Differentiation of Specific and Non-Specific Microbial Protein Interactions by Isotopic Labeling and Mass Spectrometry

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OVERVIEW

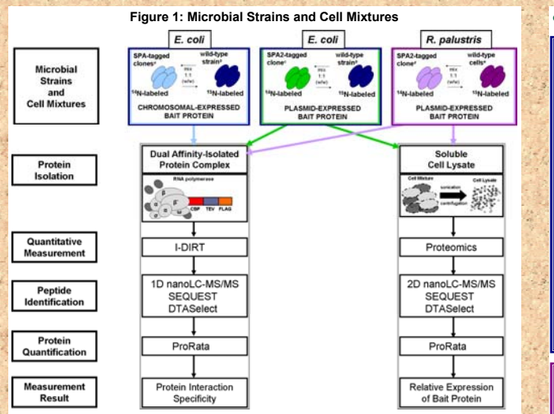
- Three different biological scenarios were created to examine chromosomal and plasmid-based expression of affinity-tagged (or "bait") proteins in two different microbial species:
  - *Escherichia coli*
  - *Rhodopseudomonas palustris*
- Quantitative mass spectrometry measurements were performed to investigate each biological scenario.

INTRODUCTION

- Affinity isolation of protein complex components coupled with qualitative peptide identification by mass spectrometry is a robust methodology for characterization of biological systems.
- However, the combination of these tools:
  - often results in non-specific (or false-positive) protein interactions among specific interactions in affinity-isolated proteins
  - have been limited to model species that use elaborate genetic systems to achieve chromosomal bait protein expression
- *I*sotopic *D*ifferentiation of *I*nteractions as *R*andom or *T*argeted[1,2] (I-DIRT) has been described to differentiate specific from non-specific protein interactions:
  - Relative measurements of protein abundance between two different cell types are performed to differentiate between specific and non-specific interactions.
- Recently, we have reported the development of a plasmid bait protein expression strategy[3] applicable to multiple Gram-negative microbial host species.
- In this study, we have used the RNA polymerase complex as a model to:
  - Examine specific and non-specific protein interactions between affinity-tagged (or "bait") protein expression strategies; and,
  - Investigate the hypothesis that plasmid-based strategies lead to over-expression of the bait and increased non-specific interactions.
- To this end, we have created three biological scenarios to examine chromosomal and plasmid-based bait protein expression of the RNA polymerase protein complex.
  - I-DIRT[1,2] measurements are performed on affinity-isolated protein complexes
  - Quantitative proteome measurements evaluate bait protein over-expression in plasmid-based scenarios.

EXPERIMENTAL

Figure 1: Microbial Strains and Cell Mixtures

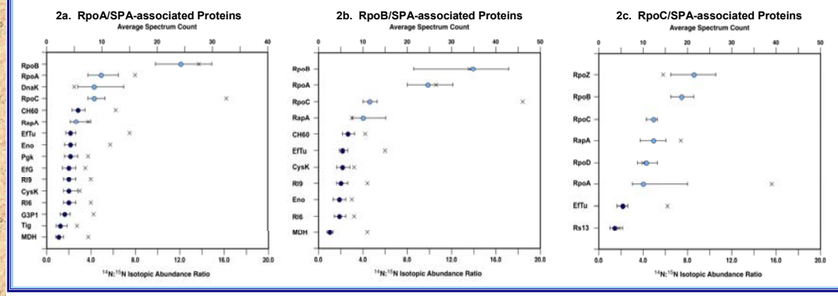


- Microbial Strains and Cell Mixtures
  - Five different microbial strains, creating biological scenarios for comparison of bait protein expression strategies, were mixed together as shown above:
    a. *E. coli* SPA[4,5] clones (RpoA/SPA, RpoB/SPA, and RpoC/SPA) were a gift from A. Emili, U. Toronto.
    b. *E. coli* K12 MG1655 (wild-type).
    c. *E. coli* clone ECK3282/SPA2 expressing the RpoA protein from the SPA2 plasmid. This plasmid was created from our vector backbone[3] to express bait proteins comparable to the original SPA system.
    d. *R. palustris* clone RPA3226/SPA2 expressing the RpoA protein from the SPA2 plasmid.
    e. *R. palustris* CGA010 (wild-type).
  - Two separate cultures of each microbial strain were grown to logarithmic phase in ^{14}N- or ^{15}N-enriched minimal medium and mixtures were made at a 1:1 (w/w) ratio by wet cell paste mass.
- Protein Isolation, Quantitative Measurement, Peptide Identification, and Protein Quantification
  - Affinity-isolated protein complexes: the RNA polymerase protein complex was isolated from each cell mixture using a combination of M2 FLAG (Sigma) and Calmodulin (GE) affinity resins, as previously described[4,5]. Tryptic peptides of the affinity isolation were separated by an automated 1D C-18 reverse phase nano-HPLC system (FAMOS autosampler, SWITCHOS, and UltiMate pump; LC-Packings/Dionex). LC-MS/MS experiments of each cell mixture were performed in quadruplicate.
  - Soluble Proteomes: soluble cell lysates were isolated from each cell mixture and digested with trypsin, as previously described[6]. Separation of tryptic peptides was performed by a 2D nano-HPLC system (strong cation exchange and reverse phase or "split-phase MudPIT") over a duration of 22 h, as previously described[6]. LC-MS/MS experiments of each cell mixture were performed in duplicate.
  - Affinity-isolated protein complexes and soluble proteome samples were ionized by a nanoelectrospray source (Proxeon Biosystems) adapted to a linear ion trap mass spectrometer (ThermoFinnigan LTQ). Tandem mass spectra were searched with the SEQUEST[7] algorithm against the predicted proteomes of each microbial species. Peptide identifications were filtered and organized by protein using DTASelect[8]. Peptide and protein abundance ratios were estimated by the ProRata program[6].

RESULTS AND DISCUSSION

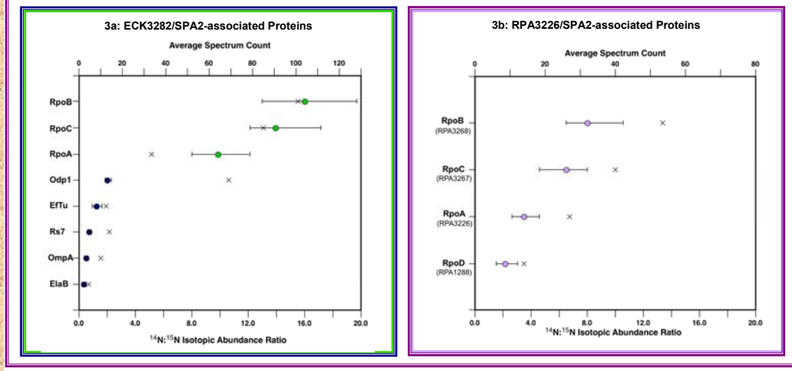
- I-DIRT[1,2] Quantitative Measurements

Figure 2: I-DIRT[1,2] of RNA polymerase from Chromosomally-Expressed Bait Proteins RpoA/SPA, RpoB/SPA, and RpoC/SPA



- Figures 2a-2c show the isotopic abundance ratio and confidence interval (error bar) for each protein; ratios are scaled along the lower x-axis.
- Specific protein interactions typically have larger abundance ratios than non-specific interactions[1,2]
- A crude absolute quantitative measurement, tandem MS spectrum count, is also provided for each protein, with values scaled along the upper x-axis.
- Interaction specificity among proteins was based upon previous studies of this complex[2,3] (legend lower right)
- Measurements of protein abundance ratios permitted successful differentiation of core RNA polymerase subunits RpoA (α), RpoB (β), and RpoC (β') from non-specific co-isolating proteins (Figures 2a, 2b, 2c)
- Consistent with published studies[2,5]:
  - complex-specific protein RapA was also differentiated in all experiments.
  - chaperone DnaK (Figure 2a), co-isolated with RpoA/SPA, yielded a specific protein abundance ratio
- Non-specific interactions were detected among high-abundance proteins:
  - elongation factor Tu (EfTu) was measured as non-specific in all experiments
  - ribosomal proteins (RI and Rs) were other common non-specifically interacting proteins (Figuers 2a, 2b, and 2c)

Figure 3: I-DIRT[1,2] of RNA polymerase from the Plasmid-Expressed Bait Protein, RpoA



- Homologs of the RpoA protein were expressed in the SPA2 plasmid to compare bait protein expression between microbial species (see Experimental).
- Measurements of protein abundance ratios permitted successful differentiation of core RNA polymerase subunits RpoA, RpoB, and RpoC from non-specific co-isolating proteins (Figures 3a and 3b)
- *E. coli* RNA polymerase (Figure 3a) was differentiated from non-specific proteins:
  - elongation factor Tu (see Figures 2a-2c)
  - OmpA and ElaB, as previously reported[2]
- Isolates *R. palustris* RNA polymerase did not yield any non-specific protein abundance ratios; however, ^{15}N-labeled proteins were qualitatively identified in each affinity isolation (data not shown).

- Quantitative Proteomics Measurements of Plasmid-Expressed Bait Relative to Native Protein

Bait Protein	^{14}N:^{15}N Isotopic Abundance Ratio	Confidence Interval	Quantified Peptides
ECK3282/SPA2	1.9	1.5 – 2.3	79
RPA3226/SPA2	0.8	0.7 – 0.9	58

- A potential drawback of plasmid-based expression scenarios is the over-expression of the bait protein.
  - The SPA2 plasmid was developed from a low-copy vector backbone for plasmid-based protein expression in multiple microbial hosts[3].
  - Here, we perform quantitative proteomics to measure potential over-expression of bait protein relative to the native, untagged wild-type protein.
- For homologs of the RpoA protein,
  - ECK3282/SPA2 was nearly two-fold above the native RpoA protein
  - Expression of RPA3226/SPA2 was slightly greater than native RpoA protein
- Bait protein over-expression observed in both species did not prevent detection of known RNA polymerase components RpoA, RpoB, and RpoC (shown in Figures 3a and 3b)

CONCLUSION

- Using the RNA polymerase complex as a model, biological scenarios of affinity-tagged (or "bait") protein expression were examined in 2 microbial species:
  - *E. coli*
    - chromosomal-expressed SPA bait proteins
    - plasmid-expressed ECK3282/SPA2 protein
  - *R. palustris*
    - plasmid-expressed RPA3226/SPA2 protein
- Quantitative measurements of protein abundance ratios:
  - differentiated specific protein interactions from non-specific interactions among affinity-isolates using I-DIRT[1,2]
    - core complex components were detected as specific protein interactions regardless of bait expression scenario
  - estimated over-expression of plasmid-expressed bait proteins at the proteome level
    - RpoA bait protein over-expression greater than two-fold was not observed
- Measurements of plasmid-expressed RpoA scenarios suggest that:
  - two-fold over-expression of bait *does not* introduce significant non-specific artifacts compared to chromosomally-expressed baits in *E. coli*
  - detection of non-specific, *background proteins are comparable* between plasmid and chromosomal bait expression scenarios in both microbial species.

REFERENCES

1. Tackett *et al* J Proteome Res 4:1752-1756 (2005)
2. Lee *et al* J Bacteriol 190:1284-1289 (2008)
3. Pelletier *et al* J Proteome Res Accepted (2008)
4. Zeghouf *et al* J Proteome Res 3:463-468 (2004)
5. Butland *et al* Nature 433:531-537 (2005)
6. Pan *et al* Anal Chem 78:7121-7131 (2006)
7. Yates *et al* Anal Chem 67:1426-1436 (1995)
8. Tabb *et al* J Proteome Res 1:21-26 (2002)

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