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Mechanisms of multispecies biofilm resistance to bactericidal agents

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ABSTRACT

Microorganisms frequently live in biofilms in both natural and artificial environments. The mechanisms through which biofilm-dwelling cells exhibit better tolerance to antimicrobial drugs than free-living cells have been extensively investigated utilising single-strain axenic models. Interspecies interactions, yet, may significantly affect how the community reacts to such harmful exposure, according to mounting scientific data. We offer an overview of the research on multispecies biofilm resistance to disinfectants in this study, paying particular attention to how local surface flora protects pathogenic species against disinfection procedures. Interspecies signalling, dispute among biocides molecules and public goods in the matrix, or the physiology and genetic plasticity related with a structural spatial arrangement are some of the mechanisms involved in this protection.

Keywords: Biofilm, Biocides, Disinfectants, Pathogens, Interspecies interactions

INTRODUCTION

In the environment, bacteria tend to be attached to surfaces and embedded within self-produced extracellular polymers that retain them bound to one another to create biofilms (Costerton et al., 1995). These formalised communities are necessary to maintain ecological balance because the ability to endure adverse conditions, such as desiccation or nutrient

starvation, and to participate in the global biogeochemical cycle are characteristics of biofilm dwellers (Burmlle et al., 2012).

Biofilms are also present in man-made environments, and have been found to be linked to nosocomial infections, food spoilage, and pipeline damage in industries (Flemming, 2011). The mechanisms that regulate this multicellular behavior, such as the production of matrix polymers, cell-cell communication, or the development of various cell types within the biostructure, have been the subject of extensive research for more than 30 years (Bridier et al., 2014). Multispecies communities are by far the most prevalent in natural biofilms, making simple laboratory models rarely reflective of these environments (Hall-Stoodley et al., 2004).

Ecological impacts in a biofilm can be determined by interactions between organisms. In particular, developing a mixed biofilm facilitates Pseudomonas sp.'s uptake of the waste products released by *Burkholderia* sp. when the contaminant chlorobiphenyl is present (Nielsen et al., 2005). The invasion of native tissues by multiple pathogenic species, such as the lungs of cystic fibrosis patients, chronic wounds, or the urinary tract frequently induces more severe and recalcitrant infections (Wolcott et al., 2013). This anthropocentric negative impact of interactions between species is reflected in biofilms related to chronic infections. In one instance, co-infection by *Pseudomonas aeruginosa* and *Staphylococcus aureus* promotes a host inflammatory response and reduces the healing of wounds (Pastar et al., 2013). Likewise *P. aeruginosa* and *Candida albicans* both enhance the virulence of *S. aureus* (Peters et al., 2010), and *P. aeruginosa* demonstrated more virulence in a Drosophila model when it was co-inoculated with Gram-positive bacteria (Korgaonkar et al., 2013).

Pathogen persistence on inert surfaces in medical or industrial environments is similarly influenced by multispecies interactions. In these situations, despite frequent and thorough cleaning and disinfection measures, equipment biocontamination is linked to nosocomial and foodborne diseases (Bridier et al., 2014). Disinfectants are multi-target agents (e.g., cell wall, proteins, DNA, and RNA) that frequently cause disruption of the bacterial membrane, in contrast to antibiotics, which normally have a single target (Maillard, 2002). Some of the mechanisms that affect the tolerance of biofilm-dwelling cells to disinfectants include extracellular polymers that block their diffusion or response, as well as differences in physiological status depending on the biofilm stratum (Stewart and Franklin, 2008). The greater resistance to disinfectants seen in single-strain biofilms is further

supported by growing proof that interactions between various species within the matrix play a role (Wang et al., 2013). The resistance to disinfectants is caused by some of the mechanisms that render biofilm cells resistant to antibiotics. In order to safeguard pathogenic species, this analysis specifically focuses on the mechanisms behind multispecies biofilms' tolerance to and resistance to disinfectants.

Species associations leading to increased biocidal resistance in biofilms

Increased evidence points to the fact that social interaction within a mixed community provides bacterial resistance to environmental challenges, including the effects of disinfectants.

When compared to their single-strain counterparts, four species isolated from a marine alga developed a multispecies biofilm with higher biomass and an eight-fold improvement in their resistance to hydrogen peroxide (Burmlle et al., 2012). Similar to this, the association of two species that are frequently isolated on surfaces in the food processing industries, *Bacillus cereus* and *Pseudomonas fluorescens,* led to a remarkable increase in their tolerance to two commonly used disinfectants, chloride dioxide and glutaraldehyde (Simes et al., 2009). In various circumstances, it has been demonstrated that the local surface flora shields pathogens from the effects of biocides. In one instance, *Veillonella parvula* in an oral biofilm allowed *Streptococcus mutans* to survive five different antimicrobial treatments with a 50% higher survival rate (Luppens et al., 2008). The significance of resident flora in foodborne or nosocomial infections is frequently overlooked as these strains are typically non-virulent. But because of adaptive mechanisms linked to regular exposure to biocides, they might be very persistent and offer an ideal environment for pathogenic bacteria (Pastar et al.,2013). In one case, an investigation revealed that a strain of *Bacillus subtilis* collected from an endoscope washer-disinfector, that was especially susceptible to the excessive amounts of oxidative disinfecting agents utilised regularly in these devices, was able to shield S. aureus from the action of peracetic acid within a multispecies biofilm (Bridier et al., 2012). Examples of some bacteria in biofilm showing resistance against biocides are presented in table 1.

These interesting cases shouldn't make us think that bacterial defence in multispecies biofilms is a common characteristic. So, *Salmonella enterica* and *P. putida*, however, cannot protect the food-borne pathogen L. monocytogenes against biocide action in a mixed biofilm

(Kostaki et al., 2012). And when cultured with two oral bacteria, *Enterococcus faecalis* was likewise discovered to be more sensitive to sodium hypochlorite (Yap et al., 2014).

The antimicrobial's inability to break through the biofilm

One of the traits that sets biofilms apart is the formation of an exopolysaccharide matrix, also known as a glycocalyx. It has been proposed that this matrix has a variety of purposes, one of which is to block antibiotics from reaching the bacterial cells that make up the community. A biofilm should not be a barrier to the diffusion of many antibiotics, however certain investigations have revealed an apparent inability of some antimicrobial drugs to permeate the biofilm. A chlorine-detecting microelectrode was used to assess the concentration of the common disinfectant chloride in a biofilm that contained both *Klebsiella pneumoniae* and *P. aeruginosa* (de Beer et al. 1994).

Table:1. Resistance shown by biofilm species against some biocidal agents

Biofilm species	**Bactericidal agents**	Reference
E. coli, S. Typhimurium	Chlorine	Wang et al.(2013)
S. Typhimurium, P. fluorescens		
E. coli, P. aeruginosa Stenotrophomonas maltophilia, E. cloacae		Stewart and Franklin, (2008)
Kocuria sp., Brevibacterium linens, S. sciuri		Wang et al.(2013)
A. calcoaceticus, B. cepacia, Methylobacterium sp. Mycobacterium mucogenicum, Sphingomonas capsulata, Staphylococcus sp	Sodium hypochlorite	Yap et al. (2014)
Methylobacterium phyllosphaerae, Shewanella		Burmolle et al.(2012)

japonica, Dokdonia donghaensis, Acinetobacter lwoffii	Hydrogen peroxide	
S. mutants, V. parvula		Leriche et al.(2003)
B. subtilis, S. aureus	Peracetic acid	Bridier et al.(2012)
Listeria innocua, P. aeruginosa		Bridier et al.(2012)
L. monocytogenes, Lb. plantarum		Wang and Wood, (2011).
S. mutants, S. aureus, P. aeruginosa	Chlorhexidine	Luppens, et al. (2008)
S. mutants, V. parvula		Canton and Morosini, (2011).

Bacteria's interspecies protection techniques

Multispecies communities can benefit from some of the same mechanisms that allow axenic biofilm-dwelling organisms to withstand the effects of disinfectants. However, in the majority of cases, the unique interactions between several species make it important to take into account additional mechanisms that are not seen in single-strain biofilms.

a. The Biofilm Matrix as a Community Asset for Interspecies

Extracellular polymeric materials that biofilm cells develop help retain them collectively and promote their multifaceted spatial organisation (Branda et al., 2005). While polysaccharides, proteins, lipids, and DNA are the main components of the biofilm matrix, the composition can vary significantly between species, environmental factors, and even between different strains of the same species (Combrouse et al., 2013).

Specific components made by one species that are beneficial to the entire population may be linked to the matrix's protective role (Flemming, 2011). This is particularly true with some enzymes that one strain produces in the matrix that may change how reactive the biocide is; for instance, it was discovered that *P. aeruginosa's* secretion of a certain hydrolase

conferred tolerance to SDS on a mixed community (Lee et al., 2014). Amyloids, a particular type of highly aggregated proteins linked to many bacterial functions like adhesion, cohesion, and host interactions (Blanco et al., 2012), are additional matrix components with protective roles. TasA in *B. subtilis,* FapC in *Pseudomonas* species, and curli in E. coli or Salmonella species are the biofilm-associated amyloids that have been most thoroughly described (Dueholm et al., 2013). The communities produced by S. enterica and E. coli, two species able to work together and share curli subunits in vivo in the context of a process known as cross-seeding, have been found to include amyloids (Zhou et al., 2012).

It has been demonstrated that the BslA amphiphilic protein generated by B. subtilis forms a protective layer at the point where a macrocolony on agar and air meet. This hydrophobic coating shields the occupants of the matrix from biocides' penetration (Kobayashi and Iwano, 2012). Due to the abundance of biocide-interfering organic material caused by these specific protective components, which coexist in the matrix with other species (Simoes et al., 2009), more producing cells or a specific polymer may be produced. This is evident of the *Bacillus subtilis* TasA amyloid matrix protein, which is typically overproduced when other strains of the Bacillus species are present (Shank et al., 2011). Coaggregation between bacteria of different species can promote matrix synthesis, the overall biofilm population and tolerance to biocides, e.g., the oral pathogen *S. mutans* was found to coaggregate with the early coloniser *V. parvula* and this resulted in a multispecies biofilm that produced more matrix and was more tolerant to chlorhexidine and five other biocides than the corresponding axenic biofilms (Luppens et al., 2008). Additional approach is metabolic cross-feeding amongst species, which can boost biofilm dwelling cells' ability to survive when exposed to biocides and accelerate their growth (Stacy et al., 2014).

Through the selection of particular mutations, populations of cells that overexpress biocide-interfering components can also appear in the community (Singh et al., 2010). Under multispecies circumstances, this emergence of genetic variations might be encouraged. In the presence of a strain of *Acinetobacter* sp., *P. putida* variations developed phenotypically diverse morphologies that led to a more stable and fruitful community (Hansen et al., 2007).

b. Genetic adaptability in Multispecies Biofilms

An effective source of genetic material that can be shared between species is the intercellular space of a biofilm. Horizontal gene transfer (HGT) between species is made easier by the physical proximity and presence of extracellular DNA (eDNA) in the matrix

(Hausner and Wuertz, 1999). In a mixed biofilm with *C. albicans*, it has been shown that *S. epidermis* produced more eDNA, which increased the biofilm's biovolume and improved infection in an in vivo model (Pammi et al., 2013). In multispecies biofilms, it has been shown that HGT in *Vibrio cholera* can be produced in response to AI obtained from other *Vibrio* species (Antonova and Hammer, 2011). As has been hypothesised to happen in a biofilm formed by curli-producing and non-producing strains, genetic factors governing biofilm formation can also be transferred across *E. coli* and *S. enterica* (Wang et al., 2013). The acquisition of new genetic material that confers antimicrobial resistance and other characteristics that can support their persistence in natural habitats is made possible by HGT, which is a common driving mechanism for bacteria (Wiedenbeck and Cohan, 2011). Under stressful circumstances, such as being exposed to antimicrobial drugs, resistant mutants can also develop spontaneously in the population (Canton and Morosini, 2011). As *P. aeruginosa* was shown to do when *C. albicans* was present in mixed biofilms, interactions and competition between species can promote the formation of genetic variations.

c. Cell to cell communication

In order to modify the biocidal resistance of biofilms, certain genes may be regulated via cell-to-cell communication pathways (Hassett et al., 1999).

Acyl-homoserine lactones (AHLs) in Gram-negative microorganisms and modified oligopeptides in Gram-positive microorganisms are examples of quorum sensing (QS) signals, also known as autoinducers (AI), which can be used for intra-species cell-to-cell communication (Miller and Bassler, 2001). They cause coordinated reactions that affect the pathogenicity of an organism, the development of genetic competence, and the creation of biofilms (Jayaraman and Wood, 2008). Numerous bacteria isolated from chronic wounds have been found to generate and manufacture AI-2 (Rickard et al., 2010). According to studies by West et al. (2012) and Pereira et al. (2013), the universal language molecule autoinducer-2 (AI- 2) is highly adapted for interspecies communication between microbes. Hence, in a biofilm, one species may disrupt another's signaling pathway by enhancing, suppressing, or inactivating QS signals (Rendueles and Ghigo, 2012). The physiological functions of the cohabitants may be directly impacted by these interferences, which may change gene expression or go beyond a "simple message" (Schertzer et al., 2009). Additionally, signalling has been observed in a community of oral bacteria composed of two species (Egland et al., 2004). These investigators demonstrated that *Veillonella atypical*

provided a signal that increased the expression of the -amylase gene in *Streptococcus gordonii.*

One of the main defence mechanisms of bacteria is thought to be their capacity to create enzymes that disrupt the communication system of other species (Chen et al., 2013). In the same way as the auto-inducer CAI-1 generated by Vibrio cholerae has been described, QS molecules may also possess antibacterial qualities. In a concentration-dependent manner, this QS signal inhibits *P. aeruginosa* in two different ways. While at low concentrations it was observed to inhibit *P. aeruginosa* QS, at higher concentrations this AI caused pore formation in the *Pseudomonas* membrane, which resulted in cell death (Ganin et al., 2012). Recent research has shown a signalling function for the exopolysaccharides made by the B. subtilis eps operon in addition to the traditional QS mediators. The extracellular domain of a tyrosine kinase recognises this polymer and activates its own synthetic pathway as a result (Elsholz et al., 2014).

d. Cellular physiology in heterogeneous communities is spatially regulated

Within a multispecies biofilm, microorganisms are not arranged randomly but rather adhere to a pattern that improves the fitness of the entire community (Robinson et al., 2010). i.e., species are layered, clustered, or well-mixed, (Elias and Banin,2012).

When the biofilm is subjected to hazardous substances, its spatial organization somewhat influences bacterial survival (Simes et al., 2009) regarding nutritional, oxygen, and metabolite gradients. This greatly relies on the relationships among the species and the regional microenvironments in the matrix (Stewart and Franklin, 2008). *Kocuria* sp. was shown to protect *Staphylococcus sciuri* from chlorine exposure, and oxygen deprivation in regionally organised multispecies biofilms was proposed as an explanation (Leriche et al., 2003). A better rate of survival after chlorine exposure was also caused by the organised relationship of *Burkholderia cepacia* and *P. aeruginosa* and their associated cell physiologies (Behnke et al., 2011). According to Lewis (2010), a particular subpopulation of cells referred to as persisters correlates to physiological forms that occur in modest amounts in the biofilm but are extremely resistant to being killed by biocides. It has yet to be extensively studied how persister cells develop in multispecies biofilms. Still, it is known that they do so in response to adverse conditions such as food deprivation or oxidative stress (Wang and Wood, 2011). It has been established that P. aeruginosa secretes the siderophore pyocyanin to cause oxidative stress and thereby competes against other bacterial species (Tomlin et al., 2001).

Exogenous pyocyanin has thus been demonstrated to cause *Acinetobacter baumannii*, an emerging pathogen isolated from the same sites of infection as *P. aeruginosa* and capable of forming mixed biofilms with it, to develop a sub-population of persister cells (Bhargava et al., 2014).

Conclusion

A growing number of studies have shown instances of cross-resistance between various antimicrobials, such as disinfectants and antibiotics, as society becomes more conscious of the rise in bacterial resistance to antibiotics (Lee et al., 2014). Interspecies bacterial interactions in spatially organised biofilms are one process that gives rise to the tolerance of bacteria to chemical disinfectants and has been largely underappreciated in recent years (Yap et al., 2014). One major issue with these biological connections is the increase in infection persistence, which is encouraged by the local flora's protection.

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