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Deciphering the iron response in *Acinetobacter baumannii*: A proteomics approach

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ABSTRACT

Iron is an essential nutrient that plays a role in bacterial differential gene expression and protein production. Accordingly, the comparative analysis of total lysate and outer membrane fractions isolated from *A. baumannii* ATCC 19606^T cells cultured under iron-rich and -chelated conditions using 2-D gel electrophoresis-mass spectrometry resulted in the identification of 58 protein spots differentially produced. While 19 and 35 of them represent iron-repressed and iron-induced protein spots, respectively, four other spots represent a metal chelation response unrelated to iron. Most of the iron-repressed protein spots represent outer membrane siderophore receptors, some of which could be involved in the utilization of siderophores produced by other bacteria. The iron-induced protein spots represent a wide range of proteins including those involved in iron storage, such as Bfr, metabolic and energy processes, such as AcnA, AcnB, GlyA, SdhA, and SodB, as well as lipid biosynthesis. The detection of an iron-regulated Hfq ortholog indicates that iron regulation in this bacterium could be mediated by Fur and small RNAs as described in other bacteria. The iron-induced production of OmpA suggests this protein plays a role in iron metabolism as shown by the diminished ability of an isogenic OmpA deficient derivative to grow under iron-chelated conditions.

1. Introduction

Acinetobacter baumannii is a Gram-negative aerobic coccobacillus recognized for its ability to cause severe nosocomial infections including pneumonia, urinary tract and burn infections, secondary meningitis and systemic infections [1,2]. More recently, this pathogen has emerged as a threat to soldiers wounded during military operations in Iraq and Afghanistan [3,4]. The high adaptability of this opportunistic pathogen coupled with its ability to resist a wide range of antibiotics and persist in medical environments underscores the clinical threat posed by this pathogen to critically ill hospitalized patients, including those suffering from heavy trauma such as military victims or victims of natural disasters [2,5].

When colonizing a host, bacterial pathogens including *A. baumannii*, must compete with the host for essential nutrients. One of the most coveted nutrients in biological systems is iron, due to its essentiality to almost all living organisms and limited availability under physiological conditions [6]. This micronutrient plays an essential role in a diverse number of cellular processes including electron transport, nucleic acid biosynthesis, and protection from free radicals [7–10]. The iron concentration is tightly regulated within living systems because excess Fe can induce oxidative damage. Thus, in host tissues, the availability of free Fe is minimal, as most Fe is sequestered by high-affinity iron-binding proteins such as transferrin and lactoferrin [7–10]. Although low free-Fe concentration portends a non-specific host defense mechanism against infection, most successful pathogens use it as a

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stimulus to express not only active iron-acquisition systems, as it was described for *A. baumannii* [11], but also the expression of genes coding for virulence factors such as hemolysins, toxins, and proteases [12]. Iron is therefore considered to be not only an essential nutrient, but also an important signal for global regulation of gene expression in prokaryotes [13].

In Gram-negative bacteria, iron-regulated gene expression is generally under the control of the Fur (ferric uptake regulator) protein first described in *Escherichia coli* [14] and produced by a wide range of bacteria. This protein operates as a classical repressor when bound to Fe by inhibiting transcription from iron-regulated gene promoters in response to increase in Fe concentration [15]. Fur has also been shown to indirectly induce gene expression [16–18]. In *E. coli*, the positive regulatory effect of Fur on gene expression involves the Fur-mediated repression of the small RNA (sRNA) RhyB [19,20]. A similar mechanism involving the PrrF1 and PrrF2 sRNA molecules was reported in *Pseudomonas aeruginosa* [21]. Although there is a large body of information related to iron acquisition and gene regulation in Gram-negative bacteria [13], little is known on the effect of this metal on differential gene expression in *A. baumannii*. This pathogen expresses active siderophore-mediated iron-acquisition systems [11] and produces a Fur protein [22,23], which is highly related to a large number of orthologs described in several other bacterial species. This iron repressor controls *A. baumannii* differential gene expression in response to changes in free-iron concentrations in the extracellular environment [23].

The field of proteomics is gaining recognition as a reliable and reproducible high-throughput approach to examine biological processes at the molecular level that in the case of *A. baumannii* has already provided important information on its metabolic versatility [24], the composition of outer membrane vesicles [25] and the effect of antibiotics and salts in differential protein production [26–28]. In this study, we employed a global proteomic approach based on 2-D gel electrophoresis (2-DE) and mass spectrometry to examine the differential production of proteins by the *A. baumannii* ATCC 19606^T type strain when cultured under Fe-replete or Fe-chelated conditions. Our results show that free-iron availability affects the production of proteins involved in siderophore-mediated iron acquisition and storage functions, as well as proteins involved in metabolic processes. Our data also suggest that the expression of iron-regulated genes in this bacterium could be controlled by the iron repressor Fur and potential sRNA regulators.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The *A. baumannii* ATCC 19606^T strain was routinely cultured in Luria–Bertani (LB) broth or agar [29] at 37 °C. Iron-rich and -chelated conditions were achieved by adding 100 μM $FeCl_3$ dissolved in 0.1 M HCl and 100 μM 2,2′-dipyridyl (DIP), respectively. For proteomic experiments, cells were cultured with agitation (200 rpm) for 7 h at 37 °C in 1 ml of unsupplemented ($^-$Fe/$^-$DIP) LB broth or broth supplemented with 100 μM $FeCl_3$ ($^+$Fe/$^-$DIP), 100 μM DIP ($^-$Fe/$^+$DIP), or 100 μM DIP plus 100 μM $FeCl_3$ ($^+$Fe/$^+$DIP). The last three culture conditions were used to detect the production of iron-induced and iron-repressed proteins and determine whether protein changes observed in the presence of DIP were due to iron chelation, respectively. Each 1-ml culture was used to inoculate 100-ml LB broth samples of the corresponding composition that were incubated with shaking (200 rpm) at 37 °C for 14 h, at which time cultures reached stationary phase as determined by monitoring cell growth spectrophotometrically at $OD_{600\ nm}$. Five culture replicates were used for each treatment condition and the entire culture procedure was repeated three times using fresh biological samples each time. Replicate samples cultured under the same conditions were pooled together and the cells were collected by centrifugation at 7000 *g* for 10 min at 4 °C. The cell pellets were suspended in 10 ml Tris-buffered saline (TBS) [30] and stored at −80 °C as the sample stock for each treatment.

2.2. Protein extraction

Total cell (TC) protein samples were prepared by collecting cells from 1 ml of each sample stock by centrifugation at 7000 *g* for 10 min at 4 °C. Cell pellets were suspended in 0.5 ml lysis buffer [5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM EDTA, 0.5% Tween-20, 25 mM Tris–HCl pH 7.0] and lysed by sonication while keeping samples on ice. Cell debris and unlysed cells were collected by centrifugation as described above and the supernatants, representing the TC protein samples, were carefully decanted into clean tubes and stored at −80 °C. Outer membrane (OM) enriched fractions were prepared by collecting cells from 9-ml sample stocks as described above. Cell pellets were rinsed with 25 ml ice-cold HEPES buffer (5 mM $MgSO_4$, 1 mM PMSF, 2 mM EDTA, 50 mM HEPES potassium, pH 7.4) and then suspended in 10 ml ice-cold HEPES buffer and lysed by sonication on ice. After removing cell debris and unlysed cells as described above, the supernatants were centrifuged at 200,000 *g* for 1 h at 4 °C. The pellets were washed unperturbed three times with 5 ml ice-cold HEPES buffer, suspended in 10 ml ice-cold HEPES buffer containing 2% Triton X-100 and incubated on ice for 1 h. The suspension was centrifuged again at 200,000 *g* for 1 h at 4 °C and the pellet representing the OM fraction was suspended in 5 ml ice-cold HEPES buffer and stored at −80 °C until further analysis.

To precipitate proteins, one volume of ice-cold acetone was added to TC and OM fractions followed by a 30-min incubation on ice and centrifugation at 10,000 *g* for 10 min at 4 °C. The pellet was solubilized in 0.5 ml of rehydration/isoelectric focusing (IEF) buffer [8 M urea, 50 mM DTT, 4% (w/v) CHAPS, 0.2% (v/v) 3/10 ampholytes and 0.002% (w/v) bromophenol blue]. Insoluble material was removed by centrifugation at 10,000 *g* for 10 min at RT and the protein content of the supernatant was determined with the bicinchoninic acid (BCA) protein quantification assay following the manufacturer's instructions (Pierce, Rockford, IL, USA).

2.3. 2-DE fractionation and image analysis

For first dimension electrophoresis, 11-cm long pH 4–7 ReadyStrip IPG strips (Bio-Rad, Hercules, CA, USA) were