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Research Article

Comparative Study of Oxidative Stress Response in *Pseudomonas aeruginosa* (Subp01) and *Pseudomonas fluorescence* (Subp02) Upon External Stimulation by Hydrogen Peroxide

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Abstract

With a previous observation of internal oxidative damage within *Pseudomonas* cells, present study further attempted to resolve the influence of the supplementation of 3 mM hydrogen peroxide (H_2O_2) into their culture media. Furthermore, the unlikely absence of damaged aggregates in *Pseudomonas* culture medium fraction as observed earlier in case of viable but non-culturable (VBNC) *Escherichia coli* cells, current study compelled to discern the inimitable stress response among different species of *Pseudomonas*. Initially, 3 mM H_2O_2 was externally added to the late log phase of *Pseudomonas aeruginosa* (SUBP01) and *P. fluorescens* (SUBP02) and afterwards the morphological consequences and the cell culturability were examined. Compared to *P. fluorescence* (SUBP02), a relatively lower reduction in cell turbidity together with a rapid drop in the colony forming units (CFUs) on the agar plates was observed in *P. aeruginosa* (SUBP01) cells upon supplementation of 3 mM H_2O_2. Accumulation of damaged aggregates in *P. aeruginosa* (SUBP01) culture medium, consistent to their morphologically impaired cells (i.e. indicative of the VBNC state) was also noticed in this strain. In contrast, such a phenotype was not observed in *P. fluorescens* (SUBP02), indicating that a large fraction of viable and culturable cells still exist in the later species. The present investigation clearly revealed the resistance trait of *P. fluorescence* (SUBP02) against the external oxidative stimulant (H_2O_2), while the cells of *P. aeruginosa* (SUBP01) were found to be sensitive.

Keywords: *Pseudomonas aeruginosa; Pseudomonas fluorescens; Oxidative Stress; Hydrogen Peroxide (H2O2); Cell Viability*

Introduction

Microorganisms exposed to oxidative stress have been well reported to exhibit an array of metabolic and physiological dysfunctions [1-4]. The reactive oxygen species (ROS), namely superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (•OH) are generated by aerobic respiration and the accumulation of ROS beyond the cellular capacity for detoxification results in oxidative stress [5-13]. Interestingly, resistance against such stress has also been noted with a significant extent [13-19].

Compared to the actively growing cells, the starved *E. coli* cells have been found to be resistant not only against the oxidative stress but also against the temperature up shift [20], due to the expressional regulation of a set of genes especially under the σ^S (the master stress regulator, encoded by *rpoS*) and σ^E (the alternative sigma factor, encoded by *rpoE*) regulons [6,7,21-23]. A nearly similar scenario has also been observed in *Salmonella* spp. [24], *Vibrio* spp. [25,26] and in *Pseudomonas putida* [27,28]. Indeed, *Escherichia coli* and *Pseudomonas* spp. have been also reported to exhibit several unique physiological responses against oxidative stress [6,7,29-33]. In fact, several physiological and morphological changes within bacterial cells during the early stationary phase appear to be similar in *Escherichia coli* and

Pseudomonas spp. [34-36]. Earlier studies noted both *E. coli* and *Pseudomonas* spp. to employ the defense mechanisms mediated by catalase (encoded by the *katE* gene) and superoxide dismutase (encoded principally by *soda* gene, and also by *sodB*) against ROS mediated toxicity [6,10,16,32,37-43].

In cohort to the previous findings, our earlier work also pondered to the morphological impairment with a reduced viability in *P. aeruginosa* cells upon high speed aeration, possibly commencing the oxidative stress [1]. However, the dubious absence of damaged aggregates in the culture medium, which is in general the indication of the existence of viable but non-culturable (VBNC) cells [1,2,6,7,20] further led the possibility of the heterogeneity in stress management among different *Pseudomonas* species; i.e. some strains could be resistant while others were sensitive. Based on these suggestive notations, the present study further clarified the oxidative stress events among the *Pseudomonas* species, i.e. *P. aeruginosa* (SUBP01) and *P. fluorescens* (SUBP02). Such strategy was employed to chalk out the possible resistant strains among these two species against the oxidant H_2O_2. Both the two species were challenged with 3 mM H_2O_2 at the late log phase, and the consequences on cell culturability were assessed. The findings of the current study projected not only on the resistance phenotype of *P. fluorescens* (SUBP02) against oxidative state, but also incremented the existing knowledge on the bacterial stress management events [44-47].

Methods

Bacterial stain, medium and culture condition

Laboratory stock cultures of *P. aeruginosa* (SUBP01) and *P. fluorescens* (SUBP02) were used in this study. Nutrient agar (Hi-Media Laboratories Pvt. Ltd., India) and nutrient broth (beef extract and peptone) were used to assess the growth and viability of bacteria [1,2,48]. Pre-cultures were prepared by inoculating 5 ml nutrient broth by a loopful pure colony from the freshly prepared bacterial culture plates, and the broths were incubated at 37°C for in shaking condition (100 rpm) for 4 hours. The resultant optical density was measured at the wavelength of 600 nm (OD_{600}) and was adjusted to 0.1 by dilution with nutrient broth. A volume of 30 µL each was introduced into 2 different sets of 30 ml of nutrient broth and incubated at 37°C with shaking at 100 rpm. At 10 hour of growth, 3 mM H_2O_2 was aseptically added into one set of nutrient broth of each of the *P. aeruginosa* (SUBP01) and *P. fluorescens* (SUBP02); while and the other set was kept running without H_2O_2; i.e. control [1,21]. At every 12 hour intervals, bacterial growth was monitored by measuring OD_{600} and the formation of colony forming units (CFUs) was estimated by counting the colonies on agar plates up to 72 hours at every 24 hour intervals [1,2,6,7,20,48].

This is to be mentioned that earlier, in order to demonstrate the extent of H_2O_2 resistance in *Pseudomonas* spp., the minimal inhibitory concentration (MIC) was evaluated through broth micro-dilution procedure using different concentrations of H_2O_2 starting from 1 mM to 10 mM [4]. The minimum concentration which could inhibit the bacterial growth (as observed by the absence of turbidity), was considered as the MIC value. The study of MIC revealed that the growth of *Pseudomonas* spp. was inhibited by 3 mM concentration of H_2O_2.

Microscopy

For the examination of the bacterial cell morphology and arrangements, simple staining (Crystal Violet, Hucker's Solution) was employed as previously described by Munna., *et al* [1,2,48]. Briefly, aliquots of 10 µl from each of the growing bacterial culture suspensions were pulled out at every 12 hour intervals as stated above; and the cell size, shape, arrangement and organization were observed under the light microscope (Optima Biological Microscope G206, manufactured in Taiwan) at 100× magnification [1,2,48].

Spot dilution test

Spot tests were performed at every 12 hours of growth up to 72 hours as described previously [1,2,20,48]. Each of the bacterial culture suspensions were serially diluted with nutrient broth up to 10^{-4} fold dilution. From each dilution, an aliquot of 5 µl was carefully dropped on to the nutrient agar plates and was kept for around 15 - 20 minutes for drying off. Finally, the plates were incubated at 37°C for 18 - 24 hours.

Results and Discussion

Our earlier investigations on the impact on bacterial cells by the spontaneous generation or by imposing the oxidative stress revealed the loss of bacterial viability and culturability due to the toxicity especially posed by the reactive oxygen species (ROS) [2,6,20,43]. One of the significant findings was the demonstration of endogenous oxidative stress generation simulated by high aeration speed, which in turn significantly eliminated the living and culturable cells of *E. coli* and *Pseudomonas* spp. at 36 hours of growth. However, a discrepancy arose when the *Pseudomonas* culture was observed to be free from cell aggregates within the culture medium fraction [1]. Although the pigmentation in the *Pseudomonas* cells was noticed to be eliminated in course of time, such a discrepancy in loss of viability without formation of debris (i.e. the aggregates) led us to assume the possible presence of *Pseudomonas* cells resistant against the oxidative stress. Such a possible heterogeneity in *Pseudomonas* population would be of further interest indeed. To resolve such uncertainty, in the present study we used H_2O_2 as the oxidative stress stimulant on *P. aeruginosa* (SUBP01) and *P. fluorescens* (SUBP02) to directly distinguish between the stress-resistant species from that of the stress-sensitive one.

Resistance potential of *P. fluorescens* (SUBP02) against 3mM H_2O_2

As stated earlier, our earlier studies revealed the cessation of *Pseudomonas* culturability in terms of the formation of colonies on agar plates at 36 hour of growth at an aeration speed of 200 rpm [1]. A similar frequency of growth diminution was observed in case of *E. coli* cells not only in such endogenous stress (due to accumulation of ROS), but also when the cells were challenged with 3 mM H_2O_2 with a direct influence of exogenous oxidative stress [2]. However, in the present study, a relatively quick drop both in the cell turbidity and in the culturable cells was observed in *P. aeruginosa* (SUBP01) cells upon prolonged exposure to 3 mM H_2O_2. Interestingly at 36 hours of incubation most of the colonies of *P. aeruginosa* (SUBP01) were found to lose their pigmentation traits followed by the complete elimination of their CFUs onward (Figure 1a and 1b). Notably, even being under the same genus, no such impaired phenotypes (i.e. non-pigmented) neither the abolition of culturable cells were observed in case of *P. fluorescens* (SUBP02) (Figure 1c and 1d). Conversely, these cells were observed to retain their unique feature of yellow-green colonies with a steady growth rate up to 72 hours of incubation period.

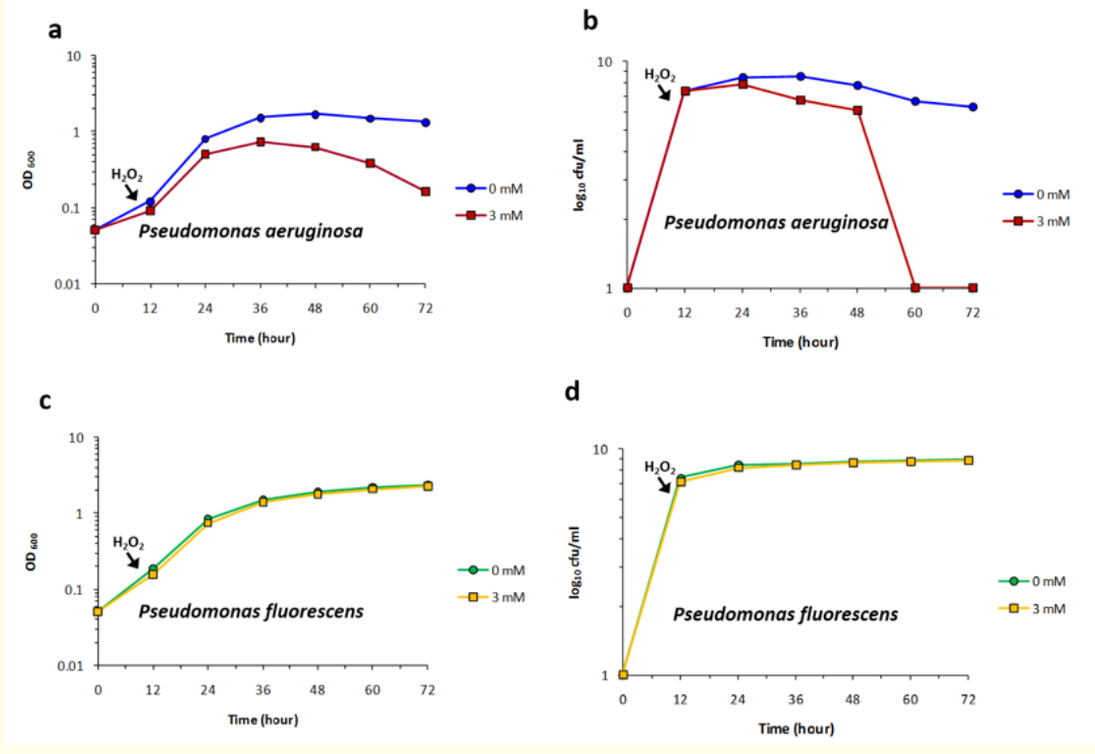

Figure 1: *Assessment of cell culturability through the examination of growth of Pseudomonas aeruginosa (a, b) and P. fluorescens (c, d) upon 3 mM H2O2 treatment. Bacterial cells were grown in nutrient broth at 37°C under 100 rpm as described in Methods. At the time points indicated, OD 600 (a, c) and CFUs (b, d) were determined. Experiments were done in triplicate, and the results were found to be reproducible. One representative data has been shown.*

Morphological impairment of *P. aeruginosa* (SUBP01) compared to that of *P. fluorescens* cells (SUBP02)

The dramatic fall in the culturable cells of *P. aeruginosa* (SUBP01) in contrast to the continual growth of *P. fluorescens* (SUBP02) (Figure 1) further led us to examine the cell morphology and arrangements of the former cells under the formed stressed condition generated by the addition of 3 mM H_2O_2 (Figure 2). Interestingly, after 36 to 72 hours of incubation periods, cell aggregates were observed within the culture media of *P. aeruginosa* (SUBP01) (Figure 2d) which were significantly comparable with the H_2O_2 treated *P. fluorescens* (SUBP02) cells (Figure 2j). In cohort with our recent findings as well from the earlier reports, the morphologically defective cells of *P. aeruginosa* (SUBP01) were suggestive of being the non-culturable or even dead cells; while the *P. fluorescence* (SUBP02) cells were noticed to sustain viability [1,27,34-37].

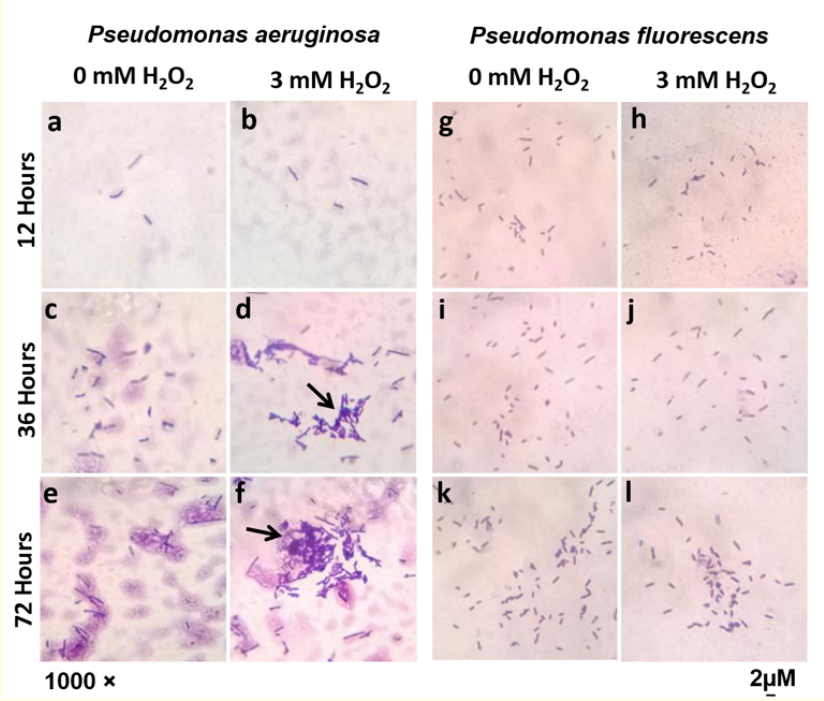

Figure 2: *Assessment of cell morphology of Pseudomonas aeruginosa (a, b, c, d, e, f; the arrowhead indicates impaired cells) and P. fluorescens (g, h, i, j, k, l) upon 3 mM H2O2 treatment. Cells were grown in nutrient broth at 37 °C under 100 rpm as described in Methods. At the time points indicated, aliquots of cell culture were removed for microscopy.*

Confirmative demonstration of loss of culturability of *P. aeruginosa* (SUBP01) cells

One of the important parameters of detecting the culturability of bacterial cells lies on the demonstration of the density of their viable inoculums within the culture suspension used for CFU formation assessment [2]. For this purpose, the measurement of viability of cells was done using the subsequent 3 dilutions followed by spotting on agar media. Such a strategy also detected the retention or loss of culturability of cells when exposed to agar [8].

In the present study, after 36 hours of incubation period, *P. aeruginosa* (SUBP01) was found to be significantly inhibited compared to that of the control; i.e. untreated with H_2O_2 (Figure 3). No spot of growth was observed after 48 hours, consistent to the growth studies as

shown in figure 1a and 1b. On the contrary, appearance of growth of *P. fluorescens* (SUBP02) was noticed up to 72 hours in all of the diluted spots (Figure 3). Thus, together with the current findings in accordance to our earlier results (Munna et al. 2014), it can be suggested that *Pseudomonas* cells tend to exhibit heterogeneity in response towards oxidative stress. However, further genetic studies on the expressional regulation of the oxidant neutralizing genes especially in the *P. fluorescens* (SUBP02) would unravel the molecular mechanism behind the resistance mechanisms against H_2O_2 [14,16,17,32,40,41].

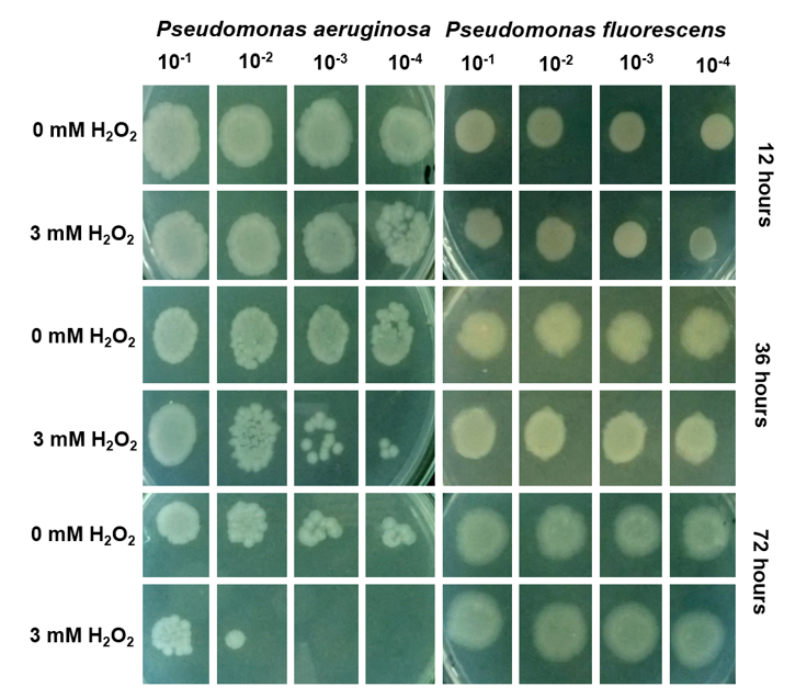

Figure 3: *Assessment of cell culturability and survival potential of Pseudomonas aeruginosa and P. fluorescens through spot dilution tests upon 3 mM H2O2 treatment. Cells were grown in nutrient broth at 37°C under 100 rpm as described earlier. At the time points indicated, aliquots of cell culture each consisting of 5 µl were used for the spot test as described in Methods. Experiments were done in triplicate and the results were reproducible. One representative data has been shown.*

Conclusions

Despite the lack of molecular study in our current investigation, the fatal impact of 3 mM H_2O_2 in *P. aeruginosa* (SUBP01) cells were clearly distinguishable from those of *P. fluorescens* (SUBP02), which has so far been conducted for the first time. The study evidently projected on the phenotypic changes upon the oxidant stimulation of different *Pseudomonas* species even belonging to the same genus, and the findings were sufficient to draw comprehensible evidence of a distinct stress defense mechanism of *P. fluorescens* (SUBP02) possibly involving antioxidant enzymes or iron-sequestering proteins, for the direct elimination of the reactive oxygen species (ROS).

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