Roles of *Pseudomonas aeruginosa* Autoinducers and their Degradation Products, Tetramic acids, in Bacterial Survival and Behavior in Ecological Niches

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*Pseudomonas aeruginosa*, an opportunistic pathogen, is known to mainly use *N*-acylhomoserine lactones (AHLs) as autoinducers. Recent progress in this field demonstrated that not only AHLs, but also their degradation products, tetramic acids, may have some biological activities. The present study examined the roles of *Pseudomonas* autoinducers and tetramic acids in bacterial survival and behavior in ecological niches. *P. aeruginosa* autoinducers and the tetramic acid derivatives were chemically synthesized, and the structure-activity correlation was investigated. Some tetramic acids derived from AHLs caused a significant reduction in the viability of *P. aeruginosa* in a concentration dependent manner (30–300 μM). The smaller the inoculum of bacteria, the stronger the bactericidal activity that was observed. The data from tetramic acid derivatives indicated the keto-enol structure of tetramic acid to be critical for the antibacterial activity. Exogenous tetramic acid did not induce significant changes in the formation of biofilm or production of exoproducts such as pyocyanin and elastase. Tetramic acid and disinfectants acted synergistically to kill *P. aeruginosa*. These results suggest the AHL-degradation product tetramic acid to be useful for bacterial control.

**Key words:** tetramic acid, quorum-sensing, *P. aeruginosa*

*Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen that causes a wide range of nosocomial infections, including sepsis, pneumonia, and urinary tract and surgical site infections (30, 31, 33). This organism is known to form biofilms, which reportedly increase bacterial resistance to phagocytic killing (25) and antibiotic killing (2, 40), and produces a variety of virulence factors, such as elastase, rhamnolipid, protease, and exotoxins (34). These virulence factors are regulated by quorum sensing (QS) which is a cell-to-cell signaling mechanism within a bacterial species (26). These systems rely on cell density-dependent transcriptional activators for controlling gene expression through production of small diffusible molecules called autoinducers. An important class of autoinducers used by Gram-negative bacteria is the family of *N*-acylhomoserine lactones (AHLs) (28, 34, 39). The QS system in *P. aeruginosa* operates via two autoinducers, N-3-oxododecanoyl-L-homoserine lactone (3-oxo-C₁₂-AHL) and N-butanoyl-L-homoserine lactone (C₄-AHL).

Recent studies have reported that competitive or cooperative communication could occur between groups of bacteria or between bacteria and hosts (13, 21, 24, 38). In particular, 3-oxo-C₁₂-AHL produced by *P. aeruginosa* possesses several functions against not only bacteria but also mammalian cells. Our previous study demonstrated that 3-oxo-C₁₂-AHL accelerated apoptosis in the neutrophil and monocytic cell lines U-937 and P388D1, but not in the epithelial cell lines CCL-185 and HEP-2 (35). On the other hand, 3-oxo-C₁₂-AHL, but not any of its analogues, was found to suppress growth and biofilm formation in *Legionella pneumophila*, whereas no bactericidal or bacteriostatic action against other Gram-negative bacteria such as *Serratia marcescens*, *Proteus mirabilis*, *Escherichia coli*, *Alcaligenes faecalis* and *Stenotrophomonas maltophilia* was found (17). 3-oxo-C₁₂-AHL may also possess some role in interspecies and inter-kingdom communication. Recent studies have found that not only AHLs but also their degradation product tetramic acid (TA1; Fig. 1): (S)-3-(1-hydroxydecylidene)-5-(2-hydroxyethyl) pyrrolidine-2,4-dione, have innate bactericidal activities against Gram-positive bacteria (15). Tetramic acids belong to a class of antibacterial agents spontaneously produced from 3-oxo-C₁₂-AHL and found in *P. aeruginosa* culture supernatants, although their exact roles in nature remain to be defined.

In this study, we examined the effect of tetramic acid and its derivatives on bacterial survival. In addition, we evaluated the effect of disinfectants on the TA-associated antibacterial activity.

**Materials and Methods**

**Bacterial strains, media and chemicals**

*P. aeruginosa* PAO1 was kindly provided by Barbara H. Iglewski. Other stains of *P. aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli* were clinical isolates from patients at the Toho
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University Omori Hospital. The isolates were stored at −80°C until used for the experiments. The incubation and growth of bacteria were performed at 35°C on Mueller-Hinton agar plates.

*P. aeruginosa* autoinducers and TA derivatives were chemically synthesized, as described previously (12, 15) (Fig. 1). The antibacterial activity of TAs was examined in bacterial suspensions in liquid medium in the presence of serial concentrations of TAs. Following incubation for specific periods, viable bacterial numbers were counted after serial 10-fold dilutions by spreading onto Mueller-Hinton agar plates.

**Effects of excessive amounts of cations on TA-associated bactericidal activity**

Stock solutions of ferric nitrate, magnesium chloride and calcium chloride (10 mM) were prepared in sterile extra-pure water. *P. aeruginosa* (10⁶ CFU mL⁻¹) in distilled water was incubated with TA1 (100 μM) in the presence or absence of the above cations (10, 100, 300 μM).

**Effects of disinfectants on TA-associated antibacterial activity**

*P. aeruginosa* (10⁶ CFU mL⁻¹) in saline was incubated in 96-well plates at 35°C with or without a sub-lethal concentration of TA1 (10 μM), in the presence of benzalkonium chloride (Maruishi Pharmaceutical, Osaka, Japan) or chlorhexidine gluconate solution (Danippon Sumitomo Pharma, Osaka, Japan). After 30 min, 2, 6 hours of incubation, 10 μL from each well was placed on Muller-Hinton agar plates and incubated overnight at 35°C. The concentration of disinfectants with no growth of bacteria was designated as the minimum bactericidal concentration (MBC).

**Effects of TA1 on biofilm formation and exoproduct production**

Biofilm formation was analyzed in polypropylene 96-well microtiter plates after 6, 24, 48, 72, 120 hours at 35°C, as described previously (29). Then, the plates were stained with crystal violet for 15 min. After a thorough washing with distilled water, the dye in the wells was solubilized in 95% ethanol. The amount of dye released was measured in a microtiter plate reader (Model 680, Bio-Rad Laboratories, Hercules, CA, USA) at an absorbance wave length of 600 nm.

The supernatants of *P. aeruginosa* cultures grown in LB broth with or without TA1 were assayed for elastases and pyocyanins. Elastase-type endopeptidase activity was determined using N-Succ(a)⁄pNA (Sigma, St Louis, MO), as described previously (14). For the pyocyanin assay, 5 mL of bacterial supernatant was extracted with 3 mL of chloroform, and then re-extracted into 1 mL of 0.2 N HCl. The absorbance of this extract was measured at 520 nm, as described previously (5).

**Statistical analysis**

Statistical significance was determined using the unpaired, two-tailed alternate Student’s *t*-test. A *P*-value of <0.05 was considered significant.

**Results**

**Anti-*P. aeruginosa* activity of TA1**

*P. aeruginosa* PAO1 was incubated in saline with C₄-AHL, 3-oxo-C₁₂-AHL, or several concentrations of TA1 at 35°C for 6 hours (Fig. 2). There was no anti-*P. aeruginosa* activity with C₄- and 3-oxo-C₁₂-AHL at a concentration of 300 μM. In contrast, decreased viability of *P. aeruginosa* was observed in the presence of TA1 in a concentration-dependent manner. TA1 at 100 μM reduced bacterial viability more than 2 log.

**Structure-activity correlations**

To examine structure-activity correlations of TAs, several TA-derivatives were chemically synthesized (Fig. 1). Several TAs at 300 μM markedly reduced bacterial numbers after 6 hours (Fig. 3). Interestingly, there was no antibacterial activity with TA2. These results suggested the keto-enol moiety of TAs to be critical to the antibacterial activity. We compared the activity of these TAs using the MBC. The strongest activity was observed with TA1 (MBC; 125 μM) and TA6 (MBC; 125–250 μM), followed by TA3 and TA7 (MBC; 250 μM), and TA4 and TA5 (MBC; 250–500 μM). It seemed likely that a long side chain was not essential for the anti-*Pseudomonas* activity of TAs.

![Fig. 1. Chemical structure of *P. aeruginosa* autoinducers (3-oxo-C₁₂-AHL and C₄-AHL) and synthetic TA1-TA7.](image)

![Fig. 2. Effect of TA1 on viability of *P. aeruginosa* PAO1. *P. aeruginosa* PAO1 was incubated in saline for 6 hours with 300 μM of the autoinducers 3-oxo-C₁₂-AHL (C₁₂) and C₄-AHL (C₄) or several concentrations of TA1. Bacterial numbers were examined after serial dilutions (n=5). * P<0.05, compared with the corresponding control.](image)

![Fig. 3. Antibacterial activities of several TAs. *P. aeruginosa* PAO1 was incubated in saline for 6 hours in the presence of 300 μM of several TAs. Then, bacterial numbers were examined after serial dilutions (n=5). The detectable limit was 2 log CFU mL⁻¹.](image)
Effects of ratio of bacteria to TA1 on antibacterial activity

Different concentrations of bacteria (approximately $10^9$, $10^8$, $10^7$, and $10^6$ CFU mL$^{-1}$) were incubated with several concentrations of TA1, and viable bacterial numbers were determined 30 min, 6 and 24 hours after the incubation. The values represent log CFU mL$^{-1}$ from three separate experiments. ○ Control, ● 30 μM, □ 100 μM, ■ 300 μM

Effects of excessive amounts of cations on TA-induced antibacterial activity

*P. aeruginosa* was incubated with or without 100 μM of TA1 in the presence of several concentrations of cationic ions, such as iron, magnesium and calcium. Iron at 10 μM did not induce any change in bacterial viability, whereas 100 μM of iron almost completely canceled out the TA-induced bactericidal activity (Fig. 5). In contrast, there were no obvious effects of magnesium and calcium ions even at 300 μM. These results suggest the TA-induced anti-*P. aeruginosa* activity to be associated with the iron-chelating activity of this compound.

Effects of TA1 on the viability of several clinical isolates of *P. aeruginosa*, *E. coli* and *K. pneumoniae*

Next, we examined TA1-induced bactericidal activity against 5 strains for each species. Two of the 5 *P. aeruginosa* isolates demonstrated more than a 1-log reduction in bacterial numbers at a TA1 concentration of 30 μM (Fig. 6A). At a concentration of 100 μM, more than a 3 log reduction in viability was observed in all *P. aeruginosa* isolates examined. In contrast, less striking effects were demonstrated with *E. coli* (Fig. 6B) and *K. pneumoniae* (Fig. 6C), compared to those with *P. aeruginosa* at a TA1 concentration of 30 μM. At higher concentrations, such as 100 and 300 μM, a drastic reduction in bacterial number was observed similar to that with *P. aeruginosa*. These results suggest TA1 to have antibacterial activity. They also demonstrate that *P. aeruginosa* may be slightly more sensitive to TA-induced antibacterial activity, than are *E. coli* and *K. pneumoniae*.
Effects of disinfectants on TA1-associated antibacterial activity

P. aeruginosa (10⁶ CFU mL⁻¹) in saline was incubated with or without TA1 (10 μM) in the presence of serial concentrations of benzalkonium chloride (Fig. 7A.) or chlorhexidine gluconate (Fig. 7B.) solution. The MBC of each disinfectant was determined after 30 min, 2 hours and 6 hours. A concentration of 10 μM of TA1 was not enough to affect the viability of P. aeruginosa PAO1 during these periods. Interestingly, the MBCs of these two disinfectants were markedly decreased in the presence of TA1. These results suggest TA1 and disinfectants to be synergistic against P. aeruginosa.

Discussion

Tetramic acid, a product of the degradation of 3-oxo-C₁₇-AHL, has antibacterial activity against Gram-positive species such as Bacillus cereus and Staphylococcus aureus (15). The degradation occurs spontaneously through an irreversible Claisen-like reaction and more importantly, tetramic acid is detected in the supernatant of P. aeruginosa cultures (15). Lowery et al. reported that tetramic acid showed an expanded spectrum of activity against some gram-positive bacteria, by dissipating membrane potential and transmembrane proton motive force (22). We also found bactericidal activity of tetramic acid derivatives against Clostridium difficile and that the outer membrane seems to be a target (37). In this study, we found that 100 μM of tetramic acid reduced the viability of P. aeruginosa, E. coli and K. pneumoniae. The compound acted very quickly, and in a dose and incubation time-dependent manner. These concentrations may be biologically meaningful in certain situations, because high concentrations of 3-oxo-C₁₇-AHL (>600 μM) have been detected in P. aeruginosa biofilms (3). However, no differences between the cells cultured with or without tetramic acid were observed in a microscopic analysis (data not shown).

Several natural products containing a tetramic acid motif, such as reutericycline, streptolygidin and tenuazonic acid, have been demonstrated to possess mycotoxic, antibacterial, antiviral, and antioxidantive activities (9, 32, 36). Reutericycline is a well known tetramic acid isolated from the sourdough isolate Lactobacillus reuteri LTH2584 (7, 11). This compound has been demonstrated to be bactericidal in gram-positive bacteria by acting as a proton-ionophore, thereby dissipating the transmembrane change in ΔpH, leading to cell lysis (8). The efficacy of this mechanism is a function of the hydrophobicity of reutericycline, favoring partitioning into the cytoplasmic membrane (6, 8). Consistent with these findings, the present results regarding structure-activity relationships clearly revealed the importance of the hydrophobic acyl side chain in tetramic acids. In addition, the presence of a keto-enol structure at the C3 position was shown to be essential for the bactericidal activity of tetramic acids against P. aeruginosa. These results further suggested that tetramic acid may destabilize membranes.

Hospital-acquired infections are responsible for significant morbidity and mortality in the healthcare environment. Recent reports have showed that several Acinetobacter clinical isolates have developed augmented resistance to multiple antimicrobials and disinfectants (16). In the present study, a synergistic effect of tetramic acid and disinfectants such as benzalkonium chloride and chlorhexidine gluconate was observed. The effect may lead to the elimination of pathogens in hospital environments. Further investigations of TAs, especially of the structure-activity relation, in addition to investigations of the mode of action, are warranted. However, the application of tetramic acids to the treatment of infectious diseases has several limitations. Critically, the antibacterial activity of tetramic acids was observed only in saline and distilled water, not in culture medium or serum-added solutions.

In the present study, we have shown that a degradation product of AHL can cause cell death among gram-negative bacteria, affecting P. aeruginosa more than K. pneumoniae and E. coli. The reason why the tetramic acid produced by P. aeruginosa has this self-killing effect is still unknown. One possible explanation is that the product constitutes a negative feedback mechanism for lowering bacterial burden. Recent studies have shown that programmed cell death also modulates bacterial numbers (4, 27). E. coli mazEF is a toxin-antitoxin “suicide module” that mediates cell death under stressful conditions caused by starvation, heat, viral infection, DNA damage, UV irradiation, and oxidative stress (4). Kolodkin-Gal et al. showed that E. coli releases a signaling molecule called extracellular death factor (EDF) that activates a programmed cell death pathway (19). Bacteria also talk to each other through chemical molecules and commit suicide under stressful conditions (1, 20). It might be worth examining the association and interaction between the self-killing effects of the AHL-degradation product and bacterial programmed cell death.

In conclusion, tetramic acid from P. aeruginosa exhibits not only antimicrobial effects on several bacteria but also self-killing action. These results suggest the AHL-degradation product to be useful for bacterial control.

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References