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FRACTIONAL ANALYSIS OF *ESCHERICHIA COLI* O157:H7 BY MASS SPECTROMETRY-BASED PROTEOMICS

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14. ABSTRACT

This report is required for the U.S. Army Edgewood Chemical Biological Center In-House Laboratory Independent Research project "Membrane Vesicles and Extracellular Proteins in Antibiotic Resistance and Virulence". Certain extracellular proteins of pathogenic bacteria have been shown to function in survival mechanisms such as host immune system modulation (Vranakis, et al., July 27, 2011, *J. Proteome Res.* DOI 10.1021/pr200422f) and biofilm formation (Ostrowski, A., et al., 2011, *J. Bacteriol. 193*:4043). To begin to address this possibility, we have analyzed *Escherichia coli* O157:H7 protein fractions by liquid chromatography-tandem mass spectrometry, followed by biochemical pathway mapping using the Kyoto Encyclopedia of Genes and Genomes. The fimbriae-specific subset included proteins involved in carbohydrate metabolism, which are important in providing energy for fimbriae motion. Moreover, inositol monophosphatase (IMP), which has a role in streptomycin synthesis, and a glucose-specific phosphotransferase system (G-PTS), involved in environmental processing, were also identified. IMP, G-PTS, and a putative stress protein, recently identified in the suspension, are expected to function as part of the bacterial survival mechanisms. This research should provide fundamental knowledge regarding extracellular proteins produced by Gram-negative bacteria. The identification of molecular level components important for survival could prove useful in arenas such as vaccine and antibiotic development.

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PREFACE

The work described in this report was authorized under the In-House Laboratory Independent Research Program. This work was started in October 2010 and completed in September 2011.

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FRACTIONAL ANALYSIS OF *ESCHERICHIA COLI* O157:H7 BY MASS SPECTROMETRY-BASED PROTEOMICS

1. INTRODUCTION

Certain extracellular proteins secreted by pathogenic bacteria have been shown to function in survival mechanisms such as host immune system modulation (*1*) and biofilm formation (*2*). In addition, Gram-negative bacteria release a subset of extracellular proteins as membrane vesicle (MV) components. Gram-negative bacteria use energy to form MVs by pinching off the outer membrane to form liposomes. MVs contain periplasmic space components including enzymes, which suggests that MVs provide a functional role for the bacteria. Pathogenic Gram-negative bacteria produce more MVs than their nonpathogenic counterparts. Not surprisingly, several studies have provided evidence for a number of roles for MVs (*3*) such as the transfer of antibiotic-resistance enzymes to other bacteria (*4*) and directed intercellular transport of virulence factors (*5*). In addition, work by Schooling and Beveridge (*6*) indicates that MVs shed by Gram-negative bacteria are a ubiquitous component in the biofilms of these bacteria. Levin and Rozen cite biofilm formation as one of three means by which bacterial populations can attain noninherited antibiotic resistance (*7*).

Our hypothesis for the larger project that includes this work is that the binding of intracellular MV proteins or secreted proteins may contribute toward mechanisms of antibiotic resistance. To address this possibility, we are characterizing the MV proteins, other extracellular proteins, and whole cell lysate proteins produced by the pathogenic Gram-negative bacterium *E. coli* O157:H7 in terms of proteomics and binding of antibiotics. The expression and antibiotic binding capability of the MV proteins and other extracellular proteins will be compared with whole cell and fimbriae protein fractions from *E. coli* O157:H7 in search of differences indicative of unique functions for these extracellular components. This report provides a comparison of the whole-cell lysate, secreted, and fimbriae protein fractions of *E. coli* O157:H7, as determined by mass spectrometry (MS)-based proteomics. This research project is expected to provide fundamental knowledge regarding MVs and extracellular proteins produced by Gram-negative bacteria. In particular, the work seeks to characterize these proteins and the role they may play with regard to virulence and resistance. In addition, this effort will further the development of a high-throughput platform for analyzing samples for the presence of biological constituents. The new knowledge will also enhance the development of nanoparticle technologies by identifying molecular level components important for recognition and encapsulation.

2. METHODS

2.1 *E. coli* O157:H7 Growth and Protein Fraction Preparation

E. coli O157:H7 was grown in an orbital shaker (125 rpm at 37 °C), containing trypticase soy broth (TSB), until the late exponential phase was reached (~10^8 cfu/mL) . The cell culture was stored at 4 °C until fractionation occurred. To isolate the whole-cell lysate and secreted protein fractions, 30 mL of culture was centrifuged at 11,300 *g* for 1 h using a Beckman J2-MC centrifuge (Beckman Coulter, Inc., Brea, CA). The supernatant was decanted to separate it from the pellet. This supernatant, containing the secreted proteins, is referred to as the *secreted fraction*. The pellet was resuspended in ~3.5 mL of 100 mM ammonium bicarbonate (ABC). This suspension was divided into three aliquots of approximately equal volume. The cell pellet suspension samples were thawed and lysed by ultrasonication (25 s on, 5 s off, 4 min total) using a Branson Digital Sonifier (Danbury, CT). The lysate was centrifuged at 14,000 rpm for 20 min at 10 °C using a Beckman GS-15R centrifuge. This is referred to as the *whole-cell lysate fraction*. In some cases, a microwave lysis procedure, in which the sample was subject to microwaves

using a Discover System (CEM Corporation, Matthews, NC), was performed at 55 °C for periods of 5, 10, and 15 min rather than using sonication.

For isolation of fimbriae, cell culture aliquots (3 × 30 mL) were centrifuged at 15,000 *g* for 30 min using a Beckman J2-MC centrifuge. Each pellet was resuspended in 7 mL of ABC. These solutions were sheared 10 times each through a 2 in., 22 gauge needle. Samples were divided into 1.5 mL centrifuge tubes and centrifuged at 15,000 *g* for 15 min using a Beckman GS-15R centrifuge. The supernatants were combined and filtered through a 0.45 µm acetate syringe filter. The filtrate was then heated at 60 °C for 1 h in a block heater. These samples are referred to as the *fimbriae fraction*. Samples were frozen at –25 °C for up to 4 days.

2.2 LC/MS Sample Preparation

Samples were prepared for LC-MS/MS in a manner similar to that previously reported (*8*). Briefly, proteins were extracted from the whole-cell lysate and secreted fractions by transferring each sample to a separate Microcon YM-3 filter unit (Millipore, Billerica, MA) and centrifuging at 14,100 *g* for 20–30 min. The filter membrane was washed with ABC and centrifuged at 14,100 *g* for 20 min. For the fimbriae fraction, the frozen samples were thawed and pipetted into Microcon YM-3 filter units (Millipore, Billerica, MA) for purification. The filters were each centrifuged three times at 14,000 *g* for 25 min with a 200 µL ABC wash in between centrifugations.

Generally, the proteins in the retentate were denatured at 40 °C for 1 h with 300 µL of 7.2 M urea and 3 µg/mL of dithiothreitol in ABC. The urea was removed by centrifugation (14,100 *g* for 30–40 min) and the retentate was washed three times with ABC (150 µL ABC followed by centrifugation at 14,100 *g* for 30–40 min using an Eppendorf centrifuge 5415C or 5415D (Eppendorf North America, Westbury, NY). The filter unit was then transferred to a new receptor tube, and the proteins in the retentate were digested overnight at 37 °C using 5 µL of sequencing grade trypsin (product no. 511A, Promega, Madison, WI) in 10 µL of acetonitrile and 240 µL of ABC. The tryptic peptides were isolated by centrifuging at 14,100 *g* for 20–30 min. Alternative digestion protocols involved adjusting the trypsin concentration, incubation time, and temperature.

2.3 LC/MS Experiments

The tryptic peptides were separated in a manner similar to that previously described (*8*) using a capillary column with the Dionex UltiMate 3000 (Thermo Scientific Dionex, Sunnyvale, CA). The resolved peptides were electrosprayed into a linear ion trap MS (LTQ XL; Thermo Scientific, San Jose, CA). Product ion mass spectra were obtained in the data-dependent acquisition mode, with a survey scan followed by MS/MS analysis of the top five most intense precursor ions.

2.4 Proteomics Analysis

A protein database was constructed as previously described (*8*) in a FASTA format using the annotated proteome sequences derived from the genomes in the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov, accessed November 16, 2010). For this task, an in-house Perl program (http://www.activatestate.com/ActivePerl, accessed November 16, 2010) was used to automatically download proteome sequences from the NCBI. The database was constructed by translating putative protein-encoding genes, and it contains amino acid sequences of potential tryptic peptides obtained by the in silico digestion of all proteins, assuming up to two missed cleavages. The acquired mass spectra were searched against this database using the SEQUEST algorithm (Thermo Scientific). The SEQUEST thresholds for searching the product ion mass spectra were Xcorr, deltaCn, Sp, RSp, and deltaMpep. These parameters provide a uniform matching score for all candidate peptides.

The generated output files, with candidate peptides, were validated using the PeptideProphet algorithm (*9*). Peptide sequences with probability scores of 95% and higher were retained and used to generate a binary matrix of sequence-to-bacterium assignments. The binary matrix was populated by matching the peptides with corresponding proteins in the database and assigning a score of one. A score of zero was assigned for a nonmatch. The column in the binary matrix represents the proteome of a given bacterium, and each row represents a tryptic peptide sequence from an LC product ion mass spectral analysis. A sample microorganism was matched with a database bacterium by the number of unique peptides that remained after filtering of degenerate peptides from the binary matrix. Verification of the classification and identification of candidate microorganisms was performed through hierarchical clustering analysis and taxonomic classification.

2.5 Biochemical Pathway Mapping

An algorithm was developed in-house for the automated comparison of proteins observed in samples from a given fraction (whole-cell, secreted, fimbriae). After sample analysis with the LC-MS/MS, this in-house algorithm was used to compare the proteins observed in the three fractionas and to determine which proteins were common between two or three fractions and which proteins were specific to a given fraction (fraction-specific proteins). Fraction-specific proteins were mapped to *E. coli* metabolic pathways using the Kyoto Encyclopedia of Genes and Genomes database (KEGG, www.genome.jp/kegg/, accessed November 16, 2011, Copyright 1995–2011, Kanehisa Laboratories).

3. RESULTS AND DISCUSSION

Three whole-cell, three fimbriae, and five secreted-fraction peptide samples were prepared for LC-MS/MS experiments and proteomics analysis. From these samples, more than 200 *E. coli* proteins were identified. The percentages of strain- and species-unique proteins identified in each fraction are shown in Figure 1, with the majority found in the fimbriae fraction. The strain-unique proteins represent biomarkers of *E. coli* O157:H7.

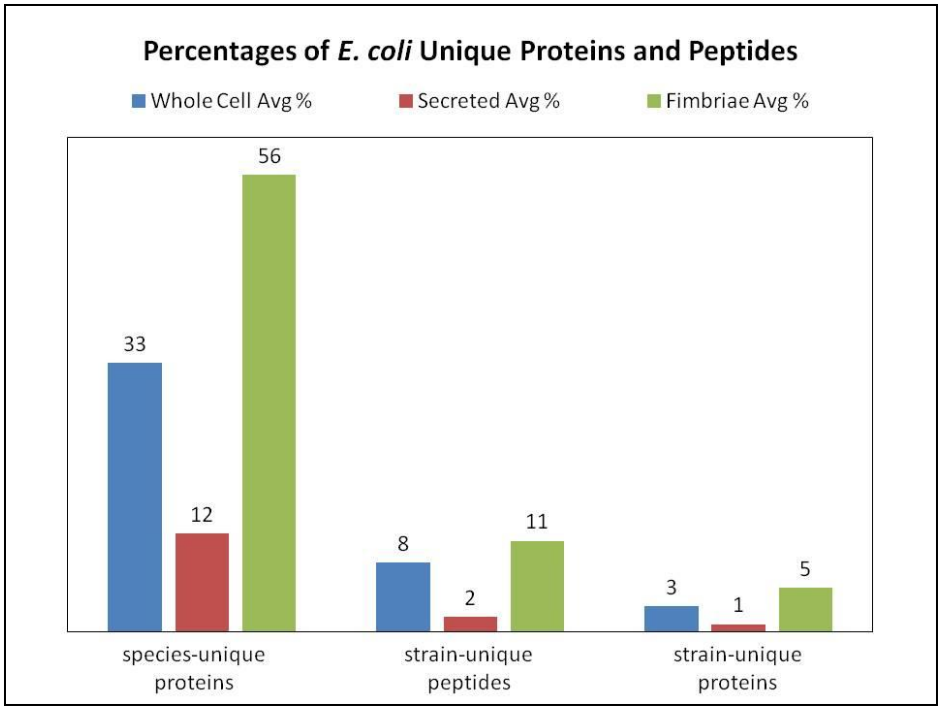

Figure 1. Average percentages of *E. coli* unique proteins and peptides identified in *E. coli* O157:H7 whole-cell, fimbriae, and secreted fractions.

Proteins present in at least two of three samples for the fimbriae and whole-cell fractions and in at least three of five samples for the secreted fraction were considered for further analysis. Of these 201 proteins, 15% were common to all fractions. In addition, of the 201 total proteins, proteins specific to each fraction were observed at 3, 5, and 29% in the secreted, fimbriae, and whole-cell fractions, respectively. The percentages of proteins localized to each fraction and shared between various fractions are shown in Figure 2.

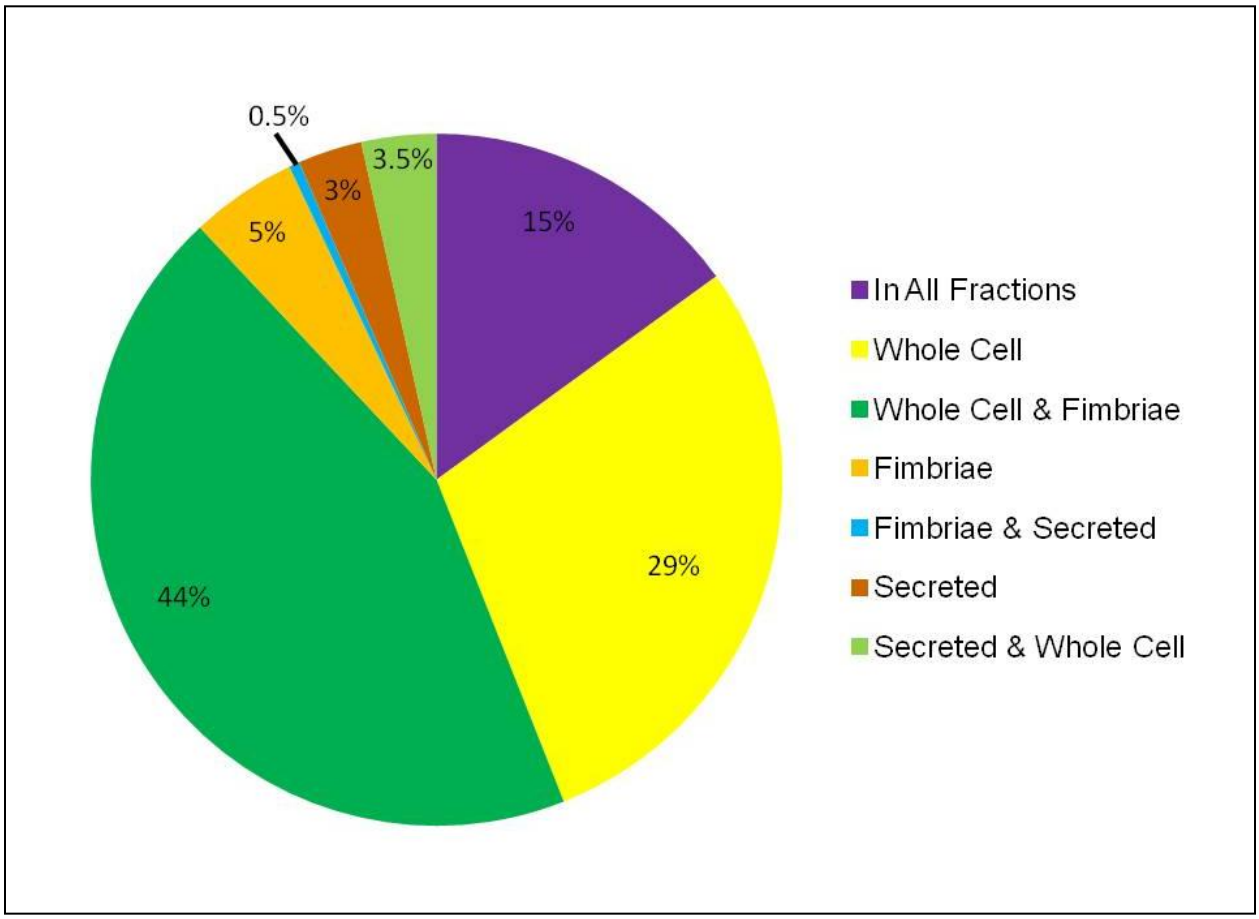

Figure 2. Percentage of observed proteins localized to various fractions of *E. coli* O157:H7.

The fraction-specific proteins were mapped to *E. coli* metabolic pathways using the KEGG. Table 1 summarizes the numbers of proteins mapped for each fraction. For a given protein, the NCBI protein identification (ID) number was used to obtain the KEGG ID number. The KEGG ID number was then used to determine the KEGG pathway identification and map. The mapping of one example protein is shown in Figure 3.

Table 1. Number of Proteins Mapped to *E. coli* Metabolic Pathways

Fraction	Number of Proteins Mapped
Whole-Cell	57
Fimbriae	14
Secreted	6
Total	77

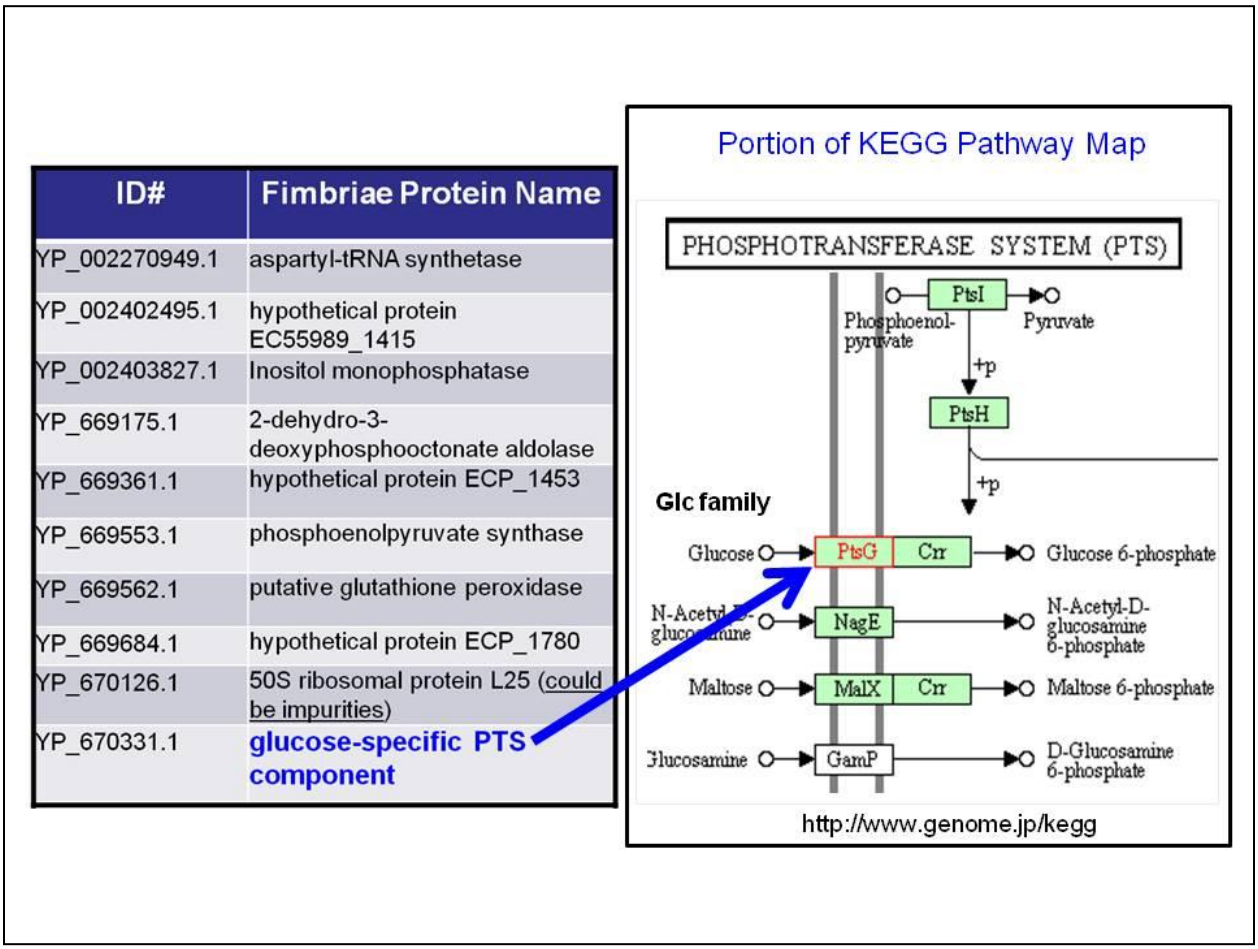

Figure 3. Mapping of a protein to its corresponding metabolic pathway.

Of particular interest were two proteins identified in the fimbriae fraction: the glucose-specific phosphotransferase system (G-PTS) component protein given in the example (Figure 3) and inositol monophosphatase (IMP). In addition, ~35% of pathway hits for the fimbriae fraction involved carbohydrate and energy metabolism, compared with ~18 and ~7% of whole-cell and secreted fraction hits, respectively.

4. CONCLUSIONS

In this study, *E. coli* O157:H7 whole-cell, fimbriae, and secreted-protein fractions were analyzed using LC-MS/MS. Proteomics analysis of the LC-MS/MS data resulted in the identification of 201 proteins, of which ~15% were common to all three fractions and ~10% were strain-unique. In addition, ~30, ~5, and ~3% were specific to the whole-cell, fimbriae, and secreted protein fractions, respectively. Biochemical pathway mapping using KEGG was carried out for 77 proteins. The fimbriae-specific subset included proteins involved in carbohydrate metabolism, which are important in providing energy for fimbriae motion. IMP, which has a role in streptomycin synthesis, and G-PTS, which is involved in environmental processing, were also identified during this study. IMP, G-PTS, and a putative

stress protein, recently identified in the suspension, are expected to function as part of the bacterial survival mechanisms. This research should provide fundamental knowledge regarding extracellular proteins produced by Gram-negative bacteria and further the development of a high-throughput platform for the analysis of samples for the presence of biological constituents. The identification of molecular level components important for survival mechanisms could prove useful in arenas such as vaccine and antibiotic development.

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ACRONYMS AND ABBREVIATIONS

ABC	ammonium bicarbonate
Crr	carbohydrate repression resistance
ECP	*Escherichia coli* common pilus
GamP	PTS system glucosamine-specific transporter
Glc	glucose
G-PTS	glucose-specific phosphotransferase system
ID	identification
IMP	inositol monophosphatase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	liquid chromatography
MalX	maltose-regulated PTS enzyme II homolog
MS	mass spectrometry
MV	membrane vesicle
NagE	N-acetylglucosamine PTS system EIIABC (Nag) component
NCBI	National Center for Biotechnology Information
PtsG	glucose phosphotransferase enzyme IIBC (Glc)
PtsH	PTS system histidine phosphocarrier protein Hpr
PtsI	phosphotransferase system enzyme I
TSB	trypticase soy broth
YP_	protein accession number prefix used by NCBI


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