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 llevó a cabo un secuenciamento genómico parcial y al azar de librerías 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 secretorios I y III, adquisición de hierro, proteasas y sobrevivencia 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, secuenciamento 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: Edwardsielliosis, 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, procoaçoadores, 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: Edwardsielliosis, genoma sequencing, virulência, pathogenesis.

Introduction

Edwardsielliosis 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 sepsemia (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 (Hirom 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$_2$O$_2$: 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 Sau3A1 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 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. Genomic DNA from ETSJ54 was also subjected to random mechanical shearing by using an ultrasonic disruptor 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 procedures.

Table 1. Strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Bacterial strains and plasmids</th>
<th>Genotype, phenotype or characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edwardsiella tarda ETSJ54</td>
<td>Wild type</td>
<td>This study</td>
</tr>
<tr>
<td><em>Escherichia coli</em> DH5a</td>
<td>F·Φ80lacI15, ΔlacZ, Δ(lacZYA-argF), U169, deoR, recA1, endA1, hsdR17 (K1, K2), phoA, supE44, λ, thi-1, gyrA96, relA1. Recipient for recombinant plasmids.</td>
<td>BRL (USA)</td>
</tr>
<tr>
<td>XL1BlueMR</td>
<td>Δ(mcrA) 183 Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac. Recipient for recombinant cosmid.</td>
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</tr>
<tr>
<td>JM109</td>
<td>recA1, supE44, endA1, hsdR17, gyrA96, relA1 thiΔ(lac-proAB) F' [traD36, proAB+, lacI lacZ ΔM15]. Recipient for recombinant plasmids.</td>
<td>Takara, Tokyo Japan</td>
</tr>
</tbody>
</table>

Cosmid and plasmids

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>SuperCos I</td>
<td>Ampicillin resistant (Apr) cosmid vector</td>
<td>Stratagene, La Jolla, CA</td>
</tr>
<tr>
<td>pGEM-T Easy Vector</td>
<td>Ampicillin resistant (Apr) lacZ cloning vector</td>
<td>Promega, Madison, WI</td>
</tr>
<tr>
<td>pUC118</td>
<td>Ampicillin resistant (Apr) lacZ cloning vector</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>pHSG398</td>
<td>Chloramphenicol resistant (Cmr) cloning vector</td>
<td>Takara, Tokyo Japan</td>
</tr>
<tr>
<td>pBluescriptII SK+</td>
<td>Ampicillin resistant (Apr), lacZ α-complementing cloning vector</td>
<td>Stratagene, La Jolla, California</td>
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</table>
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 EcoRI restriction enzyme to estimate the size of the inserted DNA fragment, followed by digestion with several restriction enzymes (i.e., BamHI, EcoRI, EcoRV, HindIII, PstI, SalI, or SacII) and the DNA fragments ligated into plasmid vectors (pUC118, pBluescript, or pHSG399) for sequencing (Figure 2). Plasmid DNA were sequenced with M13F (5′-GTAAAACGACGGC-3′) and M13R (5′-ACTATCTAGAGCGGC-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 Buckinghamham, 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 database 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).

Figure 1. Flowchart of Edwardsiella tarda ETSJ54 cosmid DNA library construction. Genomic DNA of E. tarda ETSJ54 was isolated and digested with Sau3AI 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: HindIII digested lambda DNA marker. B. Digested genomic DNA was dephosphorylated and ligated into the BamHI of Super Cos I vector. C. E. coli XL-1BlueMR cells were infected with lambda phage particles carrying the recombinant cosmid molecules.
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, polypeptide 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>
<thead>
<tr>
<th>Functional category</th>
<th>No. ORFs</th>
<th>%</th>
<th>Functional category</th>
<th>No. ORFs</th>
<th>%</th>
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<td>2 Broad regulatory functions [250]</td>
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<td>2.1 Signal transduction</td>
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<td>Total</td>
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<td>5,65</td>
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<td>3 Macromolecule metabolism</td>
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<td>1.B.1 Glycolysis</td>
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<td>3.A.1 rRNA and stable RNAs</td>
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<td>1.B.2 Pyruvate dehydrogenase</td>
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<td>3.A.2 Ribosomal protein synthesis and modification</td>
<td>4</td>
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<tr>
<td>1.B.3 Tricarboxylyl acid cycle</td>
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<td>3.A.3 Ribosome maturation and modification</td>
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<td>1.B.5 Pentose phosphate pathway</td>
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<td>and their modification</td>
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<td>1.B.7 Respiratory metabolism</td>
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<td>3.A.8 Protein translation and modification</td>
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<td>1.B.7.a Aerobic</td>
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<td>3.A.9 RNA synthesis, RNA modification</td>
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<td>1.B.7.b Anaerobic</td>
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<td>and DNA transcription</td>
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<td>1.B.8 Fermentation</td>
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<td>3.A.11 Phospholipids</td>
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<td>3.B.4 Polysaccharides</td>
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<td>3.C Cell envelope</td>
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<td>1.D Amino acid biosynthesis</td>
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<td>3.C.1 Membranes, lipoproteins and porins</td>
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<td>3.C.2 Surface polysaccharides, lipopolysaccharides and antigens</td>
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<td>1.D.2 Aspartate family</td>
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<td>3.C.3 Surface structures</td>
<td>6</td>
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<tr>
<td>1.D.3 Serine family</td>
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<td>3.C.4 Murein sacculus and peptidoglycan</td>
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<td>1.D.4 Aromatic amino acid family</td>
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<td>3.C.5 Miscellaneous periplasmic proteins</td>
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<td>1.D.5 Histidine</td>
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<td>3.C.6 Miscellaneous periplasmic proteins</td>
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<td>1.D.6 Pyruvate family</td>
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<td>4 Cell processes</td>
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<td>1.F Purines, pyrimidines, nucleosides and nucleotides</td>
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<td>4.A Transport/binding proteins</td>
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<td>4.A.1 Amino acids and amines</td>
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<td>4.A.2 Cations</td>
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<td>4.A.4 Nucleosides, purines and pyrimidines</td>
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</table>
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>
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<th>Function</th>
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<td>4.A.5 Anions</td>
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<td>Salvage of nucleosides and nucleotides</td>
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<td>4.A.6 Other</td>
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<tr>
<td>Miscellaneous nucleoside/nucleotide</td>
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<td>5</td>
<td>4.B Chaperones, chaperonins, heat shock</td>
</tr>
<tr>
<td>Biosynthesis of cofactors, prosthetic</td>
<td>1.G</td>
<td></td>
<td></td>
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<tr>
<td>groups and carriers</td>
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<td>Biotin</td>
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Total ORFs 1158

ORINOQUIA 17-1 AGOSTO 2013.indd 75
### Table 3. Putative virulence-related genes of Edwardsiella tarda ETSJ54

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Type I secretion system

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Type III secretion system

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Type III secretion system apparatus

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Iron uptake

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Proteases

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Intramacrophage survival

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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 monocytes cells that lead to cytokine production (such as TNF-a, 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 insensitive 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 appear 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 TTSS 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 mediate 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 regulator 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 edwardsielliosis remains to be elucidated.

**Proteases**

Pathogenic microorganism secretes proteolytic enzymes that mediate tissue destruction and facilitate colonization and infection. Proteases have cytotoxic activities, activate cytolytic 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 immune 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 NFkB-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* produces 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 possesses 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 *Versinia* 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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