Food Control 89 (2018) 210–218

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Carvacrol inhibits biofilm formation and production of extracellular polymeric substances of *Pectobacterium carotovorum* subsp. *carotovorum*

M.M. Gutierrez-Pacheco, G.A. Gonzalez-Aguilar, M.A. Martinez-Tellez, J. Lizardi-Mendoza, T.J. Madera-Santana, A.T. Bernal-Mercado, F.J. Vazquez-Armenta, J.F. Ayala-Zavala*

Centro de Investigación en Alimentación y Desarrollo, A.C. (CIAD, AC), Carretera a la Victoria Km 0.6, La Victoria, Hermosillo, Sonora 83000 Mexico

ARTICLE INFO

Article history:
Available online 8 February 2018

Keywords:
Erwinia carotovora
Sessile cells
Exopolysaccharides
Antibacterial activity
Terpene
Surface physicochemical properties

ABSTRACT

Pectobacterium carotovorum causes soft rot in plant food due to its ability to synthesize exoenzymes, and secrete polymeric substances to form biofilm; in addition, this aggregated form of bacteria creates resistance during disinfection. However, the biofilm formation process and composition of *P. carotovorum* is still uncharacterized, which is important to design more efficient disinfection procedures. Among the actual disinfection trends, the use of plant compounds has deserved plenty of attention; being carvacrol, a monoterpene constituent of oregano essential oil that is known to reduce biofilm formation of many bacteria. Therefore, the objective of this study was to evaluate the effect of carvacrol against biofilm development of *P. carotovorum,* EPS composition, bacterial surface charge, adhesion potential and motility. Minimal planktonic inhibitory and bactericidal concentrations of carvacrol were 2.66 and 3.99 mM; whereas, concentrations of 1.33 and 3.99 mM were needed to inhibit and eradicate biofilm, respectively. Carvacrol at 0.66 mM was chosen as the concentration to evaluate its effect on EPS secretion, motility and surface physicochemical characteristics without affecting the viability of planktonic cells. It was shown that carvacrol at this concentration decreased bacterial surface charge (−2.15 mV), adhesion potential (−1.5 mJ/m^2) and swimming motility of *P. carotovorum* (48.2 mm) compared with untreated bacteria. In addition, polysaccharides were the main components of the biofilm matrix of *P. carotovorum*, whose synthesis was inhibited by the presence of carvacrol. These results demonstrated that carvacrol could be effective against *P. carotovorum* biofilm formation and eradication, reducing adhesion, motility and synthesis of polysaccharides.

1. Introduction

Pectobacterium carotovorum subsp. *carotovorum* (before called *Erwinia carotovora*) is a plant pathogen that causes soft rot disease in cabbage, potato, onion, radish and other crops during cultivation, transportation and storage, resulting in high losses worldwide every year and considerable economic damage (Song et al., 2013). The ability of *P. carotovorum* to adhere and form biofilm is an important factor that contributes to its capacity to infect plant tissues, as well as to its resistance and persistence in different environments, especially in the field. However, this bacterium could be present also on surfaces during postharvest handling, being cause of cross-contamination and postharvest decay (Czajkowski, Pérombelon, van Veen, & van der Wolf, 2011).

A biofilm is a community of microorganisms embedded in a matrix of extracellular polymeric substances (EPS) of own production, mainly composed by polysaccharides, proteins, lipids and extracellular DNA, which can vary in composition among microorganisms and surrounding conditions (Flemming & Wingender, 2010). The biofilm formation of *P. carotovorum* is regulated by the quorum sensing (QS) system throughout the ExpI/R proteins. For most bacteria it generally happens in 5 main stages: 1) reversible adhesion, 2) irreversible adhesion, 3) microcolonies, 4) maturation and 5) dispersion (Phillips, Yang, Sampson, & Schultz, 2010). Bacterium uses flagella-mediated motility to overcome the repulsion forces between this and the target surface and once adhered they initiate their infection process, which is characterized for synthesis of plant cell wall degrading enzymes (pectate lyases, cellulases,

* Corresponding author.
E-mail address: jayala@ciad.mx (J.F. Ayala-Zavala).

https://doi.org/10.1016/j.foodcont.2018.02.007
0956-7135/

proteases), as well as biofilm formation (Prigent-Combaret et al., 2012). At this point, the composition of *P. carotovorum* biofilms is still unknown; however, it has been observed that other plant pathogens have polysaccharides as main components of their biofilm matrixes, which are related to cell-cell, cell-surface adhesion and protection against disinfectants and other stresses (Flemming et al., 2016).

Several strategies for controlling bacterial adhesion to surfaces have been proposed, including the use of natural compounds. In this sense, essential oils and their main constituents have shown potential to inhibit bacterial cells in planktonic and sessile state (biofilms) (Ortega-Ramirez et al., 2017; Tapia-Rodriguez et al., 2017). Carvacrol, a hydrophobic terpene component of oregano essential oil, has been proposed as a potential inhibitor of biofilm formation and other virulence factors of many bacteria, including *P. carotovorum* (Burt, Ojo-Fakunle, Woertman, & Veldhuizen, 2014; Joshi et al., 2016; Tapia-Rodriguez et al., 2017). Burt et al. (2014) reported that carvacrol (at subinhibitory concentrations, <0.5 mM) inhibited the biofilm formation of *Chromobacterium violaceum*, *Salmonella* Typhimurium and *Staphylococcus aureus*; whereas, Tapia-Rodriguez et al. (2017) observed that this compound (at 3.9 and 0.7 mM) caused a reduction of pyocianin and violacein, virulence factors regulated by QS system in *Pseudomonas aeruginosa* and *C. violaceum*, respectively. It has been hypothesized that the possible mechanism of action of carvacrol is through the interruption of QS mechanisms. Joshi et al. (2016) reported that after 24 h of exposure, carvacrol inhibited biofilm formation and reduced the synthesis of QS signal molecules in *P. carotovorum* subsp. *brasilense*. However, the effect of this terpene on motility, surface physicochemical properties (related with the initial adhesion of bacteria to food surfaces) and on-time EPS composition during the biofilm formation were not evaluated. In this sense, the purpose of this study was to characterize the effect of carvacrol on bacterial surface charge, adhesion potential, motility and on-time EPS composition during *P. carotovorum* biofilm formation.

2. Materials and methods

2.1. Antibacterial and antibiofilm activity of carvacrol against *P. carotovorum*

The antibacterial activity of carvacrol against *P. carotovorum* (ATCC 15713) was evaluated by the minimal inhibitory (MIC) and bactericidal (MBC) concentration (NCCLS 2001), with some modifications. For the assay, different concentrations of carvacrol (0–6.65 mM) (Sigma Aldrich, USA) were dissolved in Luria Bertani (LB) broth with ethanol (5%, to improve dissolution). Subsequently, 166 μL of *P. carotovorum* inoculum (1×10^6 CFU/mL) were added to each tube (6 mL), mixed and incubated at 28 °C for 24 h. MIC was determined as the lowest concentration of carvacrol that inhibited visible growth of the inoculated bacteria. From this experiment, a concentration of carvacrol that did not affect cell viability was determined by measuring the optical density (OD) at 600 nm in a microplate reader FLUOstar OMEGA (BMG Labtech, Chicago, IL, USA) during the incubation time at 28 °C. Samples (20 μL) from tubes without visible growth were plated in LB agar and the lowest concentration with the absence of viability was considered as the MBC.

On the other hand, the concentrations of carvacrol needed to inhibit and to eradicate *P. carotovorum* biofilm were classified as the minimal biofilm inhibitory (MBIC) and eradication concentrations (MBEC) (Chamdit & Siripermpool, 2012). For the MBIC, biofilms were grown in polypropylene coupons ($1 \times 1 \times 0.1$ cm) (Wenco®) fully immersed in 6 mL of LB broth containing carvacrol concentrations (0–2.66 mM) that did not affect planktonic growth. Tubes were inoculated with 1×10^6 CFU/mL of *P. carotovorum* and incubated at 28 °C for 24 h. Subsequently, the coupons were removed, washed with sterile saline solution (2 mL) and sonicated for 5 min (40 kHz, Branson 2510 sonicator, CT, USA) in 3 mL of sterile saline solution. Serial dilutions were made, plated in LB agar and incubated at 28 °C for 24 h. The minimum concentration where no cells were recovered from the coupons was considered as the MBIC. On the other hand, to evaluate the MBEC of carvacrol, biofilms were formed as mentioned before in LB broth without carvacrol for 24 h and washed with sterile saline solution to remove weakly adhered cells and then exposed (30 min) to carvacrol (0–6.65 mM). Coupons were sonicated in 3 mL of sterile saline solution, serial diluted, plated in LB agar and incubated at 28 °C for 24 h. The minimal concentration where no surviving cells were detected was considered as the MBEC. Experiments were performed in triplicate.

2.2. Effect of carvacrol on *P. carotovorum* motility

Swimming motility of *P. carotovorum* exposed to carvacrol was tested on plates containing semi-solid LB medium (Hossain, Shibata, Aizawa, & Tsuyumu, 2005). For the assay, 20 μL of 1×10^6 CFU/mL of *P. carotovorum* (prepared from overnight culture) were inoculated in the center of a semi-solid LB agar plate (0.3%), containing 0.66 mM of carvacrol (this concentration was selected from the antibacterial assay described in subsection 2.1) with the purpose of observe changes in motility without affecting cellular viability. Then, the plates were incubated at 28 °C and motility spread area was measured after 24 h, expressing the results in mm. The experiment was carried out by triplicate.

2.3. Bacterial surface charge of *P. carotovorum* exposed to carvacrol

Zeta potential experiments (Nano-ZS 90, Malvern instrument, Malvern, UK) were performed in order to evaluate changes on bacterial surface charge after exposure to carvacrol. For the assay, 1×10^6 CFU/mL of *P. carotovorum* were inoculated into tubes containing 6 mL of LB broth and carvacrol (0.66 mM) and incubated at 28 °C for 24 h. Then, the overnight cultures were centrifuged at 6000×*g* for 10 min, washed twice and resuspended with sterile water, suspension of non-treated cells were used as controls and results were expressed as milivolts (mV). All experiments were carried out in triplicate at 25 °C.

2.4. Free energy of adhesion of *P. carotovorum* exposed to carvacrol

The process of bacterial adhesion to abiotic surfaces can be predicted by a thermodynamic approach, calculating the change in free energy of adhesion (ΔG_{adh}) (Absolom et al., 1983), which can be estimated from the surface energy of the bacteria (γ^{bv}), the adhesion surface (γ^{sv}) and the environment where they perform this process (γ^{lv}) (the superscript *v* represents the vapor phase in which the surface energy is determined), using this formula (Kwok & Neumann, 1999):

$$\Delta G_{adh} = 2\sqrt{\gamma^{bv}\gamma^{lv}}e^{-\beta\left(\gamma^{bv}-\gamma^{lv}\right)^2} + 2\sqrt{\gamma^{sv}\gamma^{lv}}e^{-\beta\left(\gamma^{sv}-\gamma^{lv}\right)^2} - 2\sqrt{\gamma^{bv}\gamma^{sv}}e^{-\beta\left(\gamma^{bv}-\gamma^{sv}\right)^2} - 2\gamma^{lv}$$

The surface energy of the saline solution (γ^{lv}) and polypropylene (γ^{sv}) used in adhesion experiments were 65.3 and 48.17 mJ/m^2, respectively. These were determined with a tensiometer (CSC – Du Nouy, No. 70535) and by the sessile drop method using an optical goniometer CAM-Plus Micro, Tantec (Schaumburg, IL, USA) in conjunction with Neumann's equation of state (Kwok & Neumann, 2000), respectively.