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Effects of quorum sensing signal molecules on the hydrogen peroxide resistance against planktonic *Pseudomonas aeruginosa*

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The effects of quorum sensing signal molecules in *Pseudomonas aeruginosa*, *N*-butanoyl-L-homoserinelactone (C4-HSL) and *N*-(3-oxododecanoyl)-L-homoserinelactone (3-oxo-C12-HSL) on planktonic cell resistance against hydrogen peroxide were studied. In *P. aeruginosa* JP2 cells with the deletion of *lasI* and *rhlI*, the viable cell concentration decreased with time and was reduced by about 4 log after 2 h of 7.5 mM H_2O_2 treatment, while only a 2-log reduction was found for the wild type *P. aeruginosa* PAO1 cells. When cultured with 20% PAO1 spent medium, *P. aeruginosa* JP2 showed similar hydrogen peroxide resistance to that seen in *P. aeruginosa* PAO1. Culturing with 20% JP2 spent medium or with 10 μM C4-HSL and 20 μM 3-oxo-C12-HSL did not affect *P. aeruginosa* JP2 cell susceptibility to hydrogen peroxide. Although both 20% PAO1 and JP2 spent media reacted with H_2O_2 and reduced H_2O_2 to 50% of the strength of the original concentration, the remaining H_2O_2 was still sufficient to kill *P. aeruginosa* JP2. These results indicate that the difference in cell resistance against H_2O_2 between *P. aeruginosa* PAO1 and JP2 was related to the existence of gene products of the *lasI* and *rhlI* systems. However, adding synthetic homoserine lactones alone did not increase *P. aeruginosa* JP2 cell resistance to H_2O_2 as seen in the experiments adding PAO1 spent medium. Determination of the detailed relation between cascade regulation in *P. aeruginosa* and its cell resistance to H_2O_2 will require further investigation.

Key words: Homoserine lactones, hydrogen peroxide, quorum sensing, resistance

In recent years, it has become evident that many bacteria exploit a cell-to-cell communication device to regulate diverse physiological processes including bioluminescence, competence development, antibiotic biosynthesis, plasmid conjugal transfer and the production of exoenzymes [1]. Cell-to-cell communication involves a phenomenon called quorum sensing in which bacteria cells activate specific genes only at high population densities in response to chemical signals released by the bacterial cells into the growth medium [2]. Initial work was primarily focused on bacterial luminescence and demonstrated that bacteria were communicating via small molecules generically referred to as homoserine lactones (HSLs) [3]. In planktonic cultures, HSLs are produced and accumulated in parallel with increasing cell density [2]. By the time the cell density reaches a certain level, HSL concentrations build to the nM range and begin to influence gene expression patterns involving cascade-like regulatory systems controlling several genes coding for proteins participating in diverse activities [4]. Given the variety of cellular functions that appear to be controlled by such regulatory circuitry, it is thought that this represents a global control system [5]. Such global control systems represent mechanisms that 1. allow the bacterial cells to respond to environmental stimuli, altering gene expression in an attempt to adjust metabolism such that cell energy balance is maintained at optimum levels; 2. allow response to severe environmental change (e.g. heat shock); and 3. help make proper arrangements for conversion to a quiescent state (nongrowth) when the nutrient supply becomes limited.

Though cell-to-cell communication between suspended cells has been studied for many years, the effects of quorum sensing on bacterial cell resistance have not been extensively studied. For example, most studies of the physiological response to oxidative stress in planktonic cells focused on the mechanism of cell adaptation against reactive oxygen intermediates (ROIs), such as superoxide anion (O_2), hydrogen peroxide, hypochlorous acid (HOCl), and hydroxyl radical (OH·) [6,7]. ROIs usually result from the incomplete reduction of oxygen during electron transport within cells. Microbial cells may also encounter extracellular ROIs when oxidizing biocides are used as disinfectants. *Pseudomonas aeruginosa*, an

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opportunistic pathogen often found in patients with cystic fibrosis, is infamous for its antimicrobial resistance due to the production of alginate [8]. Cell-to-cell communication of *P. aeruginosa* has been known to be mediated by *N*-butanoyl-L-homoserinelactone (C4-HSL) and *N*-(3-oxododecanoyl)-L-homoserinelactone (3-oxo-C12-HSL) [9,10]. Recently, Hassett *et al* [11] reported that the expression of catalase and superoxide dismutase genes in *P. aeruginosa* was mediated by quorum sensing systems. However, the relation between cell-to-cell communication and *P. aeruginosa* oxidative stress resistance still remains unclear. In this study, we demonstrated that *P. aeruginosa* planktonic cell resistance to hydrogen peroxide was affected by the existence of quorum sensing signal molecules.

Materials and Methods

Strain and medium

The strains used in this study included a wild type of *P. aeruginosa* PAO1 [12] and a mutant of *P. aeruginosa* JP2 (Δ*lasI*::Tn10;Δ*rhlI*::Tn501) [13] which was kindly provided by Dr. Barbara H Iglewski (Department of Microbiology and Immunology, University of Rochester, New York, USA). *P. aeruginosa* JP2 is unable to synthesize C4-HSL and 3-oxo-C12-HSL. The strains were maintained on Luria-Bertani (LB; Difco, Detroit, MI, USA) agar plates with appropriate selection pressure and restreak every 4 weeks. Tryptic Soy Broth (TSB; Difco) was used throughout the experiments. To keep the maximum cell concentration at around 10^8 to 10^9 cell/mL, 1/10 strength of TSB was used in suspended batch cultures.

Suspended batch cultures

A single colony was picked for inoculation into 100 mL 1/10 strength TSB in a 250 mL flask. The flask was shaken at 200 rpm and 37 °C for 12 h and then used as the seed culture. One percent (v/v) of the seed culture was transferred to fresh 100 mL 1/10 TSB and shaken at 200 rpm and 37 °C for 12 h.

Parental spent medium preparation and C4-HSL /3-oxo-C12-HSL synthesis

The parental spent medium was obtained by separating cells from an overnight culture of *P. aeruginosa* PAO1 grown in TSB. After centrifuging at 6,000 *g* and 4 °C for 10 min, the supernatant was filtered through a 0.22 μm polycarbonate membrane (Millipore Corp., Bedford, MA, USA) and kept at 4 °C for later use. C4-HSL and 3-oxo-C12-HSL were synthesized by Dr. Timothy Kerchner (Department of Chemistry, Montana State University, Bozeman, MT, USA) as per the description of Eberl *et al* [14].

H_2O_2 treatment

After 12 h of growth, planktonic cells were challenged with 7.5 mM H_2O_2 (Sigma, St. Louis, MO, USA) for 2 h at 37 °C. One milliliter of sample was taken every 15 min and neutralized with 9-mL phosphate buffered solution containing 0.02% sodium thiosulfate (Sigma).

Cell enumeration

Planktonic samples were diluted appropriately with phosphate buffered solution. For viable cell count, at least three 0.1 mL of samples were plated on R2A agar (Difco) plates. The viable cell number was determined by averaging the number of colony formation units (CFU) counted from three plates after 18 h incubation at 37 °C. The surviving fraction was calculated by normalizing the viable cell concentration with that before H_2O_2 treatment.

H_2O_2 assay

H_2O_2 concentration was assayed as the methods of Pick and Keisari [15]. This assay is based on the horseradish peroxidase-mediated oxidation of phenol red by H_2O_2 which resulted in the formation of a compound demonstrating increased absorbance at 610 nm. Briefly, 10 μL standard or sample solution was added to 1 mL phenol red solution (0.1 mg/mL phenol red, 140 mM NaCl, 10 mM KH_2PO_4, 5.5 mM dextrose, pH 7.0) containing 85 units of horseradish peroxidase (Sigma). After sitting at room temperature for 5 min, 10 μL of 1 N NaOH was added to stop the reaction. The absorbance at 610 nm was read by a UV-visible spectrophotometer (UV-160A, Shimadzu, Japan).

Statistical analysis

All data presented were obtained from three separate experiments. At each time point in each experiment, the mean values for viable cells and H_2O_2 concentrations were recorded. Statistical analyses were performed with Sigma Plot 4.0 software (SPSS Inc., Chicago, IL, USA) and the cell enumeration results were based upon the log transformation means.

Results

Effect of *lasI* and *rhlI* genes on cell resistance

The resistance of *P. aeruginosa* PAO1 and JP2 planktonic cells in response to 7.5 mM H_2O_2 is shown in Fig. 1. In the control experiments without exposure

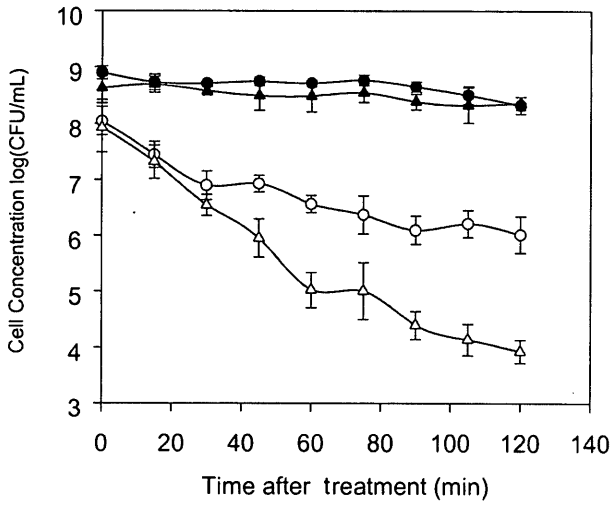

Fig. 1. Change in viable cell concentrations of *P. aeruginosa* PAO1 (△) and JP2 (○) in response to 7.5 mM H_2O_2 treatment. (▲) and (●) represent control experiment without H_2O_2 treatment for PAO1 and JP2, respectively. (n = 3; bars indicate standard errors)

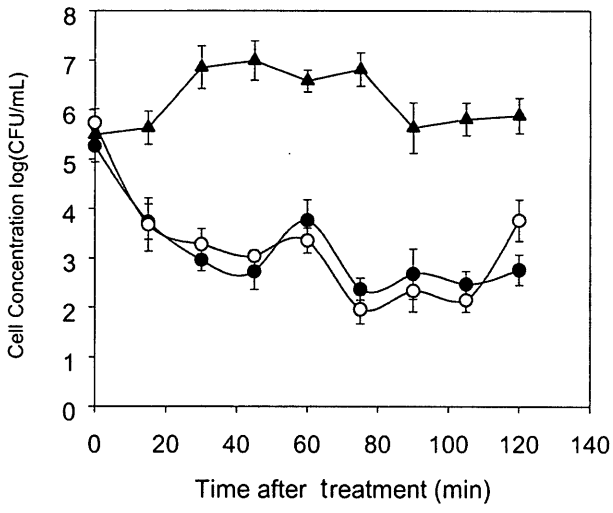

Fig. 3. Change in H_2O_2 concentration in medium containing PAO1 spent medium (●), JP2 spent medium (○) and without any spent medium (▲). (n = 3; bars indicate standard errors)

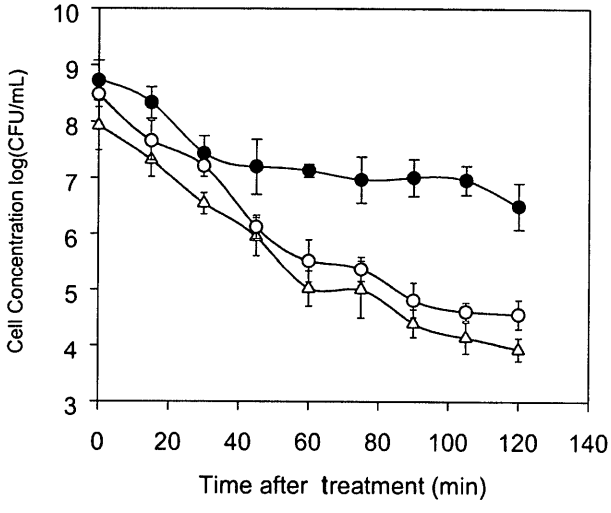

Fig. 2. Change in viable cell concentration of *P. aeruginosa* JP2 grown with PAO1 spent medium (●), JP2 spent medium (△) and without any spent medium (○) in response to 7.5 mM H_2O_2 treatment. (n = 3; bars indicate standard errors)

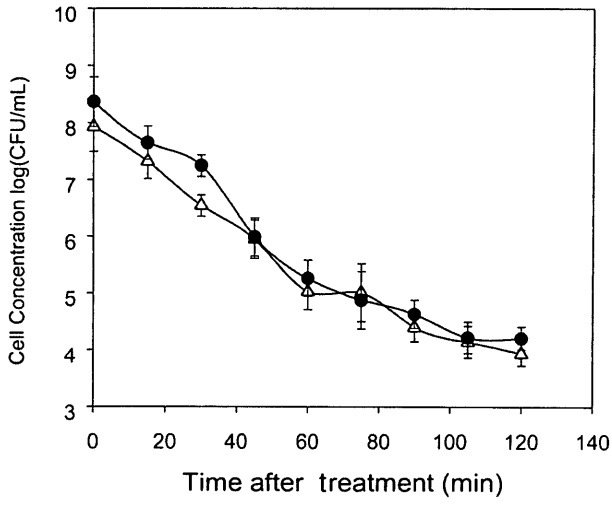

Fig. 4. Change in viable cell concentration of *P. aeruginosa* JP2 grown with 10 μM C4-HSL and 20 μM 3-oxo-C12-HSL (●) and without any synthetic HSLs (△) in response to 7.5 mM H_2O_2 treatment. (n = 3; bars indicate standard errors)

to H_2O_2, the viable cell concentrations of *P. aeruginosa* PAO1 and JP2 were constant throughout the experiments. For cells with the deletion of *lasI* and *rhlI* (JP2), the viable cell concentration decreased with time and had reduced by approximately 4 log by the end of the experiment. In comparison to the wild type cells (PAO1), there was about a 2-log reduction after 2 h of H_2O_2 treatment. These results indicate that the resistance between *P. aeruginosa* PAO1 and JP2 might have resulted from the deletion of *lasI* and *rhlI* genes.

Effect of PAO1 parental spent medium on cell resistance

In order to compensate for the deletion of *lasI* and *rhlI* genes in *P. aeruginosa* JP2, 20% PAO1 parental spent medium was added to the medium. After the growth reached stationary phase, the *P. aeruginosa* JP2 cells were treated with 7.5 mM H_2O_2. Medium containing

20% JP2 parental spent medium was used as the control experiment. Fig. 2 illustrates the response of *P. aeruginosa* JP2 grown with PAO1 and JP2 spent medium to antimicrobial treatment. The viable cell concentration of *P. aeruginosa* JP2 grown with PAO1 spent medium reduced by about 2 log after 2-h treatment, while there was about a 4-log reduction for cells grown with JP2 spent medium. These results suggest that some cell products related to *lasI* and/or *rhlI* might affect *P. aeruginosa* resistance. However, one might also suspect that the spent medium would react with H_2O_2 and hence reduce the H_2O_2 concentration. Fig. 3 shows the change of H_2O_2 concentration with 20% PAO1 and JP2 spent medium, respectively, as well as without any spent medium. The H_2O_2 concentration remained generally constant without spent medium. However, when the medium contained PAO1 spent medium, the H_2O_2 concentration dropped significantly for the first 40 min, then remained at about 3 mM thereafter. Similar phenomena were also observed in the medium containing JP2 spent medium. These results indicate that although both PAO1 and JP2 spent medium reacted with H_2O_2, the remaining 3 mM of H_2O_2 was still sufficient to kill *P. aeruginosa* planktonic cells.

Effect of synthetic C4-HSL and 3-oxo-C12-HSL on cell resistance

Similar experiments were also done using 10 μM C4-HSL and 20 μM 3-oxo-C12-HSL to replace the PAO1 spent medium. The viable cell concentration of *P. aeruginosa* JP2 grown in medium containing synthetic HSLs in response to 7.5 mM H_2O_2 is shown in Fig. 4. The results show that there was no significant difference (t-test; $p > 0.8$) in cell resistance to H_2O_2 for *P. aeruginosa* grown with and without synthetic HSLs. In other words, adding synthetic HSLs alone did not increase *P. aeruginosa* JP2 cell resistance to H_2O_2 as seen in the experiments adding PAO1 spent medium.

Discussion

Physiological activities of *P. aeruginosa* known to be controlled by the quorum sensing systems, *lasI* and *rhlI*, include expression of virulence genes [13] and *rpoS* [16], and production of multiple exoenzymes [17,18]. The physiological response to oxidative stress in planktonic cells has also been studied for decades [6, 7]. Not surprisingly, microorganisms will develop some mechanisms in response to the oxidative stress.

In this study, the experimental results suggest that the oxidative response of *P. aeruginosa* might be related to the quorum sensing signal molecules. The *P. aeruginosa* mutant (JP2) with deletion of *lasI* and *rhlI* showed greater susceptibility to H_2O_2 than the wild type (PAO1). When complemented with PAO1 spent medium, the mutant strain recovered some resistance and showed similar resistance to wild type strain in response to H_2O_2treatment. These results are consistent with the findings of a recent study by Hassett *et al* [11] that the expression of catalase and superoxide dismutase genes in *P. aeruginosa* was mediated by quorum sensing systems and consequently reduced the susceptibility to H_2O_2. In comparison to PAO1 spent medium, addition of synthetic C4-HSL and 3-oxo-C12-HSL was of little help in enhancing the resistance of JP2. This phenomenon could be attributed to the *P. aeruginosa* multilayered hierarchical quorum sensing cascade regulation system [16]. This system might involve not only C4-HSL and 3-oxo-C12-HSL but also their transcription factors, LasR and RhlR. Determination of the detailed relation between cascade regulation in *P. aeruginosa* and its cell resistance to H_2O_2 will require further investigation.

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