Unravelling a new role for bacterioferritin (BfrB) in *Pseudomonas aeruginosa*:
a step toward rational targeting of bacterial iron homeostasis

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May 19th, 2017

2017 KU Chemistry Alumni Symposium
Each year, there are about 2.3 million drug resistance cases.

In 2013, CDC published a report outlining the top 18 drug-resistant threats to the United States.
Gram-negative opportunistic pathogen

Pyocyanine and pyoverdine secretion
A complex Iron homeostasis machinery

Cell metabolism

Cell metabolism

H_{2}O_{2}

OH^{-} + OH^{-}

DNA damage

Bfd

O_{2}, H_{2}O_{2}

H_{2}O_{2} + H_{2}O

SOD

Catalase

H_{2}O + O_{2}

Fe^{3+} + Siderophore

Fe^{2+}

Fe^{3+}

Heme

O_{2}

Biliverdin

e^{-}

Heme Oxygenase

PhuS

Feo

Heme
The coexistence of two iron storage proteins in P. A.: BfrB (bacterioferritin) and FtnA (ferritin)

Less than 20% sequence similarity: very different charge distribution, packing and function.

Biochemistry, 2011, 50, 5236-5248
Biochemistry, 2010, 49, 1160-1175
Iron mobilization from BfrB need its interaction with Bfd, not for FtnA

1. In *P. aeruginosa*, *bfrB* gene is adjacent to the *bfd* gene.

2. *bfd* gene upregulated about 200-fold under low iron conditions and *fpr* (ferredoxin reductase) expression level increased for about 3 fold.

3. In *P. aeruginosa*, *ftnA* gene is adjacent to the *katA* gene, which codes for a heme catalase (KatA)
BfrB:Bfd Interaction and two hot-spot residues identified

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_d$ (µM) from SPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td>BfrB E81A</td>
<td>258.5 ± 21.5</td>
</tr>
<tr>
<td>BfrB L68A</td>
<td>298.5 ± 20.5</td>
</tr>
<tr>
<td>BfrB L68A/E81A</td>
<td>Not measureable</td>
</tr>
</tbody>
</table>

JACS 2012, 134, 13470-13481
Biochemistry 2015, 54, 6162-6175
Study the contributions of BfrB and the BfrB:Bfd interaction to bacterial iron homeostasis in-cells

<table>
<thead>
<tr>
<th><strong>P. aeruginosa strains</strong></th>
<th><strong>Description</strong></th>
<th><strong>Reference</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1</td>
<td>Wild type strain</td>
<td>Other study</td>
</tr>
<tr>
<td>PAO1 $\Delta$bfrB</td>
<td>PAO1 containing an unmarked, in-frame $bfrB$ deletion</td>
<td>This study</td>
</tr>
<tr>
<td>PAO1 $\Delta$bfd</td>
<td>PAO1 containing an unmarked, in-frame $bfd$ deletion</td>
<td>This study</td>
</tr>
<tr>
<td>PAO1 $bfrB(L68A/E81A)$</td>
<td>PAO1 a gene encoding the BfrB L68A/E81A allele at the native $bfrB$ locus</td>
<td>This study</td>
</tr>
<tr>
<td>PAO1 $\Delta$bfrB $\Delta$tn7::plac$bfrB$</td>
<td>Made by introducing pUC18-miniTn7T-LAC $bfrB$ to PAO1 $\Delta$bfrB and removing the GentR marker</td>
<td>This study</td>
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<td>This study</td>
</tr>
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</table>

Rivera et al. Metallomics, 2017, DOI:10.1039/c7mt00042a
**P. aeruginosa** store iron in BfrB, but not in FtnA

- Native PAGE gels, staining with Ferene S.
- Electrophoretic mobility of mineralized recombinant FtnA and BfrB is different.
- In WT, iron-stained bands corresponded to BfrB, not FtnA.
- When BfrB is absent, *P. aeruginosa* cells do not accumulate iron in FtnA.
- The function of FtnA in *P. aeruginosa* is unknown, which is in contrast to the findings in *E. coli*.

6-14h cell lysates from iron sufficient media
Bfd is required for the mobilization of iron from BfrB in *P. aeruginosa*

- Cells harvested at different hours post inoculation.
- **(a)** In wild type cells, the amount of iron accumulated in BfrB reached maximum at early stationary phase (~ 12 h)
- **(b and c)** In mutant Δbfd and bfrB(L68A/E81A) cells, iron can not be mobilized from BfrB
- The phenotypes of the ΔbfrB and Δbfd mutants can be restored (d-g)
Iron deficiency in $\Delta bfd$ and $bfrB(L68A/E81A)$ mutants cells

PIA plates, culturing for 22 h at 37 °C

- Iron trapped irreversibly in BfrB
- Cells senses low iron conditions
- Upregulate the synthesis of siderophores, such as pyoverdin (Pvd)
Monitor iron starvation in $\Delta bfd$ and $bfrB(L68A/E81A)$ mutants

$\Delta bfd$ and $bfrB(L68A/E81A)$ strains sense iron deprivation more acutely than wild type

Kate Eshelman

Iron levels in liquid media

There is a slow rate of iron internalization in $\Delta bfrB$ cells.
To establish a direct correlation between cellular iron levels and the observed phenotype of acute iron deprivation

Free intracellular iron is iron not stably incorporated into macromolecules (free iron pool)---whole cell EPR using a cell permeable iron chelator DFO (desferroxamine mesylate)

Total Fe are all iron in the cell, including free and stably incorporated into macromolecules---Ferrozine-Fe complex and UV-Vis spectrophotometry

Measure cellular iron levels (both total and free) in wild type and mutants
Using whole-cell EPR measure the intracellular free iron level in wild type and mutants.

- First derivative EPR signal with a $g=4.3$
- Bruker EMXplus spectrometer

![Graph showing EPR signal comparison between wild type and mutants](image)

<table>
<thead>
<tr>
<th>Rec.</th>
<th>BfrB</th>
<th>6 h</th>
<th>8 h</th>
<th>12 h</th>
<th>14 h</th>
<th>18 h</th>
<th>24 h</th>
<th>36 h</th>
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<tbody>
<tr>
<td>a</td>
<td>wt</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>ΔbfrB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>Δbfd</td>
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<tr>
<td></td>
<td>bfrB(L68A/E81A)</td>
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<td></td>
<td></td>
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</tbody>
</table>

wild type
ΔbfrB
Δbfd
bfrB(L68A/E81A)
Free iron levels in Δbfd and bfrB (L68A/E81A) cells are lower than that of wild type cells.

Wild type and Δbfd and bfrB (L68A/E81A) have similar total iron levels at 12 h and 18 h.

In the absence of BfrB, ΔbfrB cells can prevent accumulation of toxic levels of free iron in the cytosol. And the compensatory mechanism does not involve FtnA.
Dynamic equilibrium between free iron in cytosol and iron stored in BfrB

**Normal BfrB:Bfd interaction**

- $\text{Fe}^{2+}$
- $\text{Fe}-\text{utilizing proteins}$
- $\text{BfrB}$
- $\text{Fur}$

- $\text{O}_2 \text{ or } \text{H}_2\text{O}_2$ between $\text{Fe}^{2+}$ and $\text{Fe}^{3+}$

**Disrupted BfrB:Bfd interaction**

- $\text{Fe}^{2+}$
- $\text{Fe}-\text{utilizing proteins}$
- $\text{BfrB}$

- $\text{O}_2 \text{ or } \text{H}_2\text{O}_2$ between $\text{Fe}^{2+}$ and $\text{Fe}^{3+}$

**Fur: iron uptake regulator**
Iron stored in BfrB provides a source of iron for bacterial growth in iron limiting conditions.

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**Figure a**

<table>
<thead>
<tr>
<th>Rec. BfrB</th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
<th>36h</th>
<th>48h</th>
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<tr>
<td>10 μM Fe</td>
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**Figure c**

<table>
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<th>Rec. BfrB</th>
<th>6h</th>
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<td>30 μM Fe</td>
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**Figure e**

<table>
<thead>
<tr>
<th>Rec. BfrB(L68A/E81A)</th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
<th>36h</th>
<th>48h</th>
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<tbody>
<tr>
<td>10 μM Fe</td>
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<td><img src="image34.png" alt="Image" /></td>
<td><img src="image35.png" alt="Image" /></td>
</tr>
<tr>
<td>20 μM Fe</td>
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<td><img src="image38.png" alt="Image" /></td>
<td><img src="image39.png" alt="Image" /></td>
<td><img src="image40.png" alt="Image" /></td>
</tr>
<tr>
<td>30 μM Fe</td>
<td><img src="image41.png" alt="Image" /></td>
<td><img src="image42.png" alt="Image" /></td>
<td><img src="image43.png" alt="Image" /></td>
<td><img src="image44.png" alt="Image" /></td>
<td><img src="image45.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Summary

1. *P. aeruginosa* store Fe in BfrB, not in FtnA.

2. BfrB in *P. aeruginosa* store iron for subsequent utilization when cells is challenged with low iron conditions.

3. Utilization of reserved iron requires Bfd:BfrB interaction, which is necessary for the electron delivery to the cavity.

4. BfrB:Bfd interaction is of widespread importance to bacterial iron homeostasis
Acknowledgements

Dr. Mario Rivera’s research Group
Achala Punchi Hewage
Dr. Anable Soldano
Harshani Wijerathne
Thilanga Nandana
Kevin Tyner
Dr. Kate Eshelman (QuintilesIMS)

Dr. Josephine Chandler
Jacqueline Deay

Protein Structure Core Lab
Dr. Scott Lovell

Funding
NIH (AI125529 and P20GM103638)
NSF (MCB-1615767)
2014 KU Strategic grant (Level 1)