BioSystems

Cite this: *Mol. BioSyst.*, 2012, **8**, 1060–1067

www.rsc.org/molecularbiosystems

PAPER

Comparative proteomics to evaluate multi drug resistance in *Escherichia coli*†

Cristian Piras,‡[a] Alessio Soggiu,‡[b] Luigi Bonizzi,[c] Alessandro Gaviraghi,[c] Francesca Deriu,[c] Luisa De Martino,[d] Giuseppe Iovane,[d] Angela Amoresano[e] and Paola Roncada*[f]

Received 23rd September 2011, Accepted 8th November 2011
DOI: 10.1039/c1mb05385j

Drug resistance in food-borne bacterial pathogens is an almost inevitable consequence of the use of antimicrobial drugs, used either therapeutically or to avoid infections in food-producing animals. In the past decades, the spread and inappropriate use of antibiotics have caused a considerable increase of antibiotics to which bacteria have developed resistance and, moreover, bacteria are becoming resistant to more than one antibiotic simultaneously. Understanding mechanisms at the molecular level is extremely important to control multi-resistant strains and to develop new therapeutic strategies. In the present study, comparative proteomics was applied to characterize membrane and cytosolic proteome in order to investigate the regulation of protein expression in multi-resistance *E. coli* isolated from young never vaccinated water buffalo. Results highlighted differentially expressed proteins under multi drug resistance conditions giving new insights about mechanisms involved in resistance, as quorum sensing mechanisms, and suggesting possible novel bacterial targets to develop alternative antibiotic drugs.

Introduction

During the last century, after penicillin discovery, antibiotics have reduced morbidity and mortality related to infectious disease improving life quality and increasing life expectancy.[1] However, the intensive use of antibiotics[2,3] and the peculiar adaptive capacity of bacterial genome have caused the world-wide emergent phenomenon of antibiotic resistance, which poses serious threats to human and animal health. Furthermore the number of antibiotics to which bacteria develop resistance is considerably increasing and, at the same time, a growing number of bacterial species are becoming resistant to more than one antibiotic simultaneously.[4] The main aim of this study is the multi drug resistance in *E. coli*, which represents a new challenge in antimicrobial drug research. Antibiotic resistance is wide spread in *E. coli* entero-pathogen. It represents the major global cause of food- or water-borne illness and one of the most frequent causes of death in developing countries due to complications like hemolytic or uremic syndromes, which are responsible for acute renal collapse in childhood[5] especially if the *E. coli* serotype is O157 or VTEC (verocytotoxin-producing *E. coli*). In *E. coli*, multiple drug resistance to streptomycin, sulfisoxazole and tetracycline is well documented[6] and it has been associated with failures in therapy of infectious disease[7] both in human and veterinary medicine. Therefore, new strategies are required to control the diffusion and to decrease the incidence of these pathologies. Few proteomic investigations on molecular mechanisms related to one antibiotic resistance have been reported, but only limited data are available on multi-drug related molecular mechanisms. Studies on multiple antibiotic-resistance reported for *Salmonella enterica* describe the role of AcrAB–TolC efflux pumps and outer membrane proteins (OM) in antibiotics and disinfectants resistance.[4] In *E. coli* it has been well described that the outer membrane proteins (OM) are involved in streptomycin,[8] nalidixic acid,[9] chlortetracycline[10] and tetracycline resistance.[11] The role of outer membrane proteins and efflux channel is well established, but it is not clear which signals are responsible for bacteria specific activation. Interestingly, the existence of a possible link between multi-drug efflux systems and quorum sensing

[a] *Dipartimento di Scienze Zootecniche, Facoltà di Agraria, Università Degli Studi di Sassari, Sassari, Italy*
[b] *Dipartimento di Neuroscienze "B.B.Brodie", Università degli studi di Cagliari, Cagliari, Italy*
[c] *Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Facoltà di Medicina Veterinaria, Università Degli Studi di Milano, Milano, Italy*
[d] *Dipartimento di Patologia e Sanità Animale, Facoltà di Medicina Veterinaria, Università di Napoli "Federico II", Napoli, Italy*
[e] *Dipartimento di Chimica Organica e Biochimica, Università di Napoli "FedericoII", Napoli, Italy*
[f] *Istituto Sperimentale Italiano L. Spallanzani, via Celoria 10, 20133, Milano, Italy. E-mail: paola.roncada@guest.unimi.it; Fax: +39 02 50318171; Tel: +39 02 50318138*
† Presented, in part, at the 6th Annual National Conference of the Italian Proteomics Association held in Turin 21st–24th June 2011.
‡ These authors equally contributed to this work.

has also been demonstrated.[12] Typically, in Gram negative bacteria, quorum sensing involves the production, the release and the detection of auto-inducers (acylated homoserine lactone signaling).[13] Bacteria use this process for cell–cell communication and for regulation of gene expression of cell community.[14] Quorum sensing has been already described in pathogenesis and virulence[15] and in the formation of biofilm. Furthermore, in *E. coli* an increase in antibiotic resistance in mature biofilm has been observed.[16]

In the present study, an integrated approach based on two dimensional electrophoresis (2DE) coupled with mass spectrometry (MS) techniques was used to resolve *E. coli* multiresistant proteome, to investigate protein networks and to identify new putative candidates involved in resistance mechanisms.

Materials and methods

Specimen collection

Diarrheic fecal samples from young water buffalo-calves, recovered from different farms located in the province of Caserta (Southern Italy), were used in this study. All animals showed similar clinical symptoms and history of diarrhea and had never been vaccinated against *E. coli* infection. The animals were 30 days old.

Bacteriological techniques

Samples were cultured after running an enrichment broth Brain heart Infusion (BHI) incubated at 37 °C for 24 hours. Loopful from these broth cultures were then streaked onto Enterohaemolysin agar with blood (EHLY) and Sorbitol MacConkey agar (SMAC) (both from Oxoid S.p.A., Milan, Italy), and incubated at 37 °C for 24 hours. EHLY and SMAC were used to search and to isolate haemolytic *E. coli* and sorbitol-non-fermenting *E. coli* O157-H7, respectively. Suspected colonies were identified morphologically, then biochemically by the API 20E Kit system (bioMerieux, France). As positive control, *E. coli* O157 strain ATCC 43895 was used as quality control strains in each experiment.

E. coli O:157 latex test and serotyping

A latex agglutination test for the identification of *E. coli* serogroup O:157 was used (*E. coli* O:157 latex test, Oxoid). Furthermore, the determination of O:111, O:26, O:157, O:128, and O:103 antigens, employing antisera (Biolife Italiana Srl, Milano, Italy), was carried out.

Antibiotic susceptibility testing

The antibiotic susceptibility test was run in duplicate for each sample. Bacterial suspensions with a turbidity equivalent to McFarland standard 0.5 were swabbed onto Muller Hinton agar plates to determine antimicrobial susceptibility, and the Bauer and Kirby disk diffusion technique was followed. The following commercially available antibiotics discs at the indicated concentrations were used: nalidixic 1 acid (NA 30 μg), neomycin (N 30 μg) amoxicillin/clavulanic acid (AUG 30 μg), ampicillin (AMP 10 μg), apramycin (APR 15 μg), colistin sulfate (CS 10 μg), enrofloxacin (ENR 5 μg), gentamicin (CN 10 μg), tetracycline (TE 30 μg), trimethoprim/sulfamethoxazole (SXT 25 μg), ceftiofur (EFT 30 μg), lincomycin (MY 2 μg), penicillin G (P 10 μg). Plates were incubated for 24 h at 37 °C followed by measuring the diameters of the inhibition zones, including the diameter of the disc (mm).

Protein extraction for 2D electrophoresis

Three biological replicates were performed for 2D electrophoresis analysis.

Protein extraction was performed as previously described,[17] adapting protocol to *E. coli* samples. Bacteria were harvested at 9000*g*, at 4 °C, for 10 minutes and quickly washed five times with cold PBS. Cellular pellets for 2-DE analysis were suspended in lysis buffer (9.5 M urea, 4% CHAPS, 1% DTT, 10 mM TRIS) containing protease inhibitors cocktail (GE Healthcare) according to the manufacturer's instructions and disrupted by sonication 5 times for 4 min at maximum power on ice. Cell debris were removed by centrifugation at 9000*g*, at 20 °C, for 60 min. To minimize contamination by lipids, phospholipids and other cell constituents, the supernatant was cleaned by precipitation[18] using a solution consisting of tri-*n*-butyl phosphate : acetone : methanol (1 : 12 : 1), cooled on ice. 1.4 ml of this solution were added to each sample to reach a final acetone concentration of 80% (v/v) and incubated at 4 °C for 90 min. The precipitate was isolated by centrifugation at 9000*g*, for 20 minutes at 4 °C. After washing with the same precipitant buffer, it was centrifuged again and then air dried. Cellular pellets were re-suspended in 8 M urea, 4% CHAPS, 1% DTT, 10 mM TRIS and 2% Ampholine pH 3.5–10 containing nuclease mix (GE Healthcare) to remove nucleic acids. Protein concentration in all samples was determined using 2D Quant Kit (GE Healthcare).

2D electrophoresis and image analysis

Euroclone immobilized pH gradient (IPG) strips (EMP052040, 13 cm) with a linear pH range of 4.0–8.0 were rehydrated overnight in a buffer containing 8 M urea, 4% CHAPS, 1% DTT, 10 mM TRIS and 2% Ampholine pH 3.5–10. 100 μg of protein sample were loaded on each IPG strip using cup loading at the cathodic side. Isoelectric focusing was performed using an Ettan IPGphor III IEF system (GE Healthcare) at 20 °C with a current of 120 μA per strip. For IEF the following protocol was used: 30 V (4 h), 50 V (3 h), 100 V (3 h), 500 V (3 h), 1000 V (3 h), 3000 V (3 h), 4000 V (3 h), 6000 V (3 h) and 8000 V (8 h). After the first dimension, IPG strips were equilibrated twice with a solution containing 6 M urea, 2% SDS, 50 mM Tris–HCl pH 8.8 and 30% glycerol, for 15 min, under gentle stirring. For the first equilibration step was used 1% DTT and for the second 2.5% iodoacetamide. The second dimension was performed using homemade 12% acrylamide gradient vertical SDS-PAGE slab gels (13 × 13 × 0.1 cm) on a Protean II cell (BioRad). IPG strips were put on top of the SDS gels which were poured up to 1 cm from the top of the plates and then sealed with 1.5 ml of a solution containing 0.5% low-melting-point agarose diluted in hot SDS running buffer (25 mM Tris–HCl pH 8.3, 192 mM glycine, 0.1% SDS). Molecular weight protein markers (Invitrogen) were applied on one end of the IPG strips. In the second dimension, gels were run at 15 mA per gel, for 20 min and then at 40 mA per gel, until the bromophenol blue front-line came out of the gel. After runs, gels were stained with colloidal

Coomassie and digitalized with PharosFX Plus Laser Imaging System (BioRad). For 2-DE three experimental replicates were performed for each sample. Gel images were imported both into Progenesis SameSpots (v3.33.3383; Nonlinear Dynamics, Newcastle, UK) and ImageMaster 2D Platinum v6.0.1 software (GE Healthcare) for analysis. All imported images were processed to check image quality (saturation, dimension, background). The aligned images were then automatically analyzed using the 2D analysis module for spot detection, background subtraction, normalization, and spot matching, and all spots were manually reviewed and validated to ensure proper detection and matching. Statistical analysis was performed by the Progenesis Stats module on the log-normalized volumes for all spots. Student's *t*-test and one-way ANOVA were used to confirm the *p* value between different groups, *p*-values under 0.05 were considered statistically significant.

In situ digestion

Differentially expressed proteins were excised from Colloidal Coomassie-stained gel and destained by sequential washes with 0.1 M NH_4HCO_3 pH 7.5 and acetonitrile. Samples were reduced by incubation with 50 µl of 10 mM DTT in 0.1 M NH_4HCO_3 buffer pH 7.5 and carboxyamidomethylated with 50 µl of 55 mM iodoacetamide in the same buffer. Enzymatic digestion was carried out with trypsin (12.5 ng μl^{-1}) in 10 mM ammonium bicarbonate pH 7.8 with 2 hours incubation at 4 °C. Trypsin solution was then removed and a new aliquot of the digestion solution was added; samples were incubated for 18 h at 37 °C. A minimum reaction volume was used to obtain the complete re-hydration of gel. Peptides were then extracted by washing the gel particles with 10 mM ammonium bicarbonate and 1% formic acid in 50% acetonitrile at room temperature. The resulting peptide mixtures were desalted using ZipTip pipettes from Millipore, following the recommended purification procedure.

MALDI-TOF mass spectrometry

Positive Reflectron MALDI spectra were recorded on a Voyager DE STR instrument (Applied Biosystems, Framingham, MA). The MALDI matrix was prepared by dissolving 10 mg of alpha cyano in 1 ml of acetonitrile/water (90 : 10 v/v). Typically, 1 µl of matrix was applied to the metallic sample plate and 1 µl of analyte was then added. Acceleration and reflector voltages were set up as follows: target voltage at 20 kV, first grid at 95% of target voltage, delayed extraction at 600 ns to obtain the best signal-to-noise ratios and the best possible isotopic resolution with multipoint external calibration using peptide mixture purchased from Applied Biosystems. Each spectrum represents the sum of 1500 laser pulses from randomly chosen spots × sample position. Raw data were analyzed using the computer software provided by the manufacturers and are reported as monoisotopic masses.

Nano HPLC-chip MS/MS analysis

LC/MS/MS analyses were performed on a LC/MSD Trap XCT Ultra (Agilent Technologies, PaloAlto, CA) equipped with a 1100 HPLC system and a chip cube (Agilent Technologies). After loading, the peptide mixture was first concentrated and washed in a 40 nL enrichment column (Agilent Technologies chip), with 0.2% formic acid in 2% acetonitrile as the eluent. The sample was then fractionated on a C18 reverse-phase capillary column (Agilent Technologies chip) at a flow rate of 300 nl min^{-1}, with a linear gradient of eluent B (0.2% formic acid in 95% acetonitrile) in A (0.2% formic acid in 2% acetonitrile) from 7% to 60% in 50 min. Peptide analysis was performed using data-dependent acquisition of one MS scan (mass range from 300 to 1800 *m*/*z*) followed by MS/MS scans of the three most abundant ions in each MS scan. Dynamic exclusion was used to acquire a more complete survey of the peptides by automatic recognition and temporary exclusion (2 min) of ions from which definitive mass spectral data had previously acquired. Nitrogen at a flow rate of 3 l min^{-1} and heated to 325 °C was used as the dry gas for spray desolvation. MS/MS spectra were measured automatically when the MS signal surpassed the threshold of 50 000 counts. Double charged ions were preferably isolated and fragmented over single charged ions. The acquired MS/MS spectra were transformed in Mascot generic file format and used for peptides identification with a licensed version of MASCOT, in a local database.

Protein identification

Spectral data were analyzed using Analyst software (version 1.4.1) and MSMS centroid peak lists were generated using the MASCOT.dll script (version 1.6b9). MSMS centroid peaks were threshold at 0.1% of the base peak. MSMS spectra having less than 10 peaks were rejected. MSMS spectra were searched against SwissProt database using the upgrade licensed version of Mascot 2.1 (Matrix Science), after converting the acquired MSMS spectra in Mascot generic file format. The Mascot search parameters were: taxonomy "*Escherichia coli*"; "trypsin" as enzyme allowing up to 2 missed cleavages, carbamidomethyl as fixed modification, oxidation of M, pyro-Glu (N-term Q), as variable modifications, 600 ppm MSMS tolerance and 0.6 Da peptide tolerance and top 20 protein entries. Spectra with a MASCOT score of <25 having low quality were rejected. The score used to evaluate quality of matches for MSMS data was higher than 30.

Results

Antibiotic susceptibility test

An antibiotic susceptibility test was performed on all 57 *E. coli* O157 isolated. Results were interpreted according to the guidelines of the Clinical Laboratories Standards Institute breakpoints (National Committee for Clinical Laboratory Standards 2002). Susceptibility to 13 different antibiotics was evaluated on all 57 *E. coli* isolated with the following results in terms of resistance percentage (Fig. 1): augmentin (42%), ampicillin (43, 8%), nalidixicacid and apramycin (17, 5%), colistinsulfate (1%), enrofloxacine (8, 7%), gentamicin (7%), ceftiofur (3, 5%), lincomycin and penicillin (100%), tetracycline (22, 8%), neomycin (71, 9%) and sulfamethoxazole/trimethoprim (14%). All samples were found to be resistant to penicillin and lincomycin and a high prevalence of resistance to augmentin, neomycin and ampicillin was also found. Of all 57 samples 12 were selected on the basis of their resistance profiles and then included in this study. All 12 selected samples (Table 1) were resistant to lincomycin, penicillin (taking into account that *E. coli* is intrinsically resistant to penicillinG/V

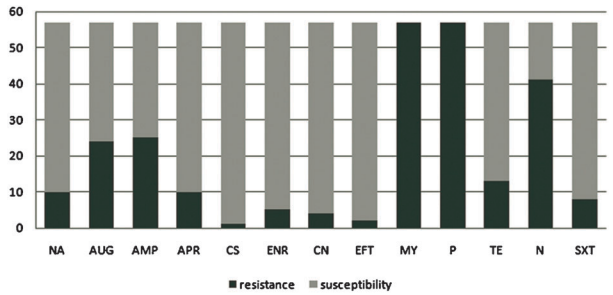

Fig. 1 Number of bacteria resistant to each tested antibiotic, considering 57 isolates. NA: nalidixic acid, AUG: augmentin, AMP: ampicillin, APR: apramycin, CS: colistin sulfate, ENR: enrofloxacine, CN: gentamicin, EFT: ceftiofur, MY: lincomycin, P: penicillin, TE: tetracycline, N: neomycine, SXT: sulfamethoxazole/trimethoprim.

and lincomycin) and N (neomycine); only one sample (no. 1) resulted to be resistant to CS (colistinsulfate). A comparative proteomic analysis was performed considering the number of antibiotics to which bacteria were resistant and grouping samples into multi resistant (MR) and control (C): the MR group includes bacteria resistant to more than five antibiotics, while the C group includes bacteria resistant to a maximum of one antibiotic, excluding P, MY and N because all bacteria were resistant.

2D electrophoresis and protein identification

Quantitative two dimensional electrophoresis was performed on the 12 selected samples with different antibiotic resistance profiles (Table 1). Image analysis highlighted 21 significantly altered spots (p-value ≤ 0.05) under MR *versus* C conditions successfully identified by mass spectrometry as shown in Fig. 2. Spots of interest were excised, destained by repetitive washings and *in situ* digested with trypsin. The resulting peptide mixtures were extracted from the gel and directly analyzed by MALDI-MS. When the identification was uncertain the peptide mixture was analyzed by nano LC/MS/MS generating sequence information on individual peptides. This information together with the peptide mass values was then used to search in protein databases leading to the identification of protein components as reported in Table 2. Identified proteins were both up and down regulated in the MR group and involved in several cellular mechanisms such as metabolism, stress-response, protein folding and trans-membrane transport and are localized either at the

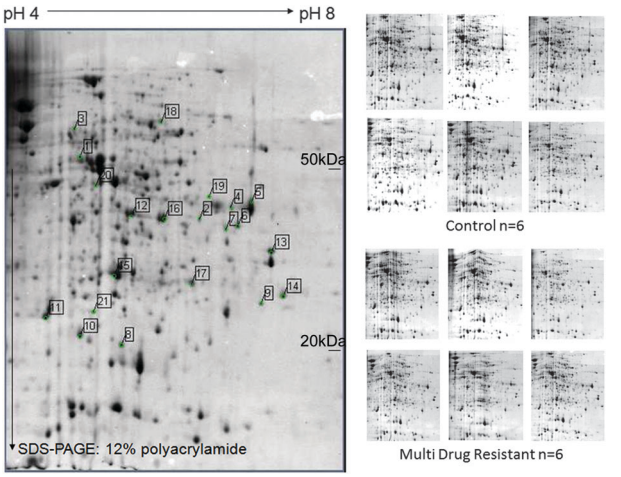

Fig. 2 *E. coli* 2D master map summarizing all differentially expressed spots, pH 4–8, acrylamide 12%; numbered spots were identified by mass spectrometry.

membrane or cytoplasmic level. In Table 3 is indicated in the first column the protein name, in the second column the protein regulation with the percentage of variation in resistant strains (MR: multi resistant) compared to non-resistant (C: control). In the third and in the fourth column, respectively, is reported the biological process where the protein is involved and the cellular localization. Results were investigated and grouped on the basis of protein function. Major differences were found in proteins involved in metabolism and transport at membrane levels as outer membrane protease, thiosulfate binding protein and zinc transport protein (Fig. 3, Tables 2 and 3). Furthermore, curved DNA-binding protein and *S*-ribosylhomocysteinelyase were up regulated under high resistance conditions. DNA-binding proteins are linked in adaptive response to environmental stress,[19] while *S*-ribosylhomocysteinelyase (Fig. 4), also called luxS protein, is involved in quorum sensing mechanisms. FKBP-type peptidyl-prolyl *cis–trans* isomerase 1 fkpa, a periplasmic isomerase involved in protein folding was down regulated. Otherwise, secreted protein hcp (major exported protein, spot 8) was apparently expressed only in the unique sample resistant to CS (Fig. 5).

Metabolic proteins

Regarding energetic metabolism, eight proteins were differentially expressed in the MR *versus* C group; fructose bisphosphate

Table 1 Antibiotic resistance profile related to samples included in this study. 'R' means resistance to the tested antibiotic. On the basis of the resistance/susceptibility test, samples were classified into C and MR groups. C group: samples 1, 2, 8, 10, 11, and 12 (resistant to a maximum of 1 antibiotic, excluding MY, P and N common to all); MR group: 3, 4, 5, 6, 7, and 9

Sample	NA	AUG	AMP	APR	CS	ENR	CN	EFT	MY	P	TE	N	SXT
1					R				R	R		R	
2									R	R		R	
3	R	R	R	R		R	R		R	R	R	R	R
4	R	R	R			R			R	R	R	R	R
5		R	R	R					R	R	R	R	R
6			R						R	R		R	
7	R	R	R	R		R	R	R	R	R	R	R	
8									R	R		R	
9	R	R	R			R	R	R	R	R	R	R	R
10									R	R		R	
11									R	R		R	
12									R	R		R	

Table 2 Protein identification by MALDI/MS and LC/MS/MS analysis. Accession number is linked to www.expasy.org

Spot	Protein name	Trend in MR *vs.* control (%)	Accession no.	MW/Da	Seq. coverage	Mascot score
1	Isocitrate dehydrogenase	↓−16	P08200	45 757	26%	66
2	Outer membrane protease ompP	↑+55	P34210	35 499	30%	99
3	Phosphoglucomutase	↑+80	P36938	58 361	15%	64
4	Terminase, ATPase subunit	↑+19	P25479	66 571	18%	63
5	Glyceraldehyde-3-phosphate dehydrogenase	↑+29	P0A9B4	35 532	54%	124
6	Curved-DNA-binding protein	↑+15	P36659	34 445	23%	65
7	Thiosulfate binding protein	↑+80	P16700	37 615	26%	54
8	Major exported protein	↑+55	Q9HI36	19 091	50%	86
9	3-Oxoacyl-[ACP] reductase	↓−35	P0AEK2	25 560	42%	79
10	*S*-Ribosylhomocysteine lyase	↑+71	Q8FEP8	19 443	43%	66
11	Enoyl-[ACP] reductase	↓−13	P0AEK5	27 864	29%	72
12	Malate dehydrogenase	↑+34	P61889	32 337	42%	151
13	FKBP-Type P–P *cis–trans* isomerase FKPA	↓−10	P65765	28 912	34%	91
14	Glutamine-binding-periplasmic protein	↓−27	P0AEQ5	27 190	58%	110
15	Triosephosphate isomerase	↑+24	P0A858	26 972	45%	145
16	Cysteine synthase A	↑+29	P0ABk6	34 490	56%	156
17	Fumarate reductase iron–sulfur subunit	↓−24	P0AC49	27 123	47%	157
18	Zinc transport protein zntB	↑+80	P64423	36 612	1 peptide	25
19	Fructose bisphosphate aldolase class I	↑+66	P0A992	38 109	1 peptide	66
20	Phosphoserine aminotransferase	↑+36	P23721	39 783	1 peptide	77
21	ATP synthase gamma chain	↓−21	P0ABA6	31 577	1 peptide	25

Table 3 Function, cellular localization and regulation of identified proteins changing their expression in MR *E. coli*

Protein	Trend in MR *vs.* control	Biological process	Cellular localization
Malate dehydrogenase	↑	Tricarboxylic acid cycle	Cytosol
Fructose-bisphosphate aldolase	↑	Glycolysis	Cytosol
Glycera1dehyde-3-phosphate dehydrogenase A	↑	Glycolysis	Cytosol
Triosephosphate isomerase	↑	Glycolysis	Cytosol
Phosphoglucomutase	↑	Glucose metabolism	Cytosol
Isocitrate dehydrogenase[NADP]	↓	Tricarboxylic acid cycle	Cytosol
Fumarate reductase iron–sulfur subunit	↓	Electron transport chain	Cytosol
ATP synthase gamma chain	↓	ATP synthesis	Plasma membrane
Enoyl-[acyl-carrier-protein] reductase [NADH]	↓	Fatty acid biosynthesis	Plasma membrane
3-Oxoacyl-[acyl-carrier-protein] reductase	↓	Fatty acid biosynthesis	Plasma membrane
Curved DNA-binding protein	↑	Molecular chaperon	Cytosol
FKBP-type peptidyl-prolyl *cis–trans* isomerase fkpa	↓	Protein folding	Periplasm
Glutamine-binding periplasmic protein	↓	Amino acid transport	Periplasm
Cysteine synthase A	↑	L-Cysteine biosynthesis	Cytosol
Phosphoserine aminotransferase	↑	L-Serine biosynthesis	Cytosol
S-Ribosylhomocysteine lyase	↑	Synthesis of autoinducer II	Cytosol
Terminase, ATPase subunit	↑	DNA packaging	Cytosol
Outer membrane protease ompP	↑	Receptor	Plasma membrane
Zinc transport protein	↑	Ion transport	Plasma membrane
Thiosulfate binding protein	↑	Ion transport/ABC complex	Periplasm
Major export protein	↑[a]	Secreted	Extracellular region

[a] Expressed only in sample 1, resistant to CS.

aldolase, glyceraldehyde-3-phosphate dehydrogenase and triosephosphateisomerase are glycolytic enzymes, phosphoglucomutase is involved in glucose metabolism, both breakdown and synthesis; malate dehydrogenase is a well known protein which takes part in the Krebs cycle. These proteins were up regulated in high resistant bacteria as shown in Table 3. Other three proteins were found to be down regulated in the MR group: fumarate reductase iron–sulfur subunit, is a part of fumarate reductase, activated during the anaerobic growth of bacteria, isocitrate dehydrogenase involved in the tricarboxylic acid cycle and ATP synthase gamma chain, involved in the production of ATP from ADP in the presence of a proton gradient across the membrane. Furthermore, 3-oxoacyl-[ACP] reductase and enoyl-[ACP] reductase were down regulated in MR; these proteins belong to the short-chain dehydrogenase/reductase family, involved in oxidation–reduction reactions and in particular in fatty acid biosynthesis. Cystein synthase A and phosphoserine aminotransferase are transferase enzymes involved in aminoacid biosynthesis, respectively, cysteine synthesis and serine synthesis. They were upregulated in MR bacteria (Tables 2 and 3).

Membrane and transport proteins

Three membrane proteins, which are responsible for transmembrane transport and alterations of cell permeability, were upregulated in the MR group (Fig. 3). Thiosulfate-binding protein is involved in trans membrane transport of sulfate/thiosulfate at outer membrane levels and zinc transport protein B mediates the efflux of zinc ions at inner membrane levels. Furthermore, outer membrane protease ompP, is a protease, belonging to the A26

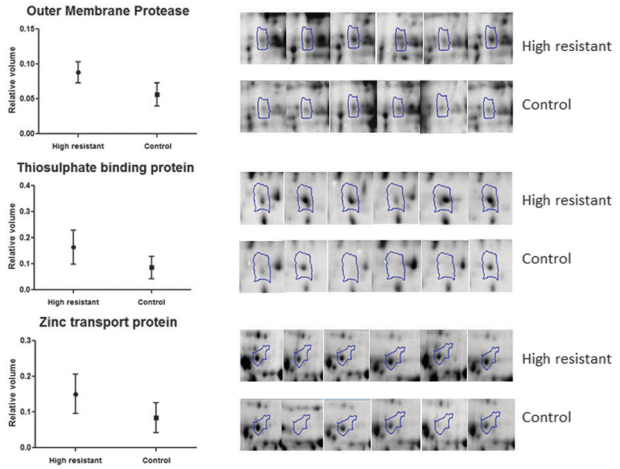

Fig. 3 Differential expression of outer membrane protease (spot 1), thiosulfate binding protein (spot 7) and zinc transport protein B (spot 18). *Y*-axis reports spot normalized volume, *X*-axis reports the expression of proteins in MR and C.

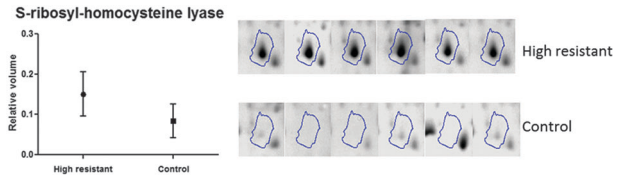

Fig. 4 Up regulation of *S*-ribosylhomocysteine lyase: MR *vs.* C.

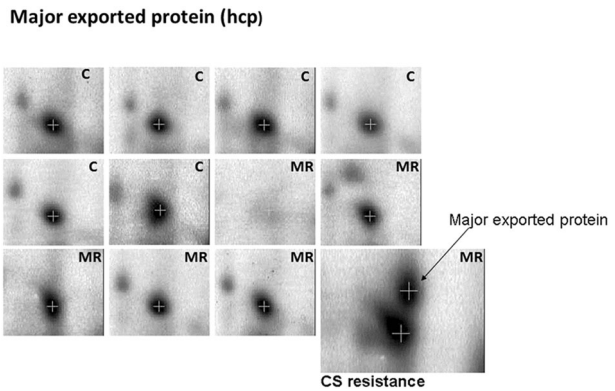

Fig. 5 Differential expression of major exported protein hcp1 in *E. coli* resistant to colistin sulfate.

peptidase family, which acts also as a receptor for bacteriophage Ox2 in the cell outer membrane; it is a trans-membrane protein. Otherwise, glutamine binding protein, involved in the glutamine transport system, belonging to the bacterial solute-binding protein 3 family, was down regulated in the MR group (Table 3).

Discussion

Multi-drug resistance in bacteria has frequently been reported, but information regarding proteomic profile changes is available only for bacteria resistant to a singular antibiotic. In this study 21 differentially expressed proteins were identified, giving new insights on antibiotic resistance in Gram negative bacteria and showing a global view of cell metabolism changes related to this condition.

The relevance of efflux pumps and outer membrane proteins

This study confirms the relevance of membrane and cell wall systems in antibiotic resistance mechanisms, as previously reported.[20,21] Thiosulfate-binding protein, over expressed in MR, belongs to the ABC (ATP binding cassette) transporter complex cysAWTP,[22] and it is involved in sulfate/thiosulfate import. This protein specifically binds thiosulfate allowing its transmembrane transport. The ABC system is responsible for transporting toxic compounds such as drugs, toxins, and detergents. Efflux pumps significantly contribute to bacterial resistance acquisition because of their broad variety of substrates recognized, their cooperation with other mechanisms of resistance and their expression in important pathogens, such as *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella enterica* sv *Typhimurium*.[23] Zinc transport protein zntB has a similar function, mediating efflux of zinc ions at the cell inner membrane level.[24] It is part of the corA metal ion transporter family (MIT). Differential expression of these proteins in MR and C is shown in Fig. 3. Few data are available on a possible role of MIT 1 in oxidative stress response in *E. coli* and *Salmonella enterica*,[25] but the role of ion transporters in drug resistance is well established.[26] Outer membrane protease ompP is a member of a unique family of bacterial endopeptidases known as omptins, which includes OmpT and OmpP of *E. coli*, SopA of *Shigella flexneri*, PgtE of *Salmonella enterica* and Pla of *Yersinia pestis*.[27] The role of outer membrane proteins is the subject of many studies on pathogenesis, virulence and resistance in bacteria; most of the omptin proteins have been implicated in bacterial pathogenesis and they are potential targets of antimicrobial drugs and vaccine development.[27]

Quorum sensing and antibiotic resistance

Interestingly, *S*-ribosylhomocysteinelyase, an up-regulated protein in antibiotic resistant bacteria (Fig. 4), is involved in the synthesis of autoinducers 2 (AI-2) which are small molecules (acylated homoserine lactone) secreted by bacteria and used to communicate both cell density and metabolic potential of the environment.[14] This regulation mechanism, called quorum sensing, plays an important role in many cellular mechanisms, such as expression of bioluminescence in marine bacteria, virulence, pathogenesis and biofilm formation (Fig. 6).[28,29] Furthermore, a possible role of quorum sensing in the regulation of multidrug efflux pumps has been described,[12] but there is no evidence regarding its possible implication in antibiotic resistance. The over expression of *S*-ribosylhomocysteinelyase in antibiotic resistance enforces previous findings which indicate quorum sensing as global regulatory systems in *E. coli*,[29] and enhances the existing knowledge suggesting its possible role in multi-drug resistance. Such a role would also be related to the formation of biofilm, which, as previously described,[16] can be involved in drug resistance.

Metabolism changes overview

This study gave a global overview of metabolism-related changes which can occur in high drug-resistant cells. In particular, most

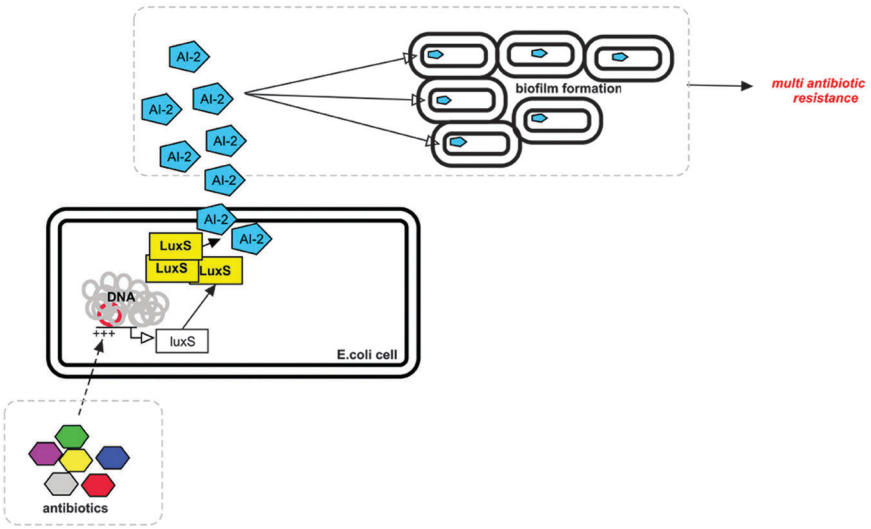

Fig. 6 *S*-Ribosylhomocysteine lyase (LuxS) involvement in quorum sensing and biofilm formation. LuxS protein (called in the text *S*-ribosyl homocysteine lyase) is involved in the synthesis of Autoinducers II that are small molecules that can be recognized by other *E. coli* cells and can promote expression of genes involved in biofilm formation.

dramatic changes are related to energetic metabolism and glycolysis, suggesting an increase of sugar metabolism, probably due to an increased need in ATP production, which is fundamental for active membrane transport systems. An ATP synthase gamma chain, involved in production of ATP from ADP in the presence of a proton gradient across the membrane, is down regulated in MR bacteria (Tables 2 and 3) and isocitrate dehydrogenase and fumarate reductase iron–sulfur subunit were down regulated: they are involved in an alternative route to glycolysis for carbon metabolism and anaerobic cellular growth. Enoyl-[acyl-carrier-protein] reductase and 3-oxoacyl-[acyl-carrier-protein] reductase are enzymes involved in lipid biosynthesis: they are down regulated in antibiotic resistant bacteria indicating a possible decrease of lipid metabolism in response to an increased sugar metabolism.

Regarding amino acid metabolism, cysteine synthase A and phosphoserine aminotransferase were up regulated in MR *vs.* C: this indicates a possible regulation also for amino acid biosynthesis. Currently, there is no reported evidence regarding such findings, but these two enzymes are co-regulated, because cysteine is synthesized from serine.

Colistin sulfate resistance

As shown in Fig. 5, secreted protein hcp (spot 8) was found only in the colistin sulfate resistant sample; this protein could be present in other gels, but under the limit of detection of the staining method used. Secreted protein hcp is one of the hemolysis co-regulated proteins (hcp1 family), described in *Pseudomonas aeruginosa* which is involved in pathogenesis and virulence associated to cystic fibrosis.[30] As reported, secreted proteins often represent important virulence factors and, interestingly, their regulation is associated to quorum sensing.[31]

Conclusions

The aim of this work was to give new suggestion to individuate molecular targets for anti-microbial drugs, using 2-dimensional electrophoresis and mass spectrometry. Results obtained with comparative proteomics gave an overview of metabolism changes and the importance of membrane outer proteins and membrane active efflux pumps in multi resistant bacteria. Moreover, this study gave new insights regarding molecular mechanisms related to antibiotic resistance suggesting a possible role of quorum sensing as a central regulatory mechanism of gene expression in multi drug resistance.

Moreover our findings improve the understanding of functional regulation of antibiotic resistance in *E. coli* and give new interesting suggestions like the involvement of quorum sensing in antibiotic resistance, a theory that was previously hypothesized, but never supported by experimental data. The strong up regulation of *S*-ribosylhomocysteinelyase, involved in quorum-sensing mechanisms, could be suggested as a possible target mechanism for developing new generation antibiotic drugs.

Acknowledgements

This work was supported by a Ministry of Health grant, W.U. Prof. Luigi Bonizzi and Proteotech srl ICT SIAI101 RES.CO.

Notes and references

1 H. Li, X. M. Lin, S. Y. Wang and X. X. Peng, *J. Proteome Res.*, 2007, **6**, 3628–3636.
2 A. C. Berge, D. D. Hancock, W. M. Sischo and T. E. Besser, *J. Am. Vet. Med. Assoc.*, 2010, **236**, 1338–1344.
3 S. E. Jones, J. M. Burgos, M. M. Lutnesky, J. A. Sena, S. Kumar, L. M. Jones and M. F. Varela, *Curr. Microbiol.*, 2011, **62**, 1139–1146.
4 K. A. Karatzas, L. P. Randall, M. Webber, L. J. Piddock, T. J. Humphrey, M. J. Woodward and N. G. Coldham, *Appl. Environ. Microbiol.*, 2008, **74**, 1508–1516.
5 M. Karmali, *Clin. Microbiol. Rev.*, 1989, **2**, 15.
6 A. Mora, J. Blanco, M. Blanco, M. Alonso, G. Dhabi, A. Echeita, E. González, M. Bernárdez and J. Blanco, *Res. Microbiol.*, 2005, **156**, 793–806.
7 M. E. de Kraker, M. Wolkewitz, P. G. Davey, W. Koller, J. Berger, J. Nagler, C. Icket, S. Kalenic, J. Horvatic, H. Seifert, A. Kaasch, O. Paniara, A. Argyropoulou, M. Bompola, E. Smyth,

M. Skally, A. Raglio, U. Dumpis, A. Melbarde Kelmere, M. Borg, D. Xuereb, M. C. Ghita, M. Noble, J. Kolman, S. Grabljevec, D. Turner, L. Lansbury and H. Grundmann, *J. Antimicrob. Chemother*., 2011, **66**, 398–407.
8 H. Li, B. C. Wang, W. J. Xu, X. M. Lin and X. X. Peng, *J. Proteome Res*., 2008, **7**, 4040–4049.
9 X. M. Lin, H. Li, C. Wang and X. X. Peng, *J. Proteome Res*., 2008, **7**, 2399–2405.
10 X. M. Lin, J. N. Yang, X. Peng and H. Li, *J. Proteome Res*., 2010, **9**, 5952–5959.
11 D. F. Zhang, B. Jiang, Z. M. Xiang and S. Y. Wang, *Int. J. Antimicrob. Agents*, 2008, **32**, 315–319.
12 S. Rahmati, S. Yang, A. L. Davidson and E. L. Zechiedrich, *Mol. Microbiol*., 2002, **43**, 677–685.
13 S. Schauder, K. Shokat, M. G. Surette and B. L. Bassler, *Mol. Microbiol*., 2001, **41**, 463–476.
14 B. M. Ahmer, *Mol. Microbiol*., 2004, **52**, 933–945.
15 S. K. Anand and M. W. Griffiths, *Int. J. Food Microbiol*., 2003, **85**, 1–9.
16 A. Ito, A. Taniuchi, T. May, K. Kawata and S. Okabe, *Appl. Environ. Microbiol*., 2009, **75**, 4093–4100.
17 S. Arioli, P. Roncada, A. Salzano, F. Deriu, S. Corona, S. Guglielmetti, L. Bonizzi, A. Scaloni and D. Mora, *Microbiology*, 2009, **155**, 1953.
18 R. Mastro and M. Hall, *Anal. Biochem*., 1999, **273**, 313–315.
19 C. Ueguchi, T. Shiozawa, M. Kakeda, H. Yamada and T. Mizuno, *J. Bacteriol*., 1995, **177**, 3894.
20 X. Z. Li and H. Nikaido, *Drugs*, 2009, **69**, 1555–1623.
21 X. Z. Li and H. Nikaido, *Drugs*, 2004, **64**, 159–204.
22 A. Sirko, M. Zatyka, E. Sadowy and D. Hulanicka, *J. Bacteriol*., 1995, **177**, 4134–4136.
23 F. Van Bambeke, E. Balzi and P. Tulkens, *Biochem. Pharmacol*., 2000, **60**, 457–470.
24 D. Ciavardelli, S. Ammendola, M. Ronci, A. Consalvo, V. Marzano, M. Lipoma, P. Sacchetta, G. Federici, C. Di Ilio and A. Battistoni, *Mol. BioSyst*., 2011, **7**, 608–619.
25 J. Sermon, E. Wevers, L. Jansen, P. De Spiegeleer, K. Vanoirbeek, A. Aertsen and C. Michiels, *Appl. Environ. Microbiol*., 2005, **71**, 6515.
26 M. A. Seeger, K. Diederichs, T. Eicher, L. Brandstatter, A. Schiefner, F. Verrey and K. M. Pos, *Curr. Drug Targets*, 2008, **9**, 729–749.
27 V. Hritonenko and C. Stathopoulos, *Mol. Membr. Biol*., 2007, **24**, 395–406.
28 S. Schauder, K. Shokat, M. Surette and B. Bassler, *Mol. Microbiol*., 2001, **41**, 463–476.
29 V. Sperandio, A. Torres, J. Giron and J. Kaper, *J. Bacteriol*., 2001, **183**, 5187.
30 C. Stover, X. Pham, A. Erwin, S. Mizoguchi, P. Warrener, M. Hickey, F. Brinkman, W. Hufnagle, D. Kowalik and M. Lagrou, *Nature*, 2000, **406**, 959–964.
31 C. Arevalo-Ferro, M. Hentzer, G. Reil, A. Gorg, S. Kjelleberg, M. Givskov, K. Riedel and L. Eberl, *Environ. Microbiol*., 2003, **5**, 1350–1369.