

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

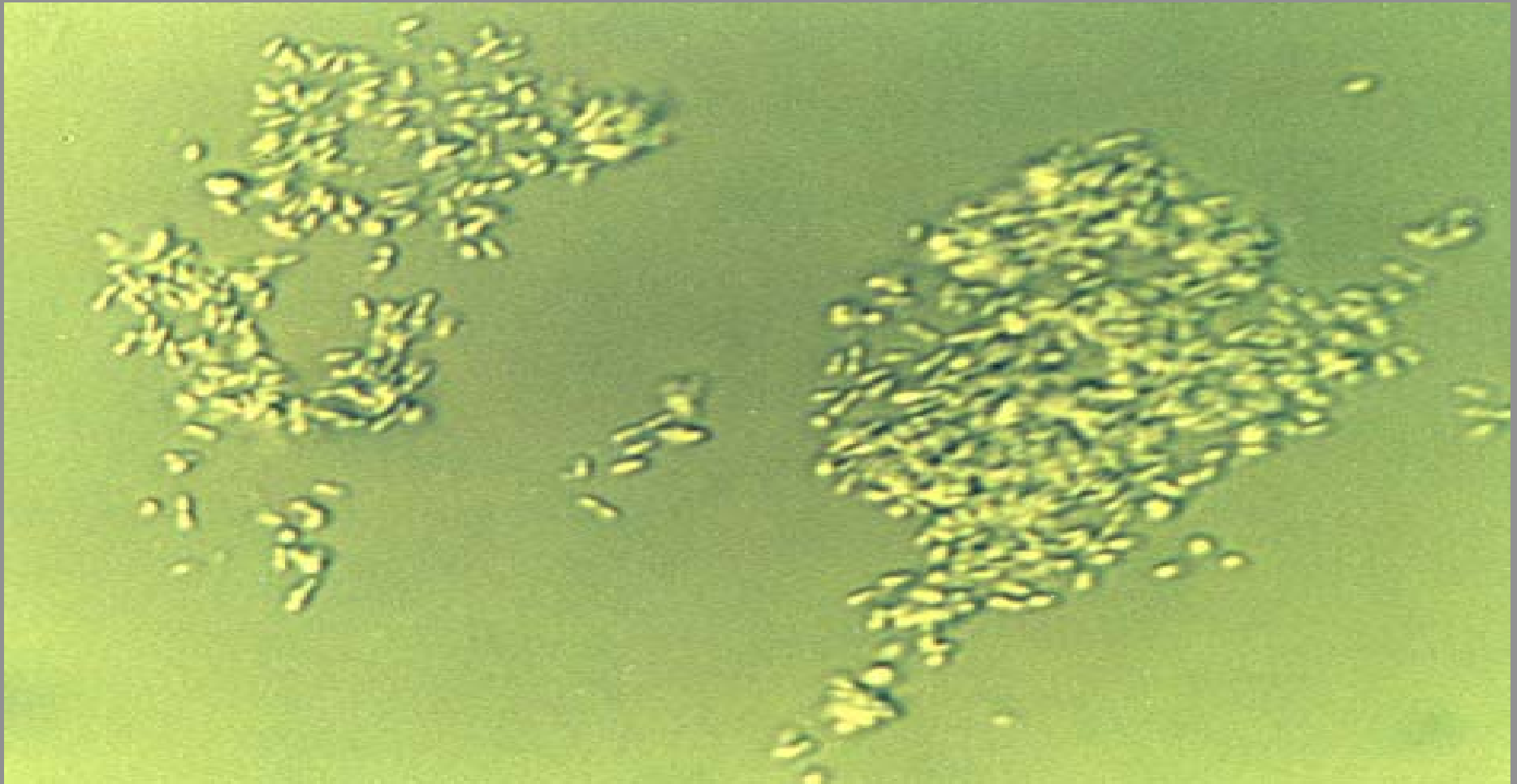
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Role of the biofilm matrix

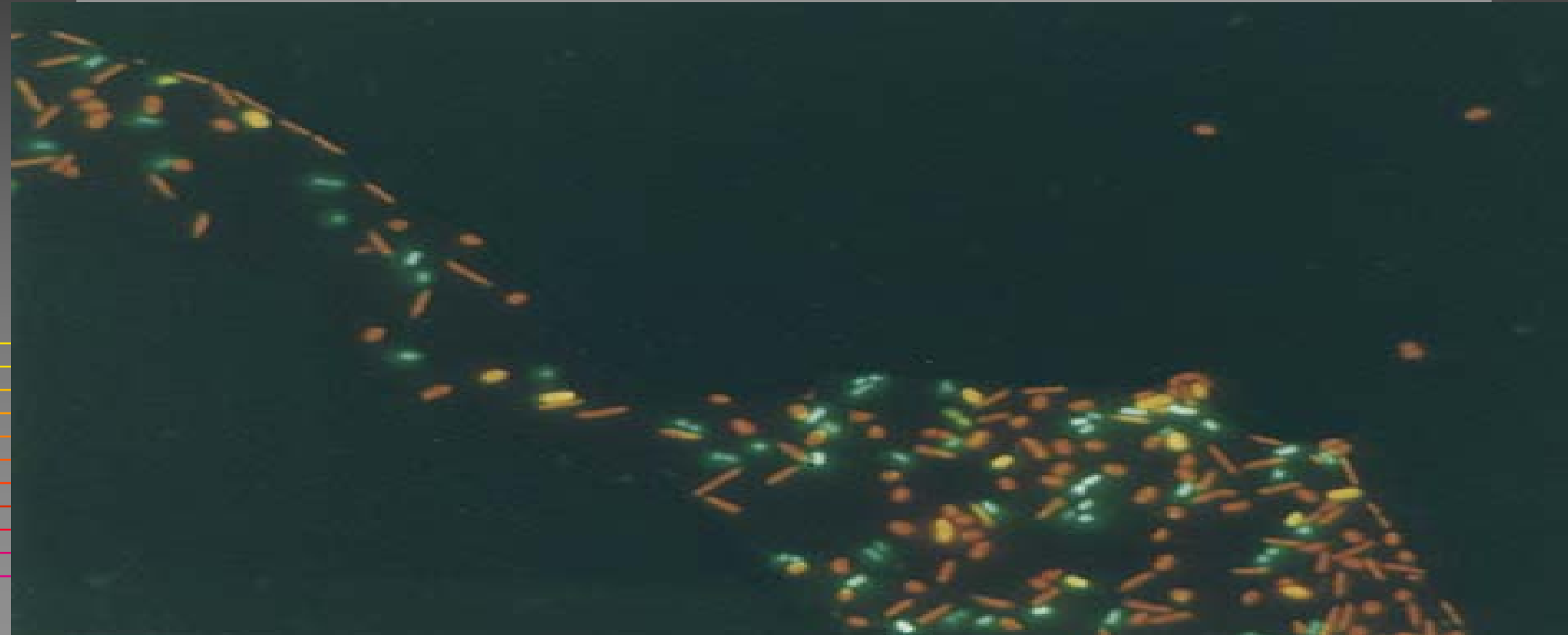
- Biofilm matrix performs various functions:
- Stabilises the biofilm
- Plays a role in biofilm attachment
- Protects the microbial cells
- Interacts with ions & macromolecules
- Interaction with protozoa & phagocytes

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

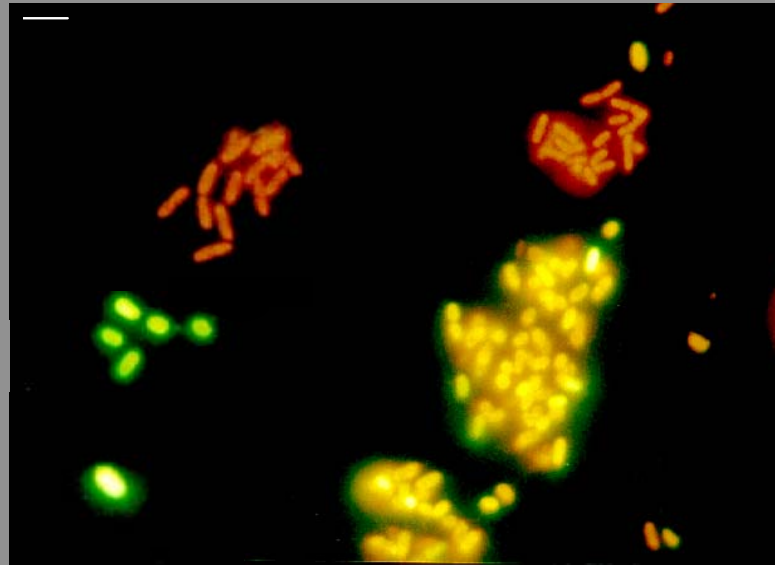


Dual Species Enteric Biofilm stained with propidium iodide

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



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

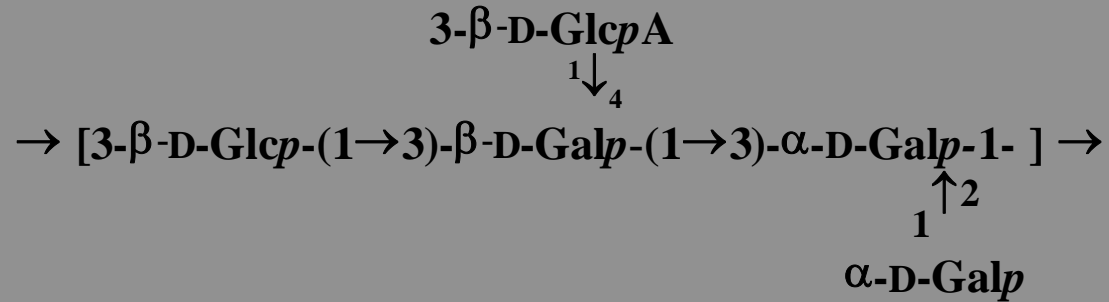
i.e. Structurally related Exopolysaccharides

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

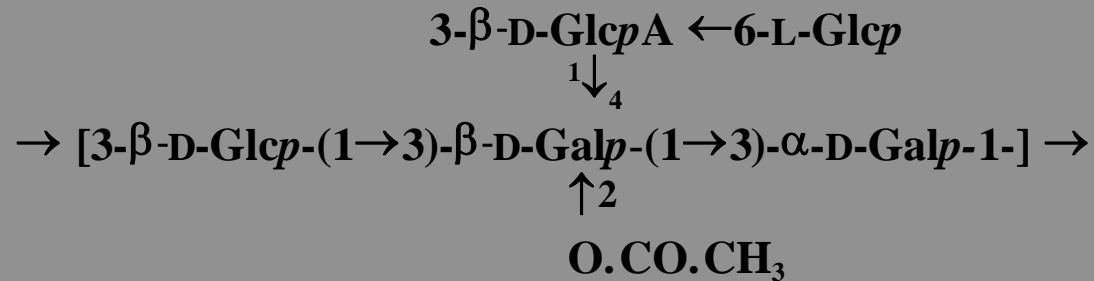
Mutan the homopolysaccharide from *Streptococcus mutans*

- 3)- α -D-Glcp-(1,3)- α -D-Glcp-(1,3)- α -D-Glcp-(1,3)- α -D-Glcp-(1

EPS from *Klebsiella* K8 and K82



Klebsiella aerogenes K8



Klebsiella K82

Haloferax mediterranea EPS



Staphylococcus epidermidis Biofilm Exopolysaccharides

■ Polysaccharide I

■ [6-β-D-GlcpNAc-(1→6)-β-D-GlcpNAc-(1→6)-D-Glcp-N-(16)-β-D-GlcpNAc-(1→6)-β-D-GlcpNAc-(1→

■

■ Ratio: GlcpNAc : GlcpN c. 5:1 (Cationic)

■ Polysaccharide II

■ [6-β-D-GlcpNAc-(1→6)-β-D-GlcpNAc-(1→6)-D-Glcp-N-(16)-β-D-GlcpNAc-(1→6)-β-D-GlcpNAc-(1→

■

■ Ratio: GlcpNAc : GlcpN 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

- $\rightarrow 3\text{-}\beta\text{-D-Glcp}\text{-}(1\rightarrow 3)\text{-}\beta\text{-L-Fucp}\text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-Fucp}\text{-}(1\rightarrow$
- $1\uparrow 4$
- $\beta\text{-D-Galp}$
- $1\uparrow 3$
- $\beta\text{-D-GlcpA}$
- $1\uparrow 4$
- $\beta\text{-D-Galp } 34 = \text{Pyr}$

The exopolysaccharide from benzene tolerant *Rhodococcus* sp. 33

- Pyr
- $4\downarrow 6$
- $\rightarrow 4)\text{-}\beta\text{-D-Galp-(1}\rightarrow 4)\text{-}\beta\text{-D-Glcp-(1}\rightarrow 3)\text{-D-Man } p\text{-(1}\rightarrow 4)\text{-}\beta\text{-D-GlcpA(1}\rightarrow$
- Results of Urai, M., Aizawa, T. *Carbohydr. Res.* **2006**, 341, 616-623.

Pseudomonas aeruginosa alginate

- [-4--D-ManpA-(1 4)--D-ManpA-(1 4)--D-ManpA-(1 4)--D-ManpA-(1 4)--D-ManpA-(1 4)--D-ManpA-(1 4)--L-GulpA (1)]
- No GulA-GulA sequences
- O-Acetyl groups are attached to many of the D-mannuronosyl residues

Typical Molecular Weights of EPS

<u>Polysaccharide</u>	<u>Molecular Weight</u>
■ Alginate	$5 \times 10^5 - 2-3 \times 10^6$
■ 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

<u>Bacterium</u>	<u>Polysaccharides produced:</u>	
<i>Azotobacter chroococcum</i>	Alginate	<u>and</u> Type specific EPS
<i>Erwinia amylovora</i>	Type specific EPS	<u>or</u> Levan ⁺
<i>Escherichia coli</i>	Type specific EPS*	<u>or</u> Colanic acid*
<i>Ent. geroviae</i> 1.15	Colanic acid type*	<u>and/or</u> Glucomannan*
<i>Pseudomonas</i> spp.	Alginate	<u>or</u> Levan ⁺
<i>Rhizobium meliloti</i>	Succinoglycan*	<u>or</u> Galactoglucan*
<i>Rhizobium/Agrobacterium</i> spp.	Succinoglycan*	<u>or</u> Curdlan*
<i>Staphylococcus epidermidis</i>	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

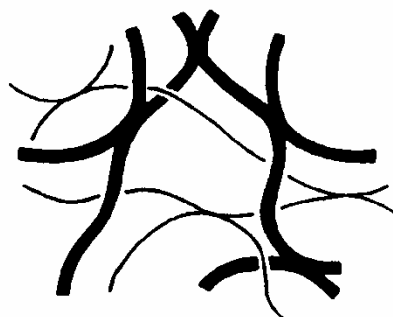
- Insoluble – bacterial cellulose, mutan
- Soluble (but highly viscous) – colanic acid, bacterial alginate
- Highly soluble – various low mass polysaccharides

Determinant physical properties of EPS in the biofilm matrix

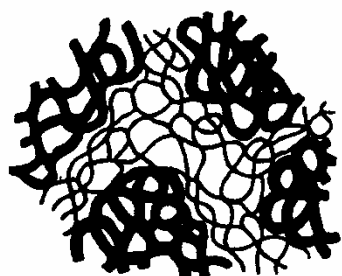
- Polysaccharide/Polysaccharide interactions
- Self; polymer/polymer
- Gel formation
- Viscoelasticity (Shear thinning)
- Intrinsic viscosity



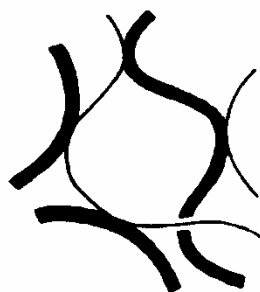
(a)



(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

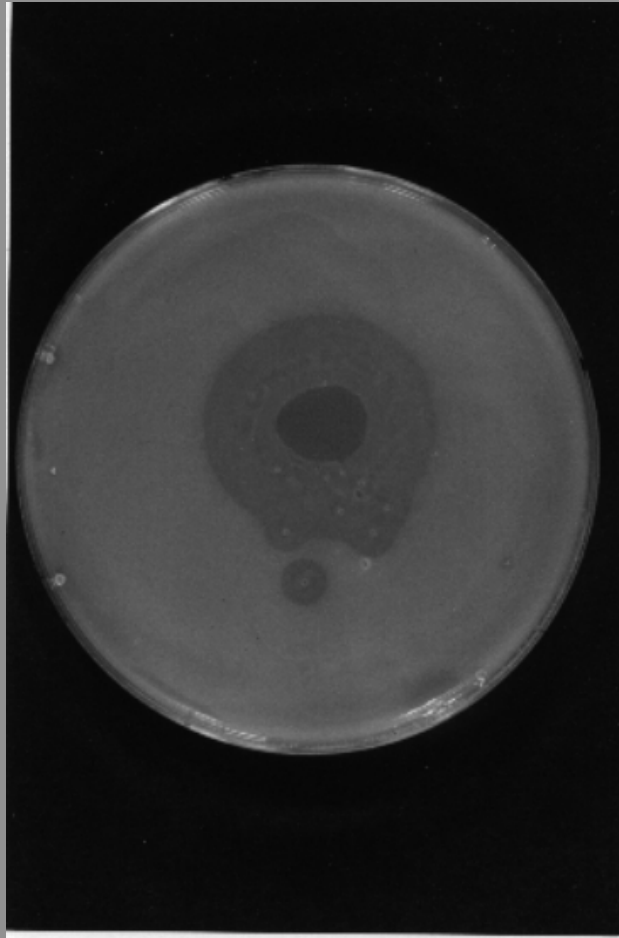
Interactions of matrix polysaccharides

- 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)