The Presence of Capsule Interferes with Biofilm Formation by *Pasteurella multocida* serogroup A

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Diseases caused by *Pasteurella multocida*

- Bovine Respiratory Disease (BRD)
- Avian Cholera
- Atrophic Rhinitis (pigs)
- Porcine Respiratory Disease Complex
- Bite wounds of humans (zoonosis)
- Human systemic infections

Outbreak at the Great Salt Lake: **Loss of 15,000 Eared Grebes**
*Utah Division of Wildlife 2007*
Human Pasteurellosis

Cat-bite wound infection of second proximal interphalangeal joint due to Pasteurella multocida. Failure of cephalaxin therapy resulted in septic arthritis.

Dog bite wound infected due to Pasteurella multocida and anaerobic bacteria.

Chronic respiratory infections

Complications of Pasteurella Infection (in 40 %)
- Local septic arthritis
- Osteomyelitis
- Tenosynovitis
- Bacteremia
**Pasteurella multocida: History**

**The Birth of Immunology**

Louis Pasteur first isolated the causative agent of Avian Cholera in 1879

Mass economic loss for chicken breeders

Discovered that ‘old’ cultures lost their virulence

Inoculated chickens with ‘old’ cultures, and coined the term ‘Vaccination’ for his process

Vaccinated chickens were healthy, but excreted virulent bacteria

(Berche, 2012)
Colony examination under incandescent lighting

- Strain forms iridescent colonies
  - Highly Virulent (50% mortality)

- Strain forms colonies of intermediate iridescence
  - Virulence ranging from 0 – 60% mortality

- Strain forms “blue” colonies
  - Avirulent (0% mortality)
  - Infected birds may become carriers of virulent variants
  - Survive in the nasal clefts of birds for up to 65 days

(Hughes et al., 1930 – 1931)
## P. multocida strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Common name</th>
<th>Serotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. multocida subsp. gallicida P1059</td>
<td>WT P1059</td>
<td>A:3</td>
<td>National Animal Disease Center</td>
</tr>
<tr>
<td>P. multocida subsp. gallicida P1059ΔhyaE</td>
<td>P1059ΔhyaE</td>
<td>Noncapsulated</td>
<td>This study</td>
</tr>
<tr>
<td>P. multocida subsp. gallicida P1059-R8</td>
<td>P1059-R8 (NC)</td>
<td>Noncapsulated</td>
<td>This study</td>
</tr>
<tr>
<td>P. multocida subsp. multocida P1062</td>
<td>WT P1062</td>
<td>A:3</td>
<td>National Animal Disease Center</td>
</tr>
<tr>
<td>P. multocida subsp. multocida P1062ΔhyaE</td>
<td>P1062ΔhyaE (NC)</td>
<td>Noncapsulated</td>
<td>This study</td>
</tr>
<tr>
<td>P. multocida X73</td>
<td>WT X73</td>
<td>A:1</td>
<td>National Animal Disease Center</td>
</tr>
<tr>
<td>P. multocida X73ΔhyaE</td>
<td>X73ΔhyaE (NC)</td>
<td>Noncapsulated</td>
<td>This study</td>
</tr>
<tr>
<td>P. multocida C0513</td>
<td>WT C0513</td>
<td>A</td>
<td>Experimental calf infection</td>
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<tr>
<td>P. multocida C0513-P5</td>
<td>C0513-P5</td>
<td>Capsule-deficient</td>
<td>This study</td>
</tr>
</tbody>
</table>

Plus many clinical isolates
Inverse correlation between biofilm formation and the amount of capsule produced; mucoid strains passed in vitro lose capsule and enhance biofilm formation.

Crystal violet assay for biofilm formation

Blue colonies of Congo Red agar
SEM images of *P. multocida* strain P1059 and mutants

A) WT P1059; B) P1059ΔhyaE; and C) P1059-R8 biofilm formation after 48 hours on glass coverslips. Note enhanced density and cross-linking of cells by matrix material.
CSLM z-stack showing live/dead staining of WT P1059 during biofilm formation

<table>
<thead>
<tr>
<th>Strain</th>
<th>Biomass (µm³/µm²)</th>
<th>Thickness (µm)</th>
<th>Maximum Thickness (µm)</th>
<th>Roughness Coefficient (0-2)</th>
<th>Surface area to Bio-volume ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT P1059</td>
<td>0.0449 ± 0.0032</td>
<td>0.001719 ± 0.0003</td>
<td>4.5</td>
<td>2</td>
<td>9.92</td>
</tr>
<tr>
<td>ΔhyaE</td>
<td>21 ± 3.23</td>
<td>29.79 ± 4.73</td>
<td>34.31</td>
<td>1</td>
<td>5.05</td>
</tr>
<tr>
<td>P1059-R8</td>
<td>61.49 ± 2.02</td>
<td>64.8 ± 1.08</td>
<td>66</td>
<td>0.02286</td>
<td>0.554</td>
</tr>
</tbody>
</table>

A) WT P1059; B) P1059ΔhyaE; C) P1059-R8. Live stain (left column), dead stain (center column), live-dead overlay (right column).
Effect of hyaluronidase on biofilm formation of encapsulated strains during growth
Structural analysis of the *P. multocida* serogroup A EPS

a) GC-MS chromatogram of partially methylated and acetylated alditols; b) proton spectrum of the intact EPS; c) proton spectrum of the EPS after overnight pullulanase digestion. * impurity.
Enzyme digestion of biofilm matrix

Prior to inoculation of the culture media with *P. multocida*, 0.1 mg/ml of α-amylase or Proteinase K was added, followed by incubation for 48 hrs. Biofilms were rinsed, stained with CV, solubilized with ethanol and the A\textsubscript{562} determined. White bars, no treatment; Light grey bars, treated with Proteinase K; Dark grey bars, treated with α-amylase. Significance values are based on comparison to the sample with no enzyme added. *, \( p \leq 0.05 \); **, \( p \leq 0.01 \); ***, \( p \leq 0.001 \); ****, \( p \leq 0.0001 \); ns, not significant.
Putative genes that may be responsible for EPS, and quantitative real time PCR to identify genes of importance for biofilm formation

Putative glycogen synthesis locus in the *P. multocida* genome

<table>
<thead>
<tr>
<th>Locus tag</th>
<th>Gene symbol</th>
<th>Putative gene producta</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM_RS02805</td>
<td>malQ</td>
<td>4-alpha-glucanotransferase</td>
<td>2,094</td>
</tr>
<tr>
<td>PM_RS02810</td>
<td>glgB</td>
<td>1,4-alpha-glucan-branching protein</td>
<td>2,193</td>
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<tr>
<td>PM_RS02815</td>
<td>glgX</td>
<td>glycogen debranching protein</td>
<td>2,019</td>
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<tr>
<td>PM_RS02820</td>
<td>glgC</td>
<td>glucose-1-phosphate adenyllyltransferase</td>
<td>1,308</td>
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<tr>
<td>PM_RS02825</td>
<td>glgA</td>
<td>glycogen synthase</td>
<td>1,443</td>
</tr>
<tr>
<td>PM_RS02830</td>
<td>glgP</td>
<td>glycogen phosphorylase</td>
<td>2,457</td>
</tr>
</tbody>
</table>

None of these genes were upregulated, suggesting that the glycogen EPS is constitutively expressed, but is unable to promote biofilm formation in the presence of capsule.

<table>
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<th>Gene</th>
<th>Function</th>
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<tr>
<td><em>csrA</em></td>
<td>Carbon storage regulator</td>
</tr>
<tr>
<td><em>pldB</em></td>
<td>Lysophospholipase</td>
</tr>
<tr>
<td><em>sgbU</em></td>
<td>L-xylulose 5-phosphate 3-epimerase</td>
</tr>
<tr>
<td><em>xylB</em></td>
<td>Carbohydrate kinase FGGY</td>
</tr>
</tbody>
</table>
**Pasteurella multocida** strains that form LESS prominent biofilms are MORE virulent

- All 10 Birds survived until euthanized
- Displayed signs of chronic pneumonia
- Significantly less virulent than X73 ($p=0.0143$)
- Strain 756 is a type F clinical isolate that is capsule-deficient

- 14/15 Birds survived until euthanasia
- Displayed signs of chronic pneumonia
- Significantly less virulent than X73
- $P=0.0027$
- Strain 755 is a type A clinical isolate that is capsule-deficient

- 3/15 birds survived until euthanasia
- 12 birds succumbed to acute infection
- 3 Birds showed signs of chronic pneumonia

*P. multocida* was isolated from all birds at euthanasia
Biofilm formation in vivo by less virulent (encapsulated) *P. multocida* strains

Yellow arrows – putative biofilm matrix

Non-Infected Control  
X73 Deficient Biofilm Former  
756 Proficient Biofilm Former  
775 Proficient Biofilm Former

Black arrows – site of inflammation around a blood vessel
Encapsulation VS exopolysaccharide encasement

Bacteria that are encapsulated are isolated from other cells, and hence form poor if any biofilm. Bacteria that produce extracellular EPS that is not attached to the cell all share in the matrix and therefore form more prominent biofilms.

In addition to *P. multocida*, *Haemophilus influenzae*, *Francisella tularensis*, and others make more prominent biofilms if capsule-deficient or have less surface polysaccharide.
Conclusion and Summary

• *Pasteurella multocida* is capable of making a biofilm consisting of a newly identified amylose-like glucan as part of the exopolysaccharide matrix.

• Biofilm formation is inversely correlated with capsule production, but EPS does not appear to be upregulated during biofilm formation.

• Biofilm formation by poor biofilm forming strains can be enhanced by serial passage of the strain *in vitro*, or by enzymatic degradation of the capsule.

• The biofilm matrix is composed largely of protein and polysaccharide.

• Proficient biofilm formers are, overall, less virulent than biofilm-deficient isolates, and may be responsible for more chronic infections.

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