Journal of Proteomics xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Comparative proteomic study of *Edwardsiella tarda* strains with different degrees of virulence

Noemí Buján [a,*], Carolina Hernández-Haro [b], Lucía Monteoliva [b], Concha Gil [b], Beatriz Magariños [a]

[a] Departamento de Microbiología y Parasitología, Facultad de Biología-CIBUS, Universidad de Santiago de Compostela, Santiago de Compostela, Spain
[b] Departamento de Microbiología II, Facultad de Farmacia, Universidad Complutense de Madrid, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Spain

ARTICLE INFO

Article history:
Received 6 February 2015
Received in revised form 4 May 2015
Accepted 9 May 2015
Available online xxxx

Keywords:
Edwardsiella tarda
DIGE
Comparative proteomics
Virulence
Fish disease

ABSTRACT

Edwardsiella tarda is an enteric opportunistic pathogen that causes a great loss in aquaculture. This species has been described as a phenotypical homogeneous group; in contrast, serological studies and molecular typing revealed a wide heterogeneity. In this work, a proteomic study of differential expression of a virulent isolate from turbot cultured in the Norwest of Spain in comparison with an avirulent collection strain was performed in order to recognize proteins involved in virulence. One hundred and three proteins that presented different abundance were successfully identified and classified into 11 functional categories according to their biological processes: amino acid, carbohydrate and lipid metabolism, tricarboxylic cycle, stress response and protein fate, protein synthesis, biogenesis of cellular components, cell rescue defence and virulence, cell membrane and transport, signal transduction and purine and pyrimidine metabolism. Twenty three protein spots detected only in turbot isolate were identified. It was shown that the same proteins appeared in different spots in the two isolates. Mass spectra obtained by MALDITOF/TOF of some of these proteins and DNA sequencing explained the changes as a result of different amino acid sequences. Several proteins related with the virulence of *E. tarda* (FliC, ArnA or FeSODI) were only detected in the turbot European isolate.

1. Introduction

Edwardsiella tarda, a Gram-negative facultative aerobic pathogen belonging to the Enterobacteriaceae family, is widely distributed in aquatic environments and has a wide host range including fish, reptiles, amphibians, and humans [1]. Edwardsiellosis is the name of the disease caused by this pathogen, and is characterized by septicaemia, haemorrhages, internal abscesses and skin lesion in various fish taxa [2].

In the last few years, repeated outbreaks of edwardsiellosis in turbot (*Scophthalmus maximus*) have occurred in different geographical areas of Europe being a serious problem in the culture of this fish species [3,4] and causing enormous economic losses in aquaculture industry. Moreover, this fish pathogen has been the causal agent of an episode of mortality in sole (*Solea senegalensis*) reared in a marine farm in the Northwester of Spain [5].

Numerous studies reported that *E. tarda* constitutes a biochemically homogeneous taxon with typical characteristics of an enteric bacterium [3,6]. However, serological studies including LPS profiling and OMP patterns [3,7] revealed the existence of intraspecific antigenic variability. On the other hand, the existence of intraspecific genetic diversity has been also confirmed by the employment of methods such as Restriction Fragment Length Polymorphism (RFLP) PCR of 16S rDNA [8], PCR ribotyping of 16S–23S internal transcribed spacer (ITS) genes in rRNA operons [9], repetitive sequence-based PCR (rep-PCR) and other PCR-based genetic analyses [5]. It is reported that *E. tarda* invades epithelial cells [10], is resistant to phagocytic killing and serum [11–13] and produces virulent enzymes, such as hemolysins [12,14] and chondroitinase [14]. In addition, different quorum-sensing signal molecules have been detected in virulent *E. tarda* strains [15,16]. However, the major virulence factors remain unknown and their identification is crucial to understand *E. tarda* virulence.

E. tarda pathogenesis relies on its ability to regulate its proteome to avoid host defences, and thus, the study of the *E. tarda* proteome is important for understanding mechanisms of pathogenesis and immune evasion. In fact, Kumar et al. [17] and Srinivasa Rao et al. [13] identified proteins that may contribute to *E. tarda* pathogenesis employing proteomic information and isolates from diseased snakehead (*Ophiocephalus punctatus*).

One of the most useful techniques to study changes in proteomes with different aims [18] is the two-dimensional difference gel electrophoresis (2D-DIGE) [19]. This technology is based on the labelling of a mixture composed by up to three protein samples that can be labelled with 3 different fluorescent dyes, Cy2, Cy3 and Cy5 in the same 2-DE

* Corresponding author at: Departamento de Microbiología y Parasitología, Facultad de Biología/CIBUS, Campus Sur, Universidad de Santiago de Compostela, E-15782, Santiago de Compostela, Spain.
E-mail address: noemi.bujan@usc.es (N. Buján).

http://dx.doi.org/10.1016/j.jprot.2015.05.008
1874-3919/

gel. Including an internal standard (pool of all the samples studied), this strategy enables the monitoring of differences in protein abundance with statistical confidence [20].

In this study, we have compared the bacterial proteomes of the fish virulent strain ACC35.1 isolated from turbot versus the fish avirulent strain NCIMB2034 with the aim to identify changes in the abundance of proteins involved in pathogenic mechanisms of *E. tarda*.

2. Materials and methods

2.1. Bacterial strains and growth condition

The virulent *E. tarda* isolate ACC35.1 obtained from diseased turbot, (LD_{50} value was 1.6×10^1 cells/ml), and the avirulent strain isolated from unknown fish NCIMB2034 were cultured on Tryptone Soya Agar (TSA, Pronadisa, Madrid, Spain) or Tryptone Soya Broth (TSB, Pronadisa, Madrid, Spain). Single colonies of each strain were inoculated into 50 ml TSB at 30 °C for 24 h with shaking. Next, 100 ml of TSB was adjusted to OD_{600} 0.2 with previous culture, and were maintained at 30 °C with shaking until obtaining OD_{600} 0.8. After centrifugation at 4000 rpm for 20 min at 4 °C, the pellets were used for protein extraction. This process was repeated three more times to obtain four biological replicates.

2.2. Protein extraction

The bacterial pellets obtained were resuspended in ice-cold lysis buffer (7 M urea, 2 M thiourea, 4% v/w CHAPS and 1 mM PMSF). Then, the suspensions were sonicated and centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was collected and aliquoted. Trichloroacetic acid (TCA) at a final concentration of 10% was added and the suspensions were kept at 4 °C during 1 h to precipitate proteins. After centrifugation at 13,000 rpm for 5 min at 4 °C, the pellet was resuspended in cold acetone and stored overnight at −20 °C. The solutions were centrifuged at 10,000 rpm for 5 min at 4 °C, and the pellets containing proteins were resuspended in rehydration buffer (7 M urea, 2 M thiourea, 4% v/w CHAPS and 30 mM Tris–HCl, pH 8.5). Protein concentration was determined by the Bradford method. The protein extracts were stored at −80 °C until used.

2.3. DIGE: experimental design, protein labelling and 2-DE

Four DIGE analytical gels, containing eight individual samples generated from four biological replicates, were carried out. Each sample was fluorescent-labelled with a set of matched fluorescent dyes according to the manufacturer's protocol for minimal labelling (GE Healthcare) 400 pmol of dye for each 50 μg of protein. The internal standard (IS) pooled sample labelled with Cy2 was also present in each gel. Two replicates of each sample were labelled with Cy3 and the other two with Cy5 (Supplementary data 1).

All samples, including the IS, were diluted 1:1 with the loading buffer (7 M urea, 2 M thiourea, 4% v/w CHAPS, 200 mM v/w DTT and 4% ampholytes according to the pH). For the first dimension (IEF), 150 μg of labelled protein was resolved in 24 cm pH 3–11 nonlinear Immobiline IPG-strips (GE Healthcare) rehydrated for 8 h with 350 μl 2D rehydration buffer (7 M urea, 2 M thiourea, 4% v/w CHAPS, 2% DeStreak and 2% IPGphor buffer 3–11). The samples were applied by cup loading. Focusing was then carried out following conditions: 120 V, 1 h, step; 500 V, 2 h, step; 1000 V, 2 h, gradient; 6000 V, 6 h, gradient; 6000 V, 14 h, step (total focusing 110 KVhr). After focusing, strips were equilibrated for 12 min in reducing buffer (100 mM Tris–HCl pH 8.0, 6 M urea, 30% v/v glycerol, 2% v/w SDS and 2% v/w DTT) and followed by 5 min in alkylating buffer (100 mM Tris–HCl pH 8.0, 6 M urea, 30% glycerol, 2% SDS, 2.5% iodoacetamide and 0.002% bromophenol blue). The strips were transferred onto 12% SDS-polyacrylamide gels in fluorescent glass plates. Electrophoresis was carried out at 20 °C, 1 W/gel for 23 h using an Ettan-Daltsix unit.

2.4. Image acquisition and DIGE data analysis

Gels were scanned directly using a Typhoon 9400 scanner (GE Healthcare) with CyDye filters. For the Cy3, Cy5 and Cy2 image acquisitions, the 532 nm/580 nm, 633 nm/670 nm and 488 nm/520 nm excitation/emission wavelengths were used respectively, adjusting the pixel size to 100 μm.

Image analysis, in this study, was performed using DeCyder software ImageQuant v5.1 (GE Healthcare). For spot detection, determination of quantity, inter-gel matching and statistics, gel images were analysed using DeCyder v6.5 software (GE Healthcare).

All pooled standard/sample gel image pairs were processed by the DeCyder DIA (differential in-gel analysis) and BVA (Biological Variation Analysis). The DIA module was used to assign spot boundaries and to calculate parameters such as normalized spot volumes. Inter-gel variability was corrected by matching and normalization of the internal standard spot maps in the BVA module. The image gel with the major number of spots was used as master gel. To reduce false positives in the p-value calculation, the false discovery rate (FDR) was applied [21]. Protein spots with 2-fold as a threshold in the average ratio with p-values less than 0.05 were considered differentially abundant with statistical significance between different samples under comparison.

2.5. Protein identification by MALDI-TOF MS

Protein identification was done at the Proteomics Facility of Universidad Complutense de Madrid-Parque Científico de Madrid, Spain (UCM-PCM), a member of ProteoRed Network. The protein spots of interest were excided from 2-DE gels stained with colloidal Coomasie blue [22] and digested with trypsin according to Havlis et al. [23]. Brieftly, spots were washed twice with double-distilled water, dehydrated with 75% acetonitrile (ACN) and dried in a Savant SpeedVac. Samples were reduced with 10 mM DTT in 25 mM ammonium bicarbonate for 30 min at 56 °C and subsequently alkylated with 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 20 min in the dark. Finally, samples were digested with 12.5 ng/mL sequencing grade trypsin (Roche Molecular Biochemicals, IN, USA) in 25 mM ammonium bicarbonate (pH 8.5) overnight at 37 °C. After digestion, the supernatants were collected and 1 ml was spotted onto a matrix assisted laser desorption ionization (MALDI) target plate and allowed to air-dry at room temperature. Then, 0.5 μl of a 3 mg/ml of α-cyano-4-hydroxy-trans-cinnamic acid matrix in 0.1% TFA-50% ACN was added to the dried peptide digest spots and again allowed to air-dry again.

MALDI-TOF MS analyses were performed in a MALDITOF/TOF mass spectrometer 4700 Proteomics Analyzer (Per-Septives Biosystems, Framingham, MA). The instrument was operated in reflector positive ion mode, with an accelerating voltage of 20,000 V. All mass spectra were internally calibrated using autodigested trypsin peptides. MALDI-TOF spectra with a signal-to noise 20 were collated and represented as a list of monoisotopic molecular weights. Proteins for which peptide mass fingerprints provided an ambiguous identification were subjected to MS/MS sequencing analyses.

MALDI TOF/TOF fragmentation spectra with a signal-to noise 10 were collected by selecting the suitable precursor ions of each MALDI-TOF peptide mass map. Fragmentation was carried out using the acquisition method 1 kV ion reflector mode collision induced dissociation on and precursor mass window ±10 Da.

Peptide mass fingerprints were analysed using MASCOT version 1.9 from Matrix Science (http://www.matrixscience.com). The searches for peptide mass finger prints and tandem MS spectra were performed in the NCBI nr databases (http://www.ncbi.nlm.nih.gov/protein). The MASCOT search parameters were: (1) species: all; (2) allowed number of missed cleavages: 1; (3) fixed modification: carbamidomethyl cysteine, (4) variable modifications: methionine oxidation; (5) peptide tolerance: ±150 ppm; (6) MS/MS tolerance: ±0.3 Da and (7) charge: +1.

Please cite this article as: N. Buján, et al., Comparative proteomic study of *Edwardsiella tarda* strains with different degrees of virulence, J Prot (2015), http://dx.doi.org/10.1016/j.jprot.2015.05.008

Only identified proteins with a score of CI% ≥ 85 were accepted and the MASCOT criteria of being significant were $p < 0.05$. Reference database used for the identification of functional protein was UniProt (http://www.uniprot.org).

2.6. Stress response and motility assays

The response to stress was performed by H_2O_2 survival following the protocol described by Dan et al. [24] with some modifications. Briefly, bacterial cells were grown in TSB during 7 h until an OD_{600} of 0.5, then washed with PBS and resuspended in PBS to 10^8 CFU/ml. Ten microliters of suspension was inoculated in 1 ml of PBS with or without 4 mM H_2O_2. The tubes were incubated at 25 °C for 1 h. Aliquots of 400 μl were plated in TSA plates after dilution in TSB. These plates were incubated at 25 °C for 32 h, and the CFUs were counted.

Medium motility tubes [25] were inoculated by sting and incubated at 30 °C for 24 h. Motility was assessed by examining turbidity medium.

2.7. Evaluation of polymyxin B susceptibility

Polymyxin B concentrations ranging from 1.46 mg/ml to 0.0014 mg/ml were tested by the broth microdilution (BMD) method. Bacterial suspensions adjusted to 0.5 McFarland were grown in TSB at 25 °C during 16 h. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of polymyxin B at which no visible growth was observed. Moreover, the cell density was measured at OD_{600} with a spectrophotometer PerkinElmer Lambda 2 (Waltham, MA).

2.8. Sequencing of FeSOD gene

The presence of superoxide dismutase type I (SODI) and superoxide dismutase type II (SODII) genes associated with the pathogenic potential was evaluated. Total bacterial DNA was extracted from pure bacterial cultures using the Insta-gene matrix (Bio-Rad, Madrid, Spain) following the manufacturer's instructions. The DNA concentration was spectrophotometrically quantified and adjusted to 100 ng/ml. The primers used in the PCR reaction were SOD forward 5′-TCGCTGGAAG AGATCGTGAAGAG-3′ and SOD reverse 5′-TGAACGCGGCAAATGAGC-3′. PCR amplification was performed following this conditions: 94 °C for 2 min, followed by 35 amplification cycles, each consisting of sequential incubation at 94 °C (1 min), 56 °C (1 min) and 72 °C (1 min), followed by a final incubation at 72 °C for 5 min. The PCR products were sequenced by StabVida (Oeiras, Portugal).

3. Results

3.1. Comparative analysis of E. tarda virulent and avirulent strains

A comparison of proteomes between the virulent strain isolated from diseased turbot (ACC35.1) and the avirulent isolate (NCIMB2034) was carried out using 2D-DIGE technology. Four 2D-DIGE gels, one for each replica, corresponding to Cy3-, Cy5- and pooled IS Cy2-labelled sample images, were analysed using DeCyder software (v 6.5). DIA module analysis allowed the detection of an average of 1977 protein spots, in BVA module, an average of standardized volume ratio and unpaired Student's *t* test, were also calculated.

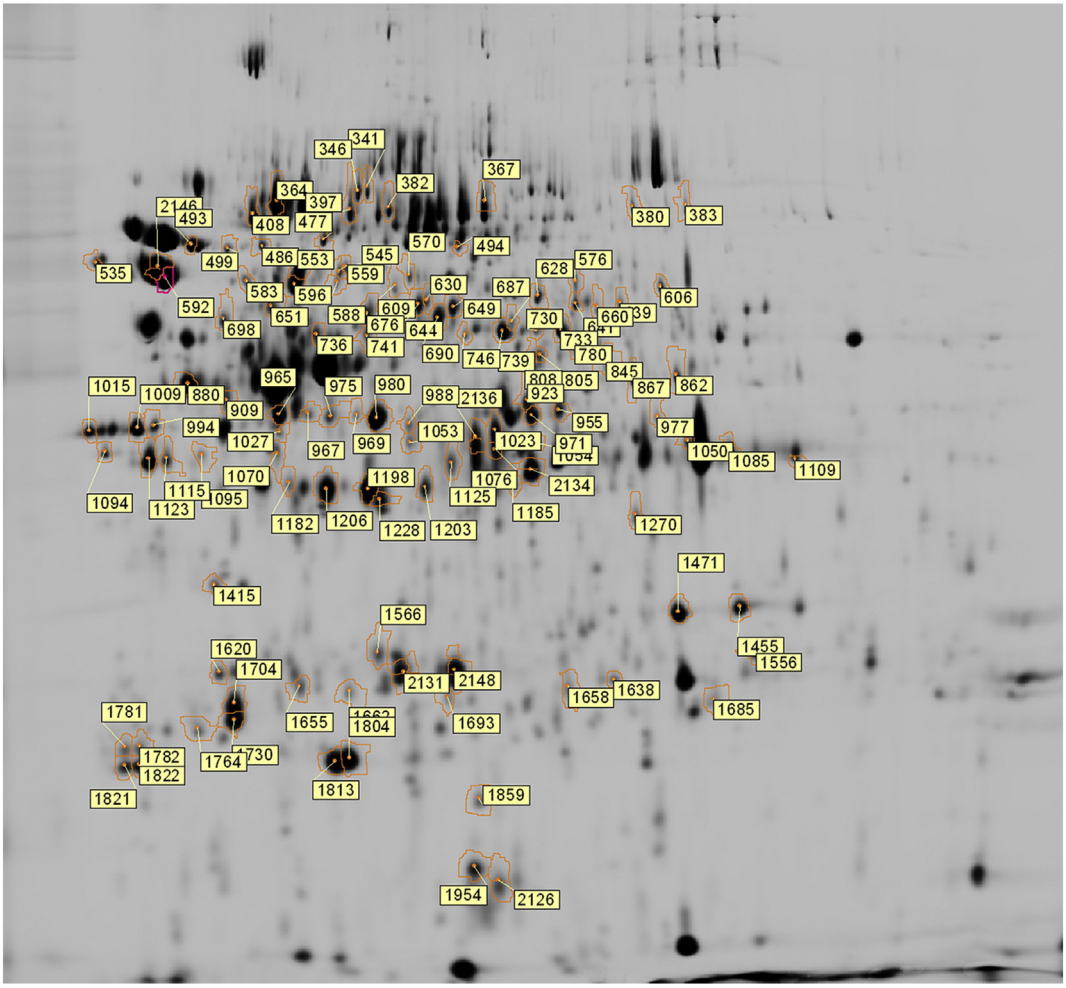

Fig. 1. Representative image 2-DE indicating the spots identified by MS.

Please cite this article as: N. Buján, et al., Comparative proteomic study of *Edwardsiella tarda* strains with different degrees of virulence, J Prot (2015), http://dx.doi.org/10.1016/j.jprot.2015.05.008

Following the statistical values with 2-fold as a threshold in the average ratio and with $p < 0.05$, 373 spots of 2148 were considered as protein spots with significant variation in abundance. For subsequent analysis, 103 protein spots (Fig. 1) were successfully identified (Fig. 2 and Tables 1 and 2) by MS (MALDI-TOF or MALDI-TOF/TOF). The MASCOT analysis determined 67 proteins represented by 102 protein species (one uncharacterized). Forty nine of these protein species belong to the virulent strain, 40 protein species to the avirulent strain and 12 protein species were detected in both, with variation in the expression (Fig. 2) However, it was observed that 23 proteins detected only in one isolate have its equivalent in the other isolate, such as spots 980 and 965 identified as phosphoglycerate kinase (Pgk), the porphobilinogen synthase in spots 967 and 969 or the heat shock protein 60 (GroEl) in spots 592 and 2146 (Fig. 3); which means that there are proteins that have the same name and function but the identification code, pI and/or mass are different (Fig. 2b and Table 2). These changes can be explained by several post-translational modifications or as a result of a difference in the amino acid sequence (Supplementary data 2), being this last hypothesis demonstrated in the protein phosphoglycerate kinase (spots 980 and 965) (Fig. 4). Furthermore, most of these protein spots, although they correspond to the same enzyme, have been identified matching with two different protein sequences (with different Uniprot codes for each of the spot) (table 2); being possible that they have different amino acids. Not all of the spots were identified, so the absence of some proteins could be related to this or to the low expression levels that made their detection difficult.

The proteomes of the two strains of *E. tarda* encoded proteins classified into 11 functional categories according to their biological processes: amino acid, carbohydrate and lipid metabolism, tricarboxylic cycle, stress response and protein fate, protein synthesis, biogenesis of cellular components, cell rescue defence and virulence, cell membrane and transport, signal transduction, purine and pyrimidine metabolism and miscellaneous.

Most of the proteins from the avirulent collection strain are involved in metabolic processes (amino acid and carbohydrate metabolism) whilst in the virulent turbot isolate the proteins detected are implicated in protein synthesis, and protein transport mechanism.

3.2. Validation of differential expression in phenotype

To confirm proteomic results, stress related proteins (heat shock protein 90 and iron-cofactored superoxide dismutase type 1), antigenic protein Et 46 (FliC) and bifunctional polymyxin resistance protein (ArnA) related with polymyxin resistance detected only in the virulent strain were evaluated. PCR amplification, H_2O_2 sensitivity, motility analysis and MIC to polymyxin B were performed to confirm their relevance in the virulent strain.

3.3. Stress response and motility

The HSP90, related to the survival under stress conditions as oxidative stress and the infection process efficiency [24], was detected exclusively in the virulent strain by proteomic analysis. The experiment performed in the presence of 4 mM H_2O_2 showed an important difference related to this enzyme between our strains. The turbot virulent isolate ACC35.1 (280 UFCs) grew two logarithms more than the collection strain NCIMB23034 (9 UFCs).

Regarding the detection of the antigenic protein Et 46, commonly known as FliC in *E. tarda* [25], the motility assay showed differences between the two strains (Fig. 5), FliC being only detected in the virulent strain.

3.4. Evaluation of polymyxin B susceptibility

The protein ArnA, involved in the polymyxin B resistance, was only detected in the turbot isolate by proteomic analysis. The MIC of polymyxin B to ACC35.1 and NCIMB2034 was evaluated by BMD, the resistance to polymyxin B being higher in the virulent strain (Fig. 6). Therefore, the MIC to ACC35.1 was 0.011 mg/ml and to NCIMB2034 was 2.85×10^{-3}.

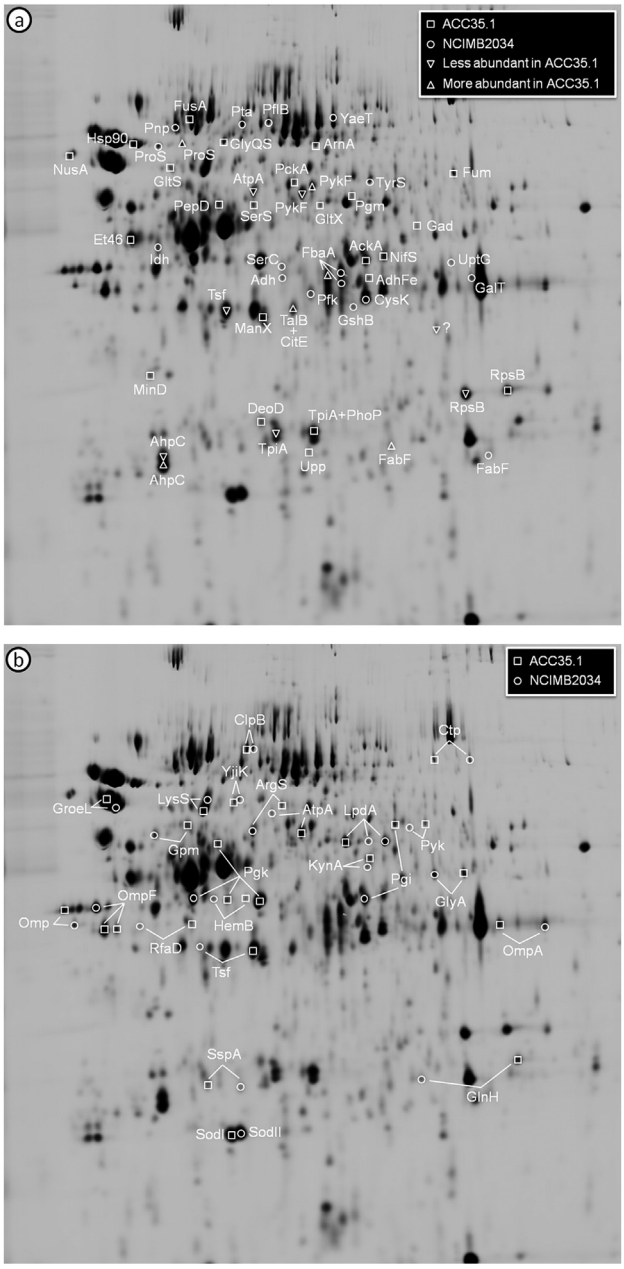

Fig. 2. 2D-DIGE maps of *E. tarda* proteins. a) Proteins detected in only one strain and proteins detected in both strains with changes in their abundance. b) Different protein species (with different mass and/or pI of the same protein detected in each strain.

3.5. SODI gene amplification

Various authors correlate the SOD types with the virulence [26,27] and our identifications concur with this hypothesis as shown in Table 2. Fifty nucleotide pairs are the difference between SODI and SODII sequences described by Yamada and Wakabayashi [26]. Taking this into account, we sequenced this gene to demonstrate that the proteomic differences between the two isolates are a consequence of the differences in the amino acidic sequence (Fig. 7).

Table 1
E. tarda identified protein spots that change their abundance between the virulent and the avirulent strains or are unique of one of them (ACC35.1 vs. NCIMB2034).

Gel N°.[a]	UniProt code[b]	Protein name	Protein description	Av. Ratio[c]	*t*-test[c]	M_r[d]	pI[d]	Score[d]	N° of pept[d]	% Cov[d]
Metabolic process										
Amino acid metabolism										
736*	D0ZDD2	PepD	MIX Aminoacyl-histidine dipeptidase K.GIHAERDAVGNILLR.K (41) R.FLAGHADELGAR.V (106) R.VTVDAAQSALLPLDAASQSR.F (107)	−2.63	0.0078	51.97	5.25	324	16/70	28
977	D4F689	CitE	Cystathionine beta-lyase	10.42	1.3E−05	38.52	6.35	179	19/65	66
955*	D0ZD86	NifS	Cysteine desulfurase	−8.66	5.5E−05	45.03	5.99	206	27/65	59
2134	D4F3E7	CysK	Cysteine synthase A	26.72	4.3E−05	34.10	5.94	326	32/65	91
780*	D0ZDS2	GadB2	Glutamate decarboxylase	−10.15	5.5E−05	52.39	5.97	99	17/65	39
1185	M0QA14	GshB	Glutathione synthase	7.01	0.00012	35.54	5.8	175	23/65	69
988	D4F722	SerC	Phosphoserine transaminase	4.56	3.1E−05	40.47	5.44	145	18/65	44
Carbohydrate metabolism										
971*	D0ZAZ8	AckA	Acetate kinase	−4.58	5.2E−05	43.45	5.78	154	21/65	57
1053	D4F0B2	Adh	Alcohol dehydrogenase NADP-dependent	3.31	6.7E−06	44.31	5.34	105	15/65	38
1054*	D0Z9Z7	AdhFe	Alcohol dehydrogenase iron-containing R.FAEGLLQTLIEEGPR.A (76) R.ALQEPENYGVR.A (66) R.VWNIHSGSDEQR.I (65)	−2.18	0.0021	42.61	5.73	310	19/70	46
367	D4F726	PflB	Formate acetyltransferase	7,59	0.00012	85.45	5.71	183	30/65	36
1023	D4F1S7	FbaA	Fructose bisphosphate aldolase K.IFDFVKPGVISGDDVQK.V (57) K.LLPWLDGLLDAGEK.H (73)	25.19	4.8E−05	41.36	5.75	220	14/72	38
1076	F0LWK4	FbaA	Fructose-bisphosphate aldolase (*Vibrio furnisii*) M.SKIFDFVKPGVISGDDVQK.V (112)	7.48	0.00035	38.80	4.92	138	6/68	15
2136▲	D0ZEE4	FbaA	Fructose-bisphosphate aldolase	−11.9	5.5E−05	39.40	5.65	141	16/65	53
397	M0Q6L6	Pta	Phospahte acetyltransferase	9.1	9.9 E−05	78.36	5.36	287	65/269	68
687*	D0ZCA1	Pgm	Phosphoglucomutase, alpha-D-glucose phosphate-specific	−11.35	1.4E−05	59.27	5.88	202	26/65	43
630*	D0ZG10	PckA	Phosphoenolpyruvate carboxykinase	−2.06	0.00094	59.37	5.55	110	15/65	29
1125	D4FAR3	PfkA	Phosphofructokinase	5.06	0.0016	35.17	5.6	144	21/65	50
1228*	D0Z7I1	ManX	PTS system mannose-specific transporter subunit IIAB	−10.84	3.9 E−05	35.03	5.4	118	23/152	66
644▼	D0Z8B8	PykF	Pyruvate kinase	6.03	0.0001	50.90	5.62	156	21/65	52
649▲	D0Z8B8	PykF	Pyruvate kinase	−11.05	0.00015	50.90	5.62	210	26/65	56
1203-	D0ZC16	TalB	MIX Transaldolase	−6.92	3.3E−06	35.04	5.61	266	25/65	70
2131▼	D4FAR2	TpiA	Triose-phosphate isomerase	8.03	5.5E−05	26.10	5.36	112	13/65	52
2126*	D0ZGV9	TpiA	Triose-phosphate isomerase	−10.88	5.6E−05	26.75	5.62	120	12/44	46
2148*	D0ZGV9	TpiA	MIX Triose phosphate isomerase	−13.17	6.7E−06	26.75	5.62	156	17/65	58
1050	D4F8D4	GalT	UTP-hexose-1-phosphate uridylyltransferase	2.58	1.4E−05	39.86	6.49	123	19/65	52
Lypid metabolism										
1658▲	D0Z9F8	FabG2	3-oxoacyl-[acyl-carrier-protein] reductase	−3.86	0.00032	25.73	6.63	90	13/65	51
1685	D4F6U3	FabG2	3-oxoacyl-[acyl-carrier-protein] reductase R.GITVNVVAPGFIETDMTR.A (150) R.SGILAEVPAGR.L (45)	19.75	4.8E−05	25.69	6.63	247	11/70	43
494*	D0ZG82	ArnA	Bifunctional polymyxin resistance protein ArnA	−10.99	0.00071	74.29	5.62	223	25/45	45
Tricarboxylic acid cycles										
1203-	D4F097	CitE	MIX Citrate (pro-3S)-lyase, beta subunit	−6.92	3.3E−06	31.15	5.14	96	11/65	48
606*	D0Z9H3	Fum	Fe-S type, tartrate/fumarate subfamily hydro-lyase subunit beta	−7.78	0.0026	60.09	6.44	232	26/65	54
909	D4F6P2	Icd	Isocitrate dehydrogenase, R.ENAEDIYAGIEWK.A (44)	16.71	4.8E−05	42.49	5.35	123	14/70	34
Stress response and protein fate										
493*	D4F335	Hsp90	Heat shock protein 90	−4.42	0.0016	71.26	4.96	267	30/65	50
408	D4F0T4	Pnp	Polyribonucleotide nucleotidyltransferase	24.34	2.1E−05	77.27	5.25	159	25/65	34
Protein synthesis										
1455*	D0ZCV1	RpsB	30S ribosomal protein S2	−29.31	1E−05	26.28	6.61	174	21/65	80
1471▼	D4F2F9	RpsB	30S ribosomal protein S2	9.96	0.00011	26.83	6.33	161	21/65	84
364*	D0ZFW5	FusA	Elongation factor G	−37.36	5.5E−05	77.52	5.17	423	44/65	73
1206▼	D4F2G0	Tsf	Elongation factor Ts	11.02	4.8E−05	30.78	5.35	203	26/65	65
746*	D0ZF30	GltX	Glutamate-tRNA ligase	−9.19	0.00062	53.86	5.64	289	31/65	57
477*	D0Z8K3	GlyS	Glycine-tRNA ligase	−5.01	0.00052	76.28	5.39	256	29/65	46
486▲	D0ZBZ4	ProS	Proline-tRNA ligase	−7.52	5.5E−05	63.39	5.17	237	27/65	51
499	D4F1C3	ProS	Proline-tRNA ligase	13.28	5.5E−05	63.73	5.03	170	22/65	41
741*	D0ZA17	SerS	Serine-tRNA ligase	−4.38	5.1E−05	48.96	5.4	182	25/65	51
535*	D0ZB73	NusA	Transcription elongation protein NusA	−4.6	0.00051	54.85	4.57	133	19/65	34
628	D4F5Q0	TyrS	Tyrosine-tRNA ligase R.LVHGAEGLQAAQR.I (49) R.LVHGAEGLQAAQR.I (25)	7.84	6E−05	15.82	5.06	103	6/69	34
Biogenesis of celular components										
1415*	D0ZH27	MinD	Septum site-determining protein MinD K.KTVVIDFDIGLR.N (16) R.TENLFILPASQTR.D (77) R.AENGDDPIKEHLLLTR.Y (44)	−10.9	3.3E−06	29.80	4.98	223	16/69	44

(continued on next page)

Please cite this article as: N. Buján, et al., Comparative proteomic study of *Edwardsiella tarda* strains with different degrees of virulence, J Prot (2015), http://dx.doi.org/10.1016/j.jprot.2015.05.008

Table 1 (*continued*)

Gel N°.[a]	UniProt code[b]	Protein name	Protein description	Av. Ratio[c]	*t*-test[c]	M_r[d]	pI[d]	Score[d]	N° of pept[d]	% Cov[d]
Cell rescue, defence and virulence										
880*	Q3V737	Et46	Antigenic protein Et 46	−65.28	9.7E−06	43.77	5.2	142	17/65	49
1704▼	M0QAN3	AhpC	Antioxidant, AhpC/TSA family	2.11	0.00035	22.41	5.12	160	18/65	81
1730▲	D0ZE70	AhpC	Antioxidant, AhpC/TSA family	−15.19	4E−05	22.41	5.12	159	17/65	78
Cell membrane and transport										
382	D4F2G9	YaeT	Outer membrane protein, Bam complex (YaeT)	4.68	0.00022	88.19	5.69	255	30/65	41
676▼	D4FAY6	AtpA	F0F1-type ATP synthase subunit alpha	3.55	0.0035	55.36	5.43	142	21/65	38
Signal transduction										
2148*	D0Z9B8	PhoP	MIX Regulation of transcription, DNA-dependent, winged helix family	−13.17	6.7E−06	25.55	5.62	155	17/65	74
Purine and pyrimidine metabolism										
583*	D0ZHD1	GlnA	Glutamine synthetase K.GGYFPVPPVDSAQDIR.S (85)	−8.31	1.4E−05	51.87	5.15	135	16/70	30
1566*	D0ZBF8	DeoD	Purine nucleoside phosphorylase DeoD-type K.HIAETFLQDVR.Q (91) R.FKDNDFAAIADFDMVR.N (93)	−4.74	5.5E−05	26.39	5.4	252	14/67	48
1693*	D0ZEX8	Upp	Uracil phosphoribosyltransferase	−17.98	3.4E−05	22.69	5.65	127	15/65	64
Others										
1270▼	D4F9M4	?	Conserved hypothetical protein	59.03	5.1E−05	34.99	8.43	112	17/65	43

X* Protein only detected in ACC35.1. X Protein only detected in NCIMB2034. X▲▼ Common proteins, increase or decrease in ACC35.1 respectively. X- mix common protein.

[a] Spot's number in master gel.

[b] UniProt identification code.

[c] Average ratio and Student's *t*-test p-value were calculated using Decyder software v6.

[d] Experimental molecular mass, isoelectric point, protein score, matched/unmatched peptides and percentage of coverage derived from MASCOT result page.

4. Discussion

Edwardsiellosis is one of the major diseases that produce economic big losses in fish farming. Numerous studies discuss the implication of different proteins in the virulence of *E. tarda* [28–30]. In fact, Sakai et al. [31] identified various proteins related to virulence such as GroEL, outer membrane protein A (OmpA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Considering that the presence/absence or the increase/decrease in specific proteins could be related to the pathogenic mechanisms, in this study, the DIGE methodology was employed to compare the cell proteome of two strains of *E. tarda* with different degrees of virulence. The aim was to observe abundance changes in proteins that could be involved in the pathogenesis.

The results showed a high number of differentially abundant proteins between the two strains, both in expression and in presence/absence. However, their identification showed that many of these were equivalent proteins with a mass and pI slightly different as the protein dihydrolipoyl dehydrogenase (see Table 2, spots 730, 733, 739). Even though these proteins perform the same function, these small changes result in the recognition of these proteins as different protein spots, by DIGE analysis, precluding a correct comparison in the levels of expression. The mass and pI changes might be due to post-translational modifications or to differences in amino acid sequence. This work described that some differences are caused by changes in an amino acid sequence (Fig. 2) being nonsynonymous changes such as phosphoglycerate kinase. A nonsynonymous variant can alter protein structure or function, however these changes can be profitable or even neutral if the changes do not affect the protein folding or the active site. Previous works associating this different electrophoretic mobility with the pathogenic potential, demonstrated that the proteins showing shifts from more acidic position are in less virulent strains [32,33]. In the present study, the electrophoretic changes did not follow a fixed pattern; the enzymes related to the virulent strain sometimes were more basic and other times more acidic. In any case this is a very relevant result, because to our knowledge, this is the first time that the proteome comparison of two bacterial strain renders too many different in proteins species with possible differences in sequences.

The metabolic and translational processes encompass most of the proteins identified which are related to the isolation of the proteins in exponential phase. Carbohydrate metabolism was the top most represented in our analysis (27.3%), followed by protein synthesis (16.7%).

An increase in glycolysis process is related to a higher virulence accordingly to various authors [34,35], which lead us to perform a detailed study of this process. The enzyme phosphofructokinase was detected in NCIMB2034 isolate displacing the pathway to glycolysis. Next enzyme, fructose bisphosphate aldolase appeared in 3 different spots in the non pathogenic and in only one of these in the turbot virulent isolate; this protein is related with conversion fructose-6P to glyceraldehyde-3P in glycolysis and in the reverse reaction in gluconeogenesis. The first step of the glycolysis pathway finishes with the creation of one molecule of glyceraldehyde-3P (G3P) and one molecule of dihydroxyacetone phosphate (DHA), the G3P continuous to the second step and the DHA, is converted to G3P by the triose phosphate isomerase. This enzyme is present in the virulent strain as 3 different isoforms whilst only one isoform was detected in NCIMB2034. This difference can produce acceleration in the step two of the glycolysis pathway and a higher amount of pyruvate in virulent strain. The conversion between glycerate 1-3P to/from G3P is regulated by phosphoglycerate kinase that is present in the virulent isolate ACC35.1 in 3 different spots against only one and in a different form in the non-pathogenic strain NCIMB2034. In summary, the proteins related to glycolysis/gluconeogenesis pathway were more abundant in the virulent strain supporting the hypothesis and explaining why ACC35.1 grows a little faster than NCIMB2034 (data not shown).

The glyoxylate cycle is well documented in many organisms: archaea, bacteria, protists, plants, fungi and nematodes [36]. This cycle is used by microorganisms in conditions of nutrient deficiency and plays important roles in their long-term persistence and infection in hosts [37–39]. However, in other microorganisms such as *Brucella suis*, the glyoxylate cycle has been shown to be unnecessary for virulence [40], being capable to use intracellular resistance strategy for colonization in hosts infection. In this work, the proteins belonging to this cycle were not detected in the *E. tarda* strains analysed. These results are in agreement with complete genome studies where it was not possible to detect genes related to this cycle [41] suggesting that the mechanism used by *E. tarda* to start the colonization can be similar to the one used by *B. suis*.

The Gram-negative envelope is composed of OMP and inner membrane proteins. There are two important groups in OMP: lipoproteins, present in the periplasm but anchored by a lipid moiety to the membrane, and β-barrel proteins, integral membrane proteins. The lipid metabolism is more represented in the strain NCIMB2034, with two spots identified as acyl carrier protein involved in lipid transport and

Please cite this article as: N. Buján, et al., Comparative proteomic study of *Edwardsiella tarda* strains with different degrees of virulence, J Prot (2015), http://dx.doi.org/10.1016/j.jprot.2015.05.008

Table 2
Identified protein spots detected in both *E. tarda* strains as different protein species (different in molecular mass or pI) (ACC35.1 vs. NCIMB2034).

Gel N°.[a]	UniProt code[b]	Protein name	Protein description	Av. Ratio[c]	*t*-test[c]	M_r[d]	pI[d]	Score[d]	N° of pept[d]	% Cov[d]
Metabolic process										
Amino acid metabolism										
862*	D0ZD92	GlyA	Glycine hydroxymethyltransferase	−10.75	0.0009	45.53	6.4	125	19/65	42
867	D4F966	GlyA	Glycine hydroxymethyltransferase	20.82	5.9E−05	45.55	6.36	188	26/65	43
805*	D0ZDI3	TnaA	Tryptophan 2,3-dioxygenase	−5.08	0.00046	53.30	5.88	194	24/65	45
808	D4F902	TnaA	Tryptophan 2,3-dioxygenase	2.15	0.0031	53.28	5.84	90	13/65	28
Carbohydrate metabolism										
1070*	D0Z956	RfaD	ADP-glyceromanno-heptose-6-epimerase	−5.66	0.00013	35.03	5.12	110	20/65	50
1095	D4F9R1	RfaD	ADP-glyceromanno-heptose-6-epimerase	3.91	0.00068	35.15	4.92	143	16/65	38
730	D4F283	LpdA	Dihydrolipoyl dehydrogenase R.CADLGLETVIVER.Y (30) K.ALAEHGIVFGEPK.T (49)	5.59	0.00017	50.93	5.91	131	12/68	22
733	D4F283	LpdA	Dihydrolipoyl dehydrogenase	10.1	8.1E−05	50.93	5.91	274	30/65	64
739*	E0T9L0	LpdA	Dihydrolipoyl dehydrogenase	−18.73	7.6E−06	51.04	5.72	312	31/65	57
641*	D0Z9W1	Pgi	Glucose-6-phosphate isomerase R.DWFLQAAGDER.H (60) R.ILPELEDAQPVR.S (57)	−7.87	0.00015	61.57	6.03	285	25/69	40
923	D4F076	Pgi	Glucose-6-phosphate isomerase	2.25	5.3E−05	61.56	5.74	163	22/65	38
736*	C5BAU6	Pgk	MIX Phosphoglycerate kinase R.LAKDYLDGVDVAQGELVVLENVR.F (75) K.KLPAVVMLEER.A (5)	−2.63	0.0078	41.07	5.45	156	14/70	34
965	D4F1S8	Pgk	Phosphoglycerate kinase K.DYLDGVDVAQGELVVLENVR.F (16) K.KLLTTCDIPVPTDVR.V (82) K.LLTTCDIPVPTDVR.V (62) K.KLPAVVMLEER.A (90)	32.51	3.9E−05	41.70	5.27	502	20/70	42
975*	C5BAU6	Pgk	Phosphoglycerate kinase	−6.39	0.0006	41.07	5.45	112	14/65	45
980*	D0ZEE5	Pgk	Phosphoglycerate kinase	−87.09	3.9E−05	41.08	5.33	154	20/65	47
651*	D0ZH99	Gpm	Phosphoglycerate mutase K.GEFHADNAIDGLQAAYAR.G (58) R.GENDEFVKPTVIR.A (87) R.AFVNADFDGFAR.R (56)	−22.28	0.0001	56.29	5.2	274	16/71	25
698	D4FAS5	Gpm	Phosphoglycerate mutase	10.23	3.3E−06	56.26	5.08	97	16/65	30
639*	D0ZH12	Pkm	Pyruvate kinase	−23.06	1.2E−05	51.85	6.3	107	18/37	37
660	D4F4C7	Pkm	Pyruvate kinase R.LNFSHGSAEDHQLR.A (38) R.HVAILGDLQGPK.I (92) R.CGEDLNYAR.R (33) R.GDLGVEIGDPELVGIQK.A (82)	3.85	0.00031	52.88	6.15	294	16/69	23
Stress response and protein fate										
383	D4F5U5	Prc	Carboxy-terminal protease	13.97	5.6E−05	76.23	6.61	147	22/65	35
380*	D0Z7S7	Prc	Carboxy-terminal protease	−14.59	0.00014	71.24	6.72	212	28/65	43
592	D4F0J3	GroEL	Chaperonin GroEL (HSP60 family)	4.59	5.6E−05	57.45	4.84	250	28/65	52
2146*	D0ZAK2	GroEL	Chaperonin GroEL (HSP60 family)	−5.75	4.8E−05	57.51	4.82	316	32/65	67
341	D4F1D3	ClpB	Heat shock protein of ClpB family	4.53	9.5E−06	95.48	5.46	266	34/65	41
346*	D0ZDN3	ClpB	Heat shock protein of ClpB family	−4.07	0.0017	95.50	5.44	303	37/65	44
1655*	D0ZBF8	DeoD	Stringent starvation protein A	−13.5	0.0003	24.52	5.34	166	21/65	65
1662	M0QCE8	DeoD	Stringent starvation protein A	14.61	3.1E−05	24.52	5.34	170	19/65	68
Protein synthesis										
1182	C5BHB7	Tsf	Elongation factor Ts	2.53	0.00012	30.69	5.44	87	14/65	32
1198*	D0ZCV2	Tsf	Elongation factor Ts	−31.54	3.3E−06	30.69	5.44	198	24/65	70
570*	D0ZGZ1	ArgS	Arginine-tRNA ligase	−9.85	5.5E−05	63.54	5.49	190	26/65	36
588	D4F498	ArgS	Arginine-tRNA ligase	3.95	0.0017	63.80	5.37	152	21/65	34
553	D4F1N9	LysS	Lysine-tRNA ligase	5.2	0.00069	48.20	5.14	186	22/65	50
596*	D0ZDX8	LysS	Lysine-tRNA ligase	−7.23	4E−05	57.97	5.22	279	31/65	62
Cell rescue. defence and virulence										
1804	Q0PD83	SodBII	Iron-cofactored superoxide dismutase M.SFELPALPYAK.N (95) K.GSEFEGKSLEEIIK.T (29)	6.86	0.00023	17.93	5.38	152	6/69	35
1813*	Q0PD76	SodBI	Iron-cofactored superoxide dismutase R.GALADAINAAFGSFAAFKDALTK.S (63) K.SAVGNFGSGWTWLVK.K (117)	−7.91	4E−05	17.90	5.55	260	10/71	77
Cell membrane and transport										
545	D4F1B5	YjjK	ABC transporter, ATP-binding protein YjiK	2.48	0.00038	61.45	5.34	160	21/65	40
559*	D0ZBY7	YjjK	ABC transporter, ATP-binding protein YjiK	−2.82	4.4E−05	62.13	5.32	267	29/65	49
1556*	D0Z7G2	GlnH	Amino acid ABC transporter substrate-binding protein	−27.95	3.2E−05	27.56	8.94	183	18/65	69
1638	D4F4P8	GlnH	Amino acid ABC transporter substrate-binding protein	36.06	5.6E−05	27.68	8.94	179	17/65	63
609	D4FAY6	AtpA	F0F1-type ATP synthase subunit alpha	8.5	0.00045	55.30	5.59	117	19/65	29
690*	D0ZHG9	AtpA	F0F1-type ATP synthase subunit alpha	−43.72	5.3E−05	55.30	5.59	312	33/65	59
1015*	Q8GF10	Omp	Outer membrane protein	−12.43	0.00033	47.28	5.27	88	12/65	29
1094	D4F5B2	Omp	Outer membrane protein K.FGNYGSIDYGR.N (90)	35.57	0.00034	41.42	4.65	96	3/71	4
1085*	D0ZFS8	OmpA	Outer membrane protein A	−5.11	0.00045	38.03	7.66	168	20/65	50

(continued on next page)

Please cite this article as: N. Buján, et al., Comparative proteomic study of *Edwardsiella tarda* strains with different degrees of virulence, J Prot (2015), http://dx.doi.org/10.1016/j.jprot.2015.05.008

Table 2 (*continued*)

Gel N°.[a]	UniProt code[b]	Protein name	Protein description	Av. Ratio[c]	*t*-test[c]	M_r[d]	pI[d]	Score[d]	N° of pept[d]	% Cov[d]
1109	D4FAY6	OmpA	Outer membrane protein A	61.65	0.0004	38.74	8.9	180	21/65	43
1009	M4TKJ8	OmpF2	Outer membrane porin F protein K.FADYGSLDYGR.N (59)	42.85	0.0001	39.92	5.03	72	4/68	11
1115*	D0ZFQ0	OmpF2	Outer membrane porin F protein K.FTSDKSDDGDHTYAR.F (55) K.FADYGSLDYGR.N (87)	−33.69	7.6E−06	40.05	5.03	197	11/69	22
1123*	D0ZFQ1	OmpF2	Outer membrane porin F protein	−98.54	9.7E−06	40.05	5.03	125	14/65	47
Others										
967	D4F9Y9	HemB	Porphobilinogen synthase K.SLYAEEAYNADGLVQR.A (74) R.VAQWLHDAEMNR.- (39)	4.15	6.7E−05	32.54	4.85	193	17/69	40
969*	D0Z9R1	HemB	Porphobilinogen synthase M.SYAFPGAFPGR.R (75) K.SLYAEEAYSSDGLVQR.A (129) K.YASCYYGPFR.D (20) R.VAQWLHDAEMTR.- (40) R.VAQWLHDAEMTR.- (23)	−8.04	3.8E−05	37.60	5.37	372	21/71	51

X* Protein only detected in ACC35.1. X Protein only detected in NCIMB2034.

[a] Spot's number in master gel.

[b] UniProt identification code.

[c] Average ratio and Student's *t*-test p-value were calculated using Decyder software v6.

[d] Experimental molecular mass, isoelectric point, protein score, matched/unmatched peptides and percentage of coverage derived from MASCOT result page.

synthesis (spots1658 and 1685, table 1). Bam complex, only detected in avirulent strain, has an important role in the transport of lipopolysaccharides and phospholipids to the outer membrane [42]. He et al. [43] performed a phenotypic study comparing various isolates of *E. tarda*, and found that the strain NCIMB2034 showed large differences in biofilm formation. The higher abundance of proteins belonging to lipid metabolism and the higher presence of various OMPs is strongly related to biofilm formation [44]. In addition, the avirulent strain forms aggregates that precipitate in liquid medium (data not show) suggesting more capacity to develop biofilm. The presence of biofilm is usually related to a virulence mechanism [45], however its presence is not always involved in it, as it is shown in this work and other [46].

Porins are the most abundant OMPs in the outer membrane of the turbot isolate ACC35.1. The high number of porins in the outer membrane increases the specific permeability and the resistance to toxic substances like antibiotic [47]. This suggests that the virulent strain presents a more efficient and developed transport mechanism which helps in the virulence and resistance to antibiotics.

The species *E. tarda* produces hydrogen sulphide from sulphite. In this work, both strains were capable to produce H_2S and gas, but only proteins related to this pathway were found in the avirulent strain (cystathione beta-lyase and cysteine synthase A). However, the absence of this protein in the virulent strain could be explained by two reasons: its expression is very low and this is why it is not possible to detect or the analysis of a higher number of spots is necessary.

In addition, the virulent strain (ACC35.1) presents other proteins associated with virulence. The protein ArnA is a bifunctional protein involved in the polymyxin resistance (and other cationic antimicrobial

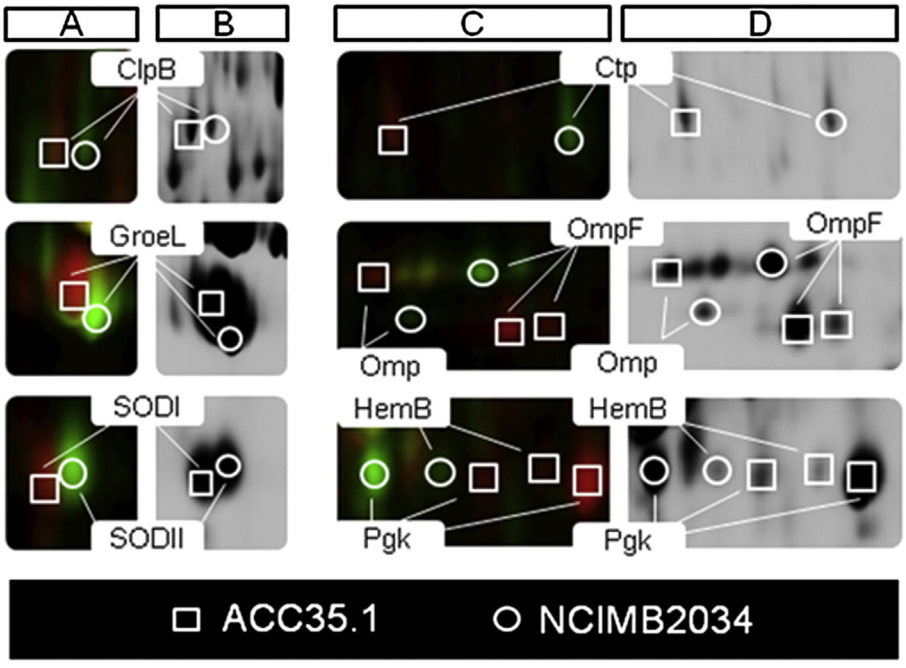

Fig. 3. Sectors showing differences in mass and/or pH between the different protein species of the same protein in each of the strains. Column A and C, DIGE gel; column B and D, Coomasie gel.

Please cite this article as: N. Buján, et al., Comparative proteomic study of *Edwardsiella tarda* strains with different degrees of virulence, J Prot (2015), http://dx.doi.org/10.1016/j.jprot.2015.05.008

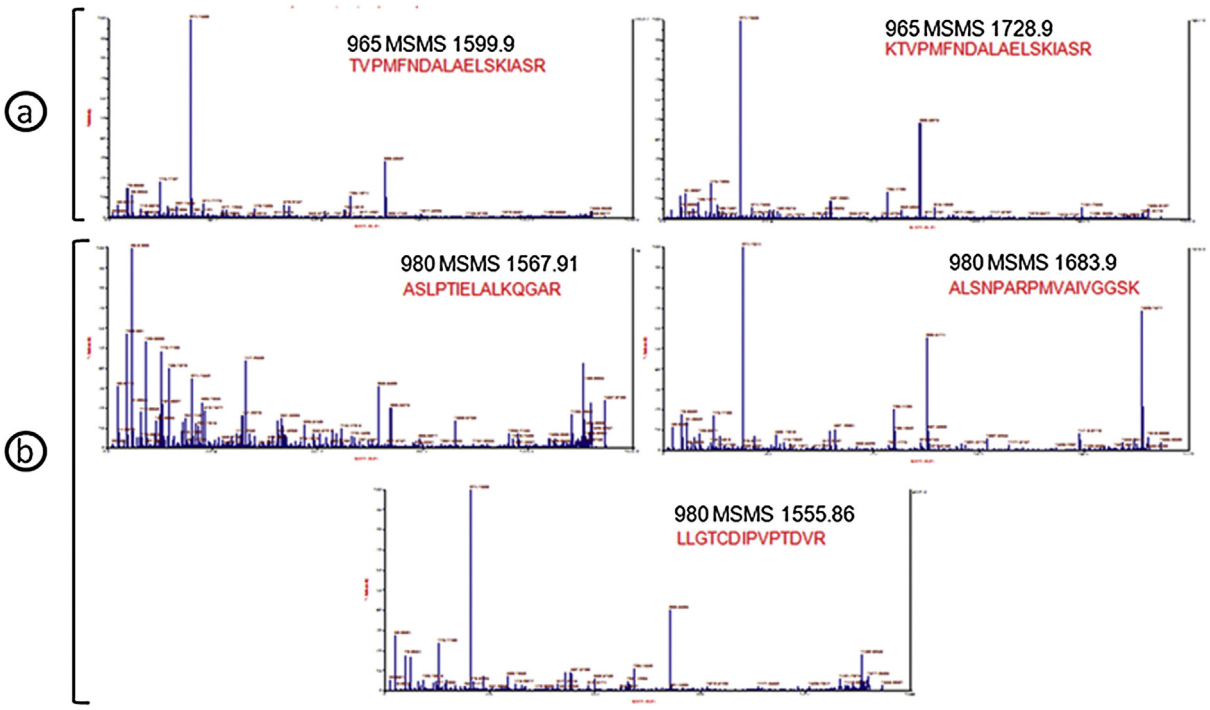

Fig. 4. Mass spectra obtained by MALDI-TOF/TOF of differential peptides between spots 965 and 980 (phosphoglycerate kinase, Pgk) corresponding to *E. tarda* NCIMB2034 a) and ACC35.1 b) protein extracts, respectively.

peptides) and the modification of lipid A [48]. This enzyme was only detected in the virulent turbot strain and polymyxin resistance experiment supports the idea that in this isolate its presence or level of expression is a differential character. Moreover, lipid A is considered a virulence factor and part of an endotoxin typical of enterobacteria [49] suggesting that this protein could be an important factor in the virulence process and will be a good candidate for mutant assays.

Heat shock protein 90 is a eukaryotic protein and the homologous in bacterial species is the heat shock protein G (HspG). The mutation in *htpG* causes slowdown in growth, reduction in the mortality rate and in the survival to high temperatures [24]. Moreover, *htpG* mutant created by Dang et al. [24] significantly reduced the ability to survive and replicate in macrophages. Changes in this process suggest that HptG is essential for efficient bacterial infection. In this work, the HspG was detected by proteomic analysis only in the turbot isolate and a major survival for this strain under oxidative stress condition was demonstrated. However, the lack of detection of Hsp90 in NCIMB2034 could mean that this protein is low expressed.

E. tarda is an enteric bacteria described as mobile with some exceptions. In this case, the study performed at 30 °C demonstrated that the motility in the virulent isolate is higher than in the avirulent strain (not mobile at <25 °C). FilC is a flagellar filament structural protein associated with virulent strains [50], and in our work it was only detected in ACC35.1. The absence of *fliC* produces a decrease in the motility ability, a decrease in biofilm formation and also defects in flagellum

Fig. 5. Growth of ACC35.1 and NCIMB2034 strains in motility medium. High motility is observed in ACC35.1 isolate with regard to the NCIMB2034 strain.

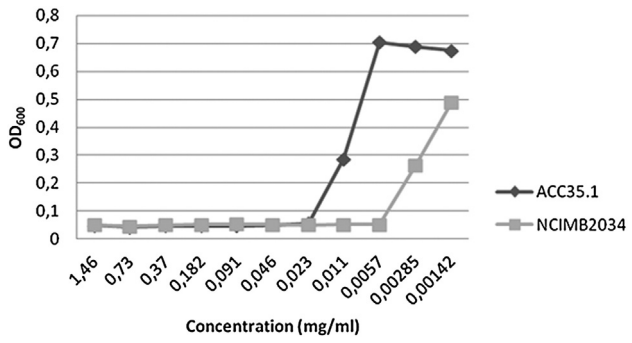

Fig. 6. Growth profiles of ACC35.1 and NCIMB2034 at different concentrations of polymyxin B.

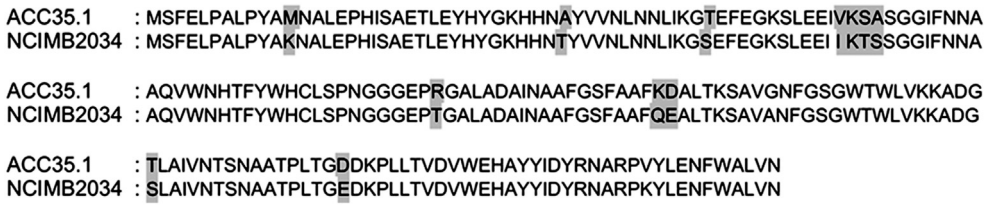

Fig. 7. Amino acid sequence alignments of SOD gene from different *E. tarda* strains. ACC35.1 corresponding to SODI and NCIMB2034 to SODII. Grey shading indicates differences between base pairs. Sequence alignment was performed with MEGA5 (http://www.megasoftware.net/).

formation [25]. The effector proteins type III secretion system (T3SS) and type VI secretion system (T6SS) of *E. tarda* decrease or disappear when the *fliC* is deleted [25] and these secretion systems were used by bacterial pathogens to excrete virulence factors into host cell [51]. The complete absence of this protein in NCIMB2034 cannot be proved, maybe the protein is present but the secondary structure is different decreasing the motility [52].

Yamada and Wakabayashi [53] using isozyme electrophoresis of the enzyme iron-cofactored superoxide dismutase (FeSOD) were able to show differences between two FeSOD types, FeSOD type 1 (SODI), associated with virulent strains and FeSOD type 2 (SODII), associated with avirulent strains. SOD protects cells from oxidative stress-induced damage, converting superoxide radical O_2^- into molecular oxygen and hydrogen peroxide. For this process, SOD is known to be a virulence factor in bacterial pathogens [35]. SOD is classified according to the associated metal ion; in this case the SOD identified is iron-cofactored SOD (FeSOD). The sequencing of this gene showed differences between pathogenic (FeSODI) and innocuous (FeSODII) strains, and these changes are in most cases non-synonyms [26]. FeSODI inhibits macrophage activation and inflammatory response in *E. tarda* [27] and these results establish a clear role for FeSODI as a virulence factor. The results obtained in this work corroborate this previous study published by Yamada and Wakabayashi [53], FeSODI was detected in the turbot virulent strain and FeSODII in the avirulent strain.

In conclusion, this work shows that 2D-DIGE is an important tool for identifying proteins involved in the pathogenesis of *E. tarda*. Comparison of proteomes between the turbot virulent isolate ACC35.1 and the non-pathogenic strain NCIMB2034 enlightened that proteins FliC, FeSODI, ArnA and Hsp90 related to virulence were only detected in the strain ACC35.1 isolated from disease turbot.

Acknowledgements

This work was supported by AGL2012-31049 from the Ministerio de Economía y Competitividad (Spain). N.B. acknowledges the Secretaría de Estado de Investigación, Desarrollo e Innovación, Ministerio de Economía y Competitividad (Spain) for a research fellowship. The proteomic analysis was performed in the proteomic facility of the Complutense University-Scientific Park of Madrid (UCM-PCM) that belongs to ProteoRed, PRB2-ISCIII, supported by grant PT13/0001/0038 from the ISCIII.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2015.05.008.

References

[1] J.M. Janda, S.L. Abbott, Infections associated with the genus *Edwardsiella*: the role of *Edwardsiella tarda* in human disease, Clin. Infect. Dis. 17 (1993) 742–748.
[2] B.R. Mohanty, P.K. Sahoo, Edwardsiellosis in fish: a brief review, J. Biosci. 32 (2007) 1331–1344.
[3] N. Castro, A.E. Toranzo, J.L. Barja, S. Núñez, B. Magariños, Characterization of *Edwardsiella tarda* strains isolated from turbot, *Psetta maxima* (L.), J. Fish Dis. 29 (2006) 541–547.
[4] F. Padrós, C. Zarza, L. Dopazo, M. Cuadrado, S. Crespo, Pathology of *Edwardsiella tarda* infection in turbot, *Scophthalmus maximus* (L.), J. Fish Dis. 29 (2006) 87–94.
[5] N. Castro, A.E. Toranzo, A. Bastardo, J.L. Barja, B. Magariños, Intraspecific genetic variability of *Edwardsiella tarda* strain from culture turbot, Dis. Aquat. Org. 95 (2011) 253–258.
[6] B. Austin, D.A. Austin, Characteristics of the diseases, Bacterial Fish Pathogens: Diseases of Farmed and Wild Fish, 3th ed.Springer Praxis, Chichester 1999, pp. 13–15.
[7] G. Kumar, G. Rathore, U. Sengupta, V. Singh, D. Kapoor, W.S. Lakra, Isolation and characterization of outer membrane proteins of *Edwardsiella tarda* and its application in immunoassays, Aquaculture 272 (2007) 98–104.
[8] M. Acharya, N.K. Maiti, S. Mohanty, P. Mishra, M. Samanta, Genotypimg of *Edwardsiella tarda* isolated from freshwater fish culture system, Comp. Immunol. Microbiol. Infect. Dis. 30 (2007) 33–40.
[9] V.S. Panangala, V.L. Santen, C.A. Shoemaker, P.H. Klesius, Analysis of 16S–23S intergenic spacer regions of the rRNA operons in *Edwardsiella ictaluri* and *Edwardsiella tarda* isolated from fish, J. Appl. Microbiol. 99 (2005) 657–669.
[10] S.H. Ling, X.H. Wang, L. Xie, T.M. Lim, K.Y. Leung, Use of green fluorescent protein (GFP) to study the invasion pathways of *Edwardsiella tarda* in in vivo and in vitro fish models, Microbiology 146 (2000) 7–19.
[11] P.S. Srinivasa Rao, T.M. Lim, K.Y. Leung, Opsonized virulent *Edwardsiella tarda* strains are able to adhere to and survive and replicate within fish phagocytes but fail to stimulate reactive oxygen intermediates, Infect. Immun. 69 (2001) 5689–5697.
[12] I. Hirono, N. Tange, T. Aoki, Iron-regulated haemolysin gene from *Edwardsiella tarda*, Mol. Microbiol. 24 (1997) 851–856.
[13] P.S. Srinivasa Rao, Y. Yamada, K.Y. Leung, A major catalase (KatB) that is required for resistance to H_2O_2 and phagocyte-mediated killing in *Edwardsiella tarda*, Microbiology 149 (2003) 2635–2644.
[14] Janda JM, Abbott SL, Kroske-Bystrom S, Cheung WK, Powers C, Kokka RP, Tamura K. Pathogenic properties of *Edwardsiella* species. J. Clin. Microbiol. 199;29:1997–2001.
[15] T. Morohoshi, T. Inaba, N. Kato, K. Kanai, T. Ikeda, Identification of quorum-sensing signal molecules and the LuxRI homologs in fish pathogen *Edwardsiella tarda*, J. Biosci. Bioeng. 98 (2004) 274–281.
[16] Y. Han, X. Li, Z. Qi, X.H. Zhang, P. Bossier, J. Detection of different quorum-sensing signal molecules in a virulent *Edwardsiella tarda* strain LTB-4, J. Appl. Microbiol. 108 (2010) 139–147.
[17] G. Kumar, P. Sharma, G. Rathore, D. Bisht, U. Sengupta, J. Proteomic analysis of outer membrane proteins of *Edwardsiella tarda*, Appl. Microbiol. 108 (2010) 2214–2221.
[18] J.A. Reales-Calderón, L. Martínez-Solano, M. Martínez-Gomariz, C. Nombela, G. Molero, C. Gil, Sub-proteomic study on macrophage response to *Candida albicans* unravels new proteins involved in the host defense against the fungus, J. Proteomics 75 (2012) 4734–4746.
[19] M. Unlu, M.E. Morgan, J.S. Minden, Difference gel electrophoresis: a single method for detecting changes in proteins extracts, Electrophoresis 18 (1991) 2071–2077.
[20] L. Monteoliva, J.P. Albar, Differential proteomics: an overview of gel and non-gel based approaches, Brief. Funct. Genomic. Proteomic. 3 (2004) 220–239.
[21] N.A. Karp, P.S. McCormick, M.R. Russell, K.S. Lilley, Experimental and statistical considerations to avoid false conclusions in proteomics studies using differential in-gel electrophoresis, Mol. Cell. Proteomics 6 (2007) 1354–1364.
[22] V. Neuhoff, N. Arold, D. Taube, W. Ehrhardt, Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250, Electrophoresis 9 (1988) 255–262.
[23] J. Havlis, H. Thomas, M. Sebela, A. Shevchenko, Fast-response proteomics by accelerated in-gel digestion of proteins, Anal. Chem. 75 (2003) 1300–1306.
[24] W. Dang, Y.-H. Hu, L. Sun, HptG is involved in the pathogenesis of *Edwardsiella tarda*, Vet. Microbiol. 152 (2011) 394–400.
[25] Y. He, T. Xu, L.E. Fossheim, X.H. Zhang, FliC, a flagellin, is a essential for the growth and virulence of fish pathogen *Edwardsiella tarda*, PLoS ONE 7 (2012) e45070, http://dx.doi.org/10.1371/journal.pone.0045070.
[26] Y. Yamada, H. Wakabayashi, Identification of fish-pathogenic strains belonging to the genus *Edwardsiella* by sequence analysis of *sodB*, Fish Pathol. 34 (1999) 145–150.
[27] S. Cheng, M. Zhang, L. Sun, The iron-cofactored superoxide dismutase of *Edwardsiella tarda* inhibits macrophage-mediated innate immune response, Fish Shellfish Immunol. 29 (2010) 972–978.
[28] S.B. Park, H.B. Jang, S.W. Nho, I.S. Cha, J.-I. Hikima, M. Ohtani, T. Aoki, T.S. Jung, Outer membrane vesicles as a candidate vaccine against edwardsiellosis, PLoS ONE 6 (2011) e17629, http://dx.doi.org/10.1371/journal.pone.0017629.
[29] J.E. Yu, A.Y. Yoo, K.-H. Choi, J. Cha, I. Kwak, H.Y. Kang, Identification of antigenic *Edwardsiella tarda* surface proteins and their role in pathogenesis, Fish Shellfish Immunol. 34 (2013) 673–682.

[30] C. Wang, Y. Liu, H. Li, W.-J. Xu, H. Zhang, X.-X. Peng, Identification of plasma-responsive outer membrane proteins and their vaccine potential in *Edwardsiella tarda* using proteomic approach, J. Proteomics 75 (2012) 1263–1275.
[31] T. Sakai, T. Matsuyama, T. Nishioka, C. Nakayasu, T. Kamaishi, K. Yamaguchi, T. Iida, Identification of major antigenic proteins of *Edwardsiella tarda* recognized by Japanese flounder antibody, J. Vet. Invest. 21 (2009) 504–509.
[32] J. Mattow, P.R. Jungblut, U.E. Schaible, H.J. Mollenkopf, S. Lamer, U. Zimny-Arndt, K. Hagens, E.C. Müller, S.H. Kaufmann, Identification of proteins from *Mycobacterium tuberculosis* missing in attenuated *Mycobacterium bovis* BCG strains, Electrophoresis 22 (2001) 2936–2946.
[33] M. Hubálek, L. Hernychová, M. Brychta, J. Lenco, J. Zechovská, J. Stulík, Comparative proteome analysis of cellular proteins extracted from highly virulent *Francisella tularensis* ssp. tularensis and less virulent *F. tularensis* ssp. holarctica and *F. tularensis* ssp. mediaasiatica, Proteomics 4 (2004) 3048–3060.
[34] D. Stead, H. Findon, Z. Yin, J. Walker, L. Selway, P. Cash, B.A. Dujon, C. Hennequin, A.J.P. Brown, K. Haynes, Proteomic changes associated with inactivation of the *Candida glabrata* ACE2 virulence-moderating gene, Proteomics 5 (2005) 1838–1848.
[35] A. Tonietto, B.A. Petriz, C.W. Araújo, A. Metha, Magalhães, L.O. Franco, Comparative proteomics between natural *Microcystis* isolates with a focus on microcystin synthesis, Proteome Sci. 10 (2012) 38.
[36] F.A. Kondrashov, E.V. Koonin, I.G. Morgunov, T.V. Finogenova, M.N. Kondrashova, Evolution of glyoxylate cycle enzymes in metazoa: evidence of multiple horizontal transfer events and pseudogene formation, Biol. Direct 1 (2006), http://dx.doi.org/10.1186/1745-6150-1-31.
[37] M.C. Lorenz, G.R. Fink, The glyoxylate cycle is required for fungal virulence, Nature 412 (2001) 83–86.
[38] D. Schnappinger, S. Erhrt, M.I. Voskuil, Y. Liu, J.A. Mangan, I.M. Monahan, G. Dolganov, B. Efron, P.D. Butcher, C. Nathan, G.K. Schoolnik, Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: insights into the phagosomal environment, J. Exp. Med. 198 (2003) 693–704.
[39] M.F. Dunn, J.A. Ramírez-Trujillo, I. Hernández-Lucas, Major roles of isocitrate lyase and malate synthase in bacterial and fungal pathogenesis, Microbiology 155 (2009) 3166–3175.
[40] S. Köhler, V. Foulongne, S. Ouahrani-Bettache, G. Bourg, J. Teyssier, M. Ramuz, J.-P. Liautard, The analysis of the intramacrophagic virulome of *Brucella suis* deciphers the environment encountered by the pathogen inside the macrophage host cell, PNAS 99 (2002) 157911–157916.
[41] Q. Wang, M. Yang, J. Xiao, H. Wu, X. Wang, Y. Lv, L. Xu, H. Zheng, S. Wang, G. Zhao, Q. Liu, Y. Zhang, Genome sequence of the versatile fish pathogen *Edwardsiella tarda* provides insights into its adaptation to broad host ranges and intracellular niches, PLoS ONE 4 (2009) e7646, http://dx.doi.org/10.1371/journal.pone.0007646.
[42] R. Voulhoux, J. Ttommassen, Omp85, an evolutionarily conserved bacterial protein involved in outer-membrane-protein assembly, Res. Microbiol. 155 (2004) 129–135.
[43] Y. He, T. Xu, Y. Han, X. Shi, X.-H. Zhang, Phenotypic diversity of *Edwardsiella tarda* isolated from different origins, Lett. Appl. Microbiol. 53 (2001) 294–299.
[44] K. Otto, J. Norbeck, T. Larsson, K.A. Karlsson, M. Hermansson, Adhesion of type 1-fimbriated *Escherichia coli* to abiotic surfaces leads to altered composition of outer membrane proteins, J. Bacteriol. 183 (2001) 2445–2453.
[45] G. O'Toole, H.B. Kaplan, R. Kolter, Biofilm formation as microbial development, Annu. Rev. Microbiol. 54 (2000) 49–79.
[46] C. Nucci, W.D. da Silveira, Corrêa S. da Silva, G. Nakazato, S.Y. Bando, M.A. Ribeiro, Pestana de Castro AF, Microbiological comparative study of isolated of *Edwardsiella tarda* isolated in different countries from fish and human, Vet. Microbiol. 89 (2002) 29–39.
[47] S.-Y. Wang, J. Lauritz, J. Jass, D.L. Milton, Role for the major outer-membrane protein from *Vibrio anguillarum* in bile resistance and biofilm formation, Microbiology 149 (2003) 1061–1071.
[48] S.D. Breazeale, A.A. Ribeiro, A.L. McClerren, C.R.H. Raetz, A formyltransferase required for polymyxin resitance in *Escherichia coli* and the modification of lipid A with 4-amino-4-deoxy-L-arabinose, J. Biol. Chem. 280 (2005) 14154–14167.
[49] W. Wang, X.H. Zhang, B. Austin, Comparative analysis of the phenotypic characteristics of high- and low-virulent strains of *Edwardsiella tarda*. 2010, J. Fish Dis. 33 (2010) 985–994.
[50] T. Matsuyama, T. Kamaishi, N. Ooseko, K. Kurohara, T. Iida, Pathogenicity of motile and non-motile *Edwardsiella tarda* to some marine fish, Fish Pathol. 40 (2005) 133–135.
[51] P.S. Srinivasa Rao, Y. Yamada, Y. Peng Tan, K.Y. Leung, Use proteomics to identify novel virulence determinants that are required for *Edwardsiella tarda* pathogenesis, Mol. Microbiol. 53 (2004) 573–586.
[52] J. Okuda, F. Murayama, E. Yamanoi, E. Iwamoto, S. Matsuoka, M. Nishibuchi, T. Nakai, Base changes in the fliC gene of *Edwardsiella tarda*: possible effects on flagellation and motility, Dis. Aquat. Org. 76 (2007) 113–121.
[53] Y. Yamada, H. Wakabayashi, Enzyme electrophoresis, catalase test and PCR-RFLP analysis for the typing of *Edwardsiella tarda*, Fish Pathol. 33 (1998) 1–5.

Please cite this article as: N. Buján, et al., Comparative proteomic study of *Edwardsiella tarda* strains with different degrees of virulence, J Prot (2015), http://dx.doi.org/10.1016/j.jprot.2015.05.008