High Performance Mass Spectrometry Facility

This report for the High Performance Mass Spectrometry Facility (HPMSF) is intended to provide a description of the research capabilities of the HPMSF, a list of all user projects, and a summary of some of the user-initiated research efforts.

This facility provides state-of-the-art mass spectrometry and separations instrumentation that has been refined for leading-edge analysis of biological problems. Challenging research in areas such as proteomics, cell signaling, cellular molecular machines, and high-molecular weight systems, in general receive the highest priority for access to HPMSF. The current research activities within the facility include proteomic analyses of whole cell lysates, analyses of organic macromolecules and protein complexes, quantitation using isotopically labeled growth media, targeted proteomics analyses of subcellular fractions, and nucleic acid analysis of RNA and DNA oligomers. The facility's capabilities of providing very high-sensitivity and high-resolution mass spectrometry and very high-resolution separations greatly benefit these areas of research and are enhanced by the equipment listed below.

The HPMSF is committed to maintaining state-of-the-art mass spectrometry and separations capabilities. To this end the facility's staff work closely with PNNL researchers to develop new capabilities such as the ion funnel, DREAMS and data analysis tools which are incorporated into the capabilities of the facility as they become available. The facility staff are highly skilled in all of the areas required for proteomics analysis, from sample preparation to analysis and data interpretation, and are available to help develop methodologies to tackle these challenging problems. As needed, PNNL staff outside of the facility can be accessed as matrixed members of the facility. Since its inception, 89 separate user projects have been undertaken by the facility with some of them extending to over a year in duration.

Instrumentation & Capabilities

Mass Spectrometers

- Triple Quadrupole Mass Spectrometer
- Ion Trap Mass Spectrometers

FTICR

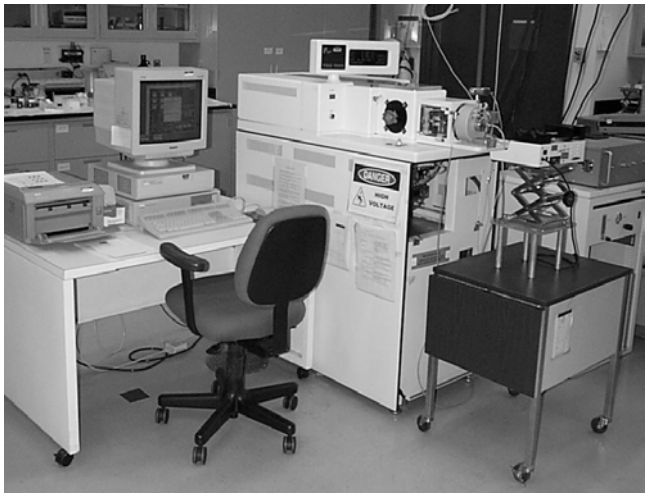
- 11.5 Tesla FTICR
- 7 Tesla FTICR
- 3.5 Tesla FTICR
- 9.4 Tesla FTICR

Additional Capabilities

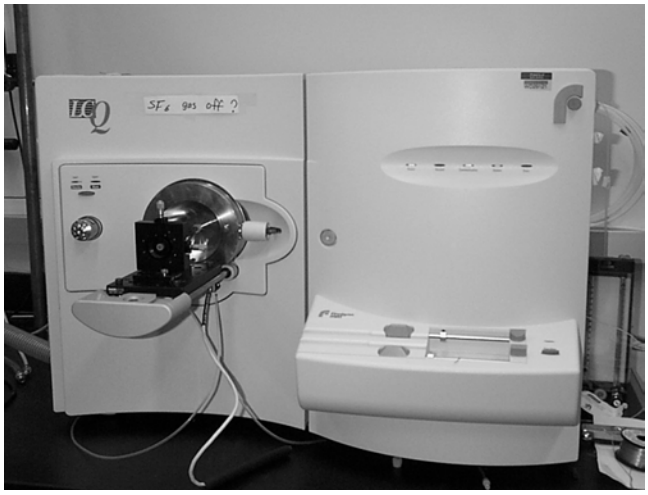
- Separations techniques

The High-Performance Mass Spectrometry Facility 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 Windows-based application that enables many of the unique instrument control functions developed in our laboratory. This same software allows automated spectral interpretation of raw FTICR data. The spectral interpretation features are integrated into our application and thus not easily transferred. As part of our efforts to disseminate our developments in mass spectrometry, this code is made available from our web site to researchers outside of EMSL. When external researchers wish to download the software, they are asked to enter their name and the name of their institution. The accompanying chart shows the monthly activity at this site and the attached spreadsheet is the list of names and institutions.

Magnetic Resonance Research Capabilities

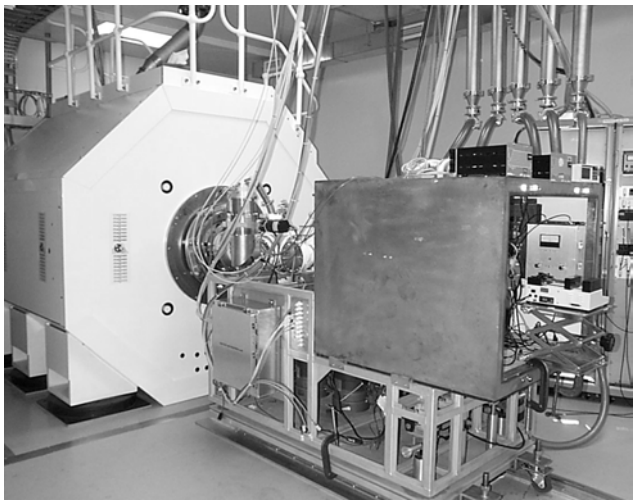


Triple Quadrupole Mass Spectrometer. The ThermoQuest TSQ 7000 is a triple quadrupole mass spectrometer designed for use with an electrospray ionization source. The TSQ has a mass range of 50-4000 m/z with peak widths as low as 0.7 Da and can be operated in MS or MS/MS mode using collision induced dissociation (CID). CID takes place in the second quadrupole at a nominal but variable gas pressure of 23 mTorr. The system can be operated in either positive or negative ion mode with the addition of SF6 as a sheath gas.

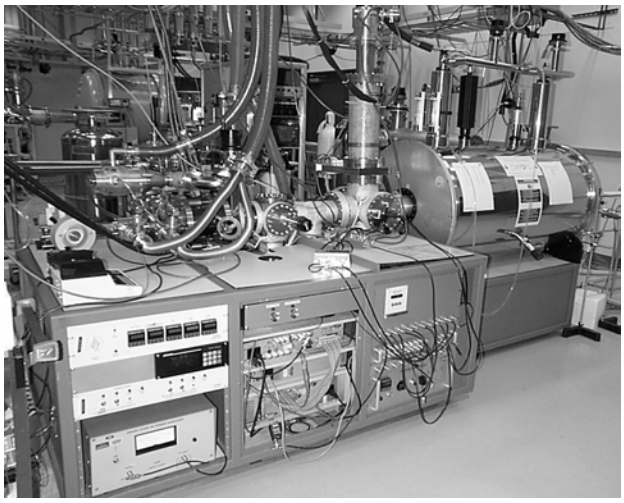


Ion Trap Mass Spectrometers. Five Ion Trap mass spectrometers from ThermoQuest are available in the facility. Two LCQ Classics, two LCQ Duos providing improved sensitivity, and one LCQ DECAXP providing the latest in sensitivity improvements from Finnigan. The Ion Trap is a three-dimensional quadrupole ion-trap-based instrument designed for use with electrospray ionization sources. These instruments are well suited to ms/ms experiments due to their very high collection efficiency for product ions. The mass range of this instrument is 150–2000 m/z but can be extended to 4000 m/z for some applications. The LCQ has 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 SF6 as a sheath gas.

Fourier Transform Ion Cyclotron Resonance Mass Spectrometers (FTICR)

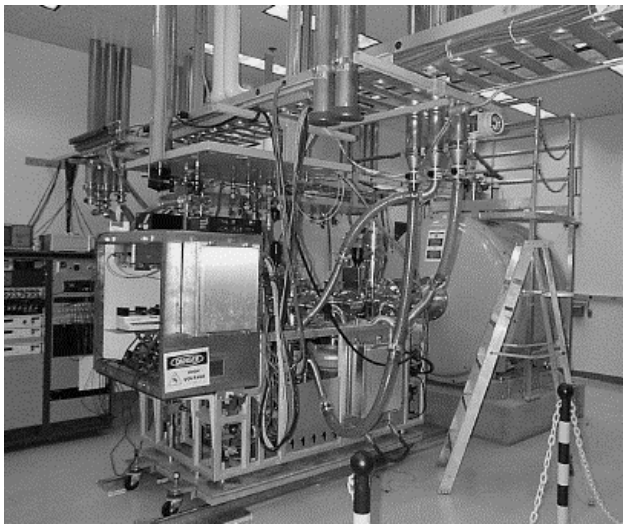


11.5 T FTICR. This ultrahigh performance Fourier Transform Ion Cyclotron Resonance Mass Spectrometer uses a wide-bore (205 mm), passively shielded 11.5 T superconducting magnet. The spectrometer is equipped with an electrospray ionization source and an ion funnel. The 11.5 T FTICR has a resolution of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



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

3.5 T FTICR. This high-performance FTICR mass spectrometer is coupled to a 3.5 T, 330-mm-bore magnet and is equipped with an electrospray ionization source. The 3.5 T FTICR has a mass resolution of 50,000 to 150,000 and a mass accuracy of 3 to 7 ppm for protein samples with molecular weights ranging from 5,000 to 20,000 Da. The ion optics on this system are the most highly developed in the facility. The spectrometer incorporates DREAMS technology and a dual channel ion funnel for simultaneous introduction of a calibrant. The detection limit of the instrument has recently been improved down to $3x10^{-20}$ moles or about 18,000 molecules (based on sample consumption).



9.4 T FTICR. The newest FTICR spectrometer that is available is a 9.4 T, 150 mm bore, actively shielded Bruker Daltonics APEX III. The original ion source has been replaced with a custom PNNL source that incorporates a dual-channel ion funnel for simultaneous introduction of calibrant ions, DREAMS technology, and automatic gain control. This permits maximum use of the spectrometers capabilities by maintaining the optimum number of ions in the ICR cell throughout a liquid chromatographic (LC) separation. The outstanding resolution of over 60,000 is maintained throughout the separation with a sensitivity comparable to the other FTICRs in the facility. This system is seamlessly integrated with PNNL's automated high pressure (5,000 psi) LC system for unattended operation 24 hours a day, seven days a week.

Separations. A signature capability of the facility is the efficient coupling of capillary separations to our mass spectrometers. Instruments for both LC and capillary electrophoretic (CE) 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 PNNL-developed LC systems that deliver constant-pressure gradient separations at up to 5,000 psi. PNNL has recently finished the development of an integrated automated high-pressure LC system. It features 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.

Quadrupole Time-of-Flight. The API QSTARTM Pulsar spectrometer is a state-of-the-art instrument that combines the robust operation of a quadrupole with the speed and resolution of a TOF spectrometer. The new LINAC™ Pulsar high-pressure collision cell, which pulses ions into the TOF analyzer, offers superior mass accuracy and sensitivity. Innovative software applications include Information Dependent Acquisition (IDA) which enables automated MS to MS/MS acquisition for maximum extraction of information from a single LC/MS run and the powerful data acquisition and processing capabilities of Analyst™ and BioAnalyst™.

FTICR Data Station Upgrades

Significant steps were taken this year to upgrade the data stations on the FTICR mass spectrometers in the facility. The data stations for the FTICR mass spectrometers located in EMSL are reaching the end of their useful lifetimes. The original vendor no longer supports the software for the data systems and the hardware is obsolete and no longer supported by SUN Microsystems. Replacing these data systems is a top priority.

A capital allocation to replace one of the three existing data stations and auxiliary control electronics was approved in June of 2002. A purchase order for a Bruker Daltonics FTMS-1560 APEX IV data station with associated control electronics was finalized on September 30, 2002. Delivery of the system is expected by February 28, 2003. The FTICR mass spectrometers in the High Performance Mass Spectrometry Facility have enhanced capabilities that are not available on commercial systems so no commercial data station will fully support those capabilities. The IDL staff in EMSL have provided the necessary support to add these enhanced capabilities to our existing data stations and they will provide the support necessary to add these enhanced capabilities to our new Bruker Daltonics data stations.

Capital Equipment Acquisitions

Bruker Daltonics Data Station including

- FTMS-1560 APEX IV console
- FTMS-1580 Infinity Cell controller
- FTMS-1590 Transfer Optics power supply
- FTMS-1570 Windows 2000 based processing system with XMASS™

Functional Genomics and Proteomics of Mitochondria

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Despite the progress made in deciphering the genetics of the more than one hundred known mitochondrial diseases, many cases remain unexplained mostly due to the fact that only 40-50% of the presumed 700–1000 nuclear encoded mitochondrial proteins have been identified to date. Recent technical advances enable a more direct study of the function of entire genomes and proteomes. A careful and considered application of these techniques should accelerate the identification of those genes. Moreover, mitochondrial genes and pathways are highly conserved across eukaryotes, and there is already an impressive list of yeast-human homologues associated with human mitochondrial disease. The objective of the proposed research is to use the tremendous experimental resources available for the study of yeast that have been partly pioneered in Dr. Davis' lab at Stanford University, in combination with the application of the advanced proteome analysis available in the EMSL lab at PNNL, to elucidate new mitochondria proteins and pathways at an accelerated pace, which will advance our understanding of mitochondria-related human diseases. To achieve the above goal, the following specific aims are being pursued.

Specific Aim 1: To identify new mitochondrial genes, high throughput proteome analysis using high-performance liquid chromatography and Fourier transform ion cyclotron resonance mass spectrometry will be applied to identify accurate mass tags for tryptic digested proteins of isolated yeast mitochondria. The sequences of the peptides analyzed will be searched against a database of known and candidate yeast mitochondria genes. Currently, 420 nuclear encoded genes have been located in yeast mitochondria. Besides these, about 400 candidate genes that have been identified at Dr. Davis' lab by the analysis of yeast knockout strains lacking each of these genes which show defects in mitochondrial functions. The new proteins identified will be further verified by making constructs fused with green fluorescence proteins (GFP) followed by in vivo localization assays. Gene expression analysis using DNA microarrays will be applied to elucidate the biological functions of these genes.

Specific Aim 2: To facilitate research on human mitochondrial pathologies, we will identify accurate mass tags for human mitochondrial proteins. Candidate genes for database searches will be generated by blasting the results from Specific Aim 1 against human sequences to identify homologous genes that may locate in human mitochondria. We will screen each gene discovered for possible mutations in a collection of cell lines obtained from patients with phenotypes known or assumed to be associated with mutations in mitochondrial proteins. These results, together with data obtained with human expression arrays, shall help identify new disease-related genes and pathways. For these proteomic studies, methods have been developed to successfully isolate highly purified yeast mitochondria membranes, as measured by antibody binding assays as well as 2D gel electrophoresis. Research is underway to achieve the same quality for isolation of mitochondria from human cell lines.

Samples of the mitochondria from *Neurospora crassa* were isolated and sent to PNNL. The membranes of the mitochondria were separated from the body of the mitochondria and the membrane proteins were extracted using protocols that had been developed at EMSL to optimize the recovery of membrane proteins for mass spectral analysis using electrospray ionization. The proteins were digested using standard protocols with trypsin. The resulting peptide mixture was separated using ultrahigh pressure (5000 psi) reversed-phase liquid chromatography. The peptides from the column were detected and identified using ESI-MS/MS. With these initial studies 492 unique proteins have been identified. This is already greater than the total number of proteins that have been identified in mitochondria from all previous studies. These initial results will permit the establishment of a set of biomarkers for subsequent rapid analysis of changes in the mitochondrial proteome.

Study of Secretory and Membrane Proteomics of *Pseudomonas aeruginosa*

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(c) Stanford Medical School, California

Pseudomonas aeruginosa is a ubiquitous gram-negative bacterium isolated from soil, water, as well as host systems including plants, insects, and vertebrates. It is one of the top three causes of opportunistic human infections, due in part to its intrinsic resistance to antibiotics and disinfectants. Over 80% of patients with cystic fibrosis acquire a *P. aeruginosa* infection that results in progressive loss of lung function and early death. It is also the most common causative organism of sepsis in burn patients, as well as of many other life-threatening infections in HIV and cancer patients. The pathophysiology of infections due to *P. aeruginosa* is complex, as shown by the clinical diversity of the diseases associated with this organism and by the multiplicity of virulence factors it produces. The objective of this study is to elucidate the strategies employed by the pathogen for successful infection and proliferation and to counter strategies used by infected hosts for self-defense in the form of immune response. These understandings will contribute to the reduction in the incidence and complications of bacterial infections associated with *P. aeruginosa* and other pathogens sharing similar mechanisms. To achieve this important and challenging goal, an interdisciplinary approach has been adopted that takes advantage of expertise from leading groups in the field of genomics and molecular biology (Dr. R.W. Davis's lab at Stanford Medical School), bacterial genetics (Dr. L.G. Rahme's lab at Harvard Medical School), proteomics (Dr. R.D. Smith's lab at EMSL/PNNL), pathology and burn wound infections (Dr. R.G. Tompkins' lab at Massachusetts General Hospital), and bioinformatics (Dr. A.P. Arkins' lab at University of California at Berkeley). In this project, we propose to sequence the genome of a common virulent strain of *P. aeruginosa*, PA14. To determine virulent factors involved in pathogenic infection, research is underway to make systematic mutations in *P. aeruginosa* and to perform infection assays. Expression analysis will then be performed on virulent and non-virulent strains to elucidate infection-related pathways. To study the host defense system, we are doing expression and mutational analysis on infected Drosophila and Arabidopsis. The results will be verified using the mouse system. Detailed analysis of the proteome of *P. aeruginosa* is essential for the success of this project.

Specific Aim 1: The advanced high throughput proteome analysis using high-performance liquid chromatography and Fourier transform ion cyclotron resonance mass spectrometry pioneered by Dr. Smith's lab will be applied to identify accurate mass tags for the membrane and secretory proteins of *P. aeruginosa.* Currently, about five hundred or so genes are implicated in the infection process, but the location of the majority of these genes is not known. However, most of these virulent factors are expected to be secretory proteins, membrane proteins, or transcription factors in the cytosol. Therefore identifying the location of these genes should provide us significant clues about their functions in pathogenicity, which will be further studied by gene knock-out experiments and infection assays.

Specific Aim 2: Comparative analysis of the proteome of the virulent wild type and non-virulent strains due to deletions of virulence-related transcription factors. By identifying the differences in secretory and membrane proteins, together with data from gene expression analysis using DNA microarray, we should gain thorough knowledge about the regulatory circuits of the infection machinery. Understandings acquired from these and other studies in this project should allow us to formulate detailed model for the mechanism of pathogen infection as well as propose novel antibiotic drug targets.

In the initial work, a method was developed for the efficient extraction, enrichment and solubilization of highly hydrophobic integral membrane proteins. This allowed a large-scale subproteomic approach to be used for the identification of this class of proteins. Further insights were gained with an affinity labeling technique to target cystein-containing very hydrophobic integral membrane proteins. Although generally efficient, previously reported large-scale proteomic analyses based on affinity labeling of cysteinyl containing membrane proteins were not intended to analyze very hydrophobic integral membrane proteins.

Both enrichment and labeling techniques were applied on a single membrane sample, which was divided in two aliquots. One aliquot was used for unlabeled sample preparation to enrich for hydrophobic proteins to identify as many membrane proteins as possible. The other aliquot was labeled using iodoacetyl-PEO biotin, tagging cysteine-containing proteins of the membrane subproteome allowing the reduction of sample complexity and the identification of lower abundance proteins.

We have been able to successfully identify 786 unique proteins from these two samples and assign 707 of them to recognized functional classes. A GRAVY value was determined for all identified proteins using the ProtParam algorithm. A total of 195 hydrophobic proteins were identified and 76 of them had a GRAVY value of >0.32, which is considered the limit for analysis by 2D gels. Using the PSORT algorithm, the number of transmembrane domains in each protein was mapped. A total of 333 proteins with one or more trans-membrane domains were identified.

Proteomic Characterization of *Yersinia pestis*

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The ability to respond adequately to a biological threat requires not only rapid and accurate detection, but also a detailed understanding of the threat agent to determine the appropriate response. Since proteins serve as the basis of all mechanisms in biological systems, an understanding of the changes in protein expression patterns of a pathogen with changing conditions is crucial to understanding the virulence factors of that pathogen. *Yersinia pestis* (Figure 1), the causative agent of plague, is a gram negative, highly communicable, enteric bacterium and is also a known bioterrorist and biowarfare threat agent. The objective of this work is the characterization of *Yersinia pestis* using a mass spectrometry–based proteomics approach, thus providing an essential bridge between ongoing efforts in the CBNP program.

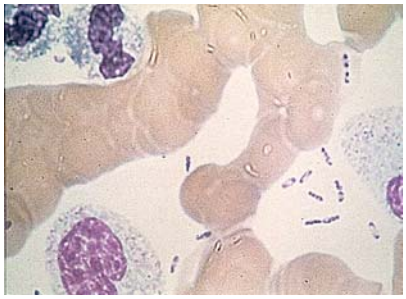

Figure 1. *Y. pestis* culture in blood. Image from www.CDC.gov.

The emerging field of proteomics offers extreme promise in addressing many aspects of biomedical issues including those related to bioterrorism. Whether to gain a more comprehensive understanding of virulence factors to design better therapeutics or investigate better biomarkers for detection, these studies are based intrinsically on the evaluation of protein expression. Unlike the static nature of the genome, the full genetic complement of an organism, the proteome (the proteins expressed in an organism at any time under any specific conditions) is constantly changing. Due to the dynamic nature of the proteome, it is extremely important to characterize the proteins that are expressed under changing conditions.

Advanced technology developed at Pacific Northwest National Laboratory has allowed for the characterization of the proteome to an unprecedented level, enabling broad range analyses on a single organism.

Specifically, the goals of this project are to expand the knowledge base of the threat, enabling better intervention and treatment after exposure. This project provides a greater understanding of the life cycle, virulence, and pathogenesis of *Y. pestis* while at the same time providing highly distinctive and specific potential biomarkers for an integrated mass spectrometry–based detection system. Additionally, the project builds a vital bridge across the gap between two established program thrusts: the determination of DNA signatures and the characterization of protein structure by pinpointing important protein targets. Additionally, the peptide identifications obtained by this study will enable new detection methodologies for existing and emerging genetically engineered biothreats.

FY02 Progress

The first phase of this work has leveraged expertise at Pacific Northwest National Laboratory and Lawrence Livermore National Laboratory to focus on the creation database of potential biomarkers for all the proteins expressed in *Y. pestis*. The virulence mechanism of *Y. pestis* and Type III secretion can be induced in culture by adjusting the physiological parameters of temperature and calcium concentration in order to mimic the environments that *Y. pestis* would encounter in going from the flea vector (26°C) to the human host (37°C). Initially, these preliminary studies have focused on the examination of growth of the organism at both ambient body temperatures of the flea vector (26°C) to the human host (37°C). The mass spectrometric analysis has provided biomarkers for at least 44% of the proteome of the organism to date (Table 1) and proteins expressed from all genetic elements have been observed. Representative proteins from all the major functional categories have also been observed (Figure 1). Of particular interest in the study of *Y. pestis*, and ultimately the target of detection devices, are the virulence factors. Although the location of many of these factors is not known, a set of proteins predicted to attribute virulence to *Y. pestis* is contained within the annotation (see http://www.sanger.ac.uk/Projects/Y_pestis). Using this list of virulence factors, the protein expression patterns from each of the culture conditions were determined. Many of the factors are observed in all the culture conditions, however some demonstrate differential expression. These experiments are still in the early stages, and the results represent only a small fraction of the information that can be distilled from the data. The logical continuation of this work—coupling the protein expression level data through comparative display experiments—will enable a much more detailed interpretation of the data leading to a better understanding of *Y. pestis* virulence. As analyses continue, this coverage of the proteome will expand, increasing the number of biomarkers available for *Y. pestis* and enabling high throughput future studies.

Table 1. Proteomic Observation of Open Reading Frames (ORFs) from *Y. pestis*

Genetic element	Total	Observed	% Observed
Total	4083	1794	44%
Chromosome	3885	1712	44%
pPMT1	100	35	35%
pCD1	89	42	47%
PPCP1	9	5	55%

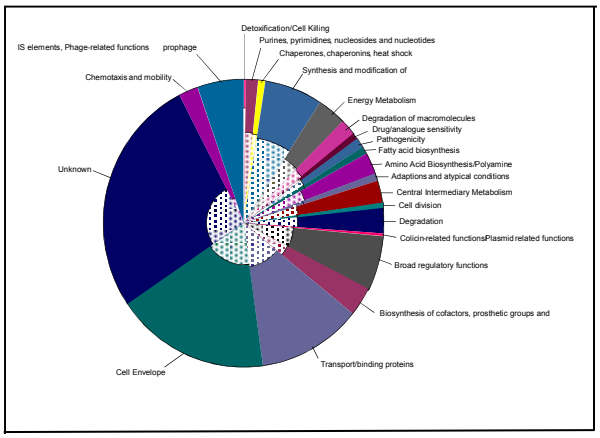

Figure 1. Illustration of the overall proteome coverage for Y. pestis. Each portion of the pie represents the percentage of proteins assigned to a particular functional category. The shaded regions of the pie represent the percentage of proteins identified for each category.

Proteomic Profiling of *Deinococcus radiodurans*

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The *D. radiodurans* strain R1 is a gram-positive non-pathogenic bacterium whose 3.1 Mbase genome consists of two chromosomes, one megaplasmid, and one plasmid [3960]. *D. radiodurans* has an extraordinary ability to tolerate both acute and chronic exposures to high levels of ionizing radiation. *D. radiodurans* can survive 5,000 to 15,000 Gy of acute ionizing radiation (depending on the culture conditions) and grow continuously under 60 Gy/hr. Our analysis used the 3116 protein-encoding open reading frames (ORFs) predicted by the TIGR annotation (ftp://ftp.tigr.org/pub/data/d_radiodurans/GDR.pep) (we exclude from this analysis 71 ORFs predicted to contain frame shifts). However, the annotation consists of the best prediction of ORFs encoded by an organism based on comparison to ORFs identified in other organisms and on the codon usage. The proteomic measurements provide a physical validation that the ORFs actually encode a protein.

Over 400 LC-MS/MS analyses of peptides from collective culture conditions yielded tentative identifications for over 70,000 peptides having a SEQUEST score above 2. Our FTICR measurements verified with high confidence 26,326 of these accurate mass and time tags (AMT). In order to confirm the presence of an ORF in our analysis, we required that at least two mass tags were identified for an ORF and that those mass tags were seen in at least three spectra in the FTICR mass spectrometer. Using these criteria, we identified 2585 ORFs from *D. radiodurans,* representing 83% of the predicted ORFs (Table 1) for *D. radiodurans,* corresponding to the broadest proteome coverage for an organism achieved to date. Over 90% of the ORFs are identified if only one mass tag is required for ORF identification. In a single FTICR analysis, the masses for ~1,500 AMTs are typically detected corresponding to ~700 ORFs (depending on the culture condition), and 15% to 20% of the *D. radiodurans* proteome.

Table 1. *D. radiodurans* proteome coverage

Category	Size	Predicted ORFs	Observed ORFs	Percent Coverage
Total	3.29 Mbp	3116	2585	83
Chromosome	12.65 Mbp	2633	2154	83
Chromosome	2412 kbp	369	304	82
Mega Plasmid	177kbp	145	100	68
Small Plasmid	46 kbp	40	27	67

The proteomic profile of *D. radiodurans* contained ORFs assigned to every functional category. Figure 1 is a representation of the distribution of proteins into these functional categories. The size of the pie slice indicates the number of proteins from the annotated genome that fall into the category indicated. Each pie piece has been shaded to indicate the percentage of proteins from that category that has been identified by the proteomic profiling. Not surprising, the categories associated with housekeeping functions, protein synthesis, amino acid synthesis, cell envelope, etc. had representation, for the most part, of over 90%. Hypothetical and conserved hypothetical proteins are proteins whose expression

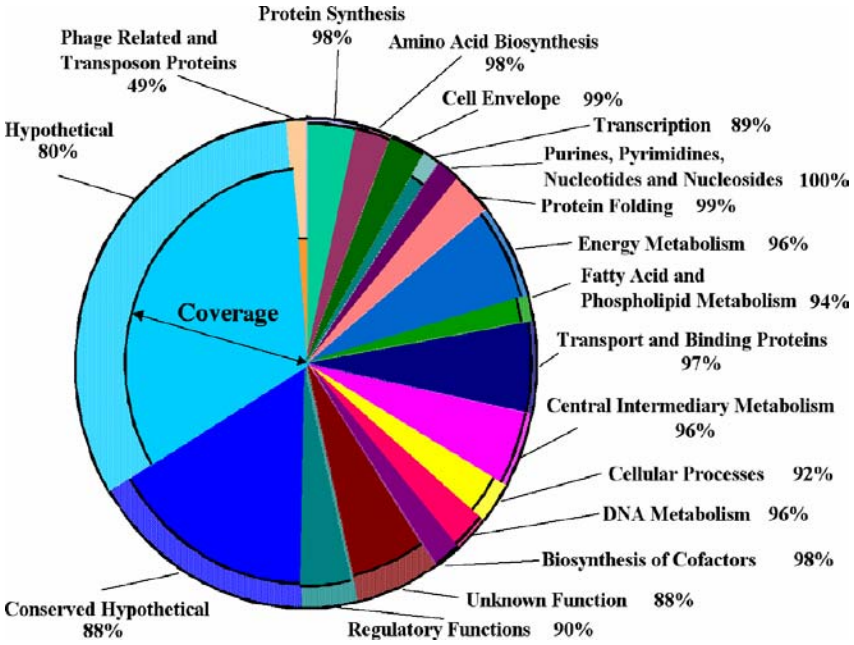

Figure 1. Distribution and coverage by TIGR annotated function for proteins identified using AMT tags from studies of *D. radiodurans*.

has not been previously confirmed by physical methods. The proteomic profiling of *D. radiodurans*, confirmed the expression of 88% and 80% of the conserved hypothetical and hypothetical proteins respectively. In confirming the expression of these proteins, these ORFs represent possible targets for further study.

Coverage of functional category members can be used to predict the expression of specific metabolic pathways. Predicted proteins involved in various *D. radiodurans* metabolic pathways were commonly identified with multiple different AMTs, suggesting their high abundance. We verified the expression of all the predicted proteins corresponding to the vacuolar type (V-type) proton ATP synthase, as well as the predicted components of the organism's TCA cycle enzymes. In addition, 80% of the predicted proteins involved in glycolysis and the pentose phosphate shunt were detected. A slightly smaller fraction of electron transport proteins were identified (several are integral membrane proteins). The patterns of expression for all the ORFs (data not shown) illustrates that many predicted proteins associated with "housekeeping" functions are expressed under all conditions evaluated. Approximately 32% and 16% of the ORFs from the *D. radiodurans* database are predicted to be hypothetical and conserved hypothetical respectively. We identified 80% of these hypothetical proteins and 88% of the conserved hypothetical proteins.

Global Analysis of *Shewanella oneidensis* Strain MR-1 Proteome Using Accurate Mass Tags

MS Lipton,[(a)] DA Elias,[(a)] MF Romine,[(a)] YA Gorby,[(a)] J Fredrickson,[(a)] and RD Smith[(a)]

(a) Pacific Northwest National Laboratory

Shewanella oneidensis strain MR-1 has potential applications in the bioremediation of metals due to its ability to enzymatically reduce and precipitate a diverse range of heavy metals and radionuclides. A thorough understanding of how MR-1 responds to changes in electron acceptor type and concentration, and the enzymatic pathways involved in these reactions is critical for effectively using metal-reducing bacteria for remediation. A critical step in this process is being able to characterize the proteome of MR-1, or the entire protein complement of the cell, expressed under a given set of conditions. The ability to conduct these analyses in a high-throughput, comprehensive and quantitative mode provides a powerful means to define regulatory and metabolic networks in bacteria. The protein complement from a single microbial genome can vary significantly as a function of cell cycle stage, cell differentiation, environmental conditions (nutrients, temperature, stress, etc.), and even association with other organisms.

We have used a new technology based on the combination of global tryptic digestion of cellular proteins, high-resolution liquid chromatography and tandem mass spectrometry, and high field FTICR mass spectrometry to define the proteome of an organism. One approach for protein identification is based upon global approaches for protein digestion and accurate mass analysis and resulted in the generation of an "accurate mass and time tags" (AMTs) database for *S. oneidensis*. Using this approach we have identified over 60% of the proteome for *S. oneidensis*. As shown in Table 1, although we have not detected a large percentage of the proteins coded on the plasmid, the majority of the proteins are coded from the chromosome, and we were able to detect a high percentage of these proteins.

Table 1. Proteins detected for *S. oneidensis*.

Category	Possible	Detected	% category coverage
Total	4943	3046	62
Chromosome	4780	2971	62
Plasmid	163	79	49

We detected proteins spanning all functional categories as shown in Figure 1. The majority of the proteins that we were able to detect, not surprisingly, were housekeeping proteins including functional categories such as the synthesis of nucleotides and nucleosides, protein synthesis, transcription, and metabolism. Much less represented is the conserved hypothetical and the hypothetical, although this finding is much less surprising since these proteins are only predicted to be expressed with no homology to any other known proteins. In an effort to analyze post translational modifications, the analysis of tryptic peptides was combined with analysis of intact proteins. A total of 546 unique tryptic peptides were detected from the three samples collected at midlog, latelog, and stationary phases of *S. oneidensis* cell growth. These peptides corresponded to a total of 287 unique proteins. The confidence of the protein identification is much greater for proteins identified through

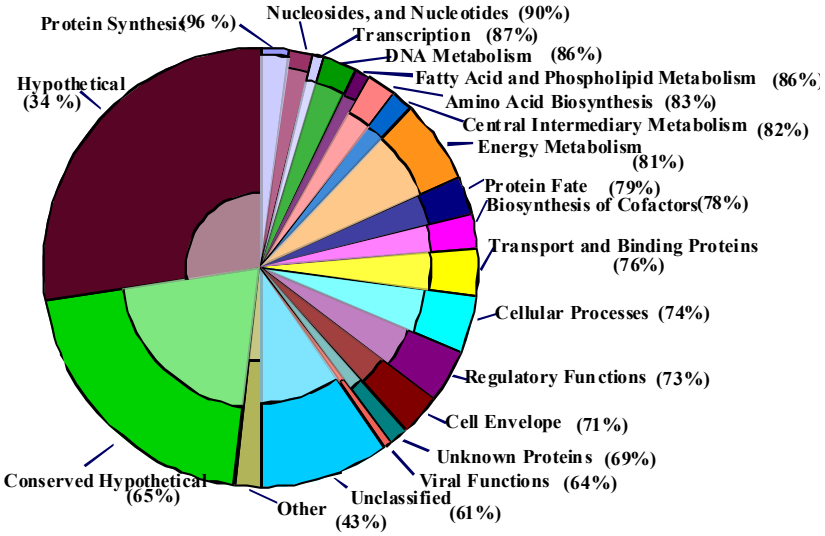

Figure 1. Distribution and coverage by TIGR annotated function for proteins identified using AMT tags from studies of *S. oneidensis*.

tryptic peptides than proteins identified as intact proteins, both because of the greatly increased mass measurement accuracy and the additional requirement of multiple MS/MS fragment ions with low mass error. Because of this, the list of proteins present in the three *S. oneidensis* samples was created from the identifications of proteins from tryptic fragments. A total of 772 unique proteins were detected from the three *S. oneidensis* samples through the analysis of intact proteins. These 772 proteins were correlated to the list of proteins identified by tryptic peptide analysis, and a total of 40 unique proteins were detected through both methods. We can identify post-translational modifications of over 25 of these proteins. Additionally, we have extended the use of this AMT database and, based upon stable-isotope labeling, to quantitatively determine the relative changes in protein abundance from MR-1 cells grown aerobically and anaerobically with fumarate as the electron acceptor in batch and continuous culture.

Characterization of Hollow Helices as Folding Nanotubes with Tunable Cavity Size by ESI-MS

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Two different classes of oligomers and polymers, consisting of benzene rings linked by amide and ethynyl groups respectively, are designed to fold into large helical structures. The backbones of these oligomers and polymers are rigidified by intramolecular hydrogen bonds. An oligomer with a backbone that is long enough folds back on itself, leading to left- and right-handed helices.

Such a backbone-based helical programming leads to helices whose folded conformation is resilient toward structural variation of the side groups that determine the outside surface properties. The interior of a helix is featured either by amide O atoms, which leads to large hydrophilic cavities, or by aromatic H atoms, which makes the tubular cavities rather hydrophobic. The internal diameters of the helices are adjustable (8 E to 40 E and larger), which represent two versatile systems of unnatural folding nanotubes with adjustable interior cavities (Figure 1). Furthermore, one class of these unnatural foldamers have backbones that are helical as well as unsaturated, features that may endow unusual and useful physical and chemical properties.

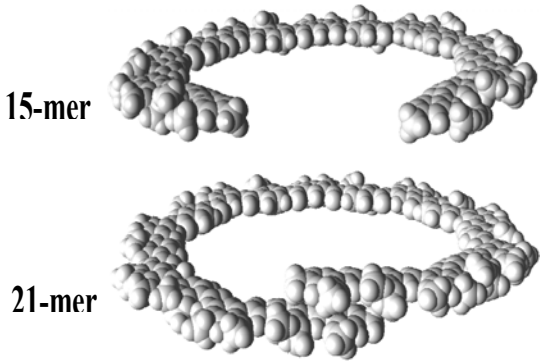

Figure 1. Models of 15-mer and 21-mer. The backbone of 21-mer is long enough to fold into helical conformation with an interior cavity >30 Å across, the largest thus far formed by the folding of unnatural foldamers.

The resulting oligomers and polymers should lead to nanoporous materials that will find numerous applications in a number of areas, such as catalysis, separation, drug carriers, nanodevice (sensors, fluidic, etc.) design, nanoscaffolds for building larger structures, and design of other nanoporous materials.

The design and synthesis of helices with superhydrophobic (fluorinated) or amphiphilic interior cavities was tested. The resolution of enantiomers and the induction of helical twist sense by chiral side chains and chiral solvents are being pursued. The photophysics, binding behavior, environment within the nanocavities, and nanofluidic properties of these folding nanotubes are being investigated by methods involving fluorescence, calorimetry, and computational studies. It is increasingly difficult to characterize large oligomers, since their structural complexity starts to reach the limit of techniques such as 1D and 2D NMR. One powerful technique for characterizing these oligomers is modern mass spectrometry, such as

electrospray mass spectrometry (ESI). To obtain high mass measurement accuracy and resolution, 7 T Fourier transform ion cyclotron resonance mass spectrometer was used.

By taking advantage of the mass spectrometry facilities at PNNL, the difficulty in characterizing the oligomers—and, in the future, polymers with high molecular weight and protein-like structural complexity—can be easily addressed (Figure 2). The most valuable information available from mass spectral measurements is the molecular weights of these large molecules, which provide a rapid means to the identification of the sythesized molecules. 9-, 11-, 15 and 21-mer were analyzed by ESI 7-T FTICR MS, spraying from chloroform solution. Positive and negative ion spectra were obtained and the composition of those compounds is confirmed.

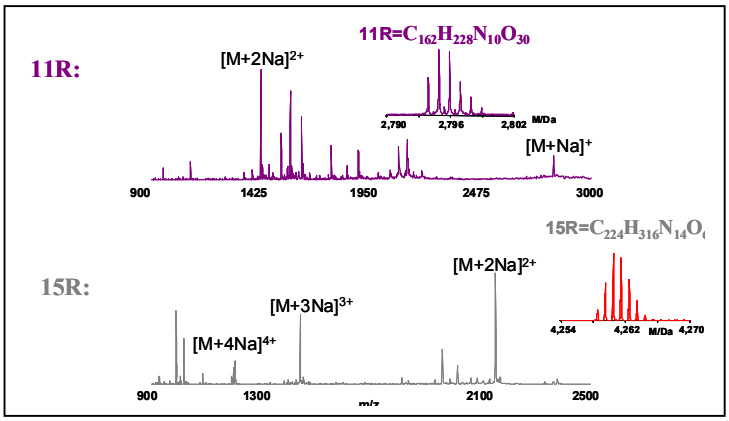

Figure 2. ESI-FTICR mass spectra of 11-mer and 15-mer, electrosprayed from choloroform. Insets show deconvoluted neutral masses of oligomers.

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Gong, B, H Zeng, J Zhu, L Yua, Y Han, S Cheng, M Furukawa, RD Parra, AY Kovalevsky, JL Mills, E Skrzypczak-Jankun, S Martinović, RD Smith, C Zheng, T Szyperski and XC Zeng. "Creating nanocavities of tunable sizes: Hollow helices." *Proc. Nat. Acad. Sci.,* **99**, 11583-11588 (2002).

Use of Isolation and Characterization of Novel Antimicrobial Peptides from the Zebrafish, *Danio rerio*

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(a) Pacific Northwest National Laboratory
(b) University of Maine, Orono

The zebrafish, Figure 1, is a dream system for scientists riding the next wave of genome-wide exploration (Fishman 2001). This photogenic creature is the first vertebrate that has proven tractable to the type of large-scale genetic screening used so successfully in fruit flies and worms.

Figure 1. Zebrafish, *Danio rerio*.

Mutations induced by chemicals, radiation, or viral insertion cause visible changes (phenotypes) that can be readily observed in this vertebrate. The lessons we learn from zebrafish will prove invaluable for those now contemplating the awesome task of interpreting similar random mutagenesis screens in the mouse. Thanks to its transparency, the zebrafish embryo facilitates analysis of mutations because changes in its phenotype can be tracked at the level of the individual cell in the living animal. The similarity of developmental programs among all vertebrates means that the zebrafish is a great model for investigating human development (or vice versa, depending on your specio-centricity).

Antimicrobial peptides have been shown to play a key role in the innate immune defense of a wide variety of organisms, ranging from bacteria to human beings. They have been demonstrated to possess anti-bacterial, anti-fungal, anti-viral, anti-parasitic, and anti-cancer properties (Figure 2). The classical understanding of antimicrobial peptide function involves the interaction of the peptide with a biomembrane, followed by disruption and eventual cell death. However, recent evidence indicates that antimicrobial peptides may be able to modulate and affect immune responses in ways more complex than these simple membrane interactions. Indeed, it is our hope that we may begin to examine some of these questions using the zebrafish model system. Antimicrobial peptides have yet to be isolated from the zebrafish, *D. rerio*. Typical genetic approaches involving EST database mining have proven difficult due to the lack of conservation at the amino acid level and the relative paucity of fish antimicrobial peptides that have already been identified. As a result, we have chosen to isolate novel antimicrobial peptides via chromatography and mass spectrometry. In preliminary experiments bioactive fractions isolated from strong cation exchange HPLC were further purified using HPLC-MS and HPLC-MS/MS. In one such experiment, over 5000 masses were detected. We hope to characterize other bioactive fractions using HPLC-MS and HPLC-MS/MS techniques and to develop a zebrafish database from which we can assign identities to specific data points. The sensitivity of the

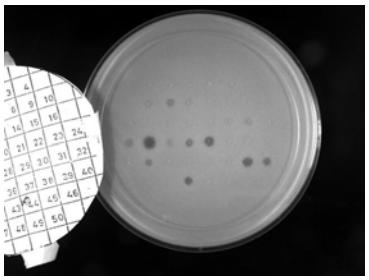

Figure 2. Antimicrobial activity of the fractions is tested on *E. coli*.

FT-ICR MS instruments will be necessary for analysis of our samples because of the small sample amounts and low concentrations.

Bioactive fractions isolated from strong cation exchange HPLC are further purified and analyzed using HPLC-MS and HPLC-MS/MS on the 7 T FTICR mass spectrometer. Having obtained MS and MS/MS data we are going to develop a zebrafish database from which we can assign identities to specific data points.

References

Fishman, MC. GENOMICS: Zebrafish – the Canonical Vertebrate. *Science*, **294**, 1290-91. (2001).

Detection of Botulinum Toxin A Proteolytic Activity

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The major objective of this project is to confirm translocation of the botulinum toxin A enzymatic unit through the lipid bilayer. Botulinum toxin A is excreted from cells and displays specific proteolytic activity in a target substrate; in this case SNAP-25 is used as the protease substrate (Figure 1).

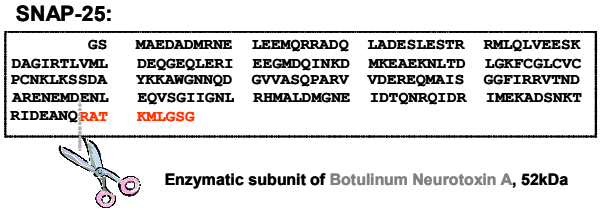

Figure 1. Sequence – SNAP-human synaptosomal-associated protein, 25kDa (SNAP25).

A synthetic fragment of SNAP-25 (17 amino acid residues) was used as the protease substrate and will be available for the calibration of the instrument. The limiting factor for the assay is expected to be the very low enzymatic subunit concentration in the solution. The study required an FTICR because of the need for high sensitivity and accuracy considering the enzymatic subunit concentration in complex solution is very low.

Project was started with direct infusion ESI-FTICRMS analysis of synthetic fragment of SNAP-25. However, it showed that the sample is much more complex than initially thought. Separation was needed and LC-7T FTICRMS and MS/MS were performed. The searched fragment was separated and identified in low concentration. Multiple LC-FTICRMS analysis of the biological sample (~ 100x more diluted) did not show the presence of the searched peptide (Figure 2).

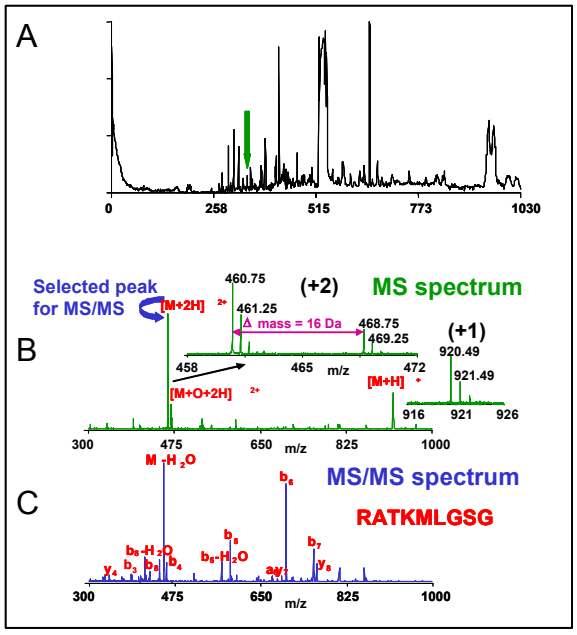

Figure 2. Multiple LC-FTICRMS analyses.

Inactivation of Monomeric Sarcosine Oxidase by a Suicide Substrate

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(b) Pacific Northwest National Laboratory

Monomeric sarcosine oxidase (MSOX) is a monomeric-protein-containing FAD that is covalently attached to a cysteine residue near the carboxy-terminus (cys315). Covalent attachment of FAD involves loss of one proton from cys315 and 1 proton from the 8-methyl group of FAD.

The kinetic mechanism of inactivation of monomeric sarcosine oxidase by N-(cyclopropyl) glycine (CPG) was recently determined. CPG is a sarcosine analog which acts as a suicide substrate and covalently modifies the flavin prosthetic group of the enzyme. Unveiling of the chemical mechanism of inactivation is of interest. The modified flavin in CPG-modified MSOX is unstable since it cycles back to unmodified flavin via a 1,5-dihydroFAD intermediate. The modified flavin can be stabilized by further reduction with sodium borohydride. The borohydride-treated CPG-modified MSOX has been crystallized. A data set has been collected at 1.85 Å, but despite the high resolution, crystallographers still have difficulty in interpreting the new electron density near the flavin and it may suggest the mixture of two different species.

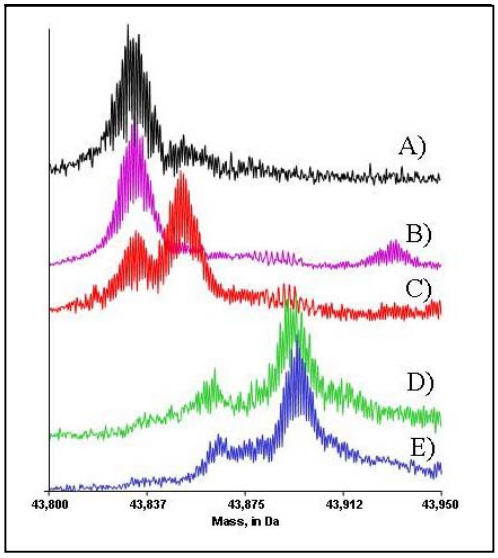

Figure 1. ESI-FTICR zero charge mass spectra of (A) unmodified MSOX; (B) MSOX treated directly with $NaBH_4$, (C) MSOX treated directly with $NaBD_4$, (D) MSOX modified with CPG, then treated with $NaBH_4$ and (E) MSOX modified with CPG, then treated with $NaBD_4$.

ESI-FTICR mass spectrometry was first used to determine a molecular weight of the borohydride-reduced CPG-modified flavin and the molecular weight of unmodified MSOX. Next, we wanted to get more details using deuterated borohydride and compare it with the nondeuterated one (see Figure 1).

Samples were analyzed under denaturing conditions keeping intact covalent modifications. The modified flavin in this construct is "stable" upon denaturation with guanidine hydrochloride in the sense that it does not revert to unmodified oxidized flavin, but small spectral changes are observed over a period of hours after denaturation. The difference in measured molecular weight indicated the mass of covalent modification and gave rise to confirmation of the structure and mechanism of formation of CPG-modified FAD in MSOX after reduction with borohydride. The proposed structure is consistent with observed mass difference of 63 Da and consistent with preliminary crystallographic results which show that C(4) of the flavin ring is tetrahedral and

that there are at least three atoms (not in a ring) attached to C(4A) of the flavin ring. Chemically, it is based on the reaction of MSOX with CPG via an initial single-electron transfer (SET) mechanism; borohydride is known to reduce imines and carbonyl functions and we have evidence that borohydride reduces the C(4) carbonyl in unmodified MSOX.

Further, modified MSOX was analyzed by ESI-FTICR MS under native conditions in order to find out if there may be a fragment of the original inhibitor that is noncovalently bound and therefore lost under denaturing conditions. This information might be especially useful to the crystallographers who are still having problems in completely interpreting the electron density (see Figure 2).

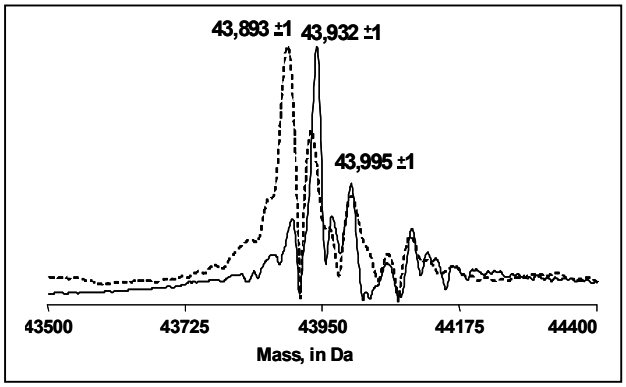

Figure 2. ESI-FTICR zero charge mass spectra of CPG-modified MSOX from native solution.

Use Characterization of a Putative Truncated Calcium-Sensing Receptor from Human Ovarian Tumors

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(a) Oregon Health & Science University, Portland
(b) Pacific Northwest National Laboratory

The wild-type calcium-sensing receptor (CaR) plays an important role in regulating the proliferative behavior of normal ovarian surface epithelial cells, the cell type responsible for ovarian cancer. In a study of 55 ovarian tumor samples from patients, a highly immuno reactive protein band of 65 kDa, which is specifically recognized by the anti-CaR antibody was observed; antibody binding is blocked in the presence of excess competitive peptide. Statistical analysis indicates a positive correlation between an increase of the protein expression and the tumor stage. This protein could represent a truncated version of the full length CaR resulting from alternate splicing, frame-shift mutation, or abnormal proteolytic processing. It is, however, also possible that it is a distinct protein expressing an epitope recognized by the antibody, generated against a synthetic peptide.

After partial biochemical purification of the full length and putative truncated CaR, the appropriate portions were size-fractionated by SDS-PAGE. Coomassie stained bands corresponding to the immunoreactive proteins at 120 kDa and 65 kDa were excised and subjected to in-gel tyrptic digestion. Tryptic peptides were separated by LC and analyzed by FTICR MS and MS/MS. Additionally, LC-MS of intact proteins (i.e., complex mixture prior to SDS-PAGE) and LC MS/MS analysis of their tryptic digest were performed. All experimental data were searched against predicted full length CaR protein sequences using the ICR-2LS program.

Parallel experiments were performed on full length CaR protein (~120 kDa) and putative truncated CaR protein (65 kDa) extracts. LC-FTICR MS data obtained from the analysis of complex mixture of proteins (prior to SDS-PAGE) trypticaly digested revealed the presence of many peptides (mainly present in higher abundances) that do not correspond to the CaR sequence. Many peptide masses corresponding to CaR were seen, but they were minor peaks in the mass spectra. Moreover, LC-MS analysis of peptides obtained by in-gel digestion of a single SDS PAGE spot (~120 kDa or 65 kDa band) gave similar results. Thus, MS-MS analysis is required for unambiguous identification of the detected peptides. Preliminary data confirm that high-abundance peptides, which are easier to dissociate in MS/MS experiment, do not correspond to tryptic peptides of CaR. Thus, the use of dynamic range enhancement methods (e.g., ejection of the high-abundance species in the quadrupole prior to ion accumulation) will be necessary for the confident identification of these low-abundance peptides that were initially assigned to the CaR protein.

Processing of the Cysteine Protease Aleurain

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(a) Washington State University, Pullman

To get a better understanding of protein trafficking from the Golgi to the vacuole via the prevacuolar compartment, a cysteine aminopeptidase aleurain has been studied. Aleurain was shown to be present in all types of plants and tissue and to be subjected to maturation from an inactive 42-kD Golgi precursor to a 33-kD intermediate to a final 32-kD vacuolar mature enzyme. Using in-vitro processing experiments in parallel with protease inhibitors, these two steps of aleurain maturation proved to be the work of two different kinds of enzymes, revealing only clues that the second maturation enzyme is a member of the cysteine protease family. By comparison to the processing hypothesis of the mammalian homologue cathepsin H (65% sequence identity), that last step could be autocatalytic. The first step not being autocatalytic made the maturation process of aleurain unique to the cysteine protease family. Past studies indicated the prevacuolar compartment as the location of aleurain maturation.

The purification of the maturation enzyme will bring light on the unique process leading to active aleurain and its mammalian homologue, cathepsin H, and will give us the first marker for the study of the prevacuolar compartment. The elaboration of three purification steps using an in-vitro assay led to partial purification of the maturation enzyme. Further studies indicated a size range for the maturation enzyme of 25-32 kD. A silver stain of a SDS-Page gel showed only five bands in the maturation enzyme size range.

We hoped that use of the Q-TOF located at the EMSL user facility would help identifying proteins in the gel bands and target a good candidate for the maturation enzyme. In-gel trypsin digest of most the bands were used on the LC Q-TOF to get peptide sequences. The data was then analyzed using Mascot search (www.matrixscience.com). Two *Arabidopsis* proteases have been identified so far, one a 52-kD pro-serine carboxypeptidase present in two bands and the other a 50-kD pro-cysteine protease present in another band. The maturation enzyme is an endoprotease and thus could not be represented by the carboxypeptidase. Until we can obtain data from the remaining band we cannot identify the cysteine protease as the maturation enzyme.

Identification of Oligoribonucleotide Products of RNA Cleavage in *Saccharomyces cerevisiae*

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Recent discoveries have shown that a class of RNA molecules called small RNAs operate many of the cell's controls, including gene regulation. These discoveries, amongst a growing realization of the importance of RNAs, led *Science* magazine to choose RNAs as the "Molecule of the Year." This project exploits the sensitivity of mass spectrometry to delve further into the metabolism of RNA to increase our understanding of the pathways affected by these very important molecules. Here we are looking for the product of RNA metabolism that is predicted to exist in all living cells but has *never* been seen *in vivo*. Newly synthesized RNAs are subject to hydrolysis by RNA polymerase II in a factor-dependent manner. This factor is a stimulatory protein that binds RNA polymerase and facilitates RNA chain elongation through difficult spots in DNA that prevent the elongation of RNAs in an unusual manner; i.e., by stimulating an endoribonuclease activity of elongating RNA polymerase. This activity, which results in the generation of a specific 9-base RNA, has been well-studied in vitro and is ubiquitous across eukaryotic organisms. Intermediates or products of this activity have never been seen *in vivo* in any organism.

A synthetic RNA oligonucleotide (p-CGUUUUUUU-OH) was used to develop the LC/MS method. The oligonucleotide was eluted from a reverse-phase C_{18} column using a water-methanol gradient containing 1,1,1,3,3,3-hexafluoro-2-propanol as an ion-pairing agent and triethylamine to improve sensitivity and suppress adduct formation in the electrospray ionization process. Negative electrospray ionization was used to introduce the LC eluent into an ion-trap mass spectrometer. Analysis was performed on ions in the mass range (m/z) of 525-2000. Estimates of reproducibility and sensitivity were obtained by analysis of triplicate injections of the standard at three concentration levels. Reproducibility was determined to be excellent and the detection limit was estimated to be around 1 ng. The major chromatographic peak was observed at ~24.6 minutes with predominate masses at m/z 1252, 834, and 625 corresponding to an oligonucleotide one base shorter than expected (p-GUUUUUUU-OH). The expected oligonucleotide was not observed, possibly due to a synthesis error or degradation. Figure 1 shows a total ion current chromatogram of a standard containing 150 ng of the synthetic oligonucleotide.

Extracts prepared from yeast will serve as experimental material. These extracts will be largely protein- and nucleic acid-free and should contain nucleotides and oligonucleotides. The use of yeast offers certain advantages in trying to identify a specific oligonucleotide product in a crude and complex mixture. The beauty of the system is that it has a negative control in a yeast strain that lacks the DNA from which RNA polymerase generates the oligonucleotides and cells that lack the protein that activates the nuclease activity in RNA polymerase, hence it is impossible to generate the oligonucleotides in the negative control. It's possible to provide extrachromosomal DNA substrates that generate a large amount of specific substrate by placing specific plasmids within cells. Specific drug treatments and

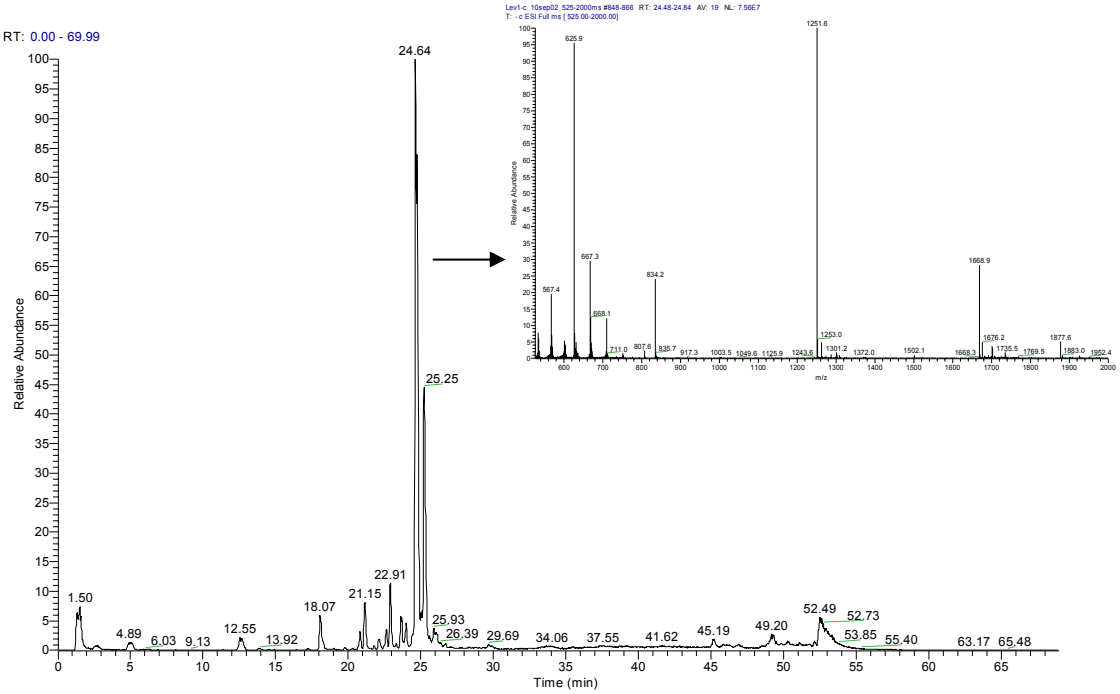

Figure 1. Total ion current chromatogram and mass spectra of a major peak at 24.6 minutes (inset) of 152 ng of synthetic oligonucleotide standard.

growth conditions of the cells should also modify the amount of substrate and product. Thus, any MS peak of interest should correlate with these other experimental manipulations. The largest perceived pitfall will likely be the positive identification of a specific oligonucleotide in a complex mixture that will no doubt contain many nucleotides and nucleotide derivatives. This is the rationale for using mass spectrometry.

After piloting the procedure, a preparative amount of nucleotide/oligonucleotide extract was generated from the yeast strain. There was 10 mL of ~100 micromolar total nucleotide in 0.33 M formic acid as estimated by absorbance at 260 nm. We are currently working on methods for cleaning up and concentrating this sample prior to analysis using the LC/MS method previously described.

User Projects

FTICR Analysis of In-Gel Tryptic Digestion of Protein that Interacts With Neuronal Calcium Sensor 1 (NCS-1)
J Andreas
Mt. Sinai Hospital, Toronto, Ontario, Canada

Determination of an Unknown Protein Implicated In Radiation Resistance In *Deinococcus radiodurans*
J Battista, A Earl
Louisiana State University, Baton Rouge, Louisiana

Amino Acid Sequence of Reaction Center Variants
J Beatty
University of British Columbia, Vancouver, closed 10/01

Identification of Proteins Differentially Expressed in Response IR
M Coleman
Lawrence Livermore National Laboratory, Livermore, California

Comparative Display of *D. radiodurians* After Exposure to Ionizing Radiation
M Daly
Uniformed Services University of Health Sciences, Bethesda, Maryland

Study of Secretory and Membrane Proteins of *Pseudomonas aeruginosa*
R Davis, W Xiao, M Mindrinos
Stanford University, Stanford, California

Mechanism of Action of G protein-coupled Receptors (GPCR) Studied by LC-FTICR Mass Spectrometry
E Dratz, P Draft
Montana State University, Bozeman, Montana

Proteomics of *Shewanella oneidensis*
C Giometti
Argonne National Laboratory, Idaho Falls, Idaho

Hollow Helices as Folding Nanotubes with Tunable Cavity Size
B Gong
University at Buffalo, Buffalo, New York

Determination of the Proteome of *Drosphila* Lipid Droplets
SP Gross, J Martinez
University of California, Irvine, California

Processing of the Cysteine Protease Aleurain
C Halls, JC Rogers
Washington State University, Pullman, Washington

Use of Mass Spectral Disassembly to Elucidate the Quaternary Structure of the Complex Heterotetrameric Sarcosi
M Jorns
MCP Hahnemann University, Philadephia, Pennsylvania

Isolation and Characterization of Novel Anti-Microbial Peptides from the Zebrafish, *Danio rerio*
C Kim
University of Maine, Orono, Maine

Accurate Mass Determination
E Lacy
Medical University of South Carolina, Charleston, South Carolina

Proteomics Analysis of Wild Type and Soil Colonization Deficient Strains of *Pseudomonas fluorescens*
S Levy, I Lopez-Hernandez
Tufts University School of Medicine, Boston, Massachusetts

Semi-Rigid Block Copolymer
A Li
Washington State University, Pullman, Washington

Whole Genome Cloning and Expression of *Treponema pallidum* Open Reading Frames
T Palzkill
Baylor College of Medicine, Houston, Texas

Identification and Relative Expression of Membrane Proteins in Breast Cancer Cell Lines
A Patwardhan
University of California, San Francisco, California

Identification of Covalent Modification of SecA
L Randall
University of Missouri, Columbia, Missouri

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