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Resistance of bacterial biofilms to disinfectants: a review

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A biofilm can be defined as a community of microorganisms adhering to a surface and surrounded by a complex matrix of extrapolymeric substances. It is now generally accepted that the biofilm growth mode induces microbial resistance to disinfection that can lead to substantial economic and health concerns. Although the precise origin of such resistance remains unclear, different studies have shown that it is a multifactorial process involving the spatial organization of the biofilm. This review will discuss the mechanisms identified as playing a role in biofilm resistance to disinfectants, as well as novel anti-biofilm strategies that have recently been explored.

Keywords: biofilm; biocide; resistance; tolerance; adaptation; spatial architecture; control

Introduction

Disinfectants are chemical agents used on inanimate objects to inactivate virtually all recognized pathogenic microorganisms (Centers for Disease Control and Prevention, USA). Unlike antibiotics, which are chemotherapeutic drugs mostly used internally to control infections and which interact with specific structures or metabolic processes in microbial cells, disinfectants act non-specifically against multiple targets (Meyer and Cookson 2010). The mode of action of disinfectants depends on the type of biocide employed, as has been extensively described in numerous reviews (McDonnell and Russell 1999; Russell 2003). Potential target sites in Gram-positive or Gram-negative bacteria are the cell wall or outer membrane, the cytoplasmic membrane, functional and structural proteins, DNA, RNA and other cytosolic components. Disinfection treatments are used in medical, industrial and domestic environments to control the biocontamination of surfaces. Although these biocide treatments eliminate most surface contamination, some microorganisms may survive and give rise to substantial problems in terms of public health. Indeed, numerous reports have highlighted the survival of microorganisms after cleaning and disinfection in food (Bagge-Ravn et al. 2003; Weese and Rousseau 2006; Stocki et al. 2007), medical (Deva et al. 1998; Martin et al. 2008) and domestic environments (Cooper et al. 2008). The resistance of microorganisms to disinfection is frequently associated with the presence of biofilms on surfaces (Bressler et al. 2009; Vestby et al. 2009). In most wet environments, microorganisms are able to adhere to a surface, producing a matrix of extracellular polymeric substances (EPS) mainly composed of exopolysaccharides, proteins and nucleic acids (Costerton et al. 1995; Branda et al. 2005; Hoiby et al. 2010). Cells embedded in the biofilm matrix are well known to express phenotypes that differ from those of their planktonic counterparts, and to display specific properties including an increased resistance to biocide treatments (Nett et al. 2008; Smith and Hunter 2008; Wong et al. 2010). The definition of 'resistance' needs to be clarified as it changes depending on whether planktonic or biofilm cells are considered. In the former case, a bacterial strain is defined as being resistant to a biocide if it is not inactivated by a specific concentration or period of exposure that usually inactivates the majority of other strains (Langsrud et al. 2003). Biofilm cells, conversely, are generally said to be resistant by comparison with their planktonic counterparts. Bacterial resistance to biocides may be intrinsic, genetically acquired or phenotypic (tolerance) (Langsrud et al. 2003; Russell 2003). Biofilm insusceptibility is sometimes considered to be a tolerance rather than a real 'resistance' since it is mainly induced by a physiological adaptation to the biofilm mode of life (sessile growth, nutrient stresses, contact with repeated sub-lethal concentrations of disinfectant) and can be lost or markedly reduced when biofilm cells revert to the planktonic state (Russell 1999). Nevertheless, stable resistant variants can appear in biofilms (see later section).

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Therefore, throughout this review, the general term of 'biofilm resistance' will be used to refer to biofilm insusceptibility when compared to the planktonic state.

As opposed to planktonic cells, for which several well-defined standards have been published (EN 1040, NF T 150), fewer standard methods are available to evaluate the susceptibility of biofilm cells to disinfectants. Standard protocols for planktonic cells can be adapted (Ntsama-Essomba et al. 1997; Meylheuc et al. 2006), or specially-designed systems can be used, such as the MBEC assay system (MBEC^{TM} assay system, Biofilm Technologies Ltd, Calgary, Alberta) (Ceri et al. 1999) which has recently been approved as an ASTM standard method (no. E2799-11). The resistance of biofilm cells can be evaluated by measuring the ratio of concentrations (Rc) or time (Rt) required to achieve the same reduction in the planktonic or biofilm population, or by comparing the reductions obtained after exposure to the same concentration for the same period of time. Examples of Rc or Rt values found in the literature for commonly used biocides are shown in Table 1. Depending on the species and the biocide considered, these values can range from 1 to 1000 and from 20 to 2160 for Rc and Rt coefficients, respectively, thus highlighting the potentially high level of biofilm resistance to different disinfectants. It should be noted that it is often difficult to compare results between studies due to the lack of standardized protocols for the testing of biocides on biofilms (Buckingham-Meyer et al. 2007).

However, the availability of this global and quantitative information on biofilm resistance is not sufficient to improve the control of surface contamination. A clearer understanding of the mechanisms involved in biofilm resistance to biocides is thus a major concern among microbiologists. While many papers have focused on the mechanisms of biofilm resistance to antibiotics (Stewart and Costerton 2001; Stewart 2002; Fux et al. 2005; Hoiby et al. 2010), there are no recent reviews that specifically deal with the mechanisms of biofilm resistance to disinfectants. In this context, the present paper first aims to review the different factors related to the physiological and structural characteristics of a biofilm that influence its resistance to disinfectants. The most recent strategies that have been proposed in the literature to overcome biofilm resistance will then be considered.

What do we know about the mechanisms involved in biofilm resistance to disinfectants?

Diffusion/reaction limitations of disinfectants in biofilms

The formation and maintenance of mature biofilms are intimately linked to the production of an extracellular matrix (Branda et al. 2005; Ma et al. 2009). The multiple layers of cells and EPS may constitute a complex and compact structure within which biocides find it difficult to penetrate and reach internal layers, thus hampering their efficacy. For example, it has been shown that the chlorine levels measured within mixed biofilms of *P. aeruginosa* and *K. pneumoniae* using a microelectrode only reached 20% of the concentrations measured in the bulk liquid (De Beer et al. 1994). Similarly, Jang et al. (2006) showed that chlorine at 25 mg l^{-1} did not penetrate beyond a depth of 100 μm into a complex dairy biofilm that was 150–200 μm thick. The restricted diffusion of molecules within the range 3–900 kDa in biofilms due to size exclusion has already been reported (Thurnheer et al. 2003). But because biocides are often highly chemically reactive molecules, the presence of organic matter such as proteins, nucleic acids or carbohydrates can profoundly impair their efficacy (Lambert and Johnston 2001) and potential interactions between antimicrobials and biofilm components seem more likely to explain the limitations of penetration into the biofilm. Indeed, interesting data were produced when measuring the mean penetration time into a 1 mm-thick mixed biofilm of *P. aeruginosa* and *K. pneumoniae*, which was eight times higher for alkaline hypochlorite (48 min) than for chlorosulfamate (6 min), even though the latter has a higher molecular weight (Stewart et al. 2001). The decreased penetration of the alkaline biocide was hypothesized to be related to its greater capacity to react with matrix constituents. It was also reported that the delayed penetration of chlorine, glutaraldehyde and 2, 2-dibromo-3-nitrilopropionamide into an artificial biofilm model (*P. aeruginosa* entrapped in alginate gel beads) was due to interactions between the biocides and constituents in the gel beads (Grobe et al. 2002). Moreover, biocide molecules may simply adsorb to the cells and matrix components in biofilms. Using fluorescence spectroscopy correlation (FCS), the diffusion capabilities of fluorescent probes (latex beads and fluorescein isothiocyanate–dextran) with different sizes and electrical charges were measured in biofilms with variable EPS contents (Guiot et al. 2002). These authors demonstrated that in the absence of any electrostatic interactions, the majority of particles tested could penetrate and diffuse into a biofilm, suggesting that nothing prevented the diffusion of antimicrobial agents as a function of their size from a steric standpoint. Conversely, the diffusion of positively charged particles within negatively charged biofilms was hindered because of electrostatic interactions, as has also been proposed for cationic cetylpyridinium chloride (Ganeshnarayan et al. 2009). During the past 10 years, the emergence of innovative optical microscopy techniques such as confocal laser scanning microscopy (CLSM), and improvements in

Table 1. Resistance coefficients of biofilm cells compared to planktonic cells, obtained from studies involving the use of commonly used disinfectants.

Biocide	Strains	Rc	Rt	References	Biofilm formation method
Benzalkonium chloride	*Escherichia coli* ATCC 10536	1000		Ntsama-Essomba et al. (1997)	Continuous flow conditions in Tygon PVC tubing
	Pseudomonas aeruginosa ATCC 15442	100		Dubois-Brissonnet et al. (1995)	Static conditions on stainless steel coupons
	Pseudomonas aeruginosa ATCC 15442	250		Ntsama-Essomba et al. (1995)	Continuous flow conditions in Tygon PVC tubing
	Pseudomonas aeruginosa ERC1		2160	Grobe et al. (2002)	Alginate gel bead substrate in agitated medium
	Staphylococcus aureus ATCC 6538	50		Luppens et al. (2002)	Continuous flow conditions on glass coupons
	Listeria monocytogenes		>20	Frank and Koffi (1990)	Static conditions on glass slides
Benzalkonium chloride C12	*Pseudomonas aeruginosa* CIP A22	10		Campanac et al. (2002)	Continuous flow conditions in Tygon PVC tubing
Benzalkonium chloride C14	*Pseudomonas aeruginosa* CIP A22	50		Campanac et al. (2002)	Continuous flow conditions in Tygon PVC tubing
Benzalkonium chloride C16	*Pseudomonas aeruginosa* CIP A22	>50		Campanac et al. (2002)	Continuous flow conditions in Tygon PVC tubing
Benzalkonium chloride C18	*Pseudomonas aeruginosa* CIP A22	>50		Campanac et al. (2002)	Continuous flow conditions in Tygon PVC tubing
Benzalkonium chloride C12	*Staphylococcus aureus* CIP 53 154	>50		Campanac et al. (2002)	Continuous flow conditions in Tygon PVC tubing
Benzalkonium chloride C14	*Staphylococcus aureus* CIP 53 154	>50		Campanac et al. (2002)	Continuous flow conditions in Tygon PVC tubing
Benzalkonium chloride C16	*Staphylococcus aureus* CIP 53 154	>50		Campanac et al. (2002)	Continuous flow conditions in Tygon PVC tubing
Benzalkonium chloride C18	*Staphylococcus aureus* CIP 53 154	>50		Campanac et al. (2002)	Continuous flow conditions in Tygon PVC tubing
Cetrimide	*Pseudomonas aeruginosa* ATCC 15442	>400		Dubois-Brissonnet et al. (1995)	Static conditions on stainless steel coupons
	Pseudomonas aeruginosa ATCC 15442	>400		Ntsama-Essomba et al. (1995)	Continuous flow conditions in PVC tubing
Chlorine	*Pseudomonas aeruginosa* ERC1		290	Grobe et al. (2002)	Alginate gel beads in agitated broth medium
	Pseudomonas aeruginosa ATCC 15442	20		Dubois-Brissonnet et al. (1995)	Static conditions on stainless steel coupons
	Pseudomonas aeruginosa ATCC 15442	5		Ntsama-Essomba et al. (1995)	Continuous flow conditions in Tygon PVC tubing
Sodium hypochlorite	*Escherichia coli* ATCC 10536	5		Ntsama-Essomba et al. (1997)	Continuous flow conditions in Tygon PVC tubing
	Staphylococcus aureus ATCC 6538	600		Luppens et al. (2002)	Continuous flow conditions on glass coupons
	Mycobacterium fortuitum (clinical isolate)	38		Bardouniotis et al. (2003)	MBEC^{TM} assay system on rocking platform
	Mycobacterium marinum (clinical isolate)	>2		Bardouniotis et al. (2003)	MBEC^{TM} assay system on rocking platform
	P. aeruginosa + *K. pneumoniae*		>60	Stewart et al. (2001)	Continuous flow conditions on stainless steel coupons
Hydrogen peroxide	*Mycobacterium fortuitum* (clinical isolate)	1		Bardouniotis et al. (2003)	MBEC^{TM} assay system on rocking platform
	Mycobacterium marinum (clinical isolate)	1		Bardouniotis et al. (2003)	MBEC^{TM} assay system on rocking platform
Peracetic acid + hydrogen peroxide	*Pseudomonas aeruginosa* ATCC 15442	40		Dubois-Brissonnet et al. (1995)	Static conditions on stainless steel coupons
	Pseudomonas aeruginosa ATCC 15442	4		Ntsama-Essomba et al. (1995)	Continuous flow conditions in Tygon PVC tubing
	Escherichia coli ATCC 10536	25		Ntsama-Essomba et al. (1997)	Continuous flow conditions in Tygon PVC tubing
Chlorosulfamate	*P. aeruginosa* + *K. pneumoniae*		>60	Stewart et al. (2001)	Continuous flow conditions on stainless steel coupons
Glutaraldehyde	*Mycobacterium fortuitum* (clinical isolate)	1.15		Bardouniotis et al. (2003)	MBEC^{TM} assay system on rocking platform
	Mycobacterium marinum (clinical isolate)	2		Bardouniotis et al. (2003)	MBEC^{TM} assay system on rocking platform
	Pseudomonas aeruginosa ERC1		30	Grobe and Stewart (2000)	alginate gel bead supports in agitated medium
	Pseudomonas aeruginosa ERC1		47	Grobe et al. (2002)	alginate gel bead supports in agitated medium
Chlorhexidine digluconate	*Staphylococcus epidermidis* ATCC 35984	4		Karpanen et al. (2008)	Static conditions in polystyrene microtitre plate
Silver nitrate	*Mycobacterium marinum* (clinical isolate)	4.2		Bardouniotis et al. (2003)	MBEC^{TM} assay system on rocking platform
	Mycobacterium fortuitum (clinical isolate)	12		Bardouniotis et al. (2003)	MBEC^{TM} assay system on rocking platform
Phénol	*Pseudomonas aeruginosa* ATCC 15442	1		Dubois-Brissonnet et al. (1995)	Static conditions on stainless steel coupons
	Pseudomonas aeruginosa ATCC 15442	1		Ntsama-Essomba et al. (1995)	Continuous flow conditions in Tygon PVC tubing
Oregano	*Staphylococcus epidermidis* ATCC 35984	4		Nostro et al. (2007)	Static conditions in polystyrene microtitre plate
Carvacrol	*Staphylococcus epidermidis* ATCC 35984	4		Nostro et al. (2007)	Static conditions in polystyrene microtitre plate
Thymol	*Staphylococcus epidermidis* ATCC 35984	4		Nostro et al. (2007)	Static conditions in polystyrene microtitre plate
	Staphylococcus epidermidis ATCC 35984	0.125		Karpanen et al. (2008)	Static conditions in polystyrene microtitre plate
Tea tree oil	*Staphylococcus epidermidis* ATCC 35984	16		Karpanen et al. (2008)	Static conditions in polystyrene microtitre plate
Eucalyptus oil	*Staphylococcus epidermidis* ATCC 35984	4		Karpanen et al. (2008)	Static conditions in polystyrene microtitre plate

Note: $Rc = C_{biofilm}/C_{planktonic}$, where $C_{biofilm}$ corresponds to the biocide concentration required to kill a given level of biofilm cells and $C_{planktonic}$ corresponds to the biocide concentration required to kill the same level of planktonic cells. $Rt = t_{biofilm}/t_{planktonic}$, where $t_{biofilm}$ corresponds to the biocide exposure time at a fixed concentration necessary to kill a given level of biofilm cells and $t_{planktonic}$ corresponds to the biocide exposure time at a fixed concentration necessary to kill the same level of planktonic cells.

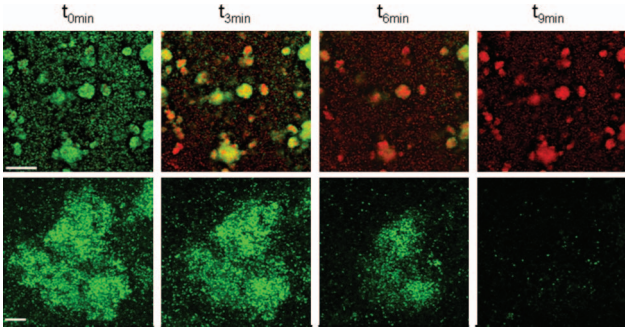

Figure 1. Visualization of cell inactivation in *S. aureus* ATCC 27217 using the BacLight Live/Dead viability kit (Invitrogen) and in a *P. aeruginosa* ATCC 15442 biofilm using the Chemchrome V6 esterasic marker (AES Chemunex) during benzalkonium chloride treatments (0.5% w/v), 0, 3, 6 and 9 min after biocide application. For *S. aureus*, total cells are stained green (Syto9) and permeabilized cells are stained red (propidium iodide). For *P. aeruginosa*, viable (non-permeabilized) cells are stained green, the loss of fluorescence corresponding to the leakage of fluorophores out of cells permeabilized by biocide activity. Each image corresponds to a horizontal section situated 5–10 μm from the substratum. Scale bar = 20 μm.

fluorescent labeling, have provided an opportunity for the direct investigation of biocide reactivity within the native structure of biofilms (Bridier et al. 2011b). A direct time-lapse confocal microscopic technique has been developed to enable the real-time visualization of biocide activity within a biofilm (Stoodley et al. 2001; Hope and Wilson 2004; Takenaka et al. 2008; Davison et al. 2010; Bridier et al. 2011a). This can provide information on the dynamics of biocide action in the biofilm and the spatial heterogeneity of bacteria-related susceptibilities that are crucial to a better understanding of biofilm resistance mechanisms. Experimentally, after staining with fluorescent markers to enable the real-time monitoring of cell inactivation, the three-dimensional structure of the biofilm is scanned by CLSM at regular intervals during exposure to the biocide and then spatial and temporal patterns of biocide action are visualized in the structure (Figure 1).

This method enabled the demonstration that the penetration of QAC to the center of an *S. epidermidis* biofilm cluster took 60 times longer than the time estimated for diffusive access in the absence of sorption (Davison et al. 2010). In *P. aeruginosa* biofilms, different patterns of fluorescence loss were observed depending on the biocide used: peracetic acid caused a uniform and linear loss of cell viability, demonstrating that the greater resistance of biofilm cells could not be due to limitations of penetration (Bridier et al. 2011a). By contrast, the same study showed that benzalkonium chloride firstly inactivated cells located in peripheral layers of clusters. The positive charge and hydrophobic nature of the biocide could therefore explain the delayed penetration observed. In a *P. aeruginosa* biofilm, the level of bacterial resistance to benzalkonium chloride increased with the C-chain length of the quaternary ammonium compound (QAC from C12 to C18) (Campanac et al. 2002). This increase in the C-chain length, leading to an increase in the hydrophobicity of the molecule, was hypothesized to limit its penetration through the hydrophilic matrix and thus cause a progressive loss of bactericidal efficacy within the biofilm. More recently, the role of the C-chain length in the binding of QAC to biofilm components, probably through hydrophobic interactions, has also been proposed (Sandt et al. 2007). In another recent paper, it was reported that bacterial cell wall hydrophobicity could alter the diffusion of nanoparticles within a biofilm (Habimana et al. 2011), suggesting that cell wall interfacial components such as peptidoglycan, fimbriae, capsules and the S-layer could also affect diffusion of compounds within the biofilm. Moreover, other components such as enzymes are present in the extracellular matrix and may play a role in neutralizing toxic compounds. For example, hydrogen peroxide was shown to be able to penetrate and partially kill cells only in a biofilm formed by catalase-deficient *P. aeruginosa* (Stewart et al. 2000). In a wild-type biofilm, the bacteria were protected from H_2O_2 penetration by catalase-mediated destruction of the biocide.

These studies illustrate that transport limitations may be a mechanism that contributes to the resistance of biofilms to disinfectants. This seems to be related mainly to physicochemical interactions between the biocide and EPS or bacterial cells rather than steric hindrance inside the biofilm. Nevertheless, although diffusion/reaction problems can partly explain the resistance of biofilms, some results have shown that despite an effective penetration of a biocide into a biofilm, only a low level of inactivation was achieved (Stewart et al. 2001). Moreover, the resistance of a *S. aureus* biofilm to a QAC could, to a great extent, be attributed to phenotypic modifications to cells rather than the protective presence of an EPS matrix (Campanac et al. 2002). These findings highlight the existence of additional mechanisms involved in biofilm resistance that will be presented in the next sections.

Phenotypic adaptations of biofilm cells to sublethal concentrations of disinfectants

During a disinfection process, the reaction-diffusion limited penetration of biocides into a biofilm may result in only low levels of exposure to the antimicrobial agent in deeper regions of the biofilm. Biofilm cells

will therefore develop adaptive responses to sublethal concentrations of the disinfectant. Increased survival following the same QAC shock was reported in adapted *Pseudomonas aeruginosa*, alongside concomitant modifications to membrane composition (Jones et al. 1989; Mechin et al. 1999). Adaptation depends on the disinfectant being effective in the presence of QACs, contrarily to sodium dichloroisocyanurate or tri-sodium phosphate (Guérin-Méchin et al. 1999). Moreover, cross-resistance to other QACs (Mechin et al. 1999) or to antibiotics (Braoudaki and Hilton 2004) has been reported for adapted cells. The adaptation of biofilm cell populations to disinfectants was first reported in *Salmonella* (Mangalappalli-Illathu et al. 2008): biofilm cells displayed better adaptation to benzalkonium chloride than their planktonic counterparts after continuous exposure. In that case, the up-regulation of specific proteins involved in energy metabolism, protein biosynthesis, adaptation (CspA) and detoxification (Mangalappalli-Illathu and Korber 2006), together with a shift in the fatty acid composition (Mangalappalli-Illathu et al. 2008) suggested that biofilm-specific adaptation conferred better survival on the biofilm-adapted population.

Moreover, the conditions prevailing during initial adhesion to a substratum may play a key role in biofilm resistance to a disinfectant as it is the initial step in the construction of biofilm architecture (Dynes et al. 2009). Cell morphology, spatial distribution and the relative amounts of exopolymer matrix in *Pseudomonas* biofilms were shown to differ in the presence of sublethal doses of chlorhexidine, benzalkonium chloride or triclosan. Chlorine dioxide at sublethal doses has also been shown to stimulate biofilm formation in *Bacillus subtilis* (Shemesh et al. 2010). These authors demonstrated that transcription of the major genes responsible for biofilm matrix production was enhanced in the presence of chlorine throughout activation of the membrane-bound kinase KinC. The ability of chlorine to collapse membrane potential has been proposed to provoke activation of this kinase.

Phenotypic adaptations of cells in a biofilm environment

From the attachment of cells to the development of a three-dimensional structure, the growth of a biofilm is associated with physiological adaptations of cells that may lead to an increase in resistance to biocides. These phenotypic adaptations result from the expression of specific genes in response to their direct micro-environmental conditions. Comparisons of gene expression profiles, and proteomic analyses of planktonic and biofilm states in different species, support this idea (Prigent-Combaret et al. 1999; Whiteley et al. 2001; Sauer 2003; Vilain et al. 2004; Shemesh et al. 2007). For example, some studies have shown that just after a cell reaches a surface, genes coding flagellar proteins are repressed and other genes coding for EPS and adhesin proteins such as curli are induced (Davies et al. 1993; Vidal et al. 1998; Prigent-Combaret et al. 2000, 2001; Sauer and Camper 2001). These changes induced by cell adhesion can lead to the appearance of more resistant phenotypes, as suggested by studies reporting the greater resistance of cells that are merely adhered to a surface when compared with their planktonic counterparts (Frank and Koffi 1990; Chavant et al. 2004; Kamgang et al. 2007).

Following the adhesion step, bacteria start to develop into a biofilm with a three-dimensional structure. A direct consequence of the growth of this structure is the emergence of chemical gradients within the biofilm. Cells located at the periphery of the cluster have access to nutrients and oxygen, while bacteria in internal biofilm layers experience nutrient-poor micro-environments where the concentrations of metabolic waste products are higher. This chemical heterogeneity governs the onset of physiological heterogeneity (Xu et al. 1998; Stewart and Franklin 2008). Two Green Fluorescent Protein (GFP) gene constructs were used to demonstrate the existence of stratified patterns of growth and protein synthesis in *P. aeruginosa* biofilms (Werner et al. 2004). Protein synthesis and active cell growth were restricted to the zone where oxygen was available and represented a narrow band in contact with the medium. Cells with distinctive metabolic rates were present throughout the three-dimensional structure, thus constituting a physiologically heterogeneous population. Alterations to growth and activity rates induced modifications to membrane composition and the expression of defense mechanisms that could lead to an increased resistance of bacteria to biocides (Stewart and Olson 1992; Lisle et al. 1998; Saby et al. 1999; Taylor et al. 2000; Sabev et al. 2006). Indeed, it is now widely accepted that the development of a stress response is an important feature of the life cycle of biofilms (Beloin and Ghigo 2005; Coenye 2010). For example, it was reported in *P. aeruginosa* that RpoS, which is the principal regulator of a general stress response, was three times more strongly expressed in 3-day old biofilm cells than in stationary planktonic cells (Xu et al. 2001). Different genes involved in the oxidative stress response have also been shown to be induced in biofilms of *L. monocytogenes, P. aeruginosa, E. coli* or *Tannerella forsythia* (Sauer et al. 2002; Tremoulet et al. 2002; Ren et al. 2004; Pham et al. 2010) and may afford protection for bacteria against the activity of oxidizing agents. Furthermore, the up-regulation or induction of genes coding to multidrug efflux pumps in biofilms may be

another possible mechanism to explain bacterial biocide resistance, as already shown for antibiotics (Gillis et al. 2005; Kvist et al. 2008). Efflux pumps are systems that enable cells to rid themselves of toxic molecules and allow bacteria to survive in the presence of such substances. One example of a well-known system specific to biocides is the QAC efflux system of *S. aureus* which is responsible for its high level of resistance to QAC and cationic biocides (Mitchell et al. 1998; Smith et al. 2008). Similar systems have been identified in other species and also for other biocides such as triclosan or chlorhexidine (Poole 2005; Villagra et al. 2008). However, the induction of biocide efflux pumps in biofilms has not yet been clearly demonstrated and further research is necessary to determine whether this phenomenon plays an important role in biofilm resistance.

The appearance of a biofilm-specific phenotype has been shown to be at least partly induced by quorum sensing. Indeed, cell-to-cell communication has been identified as controlling biofilm development in a number of bacterial species (Parsek and Greenberg 2000; Huber et al. 2001; Cvitkovitch et al. 2003; Labbate et al. 2004; Waters et al. 2008). Interestingly, it was observed that a *lasI* signaling *P. aeruginosa* mutant formed a biofilm with a flat architecture when compared to the wild-type, and also displayed evidence of its increased susceptibility to SDS (Davies et al. 1998). Similarly, *lasI* and *rhlI P. aeruginosa* mutants exhibited increased sensitivity to hydrogen peroxide and phenazin methosulfate (Hassett et al. 1999). Moreover, these authors demonstrated that the expression of catalase and superoxide dismutase genes coding to protective enzymes against oxidizing stress were under the control of quorum sensing. Consistent with these findings, regulation of the stress response by quorum sensing has more recently been reported in other species (Lumjiaktase et al. 2006; Joelsson et al. 2007; Pontes et al. 2008).

A final illustration of the adaptation of specific phenotypes that may contribute to the bacterial resistance observed in biofilms is that a small fraction of the population may enter a highly-protected state displaying dramatic resistance and referred to as persisters (Harrison et al. 2005; Lewis 2005). These cells are phenotypic variants but not genetic mutants and have also been identified in planktonic bacterial populations (Lewis 2001; Shah et al. 2006). One assumption is that persisters develop more frequently in a biofilm than in a planktonic culture, perhaps induced by the specific environmental conditions prevailing within the structure, and may therefore contribute to better antimicrobial protection in the biofilm (Stewart 2002; Roberts and Stewart 2005).

Gene transfers and mutations

Lateral gene transfer participates in microbial adaptation to the environment through the exchange of genetic sequences including plasmids, transposons or integrons that confer specific phenotypic traits on cells such as their metabolic capabilities, virulence expression and antimicrobial resistance (Top and Springael 2003; Kelly et al. 2009; Hannan et al. 2010). For example, QAC resistance genes carried by transferable genetic elements have been widely identified (Bjorland et al. 2001; Gillings et al. 2009; Elhanafi et al. 2010). Different studies have generated evidence suggesting that biofilms may constitute an optimum environment for the exchange of genetic material (Hausner and Wuertz 1999; Maeda et al. 2006; Ando et al. 2009; Nguyen et al. 2010), leading to the dissemination of biocide resistance cassettes within the population. Indeed, high cell density, the presence of a matrix, the release of large quantities of DNA or nutrient conditions within biofilms may promote conjugation and transformation processes. Another consideration is that biofilm growth can lead to the emergence of extensive genetic diversity within a bacterial population. Driffield et al. (2008) showed that cells in a *P. aeruginosa* biofilm displayed an increase of up to 105-fold in mutability when compared to a planktonic culture. It was observed that *P. aeruginosa* mutations mostly occurred in microcolonies but not elsewhere in a biofilm or in planktonic cultures, showing that these dense areas of biofilm could indeed favor mutations (Conibear et al. 2009). Different studies have reported the appearance of genetic variants in biofilms that display distinctive phenotypic traits (Boles et al. 2004; Kirisits et al. 2005; Allegrucci and Sauer 2007). The production of variants may lead to the appearance of more resistant subpopulations that will enhance the fitness of the entire population under stressful conditions. For example, when *P. aeruginosa* was grown in a biofilm for 5 days, three different stable colony morphologies, called typical (wild-type colony), mini (small variant colony) and wrinkly (rough variant colony), appeared after plating on Petri dishes, whereas the initial inoculum (broth culture) produced only one colony morphology (typical) (Boles et al. 2004). Using CLSM, these authors demonstrated that the wrinkly variant displayed greater ability to form a biofilm and with larger cell clusters when compared to the wild-type strain. Moreover, the presence of a wrinkly subpopulation was responsible for the better resistance of the biofilm to hydrogen peroxide because this population constituted $>98\%$ of the biofilm cells after exposure to the biocide, whereas it had only reached 12% prior to treatment. In addition, the authors showed that a biofilm composed only of wild-type strains (typical

colony) demonstrated a high level of susceptibility to the biocide. These results therefore reveal how genetic mutations induced by biofilm formation can lead to improved resistance to a biocide. One issue that nonetheless remains following these observations concerns the mechanisms involved in the production of genetic variants within a biofilm. Spontaneous mutations related to replication errors are a natural explanation. However, it was found that endogenous oxidative stress provoked double-stranded DNA breaks that caused the emergence of variants when these breaks were repaired by recombinational DNA repair genes (Boles and Singh 2008). In a previous study, Ciofu et al. (2005) also reported that the occurrence of hypermutable *P. aeruginosa* was linked to oxidative stress in cystic fibrosis infection. In addition, the endogenous production of reactive oxygen intermediates within biofilm microcolonies has already been reported (Mai-Prochnow et al. 2008). Taken together, these observations suggest that the oxidative stress induced in a biofilm by a harsh microenvironment may cause the emergence of biocide resistant variants through the enhancement of genetic mutations.

Pathogen protection in multispecies biofilms

In their natural environments, it is clear that biofilms are complex mixtures of different species rather than the model single species biostructures studied by the majority of laboratories (Lyautey et al. 2005; Simoes et al. 2008; Burmolle et al. 2010; Zijnge et al. 2010) (Figure 2). In these complex consortia, species interactions can lead to the emergence of specific biofilm phenotypes. A recent study reported that the food pathogen *E. coli* O157:H7 formed a biofilm with a 400-fold higher biovolume when it was grown in association with *Acinetobacter calcoaceticus*, a meat factory commensal bacterium, rather than in a monoculture (Habimana et al. 2010). It was also shown that four strains isolated from a marine alga interacted synergistically in a biofilm to produce more biomass (Burmolle et al. 2006). Moreover, the mixed four-species biofilm displayed markedly higher resistance to hydrogen peroxide than any of the single-species biofilms. Indeed, numerous studies have demonstrated that multi-species biofilms are generally more resistant to disinfection than mono-species biofilms (Luppens et al. 2008; Simoes et al. 2009, 2010; Van der Veen and Abee 2010). Unfortunately, the mechanisms involved remain unclear. The specific nature and composition of a multi-species biofilm matrix is one of the explanations proposed. It has been suggested that chemical interactions between the polymers produced by each species may lead to a more viscous matrix (von Canstein et al. 2002; Burmolle et al. 2006) and thus reduce the permeation of biocides. Similarly, because a biocide can be inactivated in a biofilm matrix by enzymes, as previously suggested regarding the catalase-mediated inactivation of hydrogen peroxide in a *P. aeruginosa* biofilm (Stewart et al. 2000), the enzymes produced by the different species may act synergistically against toxic compounds so that non-productive species will benefit from the association through enzyme complementation (Shu et al. 2003). Another explanation is that because of the specific spatial arrangement of certain bacterial species within a biofilm, some strains may be protected from a biocide by their aggregation with others within the three-dimensional structure (Figure 2A and B). It was reported for instance that *Staphylococcus sciuri* was protected from chlorine treatment because of its association with microcolonies formed by *Kocuria* sp., a more resistant strain (Leriche et al. 2003). As well as these possible interactions with other bacterial species, bacteria in a biofilm can also be protected by eukaryotic microorganisms (Figure 2C and D). Many bacterial species have been shown to survive within various amoebal species (for a review see Thomas et al.

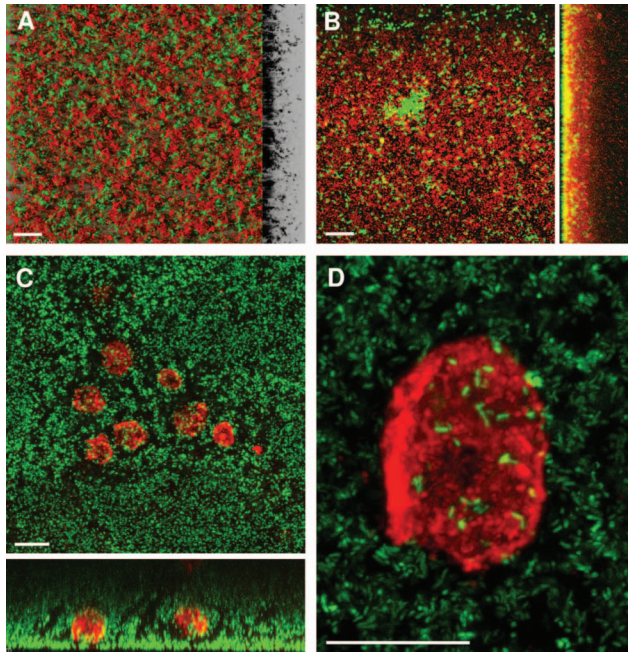

Figure 2. Confocal imaging of mixed biofilms. (A) Three-dimensional projection of a mixed 24 h-biofilm of *E. coli* mCherry (red) and *P. aeruginosa* GFP (green). (B) Section of a mixed 24 h-biofilm of *S. aureus* mCherry (red) and *P. aeruginosa* GFP (green). (C) Section of a mixed 24 h-biofilm of *P. aeruginosa* GFP (green) and the ciliate protozoan *Tetrahymena pyriformis* (red). (D) Higher magnification of the mixed biofilm showing the presence of *P. aeruginosa* (green) in *T. pyriformis* (red). Scale bar = 20 μm.

2010). Trophozoites are the actively dividing forms of amoebae; increased resistance to disinfection has been reported for bacteria internalized within trophozoites. The survival and resistance of a range of intracellular bacterial pathogens when challenged with free chlorine were investigated and it was concluded that *Acanthamoeba castellanii* trophozoites played a predominant role in the survival of these pathogens (King et al. 1988). Similar studies have reported *Burkholderia pseudomallei* as being more resistant to monochloramine, chlorine and UV once it is protected in *Acanthamoeba astronyxis* trophozoites (Howard and Inglis 2005). A decreased efficacy of silver and copper was reported against *Legionella pneumophila* and *Pseudomonas aeruginosa* within *Acanthamoeba polyphaga* trophozoites (Hwang et al. 2006). Growth in different amoebal hosts may also influence the biocide susceptibility of a particular bacterial strain; this was recently evidenced with *L. pneumophila* replicated from *Hartmannella vermiformis* which displayed greater resistance to chlorine than cells replicated from *A. castellanii* (Chang et al. 2009). Cysts are the dormant stage of amoebae and form in the event of unfavorable conditions such as nutrient depletion and various physical and chemical stresses, including biocidal treatments. The encystment of amoebae is preceded by the expulsion of food vacuoles and vesicles (Schuster 1979). These vesicles may contain bacteria that are protected from the effect of biocides (Berk et al. 1998). The cysts of several amoebal species (mostly *Acanthamoeba* spp.) have been demonstrated to resist extremely high concentrations of biocides used for a variety of applications (Coulon et al. 2010; Thomas et al. 2010). Various bacterial species, including *L. pneumophila* (Kilvington and Price 1990), *Legionella micdadei* (Fallon and Rowbotham 1990), more than 15 mycobacterial species (Adekambi et al. 2006), *Francisella tularensis* (Abd et al. 2003) and *Vibrio cholerae* (Thom et al. 1992; Abd et al. 2005) have been reported to survive within amoebal cysts, thus benefiting from the extremely efficient protection they afford.

What are the prospective strategies to eradicate biofilms on industrial and medical devices?

From the studies reviewed in this paper, it is clear that biofilm resistance to disinfectants is a multifactorial process resulting from different mechanisms and causing the inefficiency of antimicrobials, even at the usable concentrations of commercial solutions (Krolasik et al. 2010). New control strategies are needed to overcome these limitations. Another consideration is that the regulatory landscape is changing and some disinfectants that are standard today will probably be banned during the next few years (Reach, EU Directive on Biocides, 98/8/EC). It is therefore becoming crucial to find alternative 'green' molecules or processes that are efficient in eradicating surface contamination. The next part of this review highlights some potential methods that might improve anti-biofilm strategies.

Targeting the EPS to denature the spatial organization of biofilms

The diffusion/reaction limitation within a biofilm structure is one of the main mechanisms implicated in its resistance to disinfectants. Optimizing the eradication or breakdown of the matrix will thus be essential to improving the disinfection process. It is well known that mechanical action can be effective in eliminating biofilms (Maukonen et al. 2003) by disrupting the EPS in the matrix and rendering microorganisms more accessible. In this context, the use of enzyme-based detergents could be a helpful tool to improve the cleaning process. However, it is first necessary to elucidate the precise composition of the biofilm matrix so that appropriate enzyme treatments can be applied. As a general rule, a biofilm matrix is mainly composed of polysaccharides and proteins (Tsuneda et al. 2003) associated with lipids or nucleic acids (Flemming and Wingender 2010), but its composition may display qualitative and quantitative variations depending on the strains and the growth conditions involved (Branda et al. 2005). For example, cellulose has been shown to be a crucial component in the extracellular matrix of *Salmonella* and *Escherichia coli* (Zogaj et al. 2001), and poly-N-acetylglucosamine is the major component of staphylococcal biofilms (Jabbouri and Sadovskaya 2010). Mucoid strains of *Pseudomonas aeruginosa* mainly produce alginate polymers, and non-mucoid strains produce distinct carbohydrate-rich polymers (Branda et al. 2005). Depending on the composition of the biofilm matrix, different enzymes are more appropriate, such as proteases, cellulases, polysaccharide depolymerases, alginate lyase, dispersin B or DNAse (Xavier et al. 2005; Orgaz et al. 2007; Jabbouri and Sadovskaya 2010). In industrial or medical environments, numerous microbial species grow within the same biofilm, thus increasing the biochemical heterogeneity of the matrix. Commercial enzyme formulations contain mixtures of enzymes with different substrate spectra. These enzymatic processes have the advantage of disaggregating biofilm clumps rather than just removing them from the surface, as is the case with mechanical action.

One possible way to utilize enzymatic processes could be to promote a natural degradation of the

biofilm matrix. When nutrients are depleted in the bulk of the biofilm, *P. fluorescens* naturally produces enzymes which degrade its EPS in order to become disseminated to a more favorable environment (Allison et al. 1998). Specific compounds could be developed to interact with the regulation of the genes controlling the self-destruction pathway of the biofilm.

As well as enzymes, some small molecules may also be efficient in assisting with the dispersal of biofilms. Recently, D-amino acids were shown to prevent and break down *Bacillus subtilis* biofilms by interfering with the integrity of the EPS matrix (Kolodkin-Gal et al. 2010). In addition, biosurfactants, such as rhamnolipids and short-chain fatty acids (eg *cis*-2-decenoic acid) may also promote biofilm disruption (Davies and Marques 2009; Dusane et al. 2010). Combinations of EPS treatments have also proven useful. For example, ultrasonic waves (Oulahal-Lagsir et al. 2003) or a surfactant (Parkar et al. 2004) were reported to enhance the efficacy of proteolytic enzymes.

These processes which denature EPS integrity are designed to disperse the bulk of surface contamination but are generally not efficient in killing bacteria. Pathogens may eventually be redeposited elsewhere and initiate a new biofilm cycle, thus emphasizing the importance of complementary antimicrobial strategies.

Towards natural antimicrobial strategies?

It is necessary for research on new antimicrobial strategies to focus on processes that display high lethal activity against pathogens, are efficient in penetrating the biofilm structure and are easily degraded in the environment. Recent years have seen the emergence of studies on the use of natural antimicrobials as anti-biofilm compounds. Plants are a rich source of active molecules with antimicrobial properties (Lewis and Ausubel 2006). Some compounds extracted from aromatic plants, which are natural and 'generally recognized as safe', have demonstrated their antimicrobial activity on planktonic bacteria. Some are now being evaluated for their potential in eradicating biofilms. Examples include carvacrol, a natural terpene extracted from thyme or oregano (Knowles et al. 2005), casbane diterpene, isolated from the ethanolic extract of a Brazilian native plant *Croton nepetaefolius* (Carneiro et al. 2011), thymoquinone, an active principle of Arabian *Nigella sativa* seed (Chaieb et al. 2011), and a naphthalene derivative isolated from *Trachyspermum ammi* seeds (Khan et al. 2010) which limit the formation of biofilms of various bacterial species. More interestingly, some of these compounds have been tested for their bactericidal activity on established biofilms. The ratio of concentrations (Rc) required to achieve the same reduction in a planktonic or biofilm *Staphylococcus epidermidis* population is about 4 for oregano oil, thymol or carvacrol (Nostro et al. 2007), which compares well with that of most chemical agents. Eucalyptus oil, tea tree oil or α-terpineol have also displayed considerable efficacy in eradicating biofilms (Karpanen et al. 2008; Budzynska et al. 2011). A promising method for the application of anti-biofilm essential oils is to vaporize these volatile compounds so as to enhance their access to the biological targets. For example, the vaporization of allyl isothiocyanate, cinnamaldhehyde, and carvacrol has been shown to markedly inactivate *E. coli* O157:H7 attached to the surface of lettuce leaves (Obaidat and Frank 2009).

There is also renewed interest in controlling biofilms through the use of bacteriophages. Phages are viruses that infect and lyse bacteria. Phages easily diffuse through the EPS (Briandet et al. 2008) and are active on established biofilms (Donlan 2009). For example, it has been shown that the φIBB-PF7A phage was highly efficient in removing a *P. aeruginosa* biofilm within a short period of time (Sillankorva et al. 2008). Moreover, many phages produce depolymerases that hydrolyze the extracellular polymers in a biofilm and trigger its disruption. The drawbacks of phages are their narrow host range, but phage mixtures or engineered phages could provide interesting solutions. For example, a phage expressing a biofilm-degrading enzyme was engineered by one team (Lu and Collins 2007) and demonstrated efficacy on *E. coli* biofilms, reducing in the biofilm cell counts by 99.997%. Recent studies have also proposed the use of phage lysin against *S. aureus* as an alternative agent for skin decontamination (Fischetti 2008). In addition, because cell-to-cell communication is fundamental to biofilm signaling, novel antimicrobials that target quorum sensing are now emerging. Several quorum-sensing inhibitors, such as brominated furanones, have succeeded in interfering with biofilm formation (Ni et al. 2009; Sintim et al. 2010). Similarly, the cyclic-di-GMP pathway that has been shown to regulate diverse cellular processes involved in biofilm formation and virulence could be a promising antimicrobial target (Romling and Amikam 2006; Sintim et al. 2010). Other authors have proposed targeting of the iron uptake pathway to prevent *E. coli* from developing biofilms through the addition of competitive Zn^{2+} or Co^{2+} cations (Hancock et al. 2010).

Combining strategies to optimize biofilm control

One strategy to prevent the induction of bacterial adaptation to disinfectant within biofilm structures could be to substantially increase the concentration of the antimicrobial agent. However, this approach might

not guarantee biofilm eradication and it would be costly and not environmentally-friendly. Moreover, microbial communities can be comprised of several microorganisms with distinct mechanisms of resistance. Thus, the eradication of biofilms could be achieved through the combined use of treatments with different spectra and modes of action. In this respect, synergistic actions have been reported in numerous papers between two or more processes, when the effect observed is stronger than might have been predicted by adding the effects exerted by each process separately (Nazer et al. 2005). One method to assess a synergistic effect in bactericidal activity is to calculate the Fractional Bactericidal Concentration (FBC) (Harrison et al. 2008). Numerous processes have thus been evaluated, associating chemical, natural or physical treatments. For example, combinations of sodium hypochlorite and hydrogen peroxide, Cu^{2+} ions and quaternary ammonium compounds, eucalyptus oil and chlorhexidine, silver and surfactant, or bacteriophage and alkaline cleaner can all act synergistically to eradicate established biofilms (Sharma et al. 2005; DeQueiroz and Day 2007; Harrison et al. 2008; Hendry et al. 2009; Rivardo et al. 2010). Physical treatments can also be employed in association with chemical disinfectants; low-intensity ultrasonic or sonic agitation enhances the action of chlorhexidine against biofilm bacteria (Shen et al. 2010) and a combination of ultraviolet light with chlorine dioxine was shown to be more effective in eradicating drinking water biofilms than the two treatments applied separately (Rand et al. 2007).

Conclusions

Because biofilms constitute a privileged way of life for bacteria, a clearer understanding of the processes involved in their marked resistance to disinfectants is of crucial importance for their control. From the studies reviewed in this paper, it is now evident that biofilm resistance to disinfectant is: (i) intimately related to the three-dimensional structure of the biofilm, (ii) heterogeneous within the biostructure and (iii) multifactorial, resulting from an accumulation of different mechanisms. In view of the observed resistance of biofilms to disinfectants, it is now crucial that regulatory standards which focus on assessing the efficacy of a disinfectant must take account of the 'mode of life' of biofilms.

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