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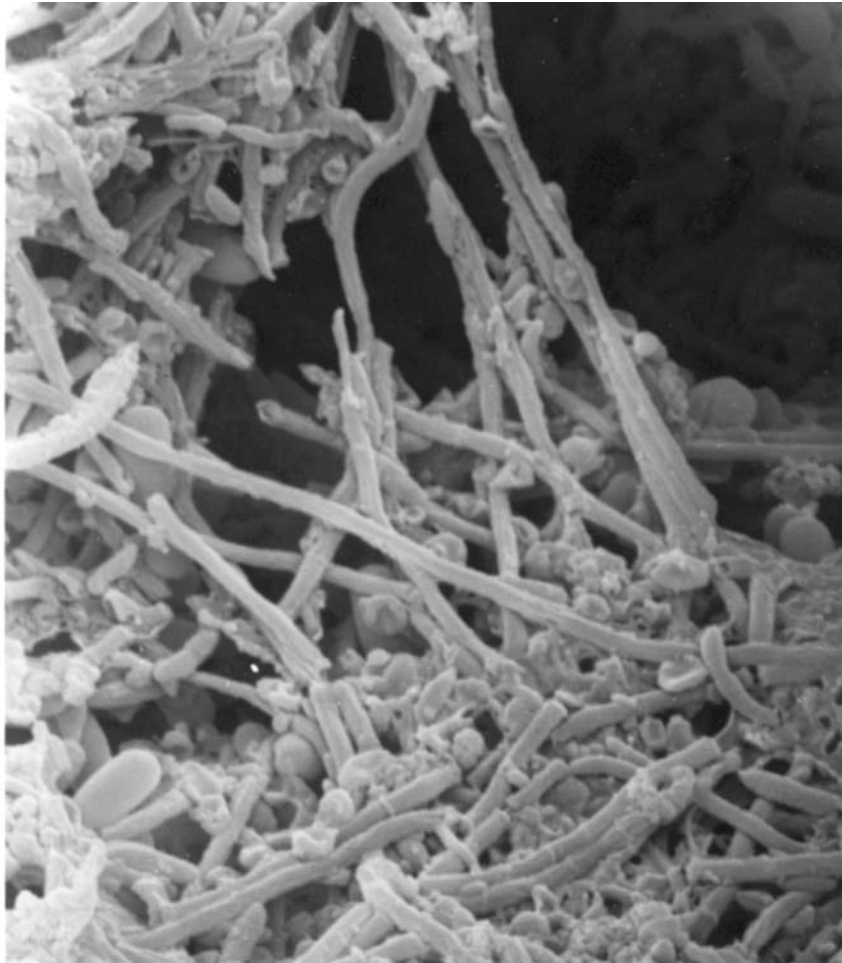
Hobart Tools of the Trade Dinner Meeting
October 1st 2008



Super Bugs



Size Doesn't Matter



BIOFILMS

Dr Tara Anderson
1 October 2008

<http://commtechlab.msu.edu/sites/dlc-me/zoo/microbes/media/biofilm.jpg>



http://toxics.usgs.gov/photo_gallery/photos/metals_variation/HighOreCreekBiofilm_1.jpg



<http://nymag.com/daily/intel/20070606sludge.jpg>

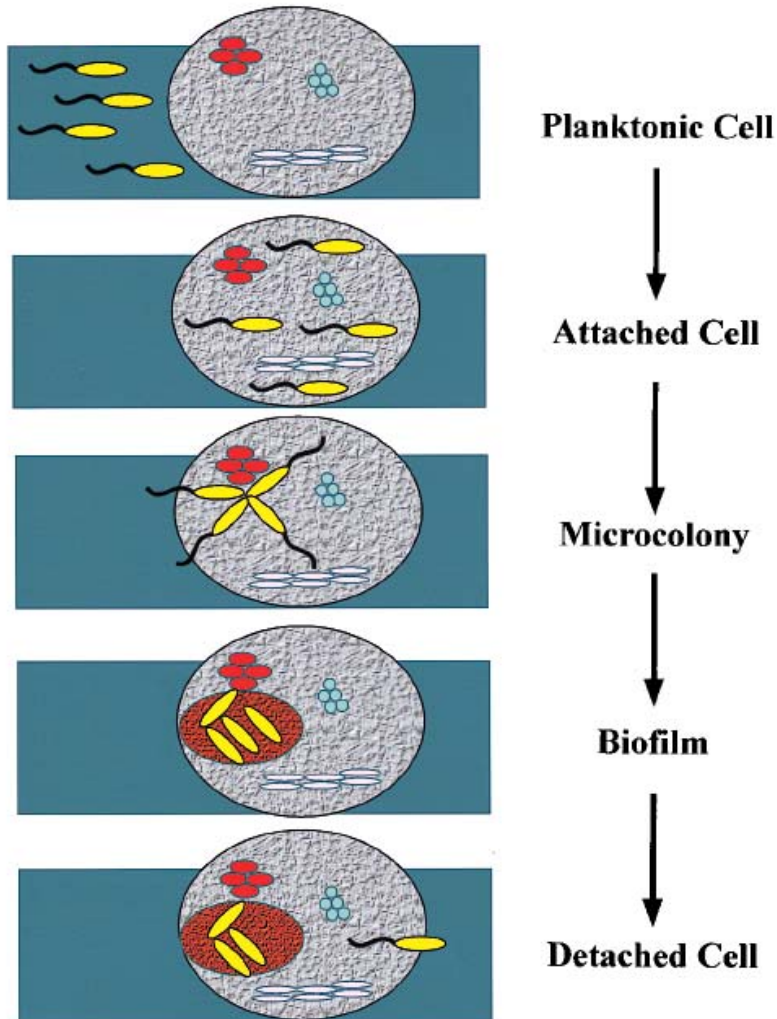


Figure 1. Scanning electron micrograph depicting a developed biofilm (*A*), the substratum (*B*), and an attached cell (*C*). (Image by Rodney Donlan and Donald Gibbon, from the American Society for Microbiology MicrobeLibrary and used with permission of the authors.)

Bacterial Phases

- Planktonic or free-swimming phase

- Biofilm
 - Complex, highly differentiated, multicultural community of cells growing on a surface and enclosed within a self developed extracellular polysaccharide matrix
 - Level of activity similar to ‘bustling city’
 - Protected microenvironment with water-filled channels allowing transport of nutrients, waste products and interbacterial communication



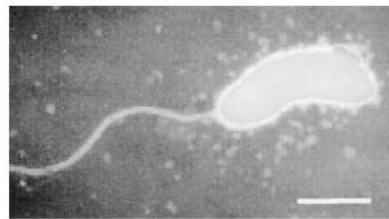
Initial phase

- Reversible adherence to surface

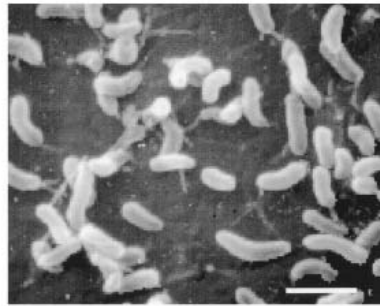
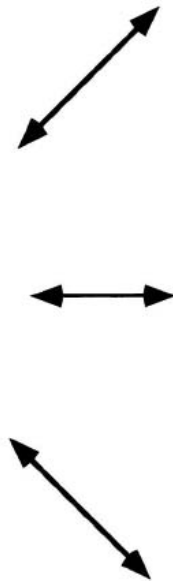
Second phase

- Intercellular adhesion
→ microcolony formation
- Exopolysaccharide formation
→ biofilm formation

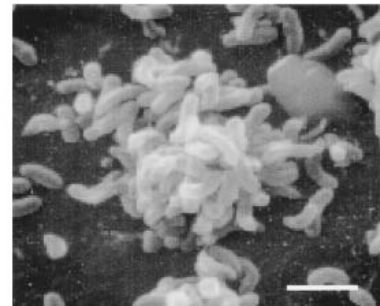
FIG. 1. A schematic representation of the steps a new bacterial species takes in forming a biofilm on a rock previously colonized with multiple species of bacteria. The yellow bacteria represent an aquatic species that swims towards the rock using polar flagella, forms random loose attachments to the rock, migrates over the surface to form a microcolony, and finally produces exopolysaccharide to form a three-dimensional biofilm. When environmental conditions become unfavorable, some of the bacteria may detach and swim away to find a surface in a more favorable environment.



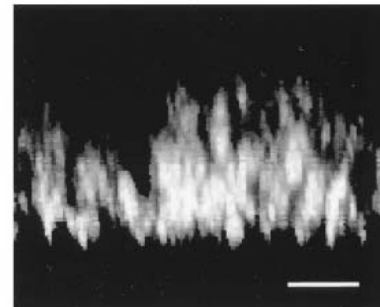
Planktonic Cell



Attached Cell



Microcolony



Biofilm

FIG. 2. A microscopic study of the steps in biofilm formation by *V. cholerae*. The planktonic bacterium was visualized by transmission electron microscopy (bar = 1 μ M), the attached cells and microcolony were visualized by scanning electron microscopy (bar = 2 μ M), and the biofilm micrograph represents a vertical section through a 20- μ m biofilm taken by confocal scanning laser microscopy (bar = 10 μ M).

Genetic Basis Of Biofilm Formation

- Surface colonisation
 - Pili and flagellar (*P. aeruginosa*)
 - Extracellular polymeric substances (EPS) production
 - Colanic acid (*E. coli*)
 - Alginate (*P. aeruginosa*)
 - Polysaccharide intercellular adhesin (PIA) (CNS, *S. aureus*)
 - Extracellular matrix binding protein (embp) (CNS)
 - Chitinase and chitin binding genes (*Vibrio* spp.)
- Specific co-aggregation of bacteria
 - Heterogenous environment
 - Bacteria distribution according to survival needs

Intercellular Communication

- Quorum-sensing molecules
- Single species biofilms
 - Acyl-homoserine lactones (acyl-HSLs)
 - *Pseudomonas* spp.
 - Mediators of surface attachment
 - Define separations between bacterial pillars
- Multispecies biofilms
 - Less well defined
 - Alter distribution of specific species in biofilms
 - Alter protein expression in neighbouring cells
 - Introduce new genetic traits into neighbouring cells

Advantages Of Biofilm Living

- Ability to acquire transmissible, genetic elements at accelerated rates
 - Emergence of new pathogens by acquisition of antibiotic resistance, virulence factors and environmental survival capabilities

- Protection from host defences

- Resistance to toxic substances (eg antibiotics, chlorine, detergents)
 - Decreased diffusion into biofilm
 - Decreased bacterial growth rate in biofilm
 - Biofilm-specific substances eg exopolysaccharide
 - Quorum-sensing specific effects

“Bacterial biofilms reported to be up to 500 x more resistant to antibiotics than planktonic cells”

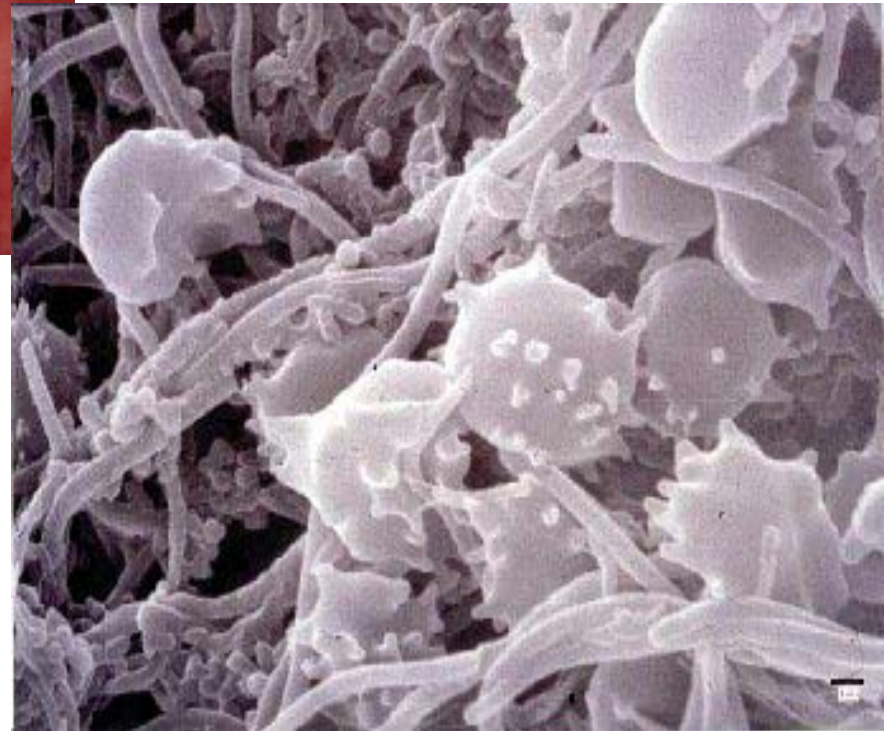
Infections Caused By Biofilms

- **>60%** of all infections are caused by biofilms

- Examples:
 - Urinary tract infections
 - Otitis media
 - Dental plaque and gingivitis
 - Wound infections
 - Tonsillitis
 - Infections involving foreign bodies – catheter-related infections, prosthetic joints, prosthetic heart valves
 - Pulmonary infections in cystic fibrosis patients



<http://www.edwardbyrne.com/perio05.jpg>



http://www.textbookofbacteriology.net/dental_plaque.jpeg

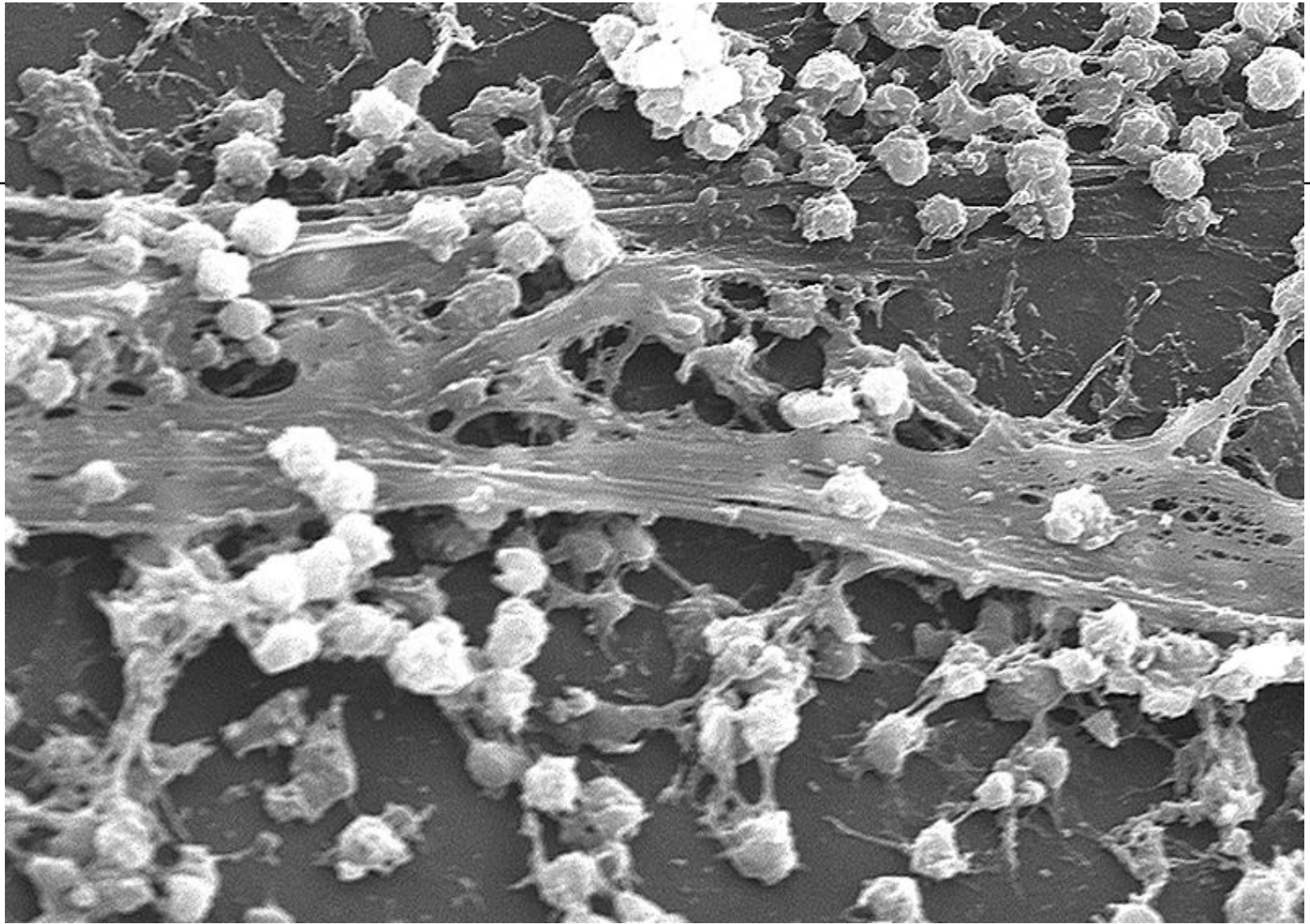
Indwelling Medical Devices

Table 1. Indwelling medical devices on which biofilms may develop.

Central venous catheters
Central venous catheter needleless connectors
Contact lenses
Endotracheal tubes
Intrauterine devices
Mechanical heart valves
Pacemakers
Peritoneal dialysis catheters
Prosthetic joints
Tympanostomy tubes
Urinary catheters
Voice prostheses

Table 2. Biofilm-associated microorganisms commonly isolated from selected indwelling medical devices.

Indwelling medical device	Organisms
Central venous catheter	Coagulase-negative staphylococci, <i>Staphylococcus aureus</i> , <i>Enterococcus faecalis</i> , <i>Klebsiella pneumoniae</i> , <i>Pseudomonas aeruginosa</i> , <i>Candida albicans</i>
Prosthetic heart valve	Viridans <i>Streptococcus</i> , coagulase-negative staphylococci, enterococci, <i>Staphylococcus aureus</i>
Urinary catheter	<i>Staphylococcus epidermidis</i> , <i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> , <i>Enterococcus faecalis</i> , <i>Proteus mirabilis</i>
Artificial hip prosthesis	Coagulase-negative staphylococci, β -hemolytic streptococci, enterococci, <i>Proteus mirabilis</i> , <i>Bacterioides</i> species, <i>Staphylococcus aureus</i> , viridans <i>Streptococcus</i> , <i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i>
Artificial voice prosthesis	<i>Candida albicans</i> , <i>Streptococcus mitis</i> , <i>Streptococcus salivarius</i> , <i>Rothia dentocariosa</i> , <i>Candida tropicalis</i> , <i>Streptococcus sobrinus</i> , <i>Staphylococcus epidermidis</i> , <i>Stomatococcus mucilaginous</i>
Intrauterine device	<i>Staphylococcus epidermidis</i> , <i>Corynebacterium</i> species, <i>Staphylococcus aureus</i> , <i>Micrococcus</i> species, <i>Lactobacillus plantarum</i> , group B streptococci, <i>Enterococcus</i> species, <i>Candida albicans</i>



<http://www.marianameyer.com/imgs/catheter.jpg>

Why Does Antibiotic Therapy Fail?

- Restricted penetration
 - Exopolymer matrix (negatively charged)
 - Restricts large molecules eg antimicrobial proteins and smaller antimicrobial peptides
 - Restricts positively charged antibiotics eg aminoglycosides
 - Synergistic relationship between restricted diffusion and degradation eg beta-lactamase
 - *Pseudomonas aeruginosa*

Why Does Antibiotic Therapy Fail?

- Reduced growth rate
 - Slower growth probably because of limited nutrients and oxygen depletion
 - Antimicrobials are more effective in killing rapidly growing cells
 - Rate of killing proportional to rate of growth



Why Does Antibiotic Therapy Fail?

- Acquired resistance
 - Plasmid transfer between different bacteria
 - Plasmids may encode resistance to a number of different antimicrobials

Why Does Antibiotic Therapy Fail?

- Presence of ‘persisters’
 - Slow-growing subpopulation of cells
 - Restricted access of bacteria to the immune system
 - Relapsing nature of biofilm infections
 - ‘Persister genes’ identified

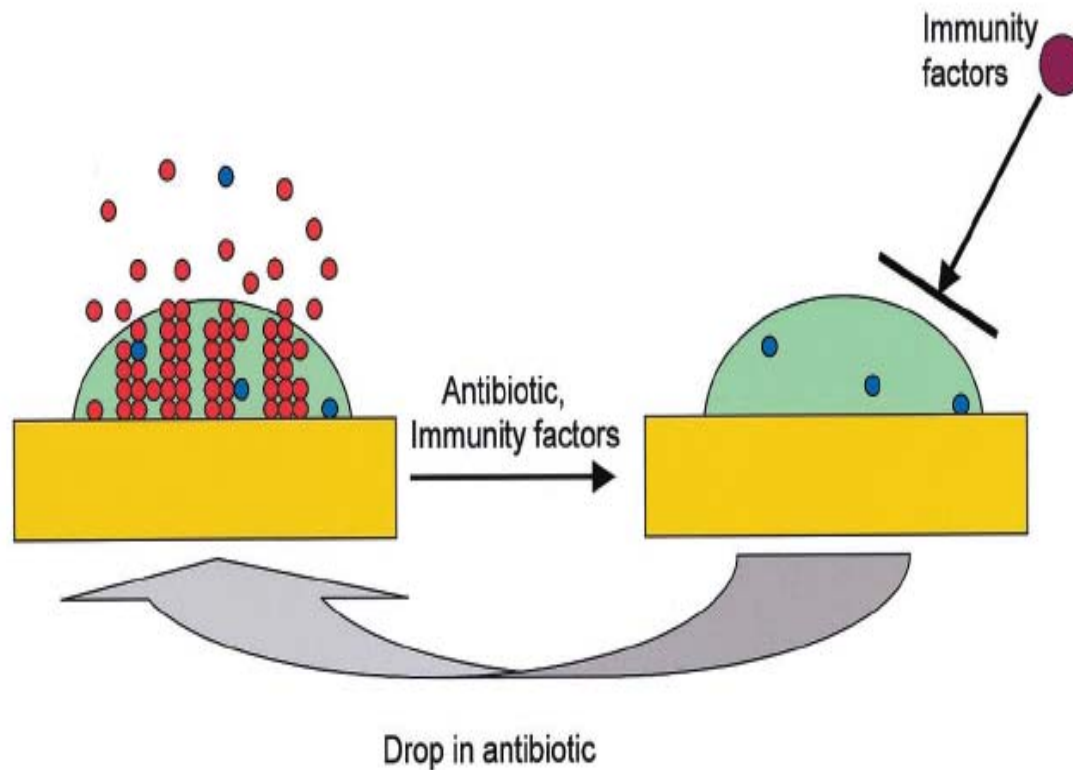


FIG. 2. Model of biofilm resistance based on persister survival. An initial treatment with antibiotic kills planktonic cells and the majority of biofilm cells. The immune system kills planktonic persisters, but the biofilm persister cells are protected from host defenses by the exopolysaccharide matrix. After the antibiotic concentration drops, persisters resurrect the biofilm and the infection relapses (33).

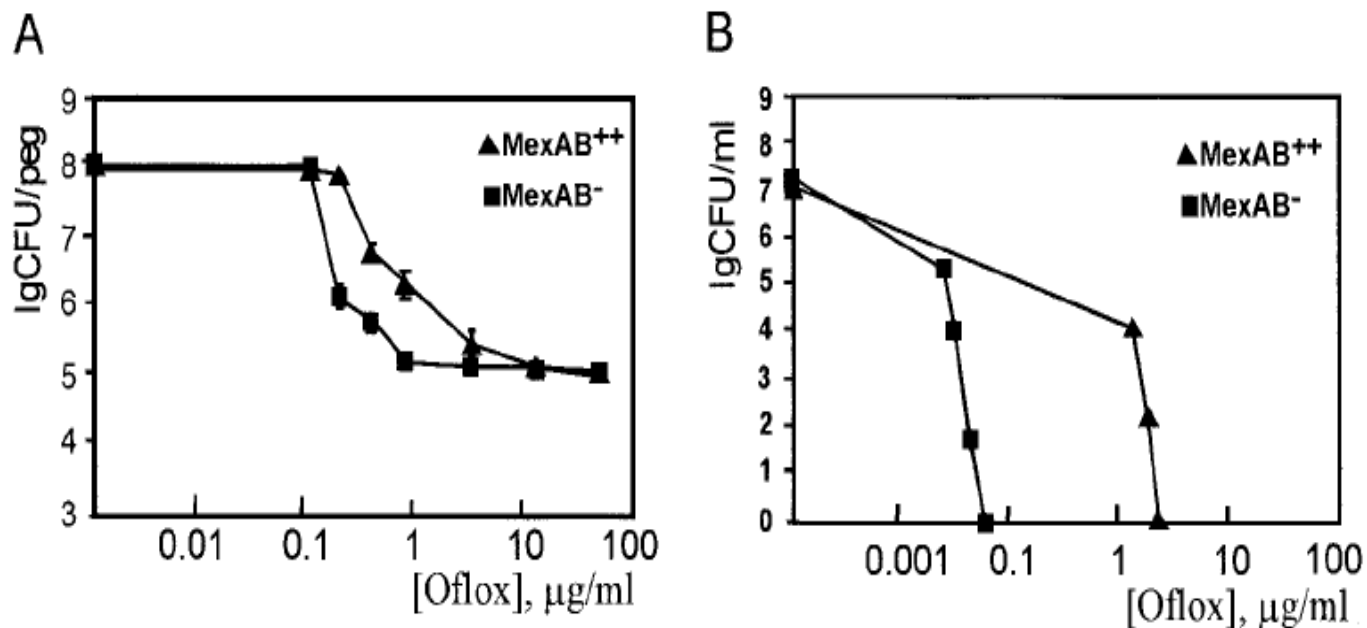


FIG. 1. *P. aeruginosa* persists surviving in a biofilm treated with ofloxacin (Oflox). (A) Biofilms were formed on pegs of a Calgary Biofilm Device (14) and were then treated with a given concentration of antibiotic in Mueller-Hinton broth for 6 h, rinsed, and dislodged by sonication. Live cells were then counted by plating. The number of live cells recovered from a single peg is expressed as the number of CFU per peg. A strain that overexpressed the main MDR pump that extrudes fluoroquinolones (MexAB⁺⁺) and a strain that lacked the pump (34) were used in this experiment. The contribution of the pump to resistance is evident at low concentrations of the antibiotic but has little effect on the survival of persisters. (B) Planktonic cells were treated similarly with ofloxacin and plated for determination of the cell count. The apparent absence of persisters is due to the low density of the population and the detection limit of the experiment; at higher densities, persisters are evident at low levels in a planktonic population (A. Spoering and K. Lewis, unpublished data). Adopted from reference 12, with permission.



Susceptibility Testing

- Standard testing performed on planktonic cells
- Not accurately predicting efficacy of an antibiotic against a biofilm associated organism

WOUNDS

Significance Of Bacteria And Biofilms

Bacterial Profiles Of Chronic Wounds

- Microflora of wounds usually polymicrobial
- Mean number of bacterial species per wound range from 1.6-4.4
- 86% of wounds with no clinical signs of infection contain >1 bacterial species

A review of the microbiology, antibiotic usage and resistance in chronic skin wounds.

Howell-Jones et al. Journal of Antimicrobial Chemotherapy 2005;55:143-149

Aerobic Bacteria

- *Staphylococcus aureus*
- Coagulase-negative staphylococci
- Enterobacteriaceae
- *Pseudomonas aeruginosa*



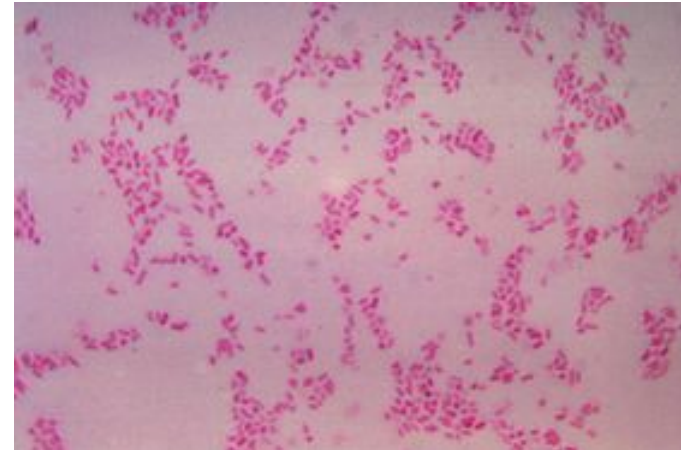
Anaerobic Bacteria

“Invisible Villains”

- ❑ **38-48% of total number of microbial isolates**

- ❑ **Often overlooked**
 - Specimen collection and transport
 - ❑ Critical for viability
 - Culture, isolation and identification
 - ❑ Time-consuming, labour intensive and \$\$

Bacteroides fragilis



http://de.wikipedia.org/wiki/Bacteroides_fragilis



<http://www.bdj.co.jp/micro/products/1f3pro000005jb2-img/1f3pro000005jbs.jpg>

Bacterial Isolates From Acute And Chronic Wounds

Aerobic and facultative anaerobic organisms	Anaerobic bacteria
Coagulase negative staphylococci	<i>Peptostreptococcus</i> spp.
<i>Micrococcus</i> spp.	<i>Clostridium</i> spp.
<i>Staphylococcus aureus</i>	<i>Eubacterium limosum</i>
Beta-haemolytic streptococcus (group C and G)	<i>Propionibacterium acnes</i>
Viridans streptococci	<i>Bacteroides</i> spp.
<i>Corynebacterium</i> spp.	<i>Prevotella</i> spp.
<i>Bacillus</i> spp.	<i>Porphyromonas</i> spp.
<i>E. coli</i>	<i>Fusobacterium necrophorum</i>
<i>Serratia</i> spp.	<i>Veillonella</i> spp.
<i>Klebsiella</i> spp.	
<i>Enterobacter</i> spp.	
<i>Citrobacter</i> spp.	
<i>Proteus</i> spp.	
<i>Providencia</i> spp.	
<i>Morganella</i> spp.	
<i>Acinetobacter</i> spp.	
<i>P. aeruginosa</i>	
<i>Stenotrophomonas maltophilia</i>	
<i>Candida</i> spp.	

Adapted from Table 2. Bowler et al. Clin Microbiol Rev 14;244-269

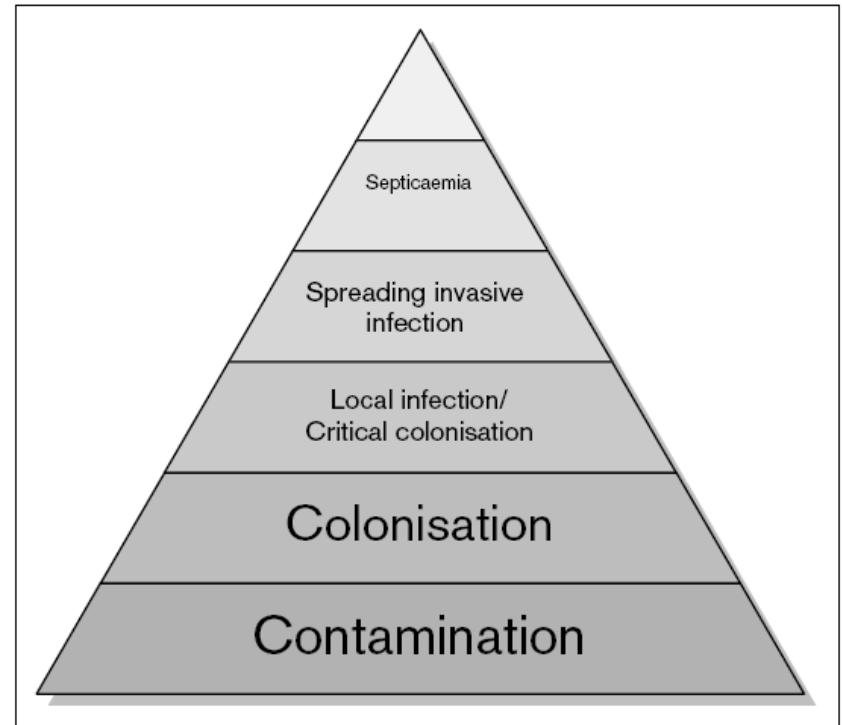
Table 1. Effects of bacterial colonisation and infection on wound healing

Phase of wound healing response	Mediators	Effect of wound colonization	Effect of wound infection
Inflammation	Complement cascade proteins activate platelets, causing release of inflammatory mediators, growth factors and fibronectin Cellular response: particularly neutrophils, macrophages	Enhanced white cell accumulation, chemotactic and bactericidal activity	General effect of infection is increased consumption of complement proteins, resulting in decreased chemotaxis, plus depletion of platelets [37] White cell function impaired in the presence of infection, including by short chain fatty acid products by anaerobic bacteria [18]. Increased production of cytotoxic enzymes and free oxygen radicals increase tissue damage [42]. Localized thrombosis and release of vasoconstrictive metabolites increase tissue hypoxia and promote bacterial proliferation and tissue destruction [37]
Granulation tissue formation and angiogenesis	Macrophages as stimulus to angiogenesis and granulation tissue formation	Increased granulation tissue formation and angiogenesis	Further increased turnover, partly due to production of bacterial enzymes, resulting in oedematous, haemorrhagic, friable granulation bed and excessive scar formation. Endotoxins in wounds stimulate production of interleukins and TNF which induce production of MMPs [34,35]. Imbalance between MMPs and their tissue inhibitors and decrease in growth factor production
Epithelialization			Decreased if bacterial load over 10^5 as bacterial metabolites inhibit migration of epithelium and digest dermal proteins and polysaccharides [43,44]. Also increased production of neutrophil proteases damage vulnerable epithelium [45]
Collagen production		Increased tensile strength of wound	Reduced numbers and proliferation of fibroblasts, with disorganized collagen production. Both increased production and breakdown of collagen, with overall effect of decreased wound strength [37]. Endotoxins decrease collagen deposition and cross linking and associated with surgical dehiscence [46]
Wound contraction			Significantly delayed [37]

TNF, tumour necrosis factor; MMP, matrix metalloprotease.

Bacteria And Chronic Wounds

- Microbial load correlates with delay in wound healing and wound infection
 - Decubitus ulcer healing
 - Skin graft survival
 - Pressure ulcer healing
 - Delayed closure of surgical wounds
- Acute or chronic wound infection exists when the microbial load $>10^5$ CFU/g tissue
- Bacteria $\geq 10^4$ CFU/g tissue must be present to cause infection in complex extremity wounds



Distribution, Organization, and Ecology of Bacteria in Chronic Wounds[▽]

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Between 1 and 2% of the population in the developed world experiences a nonhealing or chronic wound characterized by an apparent arrest in a stage dominated by inflammatory processes. Lately, research groups have proposed that bacteria might be involved in and contribute to the lack of healing of these wounds. To investigate this, we collected and examined samples from chronic wounds obtained from 22 different patients, all selected because of suspicion of *Pseudomonas aeruginosa* colonization. These wound samples were investigated by standard culturing methods and peptide nucleic acid-based fluorescence in situ hybridization (PNA FISH) for direct identification of bacteria. By means of the culturing methods, *Staphylococcus aureus* was detected in the majority of the wounds, whereas *P. aeruginosa* was observed less frequently. In contrast, using PNA FISH, we found that a large fraction of the wounds contained *P. aeruginosa*. Furthermore, PNA FISH revealed the structural organization of bacteria in the samples. It appeared that *P. aeruginosa* aggregated as microcolonies imbedded in the matrix component alginate, which is a characteristic hallmark of the biofilm mode of growth. The present investigation suggests that bacteria present within these wounds tend to be aggregated in microcolonies imbedded in a self-produced matrix, characteristic of the biofilm mode of growth. Additionally, we must conclude that there exists no good correlation between bacteria detected by standard culturing methods and those detected by direct detection methods such as PNA FISH. This strongly supports the development of new diagnostic and treatment strategies for chronic wounds.

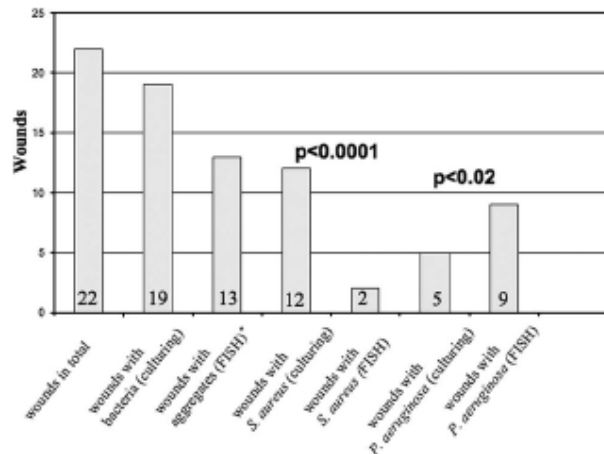


FIG. 3. Number of wounds in each category. Among the wounds with evidence of *P. aeruginosa* colonization, *P. aeruginosa* was detected more frequently with PNA FISH ($P < 0.02$). This was in contrast to the higher frequency of *S. aureus* detected by culture growth ($P < 0.0001$) from the wounds with evidence of *S. aureus*. *, aggregates as described in the legend to Fig. 1.

- No correlation between organisms identified in wound swab and biopsy
- Wound swab may miss bacteria embedded in biofilms – *P. aeruginosa*

Bacteria play a major role in the lack of healing for otherwise optimally treated chronic wounds. Novel diagnostic and therapeutic approaches required.

Eradication Of Biofilms

- Prevention of bacterial attachment
 - Incorporation of antimicrobials into the material of indwelling catheters
- Prevention of biofilm formation
 - Deficiency of iron (*P. aeruginosa*)
 - Lactoferrin (iron sequestration)
 - Iron chelator – EDTA, citrate
 - Gallium
- Disruption of biofilms to allow penetration of topical antimicrobial agents
- Interference with quorum sensing
 - Quorum-sensing blocker eg garlic-based compounds
- Enhancement of bacteria dispersion from biofilms to planktonic state
 - ‘Biofilm-dispersing activity’ – *P. aeruginosa*
- Predatory/parasitic bacteria
 - *Bdellovibrio bacteriovorus* – *E. coli*, *P. aeruginosa*
 - *Micavibrio aeruginosavorus* – *P. aeruginosa*
- Block ‘persister genes’
- Physical approach – electromagnetic field, ultrasound



References

JOURNAL OF BACTERIOLOGY, May 2000, p. 2675–2679

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MINIREVIEW

Biofilm, City of Microbes

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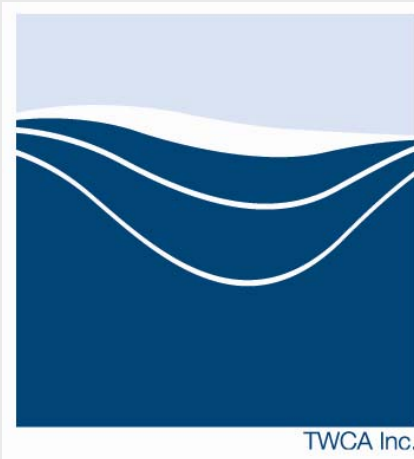
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MINIREVIEW

Riddle of Biofilm Resistance

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