

# Top-down MS Analysis of Microbial Growth States from *Rhodopseudomonas palustris* by Off-Line FPLC Fractionation and Capillary HPLC Interfaced to FTICRMS

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## OVERVIEW

- Purpose**
  - To integrate HPLC methods with a high resolution FTICR-MS to perform a global top-down analysis of *Rhodopseudomonas palustris* under two growth states
  - To evaluate the LC-FTICR-MS methods on intact proteins from off-line FPLC fractionation
  - To identify isoforms and PTMs of microbial proteins from *R. palustris*
- Methods**
  - Off-line ammonium acetate anion exchange FPLC fractionation was performed on whole cell extracts from aerobic and anaerobic *R. palustris*
  - Capillary LC-FTICR-MS was performed on intact microbial proteins from *R. palustris*
  - Integrated top-down and bottom-up MS characterization was performed on all anion exchange fractions
- Results**
  - Rhodopseudomonas palustris* protein isoforms and PTMs were identified from anaerobic and aerobic growth states
  - 200 - 400 total intact proteins were identified by the integrated top-down and bottom-up approach from the two growth states
  - Off-line anion exchange FPLC fractionation provided sufficient protein quantities for integrated top-down and bottom-up approach

## INTRODUCTION

- Intact protein or "top-down" mass spectrometry provides information on the natural state of the protein [2,3]**
  - This technique yields details about post-translational modifications (PTMs), truncations, mutations, signal peptides, and isoforms
- Intact proteins vary considerably more than peptides in terms of molecular mass, hydrophobicity, and stability**
  - Due to this difference, the interfacing of HPLC on-line with MS provides a significant advantage for the top-down MS measurements
- The dynamic range, sensitivity, and mass accuracy offered by high performance FTICR-MS affords**
  - Unambiguous protein identification in many cases
  - Detailed information about protein modifications
- This approach has been developed and evaluated for characterizing native and modified proteins from the microbe *Rhodopseudomonas palustris* in the anaerobic and aerobic growth states (Figure 1).**

## EXPERIMENTAL

### *R. palustris* Cell Growth and Protein Preparation

- Wild type *R. palustris* was grown under aerobic and anaerobic growth states (Figure 1)
- 4-5 liters of cells were grown and pooled together for each growth state
- The cell pellet from each growth state was French Pressed to yield 60-120 mg of protein for each of the two growth states
- Cell extract was centrifuged at 10,000g for 35 minutes in Sorvall centrifuge to remove all unbroken cells
- Protein extract was used for off-line anion exchange FPLC fractionation

### Off-line Anion Exchange FPLC

- To perform off-line anion exchange chromatography 60 mg of protein was injected onto a 5 ml HiTrap SP HP, Amersham Pharmacia) ion exchange column connected to a AKTA (Amersham Pharmacia) FPLC system
- After protein injection a 30 minute ammonium acetate gradient was run from 0.2 M to 2 M (Figure 2)
- 20 fractions from each growth state (total of 40 from 2 growth states) were determined to have sufficient protein concentrations (400 µg) by a Bradford protein assay

### Capillary LC-FTMS

- All Capillary LC-FTICR-MS experiments were performed with an Ultimate HPLC (LC Packings, a division of Dionex, San Francisco, CA) coupled to a IonSpec FTICR (9.4 T magnet) mass spectrometer (Lake Forest, CA) equipped with an Analytica electrospray source (Figure 3). Injections of 20-50 µg of total protein were made onto a 100 µl loop. Flow rate was ~4 µl/min with a 75min gradient.
- A VYDAC 214MS5.325 (Grace-Vydac, Hesperia, CA) C4 column (300µm id x 250 mm, 300 Å with 5 µm particles) was directly connected to the Analytica electrospray source with 100 µm i.d. fused silica.
- For all mass spectra acquired, 2 scans were signal averaged with 1024k data points and a 2 second hexapole ion accumulation time. Mass accuracies to 5 ppm were achieved with mass resolutions of 50,000 to 100,000 FWHM.

### Integrated Top-down and Bottom-up Approach

- All collected FPLC fractions were divided in half, with one half going to top-down and the other half going to bottom-up analysis (Figure 4)
- Bottom-up experiments provide a comprehensive list of proteins in each fraction against which the top-down data can be searched
- Bottom-up experiments were performed using trypsin digestion with 1D-nano-HPLC on a Deca XP quadrupole ion trap. Peptide searching was performed with SEQUEST.



Figure 1: *Rhodopseudomonas palustris* liquid and plate cultures. *R. palustris* is a widely distributed bacteria exhibiting remarkable metabolic diversity.

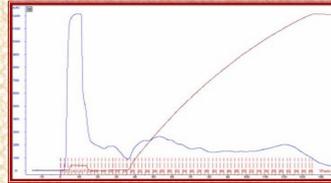


Figure 2: Ammonium acetate anion exchange FPLC chromatogram of *R. palustris* from the aerobic growth state



Figure 3: IonSpec 9.4T FTICR-MS

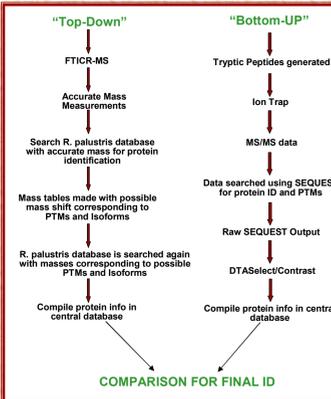


Figure 4: Flow chart of integrated top-down bottom-up approach

### Off-line FPLC Fractionation Followed by On-line HPLC FTICR-MS

- Ammonium acetate anion exchange fractionation was performed for an anaerobic and an aerobic growth state
- UV absorbance and conductivity were plotted across the entire chromatogram. The location of individual fractions is shown at the bottom of the FPLC chromatogram (Figure 5)
- 20 fractions from each growth state were further analyzed by on-line HPLC FTICR-MS
- The total ion chromatograms from aerobic FPLC fraction 28 and anaerobic FPLC fraction 27 are shown with an individual mass spectrum labeled from each TIC (Figure 5)

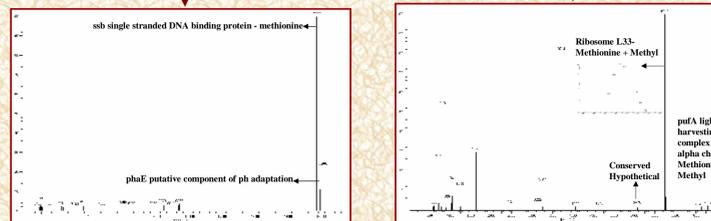
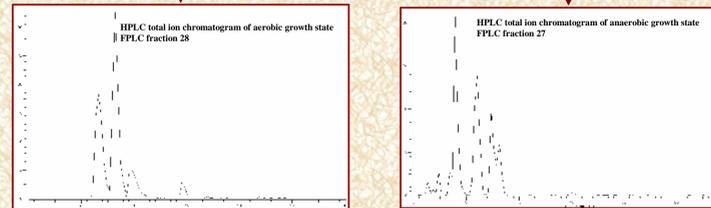
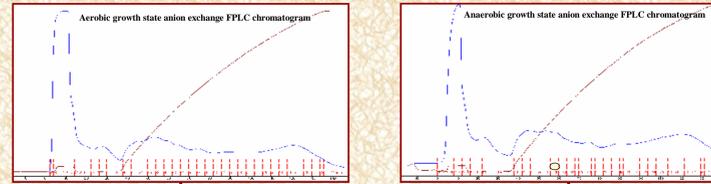


Figure 5: Anaerobic and aerobic growth states, FPLC chromatograms with an individual fraction HPLC-TIC and mass spectrum

### Hypothetical proteins

- Total ion chromatogram of aerobic growth state FPLC fraction 28 with corresponding mass spectrum 29
- Mass spectrum 29 shows a number of different hypothetical proteins tentatively identified (Figure 6)

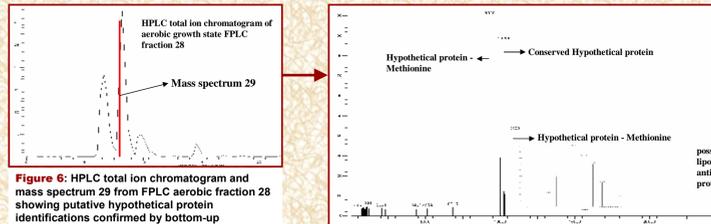


Figure 6: HPLC total ion chromatogram and mass spectrum 29 from FPLC aerobic fraction 28 showing putative hypothetical protein identifications confirmed by bottom-up

## RESULTS

### Identification of large intact proteins from the aerobic and anaerobic growth states

- Intact proteins ranging in size from 5-50 KDa were tentatively identified by HPLC FTICR-MS for both the aerobic and anaerobic growth states
- Examples include the putative identification of arsB arsenical pump protein at 44457.058 Da (Figure 7) found in anaerobic FPLC fraction 4 spectrum 49
- Also tentatively identified are intact proteins in the 20-30 KDa mass range from both the aerobic and anaerobic growth states
- In spectrum 28 from aerobic FPLC fraction 5, several large intact proteins ranging from 19-23 KDa are present. These include the superoxide dismutase protein at 22385 Da and a conserved unknown protein at 21209 Da (Figure 8)
- Mass Spectrum 30 from anaerobic fraction 25 shows the FUR protein that has a mass of 16719 (ferric uptake regulator protein) with a methionine truncation (Figure 9)

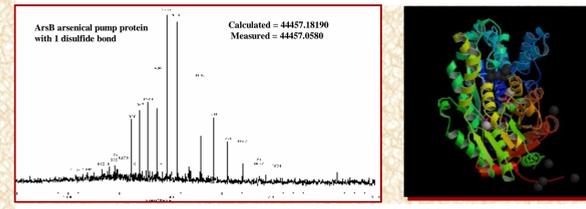


Figure 7: Mass spectrum 49 from anaerobic FPLC fraction 4 showing the putative identification of ArsB arsenical pump protein with one disulfide bond. Structure of *E. coli* ArsB is also shown (PDB 1F48).

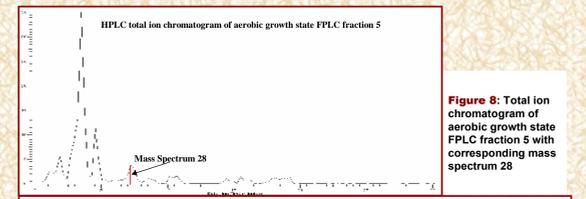


Figure 8: Total ion chromatogram of aerobic growth state FPLC fraction 5 with corresponding mass spectrum 28

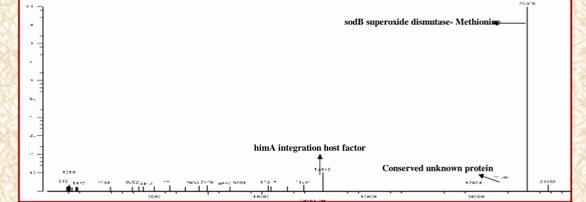


Figure 9: Mass spectrum 30 from FPLC anaerobic fraction 25 showing the FUR protein with a tentative methionine truncation. The structure of the analogous *P. aeruginosa* FUR is also shown (PDB 1M2B).

### Anaerobic growth state reveals a unique operon containing a series of five hypothetical proteins [1]

- Four of the five unique unknown proteins are identified by top-down and bottom up analysis
- Two of the unknown proteins from the unique operon were found in top-down analysis with putative PTMs and isoforms
- RPA 2335 is found within one mass spectrum with four isoforms - native, 1 methylation, 2 methylations, 3 methylations (Figure 10-A)
- RPA2336 was also identified by top-down analysis with two isoforms - native, 1 methylation (Figure 10-B)

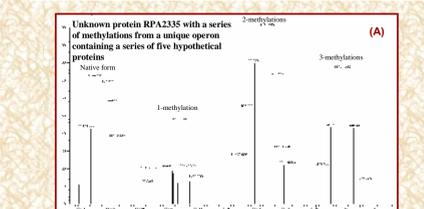


Figure 10: (A) Mass spectrum of RPA2335 from a unique anaerobic unknown operon showing the native, 1 methylation, 2 methylations, and 3 methylations isoforms. (B) Mass spectrum of RPA2336 from a unique anaerobic unknown operon showing the native protein and isoform with 1 methylation

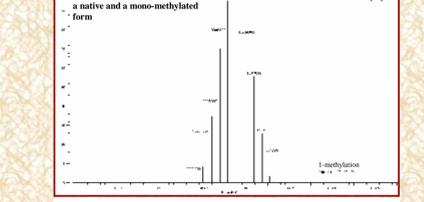


Figure 11: Unknown protein RPA1495 found only in the anaerobic growth state

### Modified intact proteins found only in one growth state

- 6 intact proteins were found only in the anaerobic growth state (Table 1)
- All six proteins have putative PTMs and isoforms
- Unknown proteins RPA1495 is found only in the anaerobic growth state and is methionine truncated (Figure 11)

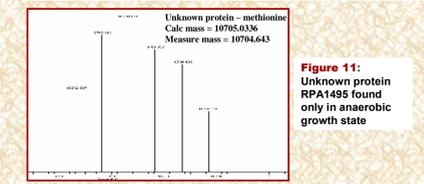


Figure 11: Unknown protein RPA1495 found only in the anaerobic growth state

Protein	Putative PTMs
RPA2334	Methionine truncation
RPA2335	1,2,3 methylations
RPA2336	1 methylation
RPA2338	Methionine truncation
RPA1495	Methionine truncation
RPA1620	Methionine truncation, 1 methylation

Table 1: Table of unknown proteins found only in the anaerobic growth state

## CONCLUSION

- 200-400 non-redundant intact proteins were preliminarily identified by top-down analysis from the two growth states
  - Aerobic - 219 correlated with bottom-up
  - Anaerobic - 426 correlated with bottom-up
- Off-line anion exchange FPLC fractionation provided sufficient protein quantities for top-down intact protein analysis
  - 20 FPLC fractions from each growth state were analyzed by an integrated top-down and bottom-up approach
- Intact hypothetical proteins were identified by top-down MS to initiate studies into function and location of the proteins
- Large proteins in the 20-50 KDa range were tentatively identified with HPLC FTICR-MS
  - ArsB arsenical pump protein at 44457 DA was tentatively identified with a disulfide bond
  - The ferric up-take regulation protein (FUR) was also identified with a methionine truncation
- An unique operon containing a series of five hypothetical proteins in the anaerobic growth state was identified by top-down MS
  - The native form and 3 isoforms with methylations were putatively identified for RPA2335 from the unique unknown operon
  - The native form and 1 isoform containing a methylation was identified for RPA2336 from the unique unknown operon
- Six unknown proteins found only in the anaerobic growth state were tentatively identified with a series of PTMs

## ACKNOWLEDGMENTS

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