

Pressure Cycling Technology (PCT) Cited at Scientific Meetings by Third Party Scientists

Poster Presented at the American Society for Microbiology ASM 2011

Composition of Microbial Communities in Petroleum Deposits

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Introduction

Microbial communities in the subterranean environment are unique in that they are completely isolated from biological communities that rely on photosynthesis, Earth's atmosphere, or oceans. Interestingly, bacteria found in petroleum deposits are in a dormant form known as Ultramicrobacteria because petroleum deposits are very limited in nitrogenous and phorous-containing nutrients. These are unique populations as compared to other subsurface microbial communities and might consist of novel species, new metabolic capabilities, and undiscovered adaptive microbial mechanisms. They are cultured using methods for oligotrophic organisms in the lab. The goal of the current study was to isolate and identify cultivable UMBs based on their hydrocarbon-utilizing properties and also to evaluate microbial community structure in petroleum deposits using 16S rDNA techniques such as ARDRA (amplified ribosomal DNA restriction analysis) of the environmental DNA extracted from the cores.

Continued on Page 2

Abstracts of Posters Expected to be Presented at

the 59th ASMS Conference on Mass Spectrometry and Allied Topics, to be held June 5 - 9, 2011 at the Colorado Convention Center in Denver, CO.

The Evaluation of Pressure-Assisted Enzymatic Digestion for the Optimal Digestion of Monoclonal Antibodies

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MP11 - LC-MS: Sample Preparation - Peptides, poster number: 201,
Monday, Poster Hall

Novel Aspect: Reducing the time required for the characterization of therapeutic mAbs by bottom-up approaches.

Introduction

The characterization of monoclonal antibodies (mAbs) using peptide mapping is achieved through a combination of top-down and bottom-up approaches. For bottom-up studies, the mAb is typically digested with either trypsin or Lys-C, and the resulting peptides analyzed by HPLC-MS/(MS). In-solution digestion is the most common approach to achieve this goal, as sequence coverage and the assessment of low-level post-translational modifications is often required. Although effective, the in-solution digestion protocol is time consuming and overnight digestion is usually required. Pressure-assisted enzymatic digestion (PAED) is an emerging technology used to achieve comparable, or in some cases better, digestion efficiency in significantly less time. In this study, the effectiveness of PAED is evaluated at different conditions using an anti-Respiratory Syncytial Virus (anti-RSV) mAb.

Methods

The PAED was performed using a Pressure Biosciences NEP2320 Barocycler under two different conditions. In the first condition (native), the samples were mixed with enzyme, while in the second condition, the samples were first reduced and alkylated (R&A) on the bench using standard protocols, then mixed with enzyme. Replicate samples were digested in the Barocycler for various intervals (2 hr max) using defined sequences of alternating high and low

Continued on Page 2

CALENDAR OF PBI EVENTS

59TH ASMS CONFERENCE ON MASS SPECTROMETRY AND ALLIED TOPICS	Midwest LIRG Meeting Automation in Translational Research: From Applications to Regulations
JUNE 5 - 9, 2011	JUNE 9, 2011
DENVER, CO	MADISON, WI

Composition of Microbial Communities in Petroleum Deposits: Continued from Page 1

Method

Metagenomic DNA:

The PowerMax Soil DNA Isolation Kit (MoBio Laboratories, CA) was adapted for use with the Pressure Cycling Technology (PCT) Barocycler Instrument (Pressure BioSciences, MA) (2). One gram of the crushed core material was vortexed with PowerBead buffer then mixed with lysis buffer and vortexed to mix. The PULSE Tube (Pressure BioSciences, MA) was loaded into the Barocycler and run for 80 cycles (35 kpsi for 20 sec at pressure, and 5 sec at ambient pressure which lysed bacteria using high pressure. DNeasy spin column (QIAGEN) was used to purify the DNA from the cell debris. The DNA was quantified and diluted with water for PCRs.

Results

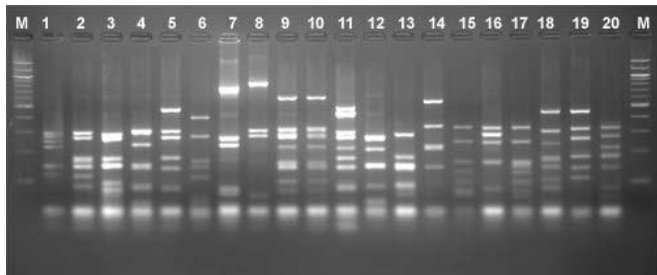


Figure 2. ARDRA gels showing *RsaI* digestion pattern of bacterial 16S rDNA clones

Discussion

- The core was populated with an unexpectedly diverse range of bacteria.
- PCT technology proved invaluable in isolation of metagenomic DNA.
- BLAST searches indicated much of the core population is similar to the microbial population from the Yellow Sea, followed by similarity to microbial mats of hypersaline lakes and also to some gut microflora.
- Several phylotypes belonged to α -proteobacteria, which commonly exist in all environments.

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The Evaluation of Pressure-Assisted Enzymatic Digestion for the Optimal Digestion of Monoclonal Antibodies: Continued from Page 1

pressure. Replicate 18 hr in-solution digestion was also performed under both native and R&A conditions (with or without denaturation) as controls. All digests were analyzed using a Waters Acquity UPLC coupled with a Waters Premier Qq/TOF in DDA mode. Sequence coverage was determined by MASCOT search against an in-house database.

Preliminary Data

The preliminary results indicate the superiority of the PAED approach for the rapid enzymatic digestion of proteins. Sequence coverage of greater than 90% was achieved from both the native and the R&A samples within 60 min in the Barocycler. The results obtained using the 60 min PAED approach were comparable to those obtained from the control sample that was denatured, reduced and alkylated followed by overnight digestion. When the model mAb sample was only reduced and alkylated, without the denaturing step, no peptides were identified under the same database search criteria. This indicates the effectiveness of the PAED approach in reducing the total cycle time required for the characterization of mAbs. Moreover, the reproducibility of the PAED approach was confirmed by replicate analysis. In this study, the effect of the different experimental conditions on various success criteria, including but not limited to, sequence coverage, number of missed cleavages and the number of unmatched peptides, was investigated and will be presented.

Does LC Separation of Intact Proteins Hinder Identification of Bacterial Markers?

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MP33 - Intact Proteins: Sequence Analysis, poster number: 564,
Monday, Poster Hall

Novel Aspect: Comparison of detected intact masses, top-down, and bottom-up for identification of strain specific bacterial markers.

Introduction

Intact protein expression profiling and subsequent identification of marker proteins is a powerful tool for bacterial strain differentiation. Advances in protein chromatography and deconvolution software make it possible to generate reproducible accurate mass – retention time profiles of > 500 features per strain. Advances in top-down technology and informatics make it possible to identify > a hundred proteins per sample by online LC-MS/MS, while maintaining intact mass for identification of SNP based biomarkers.

Continued on Page 3

Abstracts of Posters Expected to be Presented at the 59th ASMS: Continued from Page 2

However, protein level LCMS biases against classes and sizes of proteins and bottom-up proteomics identifies ten times the number of proteins. We investigate limits to mapping intact masses to bottom-up protein identifications, SNP identification by top-down versus bottom-up, and which proteins are lost with protein chromatography.

Methods

Salmonella proteins are extracted with a Barocycler (Pressure Biosciences) and separated with a Prosphere P-HR column (150mm x 2.1mm). LCMS was performed on a QTOF Premier (Waters) and deconvoluted to accurate mass, retention time, and intensity profiles with ProteinTrawler6 (BioAnalyte). Top-down and bottom-up MS/MS are performed on an Orbitrap (ThermoFisher) using a TriVersa Nanomate source (Advion Biosciences). LCMS fractions were infused for top-down identification or digested and analyzed by LC-MS/MS. On-line top-down LC-MS/MS was also performed using the same LC gradient to match retention times. The identical lysate was run on a 1D SDS-PAGE gel, digested and analyzed by LC-MS/MS. Bottom-up spectra were searched with the Mascot algorithm and top-down spectra were searched with the ProSightPC algorithm.

Preliminary Data

LCMS data show good protein level separation and reproducible extraction of protein molecular weight and retention time profiles, as well as the presence of type specific bacterial marker masses. The one hundred most abundant proteins from intact protein profiles have been identified by on-line and off-line top-down MS/MS. Bottom-up digestion of the corresponding LC fractions yields significantly more identifications, but database deficiencies mean those identifications can not always be mapped back to the intact mass. This can complicate definitive identification of differential protein profile marker masses. However, bottom-up analysis of fractions has the benefit of identifying proteins that may be chromatographically separated but not detected or resolved by intact protein LCMS. Both of these experiments limit bacterial strain analysis to the proteins that are amenable to intact protein chromatography. GeLC analysis from SDS-PAGE digestions yielded many additional identifications from the bottom-up, most of which were in a mass range not amenable to top-down MS/MS, were present at an abundance that is too low for intact protein mass spectrometry, or did not separate on the column. However, while SDS-PAGE maintains an approximate link to the intact protein mass, the comparatively small mass shifts induced by SNP differences between different strains can not be resolved by the gel. Without an intact mass measurement, we are dependent on the database and peptide sequence coverage for detection of the presence of SNPs.

Continued on Page 3 Column B

Does LC Separation of Intact Proteins Hinder Identification of Bacterial Markers? (continued from Page 3 Column A)

Considering the diversity of bacteria, relatively few strain specific bacterial proteins are found in protein sequence databases. On the other hand, the mass range and total number of proteins identified by GeLC analysis surpasses the number of proteins amenable to intact protein HPLC separation. We will also investigate the difficulty in referencing proteins identified by searching bottom-up data against incompletely annotated databases back to an intact mass.

Development of a 20 kpsi Enzymatic Digester for High Throughput Proteomic Analysis and Its Application to Membrane Proteomics

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ThP11 - Drug Metabolism: High Throughput/Robotics, poster number: 211, Thursday, Poster Hall

Novel Aspect: A new automated high pressure online enzymatic digestion reactor provides comparable digestion to overnight methods in just 20 min.

Introduction

Digesting proteins into peptides is an essential step in bottom-up proteomics. Recently, pressure assisted digestion using pressure cycling technology (PCT) was demonstrated to significantly speed time needed for digestions from hours to minutes. In this work, we developed a novel ultra-high pressure PCT reactor that couples to a 2-column LC-MS platform. The reactor consists of a pressure generator, a temperature controlled flow-through pressure cell, isolated from the flow path by high pressure valves. The system was tested initially with a pool of standard proteins and later with a set of digestion resistant proteins, including the hydrophobic membrane-spanning protein bacteriorhodopsin (*Halobacterium halobium*) and a water-insoluble fraction of *Shewanella oneidensis* proteins.

Methods

The reactor was coupled inline between the injection valve and a C18 trapping column on the LC system. Samples were mixed with trypsin and split into aliquots: part of the sample was subjected to an overnight digestion (control), while the 5 uL aliquot was passed into the PCT reactor and pressurized at 20,000 psi for 20 min, after which the digested sample was either captured on a C18 trapping column and subjected online to LC-MS/MS using an LTQ-FT or collected for subsequent LC-MS/MS analysis. A commercial four protein standard, bacteriorhodopsin, and an insoluble fraction of *Shewanella oneidensis* were used in this study. Peptide identification rates were used to evaluate the digestion efficiency.

Continued on Page 4

Abstracts of Posters Expected to be Presented at the 59th ASMS

Development of a 20 kpsi Enzymatic Digester for High Throughput Proteomic Analysis and Its Application to Membrane Proteomics: Continued from Page 3 Column B

Preliminary Data

The high pressure reactor has been successfully integrated into the flow path of the LC-MS system for automated sample handling and pressure-enhanced digestion. Computer control of pressure cycling parameters and valve operation was developed using LabView software, which facilitated communications with the LC-MS system. The PCT reactor was tested first using the commercial protein standard containing ribonuclease A, cytochrome C, holo-transferrin, and apomyoglobin. Proteins were trapped online after digestion and peptides then released for MS analysis. Successful digestion was achieved in all cases and no significant differences in the number of identified peptides and sequence coverage were observed compared to overnight digests. Bacteriorhodopsin, a well-characterized hydrophobic membrane protein resistant to proteolysis, was used to evaluate the efficacy of this method for digestion of integral membrane proteins in a detergent-free environment. The protein was first solubilized in 30% MeOH with 50 mM ammonium bicarbonate, mixed with trypsin, injected into the system, trapped in the transfer cell and pressurized at 20,000 psi for 20 min. The digested products were collected, dried, and analyzed by LC-MS/MS analysis. In this case, the method was compared with an overnight digestion control, and results in terms of number of peptide identifications and coverage were comparable for both methods. The effect of PCT on the hydrophobic domains of the protein may account for the slightly higher coverage achieved by the pressurized method. As a final evaluation, the insoluble portion of the *S. oneidensis* proteome was digested and analyzed using LC-MS/MS. Again, results were compared to those obtained with an overnight digestion. Noticeably, most of the identified proteins corresponded to inner and outer membrane proteins; however, several cytoplasmic proteins were also identified.

Identification and Validation of Vaccine Candidate Proteins by 2-Dimensional Mass Spectrometry Analysis of Group A Streptococcus

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Merck and Co., West Point, PA
ThP35 - Protein Therapeutics: Quantitative Analysis, poster number: 634, Thursday, Poster Hall

Novel Aspect: A comparative proteomic approach for identifying and validating bacterial surface-exposed proteins by mass spectrometry for a vaccine against GAS.

Introduction

Surface proteins of bacteria are involved in adhesion and invasion of host cells. Since surface proteins likely interact

Continued on Page 4 Column B

Identification and Validation of Vaccine Candidate Proteins by 2-Dimensional Mass Spectrometry Analysis of Group A Streptococcus: Continued from Page 4 Column A

with the host immune system, they are important for vaccine discovery research. We employed on-line multidimensional protein identification technology to characterize the surface of group A Streptococcus (GAS). Surface proteins were selectively digested with trypsin after mutanolysin and lysozyme treatments of intact bacteria. The obtained surface proteome was compared to the whole cell proteome obtained by analyzing total cell lysate. PSortB, a protein prediction algorithm for subcellular localization, was applied to demonstrate enrichment of proteins predicted to be surface localized. We show that identified surface proteins include the most protective antigens reported in literature and 10 newly discovered potential vaccine candidates.

Methods

For surface digestion, strains M1 or M3 GAS cells were brought up in 20% sucrose in Tris-EDTA pH8.0. They were treated with mutanolysin and lysozyme, followed by digestion with trypsin for 0.5h at 37°C. For total cell lysate digestion, strain M1 GAS was lysed using pressure cycling technology (Pressure Biosciences NEP2320). Extracted proteins were reduced and alkylated, then digested in urea using lysC, followed by trypsin. MS data was acquired on a LTQ ion trap instrument (Thermo Fisher) operated in profile mode. 5µg of peptides were separated on-line by strong cation exchanger chromatography followed by C18 reversed phase nano-LC using 10 discrete salt steps. MS/MS scans were performed on the top 7 precursor ions in reverse order.

Preliminary Data

690 proteins were identified in the whole cell lysate of M1. 4.9% of total MS signal intensity was comprised of signals from proteins predicted by PSortB as cell wall or non-cytoplasmic proteins. 12.7% of the total MS signal intensity was comprised of signals from proteins of unknown localization. A total of 305 and 334 proteins were identified in the surface digest of strains M1 and M3, respectively. The portion of mass spectrometry signal contributed by surface proteins identified by PSortB was 41% for the M1 strain and 27% for the M3 strain. The >8-fold enrichment of surface proteins for GAS M1 was reproduced with a > 5-fold enrichment of surface proteins for GAS M3. The M protein, type 1 (NT01SP1656) has been described in the literature to be the highest abundant surface protein in group A Streptococcus. M protein was also identified as the highest abundant protein for both GAS strains M1 and M3 in our measurements. The described comparative proteomics method identified ten potential vaccine candidates and allowed us, in addition, to follow the expression state for eleven proteins that had been previously identified by molecular biology.