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Inhibition of quorum sensing-controlled virulence factors and biofilm formation in *Pseudomonas fluorescens* by cinnamaldehyde

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ABSTRACT

Pseudomonas fluorescens, an important food spoiling bacteria, uses quorum sensing to control biofilm formation and motility. To date, only a few compounds targeting the LuxR-based quorum sensing system of *P. fluorescens* have been identified. In the present study, the quorum sensing inhibitory effect of cinnamaldehyde at sublethal concentrations was investigated in terms of inhibition of the extracellular protease, biofilm formation, and swimming and swarming motility. The total volatile basic nitrogen value was also measured to evaluate the effect of cinnamaldehyde on quality preservation of turbot fillets stored at 4 ± 1 °C for 15 days. The results showed that cinnamaldehyde significantly inhibited quorum sensing-dependent factors in *P. fluorescens* and extended the storage life of turbot. Unexpectedly, cinnamaldehyde did not interfere with production of AHLs (*N*acylhomoserine lactones) by *P. fluorescens*, as shown by measurement of AHL production using GC–MS. Molecular docking analysis revealed that cinnamaldehyde can interact with the LuxR-type protein of *P. fluorescens*, which could constitute the molecular basis of the quorum sensing inhibition observed. These findings strongly suggest that cinnamaldehyde is a quorum sensing inhibitor with great potential for the preservation of aquatic products to guarantee food safety.

1. Introduction

Pseudomonas fluorescens is a food spoilage bacterium largely responsible for the deterioration of aquatic products. Compared with other spoilage bacteria, *P. fluorescens* can grow well at low temperatures, which facilitates the spoilage of aquatic products in cold-chain transportation (Packiavathy et al., 2014). In order to prevent the problems caused by *P. fluorescens*, several policies and measures have been implemented. Unfortunately, the overuse of antibiotics and preservatives has accelerated the development of antimicrobial resistance among bacteria worldwide (Ouyang et al., 2016). The increasing demand of high-quality fresh food has intensified the search of new approaches to inhibit food spoilage. Recent studies have shown that interfering with or blocking the quorum sensing (QS) system, which regulates the genetic expression of specific targets, controls several physiological processes, and plays a vital role in food spoilage by bacteria, might provide an alternative strategy for disease control improving food safety (Hentzer et al., 2002).

Many bacteria regulate their collective behaviors in response to changes in the environment through a cell density-dependent communication process of cell-to-cell signaling named the QS (Ding et al., 2014). In *P. fluorescens* the QS regulates the collective behaviors *via* small secreted signal molecules named Nacylhomoserine lactones (AHLs), which are very common in gram-negative bacteria (Brachmann et al., 2013). AHLs bind to receptor molecules, such as LuxR, to form a receptor-AHL complex when the concentration of AHLs reaches a certain threshold level. In response to unfavorable environmental conditions, the receptor-AHL complex triggers the expression and secretion of a series of virulence factors, and regulates various physiological functions such as bioluminescence, antibiotic biosynthesis, pigment production, biofilm formation, secretion of extracellular proteases (ECP), and swimming and swarming motility (Parsek and Greenberg, 2000). Interestingly, the QS signaling molecules have been detected in beef, bean sprouts, and fresh products such as tomatoes, large yellow croaker fillets, and milk. Moreover, the concentration of the signaling molecules has been found to increase with the degree of food spoilage (Drenkard, 2003; Lu et al., 2005). Therefore, it is important to explore the use of QS inhibitors (QSI) in food preservation since they have been shown to be highly specific against the QS system and to have no effect on food microbiota (Abraham, 2016).

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Plant-derived compounds have been used in the treatment of microbial infections and food spoilage for many years because of their low toxicity (Gibot, 2004). However, researches on these compounds have mainly focused on their antibacterial activities (Savoia, 2012). Further research is needed to evaluate their use as QSI (Koh and Tham, 2011). Cinnamaldehyde is a major constituent of Chinese cinnamon, which has been traditionally used as a food additive in food, beverages, and chewing gum at concentrations ranging from 4 ppm to > 300 ppm. Cinnamaldehyde shows good safety profiles, and highly active characteristics have been associated to it (Subash et al., 2007), including a QSI function (Niu et al., 2006) that increased the susceptibility of bacterial biofilms to antibiotics (Brackman et al., 2011). However, the effect of cinnamaldehyde on the QS system of *P. fluorescens* has not been fully studied. In the current study, we have assessed the antibiofilm and other QS-inhibitory properties of cinnamaldehyde in *P. fluorescens* and its use in the prevention of food spoilage. Finally, we have also investigated the molecular basis of cinnamaldehyde QS inhibition using *in silico* molecular docking.

2. Materials and methods

2.1. Materials and bacterial strains

Cinnamaldehyde (> 95% purity) and AHLs standards (C_4-HSL, C_6-HSL, C_8-HSL, C_{10}-HSL, C_{12}-HSL, C_{14}-HSL) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The stock solution was stored at 4 °C and prepared by dissolving 100 μL of cinnamaldehyde or 20 mg of different AHLs standards in 10 mL sterile water or 10 mL methanol, respectively. Working solutions were prepared from the stock solution by diluting in sterile water or methanol. The chemicals used in the study were analytical grade. The test strain *P. fluorescens* (ATCC 13525) was originally isolated from putrid turbot. *Chromobacterium violaceum* 026 (CV026), a mini-Tn5 mutant derived from *C. violaceum* ATCC 31532 and deficient in the AHLs synthase CviI, was generously provided by Dr. Yang (Xinjiang Shihezi University, Xinjiang, China).

2.2. Culture conditions

CV026, used as a reporter organism in the detection of QS molecules, was cultured overnight in Luria-Bertani (LB) broth (Beijing aoboxing bio-tech CO., LTD, China) containing 20 μg/mL kanamycin, at 160 rpm and 28 °C. The test strain *P. fluorescens* was cultured under the same conditions without kanamycin until an optical density at 600 nm (OD_{600}) of 0.4 was reached.

2.3. QS inhibition tests

2.3.1. Antibacterial activity of cinnamaldehyde against P. fluorescens and CV026

The minimum inhibitory concentrations (MIC) of cinnamaldehyde against *P. fluorescens* and CV026 were determined using the Oxford cup assay according to the method described by Diao et al. (2014). Briefly, cinnamaldehyde was diluted in sterile water to the desired concentrations (0.2, 0.175, 0.15, 0.125, 0.1, 0.075, 0.05, and 0.025 μL/mL). Then, overnight culture of CV026 or *P. fluorescens* diluted 1:100 in 20 mL of LB nutrient agar were poured into two plates containing autoclaved Oxford cups. Once the medium had solidified, 200 μL of cinnamaldehyde were added to the wells and the plates were incubated for 36 h at 28 °C. After the incubation, the diameter of the zone of growth inhibition was measured and the MIC was determined as the lowest concentration with no visible growth. A control test was performed using 200 μL of sterile water only.

2.3.2. Violacein inhibition assay

The violacein inhibition assay was performed following the method of Issac Abraham et al. (2012), with minor modifications. Overnight cultures of CV026 were diluted 1:100 in 10 mL of LB agar containing 100 μL of exogenous signal molecules (2 mg/mL C_6-HSL). After the medium had solidified, wells of 6 mm of diameter were made on the plates and cinnamaldehyde (200 μL) at sub-MIC concentration (0.2, 0.175, 0.15, 0.125, 0.1, 0.075, 0.05, 0.025 μL/mL) was added. The plates were incubated at 28 °C for 24 h and the inhibition on violacein production was determined by measuring the color-changing zone. Sterile water (200 μL) was used as negative control.

2.3.3. Quantitative analysis of violacein production

The quantitative analysis of violacein production was based on the procedure employed by Kato et al. (2007). Briefly, 100 μL of an overnight culture CV026 were inoculated in 10 mL of LB broth containing different concentrations of cinnamaldehyde (0.1, 0.075, 0.05, 0.025 μL/mL) and 20 μg/mL of C_6-HSL. The cultures were incubated at 28 °C and 160 rpm for 48 h and violacein was extracted according to the method of Zhang et al. (2014). Briefly, bacterial cultures were lysed by mixing 300 μL of each culture with 150 μL of 10% sodium dodecyl sulfate (SDS) for 10 s, followed by addition of 600 μL of butyl alcohol and mixing for 5 s to extract the violacein from the lysates. The final solution was centrifuged at 8900 × *g* for 5 min and 200 μL of the organic (upper) phase containing violacein were transferred to a 96-well microtiter plate and the OD_{595} was measured.

2.3.4. Biofilm formation assay

Biofilm formation was measured using the method of Rode et al. (2007). Briefly, an overnight culture of *P. fluorescens* (OD_{600} = 0.4) was diluted 1:100 in fresh sterile LB broth and 1 mL aliquots were removed to sterilized micro centrifuge tubes (polypropylene) and incubated without agitation for 48 h at 28 °C in the presence or absence (control) of cinnamaldehyde at final concentrations of 0.1, 0.075, 0.05, 0.025 μL/mL, or C_6-HSL at 20 μg/mL (positive control). Next, the suspension culture was removed, the tubes were washed thrice with sterile water and air dried for 30 min, followed by staining of the cells adhered to the tubes with 1 mL of 0.1% (*w/v*) crystal violet for 15 min. After washing with sterile water, the biofilm-associated dye was extracted with 33% acetic acid, transferred to 96-well microtiter plates, and the biofilm biomass was determined by measuring the OD_{595}. Three independent measurements were taken and the mean and standard errors were calculated.

2.3.5. Analysis of biofilms by scanning electron microscopy (SEM) and light microscopy

For the SEM analysis, a piece of polished zinc (5 mm × 5 mm × 0.2 mm) was immersed in 10 mL of LB broth containing cinnamaldehyde (0.1, 0.075, 0.05, 0.025 μL/mL) or C_6-HSL (20 μg/mL). Next, 100 μL of an overnight culture of *P. fluorescens* (OD_{600} = 0.4) were added and the cultures were incubated statically at 28 °C for 48 h to favor biofilm formation. After the incubation, the zinc piece was removed, washed thrice with sterile water, immersed in 2.5% (*v*/v) glutaraldehyde (precooled at 4 °C) for 4 h, and washed for 10 min with 50%, 70%, 80%, and 90% (v/v) ethanol, and for 15 min with 100% ethanol twice. Finally, the zinc pieces were incubated for 15 min in isoamyl acetate twice, air dried, and the biofilms were observed by SEM.

For examination by light microscopy, *P. fluorescens* was allowed to form biofilms on glass coverslips in the absence or presence of cinnamaldehyde (0.1, 0.075, 0.05, 0.025 μL/mL) or C_6-HSL (20 μg/mL) as positive control. After 48 h incubation, the coverslips were washed with sterile water to remove planktonic bacteria and the remaining adherent biofilm cells were fixed with methanol for 15 min followed by staining for 5 min with a 2% crystal violet solution. Finally, the biofilms were visualized under the light microscope after washing the coverslips with sterile water to remove the excess dye and drying them for 30 min at 30 °C.