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ORIGINAL ARTICLE

Proteomic response of *Escherichia coli* to the alkaloid extract of *Papaver polychaetum*

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Abstract The cellular response of *Escherichia coli* exposed to alkaloids extracted from a biennial endemic plant, *Papaver polychaetum*, was explored using proteome analysis. Following determination of the minimum inhibitory concentration of the berberine-containing plant extract as 1,250 μg/mL, *E. coli* cells were grown in the presence of 750 μg/mL extract. The response of the bacteria to the extract, with berberine found as the major alkaloid, was analyzed on two-dimensional gels. The differentially expressed proteins in the presence of 750 μg/mL extract were identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry. These proteins included those that play vital roles for maintenance such as protein synthesis (elongation factor-Ts), transport (oligopeptide-binding protein A, uncharacterized amino-acid ABC transporter ATP binding protein YECC), energy metabolism (alpha-subunit of ATP synthase, pyridine nucleotide transhydrogenase STHA) and regulation. These results provide clues for understanding the mechanism of the alkaloid extract-induced stress and cytotoxicity on *E. coli*. The altered proteins can serve as potential targets for development of innovative therapeutic agents.

Keywords Proteomics · Antimicrobial · *Escherichia coli* · *Papaver polychaetum* · Berberine

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Introduction

Widespread bacterial drug resistance to current cheap and effective first-choice drugs raises the number of untreatable bacterial infections and adds urgency to the search for new infection-fighting strategies (World Health Organization 2002; Vlieghe et al. 2009). In addition to careful use of existing antimicrobials and better hygiene conditions, development of novel therapeuticals is the key to fighting the remarkable adaptability of microorganisms. The challenge in the search for new antimicrobial classes lies in the timely knowledge of the molecular mechanism of action of the drug and the related bacterial response (Bandow et al. 2003).

Use of plants for medicinal purposes has been practised for many centuries (Summer 2000; Hanson 2005; van Wyk and Wink 2005). Due to their unmatched availability of chemical diversity, plant extracts, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drugs to counter multi-resistant microorganisms (Cos et al. 2006). *Papaver polychaetum*, belonging to the plant kingdom Papaveraceae, is a biennial endemic species (southern Turkey) possessing antimicrobial activity (Ünsal et al. 2007, 2009). The alkaloid found in *P. polychaetum* is berberine (Sariyar 2002) that is commonly used for various

medicinal purposes and is effective against a broad range of bacteria, protozoa, fungi and viruses. Evidence from piecewise descriptions of individual interactions indicated that the effect of berberine is on nucleic acids since it intercalates into DNA molecules (Burbaum and Tobal 2002). However, if evaluated by the global analysis of changes in the protein expression profiles, the cellular response to either pure berberine or berberine in an extract will be expected to be of different complexities.

Proteins constitute the vast majority of drug targets against which pharmaceutical drug design processes are initiated. In this respect, proteomics is gaining widespread use in drug discovery and drug development programs allowing the dynamic study of interrelationships between proteins in microbial systems following drug treatment. This, in turn, contributes important insight for understanding the mechanistic basis for drug action (Yoshida et al. 2001). There are a couple of reports concerning the antimicrobial effects of berberine, but we are unaware of any published studies on proteomic analysis of bacteria exposed to berberine or berberine containing plant extracts.

In this study, *Escherichia coli* served as a model to investigate the physiological changes caused by *P. polychaetum* alkaloid extract. *E. coli* is the most widely studied bacterium in the world, and has an extreme importance as a model organism in many research fields due to its rapid growth rate and simple nutritional requirements (Ingledew and Poole 1984). In addition to these, in contrast to many other organisms, most of its gene products have been functionally assigned making it ideal for proteomic studies (Nandi et al. 2004; Herrmann and Ruppert 2006). The method used in this work will facilitate the identification of target proteins involved in key biological processes in *E. coli* that may serve as potential drug targets.

Materials and methods

Bacterial strain and growth conditions

Escherichia coli ATCC 29425 was routinely cultured and maintained in Luria-Bertani (LB) broth (per liter: 10 g tryptone, 5 g yeast extract, 10 g NaCl) or on LB agar plates (Cho et al. 2007) at 37°C for 18 h.

Plant material, extraction and analysis of the alkaloids

Papaver polychaetum was collected from İçel-Arslanköy (southern part of Turkey) in August 2005 (altitude 2,100 m). Voucher specimens were identified by N. Sadıkoğlu and are deposited in the Herbarium of the Faculty of Pharmacy, Istanbul University (ISTE 83225). Aerial parts of *P. polychaetum* (300 g) were extracted with MeOH and the extract was concentrated under reduced pressure. The MeOH extract was partitioned between $CHCl_3$-H_2O (1:1) to afford a $CHCl_3$-soluble fraction (Fr. A, 1,383 mg) and dried over anhydrous Na_2SO_4 (Chen et al. 1999). TLC examination of Fr. A in different solvent systems revealed the presence of only one alkaloid. Next, 60 mg of Fr. A were separated on preparative TLC yielding 28 mg of alkaloid. The structure of this alkaloid was identified as berberine by comparing its physical and spectral data and TLC R_f values with an authentic sample.

Stress treatment with the alkaloid extract and survival test

Minimum inhibitory concentration (MIC) determination

Minimum inhibitory concentration (MIC) was defined as the lowest concentration of *P. polychaetum* alkaloid extract which completely inhibited *E. coli* growth at 37°C after incubation for 18–24 h (Amsterdam 1996). The broth dilution method (microdilution) was used to determine MIC. Serial two-fold dilutions of the plant extract and berberine dissolved in DMSO were prepared in sterile 96-well U-bottomed, microtiter plates; 50 μL of the cell culture (10^5/mL) was also deposited in each well. MIC was evaluated with reference to control cells, mixed with DMSO or fresh growth media.

Cell culture conditions

Escherichia coli cells (50 μL), grown to 0.7 at OD 600 nm were used to inoculate 50 mL of fresh media in 250-mL erlenmeyer flasks. Cells were incubated at 37°C and 180 rpm. Final concentration of the extract or berberine in growth media was 750 μg/mL. In addition to the control group, culture with DMSO supplement was maintained to evaluate the differences caused by the solvent. To keep growth volume constant, water was added to the control culture. Cell growth was monitored spectrophotometrically at OD 600 nm.

Viable cell count was determined by plating samples of liquid cultures.

Sample preparation: protein extraction

Following 18 h of incubation, cells were collected by centrifugation at 4°C and 6,000 rpm for 20 min. Supernatants were discarded and the cells were washed twice with 50 mM Tris buffer (pH 7.8). ProteoPrep® Sample Extraction Kit (SIGMA PROT-TOT) was used for the extraction of cellular proteins using the protocol provided by the manufacturer. Based on cell yield, 150–250 mg of cell pellets were resuspended in 1.5 ml of extraction buffer and incubated for 15 min at room temperature. Liquid nitrogen was used for multiple cycles of freeze–thaw for cell lysis without oxidation. Suspensions containing whole cell proteins were centrifuged at 15,000 *g* for 10 min at 15°C. Supernatants containing the cellular proteins were decanted

into clean tubes and tributylphospine was added to a final concentration of 5 mM as a reducing agent. The mixture was incubated 1 h at room temperature. Proteins were alkylated with 15 mM iodoacetamide for 1.5 h at room temperature. Protein samples were centrifuged at 20,000 *g* for 5 min at room temperature to prevent insoluble material contamination and the supernatants were aliquoted and stored at −80°C for further applications.

Protein concentration was estimated by the Bradford protein assay (Bradford 1976).

Two-dimensional gel electrophoresis

Non-equilibrium pH gradient electrophoresis (NEpHGE) technique (Klose and Kobalz 1985) has been used for the two-dimensional separation of bacterial proteins. Samples were separated in the first dimension on capillary rods using polyacrylamide gels containing 9 M urea, 3.5% acrylamide, 0.3% piperazine diacrylamide and 4% ampholyte mixture (pH 2–11). Next, 300 and 60 μg protein-containing samples were loaded onto the anodic side of the tubular gels for colloidal coomasie brilliant blue (CBB)-G250 and silver staining, respectively. Optimized running voltages were 100 V, 60 min; 200 V, 60 min; 400 V, 990 min; 600 V, 60 min; and 1,000 V, 30 min. At the end of each run, gels were incubated 10 min at room temperature in 1% DTT solution. The second dimensional separation was performed using 12% acrylamide gels (Laemmli 1970) at 120 mA for 15 min and 150 mA for 135 min. Protein spots were visualized either with silver or colloidal CBB G-250 staining prior to mass spectrometric analysis. Stained gels were scanned and the ProgenesisSameSpots software (free trial version) was used to detect and match spots.

In-gel tryptic digestion of proteins in CBB-G250 stained gels

The entire gel slab was rinsed with HPLC grade water and the spots of interest were excised with a clean scalpel. For destaining, these spots were transferred to clean tubes and incubated for 30 min in 100 μL 1:1 (v/v) 100 mM ammonium bicarbonate/acetonitrile mixture with occasional vortexing. Gel pieces were shrunk by dehydration in acetonitrile. Dried gel pieces were then swollen with sufficient trypsin containing buffer (6 ng/μL trypsin in 50 mM ammonnium carbonate) for 30 min on ice. Gels were further incubated at 37°C overnight to have sufficient peptide recovery (Shevchenko et al. 2007).

Mass spectroscopy and databank searching

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) analysis was performed with slight modifications of the protocol described by Hooven and Baird (2008). Peptides formed by tryptic cleavage reactions were mixed with 1 μl saturated alpha-cyano-4 hydroxycinnamic acid matrix solution (0.5 mg/mL alpha-cyano-4 hydroxycinnamic acid diluted with 0.1% trifluoroacetic acid in 49.5% ethanol and 49.5% acetonitrile), and 1 μL of the resulting peptide–matrix mixture was spotted onto the MALDI target plate. Alcohol dehydrogenase was used as the calibration standard and Glu-fibrinopeptide was used as the standard. Mass spectrometric analysis of the peptides was performed on a MALDI-time-of-flight (TOF) micro LR (Waters, Manchester, UK), equipped with a pulsed nitrogen laser (k=337 nm). The instrument operated in positive ion reflectron mode with the source voltage set to 15,000 V. The pulse voltage was optimized at 3,250 V, the detector and reflectron voltages were set to 1,850 and 500 V, respectively. Measurements were performed in the mass range m/z 500–3,000 with a suppression mass gate set to m/z 500 to prevent detector saturation from matrix cluster peaks and an extraction delay of 600 ns. All spectra were processed and analyzed using the MassLynx 4.0 software (Waters, Milford, MA, USA). MASCOT search engine (Matrix Science, London, UK, http:www.matrixscience.com) was employed to assign monoisotopic peptide masses. Peptide mass tolerance was set at 1 Da. Maximum number of missed cleavages was set to 1 with trypsin as the protease. Taxonomy to be searched was selected as *E. coli* to increase the specificity of the results.

Statistical analysis

Experiments were performed at least five times. All data were plotted as the mean ± standard deviations, and statistically significant differences were determined using the *t* test (MS-office Excel). The gels have been compared using the tools in Progenensis software. Following scanning, the images have been automatically aligned and samespots have been detected with background subtraction, normalization and matching. The lists of spots statistically ordered by *p* value from the one-way ANOVA analysis were then viewed. By going through the spot rank table, top ranked spots have been accepted for further study.

Results

Survival of *E. coli* under the alkaloid extract stress

Initial efforts involved the minimization of the amount of toxic solvent DMSO. Consequently, concentration of the extract and berberine in the stock solution was fixed to 9.0 mg/mL.

MIC of *P. polychaetum* alkaloid extract was found as 1,250 μg/mL for *E. coli*. For the same cells, the MIC value

obtained with pure berberine was identical. This was not unexpected since the only alkaloid found in *P. polychaetum* was berberine. Taking this value as the upper limit, concentration of *P. polychaetum* extract was adjusted to 750 μg/mL to investigate the alterations the extract enforces on *E. coli*. The results were evaluated with reference to control cells, also following the changes caused by DMSO.

Figure 1 revealed that DMSO significantly retarded cell growth and reduced growth rate. Toxic effects of the extract and berberine became apparent only after 2 h of growth.

Lag phase was clearly longer for the drug-treated cells. Nevertheless, all *E. coli* cultures grown in the presence of DMSO (w/o drug) entered stationary phase at around the 10th hour of growth. Growth period in the presence of the drugs was shorter. The OD 600 nm was as low as 1.0 for the drug-treated cells whereas it was 2.2 for the cells grown under the effect of DMSO only.

Further analysis was performed to correlate optical density measurements to cell viability. Number of colony forming units in cultures after 11, 14 and 16 h of growth have been plotted in Fig. 2.

During the course of growth between 11 and 16 h, the viable cell number remained relatively constant versus the control and the DMSO supplemented cultures. In contrast, the presence of the extract caused a sharp reduction in cell viability after 14 h of growth.

Effect of plant alkaloid extract on global protein expression profiles of *E. coli*

Comparative proteomic analysis has been used to identify target-related proteins which will lead to the elucidation of underlying mechanisms of cell death upon exposure to *P. polychaetum* alkaloid extract. Differences were considered as significant when they were due to drug treatment but not DMSO. Approximately 600 protein spots were detected on the 2-DE gel with isoelectric focusing from pH 4.5 through 8.0. Representative 2-D analytical gel images are shown in Fig. 3.

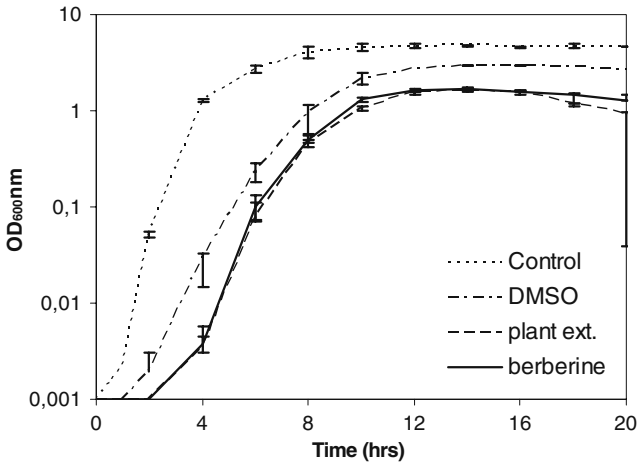

Fig. 1 Growth profiles of *E. coli* in the presence of DMSO, berberine and, alkaloid extract with reference to control culture

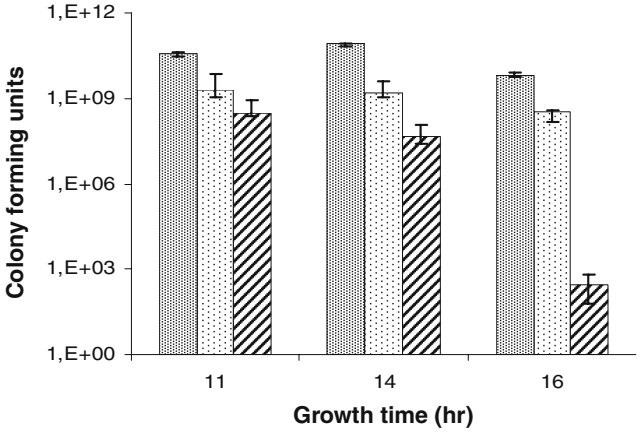

Fig. 2 Number of viable cells; control (*vertical shading*), DMSO supplemented (*dotted shading*), and alkaloid extract (*diagonal shading*) supplemented

The images from *P. polychaetum* alkaloid extract and berberine-exposed cells were almost identical, showing that the effect of the antimicrobial alkaloid extract and berberine were very similar and that the trace amounts of organic acids, phenolic compounds or pigments remained in the extract had no significant effect. Silver-stained gel images were analyzed to find a total of 28 differentially expressed proteins due to drug treatment.

Spots that exhibited significant increase or decrease in abundance due to drug treatment, but not DMSO, have been indicated in magnified views of control and extract treated cultures on Fig. 4.

Identification of differentially expressed proteins

Peptide mass fingerprinting using MALDI-TOF was performed to identify the proteins that demonstrated altered expression in 2-DE. Peptide mass fingerprints (PMFs) were obtained for the selected protein spots and all PMFs were searched with MASCOT software in Swissprot database for identification. The result has high confidence if the protein was ranked at the best hit with a significant score and high sequence coverage.

Finally of the 28 differentially expressed protein spots, 10 of them showed >2-fold increase and, out of these 10 spots, for 9 of them significant matches were obtained from the protein database. Results of the selected proteins are presented in Table 1.

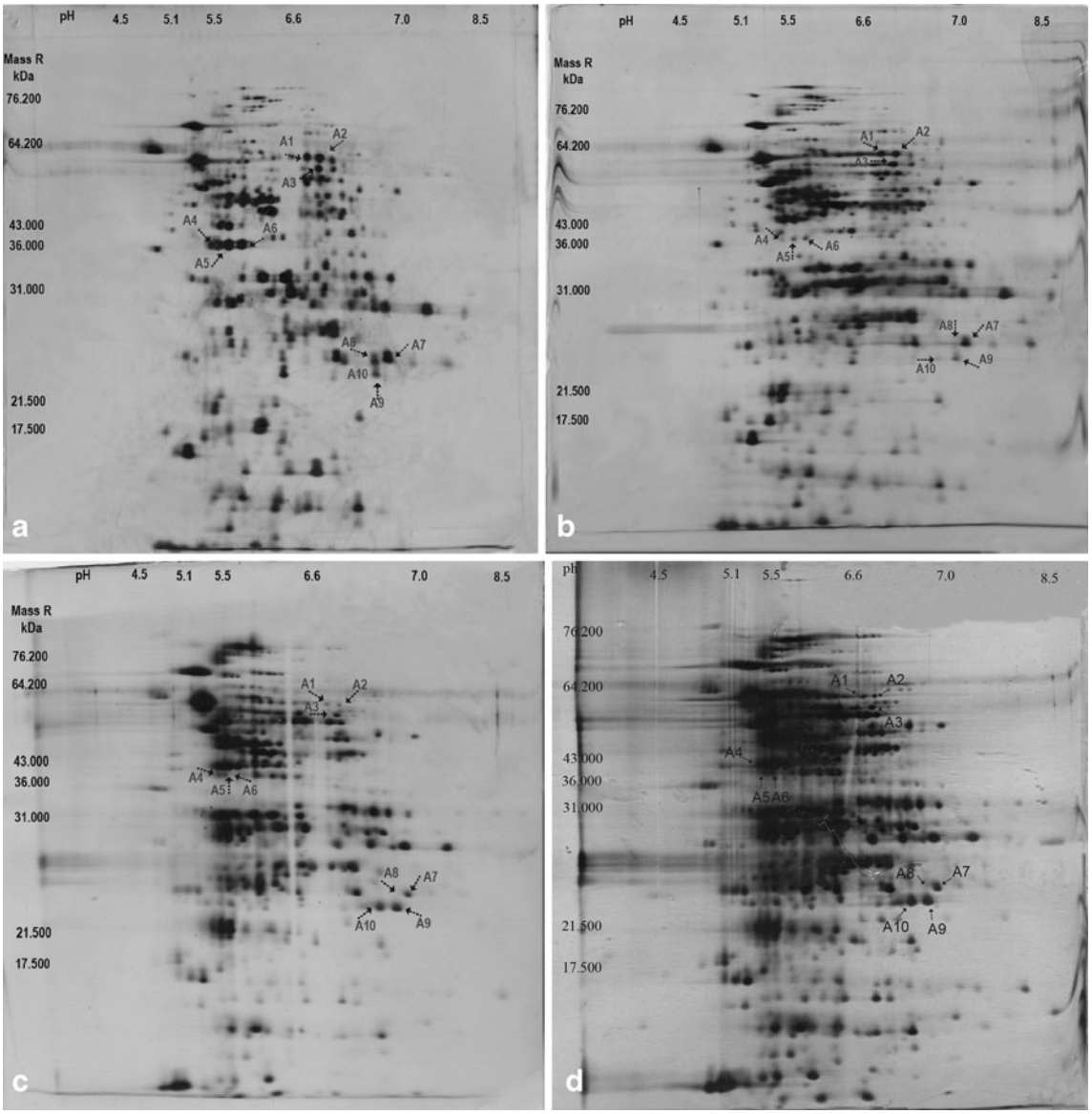

Fig. 3 Proteome maps of cultures in the presence of DMSO (**b**), 750 μg/mL plant extract (**c**), and 750 μg/mL berberine (**d**) with reference to control culture (**a**). The images are representative of five replicate gels

Table 1 shows MALDI-TOF MS analysis of the proteins. The accession number, theoretical and predicted molecular weight and pI, sequence coverage and score of each protein spot are given.

Discussion

For decades, plants of the genus *Papaver* have known to accumulate a rich spectrum of different alkaloids (Preininger 1986; Sariyar 2002; Ziegler et al. 2006). These plants have been regarded as important sources of narcotic alkaloids, such as morphine, codeine and thebaine (Ziegler et al. 2006; Salehi et al. 2007). More recent studies have shown that *Papaver* species also process significant antimicrobial activities (Ünsal et al. 2007, 2009). The antimicrobial activity of the alkaloid berberine from *P. polychaetum* has been attributed to its specificity for the minor groove of AT-rich duplexes in DNA sequences (Saran et al. 1995; Choi et al. 2001; Sriwilaijareon et al. 2002; Mazzini et al. 2003; Chen et al. 2004; Qin et al. 2006). In addition to this information, Sun et al. (1988) reported that berberine chloride blocked adhesion by reducing the synthesis of fimbrial subunits and the expression of assembled fimbriae (1988). This could be associated with the antimicrobial property of berberine since adhesion to the tissue surface is the first step for bacteria to establish infection. Unfortunately, they observed that *E. coli* growth was reduced by

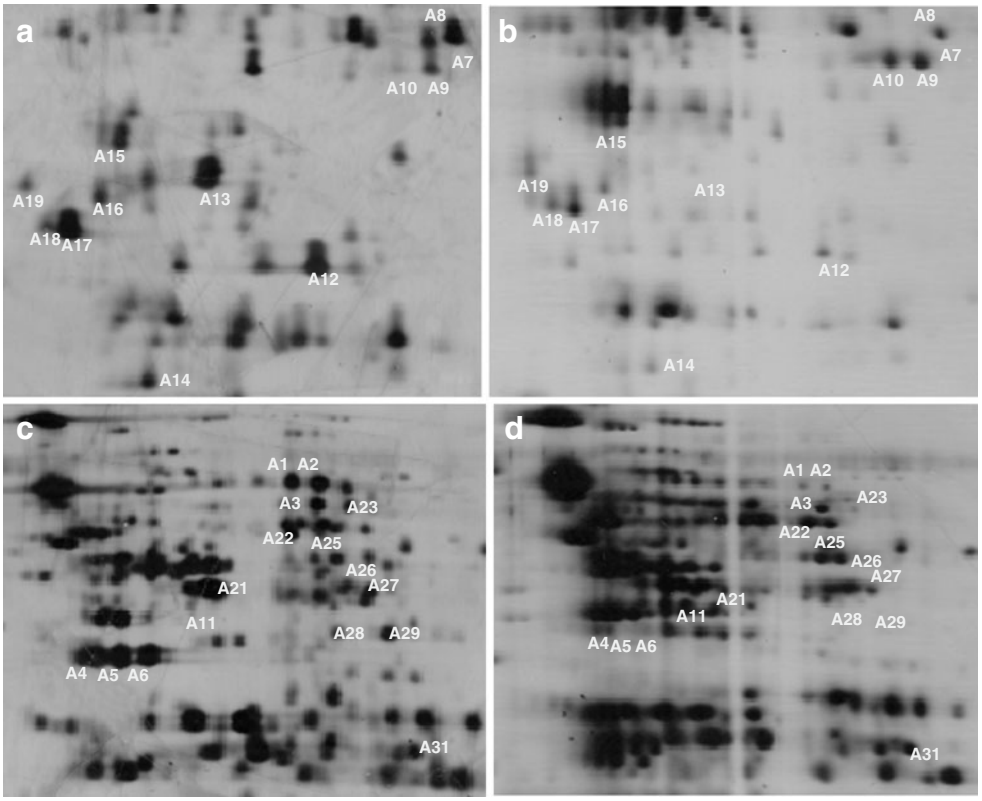

Fig. 4 Magnified views of the proteome map of the plant extract treated culture (**a**,**c**) with reference to culture control (**b**,**d**)

only 10% in the presence of 300 μg/mL berberine chloride, although 90% of its ability to adhere was lost (Wang et al. 2008). Stermitz et al. (2000) reported that berberine is pumped out by bacterial multi-drug resistance (MDR) pumps. However, it could be as effective as other antimicrobial agents against *E. coli* when administered together with an MDR inhibitor (Stermitz et al. 2000). Since information for this antimicrobial alkaloid was

Table 1 Identification of differentially expressed protein spots in the presence of *P. polychaetum* alkaloid extract

Spot no./protein description			Accession number	Sequence coverage (%)	Protein score	Theoretical molecular mass (kDa)/pI	Experimental molecular mass (kDa)/pI
Transport and binding							
Amino acid/peptide							
1	OPPA_ECOLI	↓	P23843	22	47	60.9/6.05	66/6.3
10	YECC_ECOLI	↑	P37774	35	43	27.7/8.89	25/6.6
Sugar							
6	MALE_ECOLI	↓	P02928	22	66	43.4/5.53	40/5.6
Membrane repair and maintenance							
9	BLC_ECOLI	↑	P39281	24	31	19.8/8.81	22/6.6
Protein synthesis							
4	EFTS_ECO24	↓	P02997	28	54	30.5/5.22	34/5.3
Energy metabolism							
3	ATPA_ECOLI	↓	P00822	30	103	55.2/5.80	55/5.3
2	STHA_ECO24	↓	P27306	21	41	51.6/6.09	66/6.4
Regulation							
DNA synthesis							
8	FRMR_ECO24	↓	A7ZIA5	29	37	10.3/5.84	20/6.6
Replication							
5	INTD_ECOLI	↓	P24218	16	30	45.1/9.76	36/5.6

limited, an exhaustive study with the alkaloid extract of *P. polychaetum* was conducted for the comparative analysis of cells exhibiting diverse phenotypes under drug stress.

Survival of *E. coli* exposed to *P. polychaetum* alkaloid extract

Cell growth in the presence of the extract indicated that the extract or berberine can be considered to be an antimicrobial agent against *E. coli* only when available at higher concentrations than the agents generally regarded as antimicrobials. MIC is usually reported to be in the order of <50 μg/mL range for effective antimicrobials.

Due to the operation of MDR pumps, this value is as high as 1,250 μg/mL for the extract. The high MIC value has not hampered the progress of this study since the motive was to identify the modifications in the levels of proteins to correlate varied protein abundances with the drug action mechanism. This information would be valuable for the development of new therapeuticals.

Analysis of 2-DE protein profiles

The expression of the seven proteins identified decreased markedly or they were not expressed at all and the expression of two proteins was induced under the influence of the plant extract. These proteins were involved in transport and binding, membrane repair and maintenance, proteins synthesis, energy metabolism, regulation and replication.

Among the transport and binding proteins, a component of the oligopeptide permease periplasmic oligopeptide-binding protein OPPA was down-regulated. Besides its function as a carrier for peptides up to five amino acids long, there is evidence that OPPA may act as a carrier for aminoglycoside antibiotics (Acosta et al. 2000). Complete disappearance of OPPA under *P. polychaetum* alkaloid extract stress may indicate that OPPA could act as a carrier for berberine as its structure has a resemblance to amino acids and aminoglycosides. In contrast to OPPA, the expression of the uncharacterized amino-acid ABC transporter ATP-binding protein YECC was induced. This protein is located in the cell inner membrane and belongs to the ABC transporter superfamily. It is probably part of a binding protein-dependent transport system yecCS for an amino acid, responsible for energy coupling to the transport system (Blattner et al. 1997). The repression of the periplasmic oligopeptide permease may be compensated by induction of this system for amino acid uptake.

Another protein belonging to the transport and binding family, maltose-binding protein MalE, was down-regulated. This is located in the periplasm and is involved in the high-affinity maltose membrane transport system malEFGK (Duplay et al. 1984).

Upon exposure to *P. polychaetum* alkaloid extract, proteins from energy metabolism, soluble pyridine nucleotide transhydrogenase STHA and alpha-subunit of ATP synthase ATPA, were down-regulated. STHA is proposed to be localized in the cytoplasmic space and involved in the conversion of NADPH to NADH. The down-regulation of STHA may be an indication of the repression in the respiratory activities of the cells under stress. The alpha subunit of ATP synthase is a peripheral membrane protein found integrated to the membrane. The ATP synthase complex uses the proton gradient across the membrane to drive ATP synthesis from ADP and inorganic phosphate. Under fermentative conditions, it energizes the inner membrane by catalyzing the extrusion of protons at the expense of ATP hydrolysis (Futai and Kanazawa 1983). The alpha-subunit has an essential role in the catalytic mechanism of the complex. *P. polychaetum* extract represses AtpA expression which in turn results in altered levels of free energy transduction.

Energy limitation caused by the repression of these two enzymes may be coupled to the disappeance of MalE. During energy crisis, carbon import via periplasmic binding proteins is essential for *E. coli* cells (Wang and Crowley 2005). Easton et al. (2006) suggested that use of low energy-requiring transports for carbon uptake may in turn result in the rapid consumption of such transporters (Ayudhya et al. 2009). Hence, energy limitation in the presence of the extract could eventually cause consumption of MalE.

Papaver polycaetum alkaloid extract affected the protein biosynthesis machinery by repressing the cytoplasmic elongation factor Ts, EFTS. Elongation factors interact with ribosomes and catalyze formation of the acyl bond between the incoming amino acid residue and the peptide chain to extend the nascent polypeptide chain during the elongation stage of bacterial translation (Alberts et al. 2002; Jayasekera et al. 2004). Because of its essential functions, EFTs has been regarded as a possible drug target (Jayasekera et al. 2004).

The stress of the plant extract induced the expression of the outer membrane lipoprotein, lipocalin (Blc). Normally, expression of the blc gene starts at the beginning of stationary phase and serves as a starvation response function in *E. coli*. Structural analyses of the purified Blc protein suggest a possible role in membrane repair or maintenance requiring lipid storage or transport (Valerie et al. 2004) and in phospholipid binding (Bishop 2000). Since berberine reduces the expression of fimbrial subunits in *E. coli*, as reported by Sun et al. (1988), the induction of Blc may be a predictable consequence based on its membrane repairing role. Some experiments suggest that bacterial lipocalins may play a significant role in resistance to antibiotics (Bishop 2000). Therefore, induction of Blc can also be correlated with the strong resistance to the extract.

Exposure to the plant extract repressed the expression of two other proteins, prophage dlp12 integrase (INTD),

involved in replication, and transcription repressor frmR (FRMR), involved in regulation of DNA synthesis. The integrase INTD is essential for integration of the phage into the host genome by site -specific recombination. In conjunction with excisionase, integrase is also a necessary element for excision of the prophage from the host genome (Lindsey et al. 1989). Transcriptional repressors are proteins that bind to specific sites on DNA to cause looping and prevent transcription of nearby genes. Looping alters the topology of the DNA and may thereby prevent formation of the closed or open RNA polymerase complex, activator binding or elongation (Alberts et al. 2002). The down-regulation of this protein may imply that the sites for the transcription repressor were already occupied by berberine, due to its affinity for DNA, and transcription was blocked in the absence of frmR.

Conclusion

The proteome profiling technique provided an effective approach to identify global changes in protein profiles under the influence of *P. polychaetum* alkaloid extract. To our knowledge, this is the first report on proteomic analysis of bacteria exposed to an alkaloid extract as an antimicrobial agent derived from *P. polychaetum*. In summary, the plant extract changed the levels of proteins which play vital roles for maintenance such as protein synthesis, antimicrobial resistance, amino acid uptakes, and ATP synthesis. The altered proteins identified by this approach can further be characterized as potential drug targets. The experimental findings of this study shed light on the mechanism of *P. polychaetum* extract and berberine from a molecular perspective. Further studies should lead to a better understanding of the antimicrobial mode of action of the plant extract, specifically berberine, and will contribute to the development of novel plant-based therapeutic drugs. A still open question is how beberine enters the bacterial cells to kill them. In this respect, a significant finding could be obtained by analyzing the interaction between berberine and oligo-peptide permease.

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