



The Open Microbiology Journal

Content list available at: www.benthamopen.com/TOMICROJ/

DOI: 10.2174/1874285801711010012



RESEARCH ARTICLE

Ozone Sensitivity and Catalase Activity in Pigmented and Non-Pigmented Strains of *Serratia Marcescens*

José de Ondarza*

Department of Biological Sciences, Plattsburgh State University of New York, NY, USA

Received: August 06, 2016

Revised: January 16, 2017

Accepted: January 28, 2017

Abstract:

Background:

Ozone exposure rapidly leads to bacterial death, making ozone an effective disinfectant in food industry and health care arena. However, microbial defenses may moderate this effect and play a role in the effective use of oxidizing agents for disinfection. *Serratia marcescens* is an opportunistic pathogen, expressing genes differentially during infection of a human host. A better understanding of regulatory systems that control expression of *Serratia*'s virulence genes and defenses is therefore valuable.

Objective:

Here, we investigated the role of pigmentation and catalase in *Serratia marcescens* on survival to ozone exposure.

Method:

Pigmented and non-pigmented strains of *Serratia marcescens* were cultured to exponential or stationary phase and exposed to 5 ppm of gaseous ozone for 2.5 – 10 minutes. Survival was calculated via plate counts. Catalase activity was measured photometrically and tolerance to hydrogen peroxide was assayed by disk-diffusion.

Results:

Exposure of *S. marcescens* to 5 ppm gaseous ozone kills > 90% of cells within 10 minutes in a time and concentration-dependent manner. Although pigmented *Serratia* (grown at 28°C) survived ozonation better than unpigmented *Serratia* (grown at 35°C), non-pigmented mutant strains of *Serratia* had similar ozone survival rates, catalase activity and H₂O₂ tolerance as wild type strains. Rather, ozone survival and catalase activity were elevated in 6 hour cultures compared to 48 hour cultures.

Conclusion:

Our studies did not bear out a role for prodigiosin in ozone survival. Rather, induction of oxidative stress responses during exponential growth increased both catalase activity and ozone survival in both pigmented and unpigmented *S. marcescens*.

Keywords: Catalase, oxyR, ozone, pigment, peroxide, prodigiosin, *Serratia*.

INTRODUCTION

Ozone is a potent oxidizing agent that occurs naturally in the atmosphere at low (< 50 ppb) concentrations, but can rise to levels as high as 200 – 300 ppb in smog. At such levels, ozone becomes a significant health risk associated with respiratory irritation. At yet higher levels, its oxidizing potential can be life-threatening, but also serve as a useful disinfection agent with applications in food industry [1], water quality maintenance [2], and in the health care industry [3, 4]. The efficacy of ozone as a disinfecting agent has been reported to also vary significantly based on the concentra-

* Address correspondence to this author at the Department of Biological Sciences, Plattsburgh State University of New York, NY, USA; Tel: 518-564-5156; Fax: 518-564-3175; E-mail: deondaj@plattsburgh.edu

tion, time of exposure, presence of organic materials, and type of microbe exposed, with up to a 4-log reduction in pathogen counts on seeds reported at concentrations of 2000 ppm [5], although most treatment approaches use lower concentrations of 0.1 – 10 ppm with correspondingly lower reductions in microbial counts [6, 7]. A wide variety of pathogens respond differently to ozone disinfection, raising the question of whether sensitivity to ozone can be moderated by specific microbial defenses such as catalase, superoxide dismutase, or other antioxidant systems. Indeed, microbial responses such as elevation of SOD and catalase activity have been demonstrated in *Listeria* [8] and *E. coli* [9] in response to ozone exposure. Microbial responses to hydrogen peroxide exposure are better studied and reveal much of the underlying regulatory mechanisms, including induction of catalase, alkyl hydroperoxidase, and superoxide dismutase by oxidative stress via transactivator molecules such as oxyR [10, 11], rpoS [12] and soxRS [13]. Alternatively, microbial pigmentation offers another potential defense against environmental challenges, including predation [14, 15], competition (antibiotic effects), and potentially, oxygen radicals [16 - 18]. Such pigments are often produced by bacteria as secondary metabolites via a quorum-sensing mechanism, including prodigiosin and violacein [19 - 21]. Whereas the purple pigment violacein can function as an anti-oxidant [22], such a role has yet to be demonstrated for prodigiosin, a red pigment produced by *Serratia marcescens*. *Serratia*, a Gram-negative, ubiquitous microbe and opportunistic human pathogen, produces the red pigment prodigiosin in a temperature and quorum sensing-dependent manner, and possibly benefits from such pigments due to their antibiotic properties, although the exact function of these has not been completely elucidated [17]. The pigment (prodigiosin) is thought to be located in the cell wall of the bacteria and contributes to cell surface hydrophobicity [23, 24], in a location where it could also conceivably act as a first layer of defense against external oxidative challenges. Alternately, it has been proposed that prodigiosin may act as an energy-spilling role, reducing ATP synthesis, oxidative metabolism and thus, reactive oxygen species generation [25]. We therefore sought to determine if pigmentation also offers a survival advantage and a defense against exposure to ozone. We show here that ozone kills *Serratia marcescens* in a time-dependent manner and that growth phase of the cultures, but not pigmentation or oxyR, affect survival to ozone exposure.

MATERIALS AND METHOD

Bacterial Strains

Serratia marcescens (wild type) was purchased from Presque Isle Cultures (Erie PA). All other *Serratia marcescens* strains were kindly provided by Dr. Robert Shanks (University of Pittsburgh) (Table 1).

Table 1. Strains used in this study.

Strain		Pigmentation	Reference/source
SM-R	<i>S. marcescens</i> wild type cultured at 30°C	Yes	Presque Isle Cultures
SM-W	<i>S. marcescens</i> wild type cultured at 37°C	No	Presque Isle Cultures
SM-01	<i>S. marcescens</i> wild type	Yes	Shanks Lab
SM-02	<i>S. marcescens</i> : pigA mutant	No	Shanks Lab
SM-04	<i>S. marcescens</i> Nima	Yes	Shanks Lab
SM-05	<i>S. marcescens</i> Nima: pigA mutant	No	Shanks Lab
SM-06	<i>S. marcescens</i> : oxyR mutant	Yes	Shanks Lab [11]

Culture

All strains were initially grown in Tryptic Soy Broth (Hardy Diagnostics, Santa Maria CA) prior to plating on MacConkey agar (Becton Dickinson, Franklin Lakes NJ) for ozone exposure. Wild-type *S. marcescens* was cultured for 48h at either 30°C (red-pigmented, SM-R) or 37°C, a temperature at which prodigiosin is not produced (SM-W). *S. marcescens* strains SM-01, SM-02, SM-04, SM-05, and SM-06 were cultured 6h (exponential phase) or 48h (stationary phase) at 30°C, yielding pigmentation in wild-type and oxyR mutants but not the pigA deletion mutants. Pigmentation level was assayed as the ratio of light absorbance at 535 nm/600 nm. 1 ml of each 48h cell culture was centrifuged at 13,000g to pellet cells, supernatant discarded, and pigment extracted in 1 ml acid-alcohol (95% isopropanol + 1% 2N HCl). A₆₀₀ was measured for 500 µl of cell culture resuspended in 2 ml saline; A₅₃₅ was measured for 500µl pigment extract resuspended in 2 ml dH₂O.

Ozone Exposure

Growing (6h) or stationary (48h) cultures of *S. marcescens* were diluted in TSB to an Absorbance of 0.100 at 600 nm, serial-diluted to 10^{-4} or 10^{-5} in 0.9% saline, and spread-plated onto 125 mm MacConkey agar plates. Agar plates were then exposed to 5 or 10 ppm ozone inside a 42L plexiglass chamber. Ozone was generated with an OZX-300 Ozone generator (Enaly Co, Shanghai, China) and continuously monitored with a Model 106-L Ozone Monitor (2B Technologies, Boulder CO). Concentrations were maintained to within 0.5 ppm. Exposure times ranged from 2.5 minutes to 20 minutes. Agar plates were subsequently incubated for 24 - 48h at room temperature, colonies counted, and survival calculated as a percentage of unexposed controls.

Pre-Exposure of *Serratia* to H_2O_2

Exponentially growing 5 ml TSB cultures of *S. marcescens* were subjected to oxidative stress by pre-exposure to low (200 μ M) or high (20 mM) levels of H_2O_2 for 30 min prior to ozone exposure and catalase measurements.

H_2O_2 Disk Diffusion Assay

Sensitivity of *S. marcescens* to H_2O_2 was determined by disk-diffusion assay. Pour plates were prepared by mixing 100 μ l of a 0.500 Absorbance (600 nm) culture broth of each test strain with 2 ml of melted (45°C) Top Agar (50% Trypticase soy agar/50% Trypticase soy broth) and pouring it over bottom agar (10 ml TSA in 48 mm sterile Petri plates). Once solidified, sterile 6 mm blank paper disks were placed on the agar surface and 10 μ l of 30% H_2O_2 (Sigma-Aldrich) was added to each disk. Plates were incubated for 24h at either 28°C or 37°C and zones of inhibition measured. Each assay was conducted at least 3 separate times on different days.

Catalase Assay

Catalase activity was assayed following the methods of Beers [26]. In brief, 1 ml of each SM broth culture was pelleted by centrifugation at 6,000 x g, washed and resuspended in 2 ml of 0.9% saline, and sonicated at 40% power twelve times (30s on/30s off) on ice. Cell lysates were centrifuged for 20 min at 12000 x g to pellet cell debris, and supernatants were collected. 20 μ l of each lysate, supernatant, and pellet (resuspended in 1 ml saline) were then added to 50 mM phosphate buffer (pH 7) containing 30 mM H_2O_2 . Occasionally, 100 μ l lysate samples were heated to either 50°C or 100°C for 20 minutes before assaying. Catalase activity was measured by following the change in absorbance at 240 nm in an Ocean Optics 4400 UV/Vis spectrometer and calculated as $(\Delta A_{240}/\text{min})/(43.6/\text{Mol}/\text{min} * \text{sample volume} * 0.001)$. Catalase activity was normalized to sample protein concentration when possible using Bradford protein assay.

Statistical Analysis

Pairwise comparisons were performed using Student's t-test, while multiple comparisons were conducted using one-way ANOVA with GraphPad Prism software.

RESULTS

Survival to Ozone Exposure

Exposure of 48 h cultures of *Serratia marcescens* to ozone showed a time-dependent and concentration-dependent reduction in viable cell numbers for both pigmented and unpigmented strains. Initially, brief 30 second exposures to a range of ozone concentrations were tested to determine effective concentrations for short-term (5 – 30 minute) exposures. Based on these results (data not shown), concentrations of 5 and 10 ppm were selected. Low levels of ozone (5 ppm) significantly reduced bacterial numbers over a 10 minute time period, affecting non-pigmented *S. marcescens* (SM-W) more than pigmented strains (SM-R) (Fig. 1). This effect was significant at 2.5 min exposure ($P < 0.05$), but also pronounced at 5 and 7.5 min. Similarly, ozonation at 10 ppm reduced SM-01 cell numbers, but at a faster rate (10-fold reduction in 5 min). Pigmentation in 48h broth cultures of SM-R (cultured at 30°C) was robust, attaining an Absorbance ratio (A_{534}/A_{600}) of 0.472, whereas the same strain grown at 37°C exhibited no pigmentation (A_{535}/A_{600} ratio = 0.005). To clarify whether pigmentation, or some other change brought about by lower incubation temperature, was responsible for the lower sensitivity to ozone, this experiment was repeated using two wild-type *S. marcescens* strains (SM-01 and SM-04 Nima) and their pigA deletion mutants (SM-02 and SM-05, respectively), with incubation conditions maintained at 30°C. Both mutant strains exhibited similar survival following 5 ppm ozone

exposure as their pigmented counterparts (Fig. 2), strongly suggesting that pigmentation may not be responsible for the observed difference in sensitivity to ozone.

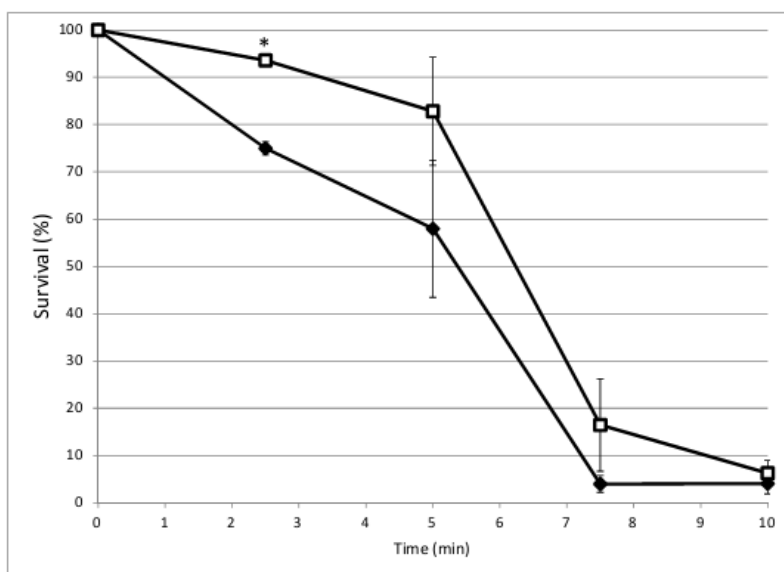


Fig. (1). Survival of *Serratia marcescens* after exposure to 2.5 – 10 min of gaseous ozone (5 ppm). Pigmented SM-R (open squares) survived ozone exposure better than unpigmented SM-W (filled diamonds); * significantly different ($P < 0.05$).

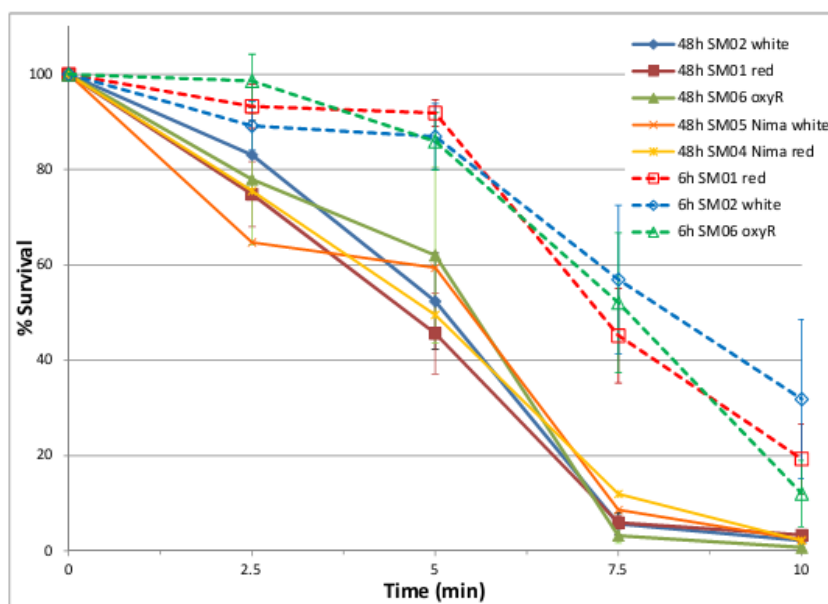


Fig. (2). Survival of wild type and mutant *S. marcescens* strains following ozone (5 ppm) exposure. *S. marcescens* strains were incubated for either 6h or 48h prior to ozonation. No significant differences were observed among strains in stationary phase (48h) or among strains in exponential phase (6h), but survival was significantly greater ($P < 0.05$) for exponential-phase cultures compared to stationary-phase cultures.

Sensitivity to Hydrogen Peroxide Exposure

To investigate whether expression of anti-oxidant enzymes such as catalase mediate this protection against ozone exposure, SM-R and SM-W were suspended in top agar and pour-plated onto a bottom agar base prior to incubation in the presence of 10µl of 30% H₂O₂ in a disk-diffusion assay. Although SM-R had slightly smaller inhibition zones when

assayed at pigment-producing temperature (28°C) than SM-W assayed at 37°C (Fig. 3), this difference was not significant. As well, SM-W also showed slightly higher ($P > 0.05$) resistance to H_2O_2 at 28°C, and greater resistance to H_2O_2 than SM-R at each assay temperature. This suggests that SM-W has better peroxide defenses (and potentially) catalase activity. A similar pattern was observed when comparing the SM-04 (Nima) strain to its *pigA* deletion counterpart (SM-05), with pigmented SM-04 having a similar, though slightly smaller, inhibition zone than unpigmented SM-05 at 28°C, or SM-04 assayed at 37°C (unpigmented). In comparison, both *E. coli* (known to have multiple catalases) and *Streptococcus lactis* (lacking catalase) have significantly smaller inhibition zones at 37°C (Fig. 3), as does an *oxyR* mutant of *Serratia* (SM-06).

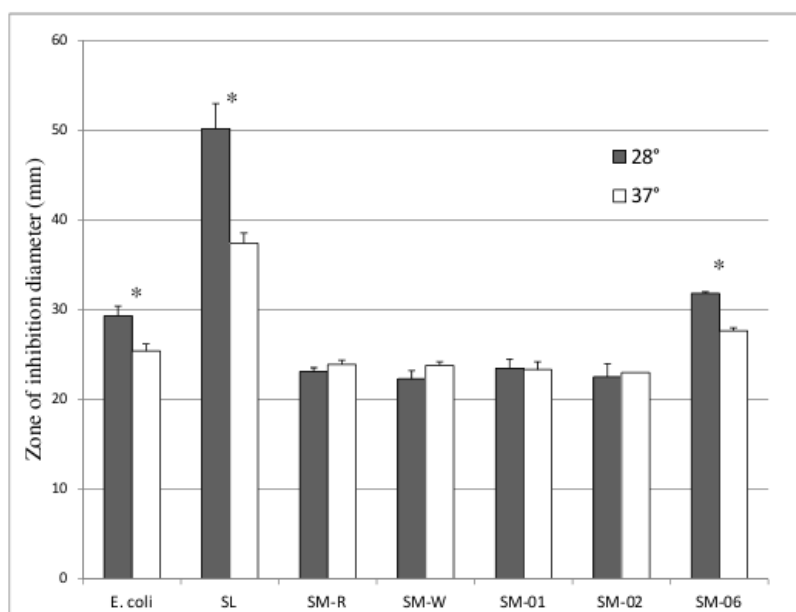


Fig. (3). Effect of assay temperature on H_2O_2 sensitivity (Zone of inhibition diameter) for *E. coli*, *Streptococcus lactis* (SL), and *S. marcescens* strains SM-01, SM-02, SM-06 (all cultured at 30°C) and SM wild type cultured at either 28°C (SM-R) or 37°C (SM-W) before exposure to 10 μ l H_2O_2 . Agar plates + H_2O_2 -impregnated disks were incubated for 24h at 28°C or 37°C. Incubation at pigment-producing temperature (28°C) slightly increased SM resistance to H_2O_2 exposure, whereas *E. coli*, catalase-lacking *Streptococcus lactis* (SL), and the *oxyR* mutant of *Serratia* (SM-06) are more sensitive ($P < 0.05$) at this temperature. * significantly different (28°C vs 37°C).

Catalase Activity in *Serratia* Strains

To assess catalase activity directly, H_2O_2 degradation was measured spectrophotometrically. Consistent with the disk diffusion data, SM-W (grown at 37°C) exhibited slightly higher catalase activity than SM-R (grown at 28°C) (Table 2). Catalase activity was detected in the cell lysate following sonication, in the soluble fraction (supernatant) of the lysate following centrifugation, in heated (50°C) lysate and in intact cells, but not in the pelleted cell debris or in boiled supernatant (Fig. 4). Heating of cell lysates to 50°C reduced catalase activity by 3% - 15% in all strains examined, whereas heating to 75°C reduced activity by 84% - 90% and boiling reduced activity essentially to background levels. Catalase activity was also shown to be slightly greater in the non-pigmented SM-02 *pigA* mutant than in the wild type strain SM-01.

Table 2. Catalase activity of *S. marcescens* cell lysates following centrifugation to separate cell debris (pellet) from soluble components (supernatant). Activity was determined in stationary phase cells (48h cultures, N = 6) or exponential phase cells (6h cultures, N = 7) as units/ml as well as normalized to sample protein concentrations (U/mg). Protein concentrations were not determined for pelleted debris due to catalase activities indistinguishable from background.

Strain	Stationary			Exponential
	Supernatant (U/ml)	Pellet (U/ml)	Supernatant (U/mg)	Supernatant (U/mg)
SM-R	103.96±3.21	2.28±0.47	779.37±204.17	ND
SM-W	123.89±21.39	4.46±1.04	852.28±111.46	ND

(Table 4) contd.....

Strain	Stationary			Exponential
	Supernatant (U/ml)	Pellet (U/ml)	Supernatant (U/mg)	Supernatant (U/mg)
SM-R	103.96±3.21	2.28±0.47	779.37±204.17	ND
SM-01	149.49±25.49	4.50±0.82	522.26±31.64	749.79±67.24 ^{a,b}
SM-02	135.32±17.35	3.81±0.79	654.1±3.94	904.63±50.28 ^{a,b}
SM-06	84.17±11.76	2.06±0.14	541.43±49.98	625.59±19.66

^a: significantly different from SM during stationary phase; ^b: significantly different from SM-06; ND: not determined

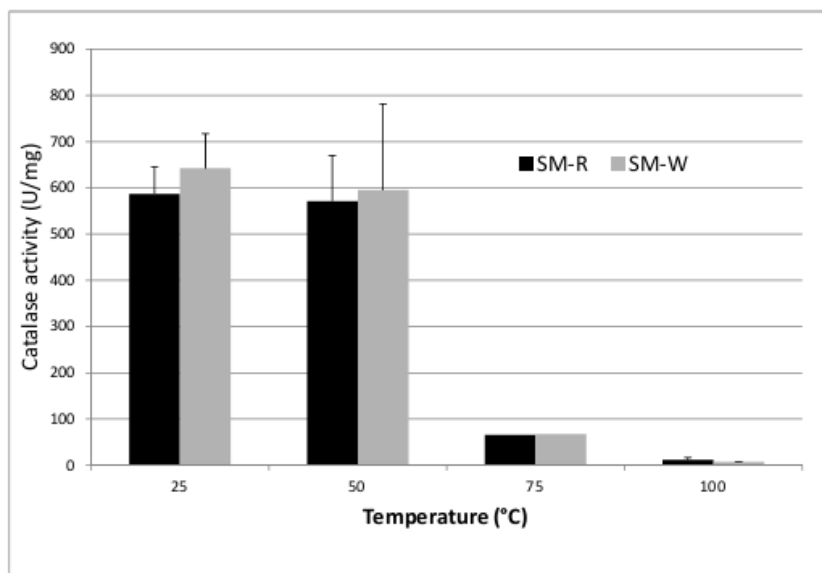


Fig. (4). Catalase activity (U/mg) of pigmented (red) and unpigmented (white) *Serratia marcescens* cell lysates, normalized to protein concentrations. Heating of lysates to 50°C did not affect catalase activity, but activity was significantly lower in lysates heated to 75°C or 100°C ($P < 0.05$). For temperature stability assays, only cell lysates were used.

Sensitivity to Ozone and H₂O₂, and Catalase Activity, of oxyR Mutant *S. Marcescens*

To further investigate the role of oxidative stress defenses in ozone sensitivity and catalase activity, an oxyR mutant strain [11] was assayed. OxyR serves as a sensor of oxidative stress in many bacteria and regulates expression of catalase [13]. 48h cultures of SM-06 (OxyR mutant) had similar ozone survival (Fig. 2) and catalase activity (Table 2) as the wild-type (SM-01) strain even while maintaining their greater sensitivity to H₂O₂ exposure in a disk-diffusion assay as previously established [11]. Interestingly, the oxyR mutant exhibited a lower sensitivity to H₂O₂ at 37°C, much like *E. coli* and *S. lactis* (Fig. 3) and unlike *Serratia* strains SM-01 and SM-02.

Effect of H₂O₂ Pre-Exposure on Ozone Sensitivity and Catalase Activity

Since the putative function of oxyR is to up-regulate microbial defenses against oxidative stress, we then repeated the ozone and catalase assays in *Serratia* strains which were cultured for 6h (exponential phase) and then pre-exposed to oxidative stress (200 μM or 20 mM H₂O₂). Catalase activity in 6h cultures was significantly greater than that of 48h cultures in SM-01 (pigmented) and SM-02 (non-pigmented), but not in SM-06 (oxyR mutant) strains of *S. marcescens* (Table 2). However, no significant differences were observed between catalase activity of H₂O₂-pre-exposed cultures and controls ($P > 0.48$). We also examined the effect of H₂O₂ pre-exposure in 6h cultures on *S. marcescens* sensitivity to ozone exposure. All strains of *Serratia* tested after 6h of incubation (SM-01, 02 and 06) exhibited a significantly greater survival to ozonation at 5 ppm than any stationary phase (48h culture) *Serratia* strains (Fig. 2). Pre-exposure to H₂O₂ had a slight but not significant effect on ozone survival, with slightly better 5 min survival rates for 6h cultures pre-exposed to 50mM H₂O₂.

DISCUSSION

Effect of Ozone Exposure on *S. Marcescens*

Ozone is a potent and highly reactive molecule which can rapidly induce cellular damage in bacteria as well as humans. Its potent antibacterial action has led to its resurgence as a disinfectant in industrial, medical and food industry settings [2]. Because it is naturally occurring, we thought it plausible that bacterial defenses against oxidizing agents will protect them from ozone disinfection. Here, we initially showed that continuous exposure to 5 – 10 ppm of gaseous ozone kills *S. marcescens* in a time and concentration-dependent manner, and that these reductions are in line with reported ozone killing efficiencies in fruits and vegetables [6, 27, 28] where microbes attached to food surfaces were effectively killed by 0.1 – 9.2 ppm of ozone. Although up to 4-log reductions were observed with dry seed surfaces [5], the above-mentioned studies reported more typical log₁₀ reductions of 0.22 – 2.89 for pathogens on moist food surfaces, depending on bacterial strain and exposure time. In the present study, *Serratia* cells were attached to a moist (agar) surface, and exposure times were similar or shorter (2.5 to 10 min vs. 5 min to 6 h) than in previous studies, but in general we attained a similar 1 – 2 log reduction in 10 min at 5 ppm (Ct value = 50 ppm.min) as with *Salmonella* and *E. coli* on bell peppers treated for 30 min at 1 ppm [27], *E. coli* on carrots and lettuce treated for 15 min at 5 ppm [28], and coliforms on papaya treated for 20 min at 9.2 ppm [6]. Ozone is thought to disrupt bacterial cell envelopes, leading to cell death by cytoplasmic leakage and cell lysis [1, 27].

Effect of Pigmentation on Survival to Ozone Exposure

Unlike most other coliforms, *Serratia marcescens* is capable of producing the red pigment prodigiosin. Expression of prodigiosin is under complex regulatory control; its genes are part of the *pig* operon (*pig* for pigment) which allows pigment production to be regulated in a temperature and quorum-sensing-dependent manner [17, 20]. Our observation of greater survival of pigmented *Serratia* cells to ozone exposure than cells of the same strain that are unpigmented (when cultured at higher temperatures) could therefore be explained either by the presence of pigment or by differential expression of other protective factors at the lower temperature. Cultures of wild-type SM grown at 30°C for 48h were intensely pigmented; this pigmentation is presumably located in the bacterial wall [17] and thus present in single cells of pigmented, but not unpigmented, cultures. Such cells consistently survived ozone exposure better than unpigmented cells of the same strain cultured at 37°C (Fig. 1). However, neither of the *pigA*-mutant strains of *Serratia* differed from their isogenic pigmented strains in term of ozone survival, and it is therefore likely that a temperature-induced change in protective genes could be responsible. The transition from environmental temperatures to that of an animal host is known to induce significant alterations in gene expression in many pathogens, including dimorphic fungi and environmental bacteria such as *Listeria* [29], *Pseudomonas aeruginosa* [30], and *Serratia*, where porin production [31], pigmentation, and possibly catalase expression [32] are changed.

Possible Role of Catalase in Ozone Resistance

Catalase expression is of particular interest, as catalase activity is affected by ozone exposure [8, 33], and we therefore examined more closely the role of catalase in *Serratia*. Genes for monofunctional (HPII; *katE*, 478 aa) and bifunctional (HPI; *katG*, 731 aa) catalase have been sequenced in *Serratia* [34, 35]. Presumably, then, both constitutive and inducible forms of catalase operate in *Serratia*. Direct measurement of catalase activity show similar levels in pigmented (SM-R, SM-01) and non-pigmented (SM-W and SM-02) strains cultured for 2 or more days (Table 2). This catalase activity is likely due to the monofunctional catalase (HPII), which, at least in *E. coli*, is increasingly expressed as cells transition into stationary phase [36]. This is consistent with detection of monofunctional catalase activity in *S. marcescens* [37, 38]. The catalase described by Zeng [38] is also relatively stable up to 70°C, consistent with our data (Fig. 2). The HPI form of catalase, in contrast, is a bifunctional catalase that is encoded by *katG* and is under control of the OxyR regulon in *E. coli* [13, 39, 40], an operon which also functions in *Serratia marcescens* [11]. Although this enzyme was not directly detected in *Serratia* in either mid-log or stationary phase cells [37], exposure of *Serratia* cells to 50 mM H₂O₂ did indeed induce *katG* expression dramatically in the recently described high oxidative stress-tolerant isolate *Serratia* sp. LCN16, a response which was absent in *oxyR* mutants [41].

Response of *S. Marcescens* to Oxidative Stress (H₂O₂ Exposure)

Here, we were unable to observe a significant increase in catalase activity following low (200 μM) or high (20 mM) level H₂O₂ pretreatments; rather, we observed a significantly higher catalase activity in 6h cultures of wild-type, but not *oxyR* mutant, *S. marcescens* as compared to 48h cultures (stationary). It is therefore possible that rapid early growth of

Serratia generates sufficient oxidative stress through aerobic growth to activate a stress response via oxyR. This can also explain the reduced resistance of oxyR mutants in H₂O₂ disk-diffusion assays [11], where the lack of an oxidative stress response makes these cells sensitive to normally sub-lethal doses of hydrogen peroxide. This oxyR-mediated protective response may well be mediated by katG. During aerobic growth of *Bradyrhizobium japonicum* [42] katG has even been shown to be the main way to remove endogenously generated H₂O₂, albeit without oxyR.

Protective Mechanisms to Oxidative Stress

The mechanism of action of ozone on bacterial cells involves oxidation of cellular components, in part through generation of reactive oxygen species such as O[·], OH[·], and hydrogen peroxide. These reactive oxygen species in turn combine with cellular lipids and proteins, causing membrane, enzyme inactivation and cell lysis [1, 27, 33]. Since hydrogen peroxide is a byproduct of ozone reactions with cells, peroxidases are reasonable means of cellular defenses against ozone [8, 33]. Indeed, defensive enzymes such as catalase have been shown to be induced by ozone exposure [9]. While our observation that exponentially growing cells have both greater ozone survival and catalase activity support a protective role of catalase, other mechanisms are certainly at work since survival of the oxyR mutant (SM-06) strain to ozone exposure was similar to that of the wild-type strain (SM-01) with a much smaller increase in catalase activity. Microbial pigments, including prodigiosin [43, 44], provide another potential protective mechanism to oxidative stress such as ozone exposure and have been shown to have antioxidant properties; although the precise mechanism of prodigiosin's antioxidant properties is unclear, pigments such as prodigiosin act as antioxidants when they donate electrons to reactive oxygen species [45]. Our studies, however, have not found this to be the case. Other protective enzymes are also activated in response to oxidative stress. Alkyl hydroperoxide reductase (ahp) plays a critical role in removing H₂O₂ in *E. coli* [46] but is saturated at low (10⁻⁵M) levels, whereas in *Pseudomonas*, ahp is part of the oxyR regulon and is more critical in detoxifying H₂O₂ in biofilms [10]. Superoxide dismutase (SOD), meanwhile, converts O²⁻ into the somewhat less toxic H₂O₂. Genes for sodA, sodB and sodC can be found in the genome of *Serratia marcescens* [34] and at least one form of SOD has been isolated [47]. However, the role of these enzymes in *Serratia*'s oxidative stress response has been studied little and remains to be elucidated. Likewise, it remains to be determined if any of these play a role in protecting *Serratia* from ozone, studies which are now beginning in our lab.

CONCLUSION

Although *Serratia*, when cultured at pigment-producing temperatures, showed increased survival to ozone and peroxide exposure than when cultured and assayed at higher temperatures, subsequent use of non-pigmented mutants showed no difference in ozone survival, catalase activity or hydrogen peroxide resistance. Neither does it appear that oxyR plays a role in protecting *Serratia* from ozone exposure, as oxyR mutants exhibit similar ozone survival as wild type cells. It is therefore likely that protective mechanisms other than prodigiosin or catalase, possibly regulated by temperature shift or quorum sensing mechanisms [48], contribute to *Serratia*'s resistance to both ozone and peroxide.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The author confirms that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

I wish to thank Dr. Robert Shanks (University of Pittsburgh) for providing many of the *Serratia* strains used in this study. Funding was provided through Plattsburgh State University of New York's Departmental of Biological Sciences, College of Arts & Sciences, and a Professional Development grant (PDIAP).

REFERENCES

- [1] Pascual A, Llorca I, Canut A. Use of ozone in food industries for reducing the environmental impact of cleaning and disinfection activities. *Trends Food Sci Technol* 2007; 18(Suppl. 1): S29-35. [http://dx.doi.org/10.1016/j.tifs.2006.10.006]
- [2] Gray NF, Percival SL, Yates MV, Williams D. Ozone disinfection. In: *Microbiology of waterborne diseases*. Jordan Hill, GBR: Academic Press: GBR 2013; pp. 599-615.
- [3] Sharma M, Hudson JB. Ozone gas is an effective and practical antibacterial agent. *Am J Infect Control* 2008; 36(8): 559-63. [http://dx.doi.org/10.1016/j.ajic.2007.10.021] [PMID: 18926308]
- [4] Edelstein PH, Whittaker RE, Kreiling RL, Howell CL. Efficacy of ozone in eradication of *Legionella pneumophila* from hospital plumbing fixtures. *Appl Environ Microbiol* 1982; 44(6): 1330-3. [PMID: 7159081]
- [5] Trinetta V, Vaidya N, Linton R, Morgan M. A comparative study on the effectiveness of chlorine dioxide gas, ozone gas and e-beam irradiation treatments for inactivation of pathogens inoculated onto tomato, cantaloupe and lettuce seeds. *Int J Food Microbiol* 2011; 146(2): 203-6. [http://dx.doi.org/10.1016/j.ijfoodmicro.2011.02.014] [PMID: 21411164]
- [6] Yeoh WK, Ali A, Forney CF. Effects of ozone on major antioxidants and microbial populations of fresh-cut papaya. *Postharvest Biol Technol* 2014; 89: 56-8. [http://dx.doi.org/10.1016/j.postharvbio.2013.11.006]
- [7] Ölmez H, Akbas MY. Optimization of ozone treatment of fresh-cut green leaf lettuce. *J Food Eng* 2009; 90(4): 487-94. [http://dx.doi.org/10.1016/j.jfoodeng.2008.07.026]
- [8] Fisher CW, Lee D, Dodge BA, Hamman KM, Robbins JB, Martin SE. Influence of catalase and superoxide dismutase on ozone inactivation of *Listeria monocytogenes*. *Appl Environ Microbiol* 2000; 66(4): 1405-9. [http://dx.doi.org/10.1128/AEM.66.4.1405-1409.2000] [PMID: 10742219]
- [9] Whiteside C, Hassan HM. Induction and inactivation of catalase and superoxide dismutase of *Escherichia coli* by ozone. *Arch Biochem Biophys* 1987; 257(2): 464-71. [http://dx.doi.org/10.1016/0003-9861(87)90591-1] [PMID: 3310892]
- [10] Panmanee W, Hassett DJ. Differential roles of OxyR-controlled antioxidant enzymes alkyl hydroperoxide reductase (AhpCF) and catalase (KatB) in the protection of *Pseudomonas aeruginosa* against hydrogen peroxide in biofilm vs. planktonic culture. *FEMS Microbiol Lett* 2009; 295(2): 238-44. [http://dx.doi.org/10.1111/j.1574-6968.2009.01605.x] [PMID: 19456869]
- [11] Shanks RM, Stella NA, Kalivoda EJ, *et al.* A *Serratia marcescens* OxyR homolog mediates surface attachment and biofilm formation. *J Bacteriol* 2007; 189(20): 7262-72. [http://dx.doi.org/10.1128/JB.00859-07] [PMID: 17675374]
- [12] Liu X, Wu Y, Chen Y, *et al.* RpoS differentially affects the general stress response and biofilm formation in the endophytic *Serratia plymuthica* G3. *Res Microbiol* 2016; 167(3): 168-77. [http://dx.doi.org/10.1016/j.resmic.2015.11.003] [PMID: 26671319]
- [13] Chiang SM, Schellhorn HE. Regulators of oxidative stress response genes in *Escherichia coli* and their functional conservation in bacteria. *Arch Biochem Biophys* 2012; 525(2): 161-9. [http://dx.doi.org/10.1016/j.abb.2012.02.007] [PMID: 22381957]
- [14] Matz C, Webb JS, Schupp PJ, *et al.* Marine biofilm bacteria evade eukaryotic predation by targeted chemical defense. *PLoS One* 2008; 3(7): e2744. [http://dx.doi.org/10.1371/journal.pone.0002744] [PMID: 18648491]
- [15] Ahmetagic A, Philip DS, Sarovich DS, Kluver DW, Pemberton JM. Plasmid encoded antibiotics inhibit protozoan predation of *Escherichia coli* K12. *Plasmid* 2011; 66(3): 152-8. [http://dx.doi.org/10.1016/j.plasmid.2011.07.006] [PMID: 21839110]
- [16] Itami C, Matsunaga H, Sawada T, Sakurai H, Kimura Y. Superoxide dismutase mimetic activities of metal complexes of L-2-(2-pyridyl)-1-pyrroline-5-carboxylic acid (pyrimine). *Biochem Biophys Res Commun* 1993; 197(2): 536-41. [http://dx.doi.org/10.1006/bbrc.1993.2512] [PMID: 8267587]
- [17] Williamson NR, Fineran PC, Leeper FJ, Salmond GP. The biosynthesis and regulation of bacterial prodiginines. *Nat Rev Microbiol* 2006; 4(12): 887-99. [http://dx.doi.org/10.1038/nrmicro1531] [PMID: 17109029]
- [18] Vaishnav P, Demain AL. Unexpected applications of secondary metabolites. *Biotechnol Adv* 2011; 29(2): 223-9. [http://dx.doi.org/10.1016/j.biotechadv.2010.11.006] [PMID: 21130862]
- [19] McClean KH, Winson MK, Fish L, *et al.* Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. *Microbiology* 1997; 143(Pt 12): 3703-11. [http://dx.doi.org/10.1099/00221287-143-12-3703] [PMID: 9421896]

- [20] Van Houdt R, Givskov M, Michiels CW. Quorum sensing in *Serratia*. FEMS Microbiol Rev 2007; 31(4): 407-24. [http://dx.doi.org/10.1111/j.1574-6976.2007.00071.x] [PMID: 17459113]
- [21] Whitehead NA, Barnard AM, Slater H, Simpson NJ, Salmond GP. Quorum-sensing in Gram-negative bacteria. FEMS Microbiol Rev 2001; 25(4): 365-404. [http://dx.doi.org/10.1111/j.1574-6976.2001.tb00583.x] [PMID: 11524130]
- [22] Konzen M, De Marco D, Cordova CA, Vieira TO, Antônio RV, Creczynski-Pasa TB. Antioxidant properties of violacein: possible relation on its biological function. Bioorg Med Chem 2006; 14(24): 8307-13. [http://dx.doi.org/10.1016/j.bmc.2006.09.013] [PMID: 17011197]
- [23] Burger SR, Bennett JW. Droplet enrichment factors of pigmented and nonpigmented *Serratia marcescens*: possible selective function for prodigiosin. Appl Environ Microbiol 1985; 50(2): 487-90. [PMID: 3901922]
- [24] Rosenberg M, Blumberger Y, Judes H, Bar-Ness R, Rubinstein E, Mazor Y. Cell surface hydrophobicity of pigmented and nonpigmented clinical *Serratia marcescens* strains. Infect Immun 1986; 51(3): 932-5. [PMID: 3512440]
- [25] Haddix PL, Jones S, Patel P, et al. Kinetic analysis of growth rate, ATP, and pigmentation suggests an energy-spilling function for the pigment prodigiosin of *Serratia marcescens*. J Bacteriol 2008; 190(22): 7453-63. [http://dx.doi.org/10.1128/JB.00909-08] [PMID: 18805986]
- [26] Beers RF Jr, Sizer IW. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J Biol Chem 1952; 195(1): 133-40. [PMID: 14938361]
- [27] Alwi NA, Ali A. Reduction of *Escherichia coli* O157, *Listeria monocytogenes* and *Salmonella enterica* sv. *Typhimurium* populations on fresh-cut bell pepper using gaseous ozone. Food Contr 2014; 46: 304-11. [http://dx.doi.org/10.1016/j.foodcont.2014.05.037]
- [28] Bermúdez-Aguirre D, Barbosa-Cánovas GV. Disinfection of selected vegetables under nonthermal treatments: Chlorine, acid citric, ultraviolet light and ozone. Food Contr 2013; 29(1): 82-90. [http://dx.doi.org/10.1016/j.foodcont.2012.05.073]
- [29] Gründling A, Burrack LS, Bouwer HG, Higgins DE. *Listeria monocytogenes* regulates flagellar motility gene expression through MogR, a transcriptional repressor required for virulence. Proc Natl Acad Sci USA 2004; 101(33): 12318-23. [http://dx.doi.org/10.1073/pnas.0404924101] [PMID: 15302931]
- [30] Grosso-Becerra MV, Croda-García G, Merino E, Servin-González L, Mojica-Espinosa R, Soberón-Chávez G. Regulation of *Pseudomonas aeruginosa* virulence factors by two novel RNA thermometers. Proc Natl Acad Sci USA 2014; 111(43): 15562-7. [http://dx.doi.org/10.1073/pnas.1402536111] [PMID: 25313031]
- [31] Begic S, Worobec EA. Regulation of *Serratia marcescens* ompF and ompC porin genes in response to osmotic stress, salicylate, temperature and pH. Microbiology 2006; 152(Pt 2): 485-91. [http://dx.doi.org/10.1099/mic.0.28428-0] [PMID: 16436436]
- [32] Crichton PV, Lazarus AS. The relationship between prodigiosin production and catalase activity. J Bacteriol 1948; 56(3): 375-7. [PMID: 16561583]
- [33] Lee YK, Mok Kim S, Han S. Ozone-induced inactivation of antioxidant enzymes. Biochimie 2003; 85(10): 947-52. [http://dx.doi.org/10.1016/j.biochi.2003.09.012] [PMID: 14644549]
- [34] Chung WC, Chen LL, Lo WS, Kuo PA, Tu J, Kuo CH. Complete genome sequence of *Serratia marcescens* WW4. Genome Announc 2013; 1(2): e0012613. [http://dx.doi.org/10.1128/genomeA.00126-13] [PMID: 23558532]
- [35] Daligault HE, Davenport KW, Minogue TD, et al. Genome assembly of *Serratia marcescens* type Strain ATCC 13880. Genome Announc 2014; 2(5): e00967-14. [PMID: 25291774]
- [36] Loewen PC, Switala J, Triggs-Raine BL. Catalases HPI and HPII in *Escherichia coli* are induced independently. Arch Biochem Biophys 1985; 243(1): 144-9. [http://dx.doi.org/10.1016/0003-9861(85)90782-9] [PMID: 3904630]
- [37] Switala J, Triggs-Raine BL, Loewen PC. Homology among bacterial catalase genes. Can J Microbiol 1990; 36(10): 728-31. [http://dx.doi.org/10.1139/m90-123] [PMID: 2253114]
- [38] Zeng HW, Cai YJ, Liao XR, Zhang F, Zhang DB. Production, characterization, cloning and sequence analysis of a monofunctional catalase from *Serratia marcescens* SYBC08. J Basic Microbiol 2011; 51(2): 205-14. [http://dx.doi.org/10.1002/jobm.201000147] [PMID: 21077118]
- [39] Brown A, Lo D, Sharpe C, et al. Increased intracellular hydrogen peroxide observed in *Escherichia coli* in the presence of two classes of antibiotics. J Exp Microbiol Immunol 2012; 16: 17-23.
- [40] Eason MM, Fan X. The role and regulation of catalase in respiratory tract opportunistic bacterial pathogens. Microb Pathog 2014; 74: 50-8. [http://dx.doi.org/10.1016/j.micpath.2014.07.002] [PMID: 25109939]

- [41] Vicente CS, Nascimento FX, Ikuyo Y, Cock PJ, Mota M, Hasegawa K. The genome and genetics of a high oxidative stress tolerant *Serratia* sp. LCN16 isolated from the plant parasitic nematode *Bursaphelenchus xylophilus*. *BMC Genomics* 2016; 17(1): 301. [<http://dx.doi.org/10.1186/s12864-016-2626-1>] [PMID: 27108223]
- [42] Panek HR, OBrian MR. KatG is the primary detoxifier of hydrogen peroxide produced by aerobic metabolism in *Bradyrhizobium japonicum*. *J Bacteriol* 2004; 186(23): 7874-80. [<http://dx.doi.org/10.1128/JB.186.23.7874-7880.2004>] [PMID: 15547258]
- [43] Gulani C, Bhattacharya S, Das A. Assessment of process parameters influencing the enhanced production of prodigiosin from *Serratia marcescens* and evaluation of its antimicrobial, antioxidant and dyeing potentials. *Malays J Microbiol* 2012; 8(2): 116-22.
- [44] Vijayalakshmi K, Jagathy K. Production of prodigiosin from *Serratia marcescens* and its antioxidant and anticancer potential. *Int J Adv Res Biosci* 2016; 3(3): 75-88.
- [45] Kim J-S, Han Y-J, Heo M-S. Free radical scavenging activity of *Zooshikella* sp. JE-34 secondary metabolites by electron spin resonance spectrometry. *J Biotechnol* 2008; 136(Suppl.): S70. [<http://dx.doi.org/10.1016/j.jbiotec.2008.07.155>]
- [46] Seaver LC, Imlay JA. Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in *Escherichia coli*. *J Bacteriol* 2001; 183(24): 7173-81. [<http://dx.doi.org/10.1128/JB.183.24.7173-7181.2001>] [PMID: 11717276]
- [47] Maejima K, Miyata K, Tomoda K. A manganese superoxide dismutase from *Serratia marcescens*. *Agric Biol Chem* 1983; 47(7): 1537-43.
- [48] Hassett DJ, Ma JF, Elkins JG, *et al.* Quorum sensing in *Pseudomonas aeruginosa* controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide. *Mol Microbiol* 1999; 34(5): 1082-93. [<http://dx.doi.org/10.1046/j.1365-2958.1999.01672.x>] [PMID: 10594832]

© 2017 José de Ondarza

This is an open access article distributed under the terms of the Creative Commons Attribution 4.0 International Public License (CC-BY 4.0), a copy of which is available at: <https://creativecommons.org/licenses/by/4.0/legalcode>. This license permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.