

Review Article

Reactive Oxygen Species in the Signaling and Adaptation of Multicellular Microbial Communities

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One of the universal traits of microorganisms is their ability to form multicellular structures, the cells of which differentiate and communicate via various signaling molecules. Reactive oxygen species (ROS), and hydrogen peroxide in particular, have recently become well-established signaling molecules in higher eukaryotes, but still little is known about the regulatory functions of ROS in microbial structures. Here we summarize current knowledge on the possible roles of ROS during the development of colonies and biofilms, representatives of microbial multicellularity. In *Saccharomyces cerevisiae* colonies, ROS are predicted to participate in regulatory events involved in the induction of ammonia signaling and later on in programmed cell death in the colony center. While the latter process seems to be induced by the total ROS, the former event is likely to be regulated by ROS-homeostasis, possibly H₂O₂-homeostasis between the cytosol and mitochondria. In *Candida albicans* biofilms, the predicted signaling role of ROS is linked with quorum sensing molecule farnesol that significantly affects biofilm formation. In bacterial biofilms, ROS induce genetic variability, promote cell death in specific biofilm regions, and possibly regulate biofilm development. Thus, the number of examples suggesting ROS as signaling molecules and effectors in the development of microbial multicellularity is rapidly increasing.

1. Introduction

Since the first observations of microorganisms by Antonie van Leeuwenhoek and their isolation and cultivation by Robert Koch, microorganisms have been traditionally viewed as simple unicellular organisms. As a result of this presumption, all microbial studies have been conducted using shaken liquid cultivations. However, during the last few decades, it has become obvious that microorganisms are able to form multicellular structures such as colonies and biofilms. More and more examples of multicellularity have been described, and it has become evident that multicellular behavior, although initially considered an exception, is instead the rule for microbes. Species of the most distant clades of both Archea and Bacteria form biofilms, as did their ancestors some 3.3 billion years ago, indicating that biofilm formation is a universal and ancient bacterial trait [1]. Biofilms and colonies are also formed by eukaryotic microbes (yeasts and molds) in different environments. Multicellular

communities are thus prevalent forms of microbial existence in natural settings.

The structural complexity and degree of organization of microbial multicellular structures vary from a simple single-layer biofilm and simple aggregates to complicated structures like the fruiting bodies of myxobacteria and slime molds [2, 3], complex natural biofilms [4] and the colonies of various microbes [5]. Importantly, cells within these structures differentiate and use various signaling molecules to coordinate and regulate the metabolism and development of the community. All these characteristics, that is, structural complexity, cellular differentiation, intercellular signaling and coordinated development, are basic attributes of true multicellularity. However, unlike conventional multicellular organisms, microorganisms retained the ability to survive and divide in their unicellular state.

The biofilm community gains a number of unique properties, especially in terms of resistance to various stresses and toxins. This is of particular importance, because

the resistance of biofilms to medical treatment is an important problem in current infection control. For this reason, biofilms have become the subject of intensive research in recent years [1, 6–10].

The production of reactive oxygen species (ROS) is an inevitable consequence of an aerobic lifestyle. Because of their reactive nature, ROS can cause oxidative damage to DNA, proteins, lipids and other cellular components, and an excess of them leads to extensive cell damage and eventually cell death. To protect themselves from the deleterious effect of ROS, cells have evolved many defensive mechanisms including, for example, enzymes capable of ROS removal, such as catalase, and various peroxidases for the removal of hydrogen peroxide or superoxide dismutase, eliminating superoxide radicals. Although ROS have been traditionally viewed as purely harmful, a more complex picture of their role in cellular physiology has been gradually emerging over the last decade. Recent data have suggested that a certain level of ROS is in fact beneficial to longevity through the adaptive mechanism called hormesis [11, 12]. During hormesis, low doses of stress or toxin induce mechanisms that protect the organism against this stressor and evoke crossadaptation to other stresses. In addition, a growing number of data suggest that ROS participate in signaling pathways in plants, animals, and fungi [13–15] and even in interspecies communication [16], and it has also been proposed that they play a role in the development of multicellularity [17]. Despite being widely accepted as signaling molecules in higher organisms, little is known about the role of ROS in microbial populations. However, the last few years have produced intriguing new data indicating that ROS-induced processes are involved in differentiation and signaling in yeast and bacterial communities.

Here, we focus on the functions of ROS in multicellular communities of unicellular yeast and bacteria. We summarize current knowledge on the possible roles of ROS and stress defense in the development of *S. cerevisiae* colonies. In the second part of this review, we summarize current knowledge on the role of oxidative stress defense and endogenous ROS production in other well-studied microbial multicellular structures—*Candida albicans* and bacterial biofilms.

2. *Saccharomyces cerevisiae* Colonies

The yeast *S. cerevisiae* is one of the most studied model organisms in genetics and molecular biology. It is also widely used for studies on the mechanisms of cellular aging, longevity, stress resistance, and adaptation. However, little is known about yeast life within multicellular communities and only a few groups (including ours) have performed pilot studies that regard development, ageing, adaptation, and differentiation of both laboratory strain colonies (e.g., [18–22]; see also below) and biofilm colonies resembling natural biofilms in various aspects (e.g., [23, 24]).

2.1. Signaling, Stress Defense, and Colony Differentiation. Yeast colonies growing on a complex agar medium with a

nonfermentable carbon source undergo several developmental stages characterized by changes in the pH of the surrounding medium, shifting from acidic to alkali and vice versa [25] (Figure 1). Alkalinization of the medium is accompanied by the release of volatile ammonia that can act as a signaling molecule that is able to induce alkalinization and ammonia production in neighboring colonies. Thus the development of colonies is synchronized [26]. After several days, the ammonia production declines and colonies enter the second acidic phase. Extensive transcriptional changes occur during the transition from the first acidic to the alkali phase (which occurs between day 7 and 11 of colony development) [27] indicating metabolic reprogramming from a typical respiratory metabolism to a different, not yet fully characterized metabolic program. Among others, genes involved in the mitochondrial TCA cycle and oxidative phosphorylation are repressed while other metabolic genes (e.g., peroxisomal β -oxidation, amino acid metabolic genes, methyl glyoxylate cycle) are induced. Interestingly, in parallel to metabolic reprogramming, the repression of a group of genes that belong to environmental stress response (ESR) genes [28] was observed. These genes also include important players in oxidative stress defense *CTT1*, *SOD1*, and *CCP1* encoding for cytosolic catalase, cytosolic superoxide dismutase, and mitochondrial cytochrome c peroxidase, respectively, and a master regulator of ESR genes, *MSN4* [27]. Later on during the alkali-to-2nd-acidic-phase transition, some ESR genes, for example, *CTT1*, are derepressed, while the expression of others such as *SOD1* is kept lower than in the 1st acidic phase. Expression changes agreed with the levels of the enzyme activities. Ctt1p and Sod1p activities decrease during the alkali phase and increase again after the alkali phase has turned into the second acidic phase [19, 22].

In parallel with metabolic reprogramming, the cells of a colony population significantly diversify. Until the beginning of the alkali phase and ammonia production, the colony population is relatively homogeneous. Upon entering the alkali phase, nondividing cells in the colony, which account for the vast majority of the colony population, start to differentiate both in a horizontal and vertical direction. This horizontal diversification leads to the emergence of a cell population of nondividing or slowly dividing chronologically aged cells in the colony center and to the cell population of the colony periphery, where colony accrual occurs and a significant number of cells still divide. Notable physiological differences were found between central and marginal cells in terms of both their metabolism and stress-related features. First, central cells produce levels of ROS several times higher than cells from the colony margin and exhibit some features of programmed cell death, also in contrast to cells from the colony margin [19, 29]. Secondly, central cells maintain a relatively high activity of oxidative stress defense enzymes Sod1p, Sod2p, and Ctt1p during the alkali phase, while a significant decrease in these activities was observed in marginal cells [19]. Nevertheless, their increased antioxidant capacity obviously does not protect some of the central cells from high ROS production and cell death. Thirdly, changes in carbon metabolism typical for the acidic-to-alkali transition are mainly induced in the marginal cells [19].

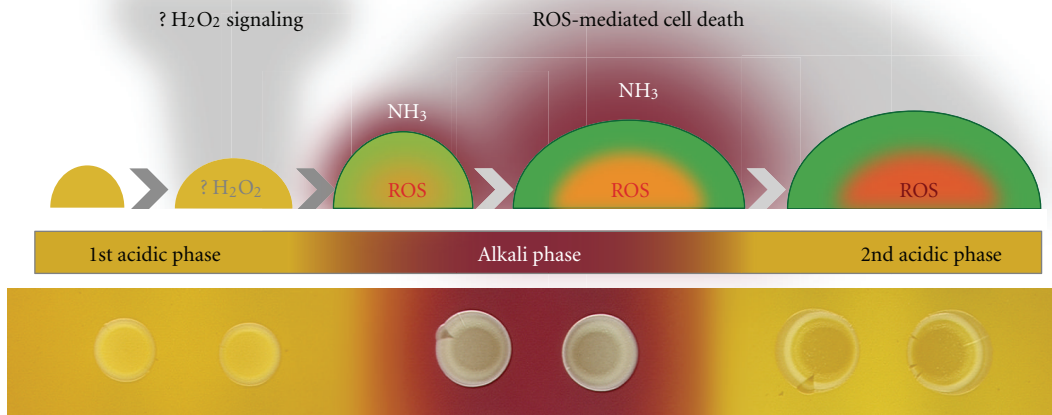


FIGURE 1: Regulatory role of H_2O_2 /ROS in the development of yeast colony. Below: development of *S. cerevisiae* colony, which passes through 1st acidic, alkali and 2nd acidic. Above: periods of hypothesized H_2O_2 and ROS involvement in the regulation of colony development. Colonies were photographed with a Hitachi HV-C20 color camera with Cosmicar lenses, Kaiser ProLite illumination system, and NIS Elements software (Laboratory Imaging).

Ammonia signaling seems to be important for this differentiation. A detailed study of *sok2Δ* strain, Sok2p being a transcription factor involved in various signaling events regulated by the Ras-cAMP-PKA pathway, showed that the center-margin differences described in wild type colonies are diminished or absent in colonies of this ammonia-signaling-deficient strain [30]. Surprisingly, a very similar colony phenotype in terms of differentiation was observed in strains lacking mitochondrial superoxide dismutase Sod2p and cytosolic catalase Ctt1p, both of which diminish ammonia signaling [19]. However, the absence of cytosolic superoxide dismutase Sod1p has a different phenotype. Colonies of the *sod1Δ* strain produce ammonia at the same time and even in slightly larger quantities than the wild type with colony differentiation even more pronounced than in the wild type [19].

The vertical differentiation observed in the central part of the colony results in two cell layers, the upper and the lower, composed of cells possessing completely different physiologies [18]. Cells on the upper layer are multiple-stress resistant and long-living, while the cells of the lower layer produce more ROS, despite activating the expression of some stress-defense genes, and slowly die.

2.2. Role of Stress-Defense Mechanisms in Colonies: Direct Defense or Regulation? Some of the results obtained using colonial populations are in stark contrast to studies on yeast liquid cultivations under starvation conditions, where the indispensability of ESR genes (including those encoding stress defense enzymes) and genes involved in mitochondrial respiration for the long-term survival was shown [31–33]. For example, strains deficient in cytosolic superoxide dismutase Sod1p are known to have a severe oxygen-dependent growth defects, including lysine and methionine

auxotrophies as a consequence of oxidative damage to the metabolic pathways synthesizing these amino acids [34]. The deletion of *SOD1* also dramatically decreases survival during aging [35]. These defects put selective pressure on the emergence of suppressor mutations compensating for these defects [36, 37]. On the other hand, the role of mitochondrial Sod2p and Ctt1p in the survival and longevity of liquid yeast populations is less evident. The absence of Sod2p has little effect during fermentative growth but its importance increases when growing on respiratory substrates, consistent with the presumed role of Sod2p in removing the superoxide radicals resulting from mitochondrial respiration [35]. The role of cytosolic catalase seems ambiguous, since its deletion leads to lower stress protection and a decreased ability to adapt to stress conditions on the one hand [38, 39], but also to increased survival during chronological aging on the other [40]. On the whole, antioxidant protection, or at least some of its components, seems to be important for the long-term survival of yeast populations in shaken liquid cultures.

The situation in colonies is almost the opposite. While *sod2Δ* and *ctt1Δ* colonies are incapable of ammonia signaling, sufficient metabolic reprogramming and differentiation and, consequently, their marginal cell population exhibit decreased survival, *sod1Δ* colonies produce ammonia, differentiate and survive in the same manner as wild-type colonies. In addition, *sod1Δ* colonies were almost free of cells with mutations suppressing the stress-sensitivity of *sod1Δ* in liquid cultivations. Altogether, *sod1Δ*, *sod2Δ*, and *ctt1Δ* behavior as well as the observed drop in some stress-defense enzyme activities during the alkali phase suggests that it is alkalization, ammonia production, and metabolic reprogramming, not stress defense and direct removal of radicals, that matter in colony differentiation and the survival of part of the population [19, 41]. In addition, from day 5 onwards, the ROS level in the colony is considerably lower

than that in liquid cultivations ([19] and unpublished data) in both wild-type colonies and colonies of the *sod1Δ*, *sod2Δ* and *ctt1Δ* strains.

Why do *sod1Δ* colonies develop normally with even more pronounced differentiation than wild-type colonies, while the deletion of some other genes involved in antioxidative defense (*SOD2*, *CTT1*) is deleterious to the colony's ability to produce ammonia and differentiate? One possibility is that a ROS signaling pathway exists that regulates colony development (Figure 2). This pathway would be activated in wild-type colonies at a particular point in their development and could regulate the beginning of ammonia production (Figure 1). As hypothesized in Figure 2, the signal would be stronger in *sod1Δ* colonies, but weaker in *ctt1Δ* colonies and even weaker or absent in *sod2Δ* colonies. The observed phenotypes cannot be simply explained by an increase or decrease in a particular ROS concentration and some more complex mechanism is likely to be involved. Superoxide dismutases catalyze the dismutation of superoxide to hydrogen peroxide and oxygen and so participate in the interconversion of various ROS types. Thus the absence of either of cytosolic or mitochondrial SOD would lead to an increase in superoxide concentration and, simultaneously, to a decrease in H_2O_2 concentration in the respective compartment. This leads to alternation in the homeostasis and/or ratio of H_2O_2 concentration between the mitochondrial matrix and cytosol. In contrast to superoxide, H_2O_2 is relatively stable and can penetrate into other cellular compartments through the membranes. Changes in its production in different compartments thus lead to changes in the H_2O_2 gradients and homeostasis and/or H_2O_2 concentration ratio between the mitochondrial matrix and cytosol. We propose that this ratio, rather than the absolute concentration of any of the ROS, is the signal that leads to some of the initial changes resulting in ammonia production, alkalization and to the physiological changes connected with it. This model is consistent with the phenotype of *ctt1Δ* colonies, since the absence of this enzyme lowers the mitochondrial-to-cytosolic H_2O_2 ratio, similarly to the *sod2Δ* strain. A possible mode of action of H_2O_2 includes the regulation of protein function through peroxiredoxins and thioredoxins, protein modifications by S-glutathiolation and direct inactivation by H_2O_2 [13, 42, 43].

Since it has been shown that different ROS trigger different adaptive responses [44], there clearly must be multiple ROS sensing pathways. That different ROS have different effects was shown in studies of the hormetic effect of superoxide and hydrogen peroxide on liquid cultivations. Both oxidants increase the longevity of the population when applied at moderate concentrations. While superoxide only induces longevity when applied during the logarithmic phase of growth, in contrast H_2O_2 only induces longevity when applied to stationary cultures [40, 45]. This would be consistent with its role in chronologically aging colonies, as proposed above. Moreover, evidence from plant research indicates that the ROS signal is often generated in short pulses [14]. This indicates that not only the amount and type of ROS, but also the precise timing of the ROS signal could be important.

Alternatively, ROS-scavenging enzymes could possess other regulatory functions, possibly independent of their antioxidant properties. For example, the function of the voltage-dependent anion channel (VDAC), a porin of the mitochondrial outer membrane, is diminished in the absence of Sod1p [46]. VDAC plays an important role in regulating mitochondrial activity and apoptosis [47] and its closing leads to a decrease in metabolite exchange and communication between the mitochondrial intermembrane space and the cytosol [48, 49]. The absence of Sod1p also affects metabolic regulation in cells [50]. Deletion of the *SOD1* gene causes a lessening of glucose repression, an important regulatory mechanism affecting nearly all aspects of *S. cerevisiae* metabolism. Moreover, *sod1Δ* cells have an increased level of mitochondrial biomass when growing in both media, with a repressing or nonrepressing carbon source. Both superoxide dismutases, Sod1p and Sod2p, were identified in a large-scale protein-protein interaction study as potential regulators of DNA repair and chromatin remodeling [51].

2.3. ROS in Programmed Cell Death in Colony. Beside their possible role in signaling leading to ammonia production and the start of colony differentiation, ROS seem to play an important role in the further development of differentiated cells (Figure 1). Programmed cell death in yeast can be induced by various signals, but their common factor is an increased ROS concentration (for reviews on yeast apoptosis, see [52–55]). ROS therefore seem to be the executioners of programmed cell death in yeast. As described above, cells in the center of a differentiated colony undergo programmed cell death, while cells at the colony margin are healthy and free of ROS [19, 29]. In contrast, the decreased center-margin differentiation observed in the colonies of non-ammonia-producing strains results in an increased cell death rate at the colony margin [19, 29]. A relatively high production of ROS occurs in the center of differentiated ammonia-producing wild-type and *sod1Δ* colonies. Notably, oxidative-stress-defense-deficient mutants with a defect in ammonia production (*sod2Δ* and *ctt1Δ*) do not exhibit increased ROS production in the colony center [19]. We propose that ROS are produced by the central cells in response to ammonia and/or alkalization and their production leads to cell death. ROS production in these cells is probably not a consequence of a low antioxidant capacity of the central cells, but rather part of a developmental program of the colony.

The dead cells in the colony center are likely to release nutrients that are then used by the cells at the edge of the colony to grow and survive [29, 56]. In addition, dead cells not only release nutrients from their biomass, but also stop consuming from the common nutrient pool. Since cells in the margin of the colony are younger and have better prospects of colonizing other localities, it makes sense if the colony invests in these prosperous cells at the expense of central cells. Opponents of the concept of programmed cell death in microorganisms could argue that cell death could bring no advantage for a unicellular organism, since the whole organism (one cell) dies and so this trait could

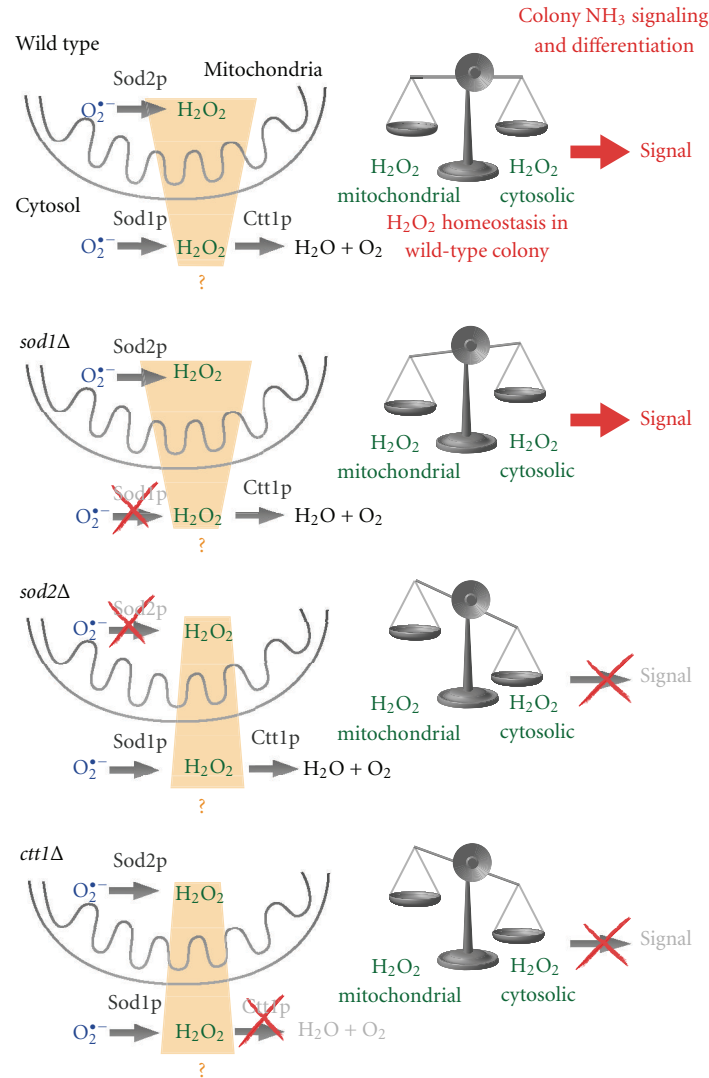


FIGURE 2: Hypothesis on the effect of H₂O₂ balance on induction of ammonia signaling. Left: predictions of changes in H₂O₂ concentration in cytosol and mitochondria of wild type and three oxidative-stress-defense-deficient strains based on reactions catalyzed by three main stress defense enzymes, cytosolic and mitochondrial superoxide dismutase Sod1p and Sod2p, respectively, and cytosolic catalase Ctt1p. Orange arrows indicate predicted H₂O₂ gradient between mitochondria and cytosol in wild-type strain and the three mutants. H₂O₂ gradient in wild-type cells is proposed on the basis of the prediction that mitochondria are the major site of ROS production in starving respiring cells from acidic-phase colonies. Right: the balances compare the mitochondrial-cytosolic H₂O₂-homeostasis of the particular mutant strain and the wild-type colony. The H₂O₂ imbalance towards the higher H₂O₂ concentration in the cytosol abolishes the induction of ammonia signaling and proper colony development and differentiation.

not be a subject to natural selection. However, when we consider unicellular organisms in terms of populations, it makes sense that the death of some cells could increase the prospects for survival of other cells in the same population. Moreover, microbial communities are often of clonal origin in nature; that is, they originate from one or a few cells and thus it is highly probable that the nutrients released by the sacrificed cells would benefit the kin of these cells, making this programmed cell death evolutionarily sustainable.

3. *Candida* Biofilms

Biofilms formed by *Candida* sp. can be serious problems in medical treatment, as they are usually highly resistant to extracellular toxins and drugs. ROS presumably play some role in *Candida* biofilms. Transcriptomic studies revealed that the biofilm population increases the expression of stress-defense genes, in particular those involved in combating oxidative stress, when compared to planktonic cultures [57].

Similar results were confirmed at the proteomic level [58]. The ROS level is dramatically decreased in biofilm when compared to a planktonic cultivation [58]. Whether this is a consequence of the activation of antioxidant mechanisms in biofilms or whether life in the biofilm *per se* results in a decreased ROS production (as with life in a colony) is unclear. It seems that a common mechanism for oxidative stress resistance and multicellular behavior exists in *C. albicans*, since cell adhesion, biofilm formation, and oxidative stress resistance are influenced by a common factor, the cell wall protein Hwp2p [59]. As the release of ROS during phagocyte respiratory burst is a crucial part of the immune response, adaptation to oxidative stress and oxidative stress defense enzymes help the yeast cells to survive respiratory burst and are thus important factors in pathogen virulence [60]. The increased oxidative stress defense of biofilms could also be responsible for their increased resistance to antifungal agents such as azoles, the toxicity of which involves the production of ROS [61].

3.1. Farnesol Signaling and ROS. Intercellular signaling by farnesol is involved in the induction of oxidative stress defense. Farnesol is a sesquiterpene alcohol produced by *Candida* sp. that acts as a quorum sensing (QS) molecule [62]. Quorum sensing is a synchronized transcriptional response of a microbial population to the presence of a small molecule called an autoinducer. Given that the autoinducer is produced continuously by all cells in the population, its concentration is proportional to the cell density.

Farnesol in *C. albicans* was shown to inhibit hyphae formation [63], to inhibit biofilm growth [64], to induce programmed cell death [65], to evoke ROS production [66], and to promote resistance to oxidative stress [67]. The latter involves farnesol-induced catalase expression via inhibition of the Ras-cAMP pathway [66] and, in parallel, farnesol-induced ROS production, which adapts cells to oxidative stress and induces protective mechanisms [68]. Farnesol thus acts as an intercellular adaptive signal that confers oxidative stress resistance to the cells within the same population. It is possible that the farnesol-induced high level of ROS and membrane-permeable hydrogen peroxide, in particular, participate in the farnesol signaling pathway, thus behaving as another intercellular signaling molecule. The possible signaling role of H₂O₂ in *C. albicans* is illustrated by the findings that low concentrations of hydrogen peroxide can induce the yeast-to-hyphal morphological transition [69], while higher concentrations induce programmed cell death [70, 71]. It was shown that H₂O₂ activates the AP-1-like stress-responsive transcription factor Cap1p, the stress-activated protein kinase Hog1p and also the checkpoint kinase Rad53p, which regulates hyperpolarized bud growth and filamentation [72–74]. Interestingly, these H₂O₂-regulated pathways are regulated and coordinated by the antioxidant enzyme thioredoxin, which appears to be a master regulator of redox signaling in *C. albicans* [72].

Farnesol also induces ROS production in other fungal and bacterial species [75–77]. Farnesol can thus act like an antibiotic, killing competing microbes and, in parallel, it

induces mechanisms (e.g., cAMP-mediated oxidative stress adaptation) that protect the producing cells from farnesol's toxic effect. A similar strategy was described in killer toxins produced by different yeast species [78]. Different outcomes described for farnesol signaling (i.e., adaptation, differentiation or apoptotic cell death) could be the consequences of various concentrations of farnesol and combination of the farnesol signal with other factors, for example, other signaling molecules, cell physiology, nutrient status, and cell location in the biofilm.

4. Bacterial Biofilms

The stages of biofilm formation, that is, attachment, maturation, and dispersal of the bacterial biofilm, are all regulated by environmental cues as well as by intercellular signaling molecules [6, 79]. The role of various QS signals, indole, and polyamine signaling in the regulation of biofilm development has been described [80–82]. Beside these signaling molecules, ROS are another possible signal involved in biofilm formation (Figure 3). The role of ROS in cell death and the generation of genetic variants within a biofilm is well-described, while ROS' signaling function and cross-talk with other signaling pathways as well as their role in microbe-microbe, host-pathogen, or host-symbiont types of interactions are slowly emerging but mostly remain to be discovered.

4.1. ROS-Induced Diversity and Differentiation of Cells within a Biofilm. Many bacterial species develop genetic variability when growing within a biofilm, but not during cultivations of planktonic cells [83–86]. Variability was demonstrated as the frequency of the different colony morphotypes, resistance to antibiotics, swimming and sliding motility and exopolysaccharide production. Since different environmental conditions require different cell adaptations, genetic variability increases the chances of the community surviving under a broader spectrum of conditions. In the biofilms of *Pseudomonas aeruginosa*, the emergence of their genetic variability is dependent on oxidative-stress-induced DNA double-strand breaks and on their repair by the RecA system, which introduces genome rearrangements [84]. Interestingly, increasing resistance to oxidative stress or adding an antioxidant to the medium significantly reduced cell variance in the biofilm, while deletion of the catalase gene increased the variance [84]. ROS- and RecA-dependent biofilm cell variation was also described in *Listeria monocytogenes* [87]. Similar results were obtained from studies of *Staphylococcus pneumoniae* biofilm phenotypic variation. In this case, the “suicide” gene *spxB* encodes for pyruvate oxidase, which produces high amounts of hydrogen peroxide and which is responsible for the unusually high death rate in *S. pneumoniae* stationary cultures and possibly also in biofilms [88]. Likewise, SpxB-mediated production of H₂O₂ induces the cell death of about 10% of the population, leading to the release of DNA from the cells in two oral bacterial species, *Streptococcus sanguinis* and *Streptococcus gordonii* [89, 90]. This extracellular DNA (eDNA)

is an important part of the biofilm extracellular matrix, it enhances cell-cell adhesion, regulates biofilm dispersal, serves as a nutrient source, and is available to be taken up and incorporated into the chromosome by competent cells [91–95]. Given that streptococcal biofilms contain a high percentage of competent cells [96], eDNA release could be an important factor in creating genetic variability in biofilms. Moreover, the mutagenic activity of H_2O_2 [97] towards eDNA even increases this variability. Interestingly, *spxB* expression is controlled by the catabolic repression regulator CcpA, linking the roles of metabolism and H_2O_2 in biofilm development [98]. Hydrogen-peroxide-induced genetic variation and cell death were also reported in biofilms of *Pseudoalteromonas tunicata*, *Marinomonas mediterranea*, *Caulobacter crescentus*, and *Chromobacterium violaceum*, depending on the presence of the lysine oxidase encoded by the *alpP* gene and its homologues [99]. This hydrogen peroxide-producing enzyme is common among bacterial species, which makes variability and cell death regulated by ROS a common bacterial trait. Interestingly, AlpP-mediated cell death is also important for dispersal of the biofilm, that is, the release of planktonic cells from the biofilm. Cell death in the biofilm center presumably provides nutrients that increase the size, metabolic activity, and phenotypic variability of the dispersed cells [100]. Remarkably, besides H_2O_2 , lysine oxidase also produces ammonia, but its possible signaling function in biofilms has not been explored. *P. aeruginosa* biofilm dispersal and cell differentiation are also regulated by the signaling molecule nitric oxide, a radical that could give rise to a spectrum of oxidants called reactive nitrogen species (RNS). Low concentrations of NO caused *P. aeruginosa* biofilm dispersal and enhanced swimming and swarming cell motilities, while higher NO concentrations induced cell death [101]. At least some of these effects are probably induced by NO-derived RNS, as RNS were detected in the biofilm. NO is produced by *P. aeruginosa* cells under anaerobic conditions through anaerobic respiration from nitrates and nitrites and is further reduced by NO reductase. Since the interior of a *P. aeruginosa* biofilm is a hypoxic environment [102, 103], the level of anaerobic NO and RNS production should be proportional to biofilm depth. In this way, cell position within the biofilm could be sensed and dispersal and cell death could be coregulated with biofilm growth [101].

Endogenous ROS production in the biofilm is the source of the high variability of biofilm cells, and ROS could act as a signal that mediates the cell death of a sensitive subpopulation in the deeper layers of the biofilm and metabolic differentiation in the upper part of the biofilm. The bacterial biofilm communities thus strikingly resemble metabolic differentiation and ammonia-regulated cell death in *S. cerevisiae* colonies described above. Whether ROS-mediated cell death is part of programmed colony/biofilm development or it is simply the inability of a sensitive subpopulation to withstand the accumulation of toxic byproducts of metabolism remains an unanswered question. The findings from yeast colonies and bacterial biofilms showing that cell death in one subpopulation leads to metabolic activity and variability in the other subpopulation,

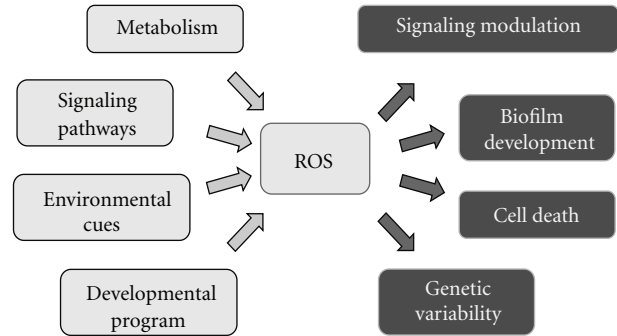


FIGURE 3: Inputs and outputs of ROS production in biofilms. Left: summary of processes inducing ROS production. Right: processes affected by ROS.

and thus increase fitness of the population as a whole, argue for the former option.

4.2. ROS-Dependent Signaling in Biofilm. Biofilm development is probably governed by both environmental cues as well as by intercellular signaling molecules. The best-studied intercellular signal is QS, which also plays a role in biofilm formation and development [6, 79]. A variety of autoinducer molecules have been identified in bacteria, of which the most studied are the species-specific acylated homoserine lactones (AHLs) found in many Gram-negative bacteria and the furanosyl borate ester AI-2 produced and recognized by both Gram-positive and Gram-negative species. It has been proposed that many autoinducers are able to induce ROS production, making ROS possible downstream signals or effectors of QS pathways [104]. QS plays an important role in the processes of biofilm formation and dispersal. For example, QS signaling through AI-2 in *Vibrio cholerae*, *Listeria monocytogenes*, *Bacillus cereus*, and *Staphylococcus epidermidis* and the autoinducer protein AIP in *Staphylococcus aureus* inhibit biofilm formation. In contrast, a positive effect on biofilm formation was described for AI-2 signaling in *Bacillus subtilis*, *Lactobacillus rhamnosus* as well as in AHL-mediated QS in *P. aeruginosa* [79, 105]. Interestingly, ROS seem to be able to modulate quorum sensing in various ways. ROS can inhibit autoinducer peptide signaling in *S. aureus in vitro* [106]. In a study of the mouse skin infection model, ROS-producing enzymes of the immune system were indispensable for defense against infection by the wild-type *S. aureus* strain, but not necessary for defense against a QS-deficient strain, indicating that QS molecules could be a target for oxidation by the immune system *in vivo* [106]. Superoxide also decreases the expression of QS locus *comQXP* in *B. subtilis* [107]. On the other hand, ROS have the potential to increase the QS signal, as certain derivatives of AHL oxidation by ROS exhibit increased biological activity [108]. In addition, QS regulates the expression of oxidative stress defense genes in various bacterial species [109–112].

Additional indications of ROS-dependent signaling pathways regulating biofilm growth have recently appeared. *Enterococcus faecalis* biofilm formation is dependent on the presence of the *xdh* gene, presumably encoding for a

selenoprotein xanthine dehydrogenase involved in purine metabolism and uric acid utilization and possibly evoking ROS production [113]. Cells in an *E. faecalis* biofilm produce high levels of ROS via a mechanism that is reliant on the presence of xanthine dehydrogenase, its cofactors selenium and molybdenum and its substrate uric acid. An intriguing model was proposed, in which uric acid in the environment is metabolized by *E. faecalis* cells with concomitant H₂O₂ production, which in turn induces biofilm formation. H₂O₂ thus would be a metabolic byproduct with a signaling function. Since uric acid is abundant in blood and urine, that is, preferred environments for *E. faecalis*, it makes sense that detecting this metabolite triggers the formation of the biofilm to successfully colonize the host [113].

In multispecies oral biofilms, streptococci produce H₂O₂ from pyruvate as a mean of biochemical warfare against other species, as well as a regulator of its own development as described above. However, the oral pathogen *Aggregatibacter actinomycetemcomitans* uses this streptococci-produced H₂O₂ as a signal that activates the expression of the complement resistance protein ApiA, which helps *A. actinomycetemcomitans* to resist the host's nonspecific immune response [114].

Some results suggest that ROS play a role in modulating the indole signaling pathway. Indole acts as an intercellular signal in many bacterial species [80]. Similarly to other signaling molecules, indole even induces a response in some species that do not synthesize it, and thus acts as an interspecies signaling molecule. In *Escherichia coli*, indole, which is synthesized by the enzymes coded by the *tnaAL* operon, inhibits biofilm growth regulates pathogenicity and the expression of multidrug resistance genes [115, 116]. Among other signals, *tnaA* expression is induced by ROS and repressed by growth in a biofilm [117, 118]. Indole was proposed to act as an oxidant in membranes and to induce membrane rearrangements [119]. Furthermore, some antibiotics induce indole production, which consequently inhibits biofilm formation in *E. coli*, via a mechanism involving hydrogen peroxide [120], showing that indole signaling and H₂O₂ cooperate in a pathway inhibiting biofilm growth. The opposite results, that is, indole-induced biofilm formation in *E. coli* and other species, were reported by others [121].

4.3. Biofilms and Oxidative Stress Adaptation. As with *C. albicans* biofilms and *S. cerevisiae* colonies, there is obviously a connection between the life of bacteria within a multicellular structure (biofilm) and their adaptation to oxidative stress. A number of genes have been identified that are important for both biofilm growth and oxidative stress resistance. Examples are the transcriptional repressor Rex and trigger factor RpoS in *Streptococcus mutans* [122, 123], *in-silico*-identified genes *uspE* and *gadX* in *E. coli* [124], posttranslational regulator CsrA in *Campylobacter jejuni* [125] and two-component systems GacS-GacA in *Pseudomonas* sp. and ColR-ColS in *Xanthomonas citri* [126, 127]. The redox-sensitive DNA-binding protein OxyR is a well-studied transcription regulator that mediates oxidative stress response in many Gram-negative bacteria [128]. OxyR

is activated by forming an intramolecular disulfide bond upon reaction with H₂O₂, which leads to the expression of OxyR-regulated genes. The role of OxyR in biofilm formation has been described in several bacterial species. In *E. coli*, OxyR induces biofilm formation by activating expression of the surface adhesin Ag43, which is responsible for cell-to-cell attachment and surface adherence [129]. Similarly, OxyR regulates cell attachment by increasing the expression of adherent fimbriae in *Serratia marcescens* and *Klebsiella pneumoniae* [130, 131] and OxyR's function in biofilm formation was also reported in *Neisseria gonorrhoeae* and *Tannerella forsythia* [132, 133].

5. Conclusions

Both endogenous and exogenous reactive oxygen species are important stress factors in the life of microorganisms. Endogenous ROS production is an inevitable consequence of microbial life in the presence of oxygen and can be even potentiated by some antibiotics that induce ROS production in sensitive microbes [134–136]. Exogenous ROS can be encountered during immune response to the presence of microbes inside the animal or plant body. In addition, many bacterial species release ROS as an oxidative weapon against competitors in multispecies populations. In these cases, ROS are produced by specialized enzymes. ROS are thus widely used as a means of biochemical warfare in nature. In order to defend against the deleterious effects of ROS, microorganisms have evolved efficient mechanisms of ROS removal. On the other hand, various pieces of data suggest that ROS could play an active and important role in processes like growth autoinhibition, cell death, and biofilm/colony development in both yeast and bacteria. In such cases, the enzymes producing ROS are tightly regulated as part of a biofilm developmental program and ROS are the effectors of some intrinsic regulation. Finally, ROS can act as signaling molecules either by targeting specific signaling pathways (e.g., kinases or transcription factors) or by, for example, modifying other signaling molecules such as quorum sensing factors. The large number of ROS-producing enzymes and the many different responses to ROS suggest that ROS-mediated processes are universal in the microbial world. Improving our understanding of the regulation and signaling driven by ROS could thus provide deeper insight into complex biological processes including the formation of biofilms, multicellular structures with important implications in medicine and other fields. The possibility of interfering with the signaling involved in biofilm formation or biofilm dispersal with ROS-producing or ROS-scavenging agents is especially attractive.

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