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Proteomic study in an *Escherichia coli* strain from seagulls of the Berlengas Natural Reserve of Portugal

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ABSTRACT

The increasing bacterial resistance among common pathogens is threatening the effectiveness of several antibiotics. This represents a serious public health problem as such bacterial strains have already been detected in domestic, wild-life animals and humans. Using *Escherichia coli* as a model organism, we applied a proteomic approach to the topic of antimicrobial resistance. In order to identify and characterize the proteome of extended-spectrum β-lactamase (ESBL) type TEM-52 producing-*Escherichia coli* strain of a faecal sample taken from Yellow-legged seagulls (*Larus cachinnans*) a bidimensional electrophoresis (2-DE) technique with an isoelectric focusing followed by a SDS-PAGE, was used. Eighty seven individualized protein spots were identified. All were suitable for peptide mass fingerprinting by a mass spectrometric technique (MALDI/TOF MS). Their identification was carried out by searching appropriate bioinformatic databases. All proteins were related to *E. coli* strains. Detection of proteins related to several *E. coli* strains linked with virulent and enterohaemorrhagic consequences in ESBL producing-*E. coli* isolates of seagull samples raises the question of how such similarities arise bearing in mind these remarkably different microbial ecosystems.

Figure 1. Seagulls (*Larus cachinnans*) in Berlengas archipelago natural reserve.

Keywords: Antibiotic Resistance; ESBL; *Escherichia coli*; Proteome; Wild Animals.

1. Introduction

Proteome analysis of innumerable biological organisms represents a major challenge for the post-genomic era and constitutes an abundant source of biological information [1]. In analogy to the genome, the proteome is used to describe a set of proteins expressed by a certain organism, under defined conditions, serving as a unique and informative readout of both its phenotypic state, which results from cell responses to physiological and environmental perturbations, and genomic information reflected in the amino acid sequences of expressed proteins. The main concern of proteomic resides, then, in the identification of proteins involved in particular cellular processes or presenting altered expression profiles as

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a consequence of different physiological conditions [2].

Currently, increasing rates of bacterial resistance exist among common pathogens that are threatening the effectiveness of the most powerful antimicrobials. The emergence and spread of antibiotic-resistant Gram-negative pathogens, such as *Escherichia coli*, can lead to serious public health issues for humans and animals [3,4]. *E. coli*, a very well characterized prokaryote, has served as a model organism for several biological and biotechnological studies increasingly so since the completion of the *E. coli* genome-sequencing project [5]. The availability of complete genome sequence databases therefore facilitates the proteomic analysis of *E. coli* using MS (Mass Spectrometry). The most common resistance mechanism in *E. coli* and other Enterobacteriaceae is through the production of β-lactamases – enzymes which inactivate certain β-lactam antibiotics [6]. Extended-spectrum β-lactamases (ESBLs) are considered a major mechanism of resistance to β-lactam antibiotics that include broad-spectrum cephalosporins and azthereonam [7,8]. In fact, nowadays, ESBL-containing bacteria represent a major threat to the human community and for hospital patients causing several outbreaks and becoming endemic in many hospitals around the world [9]. The use of antibiotics in food-producing animals could lead to selection of antibiotic-resistant bacteria that can be transferred to humans through the consumption or handling of foods of animal origin. In recent studies it was demonstrated that antimicrobial resistance in foodborne bacteria may result in prolonged illness and elevated rates of bacteremia, hospitalization and death [10]. In fact, pathogenic strains of *E. coli* can cause sepsis and infections of the nervous system, digestive and urinary tracts [11] and their presence in animals in contact with humans becomes a matter of great concern.

The traditional method of measuring protein expression is by two-dimensional gel electrophoresis (2-DE) which, combined with the mass spectrometry (MS) of protein spots, makes up one of the most prevalent techniques used in proteomics, capable of generating a portrait of the global protein expression in a given sample [12,13,14]. Two-dimensional gel electrophoresis is a long established technology by which proteins are separated according to their isoelectric point (pI) and their molecular weight (Mr) [15]. The high-throughput identification of proteins excised from 2-DE gels was demonstrated by Shevchenko and colleagues (1996), who used this procedure to identify proteins expressed in yeast, following the resolution of a cell lysate by 2-D PAGE [16]. MS-based proteomics normally involves large-scale identification, quantification, and characterization of proteins at various levels resulting in an important tool for the analysis of biological systems and the exploration of complex protein functions and interactions. The most common method of protein identification is the analysis of peptide masses resulting from enzymatic digestion (e.g. by trypsin) of proteins resolved by and excised from 2-DE, by using MALDI-TOF MS [16]. In order to make sequence data derived from several bacterial genomes accessible via public databases, proteome data are characterized by diverse data types and are stored in proprietary databases located worldwide [17,18], becoming a reliable source of information for proteomic studies.

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. In the current study, a proteomic evaluation of an *E. coli* isolate (GV5), recovered from a faecal sample of a Yellow-legged seagull (*Larus cachinnans*) from Berlengas Natural Reserve of Portugal and carrying an ESBL TEM-52, 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*

E. coli strains were obtained from faecal samples of Yellow-legged seagulls (Figure 1), randomly recovered in the beaches of Berlengas Islands National Reserve of Portugal, located at the coast of Peniche (GPS coordinates: N 39º 24' 51,77'', W 9º 30' 33,67''). They were previously characterized for antibiotic resistant genes [19]. One of these *E. coli* strains, GV5, was included in this study. This strain was proven to be a β-lactamase TEM-52 producer [20].

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 re-suspention with SDS+Tris solution (0.2 mL) [12]. 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 [21] with some specific modifications [22]. Gels were stained during 24 hours in Coomasse 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 [23].

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 [21]. 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 passively 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) [23]. 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 [24] 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 -80°C 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 [21,22]. 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 [25]. 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 25 mM 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 25 mM ammonium bicabornate was added to the dried gel pieces and the samples were incubated overnight at 37°C. 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 a-cyano-4-hydroxycinnamic acid prepared in 50% acetonitrile/ 0.1% formic acid. Aliquots of samples (0.5 µL) 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 strains included in the study were previously characterized for virulence and antibiotic resistance. The seagull's ESBL-producing *E. coli* strain GV5 presented resistance to ampicillin, cefotaxime, ceftazidime, naladixic acid, ciprofloxacine, tetracycline, streptomycin, trimethoprim-sulfametoxazole, and chloramphenicol and contained the following resistance genes: blaTEM-52 (ESBL gene encoding TEM-52) cmlA (chloramphenicol-resistance), tetA (tetracycline-resistance), sul1 and sul3 (sulfametoxazole-resistance). The intI1gene encoding the integrase of class I integrons was also identified in strain GV5, as well as the gene cassettes dfrA1 + aadA1 included in their variable region. *E. coli* GV5 was classified into the phylogenetic group D, mostly associated with virulent extraintestinal isolates [19].

In sample GV5 a total of 87 protein spots were collected and individualized in eppendorfs for their analysis using MALDI-TOF mass spectrometry. The protein spots were identified and characterized (Table 1 on supplementary ma-

terial) by correlation with bioinformatic databases (http://www.ncbi.nlm.nih.gov/). From a total of 87 protein spots, nine proteins were detected as related to *E. coli* serotype O6 and another six proteins linked with *E. coli* serotype O157:H7 (Figure 2, A and B respectively; see also Table 1). Among the proteins identified as linked to serotype O6, it is important to highlight the presence of proteins related to environmental stress conditions: the curved DNA-binding protein (spot 18), the chaperone protein (spot 35) and the GrpE protein (spot 45) [26,27,28] are responsible for the capacity of the bacteria to resist external damage (heat, osmotic and other environmental stress causes). Beta-lactamase TEM (P62593, spot 39) and dihydrofolate reductase type XV (P78218, spots 8) were related to antibiotic resistance. The trigger factor protein (or tig), on other hand, is involved in the protein exportation whereas the other proteins related to serotype O6 presented functions in important metabolic pathways essential for the bacteria. The proteins found to be related to serotype O157:H7 presented various functions, as kdsA protein (spot 60) with an important part in the external membrane and lipopolysaccharide biogenesis [27]. The curved DNA-binding protein previously identified for the O6 serotype, was also found in the O157:H7 serotype (spot 18).

The *E. coli* serotype O6 represents a heterogeneous group of bacteria which differ in the genotypic presence as well as in the phenotypic expression of virulence factors, being already detected in humans, dogs and cats [29]. On the other hand, the serotype O157:H7 is an enterohemorrhagic strain that belongs to the verotoxin-producing *E. coli* (VTEC). VTEC have emerged as food-borne pathogens related to gastroenteritis that may be complicated by hemorrhagic colitis or by the hemolytic uremic syndrome, the main cause of acute renal failure in children [30]. The transmission of these food-borne pathogens occurs through consumption of undercooked meat, unpasteurized dairy products, vegetables, water contaminated by ruminant feces as these animals can carry *E. coli* O157:H7 transiently and sporadically and pass the bacteria in their feces [30,31,32]. The proximity of the Berlengas Natural Reserve to the Continental Portuguese coastline is an important factor in considering the possible contact between seagulls and potential disease transmitters. In fact, vero cytotoxin-producing *E. coli* O157 has already been detected in the past in faecal samples from wild-birds [33] namely from gulls [34], determining probably a transmission pattern. 2-DE proteomics correlated with bioinformatic databases has already been used in analyzing and characterizing bacterial proteins, as in *E. coli* where a large number of proteins are already completely identified [5,35]. In our study a great number of proteins which were related to several functions within the cell metabolism were found, like β-lactamase TEM precursor proteins (blaT) capable of producing β-lactamases (Figure 3). The blaT proteins are prevalent in enterobacteriaceae hydrolyzing the β-lactam bond of susceptible β-lactam antibiotics like penicillins and cephalosporins [36,37]. The presence of three protein spots identified as blaT (spots 39, 40 and 79; see Table 1) confirms that our sample possesses resistance to β-lactam antibiotics. Other proteins relevant to various important and essential functions in the bacteria were found. Flagellin or FliC (spot 14) is responsible for the formation of bacterial flagella [28,38]; chaperone protein or DnaK (spots 1, 49 and 62), involved in the chromosomal DNA replication [39,40,41]; while L-asparaginase II or AspG2 (spot 53), is an important therapeutic enzyme for the treatment of leukemia [28,42].

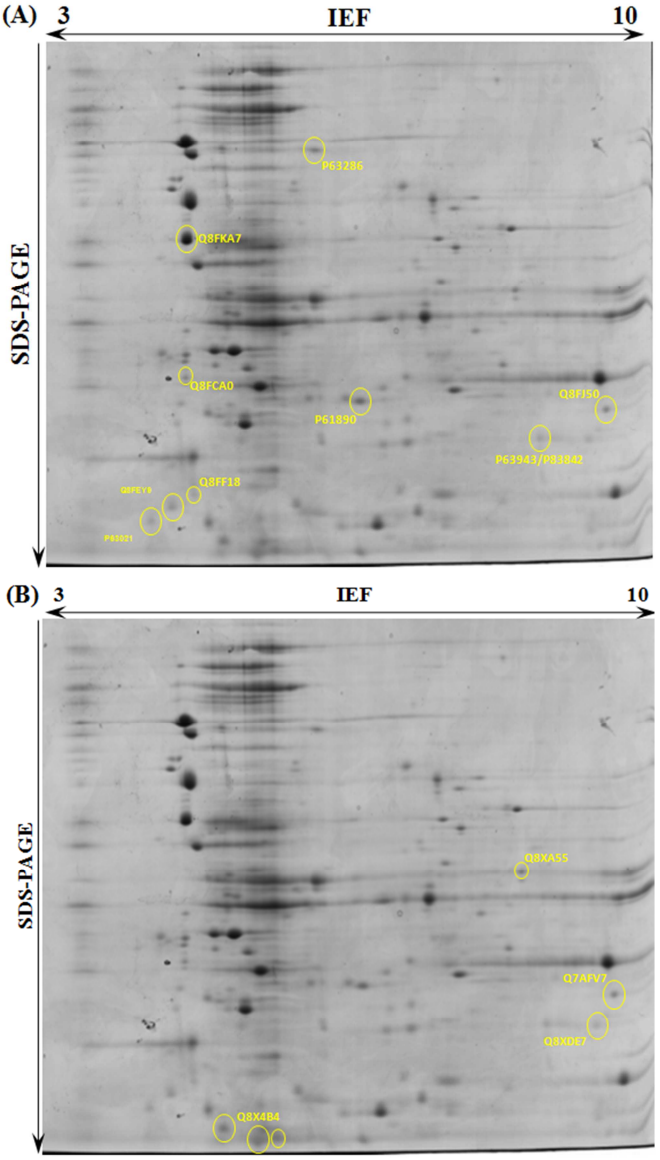

Figure 2. 2-DE gel image of *E. coli* sample GV5. (A) Accession numbers of proteins related to *E. coli* serotype O6; (B) Accession numbers of proteins related to *E. coli* serotype O157:H7.

In the case of enterohemorrhagic *E. coli* strain, four related proteins were found in *E. coli* isolates GV5 (Putative flavoprotein, Serine hydroxymethyltransferase protein 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 *E. coli* virulent strain O6 in the seagull isolate.

The presence of proteins associated with such *E. coli* strains

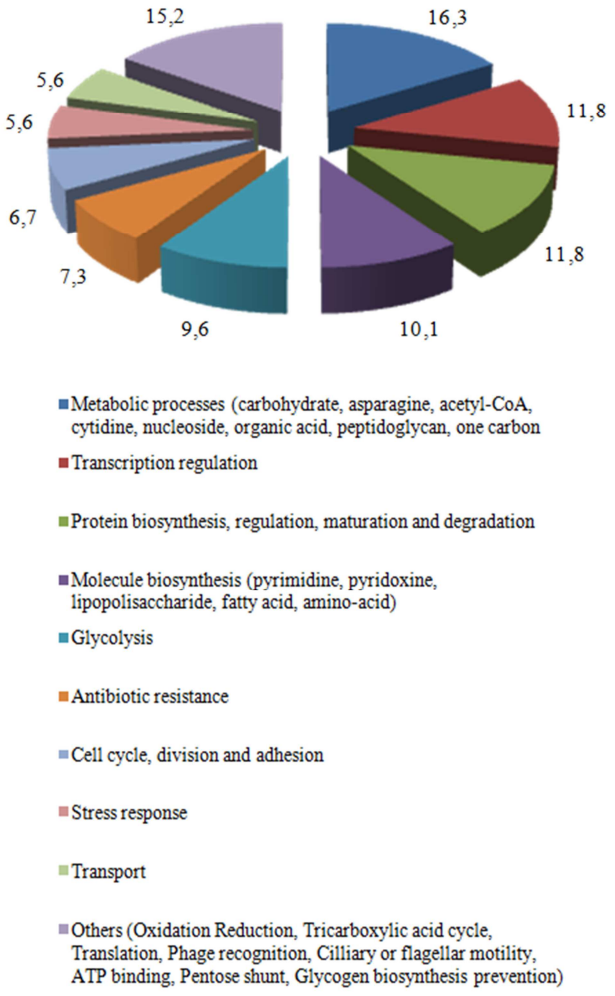

Figure 3. Distribution of the biological processes related to the protein spots found in the 2-DE gels of the *E. coli* GV5.

alongside proteins related to commensal strains in faecal samples of wild-life animals and their resistance to antimicrobial drugs represents a public health concern. The idea of antimicrobial resistance in the same extended-spectrum β-lactamase *E. coli* of very different ecosystems as microbial fauna of wild-life animals and humans with repressed imunoystems is therefore a new concern in the already problematic question of antibiotic resistance and possible transmission.

4. Concluding remarks

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. The proteome patterns obtained reveal proteins previously identified in the virulent strain *E. coli* O6 and enterohemorrhagic strain *E. coli* O157:H7. The detection of proteins related to these strains in samples of extended-spectrum β-lactamase-producing *E. coli* isolates became possible through the proteomic approach and 2-DE combined with mass spectrometry. Considering this work, it is possible to elucidate gene expression of multiresistant bacteria strains isolated from different wild ecosystems. In the future it will be important to evaluate this expression under different forms of stress. These proteins should be tested under stress conditions, for example under antibiotic pressure, in order to determine the changes in protein expression, and to test potential targets for designing new drugs to inhibit the growth of the antibiotic-resistant bacteria.

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/19/0

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