High-Performance Mass Spectrometry Facility

The High-Performance Mass Spectrometry Facility (HPMSF) provides state-of-the-art mass spectrometry (MS) and separations instrumentation that has been refined for leading-edge analysis of biological problems with a primary emphasis on proteomics. Challenging research in proteomics, cell signaling, cellular molecular machines, and high-molecular weight systems receive the highest priority for access to the facility. Current research activities in the HPMSF include proteomic analyses of whole cell lysates, analyses of organic macro-molecules and protein complexes, quantification using isotopically labeled growth media, targeted proteomics analyses of subcellular fractions, and detection of post-translational modifications (PTM) such as phosphorylation and ubiquination. More than a dozen microbial systems are currently being studied in HPMSF by researchers from throughout the country. In addition, there are several ongoing projects in higher order systems (including mammalian systems) that are investigating a broad range of biological questions from cancer screening to infectious diseases to fundamental questions of post-translational modifications and protein-protein interactions.

Capabilities

The facility features state-of-the-art liquid chromatographic (LC) separations capabilities coupled to a complete suite of MSs for proteomics analysis. The available instruments range from a group of nine ion trap spectrometers for tandem mass spectrometry (MS/MS) work to very high-sensitivity and high-resolution Fourier transform ion cyclotron resonance (FTICR) spectrometers that offer 1 ppm mass measurement accuracy. Proteomics analysis on these FTICR spectrometers is complemented by a quadrupole time-of-flight (QTOF) spectrometer that combines MS/MS analysis with mass measurement accuracy. These spectrometers are coupled with very high-resolution separations (500 peak capacity) that are highly beneficial to these areas of research. Focused research projects into biomolecular complexes and macro-molecules are supported on the facility's 7-tesla FTICR spectrometer, a flexible instrument that can be configured in many different ways.

Instrumentation & Capabilities

Mass Spectrometers

- QTOF
- Five ion trap spectrometers
- Four linear ion trap mass spectrometers

FTICR

- 7, 9.4, 11.5 and 12-T FTICR spectrometers

FT Spectrometer

- Orbitrap™

Additional Capabilities

- Twelve custom high-performance liquid chromatography (HPLC) systems
- Agilent capillary HPLC system

The HPMSF is committed to maintaining state-of-the-art MS and separations capabilities. To this end, the facility's staff work to develop and implement new capabilities such as the

ion funnel, dynamic range enhancements applied to MS (Dynamic Range Enhancement Applied to Mass Spectrometry [DREAMS]), and data analysis tools, which are incorporated into the capabilities of the facility as they become available. As part of this commitment the facility added a 12-T FTICR spectrometer that is being optimized for work on intact proteins for the identification of post-translational modifications and protein isoforms. In addition, an Orbitrap™ MS was added to the facility. This instrument provides performance similar to the FTICR spectrometers but without the need for a superconducting magnet. The members of the facility staff are highly skilled in all areas required for proteomics research, from sample preparation to analysis and data interpretation, and they are available to help develop methodologies to tackle these challenging problems. As needed, scientific staff not assigned to the facility can be accessed as matrixed members of the facility. Since its inception, more than 100 separate user projects have been undertaken in the facility, with some spanning over a year in duration.

The HPMSF has developed state-of-the-art software for the acquisition and analysis of FTICR mass spectra. This software package is called ICR-2LS. It is a Microsoft Windows-based application that enables many of the unique instrument control functions developed in the W.R. Wiley Environmental Molecular Sciences Laboratory (EMSL). This same software allows automated spectral interpretation of raw FTICR data. The spectral interpretation features are integrated into our application and, thus, are not easily transferred. The data acquired in the facility is stored and managed in an in-house-developed relational database.

Mass Spectrometry Research Capabilities. Nine ion trap MSs from ThermoElectron are available in the facility: two Finnigan LCQ Classics, two Finnigan LCQ Duos, one Finnigan LCQ DECAXP, and four LTQs that provide greater than an order of magnitude improvement in dynamic range from Finnigan. An example of one of these instruments is shown in Figure 1.

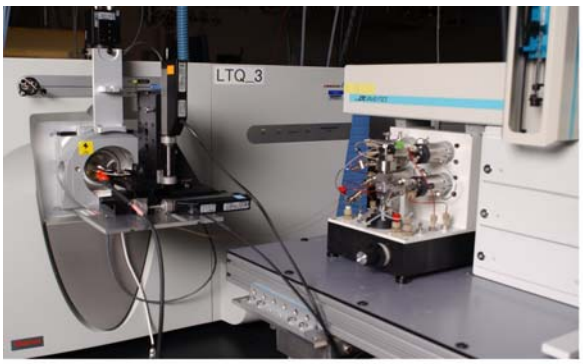

Figure 1. Ion trap MS in an HPMSF laboratory.

The ion trap instruments have either a three-dimensional quadrupole ion-trap or a two-dimensional elongated trap designed for use with electrospray ionization sources. These instruments are well-suited to MS/MS spectrometer experiments because of their very high collection efficiency for product ions. The mass range of this instrument is 150 to 2000 m/z, but the range can be extended to 4000 m/z for some applications. The ion trap instruments have a maximum resolution of 10,000 in the zoom-scan mode, and 4000 in full-scan mode. In addition, the system is easily operated in either positive or negative ion mode with the addition of SF_6 as a sheath gas.

Fourier Transform Ion Cyclotron Resonance Mass Spectrometers. This 11.5-T ultrahigh-performance MS uses a wide-bore (205 mm), passively shielded (Figure 2) superconducting magnet. The spectrometer is equipped with an electrospray ionization source and an ion funnel. The 11.5-T FTICR has a resolution of 150,000 at m/z 60,000 and a mass accuracy of 1 ppm for peptide samples with molecular weights ranging from 500 to 2000 Da. Ions are collected external to the magnet in a series of quadrupoles that allow the researcher to eliminate irrelevant ions before analysis in the FTICR spectrometer. DREAMS is a unique capability of this facility. The 11.5-T FTICR instrument can be fitted with an HPLC system and is equipped with an infrared laser for multiphoton dissociation of samples for MS/MS in the ion cyclotron resonance (ICR) cell.

Figure 2. Wide-bore, passively shielded 11.5-T FTICR MS.

The 9.4-T FTICR spectrometer (Figure 3) is a 150-mm-bore, actively shielded Bruker Daltonics APEX III. The original ion source has been replaced with a custom source that incorporates a dual-channel ion funnel for simultaneous introduction of calibrant ions, DREAMS technology, and automatic gain control. These modifications permit maximum use of the spectrometer's capabilities by maintaining the optimum number of ions in the ICR cell throughout an LC separation. The outstanding resolution of over 60,000 is maintained throughout the separation with a sensitivity comparable to the other FTICR instruments in the facility. This system is seamlessly integrated with the facility's automated HPLC (5000 psi) system for unattended operation 24 hours a day, 7 days a week.

Figure 3. 150-mm-bore, actively shielded 9.4-T FTICR instrument.

The Waters' Micromass Ultima application program interface (API) QTOF instrument is an orthogonal extraction-TOF MS that has enabled automated exact mass measurement with the ultimate performance (Figure 4) in MS and MS/MS. The instrument features optimized resolution, enhanced ion optics for enhanced sensitivity, a quadrupole mass filter, and a collision cell for MS/MS analyses. These features are also accessible over an enhanced linear dynamic range, enabling rapid and reliable quantification. This powerful combination of capabilities delivers simple exact mass measurement of precursor and fragment ions with maximum sensitivity to yield the highest confidence in structural elucidation and databank search results.

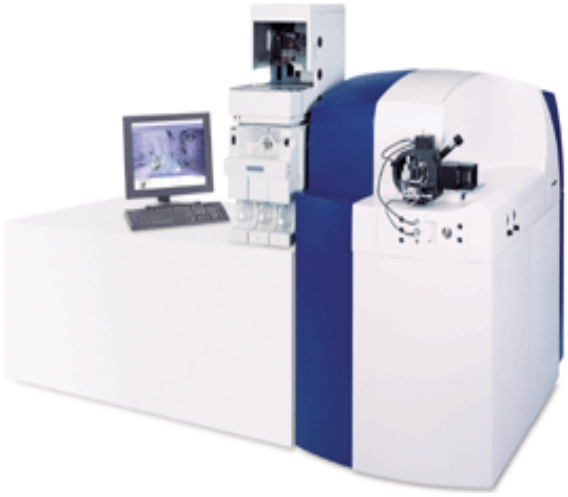

Figure 4. QTOF instrument in the HPMSF.

A signature capability of the facility is the efficient coupling of capillary separations (Figure 5) to our MSs. Instruments for both LC and capillary electrophoretic separations are available. Two ABI 270A electrophoresis systems are available along with one Agilent capillary LC and one Shimadzu capillary LC system. Unique to this facility are the in-house-developed LC systems. These systems deliver constant-pressure gradient separations at up to 5000 psi. Continuing developments of high-pressure versions of these LC systems feature a PAL autosampler with cooled sample holder, VALCO high-pressure valves, and ISCO syringe pumps. Computer software has been developed that allows the system to be configured with any of our spectrometers through DCOM communication protocols.

Figure 5. Capillary ultrahigh pressure separations capability.

The 7-T FTICR spectrometer is based on a 160-mm-bore, superconducting magnet and is equipped with a custom electrospray ionization source (Figure 6). This instrument has high-mass-resolving power (e.g., a mass-resolution greater than 2,000,000 has been obtained for insulin), while unit resolution is routinely achievable during online capillary isoelectric focusing (CIEF) experiments for proteins with mass resolutions of less than 30,000 μ; mass accuracy less than 5 ppm is typical for peptide/protein samples with molecular masses ranging from 500 to 30,000 μ. A detection limit of approximately 10 attomoles has been obtained with online LC and CIEF separations.

Figure 6. 7-T FTICR spectrometer.

The 12-T FTICR (Figure 7) has a 100-mm-bore magnet that is actively shielded. The original ion optics have been upgraded to include an ion funnel and additional quadrupole stages for enhanced ion transmission and future implementation of data-directed ion selection for MS/MS studies. The system is being optimized for high-throughput, proteome-wide, high-accuracy measurements of the molecular weight of intact proteins. Additionally a "top-down" protein characterization using a variety of dissociation schemes, such as collisionally induced and electron capture dissociation, can be performed on this spectrometer. Protein-separation schemes based on reversed-phase capillary LC, CIEF, and high-field asymmetric ion mobility spectrometry are in use and under development with this system.

Figure 7. 12-T FTICR spectrometer.

Instrument Upgrades

In Fiscal Year 2006, the following capability development and procurement activities occurred in the HPMSF:

- **Proteomics Research Information Storage and Management (PRISM) system.** This system has continued to increase in both size and capability to keep pace with EMSL's proteomics facility and the proteomics field itself. Storage servers were added to bring the online capacity of PRISM up to 20 terabytes. Several new analysis processing machines were added as well as a new SEQUEST cluster. This has brought the total number of computational processing units to more than 200.

- **X!Tandem and "theGPM."** The X!Tandem peptide identification program and "theGPM," an associated presentation web software, are both open source tools that are gaining broader acceptance in the proteomics community. X!Tandem uses a different algorithm than SEQUEST and provides both a complementary analysis capability and a welcome addition to the overall throughput of the proteomics facility. In addition, architectural changes to the Data Management System Analysis Manager program were made to allow the use of plug-in adapters to add new analysis tools to the pipeline as well as modules that pre-process the spectra collected from the mass spectrometry instruments.

- **Protein Sequences Database.** The Protein Sequences Database was implemented to solve the problem caused by an overwhelming proliferation of proteome description files (called "fasta" files, after their internal format). Research campaigns for more and more organisms have been added to PRISM, and each organism typically requires several different descriptions of its proteome. Different researchers want subtle variations on the basic versions that can be tailored to their specific experiment protocols. Additionally, there is a need to create reversed and scrambled versions of these files to support the assessment of false discovery statistics. This has led to a rapid proliferation of fasta files that were quickly becoming geometric in size. The Protein Sequences Database replaces these static fasta files with protein collections which are lightweight lists of references to protein definitions stored in a database. There is only one copy of any unique protein in the database, and protein collections simply reference them. Enhanced descriptive information is kept for each collection, making it easier to keep track of all the variations for each organism. Finally, reversed and scrambled sequences can be generated on demand by the Protein Sequences Database so that separate versions for these types of files do not need to be maintained.

- **OrbitrapTM spectrometers.** One system, acquired in September 2005, is now fully integrated into the facility. This instrument adds to the growing capability to acquire tandem mass spectrometry and accurate mass data more efficiently, providing additional capability for working on new biological systems. A second system was delivered and installed in September. These instruments are the first fundamentally new type of mass spectrometer to be developed in the last 20 years. They trap ions and detect their

harmonic oscillations, similar to an FTICR mass spectrometer, but they do not require a magnet and use only electric fields.

- **12-tesla system.** A 12-tesla system is making significant contributions to three user projects. Development of electron capture dissociation for identifying post-translational modifications will continue.

- **Triple quadrupole mass spectrometer.** A new triple quadrupole mass spectrometer was delivered to EMSL in September 2006. This instrument provides greater dynamic range than ion trap mass spectrometers and will be essential in the validation of candidate biomarkers that are discovered with global proteomics analyses.

- **LC systems.** Two new LC systems were constructed in Fiscal Year 2006. Both are configured as dual-mixer systems and use 20,000 psi Teledyne/ISCO syringe pumps. These systems are the first that are configured to use the syringe pumps in a fully automated fashion on the proteomics production line.

Future Directions

The HPMSF expects to support at least 90 user projects in Fiscal Year 2007, made up of 80 distinct users of whom 6 would be distinguished users. Every effort will be made to encourage the submittal of Science Theme proposals from each of our active users during the February 2007 call. Other expected activities in Fiscal Year 2007 include:

- **Continue efforts to maintain the ability to support the ever-growing size of the PRISM system** with available resources. Whenever possible, the existing software source control and issues tracking infrastructure is replaced by open source products that offer improved performance and increased functionality without requiring licensing fees. A collaborative information website (or wiki) was also established as the primary platform for PRISM user documentation. Wiki functionality will allow any proteomics staff member, including developers, users, and collaborators, to incrementally improve and expand the documentation bit by bit.

- **Continue to refine and further develop DREAMS.** To further extend dynamic range and sensitivity in proteome studies, the facility developed and demonstrated the DREAMS approach for high-efficiency capillary LC separations with FTICR mass spectrometry. In an initial application, this approach approximately doubled the number of detected peptides from microbial proteomes for which quantitative information can be obtained. The results indicated that the overall dynamic range of measurements was increased by at least an order of magnitude (to ~10^5 or larger). For this demonstration, a 1:1 mixture of $^{14}N/^{15}N$ labeled *S. oneidensis* cells was analyzed. In the normal set of spectra, 2485 peptides pairs were identified, which corresponded to 1272 open reading frames (ORFs). The DREAMS approach allowed the identification of 1299 additional pairs (>50% increase), pointing to 481 new ORFs (~38% increase) not previously identified. Thus, using the Accurate Mass and Time tag approach, the researchers observed (and quantified) 1753 *S. oneidensis* proteins (>36% of the predicted *S. oneidensis* proteome) as peptide pairs in a single DREAMS analysis. This coverage compares

favorably to typical coverage achievable using shotgun proteomics. Similarly, for *D. radiodurans*, a single DREAMS analysis identified 2244 peptides, covering 965 ORFs in the normal set of spectra and 2259 peptides, covering 1000 ORFs in the additional DREAMS set of spectra, for a total of 1244 non-redundant ORFs or 279 (30%) additional ORFs through the use of DREAMS

- **Use of automated gain control.** Another important improvement in FTICR performance is provided by automated gain control, a capability for data-dependent adjustment of ion accumulation time during an LC separation so as to maintain ion populations in the cell not exceeding a level that causes excessive space charge effects. The automated gain control also helps eliminate m/z discrimination in the external ion trap and improves the dynamic range of measurements and/or the mass measurement accuracy. The automated gain control capability is now commercially available and is included in the LTQ-FT purchased last year with program funds.

- **Continue development of the ability to detect post-translational modifications of proteins.** The ultimate goal will be to do this in a high-throughput fashion analogous to the existing capability for peptide detection. This will require the continued development of the new "top-down" proteomics capability that was demonstrated in Fiscal Year 2006 with the direct detection of intact protein isoforms and modified proteins from very limited amounts of user samples. Implementing the "top-down" approach on these limited samples will require nano-fractionation capabilities to be successful. This is the emerging method of choice for identifying post-translational modifications, which are very important in protein activation. This information complements the peptide-based "bottom-up" approach, which is superior for identifying the presence of protein families in a high-throughput fashion. Developing and implementing a protein fractionation protocol that can be used on eluting peaks from a separation will require substantial development and the use of infrared multiphoton heating of the ions coupled with either collision-induced dissociation or electron transfer dissociation. These efforts will be supplemented by the deployment of state-of-the-art ion trap mass spectrometer with electron transfer dissociation capability. This will allow us to effectively combine and compare analyses from the "bottom up" and "top down" approaches that use the same fundamental principles to identify the sites and nature of post-translational modifications.

Identification of Functional Pathways Associated with Clinical Tamoxifen-Resistance in Breast Cancer by Advanced Mass Spectrometry

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Not all cancers respond equally to standard cancer drugs. If a doctor can determine how a cancer will respond to a drug before treatment begins, he will know if he has to prescribe an aggressive dose or a more modest one. By examining the proteins present in tumor samples from patients with a known response history, we can identify markers that will correlate with the response and aid doctors in prescribing the correct dose.

A Umar from the Erasmus University Medical Center in Rotterdam, The Netherlands, is visiting EMSL to characterize functional pathways that lead to clinical resistance to tamoxifen (anti-estrogen) treatment in breast cancer patients.

For this study, she has used laser microdissected tumor cells from patients that either were or were not responding to tamoxifen treatment. Protein profiles from these cells were generated by state-of-the-art nanoscale liquid chromatography Fourier transform infrared cyclotron resonance mass spectrometry (nLC-FTICR-MS), and the profiles were compared. Laser microdissection has the great advantage of enabling isolation of selected subpopulations of cells, such as tumor cells, and thus overcoming the issue of tissue heterogeneity. However, this process is very time consuming, so typically only a few thousand cells amounting to a few hundred nanograms of protein can be collected per tissue. Analysis of such minute amounts of sample by conventional proteomics tools is severely constrained by detection limits. As a consequence, ultra-sensitive nLC-FTICR technology developed at EMSL is the best choice for performing comprehensive proteome analyses. The nLC-FTICR-MS technique was applied to clinical samples and showed improved proteome coverage and good reproducibility attainable for ~3000 laser microdissected breast carcinoma cells as compared to what was previously described. As a next step, Umar used this approach to analyze 50 different tumor samples. She was able to detect protein profiles that were specifically expressed in either responding or nonresponding tumors (Figure 1). Validation of these protein profiles and further functional analysis of the proteins will eventually advance our understanding of breast cancer therapy-resistance and may lead the development of new and better therapies.

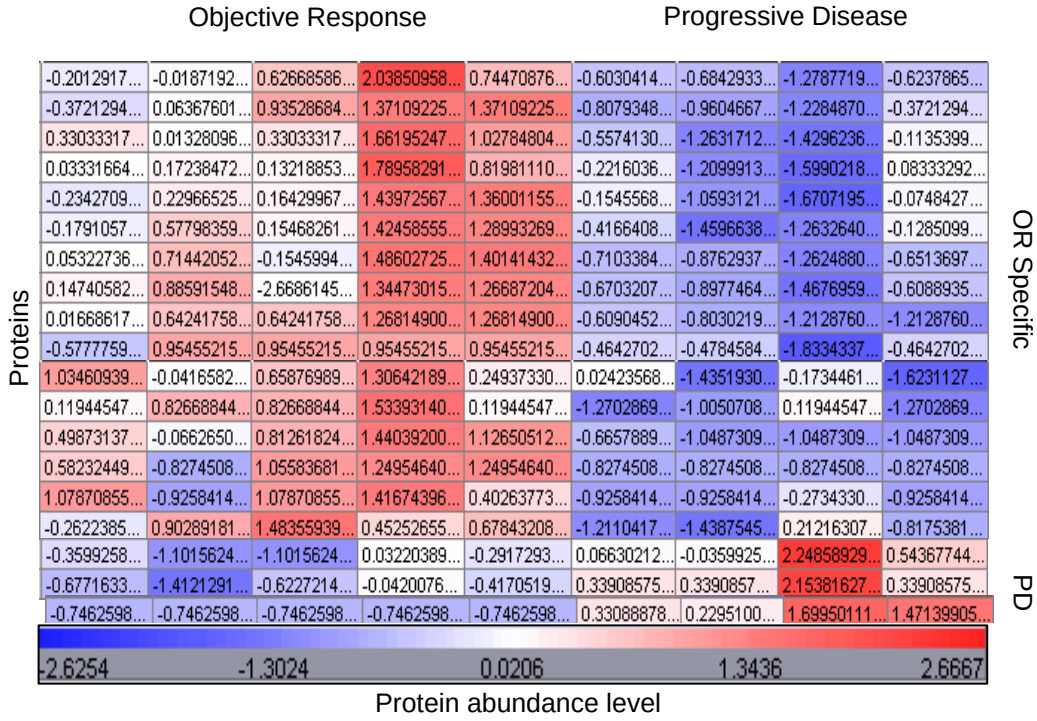

Figure 1. Detail of response-specific protein profile. Hierarchical clustering with z-scores was performed on 1999 proteins using protein abundance (obtained by averaging measured peptide abundances). The average protein abundance level (0.0206) is represented with a white background color, while the abundance levels above and below the average are represented by red and blue background colors,

Identification and Post-Translational Modification of Mitotic Regulatory Proteins

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This highlight focuses on a class of proteins known as chromosomal passengers (CP) that, because of their complex localization patterns, are thought to play multiple roles in cell proliferation and cell cycle checkpoint signaling pathways. Therefore, the study of CPs represents an area of intense research because of their importance as mitotic regulators and their potential to be cancer targets. Transient protein modification by phosphorylation plays an essential role in regulating mitosis and preventing cancer. This research is designed to identify novel phosphoproteins and post-translational modifications that regulate mitotic progression.

Spatial and temporal changes in protein localization play important roles in regulating protein activity in many cellular processes. This is most evident during mitosis when cells undergo dramatic changes in their architecture. Phosphorylation of critical mitotic

regulatory proteins is recognized as a key mechanism that controls the successful completion of mitosis with the high fidelity needed to prevent aneuploidy and possible cancer development. In our studies, we have chosen to perform targeted proteomic analyses on a subset of enriched mitotic regulatory proteins to increase the detection of very low abundance protein complexes and their post-translational modifications. We are using a phosphorylation site-specific antibody (P190) that recognizes several mitotic phosphoproteins that behave as CPs. CPs undergo dramatic changes in their localization as cells progress through mitosis, localizing on kinetochores during prophase and metaphase and at the mid-zone and mid-body at later stages of mitosis (Figure 1). Many of the known CPs, including aurora-B kinase, play critical regulatory roles during mitosis, and their functions are often compromised during cancer development.

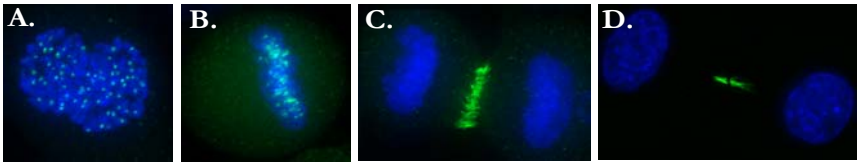

Figure 1. The P190 antibody recognizes chromosomal passenger proteins. Kinetochores are immunoreactive for the P190 antibody (green) in prophase (A) and remain labeled through metaphase (B). As the chromosomes (blue) separate, P190 labels the mid-zone during anaphase (C) and the mid-body after cell division (D).

To identify the phosphoproteins, protein complexes, and phosphorylation sites bound by the P190 antibody, we employed several mass spectrometry-based approaches to identify both peptides and phosphopeptides present in P190 immunoprecipitates (IP) (Figure 2). In one approach, we performed mass spectrometry on protein IPs from mitotic cell lysates to identify the protein complexes recognized by the P190 antibody. Using this approach, we were able to identify 298 unique proteins in the P190 IP versus 41 unique proteins in the control IP. Several known CPs were specifically present in the P190 IP, including Aurora-B, RCC1-like/TD-60, and INCENP, all of which are present in the same protein complex. We also identified other proteins that have known roles in mitosis and/or localize to the mitotic apparatus. These proteins include Aurora-A, ch-TOG, cyclin B, NuMA, cytoplasmic dynein, tubulins, actin, and actin-binding proteins. Other identified proteins include those involved in various signaling pathways that regulate mitosis such as SMAD2, Casein Kinase 1, serine/threonine protein phosphatase 2A, ALPHA-1 CATENIN, and 14-3-3 proteins. Many of the proteins specifically present in the P190 IP include those proteins present in ribosomes and spliceosomes, large protein structures that have known but poorly understood functions during mitosis. We also identified a large number of hypothetical proteins and proteins with no clearly defined cellular functions that represent candidates for novel CPs.

The P190 antibody was generated using a phosphopeptide (VLPRGL[pS]PARQLL) uniquely present in the progesterone receptor. Because the antibody cross-reacts with other phosphoproteins, we reason that the antibody only reacts with a subset of the amino acids present in the phosphopeptide that may also be present in other proteins. To determine the critical amino acid residues recognized by the antibody, we performed a tryptic digest of mitotic cell lysates followed by a phosphopeptide IP, IMAC enrichment, and identification

of phosphopeptides. The most abundant peptide from this analysis was derived from ribosomal protein L12 and had the sequence, K.IGPLGL[pS]PK.K). Other proteins present in both the phosphopeptide and protein IPs include ch-TOG (KAPGL[pS][pS]KAK), Otthump22591 (QPPLSL[pS]PAP), and Ric 8/synembrin (IQPMGM[pS]PRG).

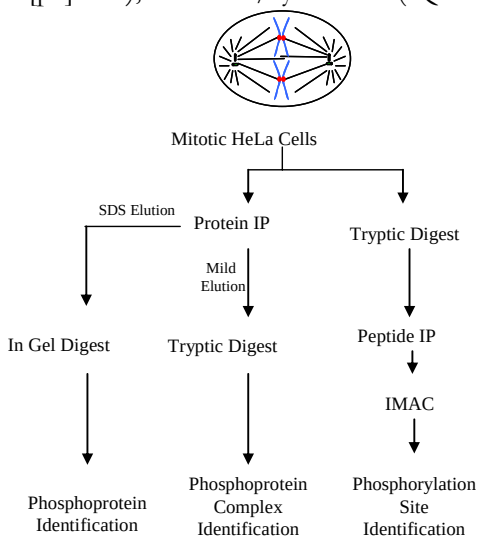

Figure 2. Strategy for identifying protein complexes and phosphorylation sites containing the P190 phosphoepitope.

From these peptides and the original starting peptide, we identified a motif of PXXLSP as the preferred binding motif of the P190 antibody. Interestingly, this motif is present in INCENP (PLPRTL[pS]PTP), the chromosomal passenger protein identified in the P190 protein IP. Other proteins in the protein IP containing this motif include SMAD2, stathmin 3, ABLIM1, Bcl9, Mystique, CAMTA1, Hira, and hypothetical protein DKFZP434K1815. Analysis of phosphorylation site databases showed that the motifs present in INCENP, SMAD2, stathmin 3, and ABLIM1 are phosphorylated *in vivo*. Functional assays are underway to address the significance of these specific phosphorylation events in regulating mitotic progression.

Proteomic Characterization of a Macaque Flu Infection Model System

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This research involves characterization of a non-human primate model system to gain more insight into the proteins that are important in the progression of flu infections.

Initial proteomic characterization of the macaque flu infection model system used a bottom-up proteomics approach with the isolation of proteins from macaque lung tissue either

infected (influenza) or mock-infected, followed by tryptic digestion to peptide form for sequence identification and relative quantitation using liquid chromatography coupled to tandem mass spectrometry analysis. Additionally, a cysteinyl-peptide enrichment step was performed at the peptide level, providing enhanced coverage of the macaque model proteome. The resulting high-quality data set of 14,100 peptides and 3,548 proteins identified in this preliminary study of the macaque model system is, to our knowledge, the first comprehensive proteomic survey performed for this model. Among the proteins detected are many candidates of particular interest based on previous clinical, pathological, and gene expression data, demonstrating the suitability of the non-human primate model for studying influenza virus pathogenesis. These include several well-known interferon-induced proteins as well as other non-cellular mediators of the innate immune response. This new macaque lung protein database creates the foundation for future research efforts aimed at applying comparative quantitative proteomics measurements to longitudinal studies of non-human primate models of influenza virus infection.

When comparing the differentially identified proteins between uninfected and influenza virus-infected lung tissue samples, consistent with previous observations demonstrating the establishment of an antiviral state in the lungs of influenza virus-infected macaques, we observed an apparent up-regulation of many proteins involved in the innate immune response. These included both interferon-induced proteins and non-cellular mediators of the innate immune response. We believe the findings reported clearly demonstrate the potential of proteomics for assisting in the determination of protein players and pathways affected by influenza virus infection.

Proteomic Profiling of *Corynebacterium glutamicum* R Incubated at Very High Cellular Densities Under Oxygen-Deprivation Conditions

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The completion of the human genome dramatically showed that there are not enough genes available to account for the diversity and complexity that we have. Thus, it is the regulation of the translation of the genome into proteins that leads to this diversity and this regulation is one of the great unsolved problems in biology. Analysis of the proteins in simpler model systems like Corynebacterium glutamicum R *will give us insights into how this regulation is controlled.*

The recent completion of the genome sequence of *Corynebacterium glutamicum* R enables the implementation of post-genomic tools to define the physiological response exhibited by this microorganism when it is subjected to various environmental conditions. We have

established and validated basic procedures for performing comparative proteomic profiles of *Corynebacterial* protein extracts. We observed a general agreement between proteomics and transcriptomics data. Further analyses could possibly reveal in this organism the presence of unknown regulatory mechanisms and global metabolic adaptations that occur when the culture is switched from the cell catalyst production phase to the product production phase.

In the quest for cost-effective production processes that would be suitable for producing a variety of commodity chemicals, such as ethanol or organic acids, the Research Institute of Innovative Technology for the Earth in Kyoto, Japan, has championed the development of novel processes that make use of very high cellular densities and oxygen-deprivation conditions. These conditions trigger the uncoupling of cell catalyst production and product production phases in anaero-tolerant and quorum-sensing sensitive organisms (Inui et al. 2004a, b; Inui et al. 2006). The complete genomic sequences of *C. glutamicum* R and *C. glutamicum* ATCC 13032 (Yukawa et al. 2006) enable us to use post-genomic tools for the global analysis of the cellular responses that *C. glutamicum* exhibits when subjected to various incubation conditions. Particularly, two-dimensional poly-acrylamide gel electrophoresis (2D-PAGE) combined to liquid chromatography/mass spectrometry (LC/MS) of *C. glutamicum* R protein extracts enabled to identify numerous enzymes of the central metabolism (Figure 1).

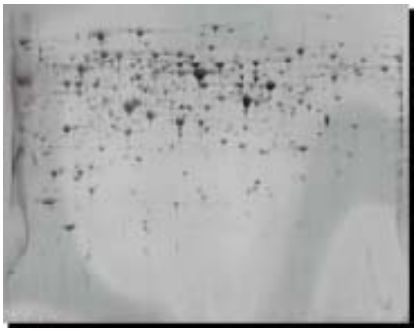

Figure 1. Two-dimensional poly-acrylamide gel electrophoresis imaging of *C. glutamicum* R protein extracts. Numerous proteins could be identified by LC/MS analysis, including key enzymes of the central metabolism (e.g., enolase, transketolase, fumarase, acetyl-CoA hydrolase, pyruvate kinase, malate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, fructose-biphosphate aldolase, triosephosphate isomerase, and fructose-1,6-biphosphatase).

Moreover, global transcriptomics analyses unraveled the genetic expression patterns in *C. glutamicum* R incubated under very high cellular densities and then subjected to oxygen deprivation (Figure 2).

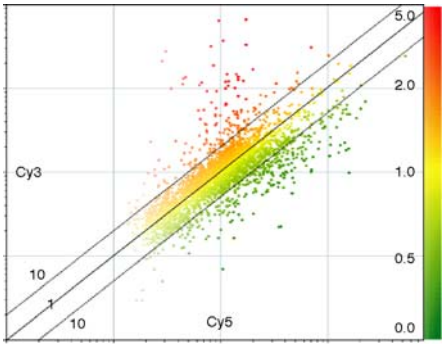

Figure 2. Scatter plot of log Cy3- and log Cy5-derived fluorescence. Each dot represents the average of six independent experiments. The RNA of *C. glutamicum* R cells grown under aerobic conditions and of similarly grown cells but reacted under process conditions was used for Cy3 and Cy5 labeling. The two outer dotted lines demarcate values for significant differences in gene expression levels (i.e., by factors of two and one-half).

While global transcriptional profiling constitutes an extremely useful technique for optimizing manufacturing strains by rational design, its main limitation is that it does not provide any relevant information regarding constitutively expressed genes or regarding those metabolic nodes that are regulated at the protein-modification level. In addition, transcriptome analysis cannot account for differential transcript stability, thus making the relationship between mRNA abundance and protein activity unclear for numerous enzymes (Yukawa et al. 2006). As a result, we applied mass spectroscopy techniques to trypsin-digested protein extracts of *C. glutamicum* R to attain both quantitative and qualitative data, in an effort to document the variations of the protein content of this organism. Specifically, we measured the relative abundance of several peptides per protein, thus uniquely characterizing that protein. This redundancy in data is important because it enables us to achieve higher data robustness. Beyond providing a complete list of such unique signature peptides, we compared the relative abundance of various proteins among samples including extracts representing protein soluble fractions, insoluble fractions, and total proteins. These fractions were isolated from both exponentially growing cells and from cells incubated under production conditions (very high cellular densities, oxygen-deprivation). As expected, a general tendency toward agreement between transcriptomic and proteomic data was observed (Figure 3).

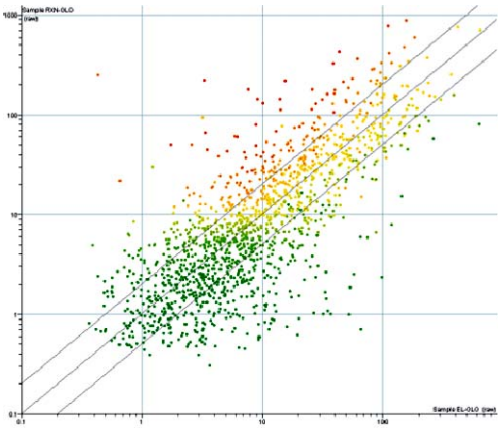

Figure 3. Scatter plot of relative protein abundance as measured by mass spectrometry of trypsin digests of total protein extracts recovered from exponentially growing cells (x axis), and from cells incubated under process conditions (y axis). The two outer dotted lines demarcate values for significant differences in protein levels (i.e., by factors of two and one-half).

This initial set of data demonstrates the robustness of the method employed, including the protein extract preparation method, and thus paves the way to detailed analyses of 1) the global metabolism of *C. glutamicum* and 2) the dynamic regulatory networks that regulate it. Furthermore, the various unique signature peptides identified in the course of these experiments will enable the study of very short proteins synthesized by *C. glutamicum*, and thus contribute significantly to enhancing the quality of the annotation of the *C. glutamicum* R and *C. glutamicum* ATCC 13032 genomes.

Citations

Inui M, H Kawaguchi, S Murakami, AA Vertès, and H Yukawa. 2004a. "Metabolic Engineering of *Corynebacterium glutamicum* for Fuel Ethanol Production Under Oxygen-Deprivation Conditions." *Journal of Molecular Microbiology and Biotechnology* 8(4):243-254.

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Yukawa, H, M Inui, and AA Vertès. 2006. "Genomes and Genome Level Engineering of Amino Acid Producing Bacteria." In *Amino Acid Biosynthesis–Pathways, Regulation, and Metabolic Engineering*, ed. V. F. Wendisch, Springer, Heidelberg, Germany.

User Projects

Zangar NAF R33

RC Zangar, SM Varnum, Pacific Northwest National Laboratory, Richland, Washington

Identification of Post-translational Modifications and Protein Complexes under Conditions of Oxidative Stress

CA Sacksteder, TC Squier, Pacific Northwest National Laboratory, Richland, Washington

Proteomic Analysis of the HMEC Mitogenic Response

BD Thrall, T Liu, DG Camp, K Waters, Pacific Northwest National Laboratory, Richland, Washington

Proteomics of *Shewanella oneidensis* MR-1 Subjected to Solar UV Radiation Stress

JM Tiedje, Michigan State University, East Lansing, Michigan

Identification of Protein Components of Vaccinia Virus Particles

B Moss, W Resch, National Institute of Health, Bethesda,, Maryland

Identifying Targets for Therapeutic Interventions using Proteomic Technology

JN Adkins, KD Rodland, N Manes, Pacific Northwest National Laboratory, Richland, Washington

HM Mottaz, Environmental Molecular Sciences Laboratory, Richland, Washington

F Heffron, S Wong, J Gustin, J Rue, R Estep, Oregon Health Sciences University/Oregon Graduate Institute, Portland, Oregon

Proteomic Characterization of Cerebrospinal Fluid (CSF) by High Resolution LC-MS/MS

S Warren, Massachusetts General Hospital East and Harvard University School of Medicine, Charlestown, Massachusetts

JM Jacobs, DG Camp, Pacific Northwest National Laboratory, Richland, Washington

Proteomic Analysis of the Cyanobacterium *Synechocystis* sp. PCC 6803

JM Jacobs, DG Camp, Pacific Northwest National Laboratory, Richland, Washington

HB Pakrasi, N Keren, Washington University in St. Louis, St. Louis, Missouri

Characterization of the Tumor Cell Lamellipodia Phosphoproteome

JM Jacobs, DG Camp, Pacific Northwest National Laboratory, Richland, Washington

RL Klemke, Y Wang, University of California, San Diego, La Jolla, California

Unraveling the Molecular Biology of Host-Pathogen Interactions

L Shi, Pacific Northwest National Laboratory, Richland, Washington

Peptide Observation Model

LJ Kangas, Pacific Northwest National Laboratory, Richland, Washington

L Wang, Washington State University Tri-Cities, Richland, Washington

Characterization of Neuropeptides Located in Neurosecretory Tissue of Cancer Borealis and Cancer Productus

KK Kutz, University of Wisconsin-Madison, Madison, Wisconsin

Protein Complex Identification using Novel Affinity Resins

NU Mayer-Cumblidge, L Shi, S Verma, TC Squier, Pacific Northwest National Laboratory, Richland, Washington

FedEx1

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Shew Strains

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Reactive Oxygen and Nitrogen Species, Produce Dynamic Protein Modifications and Protein Complexes in RAW 264.7 Macrophage Cells

HS Smallwood, Pacific Northwest National Laboratory, Richland, Washington

Identification and Post-translational Modification of Mitotic Regulatory Proteins

DL Stenoien, Pacific Northwest National Laboratory, Richland, Washington

Proteomics of HCMV

SM Varnum, JM Jacobs, Pacific Northwest National Laboratory, Richland, Washington

J Dumortier, Oregon Health Sciences University/Oregon Graduate Institute, Beaverton, Oregon

Thrust Area 1: Characterization of Purified Proteins for Distribution to Grand Challenge Participants (EMSL User Support)

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Development of High Throughput Global Metabolomics Approaches Based upon Mass Spectrometry

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Advanced Proteomics and Metabolomics Studies of Type 1 Diabetes

TO Metz, JS Zimmer, JM Jacobs, DG Camp, Pacific Northwest National Laboratory, Richland, Washington

Development of Metabolomics for Biomarker Discovery

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Proteomic Studies of Inflammation and the Host Response to Injury

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A Proteome for Specific Cell Types in *Caenorhabditis elegans*

DG Moerman, University of British Columbia, Vancouver, British Columbia, Canada

Proteomic Characterization of *in vivo* and *in vitro* Model Systems of Hepatitis C Virus Infection: Global Quantitative Proteome AMT Tag Measurements of Cellular Protein Expression

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MG Katze, DL Diamond, University of Washington, Seattle, Washington

Identification of Functional Pathways Associated with Clinical Tamoxifen-resistance in Breast Cancer by Advanced Mass Spectrometry

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Biomarker Development for Chronic Obstructive Pulmonary Disease

DL Springer, RE Johnson, JG Pounds, Pacific Northwest National Laboratory, Richland, Washington

Intra-species Proteome within a Natural Population of *Shewanella baltica*

JA Klappenbach, Rosetta Inpharmatics, LLC, Seattle, Washington

The Use of Novel Proteomics in the Plasma and Tumor Microenvironment for Class Prediction in Human Breast Cancer

LL Moldawer, SR Grobmyer, University of Florida, Gainesville, Florida

Molecular Mechanisms Underlying Cellular Adaptive Response to Low Dose Radiation

CA Sacksteder, TC Squier, MA Gritsenko, Pacific Northwest National Laboratory, Richland, Washington

Identify Biomarkers for COPD (Chronic Obstructive Pulmonary Disease) in Humans using Proteomic and Metabonomic Analysis of Serum and Urine.

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Plasma Proteomics Pilot Study to Identify Biomarkers of Stress and Nutritional Effects

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Comparative Proteomic Analysis of *Desulfovibrio vulgaris*

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Characterization of the Neurite Phosphoproteome

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Determining Proteins Expression is *Ralstonia* bacteria that Survive in Ultra Pure Water

KL Ogden, University of Arizona, Tucson, Arizona

Use of FTICR Mass Spectrometric Proteomics Analysis for the Identification of Novel Targets In Pain Research

M Yeo, W Liedtke, Duke University, Durham, North Carolina

Proteome Analysis of Members of the Family *Anaplasmataceae*

Y Rikihisa, T Kikuchi, Ohio State University, Columbus, Ohio

Fragmentation and Characteristic Study of Peptides Missed by SEQUEST

G Tan, VH Wysocki, University of Arizona, Tucson, Arizona

Proteome Analysis of Regulatory Gene Mutants in *Desulfovibrio desulfuricans* G20 and *Shewanella onedensis* MR-1

LR Krumholz, JL Groh, Q Luo, University of Oklahoma, Norman, Oklahoma

Proteomics of *Burkholderia mallei*

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The Schizophyllum Commune Proteome

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Advanced Proteomics and Metabolomics Studies of Type 2 Diabetes and Pre-Diabetes

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Proteomics of *in situ* Biostimulated Sediments

PE Long, MS Lipton, Pacific Northwest National Laboratory, Richland, Washington

Analysis of the Proteome and Transcriptome of In Vivo *Salmonella typhimurium* Infected Ileum

LG Adams, Texas A&M University, college station, Texas

Cellular Response to Virus Infection: Global Proteome Analysis of Macaque Models of HIV and Influenza Infection

MG Katze, DL Diamond, University of Washington, Seattle, Washington

Trauma-induced Reprogramming: Changes in Lipid Raft Protein Content

J Cuschieri, University of Washington, Seattle, Washington

Characterization of the SATB1 Phosphorylations

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Translation Repressor, 4E-BP2, is Covalently Modified in the Mammalian Brain

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Informatics Tool for Proteomic Biomarker Detection using Large-scale nanoLC-FT Mass Spectrometry Data

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Cyanobacteria Membrane Biology Grand Challenge: Systems Analysis of the Dynamics of Membrane Architecture, Composition, and Function- Proteomic, Metabolomic, and Metallomic Characterization

JM Jacobs, DW Koppenaal, TO Metz, Pacific Northwest National Laboratory, Richland, Washington

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Quantitative Characterization of Protein Post-translational Modifications using Mass Spectrometry

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Proteome Enabled Discovery of Growth and Survival Strategies of the High Level Nuclear Waste Actinomycete, *Kineococcus radiotolerans*

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Alpha Project Phosphoproteomics

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Pilot Study on Examination of LIRKO Islets and Serum Using Quantitative Proteomic Approaches

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Development of High Throughput Proteomic Production Operations

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Biomarkers for Early Detection and Other Stages

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High Throughput Proteomic and Metabolomic Early Biomarkers

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NBACC: Host/Pathogen Interactions

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Global and Targeted Proteomic Identification of Oxidative Modifications

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Control of Hydrogen Release and Uptake in Condensed Phases

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Cerebrospinal Fluid Proteome

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Platelet Secretome

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LC-MS-based Metabolomics for Computational Toxicology

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Identification of Metal Reductases

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Application of High-Throughput Proteomics

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Biomarker Discovery in Pathogens by Proteomics

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Secretome Analysis of Environmental Nanoparticle Induced Biomarkers

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LC-FTMS Analysis of Organophosphates

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Peptide Biomarker Analysis

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Environmental Proteomic Analysis of Anaerobic Methane Oxidizing Systems

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Proteome Analysis of *Anaeromyxobacter dehalogenans* Strain 2CP-C

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A Proteomic Dissection of the Hg(II) Toxicity Paradox

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Technology Development in Support of NCRR

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Proteomic Characterization of *Yersinia pestis*

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Low Dose

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Rhodobacter sphaerodies

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Regulated Proteolysis and Bystander Effects in Radiation

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Preliminary Work on the Proteomes of Brains and Dissected Brains Obtained from Control Mice and Treated Mice Simulating Parkinson's Disease

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Spatially Resolved Proteomic Studies of Brains Obtained from Control Mice and Treated Mice Simulating Parkinson's Disease

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Abundance of Protein Components in Photosystem II Protein Complex Purified from Mutant Cyanobacterial Cells Lacking Individual Subunit Protein

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Using Mass Spectrometry to Assist in Identifying Genes for Enzymes of Known Activity, Unknown OpenReadingFrame and Small Abundance

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Mass Spectrometric Analysis of Eukaryotic Proteosome

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Mass Spectrometry Analysis of Nucleotides/Nucleosides

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Protemic Analysis of Naturally Occurring Methane Oxidizing Archaeal Communities

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Proteomics of Membrane Protein Complexes

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Proteomic Analysis of *Deinococcus radiodurans* Under Oxidative Stress Irradiation

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Genomes-to-Life Protein Complex Isolation Capability

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Proteomics of Filamentous Fungi

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Sustrate Identification for Ser/Th Protein Phosphatase

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Identification of Functional Pathways Associated with Clinical Tamoxifen-resistance in Breast Cancer by Advanced Mass Spectrometry

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Cellular Response to Human Immunodeficiency Virus Type 1 Infection: Global Quantitative Proteome AMT Tag Measurements of Cellular Protein Expression

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Proteomic Analysis of *Shewanella oneidensis* Biofilms

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Membrane Proteins of Placental Parasites

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Combined Transcriptome and Proteome Analysis of *Shewanella oneidensis* MR-1 etrA and arcA Mutant Strains

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Proteomics of Mitochondria

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Proteomic Analysis of Arthrobacter

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Proteomic Studies of Growth and Metabolism in *Corynebacterium glutamicum* R

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