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Oxygenated monoterpenes citral and carvacrol cause oxidative damage in *Escherichia coli* without the involvement of tricarboxylic acid cycle and Fenton reaction

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ABSTRACT

Oxygenated monoterpenes citral and carvacrol are common constituents of many essential oils (EOs) that have been extensively studied as antimicrobial agents but whose mechanisms of microbial inactivation have not been totally elucidated. A recent study described a mechanism of *Escherichia coli* death for (+)-limonene, a hydrocarbon monoterpene also frequently present in EOs, similar to the common mechanism proposed for bactericidal antibiotics. This mechanism involves the formation of Fenton-mediated hydroxyl radical, a reactive oxygen species (ROS), via tricarboxylic acid (TCA) cycle, which would ultimately inactivate cells. Our objective was to determine whether *E. coli* MG1655 inactivation by citral and carvacrol follows a similar mechanism of cell death. Challenging experiments with 300 μL/L citral and 100 μL/L carvacrol inactivated at least 2.5 $\log_{10}$ cycles of exponentially growing cells in 3 h under aerobic conditions. The presence of thiourea (an ROS scavenger) reduced cell inactivation in 2 $\log_{10}$ cycles, demonstrating the role of ROS in cell death. Decreased resistance of a Δ*recA* mutant (deficient in an enzyme involved in SOS response to DNA damage) indicated that citral and carvacrol caused oxidative damage to DNA. Although the mechanism of *E. coli* inactivation by carvacrol and citral was similarly mediated by ROS, their formation did not follow the same pathways described for (+)-limonene and bactericidal drugs because neither Fenton reaction nor NADH production via the TCA cycle was involved in cell death. Moreover, further experiments demonstrated antimicrobial activity of citral and carvacrol in anaerobic environments without the involvement of ROS. As a consequence, cell death by carvacrol and citral in anaerobiosis follows a different mechanism than that observed under aerobic conditions.

These results demonstrated a different mechanism of inactivation by citral and carvacrol with regard to (+)-limonene and bactericidal antibiotics, indicating the complexity of the mechanisms of bacterial inactivation among EO constituents. Advancements in the description of these mechanisms will help in extending and improving the use of these compounds as natural antimicrobials.

1. Introduction

The growing trend to avoid synthetic food preservatives, particularly to inhibit pathogenic bacteria, control spoilage bacteria and extend product shelf life (Burt, 2004), is stimulating the scientific community and food industries to search for natural compounds as alternative antimicrobials. In this regard, plant essential oils (EOs) are attracting considerable research efforts because of their potential use as food preservatives and additives (Bakkali et al., 2008).

Application of EOs as broad-spectrum antimicrobials requires detailed knowledge about their properties, including their antimicrobial activity, which has been widely studied (see the review of Hyldgaard et al., 2012). However, the mechanism of inactivation by EOs is not well known. In fact, its study is especially complex because EOs are a mixture of low-molecular-weight organic compounds, such as terpenoids, which might differ in their ways of leading to cell death. Actually, a differential mechanism of inactivation has been revealed for different EO compounds belonging to the terpenoid family. For example, whereas sublethal injuries were detected in bacterial envelopes as a consequence of a treatment with citral or carvacrol (Ait-Ouazzou et al., 2011; Somolinos et al., 2010), the lethal action of (+)-limonene was considered under the "quantal" ("all or nothing") effect (Espina et al., 2013a), i.e. no sublethal damage was detected as a consequence of bacterial treatment with (+)-limonene. The main action of these lipophilic compounds seems related to direct interaction with the hydrophobic regions of membrane proteins and protein complexes (Sikkema et al., 1995). In this regard, Ultee et al. (1999) and Somolinos et al. (2010) showed that carvacrol and citral increased cell membrane permeability, respectively.

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In a revealing study, Kohanski et al. (2007) described the role of reactive oxygen species (ROS) in the action of bactericidal antibiotics, showing that bactericidal antibiotics would share a common mechanism of cellular death. In this common mechanism, antibiotics trigger harmful hydroxyl radical formation via tricarboxylic acid cycle (TCA cycle) and later conversion of NADH to NAD^+ through the electron transport chain. ROS formed by respiration cause leaching of iron from iron-sulfur clusters, which stimulate hydroxyl radical formation through Fenton reaction (Imlay et al., 1988; Imlay and Linn, 1986). Finally, cell death results since hydroxyl radicals are extremely toxic and will readily damage proteins, membrane lipids and DNA. Central role of ROS in bacterial killing by bactericidal antibiotics has been refuted by several authors (Keren et al., 2013; Liu and Imlay, 2013; Ezraty et al., 2013). Nevertheless, Chueca et al. (2014) attributed this disagreement to a differential mechanism of action as a function of antimicrobial concentration used, being ROS-dependent at low antibiotic concentrations and ROS-independent at high concentrations.

Recently Chueca et al. (2014) described a similar mechanism of *Escherichia coli* death for another common constituent of EOs, (+)-limonene, which is a completely different molecule from bactericidal antibiotics. *E. coli* inactivation by this compound was also due to Fenton-mediated hydroxyl radical formation that caused oxidative DNA damage.

Knowledge of the mechanism of inactivation by different EO constituents, such as occurrence of sublethal injuries in cell envelopes, has practical applications, allowing us to design combined treatments for food preservation using (+)-limonene, citral or carvacrol (Ait-Ouazzou et al., 2013a; Espina et al., 2010, 2013a,b, 2014; Somolinos et al., 2010). Consequently, and given the differences observed in previous research on the mechanism of inactivation by EO compounds, we decided to investigate whether those mechanisms would follow the common pathway described for antibiotics or (+)-limonene (Chueca et al., 2014; Kohanski et al., 2007).

The aims of this work were: (a) to study the production of hydroxyl radicals following exposure to bactericidal concentrations of citral and carvacrol; (b) to evaluate the relationship between the mechanisms of microbial inactivation by citral and carvacrol and the TCA cycle, Fenton reaction and iron source; (c) to confirm the presence of DNA damage following exposure to citral and carvacrol by disabling the DNA damage response system (SOS response); and (d) to investigate the antimicrobial effects of citral and carvacrol under anaerobic conditions.

2. Material and methods

2.1. Micro-organisms and growth conditions

The strains used, *E. coli* MG1655 (ATCC 700926) and its derived strains Δ*recA*, Δ*acnB*, Δ*icdA*, Δ*sucB*, Δ*iscS*, Δ*mdh* and Δ*tonB*, were provided by the Collins Lab from Boston University (Kohanski et al., 2007). During this investigation, the cultures were maintained in cryovials at −80 °C. Broth subcultures were prepared by inoculating a test tube containing 5 mL of sterile Luria Bertani Broth (LB; Sigma-Aldrich Steinheim, Germany) with a single colony from a plate. After inoculation, the tubes were incubated overnight at 37 °C and then diluted 1:500 in 25 mL of LB broth in 250 mL Erlenmeyer flasks. Exponential-phase cells were prepared by incubating the 250-mL flasks under agitation (130 rpm; Selecta, mod. Rotabit, Barcelona, Spain) at 37 °C in the dark until an optical density (OD_{595}) of approximately 0.3 was reached, as measured using a spectrophotometer (Biochrom, mod. Libra S12, Cambridge, England). Therefore, initial cell concentration was similar for all the strains.

2.2. Bacterial treatment with citral and carvacrol

Citral (95%) and carvacrol (98%) were purchased from Sigma-Aldrich. These compounds are practically immiscible in water, so a vigorous shaking method was used to prepare suspensions (Friedman et al., 2002).

Citral and carvacrol were added at final concentrations of 300 and 100 μL/L, respectively, to the exponential-phase cultures and were maintained under agitation (130 rpm) at 37 °C in the dark for 3 h. According to preliminary studies (data not shown), these concentrations of citral and carvacrol were chosen to inactivate more than 2 $\log_{10}$ cycles of the initial cell population in agreement with (+)-limonene results (Chueca et al., 2014).

2.3. Iron chelator and ROS quenching experiments

We added 2,2′-dipyridyl (Sigma-Aldrich) at a concentration of 500 μM. The application of iron chelators, such as 2,2′-dipyridyl, is an established means of blocking Fenton reaction-mediated hydroxyl radical formation by sequestering unbound iron (Imlay et al., 1988). Thiourea (Sigma-Aldrich) was added to achieve a final concentration of 150 mM. Thiourea is a potent ROS scavenger used to mitigate the effects of hydroxyl radical damage (Kelner et al., 1990; Novogrodsky et al., 1982; Repine et al., 1981; Touati et al., 1995). Thiourea in solid form was weighed and added to the culture, whereas stock solution of 500 mM of 2,2′-dipyridyl in ethanol (Merck, Darmstadt, Germany) was previously prepared.

The 2,2′-dipyridyl and thiourea were added to the culture simultaneously with citral or carvacrol. These concentrations were chosen according to previous research studies in (+)-limonene mechanism of inactivation (Chueca et al., 2014). No inactivation was observed in cultures added only with thiourea or 2,2′-dipyridyl (data not shown).

2.4. Experiments under anaerobic conditions

Before cell inoculation, 250 mL Erlenmeyer flasks with 25 mL sterile LB were kept for 24 h inside of an microaerophilic workstation (Don Whitley Scientific Limited, mod. MACS VA500, Shipley, United Kingdom) under anaerobic conditions (85% N_2, 5% CO_2 and 10% H_2, palladium catalyst). Cultures were prepared inside of the anaerobic chamber by the dilution of broth subcultures 1:500 in 25 mL of LB broth in the 250-mL flasks. Exponential-phase cells were prepared by incubating the flasks under agitation (130 rpm) at 37 °C until an optical density (OD_{595}) of approximately 0.3 was reached, as measured using the spectrophotometer. The addition of citral, carvacrol, 2,2′-dipyridyl and thiourea was performed inside the anaerobic chamber, and the flasks were maintained there under agitation (130 rpm) at 37 °C.

2.5. Survival counts

Samples were taken every hour for 3 h after the addition of citral or carvacrol: 100 μL of culture was collected and washed twice with filtered PBS. The samples were then serially diluted in PBS. The 100 μL samples were pour-plated onto Luria Bertani Agar (LB agar; Sigma-Aldrich). The plates were incubated at 37 °C for 24 h. Previous experiments showed that longer incubation times did not influence the survival counts.

After plate incubation, colonies were counted with an improved image analyser automatic counter (Protos; Analytical Measuring Systems, Cambridge, United Kingdom) as described by Condón et al. (1996).

2.6. Statistical analysis

Inactivation was expressed in terms of the extent of reduction in $\log_{10}$ counts after every treatment. The error bars in the figures indicate the mean ± standard deviations from the data obtained from at least three independent experiments. The software used was GraphPad PRISM® (GraphPad Software, Inc., San Diego, USA).