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Biofilm responses to oxidative stress

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Abstract

Biofilms constitute the predominant microbial style of life in natural and engineered ecosystems. Facing harsh environmental conditions, microorganisms accumulate reactive oxygen species (ROS), potentially encountering a dangerous condition called oxidative stress. While high levels of oxidative stress are toxic, low levels act as a cue, triggering bacteria to activate effective scavenging mechanisms or to shift metabolic pathways. Although a complex and fragmentary picture results from our current knowledge of pathways activated in response to oxidative stress, three main responses are shown to be central: the existence of common regulators, the production of extracellular polymeric substances and biofilm heterogeneity. An investigation into mechanisms activated by biofilm in response to different oxidative stress levels could have important consequences from ecological and economic points of view, and could be exploited to propose alternative strategies to control microbial virulence and deterioration.

Keywords

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Biofilms

The formation of biofilms - microbial communities embedded in a self-produced polymeric matrix attached to a surface- is an ancient and universal trait that enables microorganisms to develop coordinated architectural and survival strategies (Hall-Stoodley et al. 2004; Vlamakis et al. 2013). It is now largely accepted that biofilms constitute the predominant style of microbial life in natural and engineered ecosystems (Mc Dougald et al. 2011; Villa & Cappitelli 2013). Indeed, biofilm cells express specific phenotype traits that confer adaptability to environmental change (Stewart et al. 2008) and higher resistance to adverse conditions, such as limited nutrient availability, desiccation, low pH and predation (Rinaudi & Giordano 2010). The biofilm structure, its surface adhesion and the polymeric matrix provide cells with a high nutrient and water concentration and a suitable environment for signalling pathways, genetic material exchange, metabolite and enzyme interaction (Davey & O'Toole 2000).

Biofilms can colonize both biotic and abiotic surfaces, causing beneficial and/or detrimental effects to the environment, industry and human health (Costerton et al. 1987). For example, biofilm features are beneficially exploited in wastewater treatment plants (Nicolella 2000), bioremediation (Dash et al. 2013; Wu et al. 2015), biomaterial production and plant growth promotion (Davey & O'Toole 2000; Rudrappa et al. 2008; Rinaudi & Giordano 2010). Biofilms are also important in the marine environment where they can modulate the metamorphosis and/or settlement of invertebrate larvae and algal spores through diffusible or contact-mediated signals (Hadfield 2011; Shikuma et al. 2014; Thompson et al. 2015). The presence of biofilm on a host surface also modulates the host's access to nutrients, light, oxygen and toxins (Wahl et al. 2012). Nevertheless, biofilm can also be destructive, causing chronic infection in humans (Bjarnsholt et al. 2013), parasitism in animals and plants (Rinaudi & Giordano 2010), biodeterioration in engineered systems and artwork (Cappitelli et

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al. 2006), fouling of food-processing equipment (Villa et al. 2012a; Cappitelli et al. 2014) and wastewater treatment plants (Polo et al. 2014). In addition, the presence of biofilms on surfaces can modulate the attachment of macrofoulers (like plants and animals) (Clare et al. 1992). Indeed, marine organisms that maintain a foul-free surface are the main candidates for natural product antifoulants (Clare et al., 1996). Biofilm removal is usually carried out using either biocides or mechanical methods, but a complete and efficient eradication is often difficult (Bruellhoff et al. 2010; Villa et al. 2012c). Eradication problems arise because cells living in biofilm are less sensitive to antimicrobial agents than planktonic bacteria (Mah et al. 2003). In recent years, much effort has been put into addressing the development of preventive strategies that can be used to disarm microorganisms without killing them (Cegelski et al. 2008), eg targeting the early adhesion phase or interfering with cell-to-cell communication (Villa et al. 2010; Bai & Rai 2011; Villa et al. 2011).

Reactive oxygen species

Reactive oxygen species (ROS) are chemically reactive molecules produced in aerobic conditions as by-products of several metabolic processes. Molecular oxygen (O_2) is a small, nonpolar molecule that diffuses easily across biological membranes (Ligeza et al. 1998). Nevertheless, O_2 reacts poorly with cellular biomolecules. Its reactivity derives from the formation of ROS (Gerschman et al. 1954), which results from the addition of consecutive electrons to O_2, generating the superoxide (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical (•OH), and the singlet oxygen ($^1O_2$) (Imlay 2003). Indeed, O_2^- is not very reactive with biomolecules, but it does react rapidly with another molecule of O_2^- to form H_2O_2 or with nitric oxide to form a very potent oxidant and reactive nitrogen species, peroxynitrite (Pacher et al. 2007). H_2O_2 is stable, but it is a precursor of free radicals as UV radiation causes the cleavage of the oxygen–oxygen bond to form •OH through the Fenton reaction in the presence of redox metal ions (Fe^{2+} or Fe^{3+} or Cu^+). The most reactive and least selective

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species is •OH, which reacts with many biomolecules as it diffuses into the cells (Bokare & Choi 2014). $^1O_2$ is a photoexcited form of O_2, and is very dangerous as it reacts rapidly with cysteine, histidine, methionine, tyrosine and tryptophan residues, unsaturated lipids and some nucleic acids (Briviba et al. 1997).

Microorganisms routinely generate ROS when they grow in aerobic environments. The accidental autoxidation of flavoenzymes is mainly responsible for O_2^- and H_2O_2 production (Seaver & Imlay 2004). As microbial life first evolved in a world devoid of O_2 and rich in reduced iron, microorganisms evolved strategies to maintain a reducing environment and to prevent damage to essential macromolecules (Anbar 2008). When the balance between ROS and scavenger systems is disturbed, ROS accumulation within the cells leads to a condition called oxidative stress (Cabiscol et al. 2000; Green & Paget 2004; Imlay 2013). In this condition, the ROS concentration is so high that it can lead to protein, DNA, and lipid damage, an increased rate of mutagenesis, and cell death (Imlay 2013). Bacteria have evolved sensitive and specific sensors to monitor different redox signals such as the presence or absence of O_2, cellular redox state or ROS. Thus sensing mechanisms can involve redox-active cofactors, such as heme, flavins, pyridine nucleotides and iron–sulphur clusters, or redox-sensitive amino acid side chains such as cysteine thiols (Green & Paget 2004), and are tightly controlled by a complex network of regulators, including OxyR, SoxRS and RpoS.

Environmental sources of ROS

Oxidative stress is generated by both metabolic processes and diverse environmental stress factors, which are known to be sources of a ROS cascade (Kohanski et al. 2007; Arce Miranda et al. 2011). It is well established that the exposure of microorganisms to ionizing (γ) and non-ionizing irradiation (UV) leads to the intracellular formation of ROS because of the ionization of intracellular water (Sies 1997; Matallana-Surget et al. 2009). High temperatures can result in high oxidative stress, leading to damage to proteins, DNA double-strand breaks

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and cell death (Davidson et al. 1996; Murata et al. 2011; Chen et al. 2013). In addition, cold temperatures cause oxidative stress: cells of the Antarctic bacterium *Pseudomonas fluorescens*, grown at 4°C, suffer an increasing amount of free radicals and the enhanced activity of two antioxidant enzymes (Chattopadhyay et al. 2011).

Another source of oxidative stress, mainly for pathogenic bacteria, is the interaction with the host's immune system. In the presence of pathogens, plant and animal immune systems rapidly release ROS as a first-line of defence, generating the so-called "oxidative burst" (Apel & Hirt 2004). In addition, animal macrophages recognize and import bacteria into phagosomes (compartments that mature into phagolysosomes) containing ROS and reactive nitrogen species (Garin et al. 2001). Interestingly, analogous mechanisms are present in protists, such as *Acanthamoeba*, which express a respiratory burst during phagocytosis that kills ingested bacteria (Siddiqui & Khan 2012).

In the rhizosphere, ROS play an important role in the interaction between roots and microorganisms (Jamet et al. 2003), including the regulation of symbiosis (Shaw & Long 2003; Rubio et al. 2004; Fester & Hause 2005). During the early stages of plant-microorganism interactions, the plants subject microorganisms in the rhizosphere to oxidative stress, the aim being to prevent pathogen infection and establish advantageous symbiotic interactions. In return, microorganisms produce ROS scavenging enzymes in order to successfully infect the plant or down-regulate the plant ROS producing systems (Nanda et al. 2010).

Microorganisms also encounter the release of ROS-producing compounds produced by other neighbouring microorganisms. Phenazines, a large group of nitrogen-containing heterocyclic compounds, generate ROS accumulation in other microbial cells, assisting the producing bacterium in competitive survival (Mavrodi et al. 2010; Pierson LS & Pierson EA 2010). In pseudomonads, phenazines serve as an alternate electron acceptor to balance intracellular

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redox in the absence of other electron acceptors (Price-Whelan et al. 2006), and have been proposed as signalling molecules that are involved in quorum sensing (QS) regulated pathways and various stages of biofilm formation (Pierson LS & Pierson EA 2010).

In addition to natural ROS sources, the soil collects environmental pollutants, such as xenobiotics, metals and chemicals, which are able to cause oxidative stress in microorganisms (Kang et al. 2007; Pérez-Pantoja et al. 2013). Titanium oxide and silver nanoparticles are among emerging soil pollutants that cause oxidative stress in soil microorganisms (Polo et al. 2011; Mirzajani et al. 2013). Other exogenous sources of ROS are disinfectants and cleaning agents that contain peroxides, chloramines or hypochlorites (Van Houdt & Michiels 2010), and are increasingly used in a number of medical, food and industrial applications due to their broad spectrum activities and low cost (Linley et al. 2012). Their use has raised concerns about increasing resistance among pathogenic bacteria (Van Houdt & Michiels 2010) and exposing beneficial soil microbial community to oxidative stress (Ortiz de Orué Lucana et al. 2012). Whether antibiotics generate ROS to kill bacteria is an open question. In the last decade, it has been reported that the generation of ROS contributes to the efficacy of aminoglycosides, b-lactams and fluoroquinolones (Kohanski et al. 2007; Foti et al. 2012; Dwyer et al. 2014). However, the difficulty of demonstrating this thesis has highlighted (Ezraty et al. 2013; Keren et al. 2013). This issue has been dealt with in two recent and excellent reviews that summarize the data published so far (Dwyer et al. 2015; Imlay 2015).

Hormetic behaviour of ROS

Hormesis is a dose-response phenomenon characterized by low-dose stimulation and high-dose inhibition; this is represented as an inverted U-shaped dose response (Southam & Ehrlich 1943; Calabrese et al. 2011). Like many compounds exhibiting hormetic behaviour, ROS can be either detrimental or beneficial, depending on the concentration (Lewis 2008; Pan 2011). This is because exposure to low levels of the compound, or stress, can induce an

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adaptive response that protects the organism (Cap et al. 2012). When this occurs, despite lower levels of oxidatively modified biomolecules, it is possible to observe higher antioxidant, or associated enzyme, activity (Lushchak 2014).

The hormetic behavior of ROS in bacteria has important consequences in the sanitary and industrial fields because different doses of antimicrobials can either kill or increase their resistance to antimicrobials (Marathe et al. 2013). There are possible environmental repercussions to water and soil microflora exposed to low (sublethal) concentrations of oxidizing agents (Villa et al. 2012b). Though biocides are generally used at high concentrations to kill bacteria, there are sub-inhibitory biocide levels downstream from the treated area that range from the initial treatment concentration to nil (Gilbert & Mc Bain 2003; Mc Cay et al. 2010). Here, if oxidative stress is very high and so persistent as to exceed the point of no return, it can lead to cell death. However, if there are only moderate levels of stress, protective mechanisms are activated through a complex pathway involving various regulators, so that cell death is avoided (Amitai et al. 2004; Zhao & Drlica 2014). An example is the *Escherichia coli* MazE/MazF system, which generates ROS as a stress response. In response to low levels of stress, this system stimulates the activation of protective pathways, including the Cpx envelope protein stress system for the refolding or degradation of misfolded proteins in the periplasm, the inhibition of katG mRNA degradation, and MazF-mediated •OH accumulation (Pogliano et al. 1997; Raivio & Silhavy 2001; Zhao & Drlica 2014). In the case of extreme stress, the same proteins used to trigger ROS scavenging systems contribute to a cascade of ROS, and activate a programmed cell death pathway, essential to reduce the risk of hypermutation and loss of genetic integrity (Dorsey-Oresto et al. 2013). In *Bacillus subtilis*, NdoA plays the same role as the *E. coli* MazE/MazF system (Wu et al. 2011).

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Bernier & Surette (2012) has recently stated that different concentrations of antibiotics can trigger different biological responses, varying from cell death (acting as a toxin at a high concentration), adaptation (acting as a stress inducer at a medium concentration), and the shift of metabolic pathways (acting as a cue at a low concentration). Given the hormetic behavior of ROS, the above responses are also true for different levels of oxidative stress. Therefore, the effects of oxidative stress may be even more diverse and less predictable in environmental biofilms than in planktonic cells because of their chemical, physical and biological heterogeneity, and the relationships among biofilm members, each interacting with external chemicals in a particular way.

Biofilm and oxidative stress

The ability to form biofilm is a very ancient and common trait of Archaea and Bacteria, as evidenced by the observation of fossils dating back to 3.25 billion years ago (Hall-Stoodley et al. 2004). At that time, oceans and the atmosphere had a low oxygen content, as the first oxygenation events that changed the redox state of the environment occurred only 2.4 billion years ago (Anbar 2008). Microorganisms altered their metabolism and their defence strategies in order to take advantage of the accumulated oxygen and, at the same time, to avoid the damage caused by oxidative stress (Imlay 2013; Ziegelhoffer & Donohue 2009). Thus, the microbial biofilm response may have evolved alongside continuous increase of oxygen on Earth to develop a complex regulation of metabolic pathways, sensitive to the concentration, quality and durability of ROS. The authors speculate that the integration of ROS into several different signalling pathways, including the switch between planktonic and sessile forms, could have been, and still is, fundamental, from the eco-evolutionary point of view, to the survival of microbial species. We are suggesting three avenues of research for understanding the link between biofilm and oxidative stress: the existence of common regulators, the production of extracellular polymeric substances and biofilm heterogeneity (Figure 1).

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Common regulators and pathways

The first evidence of the tight connection between oxidative stress and biofilm formation is the involvement, in both processes, of the same regulators of many metabolic pathways. Through these pathways, ROS deeply influence bacterial physiology in biofilm (Cap et al. 2012), affecting its characteristics, structure and morphology (see examples in Villa et al. 2012b; DePas et al. 2013; Milferstedt et al. 2013 and in Figure 2). This may be understood as the result of the coevolution of biofilm and oxygen on Earth, which may have integrated ROS as a versatile and dynamic signal in many cellular pathways, including mechanisms regulating biofilm formation. In biofilm, cells are able not only to face oxidative stress, but also to exploit it, using ROS as a signal or cue to prepare to adapt to a changing environment. It is tempting to speculate that ROS signalling may be a driving force for the dominance of biofilm in many environmental niches. For instance, genome sequence analyses of deep-sea sedimentary bacterium *Pseudoalteromonas* sp. SM9913 which live at a very low oxygen concentration, compared to that of the closely related Antarctic surface sea-water ecotype *Pseudoalteromonas haloplanktis* TAC125, revealed a higher sensitivity to ROS, but also a potentially increased ability to form biofilm once exposed to oxygen (Qin et al., 2011). The oxidative stress response protein **OxyR** senses H_2O_2 and activates the transcription of several genes involved in antioxidative defence, eg peroxide scavengers, thiol redox buffers and enzymes that repair iron-sulfur centres and repress iron uptake genes (Storz & Imlay 1999; Zheng et al. 2011). OxyR is also involved in biofilm formation since *oxyR* mutants in various bacterial species exhibit increased auto-aggregation and an ability to form biofilms in minimal medium. In *E. coli* the process is mediated by the de-repression of *agn43*, encoding for the adhesion protein Ag43 that confers protection against H_2O_2 and stimulates bacterial biofilm formation at the microcolony stage (Danese et al. 2000; Schembri et al. 2003). Similarly, *oxyR* mutants in *Burkholderia pseudomallei* (Loprasert et al. 2000), *P.*

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chlororaphis (Xie et al. 2013) and *Porphyromonas gingivalis* (Wu et al. 2008) show increased ability to form biofilm in minimal medium and higher sensitivity to H_2O_2 and paraquat (a redox cycling agent, ie a compound able to produce ROS by changing its oxidative state). In *P. aeruginosa* biofilm exposed to oxidative stress, OxyR promotes the biofilm lifestyle to reduce metabolism and ROS production but also to encourage the dispersion of stressed bacteria (Wei et al. 2012). Indeed in *P. aeruginosa*, the oxidized form of OxyR binds both the promoter region of the bacteriophage Pf4 operon, essential for biofilm formation (Rice et al. 2009), and of *bdlA*, a biofilm dispersion locus (Morgan et al. 2006). However, the opposite effect has been described in *Serratia marcescens*, *Neisseria gonorrhoeae* and *Tannerella forsythia*, whose *oxyR* mutant strains show an impaired ability to form biofilm (Seib et al. 2007; Shanks et al. 2007; Honma et al. 2009).

RpoS is a general stress response protein that up-regulates cellular stress-related genes in response to slow growth, both in the stationary phase and under stress conditions (Hengge-Aronis 1999). In *E. coli*, RpoS is also activated in response to oxidative stress, collaborating to scavenge ROS with OxyR and SoxRS and inducing the transcription of genes involved in the protection from oxidative damage (ie *dspA*, *katE* and *sodC*) (Schellhorn & Stones 1992; Patten et al. 2004). Moreover, RpoS plays an essential role during biofilm development because it controls the expression of almost 50% of the genes that specifically induce the growth of biofilm (Collet et al. 2008). Recent studies highlight a more complex picture adding that RpoS triggers the production of extracellular structures and biofilm formation only under conditions of limited nutrient availability (Corona-Izquierdo & Membrillo-Hernandez 2002; Sheldon et al. 2012). For instance, in *Klebsiella pneumonia*, RpoS and SoxR trigger the expression of YjcC, a protein that regulates both the oxidative stress response and biofilm production by modulating the levels of the second messenger cyclic di-GMP (c-di-GMP) (Huang et al. 2013). In the food borne pathogen *Campylobacter jejuni*, it is not OxyR and

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SoxRS that regulate the genes of oxidative stress resistance, it is PerR, Fur and CosR (Atack & Kelly 2009). Under their control, AhpC, the only alkyl hydroperoxide reductase in this bacterium, negatively affects biofilm formation, maybe decreasing the oxidative stress levels in cell aggregates (Oh & Jeon 2014).

Quorum-sensing (QS) is a mechanism that enables bacteria to make collective decisions, synchronize with the rest of the population and thus function as multicellular organisms (Waters & Bassler 2005). In *P. aeruginosa*, QS-deficient mutants (*lasI*, *rhlI* and *lasI rhlI*) are more likely to suffer from oxidative stress because of the lower expression of *katA* and *sodA* (Hassett et al. 1999). In *P. aeruginosa,* QS enhances the oxidative stress response, triggering the production of scavenging enzymes; cells with an active QS system are more resistant to oxidative damage and will be selected by oxidative stress (García-Contreras et al. 2015). In *B. pseudomallei*, DpsA binds DNA and sequesters iron (Martinez & Kolter 1997) to protect DNA from damage by both acid and oxidative stress (Loprasert et al. 2004). At the same time, *bpsRI* mutants, unable to produce the QS molecules N-octanoylhomoserine lactone and N-(3-oxooctanoyl) homoserine lactone, show a reduced *dpsA* expression, and thus a higher sensitivity to organic hydroperoxides (Lumjiaktase et al. 2006). Lumjiaktase et al. (2006) also hypothesized that the control of the oxidative stress response through QS could be useful in high-density cultures, eg biofilm or stationary phase cultures, to protect DNA from oxidative damage. More recently, proteomic analysis of *B. subtilis* biofilm exposed to sublethal doses of silver nanoparticles, producing ROS, revealed a higher expression of proteins involved in stress responses (including oxidative stress proteins AhpC, SufD and thioredoxin) and quorum sensing (DegU, OppF, CotE and SrfAB), thus affecting gene expression in *B. subtilis* biofilms (Gambino et al. 2015).

The production of **phenazines** is another pathway that connects biofilm and oxidative stress. Phenazines are a large group of nitrogen-containing heterocyclic compounds with different

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chemical and physical properties depending on the functional groups present (Mavrodi et al. 2010). Mainly studied in pseudomonads, they work as an electron shuttle and are essential for long term survival under anaerobic conditions, eg in the inner part of biofilms, and they generate ROS in other organisms such as *Candida albicans* (Drago 2009). Phenazines are themselves signals capable of altering patterns of gene expression (Dietrich et al. 2008; Pierson LS & Pierson EA 2010). It has been observed in *P. chloraphis* that mutant strains deficient in phenazine are not able to form biofilm (Maddula et al. 2006). Moreover, *P. chlororaphis* produces different ratios of various phenazine derivatives, depending on the needs of the population, as each derivative has particular characteristics. For example, it has been supposed that 2-hydroxy-phenazine-1-carboxylic acid could facilitate cellular adhesion, whereas phenazine-1-carboxylic acid might allow biofilm growth, by acting as an electron shuttle within the microaerophilic community (Pierson LS & Pierson EA 2010). Phenazine production is also one of the most efficient strategies to acquire iron from the environment, a condition that significantly influences the switch from a planktonic to a sessile lifestyle in *P. aeruginosa* (Cornelis & Dingemans 2013).

Other pathways connecting oxidative stress and biofilm will surely come to the fore in the next few years. For example, *Marinomonas mediterranea*, a component of the microbiota associated with the marine plant *Posidonia oceanica*, expresses an antimicrobial protein with lysine oxidase activity (Molina-Quintero et al. 2010). This protein generates hydrogen peroxide that facilitates the subsequent dispersal of cells from biofilm, but the regulation mechanisms are not yet completely understood (Lucas-Elio et al. 2012).

The presence of common regulators and pathways between biofilm and oxidative stress could be exploited, as a novel biocide-free strategy, for biofilm control. Villa et al. (2012c) found that *E. coli* cells exposed to sublethal concentrations of zosteric acid, a natural compound from *Zostera marina*, accumulate ROS, activate scavenging mechanisms and induce a

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hypermotile phenotype, which inhibits the formation of biofilm. More recently, it has been hypothesized that this anti-biofilm compound could increase ROS accumulation by inhibiting the oxidoreductase activity of WrbA, a NADH:quinone reductase, interfering with the QS system and biofilm formation (Cattò et al. 2015). Therefore, zosteric acid seems to act as an environmental cue, warning microorganisms about environmental changes and to prepare for adversity (Villa et al. 2012c).

Extracellular polymeric substances (EPS) production

The EPS production pathway is inevitably connected to environmental stress sensors and is activated in accordance with external conditions. Among EPS, extracellular polysaccharides are often involved in the oxidative stress response. For example, increased production of polysaccharides was observed in the *Azotobacter vinelandii* (Villa et al. 2012b) (Figure 3) and *B. subtilis* biofilm matrices (Gambino et al. 2015), when exposed to sources of oxidative stress. Alginate, an extracellular polysaccharide produced by pseudomonads and *A. vinelandii,* among others, is able to scavenge hydroxyl radicals (•OH), in order to inhibit lipid and protein peroxidation (Tomida et al. 2010). Alginate is also used by *P. aeruginosa* to scavenge the H_2O_2 released to kill pathogens by macrophages, neutrophils and the hypersensitive response-plant-defence system (Mathee et al. 1999; Hay et al. 2014). The network regulating alginate production is controlled through the cross-talk of different regulators, but it the mechanisms behind the specific environmental cues that induce alginate production are unclear (Hay et al. 2014). Another example is the production of colanic acid by *E. coli* biofilm, promoted by the GGDEF protein YddV, under the regulation of *rpoS*. In addition to promoting cell aggregation and colanic acid production via diguanylate cyclase activity (Méndez-Ortiz et al. 2006), YddV also induces genes in response to oxidative and nutritional stresses (Landini 2009).

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However, EPS may be produced as a response to exogenous oxidative stress not to scavenge ROS directly but as part of the cells effort to decrease their metabolism to limit its own ROS production. This is the case of the *B. pseudomallei* succinyl-coA:3-ketoacid-coenzyme A transferase enzyme, which is down-regulated upon oxidative stress to avoid ROS production and leads to the accumulation of poly-hydroxybutyrate within cells as storage molecules (Chutoam et al. 2013).

EPS is a physical and chemical barrier for biocidal compounds and the attack of predators (Costerton & Lewandoski 1995), both of which produce ROS (see 'Environmental sources of ROS' section). Although reducing diffusion through the biofilm matrix only provides a short-term protective effect against many ROS producing compounds (Walters et al. 2003), it could be enough for sessile cells to rapidly adapt and scavenge different forms of ROS, enabling dynamic changes in ROS levels.

Biofilm heterogeneity

Biofilm represents a very heterogeneous environment both spatially and temporally, enclosing many microenvironments with different characteristics in a continuous flux of chemical gradients, which are influenced by the metabolism of resident bacteria, transport limitations (Teal et al. 2006) and the aging of the biofilm (Saint-Ruf et al. 2014). Every single cell forming a biofilm responds to environmental changes in an individual and unique way (Monds & O'Toole 2009). In every microenvironment within the biofilm, the local conditions trigger a differential response in bacteria, and select for more favorable phenotype variants. Thus, phenotype variants arise from both stochastic gene expression and genetic variation (mutation and genetic rearrangements) (Stewart & Franklin 2008). Oxidative stress is one of the main sources of heterogeneity in many bacterial biofilms (Saint-Ruf et al. 2014). In biofilm, each individual cell is exposed differentially to the surrounding environment, senses

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ROS at different levels, and activates its own ROS scavenging mechanisms, creating gradients of different ROS forms and increasing the variance of phenotypes.

In *E. coli*, exposure to iron causes ROS accumulation, and triggers the development of rugose biofilm composed of two different sub-populations, matrix- and non-matrix encased (DePas et al. 2013). Furthermore, the incubation of *E. coli* cells with paraquat induces SoxRS, which in turn determines the occurrence of several phenotypic variants able to survive fluoroquinolone antibiotics (Wu et al. 2012).

In addition, staphylococcal biofilms submitted to oxidative stress exhibit an increase in basal mutation frequency (Ryder et al. 2012). Exposure of *Staphylococcus aureus* to sub-lethal concentrations of hydrogen peroxide leads to oxidative stress adaptation of a sub-population of small-colony variants with enhanced catalase production via a mutagenic DNA repair pathway that includes a DNA double-strand break (DSBs) repair system (Painter et al. 2015). In *P. aeruginosa* biofilm, oxidative stress triggers the activation of the DNA repair system, including mutagenic DSBs, that result in higher phenotypic diversity (Boles & Singh 2008). Thus, the presence of distinct phenotypes of subpopulations within a bacterial community appears to be a common occurrence and might even be considered as an evolutionary strategy to withstand environmental stresses. This process has a high clinical relevance as it worsens the problem of antibiotic resistance (Ryder et al. 2012). Many physical, physiological and adaptive tolerance mechanisms allow biofilm subpopulations to survive and are responsible for the well-known tolerance of biofilm to antimicrobials (Bjarnsholt et al. 2013). Antibiotic resistance is also correlated with mutations and horizontal gene transfer (Martinez 2009). Both mechanisms are more frequent in biofilm because of its increased heterogeneity and the presence of a matrix that facilitates social behaviour. The presence of a matrix allows microorganisms to benefit from proximity to cells with resistance to antimicrobials because they detoxify the local biofilm environment (Conlin et al. 2014). As stated above, ROS

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enhance heterogeneity and matrix production in biofilm, increasing the number of persistent cells (Wu et al. 2012) and playing an important role in the higher tolerance of biofilm to antimicrobials. Furthermore, the resistance to antimicrobials that can arise in a biofilm is not necessarily contained to the biofilm. Any change in environmental conditions can lead to the dispersion of biofilm cells that colonize new niches with the antibiotic resistance they have already acquired.

Conclusion

The role of oxidative stress in bacterial biofilms is a topic of outstanding importance because it is relevant to the sanitary, industrial and environmental fields. The development of antimicrobial resistance in biofilms demand attention because ROS may trigger adaptive mechanisms that are more effective in biofilms than in planktonic bacteria. In this review, three avenues of research have been highlighted for further investigations into the biofilm response to oxidative stress, but others may arise with further research in the field. Unravelling the different interactions that tie biofilm response to oxidative stress will be a challenge for many years to come. Understanding the mechanisms regulating biofilm in response to different levels of ROS may shed light on both the environmental determinants for the bacterial colonization of hostile habitats and the molecular strategies used to sense environmental cues and adapt accordingly. An explanation of these pathways could be the key to identify which mechanisms lead to the colonization of habitats of ecological and economic interest. In the near future, it may also be possible to use oxidative stress in a controlled way to trigger biofilm formation and dispersal.

Disclosure

The authors report no conflicts of interest in this work.

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Figure captions

Figure 1. Emerging avenues of research to investigate biofilm response to oxidative stress.

Figure 2. Exposure to sub-lethal doses of the ROS producer caused a change in the morphology of the colony biofilm. a) Effect of hydrogen peroxide on *Burkholderia thailandensis*, b) Effect of phenazine methosulphate (PMS) on *Azotobacter vinelandii*.

Figure 3. Sublethal doses of PMS trigger the accumulation of exopolysaccharides in the matrix of the colony biofilm of *Azotobacter vinelandii*. Cryosectioning images of untreated (a) and treated (b) mature biofilms: in green, live cells were stained green with Syto9; in red, the polysaccharide component of the EPS matrix was stained with Texas Red-labelled Concanavalin. Scale bar: 100 µm.

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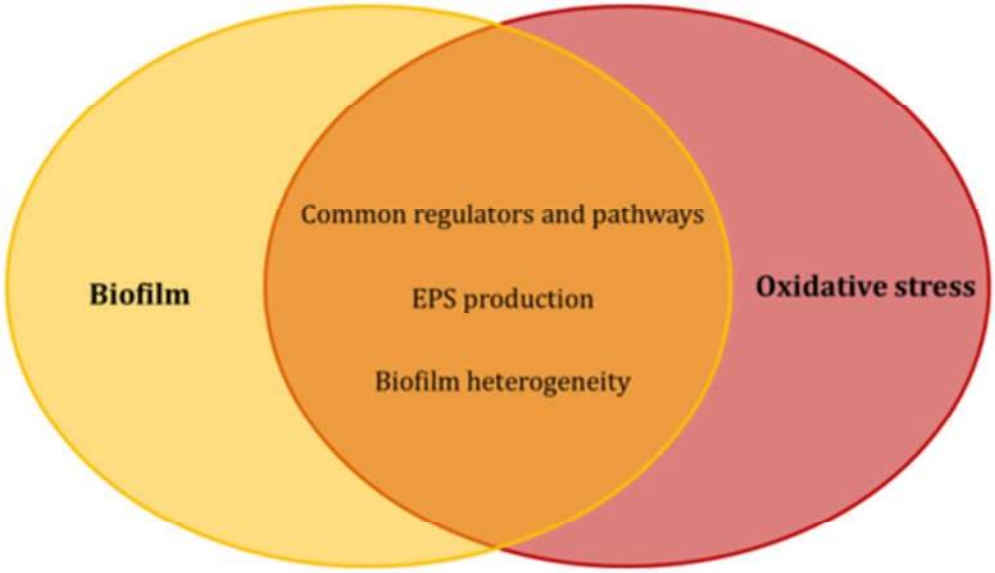

Figure 1. Emerging avenues of research to investigate biofilm response to oxidative stress.
225x141mm (72 x 72 DPI)

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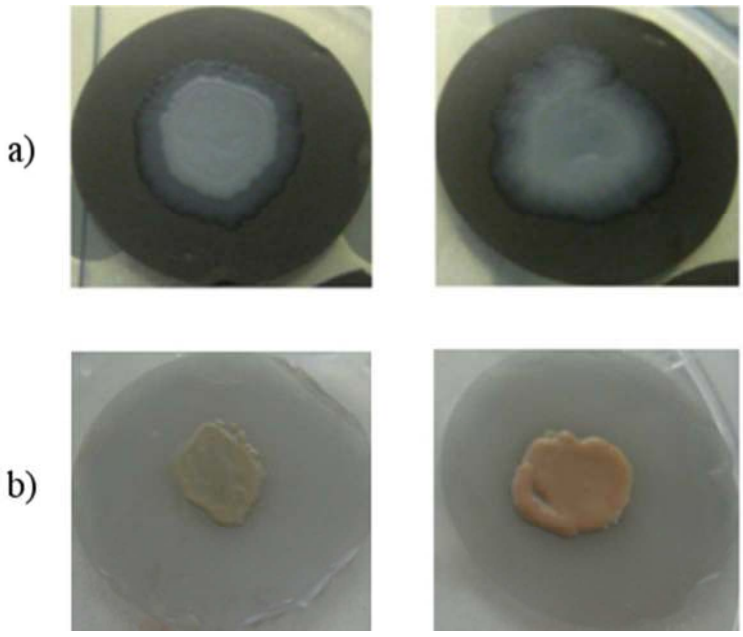

Figure 2. Exposure to sub-lethal doses of the ROS producer caused a change in the morphology of the colony biofilm. a) Effect of hydrogen peroxide on *Burkholderia thailandensis*, b) Effect of phenazine methosulphate (PMS) on *Azotobacter vinelandii*.
209x131mm (72 x 72 DPI)

For Review Only

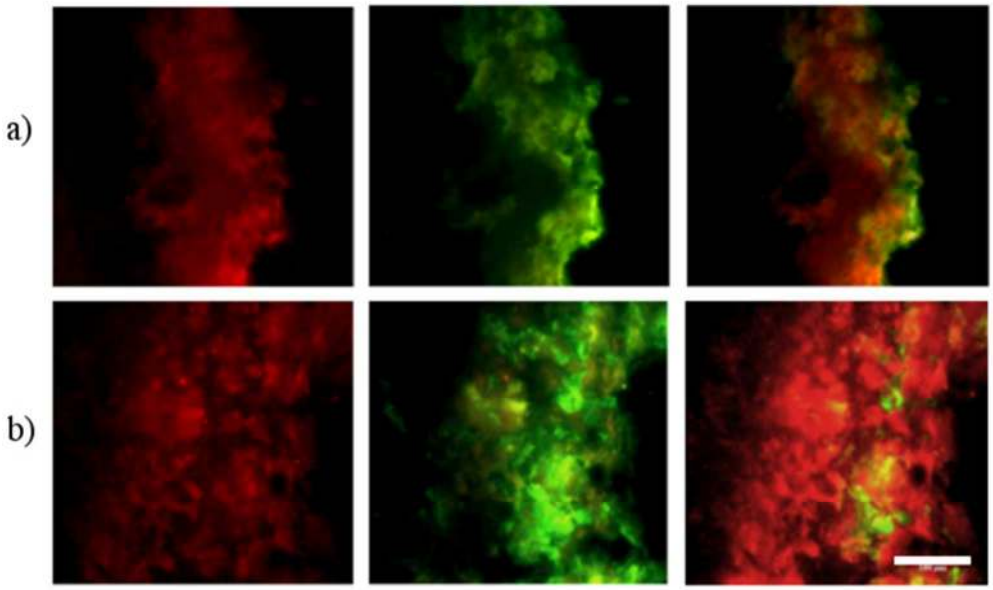

Figure 3. Sublethal doses of PMS trigger the accumulation of exopolysaccharides in the matrix of the colony biofilm of *Azotobacter vinelandii*. Cryosectioning images of untreated (a) and treated (b) mature biofilms: in green, live cells were stained green with Syto9; in red, the polysaccharide component of the EPS matrix was stained with Texas Red-labelled Concanavalin. Scale bar: 100 μm.
225x141mm (72 x 72 DPI)

view Only