# Microbial biofilms: case reviews of bacterial and fungal pathogens persisting on biomaterials and environmental substrata

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## 1. Introduction

In nature, bacteria and fungi frequently inhabit distinct environmental niches at the interface between two phases, such as air and water, or water and a substratum. In these locations, cells are anchored together by means of a multivariate combination of biomolecules which form a barrier surrounding the cells, and acts to protect against adverse conditions, such as temperature, or from chemical attack, such as chlorine in potable water. In clinical settings, human pathogens are able to survive host-mediated phagocytosis or antibiotic attack through the formation of biofilms on host surfaces such as mucous membranes, as well as medical devices such as catheters and cardiovascular stents. In the environment, biofilms are essential for the survival of many species, and have been demonstrated to provide human pathogens with a means of surviving outside of a host organism to present a vehicle for the subsequent reinfection of a susceptible population. For these reasons, biofilm microbiology has risen to prominence when assessing patient care and when investigating outbreaks of disease.

This chapter will explore four examples of microbial biofilms as models of infection and persistence. The origin of the microbes within the biofilms will be explored, as will the development of the communities, followed by treatment strategies, the consequences of poor hygiene and antibiotic stewardship, where appropriate. The four microbial models are:

1. *Escherichia coli* O157 – a human enteric pathogen capable of surviving in soil and freshwater environs; this bacterium is recognized for the possession of Shiga-like toxins which resemble the bacillary dysentery toxin and are capable of causing systemic and sometimes fatal human disease.
2. *Legionella pneumophila* – an accidental bacterial human respiratory pathogen; originally a protozoal pathogen that has colonised potable water systems to cause incidents of pneumonia-like disease.
3. *Klebsiella pneumoniae* – related to *E. coli*, this organism is capable of causing urinary tract and other infections by colonising medical devices, such as catheters, and has risen to prominence through numerous reports of resistance to an increasing number of antibiotics.
4. *Candida albicans* – one of the most frequent causes of blood borne infections, this fungus is capable of colonising medical devices such as heart stents to initiate incidents of cardiovascular disease.

## 2. Biofilms

Viable bacteria and fungi have been recovered from environmental fomites and medical surfaces after numerous cleansing treatments, where they have been shown to possess the genes for virulence factors associated with incidence of human disease. These organisms exploit an advantage unique to microbial cells: the ability to form biofilm communities. Microbial biofilms are highly organised heterogeneous communities adherent upon an artificial substratum. They are constituted of not only cells, but also of materials produced by the cells including extracellular proteins, nucleic acids, and sometimes antibiotic inactivating enzymes. The material to which a microbe or community of microbes is anchored is referred to as a substratum, which can be either abiotic, such as a cardiac stent or a stone in a river bed, or biotic in nature, including human skin or the leaf of a plant. Within the biofilms, cells present a phenotype distinct from that expressed by cells of the same species in planktonic (free-floating) culture. Two such unique features include chemical gradients, for example a move from oxygenated to anoxic regions and a marked decrease in nutrient concentration moving towards the centre of the biofilm. The exact properties of these communities are dependant upon certain factors: (i) the substratum itself, including the physical and chemical properties of the material; (ii) the number and type of cells present within the biofilm (bacterial and/or fungal cells, or different species of bacteria, for example); as well as (iii) the external physical environment, such as the water in a stream or blood in a patient. These factors dictate how the biofilm will develop and how it will respond to stresses including challenge with an antibiotic or chemical treatment. Biofilm communities present the indwelling cells with several unique advantages over their planktonic brethren, including persistence during periods of nutrient deprivation, an ability to resist and re-grow after physical, chemical and biological attack, and an ability to disseminate indwelling biofilm cells to new locations, and thus cause secondary disease or encourage persistence of the organism in a new location. Research has suggested that up to 60% of nosocomial infections involve biofilms that contaminate implants and catheters, hence research into the

development of biofilm communities, their cellular organisation, composition and architecture have become prevalent within modern microbiology.

### 2.1 Biofilm development

A biofilm can be broadly defined as an organised system of microbial cells associated with surfaces, often with unique structural phenomena, and are characterised by distinct physical and chemical gradients that affect microbial metabolic processes, that are heavily influenced by the substratum. This can be almost any material, and the ability of microbes to adhere will be determined by the deposition of molecules upon it. This scenario is the same for environmental biofilms, such as those covering stones in riverbeds, and those of medical importance, such as dental plaque and biofilms associated with catheterisation. Biofilms are ubiquitous in nature, and play an important role in the normal life cycle of many organisms. All biofilms occupy a unique environmental niche: a locality exposed to stresses different from all other sites, and can grow to include organisms that could otherwise not exist in such places by altering their environment to a more habitable niche, a phenomenon sometimes termed as the neutralisation of stresses. Interactions between the microbial glycocalyx and the surface allow for the concentration of certain ions, such as magnesium and sodium, within the biofilm which has been theorised to be associated with increased virulence. The amount of a particular chemical each organism sequesters in its environment can be related to the number of cells releasing it. Once a 'threshold' concentration of a chemical is reached (i.e. once the number of cells sequestering it reaches a 'threshold population level'), the cells register to co-ordinate cellular efforts for specific functions such the expression of virulence factors. This phenomenon is referred to as 'quorum sensing', and can be illustrated by reviewing the role of *Pseudomonas aeruginosa* in cystic fibrosis. Along the bronchial mucosa, mucoid *Ps. aeruginosa* strains secrete and grow in a calcium-dependant extracellular alginate matrix, which encourages other mucoid strains to produce alginates. This acts to protect the mucoid as well as the non-mucoid strains from the hosts' defences, and provides a physical barrier to antibiotics. This example highlights just one mechanism by which microbial cells can persist despite a treatment that would likely be effective against planktonic counterparts. If we understand that once adherent upon a surface, cells can act to protect themselves by producing a protective barrier, then it is logical to assume that the surface itself and any deposited material will influence the chemistry of what can attach to it, thus affecting biofilm development. This phenomenon is called surface conditioning, and it drastically affects the biological and chemical constitution of the biofilm.

### 2.2 Surface conditioning

A substratum quickly becomes bombarded by material native to that environ, which become attached to the surface in a process called conditioning. With medical devices, these molecules can include proteins and cell products, perhaps derived from blood or urine, for example; in the freshwater streams they can either be complex organic molecules such as proteins or inorganic compounds. Once attached, these molecules attract others, forming a thin layer of conditioning film on the surface of the material, and it is usually to this which the first micro-organisms will attach, not the substratum itself. The initial colonisers are called the pioneer species. The adherent pioneer species extrude proteins and other surface ligands that will act to bind further species into the juvenile biofilm. As the substratum material acts to select potential pioneer species by the molecules that adhere to it, pioneer organisms govern which later-colonising species adhere to them by the nature of extracellular markers they express. The physical and chemical properties of the substratum and conditioning film affect both bacterial adhesion and the properties of adherent biofilms in a number of ways.

1. *Physico-chemical properties of the substratum*
   For most micro-organisms, adhesion is associated with low surface energy. Antifouling coatings such as silicone elastomers and fluoropolymers are examples of substances with high surface energies that deter microbial adhesion. Different surfaces will have different physical and chemical properties, allowing different species to colonise different surfaces, usually measured in terms of micro-electrostatic repulsions or attractions.
2. *Action as a concentrated nutrient source*
   Particles become lodged in recesses and spaces between cells and the exopolymeric substance (EPS) matrix, allowing enzymes to digest them. Once inside the biofilm, it is unlikely that such particles will leave before the indwelling cells act upon them.
3. *Suppression of the release of toxic metal ions*
   Metal ions are retained within biofilms in the same way as nutrients. Many compounds are retained within the biofilm matrix, but some are released in a harmless form. This may lead to the removal of dissolved substances and non-essential materials that have become attached or associated with the biofilm by a sloughing process triggered by the presence of undesirable substances, such as toxic metal ions. This process can therefore act to protect non-tolerant organisms.
4. *Adsorption and detoxifying of dissolved inhibitory substances*
   It has been observed that the surface itself can adsorb a substance rendering it harmless to the biofilm-dwelling organisms. Examples include industrial chemicals such as peroxides and dichloroethane.

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5. *Supply of essential metal trace elements*
   Metallic elements can become attached to other substances, which some bacteria can more readily take up in complex rather than simple form. For example, *E. coli* produce a siderophore designated enterobactin to complex iron and supply it to the bacterial cell. Considering that biofilms can act to concentrate nutrients, the ability to complex the trace metal iron is advantageous in a nutrient poor environment such as freshwater streams as opposed to a host animal, and therefore could act to promote growth and expansion of the biofilm.
6. *Action as a sloughing mechanism*
   A shift in pH as a result of acid secretion could act to enhance the binding of organic solutes, but alkaline secretions will detach or remove such substances. During sloughing, a portion of the biofilm is detached. This can act to remove a hazardous substance from the main body of the film, and such sections have been demonstrated to attach to other surfaces after complete removal. This process can lead to the dissemination and propagation of the indwelling biofilm cells in a new location.
7. *Suppression of surface polymer effects*
   The adsorption of materials may moderate the stearic attraction or repulsion of an already established polymer, potentially leading to the release of previously adherent materials.

### 2.3 Biofilm architecture

Given the importance of the conditioning film and pioneer organisms, it has been proposed that three parts of a biofilm should be distinguished:

1. *the linking film:* this comprises the molecules that have attached to a surface, conditioning it to facilitate microbial adhesion;
2. *the basal film:* comprising the initial wave of colonising bacteria or pioneer species that adhere to the newly conditioned surface; and
3. *the surface film:* later-colonising organisms attach to the already adherent pioneer species to develop the film to create a mature, and usually a multi-species biofilm.

For bacteria, initial contact leads to the contraction of microfilaments and microtubules within a cell. These function as the skeleton of the cell, and so their contraction and expansion leads to an alteration of its shape, which is detectable by the change in the shape of their membrane to facilitate a fit to a surface niche. Once this is complete, pili and polysaccharide fibres in their glycocalyces, analogous to ligands, move to fit to surface attachment sites or receptors. If they fit, the bacterium will adhere, if not they will change shape again and the bacterium will migrate away, and are termed irreversible and reversible sorption, respectively. To create biofilms, organisms must be able to locate favourable niches with reference to nutrient and oxygen availability, and when attached, bacteria will divide to form adherent microcolonies on the substratum. The number of pioneer species flourishes after the initial formation of the biofilm, but falls-off slightly as other species attach to them. The level of later-colonising organisms starts off slowly but increases greatly as the number of potential attachment sites (i.e. adherent pioneer organisms) increases. Biofilm-dwellers secrete a range of biomolecules called exopolymeric substances (EPS) to create a complex 3-D matrix, which typically comprises 85% of the total volume of the biofilm, and to which other organisms attach creating a multi-species biofilm. After water, polysaccharides are frequently the major components of EPS matrices, but proteins, nucleic acids and lipids are often present in substantial amounts. The EPS is primarily responsible for the morphology and function of biofilms, and it is considered to be key components in determining their chemical and biological properties. For instance, *Ps. aeruginosa* EPS matrices vary greatly in their constitution. Mucoid variants are characterised by an overproduction of the viscous exopolysaccharide, alginate (a co-polymer composed of 1-4 linked D-mannuronate and $\alpha$-L-glucuronate residues).

## 3. Biofilms and disease

The ability of a pathogen to breach a host's immune system means that biofilm formation in a compromised location within a susceptible host has the potential to cause infection. A good example is the use of catheters to treat urinary tract infections. Catheterisation of the human bladder facilitates bacterial access via the lumen of the catheter or by tracking-up between the outside of the catheter and the urethra wall where the bacterium can gain access to the underlying tissue, thus circumventing the protection afforded to the host by the skin. Most urinary tract pathogens are faecal in origin, but only aerobic and facultatively aerobic species such as *Escherichia coli* or *Klebsiella pneumoniae* possess the necessary attributes to colonise the urethra. We will now review four examples of microbial human pathogen where the biofilm acts either to encourage environmental persistence of a microbe, or acts to protect the microbe from attack by a host organism or chemical shock treatment.

### 3.1 *Escherichia coli* O157

*Escherichia coli* is a member of the *Enterobacteriaceae* family of bacteria which contains several species that have made sizeable impacts on human health and the development of our society. These include *Shigella dyenteriae*, the agent of bacillary dysentery; *Salmonella typhi*, a common cause of gastroenteritis; and *Yersinia pestis*, the causative agent of plague. *E. coli* O157 is a medically important strain of *E. coli*, the characteristic feature being the expression of two verocytotoxins (VTs) and the protein, intimin, which are heavily associated with incidents of both haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS). The verocytotoxins are known as Shiga-like toxins because they share 97% amino-acid sequence homology with the *Shigella dysenteriae* toxin, which is the causative agent of bacillary dysentery. *E. coli* is a ubiquitous bacterium throughout human and mammalian populations, where it inhabits the gastrointestinal tract, usually without causing incidents of disease. In humans, *E. coli* is excreted from the host organism in the faeces, where it usually enters either the sewage treatment system and is usually killed by chlorination or a similar treatment. However, semi-treated sewage, run-off from animal grazing fields and from industrial effluent involves no treatment regime, and therefore provides a potential route for the dissemination of the bacterium into the environment, where, research has demonstrated that *E. coli* can enter the soil or water systems and persist for several years. The deposition of naturally weathered and eroded materials can transfer epilithic and epiphytic bacteria, as well as those washed into watercourses through run-off downstream, meaning that sediments removed from agricultural environments can contain high numbers of both environmental and faecal micro-organisms that may not originate from the location of the samples. Indeed, *E. coli* O157 has been recovered from mud, sludge and other sediments, including those occurring naturally along riverbeds and in lakes and seas, and the bacterium has been demonstrated to survive in cold water for up to twelve weeks. Molecular typing analyses has determined that the bacterium is capable of surviving for months and perhaps years heavily embedded within biofilm matrices, and research has demonstrated that the bacterium can persist outside of a host organism in animal faeces or faecally-derived material, on inorganic substrata such as wood or metal, and in both treated and untreated waters. Epidemiological studies have often reported that an environmental reservoir may allow for the reintroduction of a pathogen to a susceptible host population to cause additional disease after an initial epidemic or outbreak has subsided; for example outbreaks of cholera caused by waterborne strains of *Vibrio cholerae*. These data suggest the potential for *E. coli* O157 to survive beyond a host, and to facilitate the re-infection of a population after an initial outbreak of disease. Contamination with faeces is the direct and most frequent route of dissemination of *E. coli*, but this can also happen through an intermediate vector, which can be either an intermediate host or an abiotic medium such as soil or water. Indeed, tropical soil in Hawaii was able to support the replication of *E. coli* and other organisms associated with faeces, indicating the potential not only for environmental survival, but for propagation. Animal grazing and the use of untreated, manure-based fertiliser may lead to an influx of faecal bacteria to the soil. Cultivation and natural water-flow can transfer the bacteria deep into the soil, and potentially into the groundwater aquifer, which if used as a source of drinking water, can expose users to the bacteria therein. An important example of such an occurrence was the outbreak in Walkerton, Ontario, Canada in 2000, where 2300 people became ill as a result of the contamination of well-water with cattle faeces containing *E. coli* O157. Research has indicated that freshwater epilithic biofilms are complex multi-species communities. The interior layers appear to comprise mainly bacteria, while the exterior is mainly composed of algae, including diatom species such as those of the genus *Navicula*. Evidence also exists clearly demonstrating the EPS matrix amongst bacterial isolates, indicating that bacteria present are able to express exopolymeric substances in a temperate freshwater climate. Isolates belonging to environment-associated genera of the *Enterobacteriaceae*, such as *Serratia* and *Erwinia*, might be expected in soil and water samples due to the presence of plant-life. However, the recovery of *E. coli* is notable because of its association with incidents of human disease. The ability of *E. coli* O157 to survive in surface waters, soil and faeces have all been demonstrated, with molecular typing analyses indicating that either *E. coli* phenotypes are able to persist in the four freshwater epilithic biofilms over the course of months or even years, or that isolates were replaced by related isolates from the same genotype through faecal input into the streams. There is increasing evidence of environmental infection by *E. coli* O157 associated with farm and countryside visits, particularly in children. For example, an outbreak in Scotland resulted approximately 1 in 11 scouts falling ill at a campsite. The likely route of transmission was thought to be ingestion of mud and faecal material from the children's hands; sheep had grazed on the fields in the week before use by the scouts. The EU Bathing Water Directive requires that designated bathing waters must comply with microbiological standards for faecal indicator organisms. Catchment sources (surface run-off and field drainage water from fields containing grazing animals, slurry spreading, farmyard run-off, direct faecal inputs, etc.) contribute to riverine faecal bacterial loads, especially at high river discharges, because of near surface overland flow, short travel times of bacteria in streams, entrainment of stream sediment and mixing of riverine input with seawater. The sampling of sediments and water along the course of a river may reveal that different strains or phenotypes are able to form biofilms while others remain as planktonic organisms. Some species or sub-species have been shown to have an enhanced ability to form biofilms, indicating that phenotypic characteristics may favour the adherence of certain phenotypes within biofilms, while others are unable to attach and remain planktonic in the bulk phase solution, a situation which may be true for certain phenotypes of *E. coli* O157. Given that bacterial phenotype has been shown to be independent of genotype relating to virulence, it may be that the phenotype of both planktonic and adherent biofilm isolates will differ from each other, regardless of the virulent traits possessed, perhaps suggesting

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ambiguity in current detection methods for the detection of pathogenic bacteria in bodies of water. Survival studies encompassing temperate aquatic and terrestrial soil matrices would be an appropriate extension of these works. Current research suggests that *E. coli* O157 isolates are widely disseminated in the environment, probably resulting from faecal origin relating to host animal defecation and the rotation of livestock, as well as interactions with companion animals such as cats and dogs, with have contact with both human and environmental sources. Comprehensive typing analysis of the *E. coli* isolates within soil matrices on arable land may indicate that faecal shedding on the land leads to the contamination of fields, promoting the dissemination of the bacterium throughout the animal herds rotated on the grazing fields. This also leads to the possibility of one carrier species infecting another un-infected group through the contamination of soil by faecal shedding and infiltration of the soil acting as a reservoir of the bacterium where a biofilm develops between the bacterial cells and the soil particles.

*Summary*

The survival of *E. coli* O157 in the environment is an important discovery, and one which is the subject of extensive and diverse investigation. Whilst sewage is almost always treated to sufficient standard to remove the bacterium from the water, run-off from fields and storm events cannot be regulated to such a high standard. The persistence of *E. coli* O157 means that a potential threat exists to recreational water users or to consumers through abstraction for potable supplies. The environmental cycle of *E. coli* O157 is not fully understood, but research has indicated that once established within a niche, the bacterium can persist for months or even years, suggesting a source of re-infection, and for the bacterium to evade detection by concealment deep within biofilm matrices which are not routinely screened for human pathogens in favour of the over-flowing water. This example serves to highlight the importance of biofilms for the survival of an important human pathogen outside of a host organism. *E. coli* is still used as an indicator of faecal pollution, but the usefulness of this bacterium to act as a marker of faecal pollution becomes questionable given that viable *E. coli* have been recovered from environmental waters. On-going research is required to fully evaluate the threat posed by environmental biofilms as reservoirs of *E. coli* O157 before their role in the persistence and subsequent reinfection of a population by the bacterium can be understood and risks to recreational and potable water users fully evaluated. This is especially pertinent considering the number of water- and land-based leisure activities that occur on grazing pasture or bodies of water which current research suggest presents an ideal opportunity for exposure to this important bacterial human pathogen.

### 3.2 *Legionella pneumophila*

*Legionella pneumophila* is an incidental human pathogen that has become notorious amongst epidemiologists for its role in nosocomial and community-acquired outbreaks of pneumonia-like disease. The organism is a Gram negative nutritionally fastidious bacillus, and the type organism of the *Legionella* genus, which is the only genus in the family *Legionellae*. The bacterium is ubiquitous in natural aquatic environments where it exists primarily as a protozoal pathogen, and has also been isolated from numerous slow-flowing and stagnating bodies of water. The bacterium was first isolated after exhaustive laboratory analyses in 1977 following an outbreak of pneumonia-like disease in Philadelphia, USA. However, given the frequent isolation of the bacterium since its discovery, it is believed that the bacterium had colonised water systems for a considerable time prior to its primary isolation. The mechanisms by which the bacterium causes incidents of human disease are poorly understood, but to date disease has always resulted from the inhalation of aerosols containing viable bacteria. At present, no reports of illness resulting from drinking infected water have been reported, nor to suggest that horizontal transmission can occur. Human illness presents as one of two states: Pontiac Fever, a milder form of legionellosis presenting as a febrile disease resembling influenza which is not usually terminal; or the more severe Legionnaire's disease characterised by pneumonia-like symptoms, and can be fatal in up to 30% of patients. However, the severity of the disease and duration of illness depends on the individual circumstances of the patient, including immuno-compromisation, drug intake, as well as the pathogenicity profile of the bacterium. *L. pneumophila* and related species are able to cause disease because the bacterium is capable of producing a toxin, referred to as a Macrophage Infectivity Potentiator (MIP) toxin, the function of which is to internalise the bacterium within human alveolar macrophages where it is capable of resisting destruction by the macrophage, where it can metabolise and reproduce. The *mip* gene is believed to be the single most important virulence factor for this bacterium, but there are several other important ones currently under investigation, including phospholipases, proteases, heat shock proteins and a type two secretory system. Potable water is generally assumed to be sterile, but it in fact contains a myriad of bacterial species able to resist the chlorine added by the water provider. The bacterium can gain entry to potable water systems through breaks in the delivery pipes, where negative pressure draws sediment and water that has not undergone treatment into the potable reservoir. The chlorine added into potable supplies generally acts to keep the number of bacteria below the threshold level required to cause incidents of human disease. This technique is generally acceptable for the purpose of human consumption, but research has demonstrated that it is inappropriate for the eradication of *Legionella* where the bacterium can cause incidents of human disease if the water is used for showering, for example, where aerosolised viable cells can be inhaled. Planktonic *Legionella* are not particularly resistant to chlorine, and the survival of *Legionella* in potable supplies is facilitated by the endurance of viable cells within the biofilm inside the pipe system. The organic and inorganic matter present within the water combines with sediment

pulled in to form a complex deposit within the pipe which can develop to become many centimetres thick over time. This deposit will also contain a heterogeneous mix of viable and dead cells from a range of species. The bacteria will act to produce the EPS matrix, and then to reproduce deep within the sediment where they will gain protection not only from this but also from the sediment itself. Once established within a water system, *Legionella* is notoriously hard to eradicate. The persistence of viable cells within the sediment biofilm is responsible for the longevity of the species despite treatment procedures such as hyper-chlorination (a significantly higher dose of chlorine in the form of sodium hyperchlorite, for example), extreme high temperature (for example 60°C for one hour) or the use of antibiotics (such as the fluorquinolone ciprofloxacin). Water companies are required to routinely test for the presence of the bacterium in potable supplies. The majority of research suggests that after a treatment, the bacterium can become undetectable for several days or even weeks using conventional culture and molecular techniques such as PCR. However, a body of evidence exists demonstrating that regardless of single or even multiple treatments such as hyperchlorination, the bacterium reappears within a system to eventually reach the numbers present prior to the treatment cycle. These data suggest that the currently employed treatment technologies effect only a transient reduction in bacterial numbers, rather than eradicating them. For this reason, *Legionella* monitoring strategies refer to control rather than eradication of the bacterium. Research has revealed that within potable water pipes that were subject to constant chlorination, both viable and non-viable *Legionella* cells existing within the sediment, suggesting not only persistence but perhaps indicates population turnover within the biofilm. This is probably due to a combination of sheer flux and exposure to chemical stresses in the water reducing cell numbers, and of bacterial reproduction and an influx of new cells repopulating the community. Incidents on oil rigs have demonstrated the hazards that present when control measures are not strictly adhered to, where water systems are used for all needs, including drinking and washing (including showering). Numerous workers reported illness in the form of a mild influenza-like febrile disease, with some workers reporting suffering from more than one instance of the disease. Testing of patient samples revealed infection by *Legionella pneumophila*, with the water system testing positive for colonisation by the bacterium. This suggests that the bacterium can not only persist within a water system, but can present as a constant source of infection to susceptible water users.

*Summary*
If biofilms are understood to house a population of viable bacterial human pathogens that can be reduced in number but not eradicated by control measures, then the implications for industrial and municipal organisations are immense. Current guidelines require municipal authorities to constantly monitor potable supplies, and to act to control numbers of *L. pneumophila*. For industry, equipment such as water cooling towers cannot constantly be replaced; hence treatments are employed monthly, sometimes regardless of the positive isolation of *L. pneumophila* in order to keep the water safe for human use. Control measures need to be rigorously enforced to restrict the exposure of water users to a potentially fatal human pathogen. Research suggests that once the bacterium becomes entrenched within the water system, it becomes a recalcitrant problem which might prove difficult if not impossible to remove. *Legionella pneumophila* is an example of a human pathogen that has been able to exploit advances in human engineering to colonise new environmental niches and to present as a source of human disease through adaptation to man-made devices to which it should not be able to gain entry.

### 3.3 *Klebsiella pneumoniae*

*Klebsiella pneumoniae* is a Gram negative bacillus, and is a member of the *Enterobacteriaceae* family related to *E. coli*. The bacterium is a member of the normal human microbiota, where it inhabits the respiratory and gastro-intestinal tracts of healthy individuals. This bacterium is notable because it possesses a large polysaccharide capsule which research suggests prevents phagocytosis by the host, which is seen as one of its most important virulence factors. There are increasing reports of antimicrobial resistance by *K. pneumoniae* to first-line drugs, an example being the expression of extended spectrum beta-lactamase enzymes (ESBLs) and the development of resistance to carbapenems, suggesting that infection by some strains may be approaching untreatable levels. A large number of *K. pneumoniae* infections are reported to occur in the hospital setting, with intensive care units (ICUs) being one of the primary sites. One of the main reasons for this is that patients in ICU wards often require invasive medical procedure such as catheterisation. The penetration of the skin leads to a clear opportunity for foreign bodies and detritus to circumvent the natural defences of the body to take up residence within the patient. Once inside the body, bacterial cells can adhere to the tissues and lining of the blood vessels and to the catheter itself. The nutrient rich environment and the bacterium's ability to protect against host-mediated immune response can create an intractable colonisation, which is difficult to treat with standard antibiotic chemotherapy. Further, the cells are likely to begin expressing quorum-sensing molecules. Here, the bacterial cells will use the materials contained within the hosts' fluids and tissues (e.g. cells and products from lysed cells; blood proteins, sugars and other metabolites). The bacterial cells can utilise these substances for their own purposes; for example, to metabolise and reproduce, or to constitute an EPS matrix. Catheters are medical devices used either to drain fluid such as urine from a patient's bladder, or to deliver a substance, such as an antibiotic or nutrients directly into a patient's bloodstream. Incidents of catheter-associated infections are becoming increasingly common, and are frequently reported as one of the highest causes of morbidity and mortality across the world. Outbreaks of ESBL *K. pneumoniae* are frequently reported, and research indicates that isolates are increasing in the breadth of their resistance.

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Recently, it has been advocated that antibiotic stewardship be adjusted to better reflect the needs on hospital wards and to avoid prescribing drugs where resistance is increasing unless absolutely necessary, rather than a blanket empirical therapy. A report published in 2003 recommended that health care workers should be required to wear and change gloves between patients be implemented alongside weekly cleaning of the ICU and sinks and drains with 1% sodium hypochlorite in order to reduce the colonisation and persistence of bacterial human pathogens, including *K. pneumoniae*. Microbiological analyses of the staff and patients revealed that all faecal cultures tested negative for *K. pneumoniae*, as did cultures from the environment of the ICU ward itself. Implanted polymer devices are now common in post-operative medical care. In the case of *K. pneumoniae*, the bacterium is capable of colonising urinary catheters to present a significant increase in the chance of the patient acquiring a nosocomial infection and associated increased morbidity and mortality. Research suggests that the bacterium utilises type 3 pili to adhere to surfaces, and accordingly that pili alongside the polysaccharide capsule are recognised as two of the most important virulence factors for *K. pneumoniae*. Once attached, the cells produce extracellular substances which coalesce to form a matrix around and above the cells. This allows the cells beneath to multiply, where increased cell densities evolve a multi-layered biofilm presenting with increased resistance up to 1000 fold for antibiotics, indicating that *K. pneumoniae* biofilms are an intransigent phenomenon. Indeed, treatment is often only capable through the removal and replacement of these devices. However, this must be performed frequently as treatment once biofilms become established yield the potential for bacterial colonisation and infection of the underlying tissues. Theory suggests that these biofilms are capable of survival despite treatment with hypochlorite or other reactive oxygen species or antibiotics due to limited penetration into the biofilm, thus affecting only the surface layers, but also by the presence of metabolically slow persister cells residing in the basal layers of the film. These cells occur due to physical and chemical gradients changing the microenvironment around them into one of reduced oxygen and nutrient concentration, which has a strong impact upon the physiology of the cells therein. Further, the diffusion of antibiotics into the film can be retarded or halted by the dissemination of antibiotic inactivating enzymes such as ESBLs, which act to further exacerbate the situation, and gives biofilm-coated devices the potential to act as a source of reinfection for the patient unless the devices are continually replaced. Curiously, there is a developing body of research suggesting that the presence of pili is not crucial for cellular adhesion; rather, it is suggested to be related to the presence and functionality of the capsule and the possession of ESBL-coding plasmids. This is an interesting finding as it suggests that *K. pneumoniae* possessing impaired capsules but possessing ESBL-coding plasmids adhere to surfaces more strongly than their wild type counterparts.

*Summary*

It is clear that further research is required to understand the role that specific virulence factors such as pili, plasmids and capsules play in the development and resilience of *K. pneumoniae* biofilms associated with biomedical devices. *K. pneumoniae* infections are one of the most important nosocomial infections currently facing health care professionals today. The bacterium displays an alarming ability to persist in health care settings despite hygiene and treatment protocols. For this reason, therapeutic regimens are being adapted to account for the potential survival of this organism either through the dissemination of the bacterium into a hosts' body, or through its survival in the environment. It is important to note that once the bacterium becomes entrenched within a host's body, through, for example, the insertion of a catheter, the resultant colony often forms an intractable entity through the formation of a biofilm, which acts to protect the bacterium from host-mediated phagocytosis and from antibiotic therapy. Mortality and morbidity resulting from *K. pneumoniae*-associated infections is often the product of bacteraemia where part of the biofilm can become dislodged from the main entity to form a secondary site, or where cells detached from the biofilms can cause such diseases as septic shock, and respiratory and acute renal failures.

### 3.4 *Candida albicans*

*Candida albicans* is the most prevalent cause of fungal infection in humans, with incidents of candidiasis being reported globally. *C. albicans* is an opportunistic pathogen, usually existing as part of the normal microbiota of the oropharangeal and gastrointestinal tracts, the skin, oral and vaginal cavities. *Candida* are small, asexual fungi, which intermittently cause incidents of disease when physiologic imbalance or physical trauma affects the patients' biochemistry to upset the balance of the normal flora. For example, changes in metabolic activity can alter the pH of blood, having a direct effect on the ability of skin flora to adhere or survive, such as *C. albicans* and Lactobacilli in the genito-urinary tract. Notable infections include balanitis, thrush, mediastinitis, and mycotic aneurysm. The morbidity and mortality rates for candidiasis are alarmingly high, and the progression of the disease has a fatal prognosis of up to 80%, even after the commencement of treatment. The ability of *C. albicans* to colonise and multiply on biomedical devices such as shunts & stents is well established, and include devices are made from a range of materials such as steel and silicone rubber. This research indicates that the fungus is capable of not only adhering to these surfaces, but that they are capable of resisting host immune defences to persist within the patients' body, where they present the one of the highest colonisation-to-infection rates for any micro-organism. Biofilm formation by *C. albicans* is of major clinical importance as these entities present with enhanced antifungal drug resistance, meaning that infections can become recalcitrant, and can also affect the host by impacting physiologic function. A pertinent example here would be the colonisation of an intravascular stent by viable *C. albicans* during an operation. The colonised stent would be

subsequently transferred into the host where it would be able to utilise the nutrients present in the hosts' blood, thus providing the fungal cells with the opportunity to reproduce and to express extracellular moieties such as those constituting the EPS matrix. This response will confer a degree of protection against subsequent host immune response. In such situations the surgical removal of the device does not always resolve the problem. It has been shown that secondary infections can result from the sloughing off of viable fungal cells from the primary site leading to the colonisation and infection of other tissues. Blood vessels, such as arteries, can become blocked by proteins and other materials carried in the blood due to poor diet and lifestyle choices influencing the patients' biochemistry. Often, a surgical procedure is required to remove the blocked section of a blood vessel, and a stent is inserted. Stents can typically be made from ceramics or stainless steel, and are designed to facilitate blood flow through damaged sections of tissue, and to encourage cell growth over the damaged area in an attempt to promote host cell-mediated repair of the damaged tissue. Restenosis is the re-blocking of a blood vessel following a surgical procedure; in other words the re-emergence of the primary condition to which the stent was inserted to attempt to treat, and can be due to blood products clogging the vessels or due to post-operative infection. Research has demonstrated that the high flow rate and associated sheer stress can lead to a faster-growing biofilm than static cultures, suggesting that blood-borne infections could lead to the development of a highly developed microbial community with the circulatory network and sheer stress presenting the potential for dissemination throughout the host. In humans, neointimal tissue is formed in response to stenting operations, which is formed by the migration and proliferation of smooth muscle cells in the region of the damaged site. Human smooth muscle cells produce hyaluronan of varying molecular weights in numerous sites of the body, and the specific function varies accordingly. Hyaluronan is a linear polysaccharide composed of D-glucuronic acid and *N*-acetyl-D-glucosamine, which are joined by β1-3 and β1-4 links. The polymer is usually found anchored to cells, and shows evidence of a lubricating function as it demonstrates both viscoelasticity and shear dependence. Research has suggested three generalised functions of the hyaluronan: water homeostasis, where it facilitates an exponential increase in osmotic pressure; a transport function, where it acts to sieve and immobilise large particles whilst allowing smaller ones through; and finally an exclusion function, where hyaluronan acts to regulate chemical partition and in affecting physiologic function. These data suggest that the polysaccharide will be near ubiquitous in the body, especially in sites where transport or chemical interaction occur, making the bloodstream a logical place for human cells to express it. Research has revealed that the polymer is involved in cell migration, wound healing and tissue turnover, perhaps making it an ideal target for degradation by an invading pathogen. Research suggests that *C. albicans* is also capable of producing hyaluronan, although only of one specific molecular weight. The function of the fungal hyaluronan is not fully understood, but is believed to be analogous to that of the human isoforms. Research has further revealed that *C. albicans* can produce extracellular enzymes called hyaluronidases, which act to cleave the hyaluronan, and could thus act to remove its ability to perform its physiologic function. The exact purpose to which the fungus acts to cleave the human hyaluronan is unclear; however, one potential explanation exists. If the fungal hyaluronan forms part of the protective biofilm barrier against host immune response, then it is possible that the fungus uses hyaluronidase enzymes to cleave the human polysaccharides and to incorporate them into their own biofilm matrix. If this theory is correct, then it would suggest that *C. albicans* is capable of utilising the hosts' own consitution to further its own survival. In the vagina, hyaluronan occurs as a natural product. However, research has indicated that in episodes of vulvovaginal candidiasis, levels of the polymer were found to be significantly increased and associated with incidents of burning. These data suggest either that the fungus is producing copious amounts of the polymer itself, or that the host is forced to produce hyaluronan in response to the infection. For *C. albicans* to cause infection, the fungus must migrate across the host cell surface, indicating that adherence to the endothelium is essential for the initiation of a disease state. The exact mechanisms of pathogenesis are unclear. However, it is clear that the fungus utilises several key virulence factors which enable the fungus gains access to the host tissues. Particular examples include a metallopeptidase enzyme which has been suggested to play a role in the degradation of the hosts' subendothelial extracellular matrix components. This could suggest that the fungus is able to employ a complex array of virulence factors to initiate episodes of disease.

*Summary*
Whilst the pathogenicity profile of *C. albicans* is not fully understood in relation to hyaluronan and hyaluronidase enzymes, theory suggests that the fungus exploits a unique ability to convert host extracellular material in to biofilm material to protect the fungal cells from a host-mediated immune response. This final model of a microbial biofilm is an unusual situation because it means that the fungus is able to commandeer part of the human body to prevent the immune cells attacking it. This phenomenon is not fully understood, and merits further research before the roles of the polymer and *C. albicans* virulence factors can be adequately explained within the remit of fungus-associated ischaemic disease.

## 4. Future treatment strategies

Overall, no single mechanism has been proposed that satisfactorily accounts for cell resistance and survival in biofilm matrices. Rather, it is believed that multiple resistance mechanisms may act synergistically, conferring the reduced

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susceptibility to antimicrobial compounds frequently manifested by biofilms. The following four sections outline current and possible future treatment strategies for the treatment of biofilm-associated infections.

**1. Antibiotic treatment strategies**

Multispecies biofilms often contain at least one antibiotic inactivating enzyme (such as a β-lactamase which inactivates β-lactam antibiotics including penicillin), or that the drugs used are ineffective against biofilm cells which are said to have an altered, usually slowed, metabolism. Both factors mitigate against β-lactam activity, but when used in combination with macrolides, aminoglycosides, or quinolones, β-lactams may prove useful because they are broad-spectrum (target more than one species), have shown to sometimes prevent cell adherence & biofilm formation, and can be incorporated in polyurethane materials, for example, which are used to make catheters.

**2. Prevent initial attachment**

Technology to alter the surfaces of materials shows promise, and can be used for some biomedical devices, for example the incorporation of antibiotics as a means of preventing initial biofilm attachment to surfaces, with differing successes depending of the material and the antibiotic drug, respectively. Examples include: materials coated with silver ions or within which silver ions are incorporated; materials containing antibiotics which are released slowly; materials in which intrinsic properties (e.g. surface hydrophobicity) have been altered; and the use of anti-adhesive surfaces (e.g. heparin coatings).

**3. Minimising biofilm formation**

The cleaning and manufacture of devices can alter the surface properties of the material, and can thus effect cell attachment, leading to a reduced adhesion rate of hours rather than minutes. However, this can still be unfeasible where frequent cleaning or change of devices such as catheters or wound dressings is required. Selected techniques include the use of cleaning using mechanical forces that do not generate aerosols which may further spread bacteria (e.g. foam), as well as the use of sanitizers that will result in a higher bacterial kill when used in conjunction with mechanical methods, have been advocated.

**4. Quorum sensing and biofilm disruption**

Current research indicates that the disruption of microbial communication systems can generate biofilms that are less resistant to antimicrobial agents, and hence present the potential for a more rapid and more effective treatment strategy. Two possible strategies here include the use of sonic waves and electrical pulses to disrupt adherent communities. These technologies are currently in the early stages of development, however, evidence indicates that both techniques act to dislodge adherent cells from a surface and impacts their physiology to create a population of viable but non-culturable cells. This suggests that such cells might not be able to reproduce or express EPS substances, suggesting complementary antibiotic therapy might be an appropriate adjunct to eradicate the infection, or even to prevent the colonisation of surfaces of the replication of adherent cells.

## 5. Closing statement

Biofilms can be seen as shells protecting the living cells beneath, but this description belies the extreme complexity and innumerable functions of what are truly remarkable biological constructions. In addition to the physical protection they provide against predation or chemical attack, their heterogeneous constitution allows them to also provide a medium for intracellular communication, nutrient flow, the transfer of genetic material, as well as a means to disseminate viable cells to other locations in the environment or within a host where the cells can reproduce, thus facilitating the survival of the cells within. In all instances, microbial biofilms are constructed for one purpose: the protection of viable cells. Organisms are capable of producing EPS matrices and alter their metabolism making them more resistant to resist external selection pressures, including biological threats, such as predation; chemical shock, such as chlorination; and from physical stresses such as such as temperature flux or being transferred between host and environmental niches. Biofilms are believed to represent the ultimate microbial survival strategy, and are accordingly the focus of a large proportion of on-going research. Current trends include techniques to disrupt quorum sensing communication and strategies to facilitate greater antibiotic penetration into the biofilms to dislodge the basal layers of cells as well as the surface film. Such strategies have shown promise singly, but research has demonstrated that combinations of techniques often prove more effective than lone strategies. Biofilm microbiology is likely to continue to become ever more prominent as our understanding of the nature and functions of biofilms increase, and the practice of antibiotic chemotherapy advances to meet this challenge.

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