DH5α	F-,Φ80d lacZ ΔM15, Δ(lacZYA-argF), U169, deoR, recA1, endA1, hsdR17 (rk-,mk+), phoA, supE44, λ-, thi-1, gyrA96, relA1. Recipient for recombinant plasmids.	BRL (USA)
XL1BlueMR	Δ(mcrA) 183 Δ (mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac. Recipient for recombinant cosmid.	Stratagene, La Jolla, California
JM109	recA1, supE44, endA1, hsdR17, gyrA96, relA1 thi Δ(lac-proAB) F′ [traD36, proAB+, lacIq lacZ1ΔM15]. Recipient for recombinant plasmids.	Takara, Tokyo Japan
Cosmid and plasmids		
SuperCos I	Ampicillin resistant (Apr) cosmid vector	Stratagene, La Jolla, CA
pGEM-T Easy Vector	Ampicillin resistant (Apr) lacZ cloning vector	Promega, Madison, WI
pUC118	Ampicillin resistant (Apr) lacZ cloning vector	Pharmacia
pHSG398	Chloramphenicol resistant (Cmr) cloning vector	Takara, Tokyo Japan
pBluescriptII SK+	Ampicillin resistant (Apr), lacZ α-complementing cloning vector	Stratagene, La Jolla, California

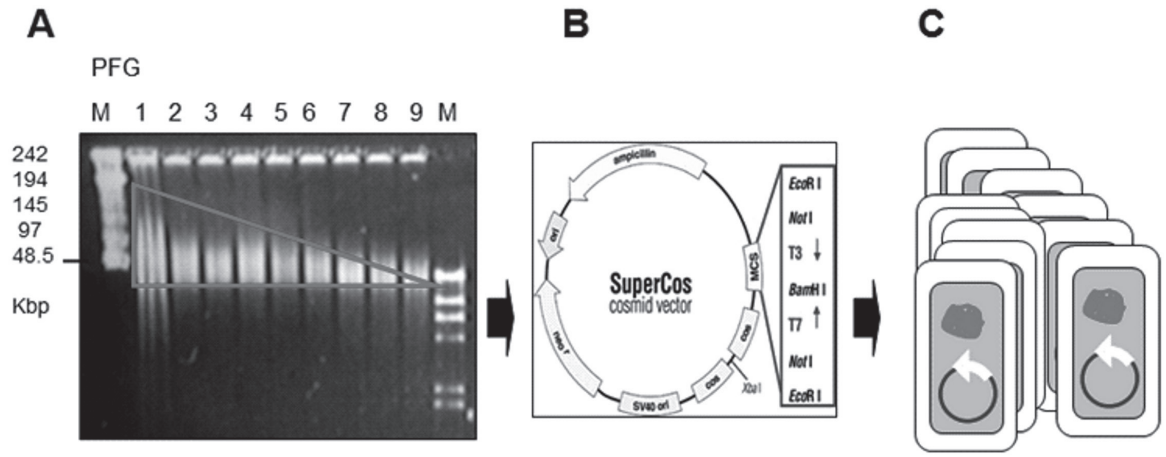


Figure 1. Flowchart of *Edwardsiella tarda* ETSJ54 cosmid DNA library construction. Genomic DNA of *E. tarda* ETSJ54 was isolated and digested with *Sau*3AI restriction enzyme for 30s, 60s, 90s, 2', 3', 5', 7', 10' and 15' and analyzed in 1% agarose by pulsed field gel electrophoresis (Lanes 2-9). Lane 1: undigested genomic DNA. PFG-M: PFG DNA ladder marker. M: *Hin*dIII digested lambda DNA marker. B. Digested genomic DNA was dephosphorylated and ligated into the *Bam*HI of Super Cos I vector. C. *E. coli* XL-1BlueMR cells were infected with lambda phage particles carrying the recombinant cosmid molecules.

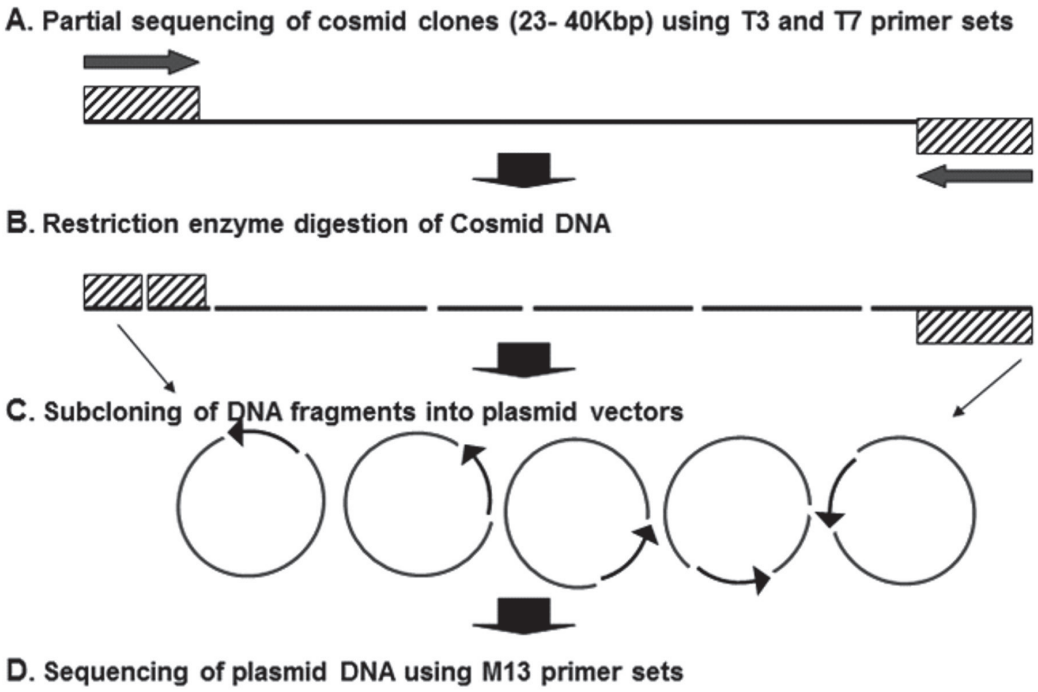
procedures (Sambrook and Russell 2001). Cosmid and plasmid DNA libraries were amplified and stored at -80 °C until use.

Subcloning and nucleotide sequence determination

Cosmid and plasmid libraries were cultured in LB agar plates with ampicillin and single colonies were randomly isolated and grown in 2 x 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 to identify putative ORF flanking the *E. tarda* DNA fragments. Cosmid DNA was digested with *Eco*RI restriction enzyme to estimate the size of the inserted DNA fragment, followed by digestion with several restriction enzymes (i.e, *Bam*HI, *Eco*RI, *Eco*RV, *Hin*cII, *Hin*dIII, *Pst*I, *Sac*I, or *Sac*II) and the DNA fragments ligated into plasmid vectors (pUC118, pBluescript, or pHSG399) for sequencing (Figure 2). Plasmid DNA were sequenced with M13F (5'-GTAAAACGACGGCCAGTACG-3') and M13R (5'-ACTATCTAGAGCGGCCGCTT-3') primer sets. 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). Specific oligonucleotides primers were designed to amplify some of the putative open reading frames (ORFs). The PCR products were ligated into pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced. Details for any technique will be provided if required.

Gene annotation and classification

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, 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 and its classification. The functional classification of *E. tarda* DNA sequences followed that used for other pathogens such as *Yersinia* and *Salmonella* species database of the Sanger Institute (www.sanger.ac.uk/Projects/Microbes/), or those reported in the Microbial Genome Database (http://mbgd.genome.ad.jp). The putative virulence-related genes of *E. tarda* ETSJ54 were submitted to the GenBank data base and the data included the closest original hits obtained when no *E. tarda* genome was known. Here, we provide an updated comparison of the predicted amino acid sequence of the ETSJ54 ORFs using the BLASTP (Version 2.2.28+) software (Altschul *et al.,* 1997).

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ATTGATAATACCCTGTTTCCTCACCAGGCTGACTGAAGGAGTCGTCATGACGATGCGTTTTCCCTTAACCCGATGTTTTCCCGCGCTGGTTGTCCTCAGC  100
                                          M  T  M  R  F  P  L  T  R  C  F  P  A  L  V  V  L  S
GCCCTGCTGCTGCAAGGCTGCGTCGCCGCCGTGATCGGCAGTGCGACCATGGCAACCCAGGCGGCCAGCGATCCCCGCAGCGTGGGTACCCAGGTCGACG  200
A  L  L  L  Q  G  C  V  A  A  V  I  G  S  A  T  M  A  T  Q  A  A  S  D  P  R  S  V  G  T  Q  V  D  D
ATGGTACGCTGGAGGCCCGCATCTCCAACGCCCTGAGCAAAGACGCACAGCTGAAGAAAGAGGCGCGCGTCGTGGTAACCGCCTATCAGGGGCAGGTGCT  300
  G  T  L  E  A  R  I  S  N  A  L  S  K  D  A  Q  L  K  K  E  A  R  V  V  V  T  A  Y  Q  G  Q  V  L
GCTGACCGGTCAGGCGCCAAGCCAGGCGCTGATTAGCCGCGCCAAGCAGATCGCGATGGGCGTTGAAGGCACCAAGGCGGTCTATAATGAGATCCGTTTA  400
 L  T  G  Q  A  P  S  Q  A  L  I  S  R  A  K  Q  I  A  M  G  V  E  G  T  K  A  V  Y  N  E  I  R  L
GGCCAGCCGGTCAGCCTGGGCACTGCCTCGGCCGATGCCTGGATCACCACCAAGGTCCGCTCCCAGCTGCTGGCCAGCGACCAGGTGAAATCCACCAACG  500
G  Q  P  V  S  L  G  T  A  S  A  D  A  W  I  T  T  K  V  R  S  Q  L  L  A  S  D  Q  V  K  S  T  N  V
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Figure 2. Subcloning and sequencing of plasmid DNA clones carrying *E. tarda* ETSJ54 genomic DNA fragments. Cosmid DNA was isolated and the 5' and 3' regions of the inserted DNA were sequenced using T3 and T7 primers (A). The cosmid DNA was digested with several restriction enzymes (B), and the DNA fragments ligated into pUC118 or pHSG398 plasmid vectors (C). The nucleotide sequence of the inserted DNA fragments were sequenced with M13 primers sets (D), and generated sequence data was compared with those in the Gene Bank database (See Materials and Methods).

Results

Functional classification of E. tarda ETSJ54 open reading frames (ORFs)

One thousand and one hundred fifty eight (1,158) putative ORFs of the *Edwardsiella tarda* ETSJ54 genome were identified from a total of 1,382 sequenced clones (1,056 cosmid and 326 plasmid clones). The number of putative ORFs and the coded genes revealed that there was not significant redundancy in the sequenced clones, and indicates these libraries are unique and represent an important tool for further studies. The functional classification of *E. tarda* ETSJ54 ORFs (Table 2) shows 5 major categories as follows: small molecule metabolism (256 ORFs), which constitute 22% from total ORFs and contain protein-coding genes involved in degradation of carbon compound and amino acids, energy metabolism, central intermediary metabolism, amino acid biosynthesis, polyamine synthesis, nucleosides and nucleotides biosynthesis, cofactors and fatty acid biosynthesis. The other four major categories are the broad regulatory function-related genes (65 ORFs), macromolecule metabolism (219 ORFs), cell processes (179 ORFs) and others (439 ORFs), which include insertion sequence elements and hypothetical proteins. The percentages of *E. tarda* ETSJ54 ORFs in each subcategory are shown in Figure 3.

Table 2. Functional classification of Edwardsiella tarda ETSJ54 ORFs

Functional category	No. ORFs	%		Functional category	No. ORFs	%
1 Small molecule metabolism				**2 Broad regulatory functions [250]**	65	
1.A Degradation	4			**2.1 Signal transduction**	0	
1.A.1 Carbon compounds	33			**Total**	65	5,65
1.A.2 Amino acids	7			**3 Macromolecule metabolism**		
1.B Energy metabolism				**3.A Synthesis and modification**		
1.B.1 Glycolysis	5			**of macromolecules**		
1.B.2 Pyruvate dehydrogenase	2			3.A.1 rRNA and stable RNAs	2	
1.B.3 Tricarboxylic acid cycle	3			3.A.2 Ribosomal protein synthesis and modification	4	
1.B.5 Pentose phosphate pathway	0			3.A.3 Ribosome maturation and modification	1	
1.B.5.a Oxidative branch	0			3.A.5 Aminoacyl tRNA synthetases		
1.B.5.b Non-oxidative branch	2			and their modification	13	
1.B.6 Entner-Doudoroff pathway	1			3.A.6 Nucleoproteins	0	
1.B.7 Respiration	1			3.A.7 DNA replication, restriction/modification,		
1.B.7.a Aerobic	22			recombination and repair	47	
1.B.7.b Anaerobic	18			3.A.8 Protein translation and modification	9	
1.B.7.c Electron transport	13			3.A.9 RNA synthesis, RNA modification		
1.B.8 Fermentation	0			and DNA transcription	8	
1.B.9 ATP-proton motive force	3			3.A.10 Polysaccharides (cytoplasmic)	3	
1.B.10 Glyoxylate bypass	0			3.A.11 Phospholipids	4	
1.C Central intermediary metabolism	24			**3.B Degradation of macromolecules**		
1.C.1 General	0			3.B.1 RNA	4	
1.C.2 Gluconeogenesis	3			3.B.2 DNA	6	
1.C.3 Sugar nucleotides	1			3.B.3 Proteins, peptides and glycopeptides	12	
1.C.4 Amino sugars	2			3.B.4 Polysaccharides	2	
1.C.5 Sulphur metabolism	4			**3.C Cell envelope**	6	
1.D Amino acid biosynthesis	0			3.C.1 Membranes, lipoproteins and porins	44	
1.D.1 Glutamate family	5			3.C.2 Surface polysaccharides,		
1.D.2 Aspartate family	9			lipopolysaccharides and antigens	28	
1.D.3 Serine family	3			3.C.3 Surface structures	6	
1.D.4 Aromatic amino acid family	6			3.C.4 Murein sacculus and peptidoglycan	19	
1.D.5 Histidine	1			3.C.5 Miscellaneous periplasmic proteins	1	
1.D.6 Pyruvate family	4			**Total**	219	18,68
1.D.7 Branched chain family	0			**4 Cell processes**	1	
1.E Polyamine synthesis	3			**4.A Transport/binding proteins**	32	
1.F Purines, pyrimidines, nucleosides				4.A.1 Amino acids and amines	17	
and nucleotides				4.A.2 Cations	19	
1.F.1 Purine ribonucleotide biosynthesis	8			4.A.3 Carbohydrates, organic acids and alcohols	23	
1.F.2 Pyrimidine ribonucleotide biosynthesis	3			4.A.4 Nucleosides, purines and pyrimidines	0	

1.F.3 2'-deoxyribonucleotide biosynthesis	6			4.A.5 Anions	3	
1.F.4 Salvage of nucleosides and nucleotides	2			4.A.6 Other	20	
1.F.5 Miscellaneous nucleoside/nucleotide	5			4.B Chaperones, chaperonins, heat shock	5	
1.G Biosynthesis of cofactors, prosthetic				4.C Cell division	5	
groups and carriers				4.D Chemotaxis and mobility	20	
1.G.1 Biotin	5			4.E Protein and peptide secretion	0	
1.G.2 Folic acid	4			4.G Detoxification	6	
1.G.3 Lipoate	0			4.H Cell killing	1	
1.G.4 Molybdopterin	1			4.I Pathogenicity	27	
1.G.5 Pantothenate	0			**Total**	179	15,55
1.G.6 Pyridoxine	1			**5 Other**		
1.G.7 Pyridine nucleotide	0			5.A IS elements, Phage-related		
1.G.8 Thiamine	4			functions and prophage	57	
1.G.9 Riboflavin	0			5.B Colicin-related functions	0	
1.G.10 Thioredoxin	1			5.C Plasmid related functions	0	
1.G.11 Menaquinone and ubiquinine	9			5.D Drug/analogue sensitivity	14	
1.G.12 Heme and porphyrin	7			5.F Adaptions to atypical conditions	6	
1.G.13 Cobalamin	6			5.G Antibiotic resistance	2	
1.G.14 Iron uptake and storage	5			5.H Conserved hypothetical protein	334	
1.H Fatty acid biosynthesis	10			5.I Unknown	26	
Total	256	22,06		**Total**	439	38,05
				Total ORFs	1158	100

Virulence-related genes in the E. tarda ETSJ54 strain

A total of one hundred and five (105) putative virulence-related genes of *E. tarda* ETSJ54 were annotated and deposited in the Gene Bank database. Identification was made by comparison of their nucleotide sequence with those in other bacterial pathogens, in which virulence-related genes and the coding protein have been characterized in some extent. Eighty (80) putative virulence-related genes were grouped into 8 subcategories and the GeneBank accession numbers are presented in Table 3. The subcategories in which the *E. tarda* ETSJ54 ORFs fall into were chemotaxis and motility conferred by the flagellum, capsular polysaccharide and endotoxin production, exotoxin secretion by type I and type III secretion systems, iron uptake, proteases and intramacrophage survival. A wide range of membrane proteins, lipoproteins and proteins involved in peptidoglycan biosynthesis are also components of the bacterial cell wall, and may play different roles in the pathogenesis of the disease, they were classified as ¨other virulence-related genes¨ and not included in this report. The predicted amino acid sequences coded by 80 of the ETSJ54 ORFs were compared to those in the protein sequence database and show that almost all coded proteins resemble those recently reported in *E. tarda* EIB202 (Wang *et al.,* 2009) and the *E. tarda* C07-087 (Tekedar *et al.,* 2013), however, there still differences between *E. tarda* strains and the amino acid identity may varies from 48% to 100 %. These differences may support further studies of its characterization.

Discussion

Chemotaxis and motility conferred by the flagellum

Bacteria are able to sense, respond and adapt to environmental signals that may be useful or detrimental to cell survival. Chemotaxis proteins and the flagellum are coupled to various signal transduction pathways that modulate gene expression to drive motility, cell-to-cell

Table 3. Putative virulence-related genes of Edwardsiella tarda ETSJ54

Category	Gene	Putative name/function	Accession No.	Close related sp. (March 2013)	Query cover	E-value	Amino acid identity
Chemotaxis and motility							
Chemotaxis							
1	motB	Flagellar motor protein	AB231478	E. tarda EIB202	98%	1,00E-152	99%
2	cheA	Chemotaxis protein, histidine kinase and related kinase	AB231479	E. tarda EIB202	98%	4,00E-146	99%
3	cheD	Methyl-accepting chemotaxis protein	AB231480	E. tarda EIB202	88%	2,00E-148	99%
4	tsr2	Methyl-accepting chemotaxis protein	AB231481	E. tarda FL6-60	98%	2,00E-99	99%
5		Chemotaxis sensory transducer family protein	AB231482	E. tarda EIB202	77%	1,00E-30	94%
6	cheM	Methyl accepting chemotaxis protein I	AB231483	E. tarda FL6-60	86%	6,00E-75	93%
7	flhC	Flagellar transcriptional activator	AB231484	E. tarda EIB202	100%	1,00E-51	100%
8	flhD	Flagellar transcriptional activator	AB231485	E. tarda EIB202	96%	1,00E-44	97%
9	fliZ	Putative regulator of Fli A	AB231486	E. tarda EIB202	78%	2,00E-30	98%
Flagellum							
10	fliA	RNA polymerase sigma factor for flagellar operon	AB231487	E. tarda EIB202	100%	1,00E-114	99%
11	fliC	Flagellin	AB195507	E. tarda EIB202	100%	0	99%
12	fliD	Fagella hook associated protein (HAP 2)	AB231488	E. tarda EIB202	100%	0,00E+00	99%
13	fliS	Flagellar specific chaperona	AB231489	E. tarda EIB202	100%	2,00E-91	99%
14	fliT	Repressor of class 3a and 3b operon (RFIA activity)	AB231490	E. tarda EIB202	87%	2,00E-37	86%
15	fliF	Flagellar M-ring	AB231491	E. tarda EIB202	99%	1,00E-77	98%
16	fliH	Flagellar assembly protein	AB231492	E. tarda EIB202	100%	6,00E-33	92%
17	fliI	Flagellum-specific ATP synthase	AB231493	E. tarda EIB202	100%	0,00E+00	99%
18	fliJ	Flagellar protein FliJ	AB231494	E. tarda EIB202	85%	3,00E-52	99%
19	fliK	Flagellar hook-length control protein FliK	AB231495	E. tarda EIB202	100%	5,00E-147	99%
20	fliL	Flagellar biosynthesis	AB231496	E. tarda EIB202	100%	7,00E-100	97%
21	fliM	Flagellar motor switch protein FliM	AB231497	E. tarda EIB202	100%	0,00E+00	99%
22	fliN	Flagellar motor switch protein FliN/type III SS	AB231498	E. tarda EIB202	96%	3,00E-85	99%
23	fliO	Flagellar protein fliO	AB231499	E. tarda EIB202	100%	4,00E-62	98%
24	fliP	Flagellar biosynthesis protein	AB231500	E. tarda EIB202	100%	1,00E-144	98%
25	flgE	FlgE flagellar hook protein FlgE	AB231501	E. tarda EIB202	100%	2,00E-42	100%
26	ygiY	Sensor histidine kinase QseC	AB231502	E. tarda EIB202	100%	8,00E-177	99%
LPS and capsular polysaccharides							
27	wzy	Putative O-antigen polymerase protein	AB231514	E. tarda EIB202	96%	7,00E-173	97%
28	wecB	UDP-N-acetylglucosamine 2-epimerase	AB231515	E. tarda EIB202	100%	1,00E-146	94%
29	waaQ	Heptosyl III transferase	AB231516	E. tarda EIB202	100%	9,00E-177	99%
30	gmhA	Phosphoheptose isomerase	AB231517	E. tarda EIB202	100%	1,00E-25	98%
31	waaA	3-deoxy-manno-octulosonic acid transferase	AB231518	E. tarda EIB202	90%	3,00E-124	98%
32	waaE	Cell wall biosynthesis glycosyltransferase	AB231519	E. tarda EIB202	1005	5,00E-44	99%
33	yfdH	Putative glycosyltransferase	AB231520	E. tarda EIB202	100%	5,00E-146	85%

34	wecC	UDP-N-acetyl-D-mannosamine dehydrogenase	AB231521	E. tarda EIB202	100%	3,00E-147	95%
35	kdsB	3-deoxy-manno-octulosonate cytidylyltransferase	AB231522	E. tarda EIB202	97%	2,00E-37	82%
36	waaC	Lipopolysaccharide heptosyltransferase-I	AB231523	E. tarda EIB202	100%	8,00E-90	100%
37	waaF	Lipopolysaccharide heptosyltransferase-II	AB231524	E. tarda EIB202	95%	2,00E-77	100%
38	nanE	N-acetylmannosamine 6 phosphate 2-epimerase	AB231525	E. tarda EIB202	87%	1,00E-123	100%
39	nanK	N-acetyl manosamine kinase	AB231526	E. tarda EIB202	87%	1,00E-13	100%
40	rcsF	Colanic acid synthesis regulator, OMP lipoprotein	AB231527	E. tarda EIB202	100%	2,00E-94	100%
41	yfbE	UDP-4-amino-4-deoxy-L-arabinose–oxoglutarate aminotransferase	AB231528	E. tarda EIB202	94%	5,00E-101	99%
42	yfbF	UDP phosphate 4-deoxy-4-formamido-L-arabinose transferase	AB231529	E. tarda EIB202	100%	7,00E-73	100%
43	wabN	Lipopolysaccharide biosynthesis, deacetylase	AB231530	E. tarda EIB202	48%	4,00E-50	100%
44	msbB	Lipid A biosynthesis (KDO)2-(lauroyl)-lipid iva acyltransferase	AB231531	E. tarda EIB202	100%	1,00E-81	100%
45	hldE	Fused heptose 7-phosphate kinase/ heptose 1-phosphate adenyltransferase	AB231532	E. tarda EIB202	100%	0,00E+00	100%
46	wzxE	Lipopolysaccharide biosynthesis protein	AB231533	E. tarda C07-087	94%	6,00E-48	87%
47	wecF	4-alpha-L-fucosyltransferase	AB231534	E. tarda EIB202	87%	2,00E-27	93%
48	waaG	UDP-glucose, heptosyl, LPS alpha 1,3-glucosyltransferase	AB231535	E. tarda C07-087	100%	5,00E-37	96%
49	cpsH	Capsular polysaccharide biosynthesis	AB231536	E. tarda EIB202	100%	7,00E-93	94%
50	ExoT	Capsular polysaccharide biosynthesis protein	AB231537	Pectobacterium wasabiae	34%	7,00E-04	55%
51	wbdC	Capsular polysaccharide biosynthesis glycosyl transferase	AB231538	E. tarda EIB202	89%	3,00E-106	100%
52	wcaJ	Capsular polysaccharide biosynthesis	AB231539	E. tarda C07-087	100%	1,00E-178	98%
53	bcsB	Cellulose synthase subunit B	AB231540	E. tarda EIB202	100%	8,00E-51	100%
54	bcsC	Cellulose synthase operon C domain-containing protein	AB231541	E. tarda EIB202	91%	3,00E-158	98%
Type I secretion system							
55	hlyB	Putative hemolysin activator HlyB	AB231542	E. tarda EIB202	100%	3,00E-137	100%
56	amyH	Large repetitive protein	AB231543	E. tarda C07-087	99%	3,00E-158	100%
57	ytfL	Putative hemolysin-related membrane protein	AB231544	E. tarda ATCC 23685	100%	3,00E-85	99%
58	tolC	Outer membrane protein tolC precursor	AB231570	E. tarda EIB202	97%	7,00E-87	98%
59	ompS1	Outer membrane porin F protein	AB231571	E. tarda C07-087	100%	0.0	99%
Type III secretion system							
60	esrA	spiR two-component sensor/regulator	AB231545	E. tarda C07-087	100%	2,00E-132	99%
61	esaM	Type III secretion apparatus protein	AB231546	E. tarda EIB202	100%	2,00E-65	99%
62	esaR	Type III secretion apparatus protein R	AB231547	E. tarda EIB202	100%	2,00E-112	97%
63	esaS	Type III secretion apparatus protein	AB231548	E. tarda EIB202	100%	1,00E-34	100%
64	escB	Type III secretion system chaperone protein B	AB231549	E. tarda EIB202	100%	2,00E-96	100%
65	esaC	Type III secretion system EscC protein	AB231550	E. tarda EIB202	100%	5,00E-180	100%

66	esaL	Type III secretion system apparatus	AB231551	E. tarda EIB202	88%	2,00E-130	100%
67	eseE	Type III secretion system effector protein E	AB231552	E. tarda EIB202	100%	3,00E-80	99%
68	eseD	Type III secretion system effector protein D	AB231553	E. tarda EIB202	100%	1,00E-82	99%
69	eseC	Type III secretion system effector protein C	AB231554	E. tarda EIB202	100%	3,00E-165	96%
70	acfC	Porcine attaching-effacing associated protein variant 1	AB231555	E. tarda EIB202	100%	6,00E-33	82%
Iron uptake							
71	pvsB	Siderophore synthetase component	AB231556	E. tarda EIB202	100%	4,00E-168	100%
72	pswP	4´-phosphopantetheinyl transferase, enterobactin synthetase component D	AB231557	E. tarda EIB202	100%	2,00E-86	100%
73		Ferric-enterobactin-transport protein, ABC transporter	AB231558	E. tarda EIB202	100%	4,00E-143	99%
74	pvsE	Vibrioferrin biosynthesis protein	AB231559	E. tarda C07-087	99%	1,00E-77	97%
75		TonB dependent siderophore receptor	AB231560	E. tarda EIB202	96%	3,00E-140	87%
Proteases							
76	sepA	Zinc metalloproteinase, aureolysin	AB231561	E.tarda FL6-60	95%	1,00E-129	97%
77		Chondroitin ABC lyase	AB231562	E. tarda EIB202	95%	1,00E-98	87%
Intramacrophage survial							
78	pagC	Virulence-related membrane protein	AB231563	E. tarda EIB202	100%	3,00E-129	99%
79	mgtC	Mg(2+) transport ATPase C	AB231564	E. tarda EIB202	100%	2,00E-19	100%
80	mgtB	Mg(2+) transport P-type ATPase B	AB231565	E. tarda EIB202	100%	0,00E+00	98%
Total							
80							

clumping or prevent chemotaxis (Bible *et al.,* 2012). In fish pathogens, those proteins may be advantageous in a highly dynamic environment such the water, where they may allow the bacteria to reach the host mucosal surfaces and to find an appropriate niche for colonization. The flagellum has been involved in the invasion process of *Salmonella enterica* (Stecher *et al.,* 2004) and *Burkholderia pseudomallei* in mammals (Chua *et al.,* 2003), similarly in fish, a flagellin (FliC) deficient *E. tarda* showed reduced pathogenecity, motility, biofilm formation and reduced levels of TTSS virulence-associated proteins (He *et al.,* 2012). Flagellin is the structural component of the flagellum, and a pathogen associated molecular pattern (PAMP) recognized by Toll-like receptor 5 (TLR-5), capable of activate innate and adaptive immune responses with strong adjuvant activity (Sanders *et al.,* 2009), and overexpression of flagellin may induce elevated immune responses and attenuate bacterial virulence (Yang *et al.,* 2012). We identified regulators of the chemotaxis response such as CheA and major components of the flagella structure in *E. tarda* ETSJ54 (Table 3), including flagellin, and previously we reported that a rabbit anti-*E. tarda* serum reacted with the recombinant flagellin (FliC, ET46) of ETSJ54 in Western blot analysis demonstrating its antigenic properties (Verjan *et al.,* 2005). Recently, flagellin was found in the OMP extract of *E. tarda* where it appears to mediates direct interaction of the bacteria with fish epithelial cell surface proteins (Liu *et al.,* 2012), indicating not only functions in motility but also in adhesion and invasion. The flagellum is a protein export system structurally similar to the type III secretion of virulence factors (TTSS), which appear to exist only in flagellated Gram-negative species, therefore, additional functions to this structure might be discovered in near future. Both the flagellum and TTSS were recently reported to be regulated by the two-component system QseB/QseC in *E. tarda* (Wang *et al.,* 2011), genes also found in *E. tarda* ETSJ54 (Table 3).

Lipopolysaccharide (LPS) and capsular polysaccharide

The lipopolysaccharide (LPS) is considered a major virulence factor, and is one of the most potent microbial initiators of inflammation by Gram-negative bacteria.

Three components structure the LPS molecule, the hydrophobic lipid moiety or lipid A, an oligosaccharide core attached to the lipid A, and the O-antigen (Gyorfy *et al.,* 2013). LPS mediates cell activation by a signaling pathway involving the LPS binding protein (LBP) that transfer LPS to CD14 and then to the MD-2 and TLR-4 complex (Ohto *et al.,* 2007), that form a multimeric complex on the surface of monocytic cells that lead to cytokine production (such as TNF-α, IL-1, IL-6) and a systemic inflammatory reaction that can result in multiple organ failure, shock and death (Gyles 2011). The structure of the O-polysaccharide of *E. tarda* was reported (Vinogradov *et al.,* 2005) and gives insights into the differences and relationships with other LPS molecules and their differential immunostimulatory activities. We identified a number of genes involved in the synthesis and assembly of the LPS and the capsular polysaccharide of *E. tarda* ETSJ54; however, the mechanisms of action in fish are yet to be recognized. Fish were reported to be low responders or insensitivity to the effects of LPS (Iliev *et al.,* 2005), although, there had been some reports on the immunomodulatory capacity of various LPS preparations (Sampath *et al.,* 2009; Nayak *et al.,* 2011), today the hemodynamics and vascular changes that can be induced in mammals upon LPS administration are considered absent in fish. It is accepted that LPS could induce a differential immune response in fish that appears to depend on its structure and source (Hang *et al.,* 2013; Kadowaki *et al.,* 2013), and it become necessary to evaluate the role of the LPS in the fish model of Gram-negative sepsis, as this might be different to that known in mammals. LPS and the capsular polysaccharide in *E. tarda* may also be involved in conferring additional properties to the bacterium such as serum resistance (complement mediated killing), intramacrophage survival or even have another roles not yet described.

Secretion of toxins: Type I secretion system

The bacterial type I secretion system (T1SS) is involved in the secretion of various cell toxins and adhesins such as the giant nonfimbrial adhesin of *Salmonella* (Griessl *et al.,* 2013). The pore forming toxin hemolysin (HlyA) from *E. coli* is the example of toxins inserted into the host cell membrane to form a pore or channel that leads to lysis of the host cell (Chen *et al.,* 1996). The *E. tarda* hemolysin (EthA) was characterized in early studies (Hirono *et al.,* 1997). The protein was associated with lysis of the phagocytic vacuole within macrophages (Janda *et al.,* 1991), cytotoxicity in HEp-2 cells (Strauss *et al.,* 1997), and most recently required for cell invasion and internalization of *E. tarda* by epithelial papilloma of carp (EPC) cells (Wang *et al.,* 2010).

Another toxin that may be involved in the pathogenesis of *E. tarda* infections, but not yet described is the leukotoxin or RTX (repeats in the structural toxin), an initially described cytotoxic pore-forming toxin that appears to have a broad spectrum of biological and biochemical activities (Linhartova *et al.,* 2010). It has been well characterized in *Mannheimia haemolytica*, where it shows dose-dependent activity ranging from activation, increases respiratory burst and degranulation of leukocytes at low dose of toxin, up to apoptosis and necrosis at high doses (Narayanan *et al.,* 2002). In this study, we identified in the *E. tarda* ETSJ54 genome the genes coding for the hemolysin A and the hemolysin activator protein hlyB, and a gene coding for the *Salmonella typhimurium* large repetitive protein, also called hemagglutinin/hemolysin related protein in *Ralstonia solanacearum* (Salanoubat *et al.,* 2002) or RTX family exoprotein of *E. coli* (Perna *et al.,* 2001). A functional characterization of this protein in *E. tarda* will allow us to understand more about the pathogenic mechanisms displayed by the bacterium during the induction of disease.

Type III secretion system

Plant and animal bacterial pathogens possess a type III secretion system (TTSS) that secretes bacterial virulence proteins into the host cells, capable of modulating a variety of cellular pathways (Hicks *et al.,* 2011), to generate a differential antigen-specific T cell responses (Lee *et al.,* 2012). This system consists of a secretion apparatus, regulatory proteins, toxins (effector proteins) and chaperone proteins which protect and guide the effector proteins to the TTS apparatus (Ehrbar and Hardt 2005). The TTSS is used for different purposes including attachment, internalization, invasion, multiplication within the host cells and systemic spreading (Abe *et al.,* 2005), and appear to be switched off in vitro, when the bacteria is not in contact with host cells (Gaillard *et al.,* 2011). In *E. coli* this system may induce effacement of the microvilli from intestinal epithelial cells, leading to the formation of attaching/effacing (A/E) lesions (Abe *et al.,* 2005; He *et al.,* 2004). *Yersinia* species and *Pseudomonas aeruginosa* effector proteins mediate inhibition of phagocytosis by interfering with the host cell signaling, perturbing the dynamics of the cytoskeleton, and blocking the production of proinflammatory cytokines (Navarro *et al.,* 2005; Sodhi *et al.,* 2005), whereas in *Salmonella typhimurium*, TTSS appear to mediates irreversible adhesion and invasion *in vitro* (Misselwitz *et al.,* 2012), as well as invasion to the intestinal epithelial cells and trafficking to the basolateral side *in vivo* (Muller *et al.,* 2012). A type III secretion system was previously identified and characterized in

virulent strains of *E. tarda* (Rao *et al.,* 2004; Zheng *et al.,* 2005), and in the course of this study we also found several components of the *E. tarda* type III secretion system (Table 3), however its relevance in fish cell/tissue damage needs further studies.

Genes associated with the iron acquisition system

The genome of *E. tarda* ETSJ54 like other enteropathogens possess a gene cluster that encode proteins involved in biosynthesis and utilization of siderophores, proteins that mediates iron uptake (Sudheesh *et al.,* 2012), an element involved in many biological processes such as respiration, tricarboxylic acid cycle, oxygen transport, gene regulation and DNA biosynthesis (Krewulak and Vogel 2008). The concentration of iron within the host under normal conditions is too low to permit growth of bacteria, and the pathogens are forced to express highly efficient mechanisms for iron acquisition. In fact, bacteria can acquire ferrous iron (Fe^{2+}) and accessible host iron-binding proteins (hemoglobin, transferrin, lactoferrin) by using receptor-mediated transport systems such as the FeoA-interacting G-protein-like transporter FeoB (Kim *et al.,* 2012). However, the main mechanism that contributes to the virulence is the production of iron-chelating compounds (siderophores) also called enterobactin (catecholate) and ferrichrome (hydroxamate), characterized by their high specificity and affinity towards ferric (Fe^{3+}) iron (Andrews *et al.,* 2003; Miethke and Marahiel 2007). Siderophore production appear to be regulated by the iron-responsive transcriptional repressor fur and by small RNA molecules such as RyhB (Salvail *et al.,* 2010). This study identified genes involved in the synthesis and transport of siderophores through the bacterial cell wall in *E. tarda* ETSJ54 (Table 3), that gives support to preliminary observations that suggested the presence of this iron acquisition system in this bacterium (Kokubo *et al.,* 1990), however, its role in the pathogenesis of edwardsiellosis remains to be elucidated.

Proteases

Pathogenic microorganism secretes proteolytic enzymes that mediate tissue destruction and facilitate colonization and infection. Proteases have cytotoxic activities, activate cytolitic toxins, stimulate the production of inflammatory mediators enhancing vascular permeability, promote uptake of nutrients by pathogens, and particularly, they appear to process and degrade vital molecules of the innate inmune system, including the proteins of the coagulation intrinsic pathway and complement proteins (Potempa and Pike 2009), thus proteolytic cleavage appears to be a mechanisms of antibacterial activities inactivation (Potempa and Potempa 2012). The metalloproteinase produced by *Staphylococcus aureus* (Aureolysin) is an example of zinc-dependent metalloproteinases produced as precursor (proAur) with autocatalytic activation properties (Nickerson *et al.,* 2008), and involved in the cleavage of host-plasma proteins and modulation of immunological reactions (Laarman *et al.,* 2011). We identified two proteases genes in the *E. tarda* genome, one with nucleotide sequence identity to the zinc metalloproteinase of *S. epidermidis* and the other had identity the chondroitin ABC lyase of *Proteus vulgaris,* an enzyme that has beneficial effects in reducing the chondroitin sulphate proteoglycans-mediated inhibition of central nervous system repair, following spinal cord injury (Bradbury and Carter 2011). The involvement of these proteins in the pathogenesis of the disease in fish needs specific studies of their biological function.

Intramacrophage survival

Bacterial pathogens evolved mechanism to circumvent the hostile environment within phagocytic cells, avoiding phagosome-lysosome fusion, conferring survival and an intracellular lifestyle (Grabenstein *et al.,* 2006) or enabling the bacteria to adapt to intramacrophage stresses (Thompson *et al.,* 2011). *S. typhimurium, Yersinia pestis* and *Y. pseudotuberculosis* survive within macrophages by regulating the expression of several genes of the two-component regulatory PhoP/PhoQ system. The gene products mediate survival to the bactericide cationic peptides, inhibit antigen processing and presentation and therefore, inhibit induction of specific immunity (Pujol and Bliska 2005). *E. tarda* is an intracellular pathogen, and virulent strains of *E. tarda* proliferate and increase in number inside the macrophages since 9 hr after phagocytosis, which is not observed with low virulent strains (Ishibe *et al.,* 2008). The intracellular life style and replication of *E. tarda* within murine macrophages depend on the expression of the type III secretion system, which induces an NFκB-mediated anti-apoptotic response in the infected macrophages (Okuda *et al.,* 2006). Mutations in the TTSS apparatus, chaperones, effectors and regulators of *E. tarda* were found to have decreased survival and growth within fish phagocytes (Tan *et al.,* 2005). In addition to the genes involved in survival of *E. tarda* within macrophage reported previously (Srinivasa Rao *et al.,* 2001), we identified mgtC, mgtB, molecules involved in intramacrophage survival and growth under Mg^{2+} deprived media (Alix and Blanc-Potard 2007), and pagC, another molecule regulated by the PhoP-PhoQ two-component system, found to be required

to serum resistance in *Salmonella enterica* (Nishio *et al.,* 2005), that may also contribute, although at lower levels, to this particular life style (Alix *et al.,* 2008).

Conclusions

Preliminary studies reported that *E. tarda* produce several virulence-related factors involved in the pathogenesis of edwardsiellosis. Some of the above virulence related factors were corroborated in recent studies using transposon mutagenesis. Moreover, in this study, we contribute to the understanding of the pathogenesis of *Edwardsiella tarda* infections by annotating a number of genes coding for several virulence-related factors, supporting previous observations about its virulence. This preliminary study reveals this bacterium possess a number of putative virulence-related genes associated with mobile genetic elements that mirror a high genetic flux and horizontal gene transfer, and pathogenic mechanisms similar to those displayed by *Salmonella* and *Yersinia* species in mammals. This information will be useful to initiate specific studies on the role of each gene-protein in the pathogenesis induced by this bacterium in fish and mammals.

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