Nature, Properties and Functions of EPS in the Biofilm Mode of Life

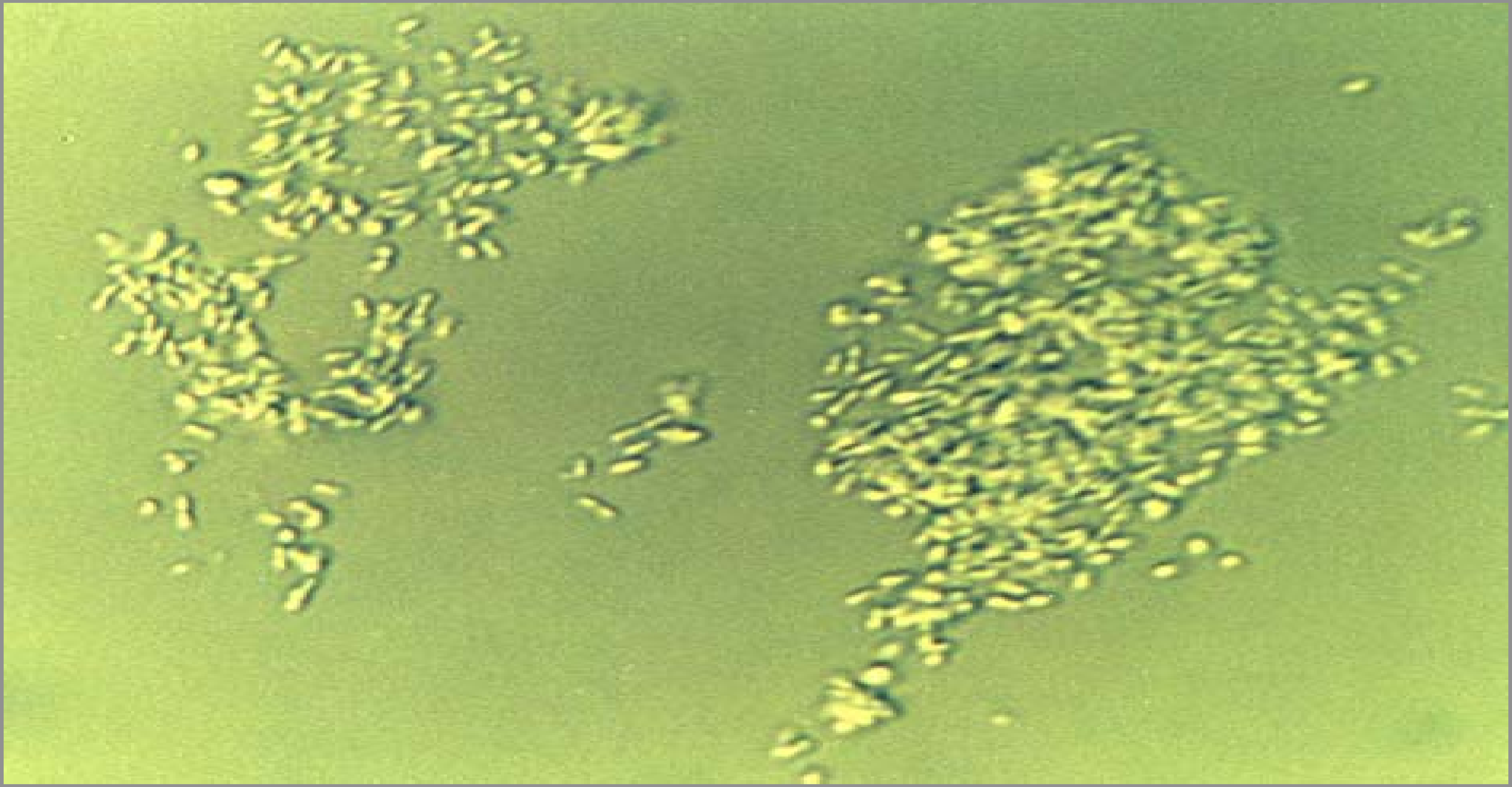
Ian W. Sutherland

Institute of Cell & Molecular Biology

Role of the biofilm matrix

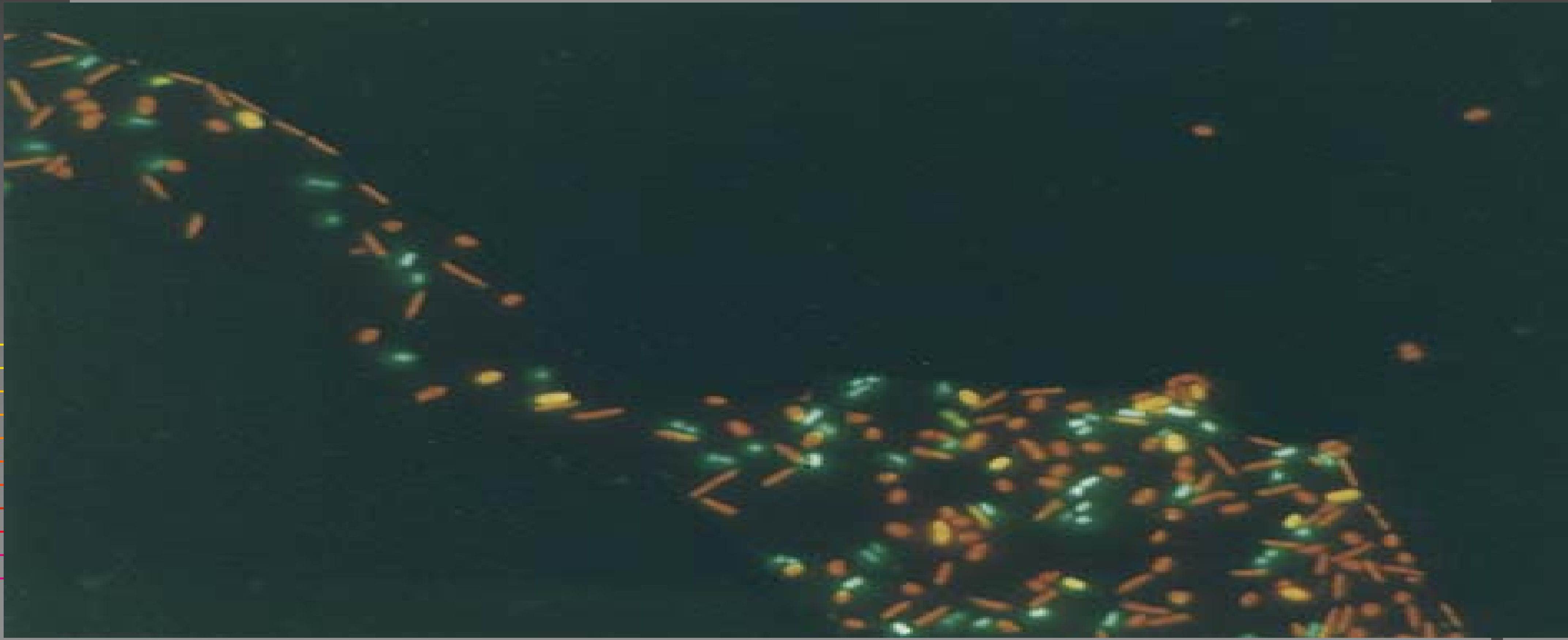
- **Biofilm matrix performs various functions**:
- Stabilises the biofilm
- Plays a role in biofilm attachment
- Protects the microbial cells
- Interacts with ions & macromolecules
- Interaction with protozoa & phagocytes

Single species Enteric biofilm on glass - microcolonies
(Skillman, 1998)



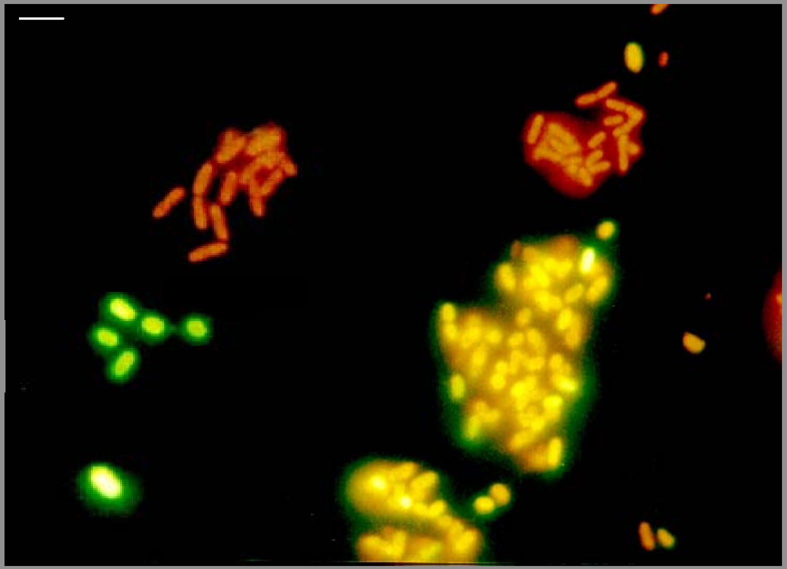
Dual Species Enteric Biofilm stained with propidium iodide

E. agglomerans GFP & *Klebsiella* G1 (Skillman, 1998)



Edinburgh University

Dual Species Biofilms



DUAL SPECIES BIOFILM SHOWING NEUTRAL INTERACTION
[L.C.Skillman,1998]

Composition of the Biofilm Matrix

- **Water**
- **Polysaccharides**
- **Proteins**
- **Nucleic acids**
- Cells
- Other cell products
- Ions

Matrix Polysaccharides

Structure and Function

Biofilm Matrix Exopolysaccharides

- Homopolysaccharides
- Heteropolysaccharides
- Acyl or other organic substituents
- Inorganic substituents
- Physical properties of polysaccharides
- Role of polysaccharides in adhesion

Microbial Exopolysaccharides

- **Carbohydrate Composition**
- **Organic and Inorganic substituents**

Microbial Exopolysaccharides

- **Carbohydrate Composition**

- **Typical monosaccharides:** **D-glucose, D-galactose, D-mannose, L-fucose, L-rhamnose, D-glucuronic acid, D-galacturonic acid, L-guluronic acid, D-mannuronic acid, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine**

- **May be other rarer sugars e.g. Aminouronic acids etc.**

Acid substituents found in Exopolysaccharides

Organic Acids

Pyruvic and Acetic - both very common (ketal & ester respectively)

Succinic – rarer (as half-ester)

Amino Acids

L-Glutamic	***Klebsiella aerogenes* K82**
L-Serine	***Pseudoalteromonas aliena*, *E. coli* K40**

Inorganic acids

Phosphate **Confers negative charge**

Common in some genera and Gram positive spp. including LAB

SulphateEster **Also confers negative charge**

Cyanobacteria; *Haloferax mediterranea*, other Halophiles

Some Biofilm Exopolysaccharides

- Neutral – Mutan (*Streptococcus mutans*)
  Bacterial cellulose
- Basic – *Staphylococcus epidermidis*
- Acid – Colanic acid (*E. coli*)
  Bacterial alginate (*Pseudomonas aeruginosa*)

Microbial Exopolysaccharides from Gram Negative Bacteria

- **Physical properties of polysaccharides**

Structural features of Exopolysaccharides

Polysaccharide Component		Effect	Example
Neutral sugars	Uncharged polymer	Insoluble	Cellulose
Uronic acids	Polyanionic	Solubility, Ion binding	Xanthan, Alginates
Pyruvate	Polyanionic	Ion binding, Transition	Xanthan, Galactoglucans
Methylpentoses	Lipophilicity	Solubility	
Acetylation	Solubility	Gelation, Reduced ion binding	Alginates, Gellan, etc
Side-Chains	Various	Solubility	Scleroglucan, xanthan
1,3 or 1,4 Linkages	Rigidity	(In)Solubility	Curdlan, Cellulose
1,2 Linkages	Flexibility	Solubility, Stability	Dextrans

Polysaccharide Heterogeneity

- Biofilm may contain more than one EPS
- Polymer may be polydisperse
- Polymer may vary in acylation
- Polymer may carry defective side-chains
- Enzymic degradation may occur

Natural families

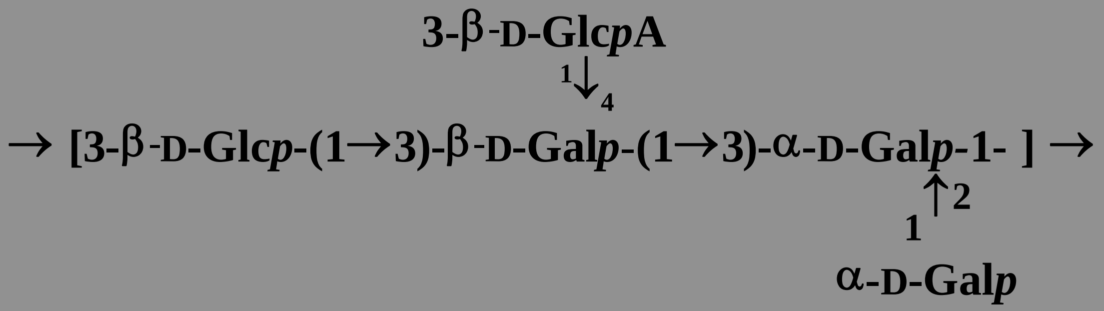
i.e. Structurally related Exopolysaccharides

- Bacterial Alginates
- Beta-Glucans (Bacterial & Fungal)
- Colanic acid
- Galactoglucans
- Succinoglycans
- Xanthans and Acetan
- XM6

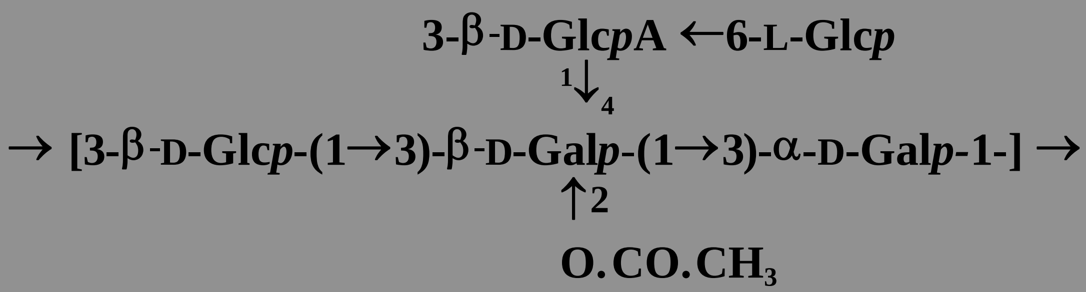
Mutan the homopolysaccharide from *Streptococcus mutans*

- **3)-α-D-Glc*p*-(1,3)-α-D-Glc*p*-(1,3)-α-D-Glc*p*-(1,3)- α-D-Glc*p*-(1**

EPS from *Klebsiella* K8 and K82



Klebsiella aerogenes K8



Klebsiella K82

Haloferax mediterranea EPS

- → [-4-β-D-Glc*p*NAcA-(1→ 6)-α-D-Man*p*-(1→ 4)-β- D-Glc*p* NacA-1-] →
  ↑3
  O-SO_3^-

Staphylococcus epidermidis Biofilm Exopolysaccharides

- **Polysaccharide I**
- **[6-β-D-Glc*p*NAc-(1 6)-β-D-Glc*p*NAc-(1→ 6)--D-Glc*p*-N-(16)-β-D-Glc*p*NAc-(1 6)-β-D-Glc*p*NAc-(1→**
-
- **Ratio: Glc*p*NAc : Glc*p*N c. 5:1 (Cationic)**
- **Polysaccharide II**
- **[6-β-D-Glc*p*NAc-(1 6)-β-D-Glc*p*NAc-(1→6)-- D-Glc*p*-N-(16)-β-D-Glc*p*NAc-(1 6)-β-D-Glc*p*NAc-(1→**
-
- **Ratio: Glc*p*NAc : Glc*p*N c. 16:1**
- **Also contains: phosphate and succinyl half esters (Anionic)**
- Results of Mack *et al.* (1996) *J. Bacteriol.* **178**, 175-83.

Colanic acid the exopolysaccharide from *E.coli*, *Salmonella typhimurium* and other bacteria

- → 3-β-D-Glc*p*-(1→3)-β-L-Fuc*p*-(1→ 4)-α-L-Fuc*p*-(1→
- 1↑4
- β-D-Gal*p*
- 1↑3
- β-D-Glc*p*A
- 1↑4
- β-D-Gal*p* 34= Pyr

The exopolysaccharide from benzene tolerant *Rhodococcus* sp. 33

- Pyr
- 4⇓6
- → 4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→3)--D-Man *p*-(1→4)-β-D-GlcpA(1→

- Results of Urai,M., Aizawa,T. *Carbohydr*. *Res*. **2006**, *341*, 616-623.

Pseudomonas aeruginosa alginate

- [-4--D-Man*p*A-(1 4)--D-Man*p*A-(1 4)--D-Man*p*A-(1 4)--D-Man*p*A-(1 4)--D-Man*p*A-(1 4)--D-Man*p*A-(1 4)--L-Gul*p*A (1]
- No GulA-GulA sequences
- O-Acetyl groups are attached to many of the D-mannuronosyl residues

Typical Molecular Weights of EPS

Polysaccharide	Molecular Weight
Alginate	5×10^5 - 2-3 $\times 10^6$
Dextran	$1 \times 10^5 - 2 \times 10^7$
Emulsan	9.9×10^5
Pullulan	$1 \times 10^4 - 1 \times 10^5$
Scleroglucan	$1.9 \times 10^4 - 2.5 \times 10^4$
Xanthan	$2 \times 10^6 - 5 \times 10^7$

Multiple Polysaccharide Production by some Gram Negative Bacteria

Bacterium	Polysaccharides produced:		
Azotobacter chroococcum	Alginate	and	Type specific EPS
Erwinia amylovora	Type specific EPS	or	Levan^{+}
Escherichia coli	Type specific EPS*	or	Colanic acid*
Ent. geroviae 1.15	Colanic acid type*	and/or	Glucomannan*
Pseudomonas spp.	Alginate	or	Levan^{+}
Rhizobium meliloti	Succinoglycan*	or	Galactoglucan*
Rhizobium/Agrobacterium spp.	Succinoglycan*	or	Curdlan*
Staphylococcus epidermidis	Polysacch.I*		Polysacch. II*

- \- * One or more sugar nucleotide precursors shared
- – + Only synthesised from sucrose

Factors affecting composition and structure of the biofilm matrix

- Microbial composition
- Nutritional environment - nutrient availability, nutrient balance
- Physical environment
- Stress! e.g. diurnal variations in light, humidity, temperature etc.

Determinant properties of EPS in the biofilm matrix

- Solubility
- Flexibility
- Polyelectrolyte activity (Weak?)
- Acyl groups
- Interactions with polysaccharides and proteins (and cells)

Hydration - the water in the matrix

- Polysaccharides may adsorb large quantities of water
- Hyaluronic acid (1kg water/gm polysaccharide); Other EPS less
- Water is not necessarily free
- Effectively provides reservoir of solutes in close association with microbial cells

Hydration and related effects

- Highly hydrated gel around biofilm
- Reservoir of water
- Reservoir of ions and other solutes
- Aqueous environment for enzyme action

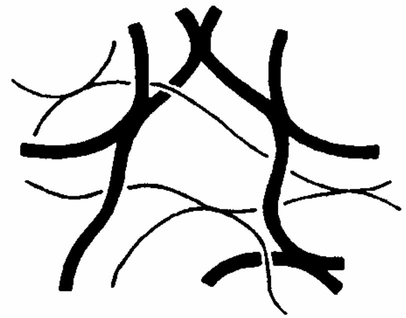
Polysaccharide Properties

- Insoluble – bacterial cellulose, mutan
- Soluble (but highly viscous) – colanic acid, bacterial alginate
- Highly soluble – various low mass polysaccharides

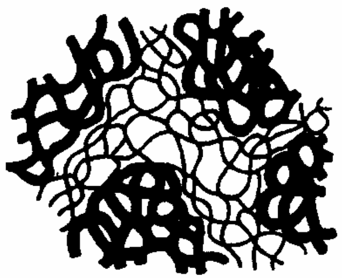
Determinant physical properties of EPS in the biofilm matrix

- Polysaccharide/Polysaccharide interactions
- Self; polymer/polymer
- Gel formation
- Viscoelasticity (Shear thinning)
- Intrinsic viscosity

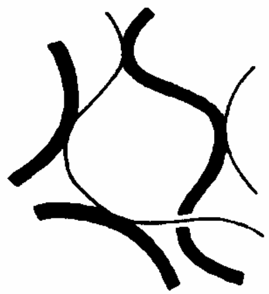
(a)



(b)



(c)



(d)

Binary networks

Four types of binary networks can be visualised. Type (a) consists of a single polymer network containing a soluble second component. Type (b) contains two independent *interpenetrating* networks. In type (c) both polymers form independent networks, but these networks are *phase separated* into distinct regions. Intermolecular binding between the two polymers to form a new type of *coupled* network is shown in (d).

Factors affecting properties of EPS in the biofilm matrix

- Polysaccharide composition
- Polysaccharide structure
- Polysaccharide mass
- Acyl groups
- Ionic environment
- pH

Effects of pyruvate ketal groups

- Increased ion binding (xanthans)
- Inhibition of synergistic gelation (xanthan)
- Decreased solubility (xanthans)
- Decreased stability (xanthan)
- None - polysaccharases, xanthan lyase

Effects of acetyl groups

- Inhibition of ion binding (alginates)
- Inhibition of lyases (gellan, alginate)
- Inhibition of gelation (XM6)
- Inhibition of crystallinity (XM6)
- Inhibition of synergistic gelation (xanthan)
- Promotion of solubility (alginates)
- Promotion of stability (xanthan)
- None - polysaccharases, xanthan lyase

Factors affecting adhesive role of EPS in the biofilm matrix

- Polysaccharide structure
- Ionic environment
- EPS solubility
- Polysaccharases
- Exposed surfaces - teeth; cells; bacteria and their products

Protein: Polysaccharide Interactions

- Segregative and Incompatible (Polymers repel each other)
- Associative (Associate to form complexes)
- Polysaccharide may bridge two protein molecules (Coacervation) - may lead to flocculation

EPS:Protein Interactions in the Biofilm Matrix

- Associative - new properties
- Incompatible or Segregative
- e.g. EPS + globular proteins induces depletion interactions; effective mutual attraction between the proteins. Depletion of polymer between neighbouring protein molecules lowers partial osmotic pressure relative to bulk pressure, colloidal particles are effectively attracted.

Biological interactions in biofilms

- Biofilm matrix is exposed to:
- Interactions with enzymes
- Interactions with bacteriocins
- Interactions with bacteria
- Interact with ions & macromolecules
- Interaction with protozoa & phagocytes
- EPS plays a role in all these interactions and adhesion and biofilm structure will be affected!

Effects of matrix/antimicrobial interaction

- Bactericidal
- Selectively bactericidal - killing some but not all species
- Inhibition by EPS components of matrix
- Failure to reach target
- Stimulation of matrix EPS synthesis!
- All these effects will affect adhesive role of matrix

Role of matrix EPS in initial adhesive interactions

- Bridging repulsion barriers
- Chemical and physical properties of the EPS will affect adhesion
- Ions and non-EPS macromolecules may either promote or inhibit adhesion
- Production of EPS by one species may indirectly affect the conditioning film and alter adhesion by another species
- EPS may provide additional epitopes for lectins or may mask those already present

Biofilm Matrix Polysaccharides

- Are they specific?
- What are the major chemical features?
- What are the main physical properties?
- How do these properties affect adhesion?
- Can we remove the biofilm matrix EPS?
- What are the consequences?

Matrix Exopolysaccharides in Biofilms

- Homopolysaccharides - e.g.. Mutan
- Heteropolysaccharides - e.g.. Colanic acid
- Cationic polymers (rare) - e.g. *Staph. epidermidis*
- Acyl & other organic substituents - e.g. Alginate, Galactoglucans
- Physical properties - viscosity, gelation
- Biological Properties

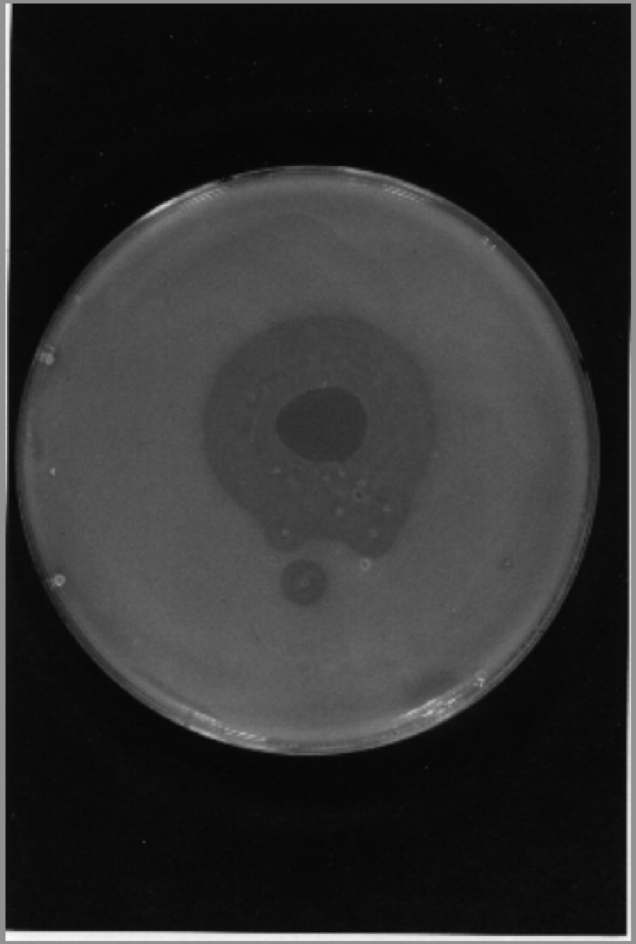
Interactions of matrix polysaccharides

- Chain entanglement yielding gels
- Ionic bridging between chains - gelation
- Hydrogen bonding of linear molecules leading to exclusion of water

Phages and the Biofilm Matrix

- Phages may destroy part or all of the matrix
- Phages may selectively destroy bacterial cells in the biofilm
- Temperate phages might add new genes to the biofilm gene pool

Polysaccharide depolymerase



Effects of phage/bacterium interaction

- Dissolution of homologous EPS e.g. *Enterobacter agglomerans* or no polysaccharide destruction e.g. *E. coli* K12 or *Pseudomonas aeruginosa*
- Possible dissolution of heterologous EPS
- Bacterial cell lysis with release of bacterial periplasmic & cytosolic contents (may include further polysaccharases and lytic enzymes)
- Localised or generalised destruction of the biofilm

Specificity & Products from Phage Enzymes

- Usually specific, occasionally several substrates if high degree of common structure
- Usually oligomers of repeat unit
- Commonly 1 or 2 repeat units
- Usually unaffected by acyl groups
- Either glycanases or polysaccharide lyases

Biofilm Properties

- Biofilms are more resistant to antimicrobial agents than planktonic cells
- Synergistic biofilms were more resistant to disinfectants than single species biofilms
- Bacteriocin-producing enteric species can co-exist in biofilms but not in mixed planktonic culture

Biological interactions in biofilms

- Natural biofilms are exposed to:
- Interactions with phages
- Interactions with bacteriocins
- Interactions with *Bdellovibrio*
- Protozoal grazing
- In all these interactions EPS (polysaccharides, proteins and nucleic acids) will play a role!

Biofilm bacterial types

- Exposed - non EPS producer
- Deep - non EPS producer
- Exposed - capsulate
- Deep - capsulate
- Exposed - slime EPS only
- Deep - slime EPS only

Biological interactions in biofilms

- Biofilms are exposed to:
- Interactions with enzymes
- Interactions with bacteriocins
- Interactions with bacteria
- Interact with ions & macromolecules
- Interaction with protozoa & phagocytes
- In all these interactions EPS will play a role and adhesion will be affected!

Effects of bacterium/antimicrobial interaction

- Bactericidal
- Selectively bactericidal - killing some but not all species
- Inhibition by EPS
- Failure to reach target
- Stimulation of EPS synthesis!
- All these effects will affect adhesive role of EPS

EPS:Protein Interactions in the Biofilm Matrix

- Localised phase separation within the matrix
- Coacervation - bridging two or more protein particles
- ? Generation of water channels with low or negligible dissolved polymers
- Localised emulsions?

Biofilm Enzyme Activities

- Polysaccharase - Localised destruction, EPS solubilisation
- Polysaccharide lyase - Localised destruction, EPS solubilisation
- Esterase - removal of acyl or other substituents - gel formation; increased interaction with cations & other polysaccharides
- Proteases
- DNases
- RNases

Tools of the Trade

- Green fluorescent protein
- Specific phages
- Phage-induced enzymes
- Bacteriocins
- Disinfectants
- Carbohydrate analysis

The Edinburgh Biofilm Team

- David Allison - the pioneer!
- (Still working with biofilms at Manchester University)
- Kevin Hughes- phage and polysaccharides
- (Now at British Antarctic Survey)
- Lucy Skillman - GFP and dual species
- (At Murdoch University, Australia)
- Karen Tait - disinfectants/antimicrobials
- (At Plymouth Marine laboratory)
- Joanne Greated - *Staph. epidermidis*
- Lynn Kennedy - everything else!
- (Lab Superintendant – Cambridge University Stem Cell Laboratory)