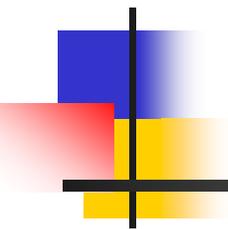


# *Mass Spectrometric Analysis of Protein Complexes Isolated from Rhodospseudomonas palustris*

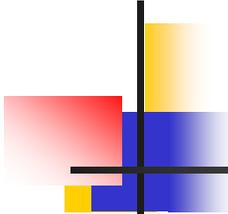


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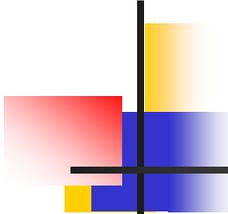
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University of Tennessee



# Overview

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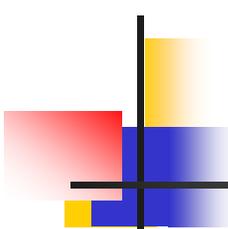
- Initial target proteins in *Rhodopseudomonas palustris* have been expressed as fusions with affinity labels to enable isolation of protein complexes.
- The GroELS and nitrogenase complexes have been affinity-isolated and characterized by MS.
- The 70S ribosome from *R. palustris* has been isolated biochemically, and its subunits characterized by “bottom-up” and “top-down” MS.



# Introduction

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- The bacterial species *Rhodospseudomonas palustris*
  - occurs widely in the environment
  - survives in a variety of conditions
    - light / dark
    - aerobic / anaerobic
- This species thus has the potential to express markedly different complements of proteins and protein complexes under different growth conditions.
- As part of a center funded by the U.S. Department of Energy Genomes To Life program [1], we are analyzing protein complexes from *R. palustris* by expressing target proteins as fusions with affinity tags to allow subsequent isolation of other proteins associated with the target [2], followed by both “top-down” and “bottom-up” mass spectrometry analysis [3]. For comparison, we have isolated the 70S ribosome from *R. palustris* and analyzed by mass spectrometry.



# Methods

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- Selected *R. palustris* genes were cloned with affinity tags and expressed in both *E. coli* and *R. palustris* using modified pDEST vectors (Invitrogen).
  - His6, His6-V5 epitope, and GST affinity tags
  - N- and C-terminal positions
- Isolation of fusion proteins
  - affinity purification with Ni-NTA, anti-V5 antibody, or glutathione-bearing agarose beads
  - Expression confirmed using 1-D PAGE and western blots.
- Isolation of 70S ribosome
  - Sucrose density gradient fractionation [4]
- “Shotgun” analysis: analysis by mass spectrometry without prior gel separation [5]
  - “top-down” (with FTICR MS)
  - “bottom-up”
    - trypsin digestion
    - Reverse-phase HPLC separation online with electrospray/quadrupole ion trap MS-MS
    - Protein ID’s: tandem mass spectral data with sequence database using Sequest .

# Strategy for Identification and Characterization of Protein Complexes

PCR amplification of target gene and cloning into modified expression vector



Transfer expression plasmid into *R. palustris*



Expression of affinity tagged proteins under various physiological conditions



capture protein complexes using affinity tag



Characterize protein complex

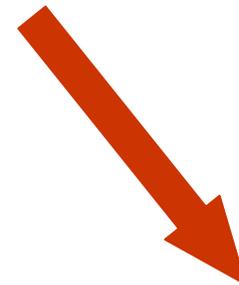
mass spectrometry



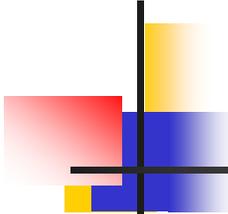
ID proteins



Stoichiometries



Functional assays



# Results: Expression of fusion proteins in *R. palustris*

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- Plasmids encoding 22 affinity-tagged fusion proteins have been inserted into *R. palustris* (**Table 1**).
- **Figures 2 and 3** show selected examples of western blots to confirm expression of fusion proteins
- Further confirmation of expression was by LC-MS-MS analysis and database searches (**Figure 4**)
- Several background proteins were common to numerous LC-MS-MS experiments. The use of two separate affinity isolations reduced this background [6] (**Figure 5**).

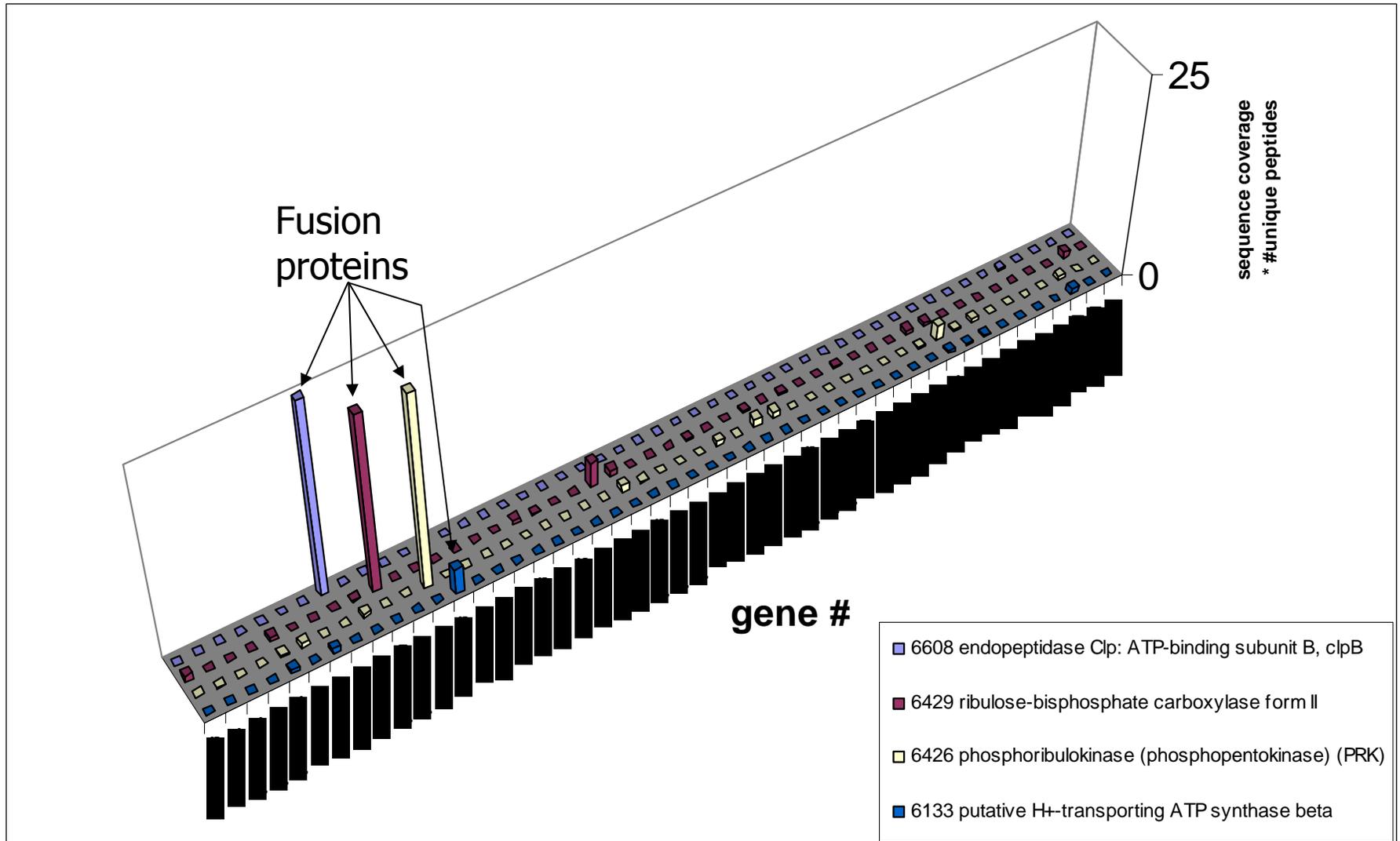




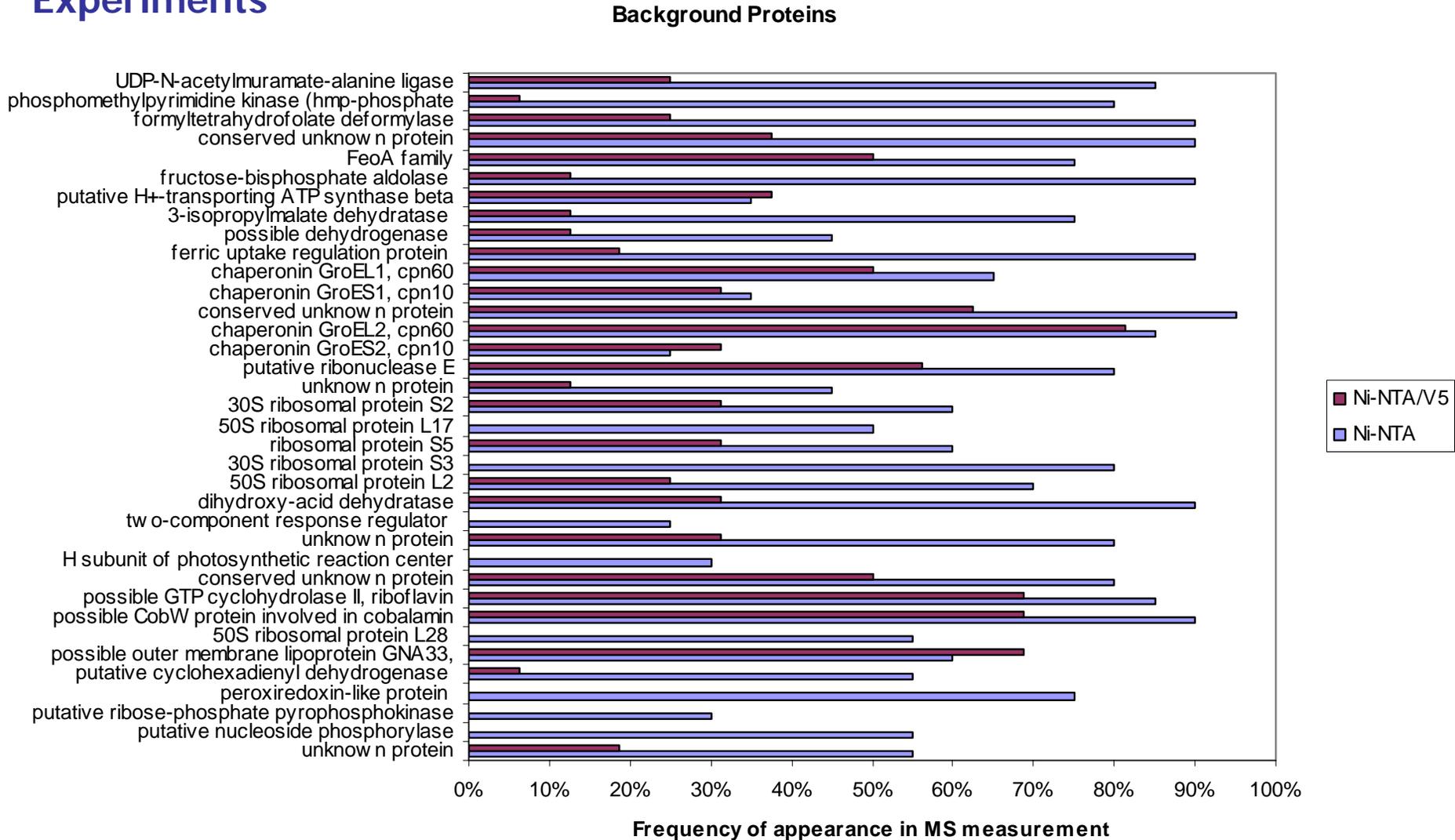
**Table 1. *R. palustris* target genes PCR amplified and cloned into destination vectors**

3306	<b>NirK</b>	Cu-containing nitrite reductase
2164	<b>GroEL-2</b>	Has ½ of CIRCE element
2165	<b>GroES-2</b>	
1140	<b>GroEL-1</b>	Has CIRCE element
1141	<b>GroES-1</b>	
4465	<b>SoxB</b>	sulfite dehydrogenase/Mn-dep. hydrolase
4464	<b>SoxC</b>	molybdopterin subunit sulfite oxidase
962	<b>HupS</b>	uptake hydrogenase small subunit
963	<b>HupL</b>	uptake hydrogenase large subunit
3147	<b>ClpA</b>	endopeptidase
4433	<b>ClpB</b>	Endopeptidase Clp: ATP-binding subunit B
4620	<b>NifH</b>	nitrogenase iron-protein
4619	<b>NifD</b>	Nitrogenase alpha chain
4618	<b>NifK</b>	Nitrogenase beta chain
4644	<b>CbbP</b>	Phosphoribulokinase
4641	<b>CbbM</b>	RuBisCo FormII
1559	<b>CbbL</b>	RuBisCo Form I
0245	<b>Ffh</b>	signal recognition particle
176	<b>AtpD</b>	ATP synthase beta chain
1548	<b>PuhA</b>	H subunit of rxn center complex
3247	<b>RplB</b>	50S ribosomal protein L2
3248	<b>RplW</b>	50S ribosomal protein L23

# Figure 4. Confirmation of Expression of Affinity-labeled Fusion Proteins by LC-MS-MS



# Figure 5. Non-labeled proteins observed by LC-MS-MS in Affinity Isolation Experiments



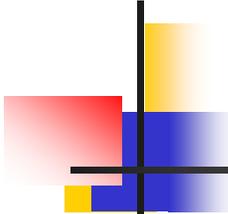
≥1 unique peptide observed.

Affinity isolation was 1-stage (Ni-NTA agarose beads) or 2-stage (Ni-NTA-agarose beads, anti-V5 antibody-agarose beads).

# Results

## Isolation of GroELS complex

- Most fusion proteins expressed to date in *R. palustris* are not normally produced under the growth conditions used.
- GroEL is an exception.
- C-terminally His6 tagged GroEL-2 was expressed and isolated using an ATP-containing buffer in order to stabilize the GroEL-GroES interaction.
- LC-MS-MS analysis of the digested isolate allowed identification of components of both versions of the GroELS chaperonin complex (**Table 2**).
- ESI-FTICR-MS of the undigested complex shows the His6 tagged GroEL-2 and the native GroEL-1 (**Figure 6**).



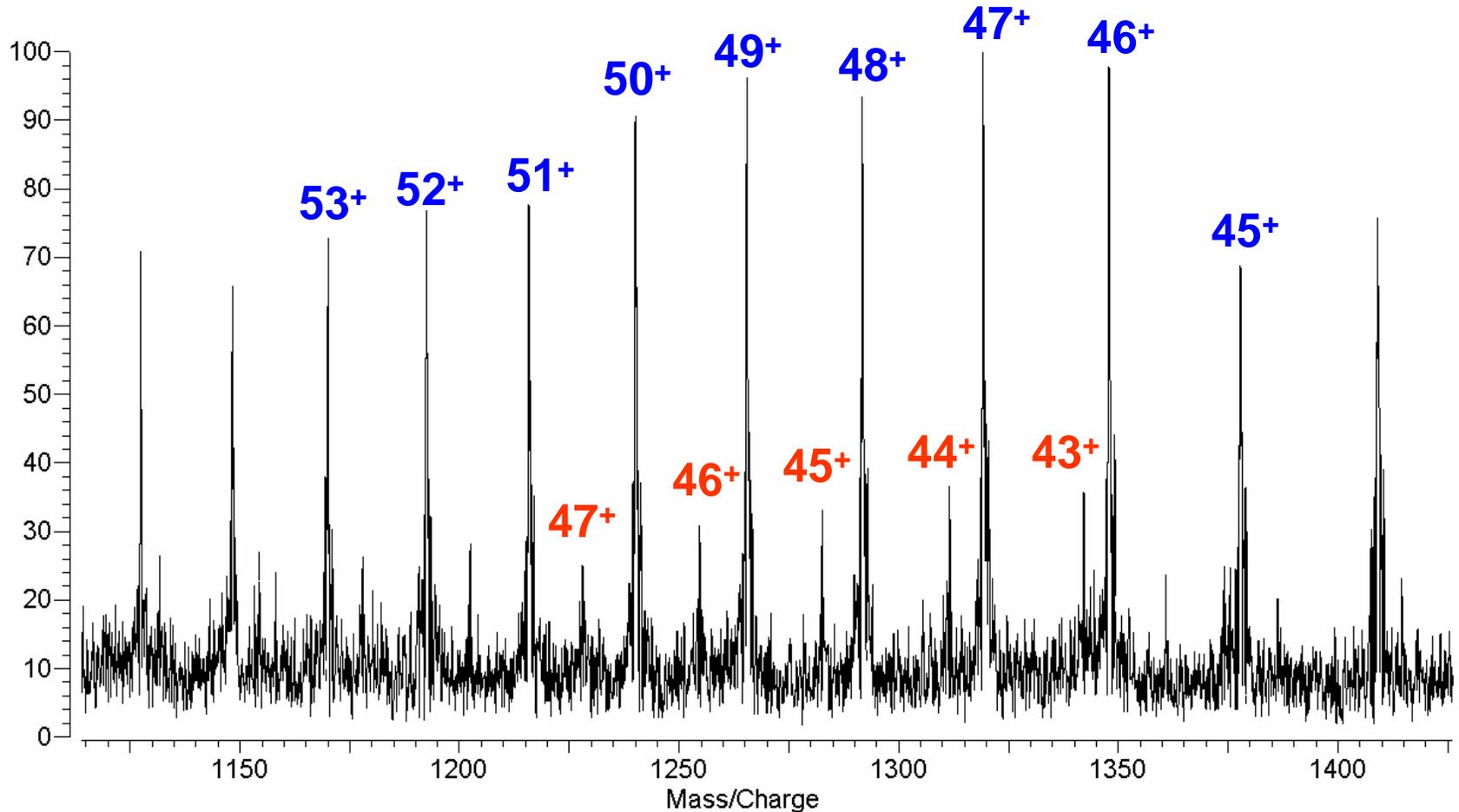
# Table 2. GroELS Complex ID

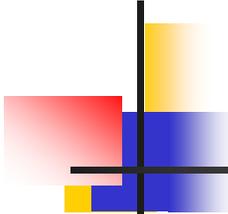
Locus	Protein Name	distinct peptide ID's	Peptides unique to protein	Sequence Coverage
RPA2165	GroES2	7	7	58%
RPA2164	GroEL2 + HIS6	48	44	76%
RPA1141	GroES1	2	2	32%
RPA1140	GroEL1	29	25	64%

# Figure 6. ESI-FTMS of GroEL-histag Pulldown

ORF 2164 GroEL2-histag (Mr = 62054.402 Da)

ORF 1140 GroEL1 (Mr = 57625.733 Da)





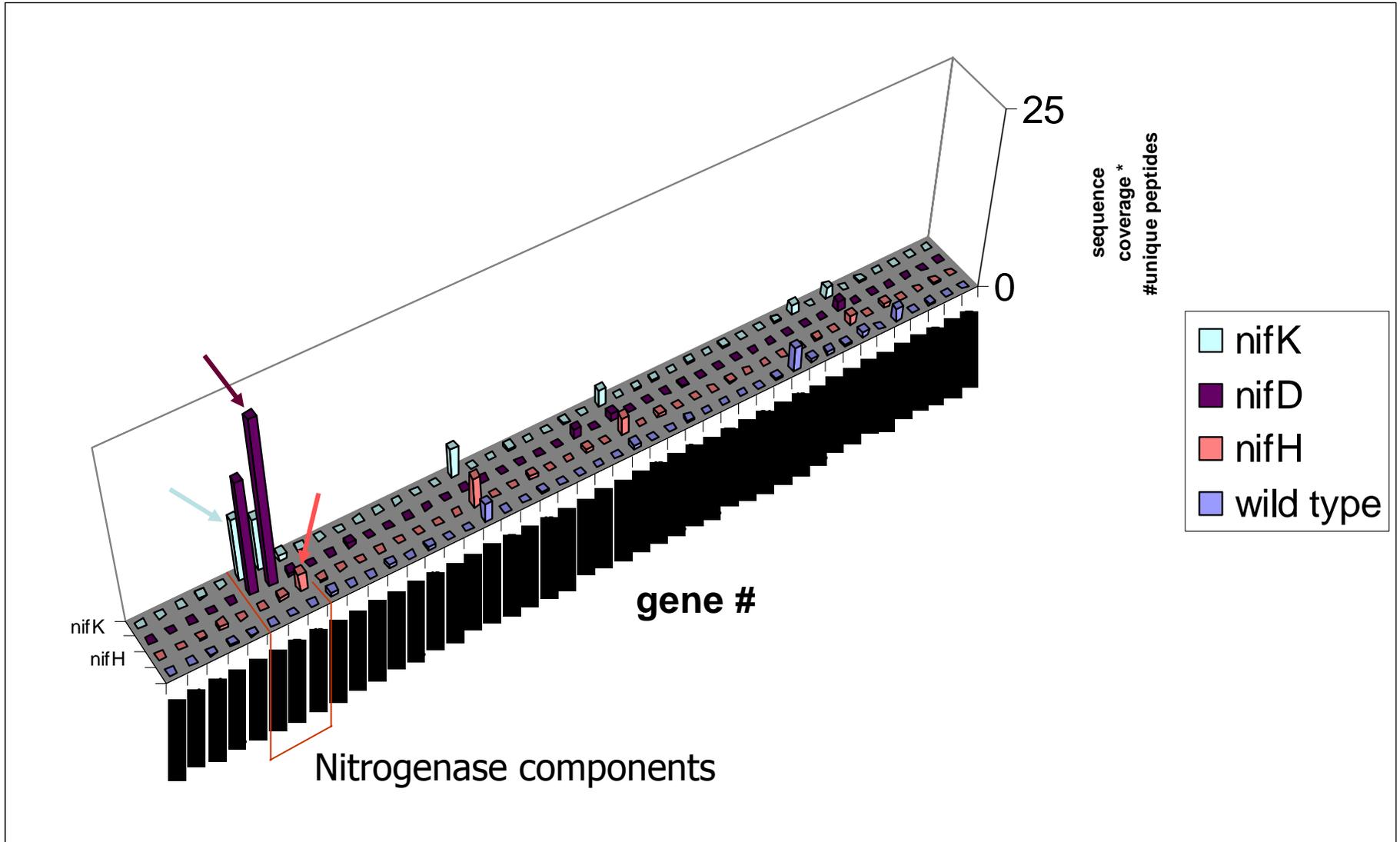
# Results

## Isolation of Nitrogenase complex

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- Three proteins in a nitrogenase complex (NifD, NifH, NifK) were each cloned with both His6 and V5 epitope tags, expressed under photoheterotrophic, nitrogen-fixing conditions, and isolated.
- LC-MS-MS analysis of the isolate from each labeled component showed evidence for all three components of the nitrogenase complex (**Figure 7**).

Figure 7. Nitrogenase Complex  
6xHis, V5 tandem purification  
photoheterotrophic nitrogen fixing *R.palustris*



Arrows indicate affinity-labeled protein

# Analysis of the 70S Ribosome from *R. palustris* [4]

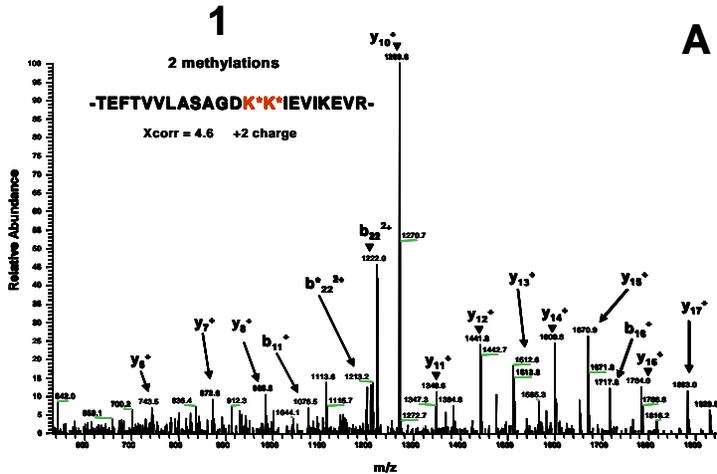
- The 70S ribosome from *R. palustris* was isolated using sucrose density gradient fractionation [4].
- We successfully identified 52 out of 54 total orthologues to *E. coli* ribosomal proteins based on tandem mass spectra of 2 or more unique tryptic peptides per ribosomal protein (**Table 3**).
- We used FT-ICR MS to measure accurately the intact masses of ribosomal proteins, including several with post translational modifications (PTM) (**Table 4**).
- For several of these PTMs we were able to locate the modification position by searching MS/MS fragmentation spectra of tryptic peptides (**Figure 8**).

# Figure 8. Ribosomal Protein L7/L12 (RRP-L7)

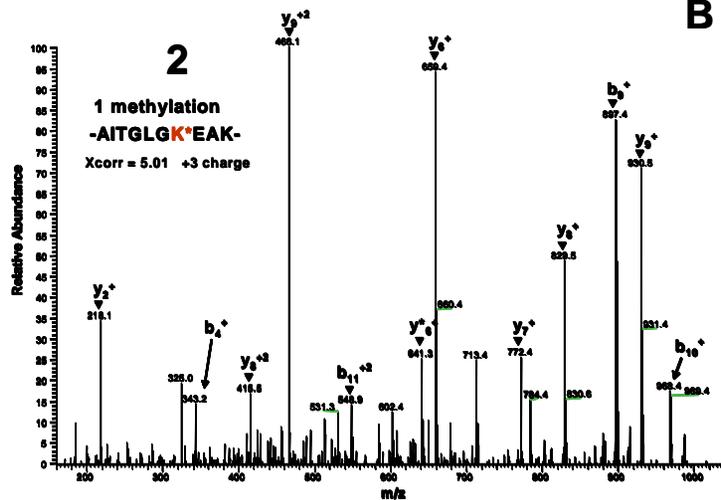
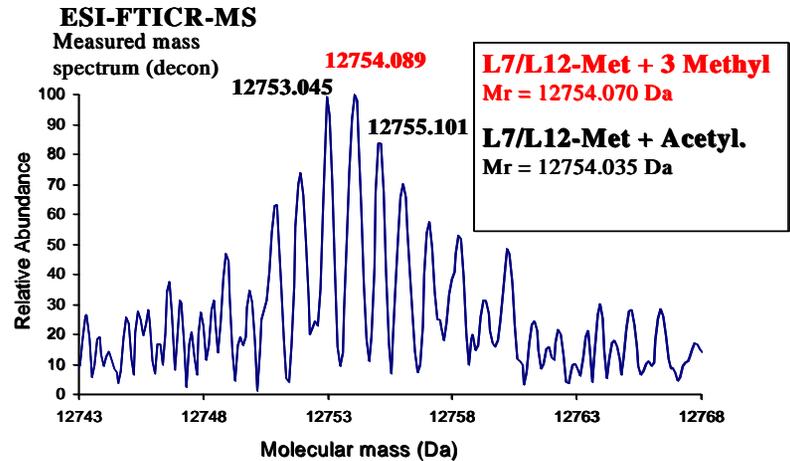
-ADLQKIVDDLSSLTVLEAAELAKLLEEKWGVSA~~AAVAVAA~~PGAGGAAAPAE~~E~~KTEFTV  
 LASAGDK\*K\*IEVIKEVRAITGLGLK\*EAKDLVEGAPKPLKEGVNKEEAEKVK~~A~~QLEKAGAKVELK

1

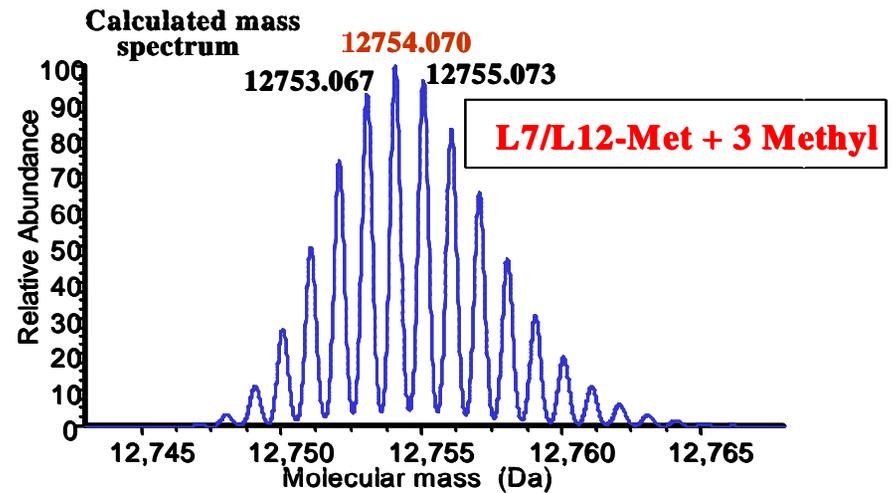
2



A



B

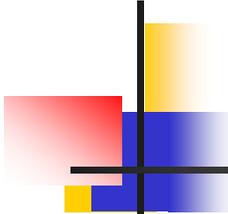


# Table 4. “Top-Down” Results

## *R. palustris* Ribosome

ribosomal protein	post translational modification	calc. mass	meas. mass	bottom-up conf.*
L1	none	23877.832	23877.449	yes
L6	loss of Met	19272.408	19272.674	yes
L7/L12	loss of Met + 3 Methyl	12754.07	12754.089	yes
L9	none	21178.022	possible?	yes
L11	loss of Met + Methyl + N-ter Acetyl	15507.107	15507.246	yes
L14	none	possible?	13487.589	
L15	none	16836.243	16836.259	yes
L17	3 Methyl	15716.353	15716.056	no
L18	loss of Met	12904.93	12905.157	no
L19	none	14296.764	14296.899	yes
L21			possible?	
L23	none	10907.949	10908.021	yes
L24	loss of Met	10998.226	10998.231	yes
L24	loss of Met + Methyl	11012.241	11012.146	yes?
L25			possible??	
L27	none	9580.016	possible?	yes
L28	none	10978.073	10978.075	yes
L29	loss of Met	7849.213	7849.239	no
L30	loss of Met	7092.967	7092.988	yes
L31	none	8566.315	8566.334	yes
L32	loss of Met	6860.73	6860.636	no
L33	loss of Met + Methyl	6248.504	6248.45	
L34			possible?	
L35	loss of Met	7415.278	7415.278	yes
L36	none	5063.971	5063.952	yes
S4	loss of Met + Methyl		23439.59	
S5	loss of Met	20522.086	20522.411	no
S7	loss of Met	17556.27	17556.629	no
S8	loss of Met	14477.6316	14477.683	yes
S8	loss of Met+Acet+4Met	14575.704	14575.619	
S9	loss of Met		17368.561	yes
S10	none	11667.363	11667.404	yes
S12	none	13874.799	13875.167	
S13	loss of Met		14343.596	no
S14	loss of Met	11331.399	11331.9	no
S15	loss of Met	10010.563	10010.562	yes
S16	loss of Met	12017.595	12017.575	yes
S17	loss of Met	9553.253	9553.316	yes
S18			possible??	
S19	loss of Met	10087.371	10087.379	yes
S20	none	9708.365	9708.281	yes
S20	loss of Met	9577.324	9577.387	yes
S21	none	10062.669	10062.722	yes

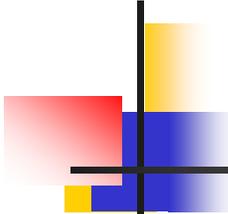
\* intact mass with PTM have been validated by fragmentation spectra of tryptic peptides



# Conclusions

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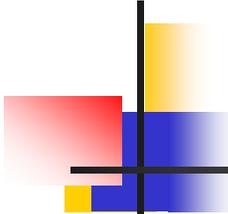
- Fusion proteins with affinity tags can be expressed in *R. palustris*
- The GroELS and nitrogenase complexes were isolated using affinity-labeled subunits.
- The 70S ribosome was analyzed in detail using “bottom-up” and “top-down” methods, verifying expected post-translational modifications.
- We are implementing higher-throughput production and analysis of fusion proteins in *R. palustris*.



# References

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5. Link, A.J. et al., "Direct analysis of protein complexes using mass spectrometry," *Nature Biotech.* **1999**, *17*, 676-682.
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