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Proteomic evaluation of Escherichia coli isolates from human clinical strains

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ABSTRACT

Acquired resistance to beta-lactams is mainly mediated by extended-spectrum beta-lactamases (ESBLs) that confer bacterial resistance to all beta-lactams except carbapenems and cephamycins, which are inhibited by other beta-lactamase inhibitors such as clavulanic acid. Although ESBLs still constitute the first cause of resistance to beta-lactams among Escherichia coli, other "new beta-lactamases" conferring resistance to carbapenems, such as metallo-beta-lactamases (MBL) and KPC carbapenemases, or to cephamycins, such as CMY enzymes, have more recently emerged and are often associated with ESBLs. In order to identify and characterize the proteome of extended-spectrum β-lactamase (ESBL) type TEM-52 and CMY-2 producing-Escherichia coli strains of human clinical origin a bidimensional electrophoresis (2-DE) technique with an isoelectric focusing followed by a SDS-PAGE, were used. Full proteomic studies were conducted in the same IEF and SDS-PAGE conditions, for two protein samples of E. coli strains with similar antibiotic-resistance profiles recovered from human clinical sources. A total of 64 and 91 spots were recovered and identified in C583 and C580 strains, respectively. Our results will be helpful for further understanding of antibiotic-resistant mechanism.

Keywords: Antibiotic resistance; ESBL; *Escherichia coli*; Proteome; Humans.

1. Introduction

Proteomics is the large-scale study of proteins, particularly their structures and functions [1]. After genomics, proteomics is often considered the next step in the study of biological systems. It is much more complicated than genomics mostly because while an organism's genome is more or less constant, the proteome differs from cell to cell and from time to time. This is because distinct genes are expressed in distinct cell types. This means that even the basic set of proteins which are produced in a cell needs to be determined. In the past this was done by mRNA analysis, but this was found not to correlate with protein content. It is now known that mRNA is not always translated into protein, and the amount of protein produced for a given amount of mRNA depends on the gene it is transcribed from and on the current physiological state of the cell. Proteomics confirms the presence of the protein and provides a direct measure of the quantity present [2].

Bacterial surface proteins are important for the host-pathogen interaction and they are frequently involved in disease pathogenesis. A wide variety of bacterial surface proteins is represented by lipoproteins which are important components of the bacterial transport system; transmembrane structures involved in the import-export of substrates, including sugars, amino acids, oligopeptides, polyamines, various metal ions and minerals. These systems contribute to many bacterial processes, such as acquisition of nutrients, stress responses

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and intercellular signalling, many of which could be vital for bacterial growth and survival within the host [3]. In the last years extended-spectrum β-lactamases (ESBLs) of the CTX-M class are dramatically spreading among human clinical E. coli isolates, mainly in those recovered from the community [4]. Escherichia coli have become one of the most important causes of nosocomial and community acquired infections. Beta-lactams (mainly extended-spectrum cephalosporins and carbapenems) and fluoroquinolones constitute the main therapeutic choices to treat infections caused by these micro-organisms. However, resistance to these compounds has been reported more and more frequently in Europe in the past years [5]. However, the increasing number of Enterobacteriaceae with ESBLs that also contain AmpCs and other new mechanisms of resistance to fluoroquinolones or aminoglycosides indicate that the recent increase of ESBL producers in Europe constitutes a complex multifactorial problem of high public health significance that deserves a deep analysis and the implementation of specific interventions at different levels.

Two-dimensional gel electrophoresis (2-D electrophoresis) is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. It allows a fast overview of changes in cell processes by analysis of the entire protein extracts in any biological and medical research projects. New instrumentation and advanced technologies provide proteomics studies in a wide variety of biological and biomedical questions. Proteomics work is being applied to study antibiotics-resistant strains and human tissues of various brain, lung, and heart diseases [6]. This technique separate proteins in two steps, according to two independent properties: the first-dimension is isoelectric focusing (IEF), which separate proteins according to their isoelectric points (pI); the second-dimension is SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins according to their molecular weights (MW). In this way, complex mixtures consisted of thousands of different proteins can be resolved and the relative amount of each protein can be determined. The procedure involves placing the sample in gel with a pH gradient, and applying a potential difference across it. In the electrical field, the protein migrates along the pH gradient, until it carries no overall charge. This location of the protein in the gel constitutes the apparent pI of the protein. There are two alternatives methods to create the pH gradient - carrier ampholites and immobilized pH gradient (IPG) gels. [7]. Mass spectrometry (MS) is a powerful tool in protein analysis. Electrospray and matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) technologies can be used to precisely detect small changes in the masses of proteins and peptides. These techniques involve the ionization of molecules into products that can be detected. The mass-to-charge ratio of gas phase ions can then be correlated with the molecular structure of the initial species. Electrospray ionization involves an electric field applied to a solution sprayed from a needle. In MALDI, gas phase ions are generated by desorption ionization of the molecule of interest from a layer of crystals formed from volatile matrix molecules. By using these techniques mutated proteins can be detected rapidly, and the precise site of the mutation can be characterized using tandem MS/MS of peptides of the protein [8]. Antibiotic resistance presents a significant challenge to scientists in the field of infectious diseases. Identification of protein determinants for resistance will not only provide markers for resistance to a particular drug but will also aid in the understanding of the mechanisms of antibiotic function and resistance. Several antibiotics act by targeting protein biosynthesis, interacting with ribosomal structural proteins, rRNAs, and ribosomal-associated proteins [9].

In the current study, a proteomic evaluation of two E. coli isolates (C583 and C580), recovered from clinical human samples and carrying a plasmidic β-lactamase of class AmpC (CMY-2) and a TEM-52 enzyme, respectively , was performed by 2-DE and subsequent protein identification by MALDI-TOF MS.

2. Material and methods

2.1 *Cell culture and purification of E. coli*

ESBL-producing E. coli strains (C583 and C580) of human clinical origin were previously studied and characterized by Vinué et al. [10] and were included in this study. Consequently, proteomic analysis was performed with E. coli strains from human clinical samples.

2.2 *Protein extraction*

Exponentially growing cells (15 mL) were harvested by centrifugation (3 min, 10,000 xg, 4ºC) and re-suspended in PBS (4 mL) at room temperature, followed by a second centrifugation and resuspension with SDS+Tris solution (0.2 mL) [11]. Cell disruption was performed by sonication (3 × 10 s, 4 °C at 100 W); cell debris was removed by centrifugation (14,000 xg, 30 min at 4ºC). The protein concentration was assayed using a 2D Quant kit (GE Healthcare).

2.3 *One-dimensional electrophoresis and staining*

One-dimensional electrophoresis was conducted with SDS-polyacrylamide gels (T=12.52%, C=0.97%) in a HoeferTM SE 600 Ruby® (Amersham Biosciences) unit, following Laemmli [12] with some specific modifications [13]. Gels were stained during 24 hours in Coomassie Brilliant Blue R-250 and washed in water overnight. It was then fixed in trichloroacetic acid (6%) for four hours and in glycerol (5%) for two hours [14].

2.4 *Two-dimensional electrophoresis and proteome analysis*

2-DE was performed according to the principles of O'Farrell (1975) but with IPG (ImmobilineTM pH Gradient) technology [12]. Protein samples of E. coli (GV5) were used in parallel with samples of E. coli C583 and C580. For IEF, precast IPG strips with linear gradient of pH 3-10 were pas-

sively rehydrated overnight (12 to 16 hours) in a reswelling tray with rehydration buffer (8M urea, 1% CHAPS, 0.4% DTT, 0.5% carrier ampholyte IPG buffer pH 3-10) at room temperature. IPG strips were covered with DryStrip Cover Fluid (Plus One, Amersham Biosciences). Lyses buffer [9.5M urea, 1% (w/v) DTT, 2% (w/v) CHAPS, 2% (v/v) carrier ampholytes (pH 3-10) and 10 mM Pefabloc® proteinase inhibitor] was added to the E. coli isolates (1:1). Samples containing a total of 100 µg of protein were loaded into 13 cm IPG strips (pH 3–10 NL, Amersham Biosciences, UK) [14]. The sample solution was then applied to the previously rehydrated IPG strips pH3-10 by cup loading and then proteins were focused sequentially at 500 V for 1 h, gradient at 1000 V for 8 h, gradient at 8000 V for 3 h, and finally 8000 V during 1 h on an EttanTM IPGPhor IITM (Amersham Biosciences, Uppsala, Sweden). Seven IEF replicate runs were performed according to Görg [15] and the GE Healthcare protocol for IPG strips pH 3-10 of 13 cm, in order to obtain the optimized running conditions, resulting in a final 13 hour run. Focused IPG strips were then stored at -80ºC in plastic bags. Before running the second dimension, strips were equilibrated twice for 15 minutes in equilibration buffer (6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS in 0.05 M Tris-HCl buffer (pH 8.8)). In the first equilibration 1% DTT was added to the original equilibration buffer and to the second, 4% iodoacetamide. Bromophenol blue was added to both solutions. The equilibrated IPG strips were gently rinsed with SDS electrophoresis buffer, blotted to remove excessive buffer, and then applied to 12.52% polyacrylamide gels in a HoeferTM SE 600 Ruby® (Amersham Biosciences) unit. Some modifications were introduced in the SDS-PAGE technique previously reported by Laemmli (1970), that allowed its resolution to be increased, with proper insertion of the IPG strips in the stacking gel [12,13]. After SDS-PAGE, the 2-DE gels were fixed in 40% methanol / 10% acetic acid for one hour and afterwards stained overnight in Coomassie Brilliant Blue G-250 [16]. Coomassie-stained gels were scanned on a flatbed scanner (Umax PowerLook 1100; Fremont, CA, USA), and the resulting digitized images were analyzed using Image Master 5.0 software (Amersham Biosciences; GE Healthcare).

2.5 *Protein identification by MALDI-TOF/TOF*

To increase experimental efficacy, four separate gels were analyzed originally representing three independent E. coli protein samples that were previously pooled together and compared. Spots of expression in all gels were manually excised from the gels and analyzed using Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF). The gel pieces were washed three times with 25mM ammonium bicarbonate /50 % ACN (acetonitrile), once with ACN and dried in a SpeedVac (Thermo Savant). 25 mL of 10 mg/mL sequence grade modified porcine trypsin (Promega) in 25mM ammonium bicabornate was added to the dried gel pieces and the samples were incubated overnight at 37ºC. Extraction of tryptic peptides was performed by addition of 10% of formic acid (FA)/50% ACN followed by three-fold lyophilisation in a SpeedVac (Thermo Savant). Tryptic peptides were re-suspended in 10 mL of a 50% acetonitrile/0.1% formic acid solution. The samples were mixed (1:1) with a matrix consisting of a saturated solution of α-cyano-4-hydroxycinnamic acid prepared in 50% acetonitrile/ 0.1% formic acid. Aliquots of samples (0.5µL) were spotted onto the MALDI sample target plate.

Peptide mass spectra were obtained on a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Europe) in the positive ion reflector mode. Spectra were obtained in the mass range between 800 and 4500 Da with ca. 1500 laser shots. For each sample spot, a data dependent acquisition method was created to select the six most intense peaks, excluding those from the matrix, trypsin autolysis, or acrylamide peaks, for subsequent MS/MS data acquisition. Mass spectra were internally calibrated with autodigest peaks of trypsin (MH+: 842.5, 2211.42 Da) allowing a mass accuracy of better than 25 ppm.

2.6 *Database search*

Spectra were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems), which uses internal MASCOT software (v 2.1.04, Matrix Science, London, UK) for searching the peptide mass fingerprints and MS/MS data. Swiss-Prot nonredundant protein sequence database (Release 57 of March 2009, 428650 entries) was used for all searches under E. coli. The database search parameters were as follows: carbamidomethylation and propionamide of cysteine (+71Da) as a variable modification as well as oxidation of methionine (+16Da), and the allowance for up to two missed tryptic cleavages. The peptide mass tolerance was 25 ppm and fragment ion mass tolerance was 0.3 Da. Positive identifications were accepted up to 95% of confidence level. Protein identifications were considered as reliable when the MASCOT score was > 70 (MASCOT score was calculated as $-10 \times \log P$, where P is the probability that the observed match is a random event.). This is the lowest score indicated by the program as significant ($P < 0.05$) and indicated by the probability of incorrect protein identification.

3. Results and Discussion

E. coli strain C583 exhibited resistance to ampicillin, amoxicillin/clavulanic acid, cefoxitin, cefotaxime, ceftazidime, naladixic acid, ciprofloxacine, and trimethoprim-sulfametoxazole and harbored the intI1 gene of class 1 integrons. A plasmidic β-lactamase of class AmpC (CMY-2) was detected in this strain. On the other hand, E. coli C580 presented resistance to ampicillin, cefotaxime, ceftazidime, naladixicacid, ciprofloxacin, and trimethoprim-sulfametoxazole but did not contain class 1 integrons. This strain was proven to be a β-lactamase TEM-52 producer [10].

A comparative analysis among the strains was carried out. The protein expressions of E. coli strains were visualized on 2-DE gels (Figure 1). The use of pH 3-10 IPG strips resulted in a well spread display of protein spots which allowed their safe and accurate excision and image identification.

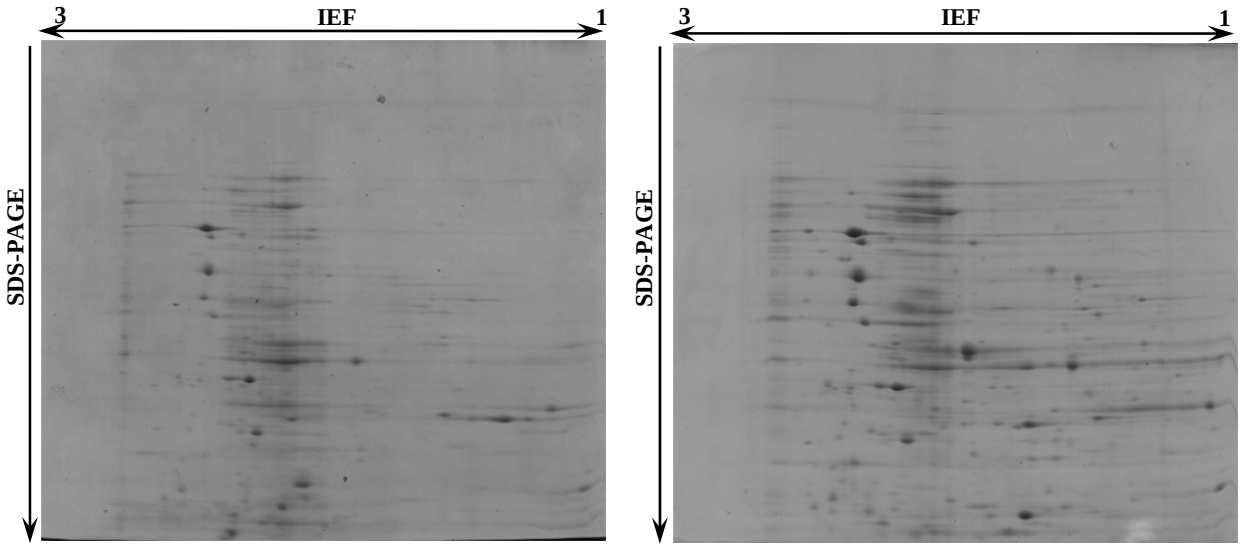

Figure 1. 2-DE gel image of E. coli samples with IPG strips pH3-10. A: C583 B: C580.

Full proteomic studies were conducted in the same IEF and SDS-PAGE conditions, for two protein samples of E. coli strains of human clinical samples C583 and C580. A total of 64 and 91 spots were recovered. All the proteins present in the 2-D gels spots were identified by MALDI-TOF MS and protein bioinformatic databases querying (Tables 1 and 2 of the supplementary material). Among the proteins identified, it is important to emphasise the presence of proteins related to biosynthesis and regulation, glycolysis, stress response, cellular metabolic processes and antibiotic resistance in both

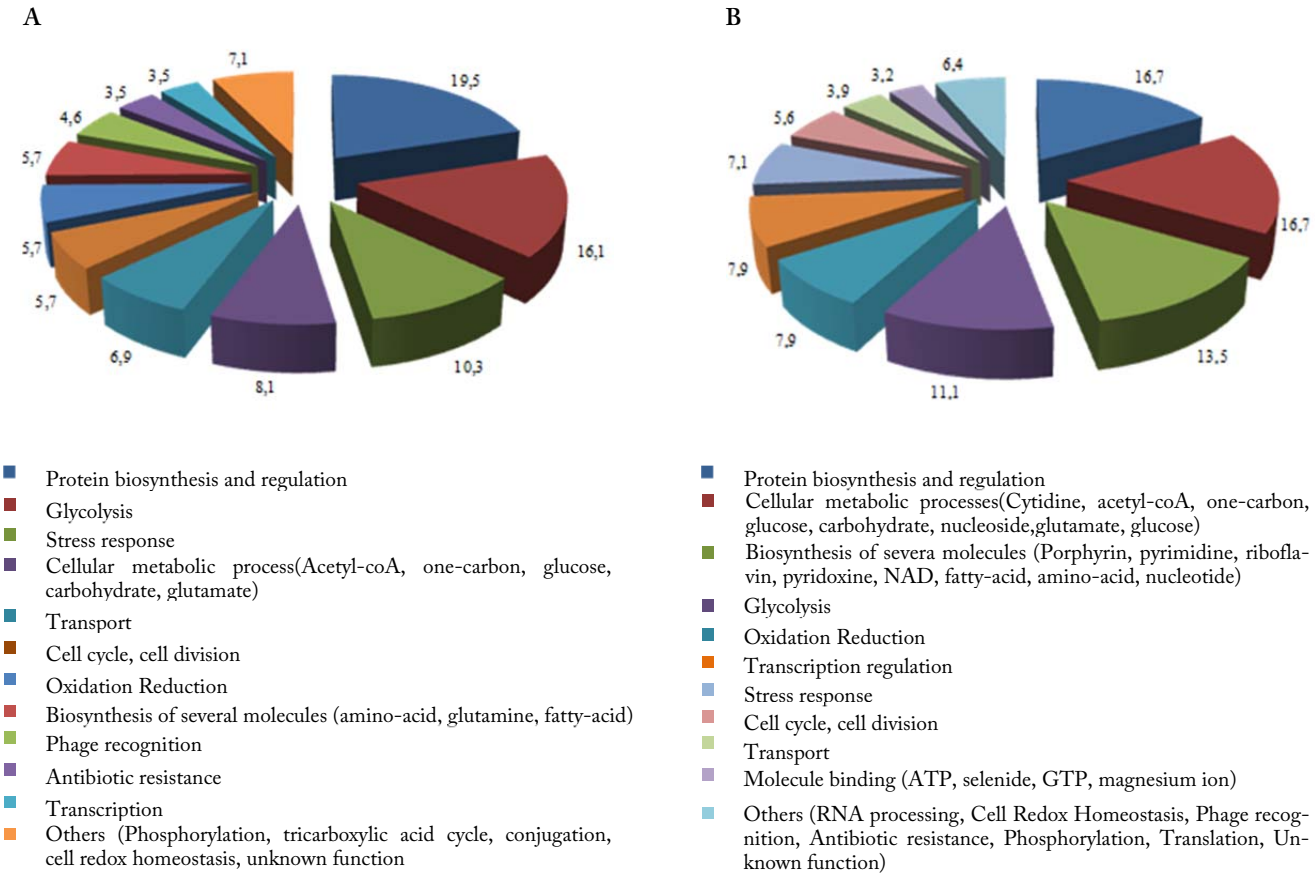

Figure 2. Distribution of the biological processes related to the protein spots found in the 2-DE gels of the *E. coli*. A: C583 and B: C580.

E. coli strains (Figure 2). Proteins related to nine different E. coli strains were found in samples of our C580 and C583 strains; namely proteins associated to three commensal strains (K12, O9:H4 and strain ATCC 8739 / DSM 1576 / Crooks), two uropathogenic (UTI89 and O6:K15:H31 (strain 536)), one avian pathogenic (O1:K1), one enterohemorrhagic (O157:H7), one enterotoxigenic (O139:H28 (strain E24377A)) and one virulent strain (O6) were identified in our strains. The presence of proteins related to one virulent strain O6 and one enterohemorrhagic strain O157:H7 is consistent with the results found in the seagull isolates previously studied in this report. Related to virulent E. coli strain O6 were identified two proteins for E. coli isolate C583 (Trigger factor protein and Chaperone protein ClpB) and three proteins for E. coli isolate C580 (Trigger factor protein, Chaperone protein ClpB and Pyridoxine 5'-phosphate synthase protein). In the case of enterohemorrhagic E. coli strain, two related proteins were found in both E. coli isolate C583 (Putative flavoprotein and Serine hydroxymethyltransferase protein) and other two were found in E. coli C580 (Putative flavoprotein and Curved-DNA binding protein). Also noticed was the detection of proteins Malate dehydrogenase and GrpE protein linked with E. coli enterohemorrhagic strain O157:H7 in our human isolates. A BLAST of a putative wrbA flavoprotein isolated from C580 sequence was released with Translate Reverse tool (Figure 3).

Uropathogenic strains are generally involved in urinary tract infections (UTI) in humans, with UTI89 strain being related to the expression of adhesive organelles (known as pili) that interact with proteins of urinary epithelial cells [17].

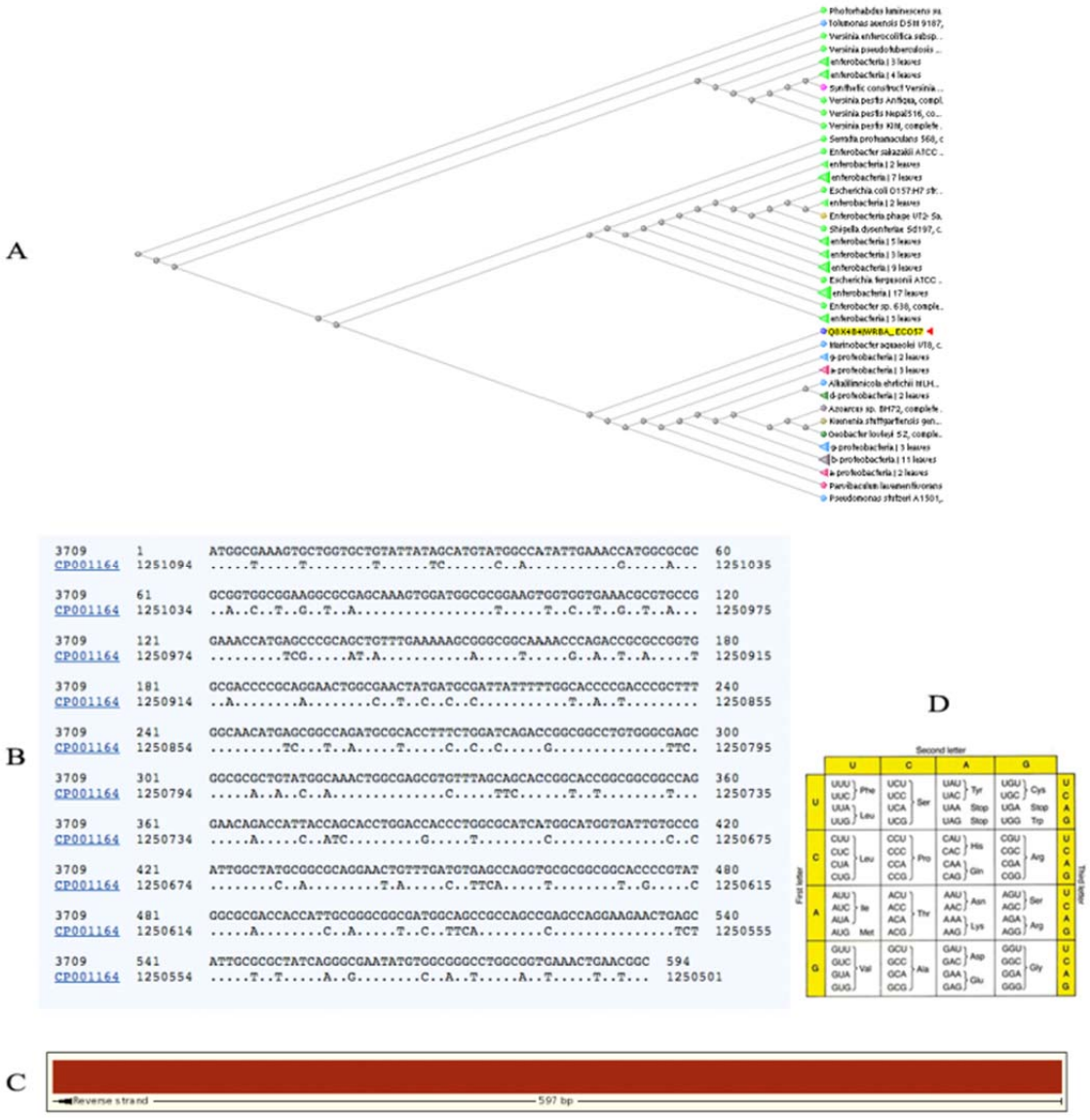

Figure 3. BLAST of a putative *wrbA* flavoprotein isolated from C580 *in silico* sequence from *Translate Reverse* tool (http://www. bioinformatics.org/sms2/rev_trans.html). A: Phylogenetic tree in terms of BLAST with BLASTn alghortim from NCBI; B: Alignment with *in silico* sequence and *wrbA* flavoprotein of *E. coli* O157:H7 EC4115; C: Analysis of a transcript of *wrbA* flavoprotein *E. coli* O157:H7 EC4115 gene with *Emsembl Bacteria* (http://bacteria.ensembl.org/ index.html); D: Genetic code.

In the case of E. coli O6:K15:H31, it is related to pyelonephritis isolates involved in ascending UTIs [18]. Enterotoxigenic strains are an important cause of intestinal infections in children and adults with genes encoding heat-labile and heat-stable properties carried on plasmids. Enterotoxin plasmids are even among the first to be associated with virulence and E. coli O139:H28 (strain E24377A) is among the current examples of this type of E. coli harmful to humans [19]. Avian pathogenic strains such as O1:K1 belong to the same highly pathogenic clonal groups as E. coli strains of the same serotype isolated in several clinical cases of UTIs, neonatal meningitis and septicemia therefore constituting a potential zoonotic risk [20]. Finally, proteins previously characterized as linked with three commensal strains were also found in E. coli isolates from both human faecal samples: K12, O9:H4 and strain ATCC 8739 / DSM 1576 / Crooks. E. coli K12 is a well-known commensal strain with two major porines OmpC and OmpF, corresponding proteins of the external membrane [21]. E. coli strain ATCC 8739 / DSM 1576 / Crooks differs from strain K12 by presenting an insertion element IS1-13 associated to an upstream promoter and to the first 114 bp of OmpC, therefore only expressing OmpF [22]. The E. coli strain O9:H4 was originally isolated from stool from a healthy laboratory scientist who had been using an undomesticated E. coli isolate for several human colonization studies. With no damage done to the hosts, this strain is normally used as a control sample for comparison of pathogenical strains [23]. Such a large number of proteins related to different E. coli strains present in the same faecal sample should cause concern as it represents a major antibiotic resistance at several levels associated with the presence of proteins found in both humans. The relationship between the resistance mechanisms in clinical strains and the understanding of its functioning is of great value for public health worldwide. The full knowledge of how antibiotics resistance evolves and are transmitted between potential hosts of different ecosystems takes on great importance. Proteomic analysis and protein identification can become important and reliable complementary tools to improve our knowledge in this field.

4. Concluding remarks

A well-defined E. coli proteome will have direct applications in biochemical, biological, and biotechnological research fields in the following ways: (i) underpins our understanding not only of the prokaryotic regulatory network but also of complex eukaryotic regulatory networks including stimulon, regulon, and cascade-like networks, (ii) provides invaluable information for designing metabolic engineering strategies to enhance production of various bioproducts, including recombinant proteins, biopolymers, and metabolites, (iii) can be used as a model system to help accelerate the development of advanced high-resolution, high-throughput, and high-sensitivity proteomic technologies [24]. The evaluation of protein profiles in response to various stress mechanisms, such as sensitivity to antibiotics or modifications related to antibiotic resistance, could represent a valid and integrating approach for the development of new therapeutic strategies [25]. In this study, the elaboration of a 2-DE electrophoresis gel of an extended-spectrum β-lactamase E. coli strain with phenotypic and genotypic profiles indicating antimicrobial resistance allowed us to identify and characterize the proteins present. Considering that the worldwide emergence of antibiotic-resistant bacteria poses a serious threat to human health, understand mechanisms of the resistance are extremely important to the control of these bacteria. Our findings will be helpful for further understanding of antibiotic-resistant mechanism(s).

5. Supplementary material

Supplementary material regarding this manuscript is online available in the web page of JIOMICS.

http://www.jiomics.com/index.php/jio/rt/suppFiles/20/0

References

1. S.D. Patterson, R.H. Aebersold, Nat. Genet. 33 (2003) 311–323.
2. E.A. Panisko, T.P. Conrads, M.B. Goshe, T.D. Veenstra, Exp. Hematol. 35 (2002) 97-107.
3. P. Roncada, F. Deriu, A. Gaviraghi, P. A. Martino, L. Bonizzi, Vet. Res. Commun. 33 (Suppl 1) (2009) S157–S160.
4. G.A. Jacoby, L.S. Munoz-Price, New England Journal of Medicine 352 (2005) 380-391.
5. F. Perez, A. Endimiani, K.M. Hujer, R.A. Bonomo, Curr. Opin. Pharmacol. 34(11) (2004) 514-521.
6. B. Wittmann-Liebold, H.-Rüdiger Graack, and T. Pohl. Proteomics 6 (2006) 4688–4703.
7. http://www.tau.ac.il/lifesci/units/proteomics/.
8. S.K. Wilcox, G.S. Cavey, J.D. Pearson. Antimicrob. Agents Chemother. 45(11) (2001) 3046–3055.
9. C.M.T. Spahn, C.D. Prescott. J. Mol. Med. 74 (1996) 423–439.
10. L. Vinué, M. Lantero, Y. Sáenz, S. Somalo,I. de Diego, F. Pérez, F. Ruiz-Larrea, M. Zarazaga, C. Torres, J. Med. Microbiol. 57 (2008) 916-920.
11. J.E. Celis, P. Gromov, Curr. Opin. Biotechnol. 10(1) (1999) 16-21.
12. U.K. Laemmli, Nature 227 (1970) 680-685.
13. G. Igrejas, Genetic, biochemical and technological factors associated to the utilization of common wheat (Triticum aestivum L.), University of Trás-os-Montes and Alto Douro 2000, Ph.D. Thesis.
14. A. Görg, C. Obermaier, G. Boguth, A. Harder, B. Scheibe, R. Wildgruber, W. Weiss, Electrophoresis 21 (2000) 1037-1053.
15. A. Görg, A. Klaus, C. Lück, F. Weiland, W. Weiss, Technische Universität München 2007.
16. A. Görg, W. Weiss, M.J. Dunn, Proteomics 4 (2004) 3665-3685.
17. D.A. Hunstad, S.S. Justice, C.S. Hung, S.R. Lauer, S.J. Hultgren, Infect. and Immun. 73(7) (2005) 3999-4006.
18. A.L. Lloyd, D.A. Rasko, H.L.T. Mobley, J. Microbiol. 189(9) (2007) 3532-3546.
19. M.K. Wolf, Clin. Microb. Rev. 10(4) (1997) 569-584.
20. M. Moulin-Schouleur, M. Répérant, S. Laurent, A. Brée, S. Miqnon-Grasteau, P. Germon, D. Rasschaert, C. Schouler, J. Clin. Microbiol. 45(10) (2007) 3366-3376.
21. K. Hayashi, N. Morooka, Y. Yamamoto, K. Fujita, K. Isono, S. Choi, E. Ohtsubo, T. Baba, B.L. Wanner, H. Mori, T. Horiuchi,

Mol. Syst. Biol. 2 (2006) 1-5.

22. A. Copeland, S. Lucas, A. Lapidus, T. Glavina Del Rio, E. Dalin, H. Tice, D. Bruce, L. Goodwin, S. Pitluck, H. Kiss, T. Brettin, J.C. Detter C. Han, C.R. Kuske, J. Schmutz, F. Larimer, M. Land, L. Hauser, N. Kyrpides, N. Mikhailova, L. Ingram, P. Richardson, Direct submission.
23. D.A. Rasko, M.J. Rosovitz, G.S. Myers, E.F. Mongodin, W.F. Fricke, P. Gajer, J. Crabtree, M. Sebaihia, N.R. Thomson, R. Chaudhuri, I.R. Henderson, V. Sperandio, J. Ravel, J. Bacteriol. 190(20) (2008) 6881-6893.
24. M. Han, S.Y. Lee, Microbiol. Molec. Biol. Rev. 70(2) (2006) 362-439.
25. D. Hiscock, C. Upton, Bioinformatics 16(5) (2000) 484-485.