

Effect of Iron Availability on the Survival of Carbapenem-Resistant *Acinetobacter baumannii*: a Proteomic Approach

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Abstract

Acinetobacter baumannii is a notorious pathogen and is known to cause hospital-acquired nosocomial infections. Presently, carbapenems are most effective antibiotics against *A. baumannii*, however, emerging carbapenem resistance by *A. baumannii* further made it difficult to treat the infections. One of the requirements for successful invasion and colonization of *A. baumannii* is the essential micronutrient iron inside the human host. However, the bioavailability of iron is sequestered due to the nutritional immunity of host, which acts as an important virulence factor. Present study is an attempt to identify membrane proteins in response to *in vitro* iron-overload and iron-limiting conditions by using Differential In-Gel Electrophoresis (DIGE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Carbapenem-resistant clinical strain of *A. baumannii* under iron-stress conditions, showed high expression of Fhu E receptor, ferric acinetobactin receptor, ferrienterchelin receptor, ferric siderophore receptor, bacterioferritin and cell division inhibitor suggesting their correlation in iron circumvention inside human host. On contrary, in presence of iron, proteins such as ATP synthase, malate dehydrogenase, acetyl-CoA carboxylase, ribosomal protein, EF-Ts etc are elevated indicating their role in facilitating the metabolism of carbohydrate, amino acid, fatty acid and nucleic acid. Present results indicate the adaptability of carbapenem-resistant *A. baumannii* to iron availability by differentially expressing some of its membrane proteins.

Keywords: *Acinetobacter baumannii*; Iron-regulated Outer membrane proteins; Differential In-Gel Electrophoresis (DIGE)

Introduction

Acinetobacter baumannii has emerged as one of the most troublesome pathogens for health care institutions globally due to its remarkable ability to up regulate or acquire resistance determinants [1-5]. This pathogen has emerged as a threat to soldiers wounded during military operations in Iraq and Afghanistan [6,7] as well as isolated from animals [8]. Because of versatile metabolism [2], *A. baumannii* has the ability to grow at wide range of survival conditions and this is also an important factor for emerging this organism as most adaptive pathogen in the hospital environment. Further the intrinsic resistance to a number of antimicrobial agents allows the *A. baumannii* to spread in the hospital settings. Severe infections with this organism occur in compromised individuals after invasive diagnostic and therapeutic procedures [9-11]. Iron is a requisite nutrient for the growth and proliferation of bacteria. Iron (Fe) can adopt either of two positively charged ionic forms i.e., ferrous (Fe²⁺) and ferric (Fe³⁺). Under aerobic conditions, it is present as ferric not ferrous (biologically available form). Iron is present in conjugation with proteins like transferrin, lactoferrin, ferritin and insoluble ferric hydroxides. Iron homeostasis is so strictly regulated that there is virtually no free iron in living organisms [12-14].

Pathogenicity of bacteria is also significantly influenced by the bioavailability of iron inside the host. Unlike higher organisms, which may obtain bioavailable iron from the diet, bacteria must acquire iron from their environment. This presents a challenging problem, as free iron availability in human blood are 10⁻¹⁸ M [15] while most pathogens require 10⁻⁶ M to 10⁻⁷ M of iron [12]. This presents a problem to invading bacteria because the majority of iron in humans is tightly bound by iron-binding proteins. As a result, the concentration of freely available iron is very less to allow the invasion of a pathogen. This limitation of the availability of free iron in host for infecting microorganisms has been termed nutritional immunity [16]. To overcome this nutritional limitation microorganisms have acquired diverse mechanisms

[12]. Two general mechanisms for iron acquisition developed by Gram negative bacteria are siderophore dependent and siderophore independent (receptor-mediated iron acquisition from host proteins) [17,18].

Iron limiting conditions during growth of *E. coli* stimulate the synthesis of several membrane proteins [19,20]. Role of iron in the differential expression of iron-regulated membrane proteins are studied in gram negative bacteria using transcriptomics [18] and proteomics [21,22]. As the bacterial membrane plays a vital role in regulating a plethora of proteins involved in various biochemical pathways, we looked in the total membrane (inner and outer) proteome of *A. baumannii*. Membrane proteome of resistance strain of *A. baumannii* under antibiotic availability has been done [2,23,24]. Membrane proteome of sensitive strain ATCC19606 under diverse iron availability has also been done using traditional two dimension electrophoresis [21]. We have investigated the membrane proteome of carbapenem resistant strain of *A. baumannii* with diverse iron availability using a highly sensitive fluorescence based DIGE method. DIGE allows multiplexing of protein sample in same gel hence controlling non-biological variation, increases dynamic range, sensitivity of traditional

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2D-PAGE and have accurate quantification with statistical confidence. Use of Internal standard in DIGE technology also allows repetitive measurements and multi-variable analyses to be quantitatively analyzed in one coordinated experiment, yielding statistically significant changes in protein expression related to different condition. Hence, we assume that DIGE have less differently expressed proteins with significance role in iron stress. Therefore, a quantitative proteomic study on characterization of iron regulated membrane proteins from a clinical strain is essential in understanding the etiology of host-pathogen interactions of *A. baumannii* during infections. In the present report, we have shown that during iron limiting conditions, clinical strain of *A. baumannii* increases the production of several membrane proteins which are directly or indirectly linked to iron homeostasis and resistance mechanism.

Material and Methods

Reagents and chemicals

MacConkey agar, Muller Hinton agar was purchased from Himedia laboratories Ltd, India and LB media was from Pronadisa Conda Laboratories. Urea, Thiourea, Tris-HCl, NaCl and Glycine were from Merck, India. Ammonium bicarbonate (mass spectrometry grade), were from Sigma chemical co. USA. Hydrochloric acid, Glacial Acetic acid, Glycerol and Methanol were from Qualigens. Acrylamide, Bisacrylamide, Ammonium persulphate, TEMED, SDS, EDTA, Coomassie Brilliant blue, β -mercaptoethanol and Bromophenol blue were obtained from Bio-Rad laboratories, USA. Mass spectrometry grade trypsin was from Promega, USA. Immobililine dry strips, dry strip cover fluid, Dithiothreitol (DTT), Iodoacetamide and fluorescent dyes (Cy2, Cy3 and Cy5) were purchased from GE Health Care. Dimethylformamide (HPLC grade) was from Spectrochem, India. CHAPS, HPLC grade acetonitrile and proteomic grade water were purchased from G. Biosciences, USA. All other routine chemicals (analytical grade) obtained from Merck, India. 2,2' Bipyridyl, DMSO and FeCl_3 were purchased from Himedia.

Bacterial strain and growth conditions

The proteomic study was carried out on carbapenem resistant clinical strain, RS307, of *Acinetobacter baumannii*. RS307 was collected from microbiology department, A.I.I.M.S., New Delhi and was confirmed up to the species level (*baumannii*) using method similar to our earlier published method [2]. Minimum inhibitory concentration of RS307 for carbapenem like imipenem and meropenem (Figure 1) were found to be $\geq 64 \mu\text{g/mL}$. This showed RS307 has high resistance to carbapenem. Luria Bertani (LB) broth media contain $<3 \mu\text{M}$. LB media with $100 \mu\text{M}$ of 2,2' Bipyridyl was used as iron deficient media [21]. LB media with $100 \mu\text{M}$ FeCl_3 was used as iron rich condition [21].

Extraction of total membrane proteins

Bacteria was grown independently in 250 mL iron deficient and iron rich LB media at 37°C to an optical density between 0.6-0.8 at 600nm and harvested by centrifugation at 7000 rpm for 15 min. Pellet was resuspended in Tris buffered saline (10 mM, pH 7.5). Total membrane proteins were extracted according to our previously described method [2]. In summary, cells were broken by sonic bursts using Misonix XL Ultrasonic processor under cooling for a total time of 5 minutes. The suspension containing the cell envelope was subjected to ultra centrifugation for 30 min at $1,00,000\times g$. The pellet containing the total membrane fraction was washed and resuspended in 0.5% CHAPS.

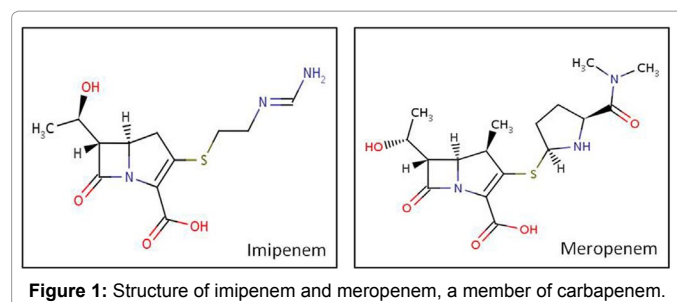


Figure 1: Structure of imipenem and meropenem, a member of carbapenem.

Differential in Gel Electrophoresis (DIGE)

Differential In-Gel Electrophoresis (DIGE) was employed for identification of proteins in iron rich and iron deficient conditions. For DIGE experiments each bacterial strain was grown three time under constant culture conditions and time. Total membrane proteins were extracted from two different conditions and three experimental sets were made according to our previous experimental design [2]. In each experimental set, the $50 \mu\text{g}$ of protein either from iron-rich culture or iron-deficient culture was labelled with the 200 pmole of fluorescent dyes Cy 3 or Cy 5 separately. 1/6 fraction of protein sample from each strain was pooled and labelled with Cy2. Cy2 labelled protein acts as internal standard and remains constant in every experiment. Protein labelling was achieved by incubating the reaction mixture on ice for 30 minutes in dark. After completion of labelling time, reaction was quenched by the addition of $1 \mu\text{l}$ of 1 mM lysine. Both the samples were mixed together along with the internal standard. The final volume of reaction mixture was adjusted to $250 \mu\text{l}$ using rehydration buffer (7M urea, 2M thio-urea, 2% CHAPS, 0.7 mg DTT, $1.25 \mu\text{l}$ IPG buffer) and rehydrated for 16 hours in dark with 13 cm, 4-7 pH IPG strip.

Two dimensional gel electrophoresis

In 2D gel electrophoresis, Isoelectric focusing (IEF) was done on Ettan IPGphor 3 IEF system and SDS PAGE analysis was carried out in SE 600 Ruby gel apparatus (GE healthcare). Rehydrated IPG strip were subjected to isoelectric focusing for 24,000 volt-hours. After IEF, strips were equilibrated in 5 mL of SDS-equilibration buffer (50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 0.02% bromophenol blue) containing first 0.05% DTT and then with 1.25% iodoacetamide for 15 min each. Second dimension electrophoresis was run on a 12% polyacrylamide gel at 4°C with 15 mA for 30 min and then at 30 mA for both the strips, till the bromophenol blue came out of the gel.

Image acquisition and analysis

DIGE gels were scanned for Cy2, Cy3 and Cy5 fluorescence labeled proteins using a Typhoon™ TRIO Variable Mode Imager (GE Healthcare, USA). Cy2 images were scanned using 488nm excitation and 520BP40 emission filter; Cy3 images were scanned using 532 nm excitation and 580BP30 emission filter; Cy5 images using 633 nm excitation and 670BP30 emission filter. All gels were scanned with a PMT of 700. Images were cropped using Image-Quant™ version 6.5 (GE Healthcare, USA) to remove areas extraneous to the gel image. The final expression levels were determined by the DeCyder software version 7.0 (GE Healthcare, USA). In DeCyder analysis, the gel images were processed by DeCyder Differential Analysis (DIA) using default setting. The estimated number of spots for each co-detection procedure was set to 1500. The spots on gels were co-detected automatically as 2DE DIGE image pairs, which intrinsically link a sample to its in-gel standard. Differential protein production between two samples in the gel was based on the ratio of standardized log abundance of the Cy3

versus Cy5 spot volume over the Cy2 spot volume. Initially, all DIA workspaces were imported to Biological Variance Analysis (BVA) workspace and the experimental setup and relationship between samples were assigned in the BVA workspace. Each individual image of Cy3 gel or Cy5 gel was assigned an experimental condition, either RS307 in presence of iron or RS307 in absence of iron according to the labeling. All Cy2 images were classified as internal standards and were used for gel-to-gel matching of the standard spot maps in all gel images. The gel with the highest spot count was assigned as the master gel. Matching between gels was performed utilizing the in-gel standard from each image pair. Matching was further improved by land marking and manually confirming potential spots of interest. The degree of difference in standardized abundance between two protein spot groups is expressed as average ratio (fold change). A fold change with a threshold value of minimum 2-fold increase or decrease was used. Student *t* test was performed for every matched spot-set, comparing the average and standard deviation of protein abundance for a given spot. Therefore, proteins which had higher value than 2-fold change and also with a significant *p*-value ($e0.05$) were considered in the present study for identification. To increase the number of down regulated proteins (with possible role in iron acquisition) some proteins with significant *p*-value ($e0.05$) but less than two fold changes are also included in the present study.

In-gel digestion and mass spectrometry

Protein spots were excised from coomassie (G-250) stained 2D gel and in-gel digestion was carried out using trypsin enzyme (Mass spectrometry grade, Promega Corp., Madison, WI). The resulting peptide mixture was separated by reverse phase chromatography (TempoTM nano-LC system, Applied Biosystems) using a Pep Map C18 column (Dionex: 75 $\mu\text{m}\times 150$ mm, 5 μm , 100 A, 300 nl/min) in combination with a C18 trap column. Peptides were separated using a 70 minute linear gradient from 5% to 98% acetonitrile in 0.1% formic acid with a total flow rate of 400 nl/min. The eluting peptides were ionized by electron spray ionization and analysed by QSTAR XL system (Applied Biosystems, MDX Sciex, Foster city, CA, USA). Nanospray ionization was carried out using an ion spray voltage of 900. The progress of each run was monitored by recording the total ion current (TIC) for positive ions as a function of time for ions in the *m/z* range of 400-1600 for MS and 140-1600 for MS/MS. The spectra was acquired in an information dependent manner utilizing the Analyst QS software 2.0 acquisition features to generate raw data in the * wiff format without merging putatively like spectra. The other parameters set were: interface temperature, 50°C; curtain gas flow, 1.13 L/min; declustering potential, 60 V; focusing potential, 280 V; declustering potential 2, 15 V. Database searching was done using Mascot (Matrix Science, UK) using setup similar to our earlier publication [2].

Results and Discussion

Resistance of *A. baumannii* against carbapenem increasing at alarming rate (36% in 2009 as compared to about 20% found five years ago in our hospital) [4]. Clinical strains with high MIC (>64 $\mu\text{g/ml}$) pose a bigger threat than the lower resistance strain [2-5,24,25]. RS307 (MIC, >64 $\mu\text{g/ml}$ for imipenem, latest β -lactam) was selected for the present study because of better survive of RS307 relative to resistance strain with lower MICs [2].

Proteomics of *A. baumannii* under iron overload with respect to iron starvation

Generally, the amount of iron present under the physiological

conditions in human blood are 10^{-18} M [15], which is very low and is not conducive for *A. baumannii* to survive. However, in experimental growth condition, to ensure the non-availability of iron to the bacterium, the iron chelator, 2,2' Dipyridyl (100 μM) was added to remove traces of iron if present any. A reduction in the OD at 600nm value was seen in cultures after overnight growth containing iron chelator. The total protein concentration as was estimated to be very low (0.74 mg/ml), in this iron deficient conditions. The other condition, the iron rich condition, was generated by supplementing the growth medium with iron by the addition of 100 μM FeCl_3 . The protein concentration was maximum (1.71 mg/ml) in iron surplus condition. The production of fewer amounts of proteins in iron deficient condition clearly shows that the growth of the bacterium is low under iron deficient conditions but has increased during iron surplus conditions.

DIGE of iron regulated membrane proteins in iron rich (+Fe) and iron deficient conditions (-Fe)

The proteomic study was done with the *Acinetobacter baumannii* clinical strain using DIGE. Before DIGE experiments, 2DE gel of total membrane protein profiles of clinical strain were generated using 3-10 pH IPG strips and 4-7 IPG strips (data not shown). The IPG strips of pH range 3-10 were used to locate all the membrane proteins, however, most of the membrane proteins were found towards acidic region in the 4-7 pH range. Therefore for better resolution and visualization, 4-7 IPG strips were used in further experiments. The overall profile of DIGE photographs of proteomics of *A. baumannii* under these two conditions

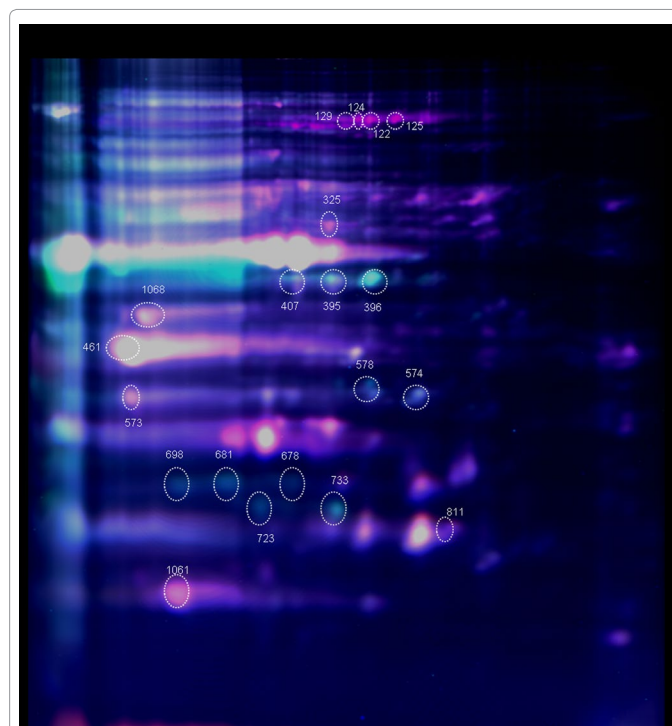


Figure 2: Differential In-Gel Electrophoresis of total membrane proteins of carbapenem resistant strain RS307 of *Acinetobacter baumannii* in presence of iron relative to its absence. Total membrane proteins of RS307 in presence of iron (50 μg), its absence (50 μg) and pooled fraction (50 μg) were labelled using Cy3, Cy5 and Cy2 dye, respectively. Isoelectric focusing (IEF) was done using 13 cm, 4-7 pH range IPG strip and second dimension was done on 16 \times 18 cm gel-electrophoresis. Gels were analyzed by Typhoon Imager. Upregulated proteins are seen as green while down regulated proteins are seen as red spots and unaltered proteins are seen as yellow.

showed differences (Figures 2 and 3). Proteins which are downregulated in presence of iron can be associated with iron acquisition and those upregulated can be associated with fast growth of the bacteria. After BVA analysis, expressions of some protein (master no. 129, 124, 122, 125, 1061, 573, 1068, 461 and 325) were found to be down-regulated in iron-rich condition (Table 1). This may be postulated that all these proteins are regulated by the iron concentrations, thus they may be regarded as the iron-regulated membrane proteins. Proteins (master no. 396, 574, 698, 678, 723, 681, 395, 733, 578) were found to be up-regulated in iron-rich condition (Table 1) and associated with fast

growth of *Acinetobacter* in the presence of iron.

Down-regulated proteins in carbapenem resistant *A. baumannii* under iron overload

Proteins which are down-regulated in the presence of iron appear to play a role in the iron acquisition. We found seven proteins which are down regulated due to excess iron (Figure 3). Four of them are receptors for the iron binding siderophore which include FhuE receptor (80.6kDa, pI5.44), Acinetobactin receptor (78.3 kDa, pI5.46), Ferrienterochelin receptor (82.9 kDa, pI 5.66), Putative ferric

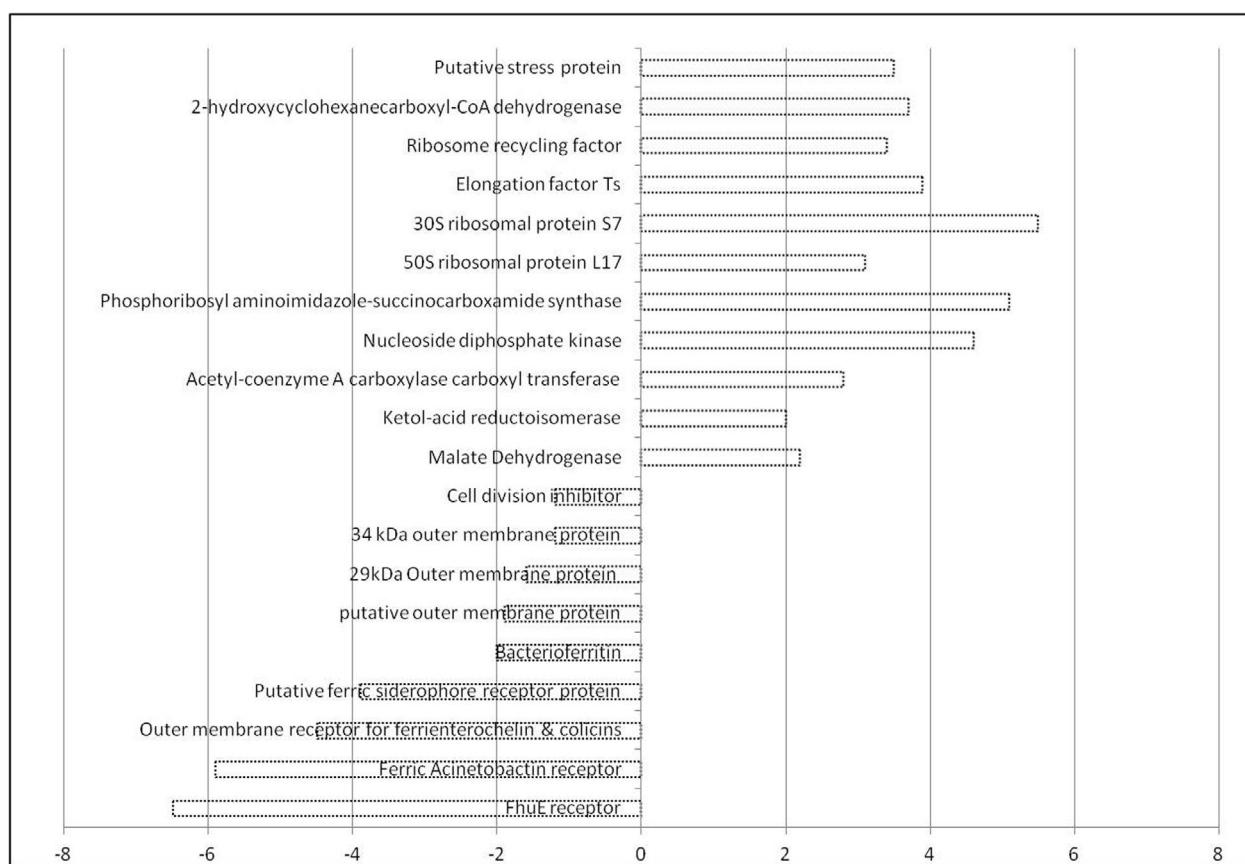


Figure 3: Schematic presentation of differently expressed proteins in the carbapenem-resistant *A. baumannii* in presence of iron relative to its absence. Only those over-expressed and down-regulated proteins which showed 2-fold and higher change with a significant *p*-value ($e0.05$) were considered in the present study. To increase the number of down regulated proteins (with possible role in iron acquisition) some proteins with significant *p*-value ($e0.05$) but less than two fold changes has also been included in the present study.

siderophore receptor and iron binding protein like Bacterioferritin (18.137 kDa, pI 5.02).

FhuE receptor is present on the cell outer membrane and plays an important role in iron acquisition by acting as a receptor and transporter for iron-siderophore complex [26]. It is also required for Fe^{3+} uptake via coprogen, ferrioxamine B and rhodotorulic acid [25-28]. Iron proficiency of *A. baumannii* also depends on the expression of the acinetobactin-iron acquisition system [29]. Acinetobactin is a novel siderophore, with both catecholate and hydroxamate functional groups isolated from iron minimal cultures of *A. baumannii* [29]. Gaddy et al found that persistence of *A. baumannii* in epithelial cell type depends on active production of acinetobactin and transport of ferric acinetobactin complexes [30]. Production of acinetobactin-

mediated iron acquisition system also allows bacteria to persist as well as to cause cell apoptosis and host killing [30]. Ferrienterochelin receptor is also identified as outer membrane receptor for ferrienterochelin (iron binding molecules) and colicins. Enterochelin (also called enterobactin) is secreted by Enterobacteriaceae under conditions of iron stress and serves to solubilise iron that would otherwise be unavailable to the cells. The uptake of ferrienterochelin is inhibited in the mutants which lack ferrienterochelin receptor [31]. Such mutants are also resistant to colicins but the mechanisms through which the colicin action and ferrienterochelin uptake systems interact are not fully understood. Other down regulated receptor is putative ferric siderophore receptor protein which is involved in active transport of Fe^{3+} chelated to molecules known as siderophores. Therefore, it is

Master No.	Fold change in DIGE	M _w (kDa)	pI	Identified Protein and Accession number	Score/ Sequence coverage (%)
129	(-) 6.5	80.6	5.44	FhuE receptor, <i>Acinetobacter baumannii</i> , gij260555105	903 / 33
124	(-) 5.9	78.3	5.46	Ferric Acinetobactin receptor, <i>Acinetobacter baumannii</i> , gij35210425	623 / 28
122	(-) 4.5	82.8	5.66	Outer membrane receptor for ferrienterochelin and colicins, <i>Acinetobacter baumannii</i> , gij184157262	313 / 11
125	(-) 3.9	85.5	7.55	Putative ferric siderophore receptor protein, <i>Acinetobacter baumannii</i> , gij169796074	1151 / 58
1061	(-) 2.0	18.1	5.02	Bacterioferritin, <i>Acinetobacter baumannii</i> , gij126640856	420 / 63
573	(-) 1.9	24.8	4.50	Putative outer membrane protein, <i>Acinetobacter baumannii</i> , gij72535025	640 / 50
461	(-) 1.6	29.0	4.61	29kDa Outer membrane protein, <i>Acinetobacter baumannii</i> , OM29_ACIBA	111 / 91
1068	(-) 1.2	32.1	4.77	34 kDa outer membrane protein, <i>Acinetobacter baumannii</i> , gij184159810	550 / 46
325	(-) 1.2	30.9	5.39	Cell division inhibitor, a membrane ATPase, activates minC, <i>Acinetobacter baumannii</i> , gij169634045	239 / 30
407	2.2	35.3	5.20	Malate Dehydrogenase, <i>Acinetobacter baumannii</i> , MDH_ACIBC	125 / 15
396	2.0	37.0	5.39	Ketol-acid reductoisomerase, <i>Acinetobacter baumannii</i> , ILVC_ACIBC	86 / 10
574	2.8	29.8	5.60	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha, <i>Acinetobacter baumannii</i> , ACCA_ACIBC	93 / 28
698	4.6	15.5	5.52	Nucleoside diphosphate kinase, <i>Acinetobacter baumannii</i> , NDK_ACIBC	257 / 51
678	5.1	27.2	5.02	Phosphoribosyl aminoimidazole-succinocarboxamide synthase, <i>Acinetobacter</i> , PUR7_ACIBAD	71 / 6
723	3.1	13.9	10.9	50S ribosomal protein L17, <i>Acinetobacter baumannii</i> , RL17_ACIBT	57 / 18
681	5.5	17.6	10.2	30S ribosomal protein S7, <i>Acinetobacter baumannii</i> , RS7_ACIBT	40 / 8
395	3.9	30.8	5.27	Elongation factor Ts, <i>Acinetobacter baumannii</i> , EFTS_ACIBC	96 / 18
733	3.4	20.6	5.66	Ribosome recycling factor, <i>Acinetobacter baumannii</i> , RRF_ACIBS	54 / 10
578	3.7	28.5	5.28	2-hydroxycyclohexanecarboxyl-CoA dehydrogenase, <i>Acinetobacter baumannii</i> , gij260557878	593 / 61
811	3.5	15.8	5.62	Putative stress protein of <i>Acinetobacter baumannii</i> gij169795610	452 / 61

Table 1: Identification of differentially expressed proteins of carbapenem-resistant strain (RS 307) of *A. baumannii* under iron overload growth condition with reference to that of iron-free condition. Differential expression is shown as fold change (p-value \leq 0.05). Negative sign of fold change showed that it is down-regulated in the presence of iron or over-expressed in the absence of iron.

hypothesized that iron siderophore receptors are over-expressed under iron depleting conditions (or down-expressed in the iron surplus), whereby siderophores are secreted by the bacteria to chelate iron from the surroundings and transport it through this transmembrane receptor.

It has been also reported that *Pseudomonas aeruginosa*, another important respiratory pathogen, can cause tissue damage by the synergistic interaction between pyocyanin and ferripyochelin [32]. This interaction occurs close to the endothelial cells and leads to the formation of highly toxic hydroxyl radicals that produce local tissue damage at the site of infection. This virulence mechanism may also explain the pathogenesis of the respiratory infections caused by *A. baumannii*. Similarly, bacterioferritin is also down-regulated which is an iron storage protein and has capacity to store very large amount of iron as Fe³⁺ mineral inside its central cavity [33]. It is also involved in storing excess of iron so as to prevent oxidative stress from iron overload [34].

Various outer membrane proteins (spot no. 573, 461 and 1068) are down regulated under iron limiting stage which might help in more iron acquisition but their role in the iron transport is not explained yet. Blast search of these three outer membrane proteins were done and results explain its association with development of carbapenem resistance. Putative outer membrane protein (spot no. 573) is identified as CarO which is associated with uptake of imipenem [35]. It is inactivated

in carbapenem resistant strain [36], therefore we can say that it is involved in the imipenem resistance [37]. CarO is also associated with the transport of a siderophore precursor [38]. Downregulated 29 KDa outer membrane protein (spot no. 461) is known to confer carbapenem resistance [39]. Similarly, downregulated 34 KDa outer membrane protein (spot no. 1068) is also associated with carbapenem resistance [40]. These results suggest that carbapenem-resistant *Acinetobacter* adopted in such a way that under iron starvation, proteins with role in carbapenem resistance also differentially regulated. Therefore, present finding also showed the relation between iron availability and carbapenem resistance.

It is not surprise to observe down-regulation of cell division inhibitor under iron deficiency which might reflect slow division or slow growth of *Acinetobacter*. Cell division inhibitor (spot no 325) of *A. baumannii* which is a membrane ATPase and activates minC. Min system prevents Z rings assembling at the poles. Min C is the primary inhibitor of the system and it appears to inhibit Z ring formation by interacting and destabilizing FtsZ polymers directly [41] thus result into slow growth.

Identification of Upregulated proteins in iron rich conditions

Upregulated proteins in iron surplus condition help in the elevated growth of bacterium and it is classified into different groups.

Protein associated with metabolism and energy production: Increased metabolic activity and energy production are required for pathogen to enhance its growth in the presence of iron. Metabolic enzymes are reported to be present in the membrane fraction [2,23,37,42-44].

Protein spot no. 407 was upregulated by 2.3 fold and identified as malate dehydrogenase (35.3 kDa and pI of 5.2) of *A. baumannii*. Malate dehydrogenase is a part of TCA cycle, an important pathway for energy production and also reported to be found in the membrane fraction [2]. Increased levels of malate dehydrogenase is an attempt to overcome the oxidative stress as seen in *E. coli* [45], during the adaptation from anaerobic to aerobic conditions and also in the biofilm formation in *A. baumannii* [46].

Amino acid biosynthesis is very important for the fast growth of the organism. Ketol-acid reductoisomerase catalyzes the conversion of 2-aceto-2-hydroxyacids to 2-keto-3-hydroxyacids and their subsequent reduction by NADPH to 2,3-dihydroxyacids [47]. This enzyme participates in branched amino acid biosynthesis like valine, leucine and isoleucine. In the presence of iron, elevated production of Ketol-acid reductoisomerase (37kDa) was observed which reflect fast amino acid biosynthesis.

Acetyl-CoA carboxyl transferase (spot no. 574) involved in the carboxylation of acetyl-CoA to malonyl-CoA [48], a precursor of fatty acid synthesis. Elevated Acetyl-CoA carboxyl transferase enhances fatty acid production during iron surplus condition which helps *Acinetobacter* to grow faster.

Enzymes involved in the nucleotide biosynthesis: Nucleotide biosynthesis is very critical for growth of bacteria in host [49]. Nucleoside-diphosphate kinases (spot no. 698) play important role in nucleotide metabolism by generating all nucleoside triphosphates from their corresponding nucleoside diphosphates [50]. Phosphoribosyl aminoimidazole-succinocarboxamide synthase catalyzes the conversion 5-aminoimidazole-4-carboxyribonucleotide to 5-aminoimidazole-4-(*N*-succinyl-carboxamide) ribonucleotide, and reaction of purine biosynthesis pathway. Both these enzymes are overproduced in the presence of iron and facilitate the high level of nucleotide biosynthesis needed for fast growth of *Acinetobacter* in the presence of iron [51].

Protein synthesis machinery: In the presence of iron, bacterium activates the protein synthesis machinery to take care of the increased metabolic activity. It was found that 50S ribosomal proteins (spot no. 723) and 30S ribosomal protein (spot no 681) of the protein synthesis machinery are overproduced. Recently, these proteins are reported in membrane fraction of *A. baumannii* [2,23] and other Gram negative bacteria [52]. Upregulation of elongation factor TS (spot no. 395) is the consequent event of upregulated ribosomal protein and promotes translation. The increased production of ribosomal proteins in the clinical strain may be one of the ways by which organism grow faster in host. In the presence of iron, ribosomal recycling factor (spot no 733) also elevated to enhance the ribosomal recycling which further increases the translation.

Proteins with other functions: 2-hydroxycyclohexanecarboxyl-CoA dehydrogenase of *Acinetobacter baumannii* (28.5kDa and pI 5.28) over-expressed in iron rich condition. This enzyme is a part of pathway for degradation of benzoyl-CoA to 3-hydroxypimeloyl-CoA during the anaerobic benzoate degradation pathway [53]. It catalyzed the oxidation of 2-hydroxy-cyclo-hexanecarboxyl coenzyme-A to

2-ketocyclohexanecarboxyl-CoA, which is a very important step for benzoate degradation [53]. Benzoate is finally converted into the acetyl Co-A [53] which can be used as energy source. Therefore it can be speculated that in the presence of iron, conversion of benzoate to acetyl CoA is promoted but its role related to iron availability is not reported.

Similarly, putative stress protein (15.8 kDa and pI of 5.62) of *Acinetobacter baumannii* showed higher expression under iron rich condition. Iron induces oxidative stress by the Fenton's reaction by producing hydroxyl radicals [34]. Hence, this putative stress protein is overproduced (3.5 fold) under iron surplus conditions to fight against oxidative stress imposed by iron surplus conditions.

Conclusions

Iron regulated membrane proteins help in the transport of iron from the external milieu under iron scarce conditions and proteins associated with the metabolism helps *Acinetobacter* to grow faster under iron surplus conditions. Differentially expressed proteins also associated with carbapenem resistance which might explain differential behaviour of carbapenem resistant strain of *Acinetobacter baumannii* under iron limiting and surplus conditions. Therefore, it can be concluded that membrane proteins and metabolism of *Acinetobacter* have diverse response with availability of iron and carbapenem-resistant *Acinetobacter* develop mechanisms to survive under nutritional immunity imposed by the host and antibiotics load.

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Supporting Information Available

Supplementary Figure: Complete protein identification data of proteins from Mascot.

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