Phenotype Array Analysis of Metabolic Differences in Yersinia pestis

A. Holtz, S. McCutchen-Maloney

May 18, 2005

The American Society for Microbiology's 105th General Meeting
Atlanta, GA, United States
June 5, 2005 through June 9, 2005
Disclaimer

This document was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor the University of California nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or the University of California. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or the University of California, and shall not be used for advertising or product endorsement purposes.
Phenotype Array Analysis of Metabolic Differences in *Yersinia pestis*

Ann E. Holtz-Morris
Lawrence Livermore National Laboratory
Plague

Ca++
Temp
Extra-
Intra-
37˚C
26˚C
Bubonic
Pneumonic
Flea
Rodent
http://phil.cdc.gov
Host-pathogen Interactions

- Low Calcium Response
  - Intracellular environment
  - Induces TTSS
  - Bacteriostasis

- Signatures for Early Detection of Exposure, Pathogen ID, Emerging and Engineered Threats, Antibiotic Resistance, Lethality, Dose
- Prevention, Detection, Containment, Treatment, Attribution
- Characterize pathogen, environmental and host factors that influence virulence
Pathogen Characterization

Real-time Expression

Virulence factor
Real-time expression
Fluorescent reporter

2D-DIGE

Y. pestis proteome
26°C high calcium (flea)
37°C low calcium (human)
>2800 proteins spots
239 differential
1D by mass spec

Proteomics

Phenotype Arrays

Vir. Factor Promoter
reporter plasmid

Y. pestis function
Phenotype differences
• Between growth conditions
• Between diverse strains

• Biochemical Pathways
• Systems Biology

Characterize pathogen, environmental and host factors that influence virulence

Mass Spec

Synthesis of Macromolecules
Nucleobase synthesis
Amino Acid Biosynthesis
Phage related protein
Energy Metabolism
Macromolecule Degradation
Broad Regulatory Functions
Central Intermediary Metabolism
Biosynthesis of Cofactors
Cell Envelope
Fatty Acid Biosynthesis
Cell Processes
Putative Protein
Conserved Hypothetical
Hypothetical Protein
Other

Collaboration with Drs. M. Lipton, D. Smith
>5000 Proteins from annotation, ~35% coverage

Holtz et al. 2004 ASCB
Forde et al. 2004 BBRC
Chromy et al. 2005 J Bact
Hixon et al. (submitted)
Forde et al. 2004 BBRC
Clatworthy et al. in prep
Systems Biology of \textit{Y. pestis}

Genomics$^{1,2,3}$

Transcriptomics$^4$

Proteomics$^5$

Functionomics$^6$

1. Parkhill \textit{et al.} 2001 Nature
Y. pestis Physiological Growth Conditions (Flea Vs. Human)

- 26 °C
- 37 °C
- High [Calcium]
- Low [Calcium]
Basic Metabolism

- Original papers (1940s to 1960s) did not account for differences due to low calcium response and subsequent TTSS.
- It is now known that significant differences in mRNA expression\(^1\) and protein expression\(^2\) occur between flea vector, mammalian extracellular, and mammalian intracellular environments.

\(^{1}\)Motin et al. 2004 J Bacteriol
\(^{2}\)Chromy et al. 2005 J Bacteriol
Expected Metabolic Activity

Hypothesis: Different metabolic pathways may be used in different environments: unique, union or intersection.
Applications of Results

- Pathogen characterization to support detection and forensics
  - Differential - flea vs. human physiological conditions
  - Strain diversity - typing and attributes
    - Eg. 2004-2005 Congo outbreak, 57 dead/130 cases - why pneumonic\(^1,2\)?
    - Eg. Possible engineered pathogens

- Biomarkers and virulence factors
  - What influences their expression?
  - Can a false negative, \(i.e\). the pathogen is present but the detection method fails because the marker is missing, occur due to changes caused by the growth conditions, as in case of F1 antigen?\(^3\)
  - Identify virulence pathways and tie to host response in next generation arrays

- Contribute to systems biology view of virulence

\(^1\)http://www.cidrap.umn.edu/cidrap/content/bt/plague/news/mar1505plague.html
\(^2\)http://www.cidrap.umn.edu/cidrap/content/bt/plague/news/feb1805plague.html
\(^3\)Mahesh \textit{et al.} 2005 Comp Immunol Microbiol Infect Dis
Outline

I. Introduction and background
II. Phenotype array technology
III. Results
IV. Analyzing data
V. Linking to virulence
VI. Future
Phenotype Array Technology

- Measures dye produced by coupled redox reaction to respiration
- Change in intensity indicates metabolism of the substrate in that well
- Inoculum is in gelling media IF-0 or IF-10
- Optics system converts color into a dye intensity value

Bochner 2003 Nat Rev Genet
Bochner et al. 2001 Genome Res
High Throughput Screening: 2000 Chemicals Simultaneously

- Minimal medium
  - Carbon sources
  - Nitrogens
  - Phosphates
  - Sulfurs
  - Amino acids
  - Dipeptides
  - Tripeptides

- Rich medium
  - Osmotic stressors
  - pH effects
  - Antibiotics
Outline

I. Introduction and background
II. Phenotype array technology
III. Results
IV. Analyzing data
V. Linking to virulence
VI. Future
Response to stressors and antibiotics varies with calcium and temperature.
Flow Chart of Reiteration

Measure Cell Respiration

Analyze data

Add metabolite

Changed phenotype
Supplement Changes Phenotype

<table>
<thead>
<tr>
<th>Negative Control</th>
<th>Phosphate</th>
<th>Pyrophosphate</th>
<th>Trimetaphosphate</th>
<th>Triethyl Phosphate</th>
<th>Hypophosphate</th>
<th>Adenosine-2'-monophosphate</th>
<th>Adenosine-3'-monophosphate</th>
<th>Adenosine-5'-monophosphate</th>
<th>Adenosine-2',3'-cyclic monophosphate</th>
<th>Adenosine-3',5'-cyclic monophosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiophosphate</td>
<td>Dithiophosphate</td>
<td>D,L-α-Glycerol Phosphate</td>
<td>β-Glycerol Phosphate</td>
<td>Carbamyl Phosphate</td>
<td>D-2-Phospho-Glyceral Acid</td>
<td>D-3-Phosphoglyceral Acid</td>
<td>Guanosine-2'-monophosphate</td>
<td>Guanosine-3'-monophosphate</td>
<td>Guanosine-5'-monophosphate</td>
<td>Guanosine-2',3'-cyclic monophosphate</td>
</tr>
<tr>
<td>Phosphoenol Pyruvate</td>
<td>Phosphoglycolic Acid</td>
<td>D-Glucose-1-Phosphate</td>
<td>D-Glucose-6-Phosphate</td>
<td>2-Deoxy-D-Glucose 6-Phosphate</td>
<td>D-Glucosamin-6-Phosphate</td>
<td>6-Phosphogluconic Acid</td>
<td>Cytidine-2'-monophosphate</td>
<td>Cytidine-3'-monophosphate</td>
<td>Cytidine-5'-monophosphate</td>
<td>Cytidine-2',3'-cyclic monophosphate</td>
</tr>
<tr>
<td>D-Mannose-1-Phosphate</td>
<td>D-Mannose-6-Phosphate</td>
<td>Cysteamine S-Phosphate</td>
<td>Phospho-L-Arginine</td>
<td>o-Phospho-L-Serine</td>
<td>o-Phospho-L-Threonine</td>
<td>Uridine-2'-monophosphate</td>
<td>Uridine-3'-monophosphate</td>
<td>Uridine-5'-monophosphate</td>
<td>Uridine-2',3'-cyclic monophosphate</td>
<td>Uridine-3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>O-Phospho-D-Tyrosine</td>
<td>O-Phospho-L-Tyrosine</td>
<td>Phosphocholine</td>
<td>Phosphoryl Choline</td>
<td>O-Phosphoryl-Ethanolamine</td>
<td>Phosphono Acetic Acid</td>
<td>2-Aminoethyl Phosphonic Acid</td>
<td>Thymidine-3'-monophosphate</td>
<td>Thymidine-5'-monophosphate</td>
<td>Inositol Hexaphosphate</td>
<td>Thymidine-3',5'-cyclic monophosphate</td>
</tr>
</tbody>
</table>

Flea: 26°C +Ca
+Met
+Cys
Chemical Bins

- **Growth conditions**
  - Compounds used at 37°C
    - Only at low calcium
    - Only at high calcium
    - Irrespective of calcium
  - Compounds used only at 26°C, irrespective of calcium
  - Compounds used at both 37°C and 26°C
    - Some better at 37°C
    - Some equally well at 26°C
  - Compounds used only in presence of calcium, regardless of temperature
  - Compounds not used
Possible inhibitory, Val-X

Compounds used only when methionine or cysteine or tryptophan supplemented
  - *eg.* Tween 20, 40, and 80 +Cys
  - *eg.* Phosphorylated substrates at 26°C +Ca +Met
Comparing Strains:

- Y. pestis KIM D27: pgm-, conditionally virulent
- Y. pestis NYC: virulent, recent bubonic clinical isolate
  - is 1000X more lethal than India 195/p in mouse model
- Y. pestis India 195/p: virulent, pneumonic clinical isolate
Strain Dependent Phenotype

- Most strains used compounds similarly.
- Methionine precursors used only by KIM D27 and only at 26°C.
The patterns of chemical utilization can differentiate strains.

<table>
<thead>
<tr>
<th>chemical</th>
<th>India 195/p</th>
<th>NYC</th>
<th>KIM D27</th>
</tr>
</thead>
<tbody>
<tr>
<td>glycerol</td>
<td>-</td>
<td>-</td>
<td>37°C {+}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>26°C {-}</td>
</tr>
<tr>
<td>N-Acetyl-Neuraminic Acid</td>
<td>37°C {+}</td>
<td>37°C {+}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>26°C {-}</td>
<td>26°C {-}</td>
<td></td>
</tr>
<tr>
<td>Methionine and precursors</td>
<td>-</td>
<td>-</td>
<td>37°C {-}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>26°C {+}</td>
</tr>
<tr>
<td>pH5.5</td>
<td>37°C {-}</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>26°C {+}</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Outline

I. Introduction and background
II. Phenotype array technology
III. Results
IV. Analyzing data
V. Linking to virulence
VI. Future
The Data Explodes

- Studies are reiterative
- Software is necessary
- 4 hours “hands on” to 40 hour data analysis
Analyzing Data: Use of KEGG$^1$ Maps

- Points out unannotated metabolic activities
- Points out activities not expressed under the conditions

$^1$Kanehisa et al. 2004 Nucleic Acids Res
Visualizing Phenotype Data

Metabolic overview map

Expression Levels
- 30.
- 13.
- 5.5
- 2.5
- 1.1
- 0.5
- 0.21
- 0.1
- 4.00e-2

No data available
Unknown enzyme
Use of Biocyc Map

- Comparing changing conditions
- Examining global relationships
- Alternative pathways

Y pestis KIM PGDB courtesy Peter Karp, SRI International
Y. pestis KIM Overview Maps

- Y. pestis KIM overview of 4 conditions
- Order
  - 26° +Ca
  - 26° -Ca
  - 37° +Ca
  - 37° -Ca
- Mass-spec protein data and substrate rate data combined
Nature Reviews Microbiology 3, 272 (2005)

“When a genome has been completely annotated, there remain almost 40% of genes — many of which are conserved among several different species — for which no function can be predicted.... Plus, experimental validation of predicted functions has lagged far behind the speed of annotation. In fact, an inverse pyramid of information is present, in which annotations of huge numbers of sequenced genes are based on a relatively tiny number of functionally characterized genes.”

We need to greatly improve the systems biology of *Y. pestis* to provide new diagnostic and therapeutic targets.
Outline

I. Introduction and background
II. Phenotype array technology
III. Results
IV. Analyzing data
V. Linking to virulence
VI. Future
Real-time Characterization of *Y. pestis* Virulence Factors

Measures activity of virulence factor promoters by fluorescent reporter system, estimating virulence factor expression

Forde et al. 2004 BBRC.
Linking YopE Expression to Phenotype

Functional phenotype changes virulence factor expression

Red is respiration; white is pYopE-GFP reporter
I. Introduction and background
II. Phenotype array technology
III. Results
IV. Analyzing data
V. Linking to virulence
VI. Future
Next Generation Signatures/Biomarkers

- Qualitative information about a pathogen
  - Specific host response to pathogens
  - Differences between closely related organisms
  - Virulence factors of the pathogen
  - Antibiotic resistance, genetically engineered

- Panel of biomarkers needed to ID exposure
  - Naturally occurring and genetically engineered

Patient with Fever/Cough

Is it plague, anthrax, SARS, flu?

Do we have to wait for this
Carbon Sources for Validating Biomarkers

Upcoming project

Carbon sources from Phenotype Arrays

No False Negatives!
Data Integration and Future

Future: integrate metabolomics to verify biochemical pathways

Why is expression so different due to carbon sources?
Summary

These studies have already found:
- Indications of incomplete genome annotation
- Changes in drug and osmotic susceptibility
- Functional metabolic changes
- Strain dependent phenotypes
- Linked virulence factor expression to carbon sources
- Opened new lines of inquiry for future research
**LLNL Biodefense Proteomics Group**

Sandra McCutchen-Maloney, Ph.D., PI

Host Pathogen Interactions:
  - Brett Chromy, Ph.D.
  - Celia Zhang, Ph.D.
  - Megan Choi
  - Vicki Kopf
  - Todd Corzett
  - Michael Derksen
  - Rachelle Bermingham

Real Time Reporters
  - Anne Clatworthy, Ph.D.
  - Cameron Forde, Ph.D. (UBC)

Statistician
  - Imola Fodor, Ph.D.

Microbiologists
  - Kris Montgomery, M.S.
  - Brent Ricks
  - Gilda Vanier

Students
  - Brian Chang
  - Nate Chongsiriwatana
  - Chris Corzett
  - Brigitte Fisher
  - Alexandra Robbins
  - Kristin Robbins
  - David Schroeder
  - Josefina Seoane

**Acknowledgements**

*With Support From*
Department of Homeland Security
Lawrence Livermore National Laboratory (LDRD)

This work was performed under the auspices of the U. S. Department of Energy by University of California, Lawrence Livermore National Laboratory under contract W-7405-Eng-48.

**Collaborators**

LLNL - P. Imbro, K. Smith, J.P. Fitch, M. Palmblad

Biocyc - Peter Karp, SRI International

Strains - E. Garcia, V. Motin, P. Imbro, Ft. Collins

UC Davis - J. Foley, N. Drazenovich

PNNL - M. Lipton, K. Hixon, D. Smith

Texas A&M - N. Clarke, G. Adams

Industry - Biolog
Host-pathogen Interaction Model (Infectomics)

**Host**
- Cells (infection routes)
  - Blood, Lung, Nasal
- Whole Blood (human)
- Animals
- Human Studies

**Pathogens**
- **Y. Pestis**
  - near neighbors: *Y. enterocolitica*, *Y. psuedotuberculosis*
- **B. anthracis**
  - near neighbors: *B. cereus, B. thuringensis*
  - (Virulent v. avirulent)

**Protein**
- 2-D DIGE
- Mass Spec.
- Phenotype Arrays

**Protein Biomarkers**
Microarray data showed different expression levels.

- **Green** are upregulated at 26°C-Ca.
- **Red** are upregulated at 37°C-Ca.

Comparing RNA expression to protein expression to metabolic activity.

Yersinia pestis Proteomic Profiling

2-D DIGE

FTICR-MS and accurate mass tags

Y. pestis soluble Proteome
26°C high calcium (flea)
37°C low calcium (human)
>2800 proteins spots
Identified 239 differential proteins


>5000 Proteins from Annotation
~35% proteome coverage
submitted

Collaboration with Drs. M. Lipton and D. Smith, PNNL, submitted
Host Response

Host Models
Cells
Whole Blood
Animals
Human Clinical
Pathogens
Y. pestis
B. anthracis
F. tularensis
Other strategic Viruses, Toxins
Y. pe, Y. ps, Y. en
Distinct response

7 pathogens, plasma
Heat map of host response in human blood

Y. pestis NYC
Y. pestis India
Y. enterocolitica
Y. pseudotuberculosis
Avirulent Y. pestis
Avirulent B. anthracis
Control

Invited review 2005 Exp Rev Prot
Zhang et al. 2005 Proteomics
Chromy et al. 2004 JPR
Chromy et al. 2004 BBRC
Foley et al. submitted Vet Med

Animal Models
Lungs – lesions, Y. pe present
? pneumonic (alveoli) with NYC
RT PCR – Cytokine response
Deer mice – not resistant to NYC
CBCs – sepsis 3 days after exposure
Oxidative stress early
Spleen – severe necrosis
Lung, liver, spleen DIGE in progress

Future – > Dipstick, Mouthswab, Breathalyzer
Other Pathogens and Models – > bovine, swine, NHP

Cytokine Arrays

4 pathogen exposures, WBCs
IMAC-Cu chip

MS Screen

2-D DIGE Plasma
Monkey, Bovine
Human
Swine,
Rodent – in progress
(Plasma, Tissue,
Saliva, Breath)
Red dye accumulates as bacteria respire.

Omnilog robot (Biolog, Hayward CA) measures dye intensity in each well every 15 minutes.

Shown here, avirulent *Y. pestis* KIM D27 grown on antibiotics plate #15A with Calcium (top) and without Calcium (bottom).
Compare to “standard” phenotype in Biolog’s diagnostic database
Analyze mutants