Journal of Aquaculture Research & Development

Tripathi et al., J Aquac Res Development 2017, 8:1
DOI: 10.4172/2155-9546.1000467

Research Article Open Access

Proteomic Analysis of Sensitive and Resistant Isolates of *Escherichia coli* in Understanding Target(s) of a Cyanobacterial Biomolecule Hapalindole-T

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Abstract

A broad spectrum biomolecule Hapalindote-T, from a cyanobacterium *Fischerella* sp. colonizing Neem tree bark was used for its targets using *Escherichia coli*. The cellular extracts of Hap-T^S (sensitive) and Hap-T^R (resistant) of *E. coli* were subjected to 2DGE. The protein spots (selected) with altered expression were analysed by LC-MS. The data obtained was matched with database of *E. coli*. Seventeen proteins were found with altered expression level. Three membrane proteins, OmpP, Agn43A and LysU, found in Hap-T^S strain were absent in the Hap-T^R strain. However, fourteen proteins, AspA, GlpK, LpdA, HslU, GlnA, SucB, YihT, GalF, MDH, RfbB, RmlB, AcrAB, FabB and GapA, related to certain metabolic pathways of the cell and overproduced in the extract of Hap-T^R strain. The seventeen screened proteins were related with vital metabolic pathways including membrane protein (Omp P), in *E. coli*. The results indicated that these proteins might be the cause of resistance in *E. coli*. These results suggested that overproduced proteins/enzymes in the resistant strain might be a survival strategy under Hap-T stress and could be used as signature protein for the development of new drugs.

Keywords: Cyanobacterium; Hap-T; Drug target; 2-DGE; *E. coli*

Introduction

Rapid emergence of resistant microbes against various drug(s) led the scientists to explore mechanisms of drug resistance in microbes [1]. Pace of lead molecules' discovery necessitates simultaneously newer drug targets to combat with increasing drug resistance in pathogenic bacteria. Methanolic extract of a cyanobacterium, *Fischerella* sp. was fractionated and the active biomolecule, with broad spectrum antibacterial and antimycobacterial activity was identified as Hapalindole-T (Hap-T) from our lab [2,3]. Twenty types of hapalindoles have already been reported from a cyanobacterium *Hapalosiphon fontinalis* [4]. An artificially synthesised 12-epi-hapalindole-Q was reported to be antibacterial as well as antimycotic [5]. Such biomolecules may also be modified to develop more potent antimicrobial agents [6]. Proteome analysis, using a combination of 2-DGE and mass-spectrometry drew much attention recently, because of its role in deciphering targets through interaction of genome database as finding cellular target(s) of streptomycin [1], kenamycin and amikacin [7] in *Mycobacterium tuberculosis*.

The cellular target(s) of Hapalindole-T has not been investigated so far. Tsherefore, non-infectious *E. coli* was used in present work to explore the cellular protein(s) related to *E. coli* resistance. A comparison of proteome of Hap-T sensitive (Hap-T^S) and resistant (Hap-T^R) strains of *E. coli* would reveal possible target(s) of Hap-T. Identification of such target(s) would facilitate development of an *in vitro* assay to screen derivatives of Hapalindole-T from drug(s) libraries.

Materials and Methods

Bacteria, media and Hapalindole-T

The overnight broth culture of Hap-T^S strain (~10^7 cells/ml) was spread on Luria Bertani (LB) agar plate containing 10-50 µg/ml Hap-T. Hapalindole resistant colonies (Hap-T^R) appeared spontaneously on plates containing 50 µg/ml Hap-T. *Escherichia coli* was grown at 37°C in LB broth and plated on LB agar plates for colony forming units (CFU). Hapalindole-T (Hap-T, $C_{21}H_{23}N_2ClSO$, Mr 386, melting point 179-182°C) was isolated from a cyanobacterium *Fischerella* sp. [2]. The minimum inhibitory concentration (MIC) inhibiting growth of *E. coli* sensitive strain Hap-T^S was 4.0 µg/ml.

Isolation of Hap-T^R strain of *E. coli* and Hapalindole-T sensitivity of Hap-T^S and Hap-T^R strains

Bacteria were grown in LB broth. Cells were washed and suspended in PBS to ~10^7 cells/ml to which specified concentrations of Hapalindole-T were added. At regular intervals, aliquots of cells were withdrawn, washed with PBS and plated after suitable dilutions on Muller Hinton medium (MH). Plates were incubated at 37°C till colonies of visible size appeared.

Two dimensional gel electrophoresis

Lysates of Hap-T^S and Hap-T^R strains were prepared according to the procedure described by O'farrell for 2-DE [8]. Cells of both strains (200 ml each) were centrifuged (10,000 rpm) at 4°C and pellet of cells was transferred 0.5 ml lysis buffer (8 M urea, 2 M thiourea, 0.01 M Tris-HCl, 1 mM EDTA, 1% w/v DTT, 5% v/v NP-40, 2% w/v CHAPS, 10% v/v glycerol, 2% ampholyte {0.8% pH 5-7 and 1.2% pH 3-10}, 0.0002% bromophenol blue and 1 mM PMSF). Cells with lysis buffer were vortexed vigorously and subjected to three rounds of freeze-thaw. After centrifugation the supernatant was separated as cell lysate and stored at -70°C. Protein was estimated by Bradford method [9].

50 µg protein was subjected to IEF. This was done in 15 × 0.3 cm

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Received July 25, 2016; **Accepted** February 15, 2017; **Published** February 17, 2017

Citation: Tripathi MK, Kumar M, Deepali S, Asthana RK, Nigam S (2017) Proteomic Analysis of Sensitive and Resistant Isolates of *Escherichia coli* in Understanding Target(s) of a Cyanobacterial Biomolecule Hapalindole-T. J Aquac Res Development 8: 467. doi: 10.4172/2155-9546.1000467

vertical glass tubes with 5% gels containing 6% ampholyte (four parts pH 3-10 and 2 parts pH 5-7). The tube gel was given a pre-run for 190 Vh before loading the samples to carry out IEF for 15,000 Vh. The tube gels were removed carefully and kept in SDS-sample buffer (10% glycerol, 5% beta-mercaptoethanol, 2.3% SDS, 0.0625M Tris-HCl, pH 6.8). It was transferred on to second dimension with resolving (10%) and stacking (4%) gels, and run at 150V continuously upto the bottom. The gels were stained with coomassie blue R-250 and analysed by Gel Doc (Bio-Rad, USA) using PD quest software. All experiments were performed in three independent replicates and only those spots present in at least two gels each of Hap-T^S or Hap-T^R lysates were taken for analysis.

LC-MS analysis of protein spots

Gel plugs and gel pieces were washed with acetonitrile (ACN, 50-100%), subjected to speed vacuum for 15 min and then reduced by dithiothretol (DTT, 10 mM) in ammonium carbonate (100 mM) and ACN (5%) for 1 h at 55°C. This was followed by dehydration with NH_4CO_3 (100 mM) for 10 min and ACN (100%) for 20 min. Now alkylation was done in dark by iodoacetamide (50 mM) in NH_4CO_3 (100 mM) at room temperature for 30 min. These gel pieces were washed again with NH_4CO_3 (100 mM) and ACN (100%) followed by drying in speed vacuum.

Gel pieces were immersed in digestion buffer (50 mM NH_4CO_3, trypsin) on ice for 45 min and kept wet by adding NH_4CO_3 (50 mM) and incubated at 37°C for 16 h. The supernatants of digested solution were collected. Gel plugs were extracted once with NH_4CO_3 (20 mM) for 20 min followed by two-times extraction with trifluoroacetic acid (TFA, 1%) in ACN (50%) followed by ACN (100%) for 20 min each. All supernatants were pooled and concentrated collectively in speed vacuum. The sample (16 µl) was injected into nano LCMS (Agilent 1100 series LC/MSD trap XCT). The data obtained was searched using MASCOT search engine with *E. coli*-limited search filter. Mass tolerance was kept 50/100 ppm.

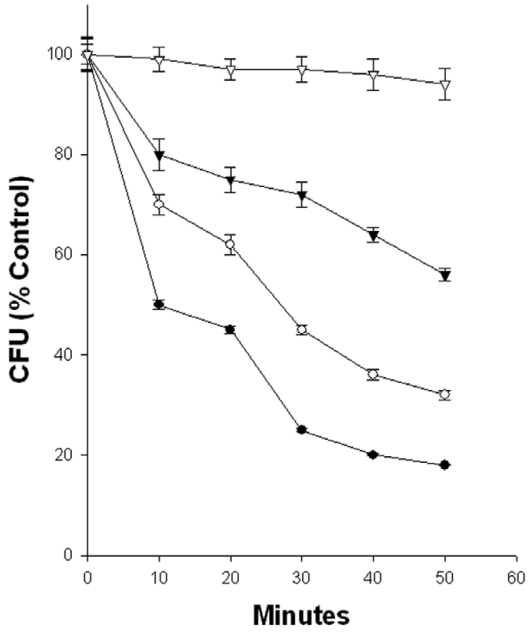

Figure 1: Colony forming ability of *E. coli* (Hap-T^R) in 50 µg/ml Hap-T (∇-∇) and Hap-T^S strain after exposure to Hap-T at different time points: 20 µg/ml (▼-▼), 25 µg/ml (○-○) and 30 µg/ml (●-●).

Statistical analysis

All experiments were carried out in triplicates with standard deviation (SD) represented in bars wherever necessary.

Results

Effect of Hap-T on survival of *E. coli* (Hap-T^S)

The colony forming ability of Hap-T^R and Hap-T^S are shown in Figure 1. Hap-T^R cells were exposed to 50 µg/ml of Hap-T to various time intervals (10-60 min.) but the strain was found resistant to Hap-T at 50 µg/ml with more than 90% survival. Hap-T^S cells were sensitive to Hap-T, as increasing concentration (10-60 µg/ml) and incubation time, the CFU of such cells decreased.

Analysis of 2-DE gels and identification of protein spots with LC-MS

Total protein of Hap-T^S and Hap-T^R cells by 2-DE, is shown in Figures 2A and 2B. The spots observed in both the gels were comparatively quite comparable and matching. Approximately 200 spots were detected after IEF in pH range 3-10 with 12% SDS PAGE. Selected spots showed altered expression of proteins as reflected by their intensities on both the gels. There were thirteen differing protein spots, but only six were numbered. The protein spots 1 and 2 were present in

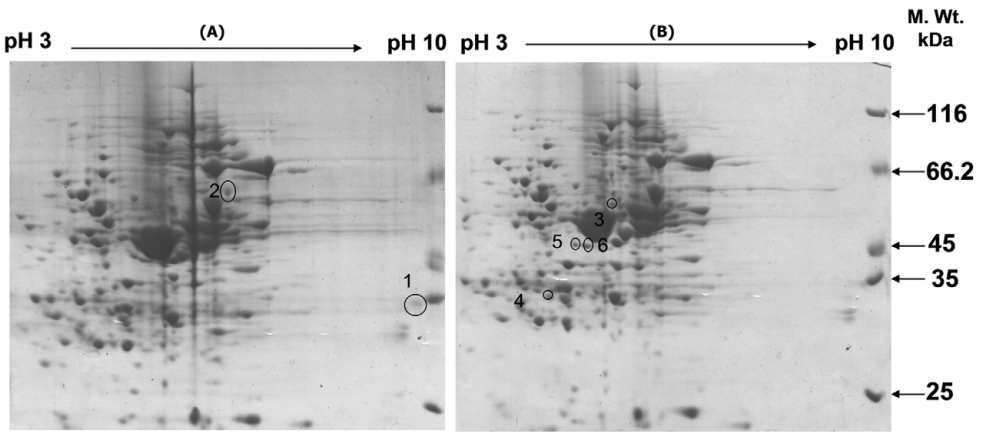

Figure 2: Two dimensional gel (pH 3-10) of *E. coli* (A) Hap-T^S and (B) Hap-T^R isolates.

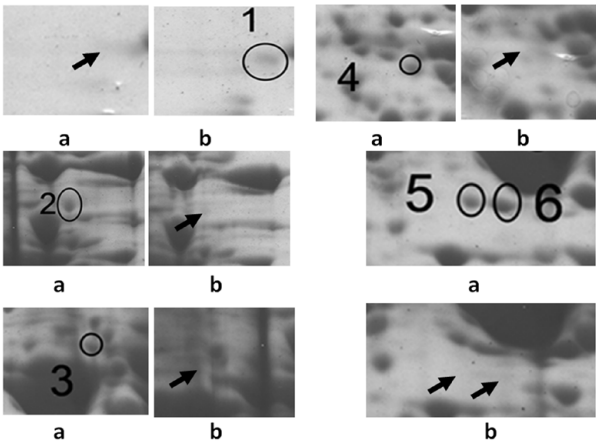

Figure 3: Magnified 2D gels with altered expression of proteins among (a) Hap- T^S and (b) Hap-T^R.

Hap-T^S only and 3 to 6 were present exclusively in Hap-T^R (Figure 3). Such spots were digested with trypsin and processed through LC-MS and data showed mixture of proteins as listed in the Table 1.

These proteins were matched with database of *E. coli.* Among these seventeen proteins three were found unique to Hap-T^S and identified as outer membrane proteinase (*ompP*) and antigen 43 (*agn43*) precursors involved in autoaggregation of *E. coli* cells. Lysyl-tRNA synthetases (*lysU*), synthesizes a number of adenyl nucleotides acting as a modulator of heat shock response. The remaining fourteen proteins were found to be associated with Hap-T^R strain. Proteins in spot 3 (Figure 3) are known to be involved in amino acid biosynthesis and biodegradation including, aspartate ammonia-lyase (*aspA*), glycerol kinase complexed (*glpK*), dihydrolipoamide dehydrogenase (*lpdA*), trigonal crystal form of heat shock locus u (*hslU*), glutamine synthetase (*glnA*) and dihydrolipoamide acetyltransferase (*sucB*). The spot 4 (Figure 3) contained putative aldolases (*yihT*), malate dehydrogenases (*mdh*), and UDP-glucose pyrophosphorylase (*galF*). These enzymes are associated with glucose metabolism. The spot 5 (Figure 3) was identified as enzyme dTDP-glucose-4, 6-dehydratase (*rfbB*) and RmlB, an enzyme with multiple participation, especially in nucleotide sugar metabolism as well as biosynthesis of non-ribosomal proteins. The third protein acridine efflux pump (*acrAB*) is linked with drug efflux system having broad substrate specificity. The spot 6 (Figure 3) possessed β-keto-acyl-ACP-synthase I (*fabB*) and gleceraldehyde-3-phosphate dehydrogenase (*gapA*/GAPDH). FabB catalyzes the elongation of fatty acid from C-10 to unsaturated C-16 and C-18 fatty acids. GAPDH maintains the reducing power of the cells under stress. Thus identification of these proteins suggested that resistance to Hap-T induced changes associated with cell membrane, amino acid biosynthesis/degradation, carbohydrate metabolism, fatty acid biosynthesis type II including non-ribosomal protein biosynthesizing enzymes.

Discussion

Natural products from cyanobacteria are likely to offer a new source of antibiotics due to the presence of unique biosynthetic mechanisms [10,11]. In this paper we have investigated the cellular targets of an antimicrobial compound Hap-T [2] by comparing total proteome of sensitive and resistant strains of *E. coli*. Since the genome of *E. coli* is sequenced and annotated therefore, it was used as surrogate to find the cellular target(s) of Hap-T. Proteome of the two *E. coli* strains indicates role of proteins in acquiring resistance against Hap-T. The differentially expressed proteins associated with the Hap-T^S and Hap-T^R strains were identified (Table 1). Action of any biomolecule/drug depended on its intracellular accumulation in organism, affecting the metabolism of the cell leading to death [12,13]. The colony forming ability of Hap-T^S decreased with the increase in dose of Hap-T and duration of exposure. However, Hap-T^R strain possessed more than 95% colony forming ability even in the highest dose of biomolecules (50 µg/ml) and longest duration of exposure (Figure 1). Such differential behaviour in the two strains of *E. coli* might be because of its altered metabolism, reflected in the proteomic analysis (Figure 2). There are reports of marked alterations in total proteome of *Mycobacterium tuberculosis* and *Orientia tsutsugamushi*, challenged with drug/biomolecules [14-16]. In fact, antimicrobial drugs are designed after keeping the point in view, that target genes must be essential for the survival of the bacterium. However, such genes are either lack in the host or not affected after exposure of such designed drugs. A comparison of proteome of Hap–T^S and Hap–T^R strains provided first global protein profile, clearly indicating altered expression of membrane associated proteins, enzymes related with protein turn over, management of carbon skeleton and energy transduction as well as FabB and aldolases. Such differences in proteins of attenuated and virulent mycobacterial strains helped in designing of novel vaccines [17].

OMPs in bacteria are well known for their role in permeability [18]. Down-regulation of OMPs in Hap-T^R *E. coli* strain indicated a mechanism of resistance through restricting permeability of Hap-T inside cell. This observation was in tune with reports of altered protein expression in outer membrane of *E. coli* resistant to chloramphenicol, ampicillin and tetracycline [19-21]. Likewise, Up-regulation of AcrAB can efflux the Hap-T rendering resistance in the strain. The importance of efflux pump is already established by use of mefloquin as inhibitor in *Pseudomonas aeruginosa* and *E. coli* [22]. Sensitivity of the two AcrA mutants of *E. coli* increased against antibiotics as MICs decreased [23]. Increased AcrAB expression was also corroborated with tigecycline resistance in *Enterobacter cloacae* [24]. However, importance of membrane targeting drugs has been also reported by Eun et al. [25] as DCAP (a broad spectrum antibiotic) killed slow growing bacteria after targeting membrane. Thus, our observation of membrane targeting by Hap-T gets support with the results. The proteins RfbB and RmlB are synonyms and named as RmlA-D instead of RfbA-D [26] and these proteins were necessary for survival of *Mycobacterium* as observed by knockout mutants [27].

Spot	Matched Proteins	Name of the Proteins	Accession No	MW (kDa)	P*I*	Score	Sequence Coverage (%)
1	One	Outer membrane proteinase (*omP*)	A36944	35.47	5.9	166	57
2	Two	Antigen 43 precursor **(*agn43A*)**	GI 7243712	106.89	5.5	629	12
		Lysyl-tRNA synthetases (*LysU*)	GI 146689	57.35	5.1	105	5
3	Six	Aspartate ammonia-lyase (*aspA*)	GI 15804731	54.71	5.5	716	30
		Chain G, Glycerol kinase (*glpK*)	GI 442946	56.34	5.3	465	21
		Dihydrolipoamide dehydrogenase (*lpdA*)	GI 15799800	50.94	5.7	440	21
		Chain A, Trigonal Crystal form of heat shock locus U (hslu)	GI 7245635	49.54	5.2	420	20
		Glutamine Synthetase (*glnA*)	GI 146157	51.98	5.2	171	11
		Dihydrolipoamide acetyltransferase (*sucB*)	GI 15800431	43.98	5.5	150	13
4	Three	Putative aldolase (*yihT*)	GI 15804467	32.2	5.7	181	17
		UDP-glucose pyrophosphorylase (*galF*)	GI 24266667	32.94	5.7	150	17
		Malate dehydrogenase (*mdh*)	GI 226907	32.41	5.6	126	8
5	Three	dTDP-glucose 4,6 dehydratase, NAD(P)-binding (rfbB)	GI 16129981	40.7	5.4	644	35
		RmlB	GI 50882468	40.68	5.2	510	28
		Acridine efflux pump (*acrAB*)	GI 15800192	42.22	7.6	318	24
6	Two	Beta-ketoacyl-ACP synthase I (*fabB*)	GI 3805908	42.95	5.3	397	30
		Glyceraldehyde-3-phosphate dehydrogenase(*gapA*)	GI 26248038	36.1	6.3	247	19

Table 1: Protein profile of spots 1, 2 of Hap-T^S and spots 3, 4, 5, 6 of Hap-T^R strains on 2-DE gels, pH 3-10.

Up-regulation of the enzymes which were involved in the management of the carbon skeleton, amino acid pool as well as energy crisis, seemed to play a key role in the development of Hap-T resistance. Overexpression of LpdA, associated with energy management was known to cause tellurite resistance in *E. coli* [28] which led the structure based drug design to control parasitic protozoa *Trypanosoma cruzi* [29]. The enzyme such as HslVU associated with supply of metabolic precursors through proteolytic activity, was used as drug target for *Plasmodium falciparum* [30]. AspA is little explored as drug target however, 3-nitropropionate acted as a competitive inhibitor to AspA of *Bacillus* sp. YM55-1 [31]. The presence of GS in human beings as well as in bacteria can be a choice for drug target because of the difference in affinity of ATP binding site in such enzyme [32]. Overexpression of SucB is also known to be associated with persistence in survival and antibiotic resistance in *E. coli,* through involvement in energy production [33] therefore, mutation in *SucB* can serve the purpose. GAPDH reduces the energy crisis of cell under stress via generating more reducing power and influencing the cell survival. However, it is also least understood as a drug target except as a neuroprotective agent [34]. The enzymes, class II aldolases (GalF, MDHs) and fatty acid biosynthetases type II (FAS II) are specific to prokaryotes [35,36]. GalF regulates the cellular level of UDP glucose which is an adaptive mode under stress management [37]. The amino acids of MDHs from *E. coli* and *Salmonella typhimurium* and mitochondrial isozyme of eukaryotes have high identity [38] therefore, seemed to be a promising drug target. Increased expression of FabB protein through introduction of multicopy plasmid in *E. coli* confers thiolactomycin (TLM) resistance [39]. Interaction of TLM and acyl enzyme intermediates of FabB and FabF have shown preferential binding towards each other in *M. tuberculosis* and *E. coli* [40].

Conclusion

The strategy of survival in a bacterium is a cascade of mechanisms, operating through a complex metabolic circuit. Over production of individual proteins may be responsible for its survival and resistance against antimicrobials. Such studies on resistant model strain (*E. coli*) against the target biomolecule in understanding the strategy of survival, justifies our 2-DE approach in the present case.

Acknowledgments

We are grateful to the Head and Programme Coordinator of Centre of Advanced Study in Botany, Banaras Hindu University and Direcctor Amity Institute of Biotechnology for laboratory facilities, We are also thankful to TCGA, New Delhi for LC-MS of 2-DE protein spots. We are grateful to Dr. B. S. Srivastava, Central Drug Research Institute, Lucknow, India for critically going through the manuscript.

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