APPLICATION NOTE

ICP - Mass Spectrometry

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Iron Content Measurement in Individual Bacterial Cells Using SC-ICP-MS

Introduction

Iron is an essential metal cofactor for diverse biological processes within the bacterial cell. It is often a growth-limiting nutrient in bacteria, and the total iron quota for a cell will depend on the cells' growth states and metabolic requirements. Bacteria have evolved complex systems to regulate iron levels within the cell.[1] Excess soluble iron within the cell is toxic due to formation of reactive oxygen species that damage cellular components, meaning that iron levels must be tightly regulated. However, the variability of iron regulation within individual cells and throughout cell populations has not been determined. Quantifying iron in bacterial cells in near real time could provide insight into the limits of bacterial iron tolerance when defining the effects of growth conditions and stress responses, including those due to antibiotics. Interestingly, some mineral clays and clay extracts that are bactericidal are high in dissolved iron, which may disrupt bacterial iron homeostasis, leading to death.[2] Moreover, monitoring iron levels within single cells will provide the distribution of cellular iron to determine the homogeneity of a population.

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Total cellular metals in bacteria can be measured from batch cultures using ICP-MS, and estimates of average iron content in individual bacterial cells are calculated from measurements of the total number of cells.[3] Errors arise from the presence of dead cells or intact cells that are non-cultivable by plate methods. However, direct measurement of the iron concentrations in individual bacterial cells has not been possible due to instrumental limitations.

Recent advances in single cell ICP-MS (SC-ICP-MS) allow for the metal concentration of individual cells to be measured directly on an individual cell basis. PerkinElmer's new Asperon™ single cell introduction system introduces individual intact cells into the plasma of an ICP-MS. The introduction system, combined with fast acquisition times of the NexION® series ICP-MS, means that the amount of iron within individual bacteria cells can be quantified. Here we quantify the amount of Fe per cell for three bacterial strains: *Escherichia coli B* (Eco), *Bacillus subtilis 168* (BAC), and *Rhodococcus jostii RHA1* (RHA), using SC-ICP-MS. This technique allowed for the quantification of the mean Fe concentration per cell, as well as the distribution of Fe levels for each strain. The Fe content correlated to the cellular size of the bacteria with the largest strain (RHA) containing the most Fe and the smallest strain (Eco) containing the least average Fe content per cell.

Experimental

Bacterial Cultures

Three different non-pathogenic bacterial species, *Escherichia coli B, Bacillus subtilis 168,* and *Rhodococcus jostii RHA1,* were analyzed in this experiment. Cell sizes were previously reported as approximately 2 µm, 4 µm and 10 µm ± 2, respectively.[4-6] Single isolated colonies from streak plates of each bacterium were used to inoculate cultures in three different 5 mL test tubes of Luria-Bertani (LB) media. Eco and BAC were grown overnight in an incubator shaker at 37 °C and 200 rpm, while RHA was grown overnight at 30 °C and 200 rpm.

An aliquot (1 mL) from each overnight culture was used to inoculate three pre-warmed flasks containing 250 mL of LB media and placed back into the incubator shaker to continue growing at the above mentioned conditions. Aliquots (1 mL) were taken from each flask periodically to monitor optical density (OD_{600}) on a Cary 50 Bio UV-Visible spectrophotometer (Varian). Eco and BAC were grown to late exponential phase, with a final OD_{600} of 1.7 and 1.4 respectively. RHA was grown to late stationary phase with a final OD_{600} of 2.3.

Cultures were aliquoted in 1 mL samples and stored in 50% glycerol and at -20 °C until SC-ICP-MS analysis was performed. Bacterial cell concentrations were determined by plating and counting CFUs per mL.

SC-ICP-MS Analysis

Bacteria cell samples (Table 1) were thawed for one min in a water bath at 35 °C, then kept on ice until diluting to 100,000 cells/mL^{-1} in 1% phosphate buffered saline (PBS) immediately prior to analysis with SC-ICP-MS. The samples were thawed separately prior to use to prevent degradation of the bacteria over time. Data was acquired for one minute at dwell time of 50 µs. Each measurement was repeated three times. A total sample volume of 100 µL was consumed for the measurements. The argon-oxide interference on ^{56}Fe was eliminated by flowing pure ammonia gas into the reaction cell (Reaction mode). SC-ICP-MS conditions can be found in Table 2.

Table 2. NexION ICP-MS operating conditions.

Parameter	Value
Sample Uptake Rate	19.5 µL/min
Nebulizer	HEN MEINHARD® glass concentric
Spray Chamber	Asperon Single-Cell
Injector	2.0 mm id Quartz
RF Power	1500 W
Neb Gas Flow	0.34 L/min
Makeup Gas Flow	0.7 L/min
Analyte	^{56}Fe
Reaction Mode	Ammonia at 0.35 mL/min, RPq=0.5
Transport Efficiency	33%

Transport efficiencies (TE) were calculated using NIST 60 nm (8013) Au particles at a concentration of 50,000 part/mL and Fluidgim 2.5 µm polystyrene beads laced with lanthanide metals (Ce, Eu, Ho, and Lu) at a concentration of 33,000 part/mL. Dissolved calibrations were performed with Fe standard at concentrations of 1, 2, and 3 ppb. All calibration standards were matrix matched with the samples and prepared in 1% PBS.

ICP-MS Analysis

Eco, BAC, and RHA bacteria were grown to the same optical density as for SC-ICP-MS analysis and centrifuged to a bacterial cell pellet. The samples were immediately frozen, freeze-dried, and homogenized. Samples (10 - 30 mg) were weighed into Savillex® PFA Vials. Closed-vessel digestion was performed on a hotplate with Optima® grade concentrated nitric acid. The samples were dried after the digestion and re-dissolved in 5 mL of 0.05 M HNO_3. National Research Council of Canada: NRCC lobster hepato-Pancreas (TORT-1) was used as a certified reference material. Reaction mode with pure ammonia was used for ^{56}Fe analysis (ICP-MS conditions found in Table 2).

Table 1. Bacteria cell samples for SC-ICP-MS analysis.

Sample Name	Description	Cell Concentration (CFU/mL)	Estimated Cell Length (µm)
Eco	Eco in LB media and 50% glycerol	6.0×10^8	2
BAC	BAC in LB media and 50% glycerol	8.5×10^7	4
RHA	RHA in LB media and 50% glycerol	9.5×10^7	10
Blank	LB media and 50% glycerol	n/a	n/a

Results and Discussion

Control Samples

Control samples of 1% PBS and a blank LB media in 50% glycerol (treated identically to the bacteria cell samples) were analyzed. Figure 1 show typical scans for control samples of 1% PBS and a blank media sample treated in the same way as the bacteria samples. In both cases, the control signals showed no signal of iron.

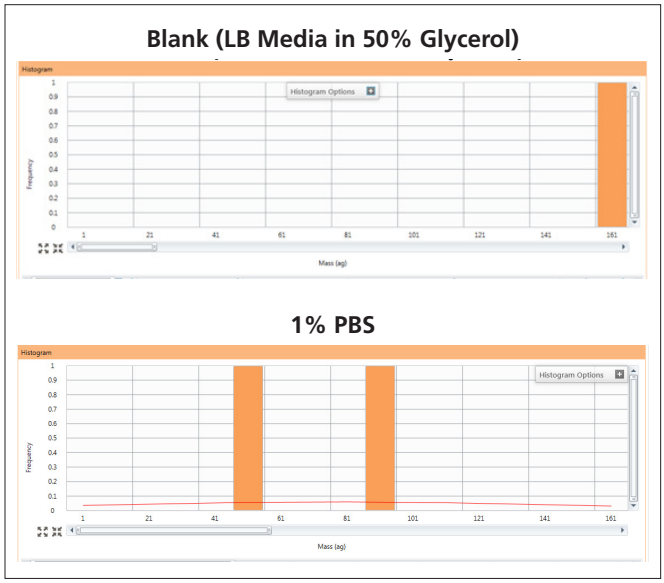

Figure 1. ^{56}Fe Scans for a blank sample of LB media in 50% glycerol treated in the same way as the bacteria samples, and 1% PBS blank.

Bacteria Measurements

Figure 2 depicts typical ^{56}Fe scans for Eco, BAC, and RHA bacteria at cell concentrations of 50,000 cells/ mL. The average amount of ^{56}Fe per bacteria differs for each strain, with Eco cells containing the least and RHA cells containing the most. The percentage variation in Fe per cell is similar for all samples. The distributions of cell lengths and concentrations of iron per cell can be attributed to the diversity found within an actively dividing population of cells. Within cell cultures, the majority of cells will be in a mature single-cell state, with a small proportion of the population undergoing division. In preparation for division, cells grow larger such that one cell can divide into two cells. The diversity within the population may account for the observed results, where most cells fit within the normal range and a small proportion of the cells are longer and contain more iron. In addition, R. *jostii* (RHA) cells are noted to be "sticky", and a small minority of the single cell measurements could show aggregates of two or more cells. To that, we observe a secondary distribution at the 160-170 ag mark corresponding to an aggregate of two cells.

Serial dilutions of the bacterial cells were measured to test for dual coincidences (two or more cells entering the plasma concurrently). Figures 3A and 3B show that the average mass of Fe per cell does not change with serial dilutions of 100,000, 75,000, and 50,000 cells/mL, whereas the number of cells counted for each dilution changes linearly, indicating no significant influence of multiple coincidences due to cell concentration.

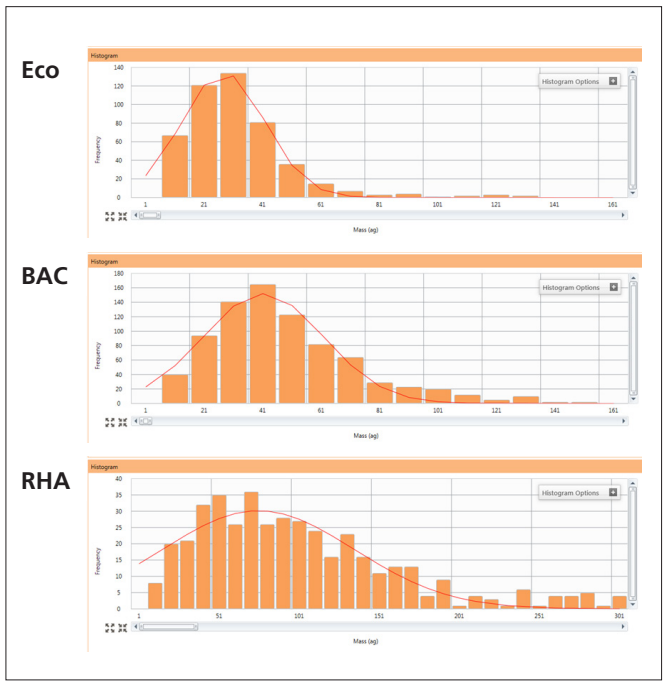

Figure 2. ^{56}Fe Scans for Eco, BAC, and RHA at cell concentration of 50,000 cells/mL showing distributions of single cell iron content. Frequency of cells containing a given amount of Fe (ag) is given for Eco, BAC, and RHA, with Eco having the lowest average amount of Fe per cell and RHA having the highest.

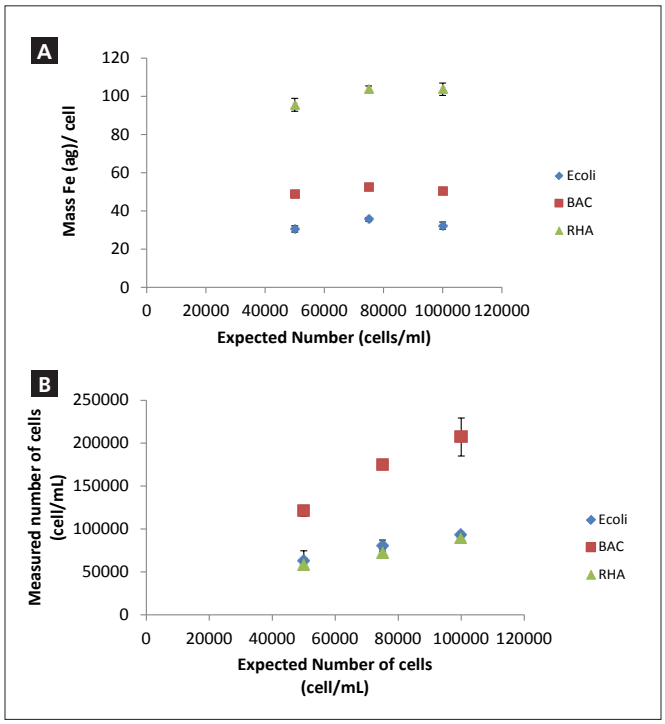

Figure 3. (A) Expected number of cells for three bacterial samples (Eco, BAC, and RHA) at dilutions of 100,000, 75,000, and 50,000 cells/mL in relation to the amount of Fe (ag) per cell. For all three bacterial samples measured, the number of cells in a given sample appears to have little effect on the amount of Fe per cell measured. (B) Expected number of cells in dilutions of 100,000, 75,000, and 50,000 cells/mL in relation to the measured number of cells for each dilution. For all three bacterial samples, there appears to be a linear relationship between the expected and measured number of cells for a given dilution.

Relating Amount of Fe to Bacterial Length

The average amount of Fe per cell for the three bacterial strains can be plotted against the estimated length for each strain. Length is used as a proxy for bacterial volume. Figure 4 shows that there is a linear relationship between the amount of Fe contained in the bacteria and their length.

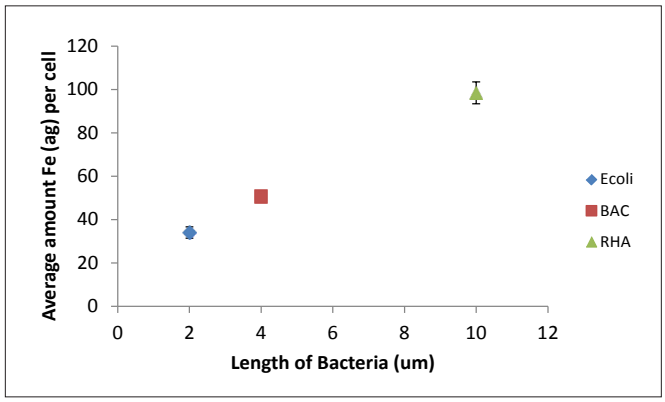

Figure 4. Length of single bacterial cells versus the average amount of Fe (ag) measured per cell. There appears to be a positive correlation between cell length and the amount of Fe measured per cell, with the longest bacterium (RHA) containing the most Fe per cell, and the shortest (Eco) containing the least.

Conclusion

This work demonstrates that Single Cell ICP-MS is able to quantify the amount of Fe in individual bacterial cells. Moreover, this method provides the distribution of Fe per cell in bacterial cultures. The data supports a model of tight regulation of total Fe in bacterial cells. SC-ICP-MS has the potential to analyze the distribution of Fe in bacterial cells grown under varying stress conditions.

References

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Consumables Used

Component	Description	Part Number
Spray Chamber Drain Tubing	Orange/red (1.30 mm id), PVC, flared, pack of 12	N0773111
Iron (Fe) Stock Standard	1000 ppm Fe, 125 mL	N9303771
50 nm Au Nanoparticles	1e7 particles/mL, 25 mL	N8151035
PFA Sample Vials	1 mL, pack of 10 2 mL, pack of 10	N0777403 N0777404

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