S. maltophilia: Mechanisms of Virulence and Multi-Drug Resistance for an Emerging Pathogen

Bryan W. Berger

Department of Chemical and Biomolecular Engineering Program in Bioengineering
Research Overview – Bryan Berger – lehigh.edu/berger

**Scalable Biosynthesis**
- Scalable, cellular biosynthesis of QDs, novel nanoparticles and biosurfactants
- Engineering novel functionalities into biosurfactants
- Engineering enzyme selectivity for polysaccharide degradation and stability

![High-yield size controlled quantum dot biosynthesis](image)

**Microbial Pathogenesis**
- *Fusobacterium* invasion in ulcerative colitis
- Multi-drug resistance in *Stenotrophomonas*

![Microbial Pathogenesis](image)

**Membrane Biophysics**
- Membrane proteases
- Neuropilin co-receptors
- Receptor modifying proteins

![Membrane Biophysics](image)
Figure 2: The number of new systemic antibiotic agents has declined since 1980, and most (75%) of these drugs are in two classes, beta-lactams and quinolones.

- Aminoglycosides
- Beta-lactams
- Lipopeptides
- Macrolides/Lincosamides/Streptogramins
- Oxazolidones
- Quinolones
- Tetracyclines
- Other

- MRSA
- VRE
- FQRP

Time Period:
- 1980-1984
- 1985-1989
- 1990-1994
- 1995-1999
- 2000-2004
- 2005-2007
Resistance Gene to Last Line of Antibiotic Defense Has Emerged

Until recently, resistance to the polymyxin class of antibiotics—the last line of microbial defense—was thought to be highly improbable. However now, Chinese scientists have discovered a new gene, called mcr-1 that is widespread among Enterobacteriaceae, a large family of Gram-negative bacteria that include a variety of human pathogens, after taking samples from pigs and patients in South China.

"These are extremely worrying results. The polymyxins (colistin and polymyxin B) were the last class of antibiotics in which resistance was incapable of spreading from cell to cell. Until now, colistin resistance resulted from chromosomal mutations, making the resistance mechanism unstable and incapable of spreading to other bacteria," explained co-author Jian-Hua Liu, Ph.D., a professor at the South China Agricultural University in Guangzhou, China. "Our results reveal the emergence of the first polymyxin resistance gene that is readily passed between common bacteria such as Escherichia coli and Klebsiella pneumoniae, suggesting that the progression from extensive drug resistance to pandrug resistance is inevitable."
1. Obligate intracellular pathogen

2. Biofilm as a defense against antibiotics
March 19 2013

Kyla Cochrane and Emma Allen-Vercoe, University of Guelph
Kyla Cochrane and Emma Allen-Vercoe, University of Guelph
Secreted Effectors that Alter Host Cytoskeletal Structure During Infection

- What are unique mechanisms used by *S. maltophilia* to alter cytoskeletal structure during infection?
- How do specific, secreted effectors contribute to unique ability of *S. maltophilia* to cause chronic infection?
Search Strategy: Identify Novel Factors Based on Sequence Variability within Identified Domains

1. Identify all CDS > 50 aa
2. Align CDS across >5 genomes
3. Calculate sequence variability
4. Sort based on sequence variability
5. Identify sequences with specific motifs

- Focus on pathogens *S. maltophilia* and *P. aeruginosa*, both of which have multiple pathogenic and reference genomes sequenced
- Vary the sequence window from 30-150 aa’s to optimize response in terms of identifying novel proteins
- Use additional tools (SignalP, JPRED2) to sort sequences based on predicted subcellular localization (cytosol, periplasm, extracellular), secondary structure and presence of repeat or other conserved sequence motifs
Evolution from Carriage to Virulent Strain Includes Signal Peptide and Additional N-terminal Domains

- In searching genome of *S. maltophilia* and comparing carriage (or environmental) versus virulent strains, a series of predicted ankyrin repeat (ANK) proteins emerged as highly variable across genomes.
- In particular, evolution to virulent strain includes unique N-terminal regions with diverse sequences resembling coiled-coil and other motifs known to be important for self-association.
- ANK-domain effectors identified for *Legionella pneumophila* and other pathogens, but lack N-terminal domains of known significance (i.e., signal peptide sequences).
Prevalence of smlt3054 in Clinical Isolates

- Pilot study with LVH to collect 50-75 isolates from patients who test positive for *S. maltophilia*
- Double-blind study to assess whether presence of predicted proteins correlate with invasiveness
- Western blot from clinical isolates confirms expression of Smlt3054 as well as secretion when overexpressed.
Ankyrin Repeats as Domains That Modulate Protein Self-Assembly

- At least 40 mammalian actin-binding proteins that contain ANK repeats (Shank, epsin, ERMs)
- ANK1 links CD44 through direct interaction to PI-K dependent signaling pathways upregulated in response to shear or other extracellular cues
- Spectrin binding to ANK modulates rate of F-actin polymerization
Smlt3054 Binds Actin and Modulates Host Cell Growth and Cytoskeletal Structure

- Co-transfect smlt3054-eGFP with actin-mRFP into A549 lung endothelial cell line
- Large, dense granules observed in center of cells
- Near complete co-localization of actin-smlt3054
- DAPI counterstain indicates multi-nucleated cells, with large, dense inclusions containing cellular actin and smlt3054
Smlt3054 Binds Actin and Modulates Host Cell Growth and Cytoskeletal Structure
Riding the Actin Wave: A Possible Mechanism of Entry into Host Cells

**Short times (2h):**

**Long times (20h):**

- Co-localization of F-actin ‘waves’ with exogenously added smlt3054 is indicative of specific binding during actin turnover/ECM rearrangement
- As smlt3054 accumulates over long times, cells exhibit numerous defects and other features suggestive of apoptosis/transformation
- Uptake is temperature dependent, suggesting an activated transport mechanism
Confocal time lapse of HEK293 cells with added smlt3054
Expression and Purification of smlT3054

SDS-PAGE:

Anti-His6 mAb:

- Subclone, express and purify using MBP-pET28a
- Moderately soluble as fusion (~ 1 mg/mL), and poorly soluble as purified protein (~0.1 mg/mL)
- Removal of N-terminal region (Start-H39) prevents aggregation and improves solubility
Mechanism of Actin Polymerization

2. ATP-bound actin forms a nucleus for actin oligomerization.
3. Nucleus formation leads to actin assembly.
4. ADP-bound actin is formed during the polymerization process.
5. The final product is a stable actin oligomer.
F-actin only  
(contains no sarkosyl)

F-actin + Smlt3054  
(contains sarkosyl)
Model for *S. typhimurium* interaction with host cells.

Galán J E, and Zhou D PNAS 2000;97:8754-8761
Conclusions

- Smlt3054 is a secreted effector from virulent strains of *S. maltophilia*, and is found predominantly in clinical strains
- Smlt3054 binds F-actin, which depends on both ANK domains and N-terminal repeat to induce morphological changes in cell
- Smlt3054 induces a multi-nucleated, ‘transformed’ phenotype
- Rate of F-actin polymerization is modulated by multiple subdomains of smlt3054

Future Work

- Identify binding partners for smlt3054: cell surface receptors in lamellapodia? Other intracellular factors?
- What is the uptake mechanism across lamellapodial membrane? Internalization, invasion or some other mechanism, since *S. maltophilia* is not known to be highly invasive?
- Optimization of ANK library to target ANK1-ANK2
Genetic Mutation Causes Drug Resistance

Non-resistant bacteria exist → Bacteria multiply by the billions → Some mutations make the bacterium drug resistant → Drug resistant bacteria multiply and thrive.

- A few of these bacteria will mutate.
- In the presence of drugs, only drug resistant bacteria survive.
Bacteria have evolved numerous antibiotic resistance mechanisms

3. Biofilms
   - Motile bacteria adhere to surface
   - Secrete extracellular polymeric substance (EPS)
     - Comprised of polysaccharides, DNA, and protein
     - Acts as diffusion barrier against antibiotics
Bacteria encased in EPS are protected from antimicrobials as well as other stress factors including phagocytosis and dehydration.
**Stenotrophomonas maltophilia** is an intrinsic multidrug resistant (MDR), nosocomial pathogen of growing concern

- Third most common nosocomial non-fermenting Gram-negative bacilli
- Third most common cause of late-onset ventilator-acquired pneumonia
- Second most common bacteria isolated from lungs of CF patients
- Associated with high mortality rate (30%) with few treatment options due to intrinsic MDR
- Capable of adhering to and producing biofilm on IB3-1 bronchial cells as well as numerous inert surfaces found in indwelling medical devices such as catheters and ventilators
- Biofilm formation in linked to virulence and MDR

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Antibiotic screening of *S. maltophilia* clinical strains received from Lehigh Valley Hospital

Biofilm producing *Stenotrophomonas maltophilia* Strain BB2 collected from Lehigh Valley Hospital
Many biological important polysaccharides contain uronic acids

- Four major groups
  - Alginates, three block types
    - Poly-\(\beta\)-D-mannuronic acid (polyManA)
    - Poly-\(\alpha\)-L-guluronic acid (polyGulA)
    - Alternating ManA and GulA blocks (polyMG)

*Pseudomonas aeruginosa* expresses a periplasmic alginate lyase (AlgL) which regulates the chain length of secreted alginate and prevents lethal build-up of alginate in periplasm.

![Diagram of alginate transport and degradation](image_url)

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Bacterial “Spreading Factors” Degrade Exopolysaccharides to Facilitate Spreading of Bacteria and Associated Toxins

- Secreted lyases are also used by bacterial pathogens to degrade host EPS
- This enables “permeabilization” of host tissue to allow bacteria to penetrate or increase rate of soluble toxin diffusion
- *Mycobacterium, Streptococcus* and other pathogens also use depolymerized sugar monomers as carbon source to persist under starvation conditions
Genomic sequencing of clinical isolate *Stenotrophomonas maltophilia* K279a revealed a putative alginate lyase (Smlt1473) absent from the genomes of environmental strains

- Strain K279a isolated from blood of cancer patient who developed an infection which did not respond to antibiotic treatment
- Smlt1473 restricted to a subset of *S. maltophilia* clinical isolates and other related pathogens such as *Achromobacter* and *Bordetella* (≥ 60% amino acid sequence identity)
- Predicted to belong to PL-5 family
- No evidence to suggest alginate is a major component of *S. maltophilia* biofilm

**Goals:**
- Characterize unique, restricted lyase
- Determine substrate specificity
- Postulate possible biological role of lyase
Gels run from the top down with ladder loaded last
(i.e. gel #1 is BB1, BB2, BB3, BB4, BB5, BB6, BB7, Ladder)

Positive band (533 bp)

BB1-7
BB8-13
BB16-18, 20-23
BB24-30
BB32-38

BB40, 43, Negative control

gDNA screen of smlt1473
Expected size: 533 bp
Number of strains screened: 36
Number of positive strains: 28
Percent positive: 77.8%
Smlt1473 was heterologously expressed in *E. coli* and purified in a one step manner via immobilized metal ion affinity chromatography.
Heterologous secretion of Smlt1473 is dependent on a predicted N-terminal lipoprotein signal.

MSLPLRLALLPTLLASASAFAS[CPAPPPGQPDIRAIG

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Heterologous expression of Smlt1473 resulted in extracellular lyase activity.
Mutation of putative catalytic residues resulted in complete or significant knockdown of lyase activity.
Residues with similar effects on substrate specificity and activity were found to be clustered together with respect to catalytic core (white) based on heat map of Smlt1473.

Applications in the rationale design of mutant lyases with a high degree of specificity and activity towards a polysaccharide of interest.
Other polysaccharides: pectic acid, mucin, acetylated alginate, heparin

**Celluronic acid:**
Primary component of plant cell wall

**Alginic acid:**
Component of bacterial biofilm

**Hyaluronic acid:**
Component of mammalian ECM
Substrate specificity analysis revealed a pH-dependence on lyase activity

- Maximum activity against
  - Hyaluronic Acid at pH 5
  - PolyGlcA at pH 7
  - Alginate based substrates at pH 9
Hyaluronic Acid

What is it?
Hyaluronic Acid is a substance that is naturally present in the human body and can help treat various joint disorders along with promoting healthy skin.

What's it do?
Hyaluronic acid acts as a lubricant in the joints and other tissues within the body.

Where to get it!
Hyaluronic Acid is found in the eyes and joints, and it can also be extracted from rooster combs or made by bacteria in the laboratory. It is also found in high concentration in NeoCell Beauty Bursts.

Fun Factoids
- Hyaluronic Acid has been promoted to prevent the effects of aging and has been referred to as a "fountain of youth".
Oxidative “burst” to generate oxygen free radicals that damage bacterial DNA

**Acidify extracellular environment to pH < 5 which is lethal to bacteria**

Release defensins and other antimicrobial peptides (AMPs)

Extravasate into deep tissue at site of infection (http://www.youtube.com/watch?v=9wxK6oLA5oc)
Extracellular Acidification as a Cue to Activate Neutrophils

Extracellular Acidification Induces Human Neutrophil Activation

AnaÍlia S. Trevani, Graciela Andonegui, Mirta Giordano, Daniel H. López, Romina Gamberale, Fernando Minucci and Jorge R. Geffner

*J Immunol* 1999; 162:4849-4857; http://www.jimmunol.org/content/162/8/4849

- Acidification can act as a trigger from damaged host endothelium to release cytoplasmic stores and lower local pH
- This triggers secondary lowering of extracellular pH by neutrophils
- Neutrophils respond to lower pH by increasing rate of free radical generation as well as protease release
- Thus, neutrophils are activated at acidic pH, where they reduce intracellular pH – this is same pH range in which Smlt1473 is most active
Biofilm Degrading Enzyme, Bacterial Spreading Factor, Both?

- Increasing mannnuronic acid (ManA) content in biofilm from *P. aeruginosa*
- Bacterial lyases purified from cystic fibrosis patients with chronic infection show pronounced preference for polyManA versus alginic acid
- Achromobacter AXX-A is polyManA with little to no HA activity, whereas Smlt1473 is multifunctional

Partial Purification and Characterization of a Polymannuronic Acid Depolymerase Produced by a Mucoid Strain of *Pseudomonas aeruginosa* Isolated from a Patient with Cystic Fibrosis

W. MICHAEL DUNNE, JR.,* AND FRANCIS L. A. BUCKMIRE

Department of Microbiology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

**Figure:**
- Extracellular depolymerase activity over time with varying concentrations of ManA.
- A gel showing molecular weight markers and fraction numbers.

**Table:**
- Kinetic parameters for depolymerase activity at different concentrations.

**Reference:**
- 0099-2240/85/090562-06$02.00/0
- Copyright © 1985, American Society for Microbiology
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