Bacterial Biofilms: Synthesis, Structure and Applications

Bryan W. Berger

Department of Chemical and Biomolecular Engineering
Program in Bioengineering
Bad Bugs, No Drugs – 10 New Antibiotics by 2020 (IDSA)

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
- Tetracycline
- Other

Graphs showing the percentage incidence of MRSA, VRE, and FQRP from 1980 to 2000.
100% BEEF*
*WE KILLED THE SALMONELLA WITH AMMONIA
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 have evolved numerous antibiotic resistance mechanisms

Planktonic bacteria

Mucoid bacteria

EPS
Bacteria encased in EPS are protected from antimicrobials as well as other stress factors including phagocytosis and dehydration.

Planktonic bacteria

EPS

Mucoid bacteria

Antimicrobial

Attachment

Growth
Figure 1: Biofilm Formation

- Reversible Adsorption of Bacteria (sec.)
- Irreversible Attachment of Bacteria (sec.-min.)
- Growth & Division of Bacteria (hrs.-days)
- Exopolymer Production & Biofilm Formation (hrs.-days)
- Attachment of Other Organisms to Biofilm (days-months)
**Table 1.** Clinical manifestations of *S. maltophilia* obtained from clinical specimens.

<table>
<thead>
<tr>
<th>Clinical manifestations</th>
<th>Number of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pneumonia</td>
<td>30 (65%)&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Urinary infections</td>
<td>3 (7%)&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Meningitis</td>
<td>1 (2%)&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cervicitis</td>
<td>1 (2%)&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Other</td>
<td>11 (24%)</td>
</tr>
</tbody>
</table>

<sup>1</sup>- in 19 cases – *S. maltophilia* was the sole isolate;  
<sup>2</sup>- *S. maltophilia* was the sole isolate.
**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

<table>
<thead>
<tr>
<th>Class</th>
<th>Name</th>
<th>Number of Resistant Strains (n=28)</th>
<th>Percentage of Resistant Strains (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lactam</td>
<td>Ampicillin</td>
<td>28</td>
<td>100</td>
</tr>
<tr>
<td>Aminoglycoside</td>
<td>Streptomycin</td>
<td>28</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Spectinomycin</td>
<td>28</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Kanamycin</td>
<td>28</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Gentamicin</td>
<td>27</td>
<td>96.4</td>
</tr>
<tr>
<td>Glycopeptide</td>
<td>Zeocin</td>
<td>28</td>
<td>100</td>
</tr>
<tr>
<td>Broad-spectrum</td>
<td>Nalidixic acid</td>
<td>7</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
<td>5</td>
<td>17.9</td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol</td>
<td>3</td>
<td>10.7</td>
</tr>
</tbody>
</table>

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

Biofilm producing *Stenotrophomonas maltophilia* Strain BB2 collected from Lehigh Valley Hospital
S. maltophilia Clustering and Biofilm Formation in Culture
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
Many biological important polysaccharides contain uronic acids

- Hydroxyl group of C6 is oxidized to form carboxylic acid
- Results in anionic polysaccharide

![Oxidation](image)

- Four major groups
  1. Alginates, three block types
     - Poly-β-D-mannuronic acid (polyManA)
     - Poly-α-L-guluronic acid (polyGulA)
     - Alternating ManA and GulA blocks (polyMG)

![ManA](image)
![GulA](image)
Many biological important polysaccharides contain uronic acids

- Hydroxyl group of C6 is oxidized to form carboxylic acid
- Results in anionic polysaccharide

\[ \text{Oxidation} \]

- Four major groups

1. Alginates, three block types
   - Poly-β-D-mannuronic acid (polyManA)
   - Poly-α-L-guluronic acid (polyGulA)
   - Alternating ManA and GulA blocks (polyMG)

\[ \text{C5 epimers} \]

ManA

GulA
Many biological important polysaccharides contain uronic acids

- Four major groups
  1. Alginates, three block types
    - Poly-β-D-mannuronic acid (polyManA)
    - Poly-α-L-guluronic acid (polyGulA)
    - Alternating ManA and GulA blocks (polyMG)

Many biological important polysaccharides contain uronic acids

- Four major groups
  1. Alginates, three block types
     - Poly-β-D-mannuronic acid (polyManA)
     - Poly-α-L-guluronic acid (polyGulA)
     - Alternating ManA and GulA blocks (polyMG)

![Chemical structures of uronic acids and calcium ions]
Many biological important polysaccharides contain uronic acids

- Four major groups
  1. Alginates, three block types
     - Poly-β-D-mannuronic acid (polyManA)
     - Poly-α-L-guluronic acid (polyGulA)
     - Alternating ManA and GulA blocks (polyMG)
Many biological important polysaccharides contain uronic acids

- Four major groups
  3. Glucuronans (poly-β-D-glucuronic acid, polyGlcA)
     - Major component of cell well in green algae
     - Component of several bacterial exopolysaccharides
Many biological important polysaccharides contain uronic acids

- Hyaluronic acid (HA)
  - Major component of extracellular matrix in nearly all connective tissue
  - Repeating disaccharide of GlcA and N-acetylglucosamine (GlcNAc)
  - Degree of polymerization up to 25,000 disaccharide units (MW = 10 MDa)
  - High viscosity acts as defense mechanism against infectious agents and secreted toxins by preventing diffusion into deep tissue
Polysaccharide lyases (PLs) catalyze the cleavage of uronic acid containing polysaccharides

- Enzymes which cleave O-glycosidic bond between sugar rings
- Classified into 21 polysaccharide lyase families (PL families) based on secondary structure fold and substrate specificity
- Reaction proceeds via a β-elimination mechanism, resulting in the formation of a double bond at the new non-reducing end
*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.

*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.

*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.

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 a blood 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)

gDNA screen of smlt1473
Expected size: 533 bp
Number of strains screened: 36
Number of positive strains: 28
Percent positive: 77.8%

(i.e. gel #1 is BB1, BB2, BB3, BB4, BB5, BB6, BB7, Ladder)
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.
Heterologous expression of Smlt1473 resulted in extracellular lyase activity.
Formation of unsaturated products allows for the monitoring of enzymatic activity by measuring absorbance at 235 nm with respect to time.

$$1 \text{ Unit} = \frac{1 \Delta Abs_{235 \text{ nm}}}{\text{min}}$$

**Specific Activity** = $\frac{\text{Unit}}{\text{mg enzyme}}$
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
Smlt1473 was found to be active against a wide variety of uronic acid containing polysaccharides.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH</th>
<th>Specific Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate</td>
<td>9</td>
<td>20.4 ± 0.7</td>
</tr>
<tr>
<td>Poly-β-D-mannuronic acid (polyManA)</td>
<td>9</td>
<td>68.5 ± 2.9</td>
</tr>
<tr>
<td>Poly-α-L-glucuronic acid (polyGulA)</td>
<td>9</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>Alternating ManA/GulA (polyMG)</td>
<td>9</td>
<td>12.8 ± 0.4</td>
</tr>
<tr>
<td>Poly-β-D-glucuronic acid (polyGlcA)</td>
<td>7</td>
<td>848.3 ± 6.3</td>
</tr>
<tr>
<td>Poly-α-D-galacturonic acid (polyGalA)</td>
<td>5,7,9</td>
<td>ND</td>
</tr>
<tr>
<td>Hyaluronic Acid (HA)</td>
<td>5</td>
<td>42.3 ± 1.3</td>
</tr>
<tr>
<td>Mucin</td>
<td>5,7,9</td>
<td>ND</td>
</tr>
<tr>
<td>Heparin</td>
<td>5,7,9</td>
<td>ND</td>
</tr>
</tbody>
</table>

No detectable activity (detection limit: 0.001 absorbance units at 235 nm per minute).
Smlt1473 was found to be active against a wide variety of uronic acid containing polysaccharides

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH</th>
<th>Specific Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate</td>
<td>9</td>
<td>20.4 ± 0.7</td>
</tr>
<tr>
<td>Poly-β-D-mannuronic acid (polyManA)</td>
<td>9</td>
<td>68.5 ± 2.9</td>
</tr>
<tr>
<td>Poly-α-L-glucuronic acid (polyGulA)</td>
<td>9</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>Alternating ManA/GulA (polyMG)</td>
<td>9</td>
<td>12.8 ± 0.4</td>
</tr>
<tr>
<td>Poly-β-D-glucuronic acid (polyGlcA)</td>
<td>7</td>
<td>848.3 ± 6.3</td>
</tr>
<tr>
<td>Poly-α-D-galacturonic acid (polyGalA)</td>
<td>5,7,9</td>
<td>ND</td>
</tr>
<tr>
<td>Hyaluronic Acid (HA)</td>
<td>5</td>
<td>42.3 ± 1.3</td>
</tr>
<tr>
<td>Mucin</td>
<td>5,7,9</td>
<td>ND</td>
</tr>
<tr>
<td>Heparin</td>
<td>5,7,9</td>
<td>ND</td>
</tr>
</tbody>
</table>

No detectable activity (detection limit: 0.001 absorbance units at 235 nm per minute).
Analysis of oligosaccharide products revealed an endolytic cleavage.
Other polysaccharides: pectic acid, mucin, acetylated alginate, heparin
β-elimination mechanism proceeds in three steps
1. Positively charged (Arg) or polar (Asp or Gln) residues neutralize negatively charged carboxyl group on substrate, lowering $pK_a$ of C5 hydrogen and priming it for abstraction.
2. A general base abstracts the C5 hydrogen

Neutralizing group

Tyrosine

Histidine

or
2. A general base abstracts the C5 hydrogen

Neutralizing group

Tyrosine

or

Histidine

or
2. A general base abstracts the C5 hydrogen
3. Electron transfer to form a double bond between C4 and C5 of substrate and proton donation by general acid, results in cleavage of O-glycosidic bond.
Primary amino acid sequence alignment with other PL-5 lyases revealed putative catalytic residues

<table>
<thead>
<tr>
<th>smlt1473</th>
<th>Primary</th>
<th>Secondary</th>
<th>Tertiary</th>
<th>Quaternary</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. xylosoxidans</td>
<td>1 MRSVSSFPRGGALPAVARWYAACLPLMLCQAIALAFF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. avium</td>
<td>1 MRRSSRGRGRRFVLRALW</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. marina</td>
<td>1 MRNPKLNNLAPTLSSL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>1 MKTSHLIRALPGALAR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>smlt1473</th>
<th>Primary</th>
<th>Secondary</th>
<th>Tertiary</th>
<th>Quaternary</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. xylosoxidans</td>
<td>44 GSVIDPALQQ-QN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. avium</td>
<td>53 SSVIDPALKA-ENKAATRFPFVGLATMDSAYIG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. marina</td>
<td>73 DKARAT-LNVKAEKFRSISDIDRTTLDKTRLQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>71 DSARAT-LNVKAEKFRSISDIDRT -</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>smlt1473</th>
<th>Primary</th>
<th>Secondary</th>
<th>Tertiary</th>
<th>Quaternary</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. xylosoxidans</td>
<td>116 MRQWMLDAVAMAYLK VHQDQA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. avium</td>
<td>130 VRQWTLGAAGIALK YTRRTA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. marina</td>
<td>145 MRKWAIGMSASLYIKFSDSHPLAQHQQAEALF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>143 MRKWAIGMSASLYIKFSDSHPLAQHQQAEALF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>smlt1473</th>
<th>Primary</th>
<th>Secondary</th>
<th>Tertiary</th>
<th>Quaternary</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. xylosoxidans</td>
<td>189 WQAGHAAAFQKGIDQIDIQDDQSLPLEMARQGRSHYHDYLAIPLVMMAEIARLGERQDWY</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. avium</td>
<td>207 LRTAQIYRKGTDIADIQADSPIEMARQGRSHYHDYLAIPLVMMAEIARLGERQDWY</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. marina</td>
<td>225 FDWMASEVKVQNGVDVFNLPLQKRQGLALYHNYALPPQAPQACNYVMDR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>223 FDWMASEVKVQNGVDVFNLPLQKRQGLALYHNYALPPQAPQACNYVMDR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>smlt1473</th>
<th>Primary</th>
<th>Secondary</th>
<th>Tertiary</th>
<th>Quaternary</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. xylosoxidans</td>
<td>267 AWFQNHGGAAQL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. avium</td>
<td>285 DWFARQAGVAKDR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. marina</td>
<td>303 DFEEENKQKQMTDLKEDM-FKAELEFPCTLYTCPDVIEKRMPOPKFTRFRGCGLTDKVDYDPSEHGR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>301 ETTEFKGIDQGDQDITKVDK -</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1QAZ</th>
<th>Primary</th>
<th>Secondary</th>
<th>Tertiary</th>
<th>Quaternary</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. xylosoxidans</td>
<td>285 DWFARQAGVAKDR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. avium</td>
<td>281 FWFQNHGGAAQL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. marina</td>
<td>303 DFEEENKQKQMTDLKEDM-FKAELEFPCTLYTCPDVIEKRMPOPKFTRFRGCGLTDKVDYDPSEHGR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>301 ETTEFKGIDQGDQDITKVDK -</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1QAZ</th>
<th>Primary</th>
<th>Secondary</th>
<th>Tertiary</th>
<th>Quaternary</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. xylosoxidans</td>
<td>285 DWFARQAGVAKDR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. avium</td>
<td>281 FWFQNHGGAAQL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. marina</td>
<td>303 DFEEENKQKQMTDLKEDM-FKAELEFPCTLYTCPDVIEKRMPOPKFTRFRGCGLTDKVDYDPSEHGR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>301 ETTEFKGIDQGDQDITKVDK -</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Homology model built from solved *Sphingomonas A1-III* alginate lyase crystal structure (PDB: 1QAZ) revealed putative catalytic residues were clustered together in a deep cleft.
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.
Neutrophil Phagocytosis to Clear Invading Bacteria from Bloodstream

- 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)

*Nature Clinical Practice Rheumatology (2006) 2, 661-670*
Extracellular Acidification as a Cue to Activate Neutrophils

Extracellular Acidification Induces Human Neutrophil Activation

Analía 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 mannuronic acid (ManA) content in biofilm from \textit{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 \textit{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

\textbf{EXTRACELLULAR DEPOLYMERASE ACTIVITY/ML}
Chemical and structural differences between substrates likely account for unique, substrate-dependent effect of mutating each residue.
Acknowledgements

Logan MacDonald (PhD Bioengineering)

Elizabeth Weiler (BS BioE)

Emily Wong, MD, PhD (LVHN)