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Global transcriptional response of *Escherichia coli* MG1655 cells exposed to the oxygenated monoterpenes citral and carvacrol

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ABSTRACT

DNA microarrays were used to study the mechanism of bacterial inactivation by carvacrol and citral. After 10-min treatments of *Escherichia coli* MG1655 cells with 100 and 50 ppm of carvacrol and citral, 76 and 156 genes demonstrated significant transcriptional differences ($p \leq 0.05$), respectively. Among the up-regulated genes after carvacrol treatment, we found gene coding for multidrug efflux pumps (*acrA*, *mdtM*), genes related to phage shock response (*pspA*, *pspB*, *pspC*, *pspD*, *pspF* and *pspG*), biosynthesis of arginine (*argC*, *argG*, *artJ*), and purine nucleotides (*purC*, *purM*). In citral-treated cells, transcription of *purH* and *pyrB* and *pyrI* was 2 times higher. Deletion of several differentially expressed genes confirmed the role of *ygaV*, *yjbO*, *pspC*, *sdhA*, *yejG* and *ygaV* in the mechanisms of *E. coli* inactivation by carvacrol and citral.

These results would indicate that citral and carvacrol treatments cause membrane damage and activate metabolism through the production of nucleotides required for DNA and RNA synthesis and metabolic processes. Comparative transcriptomics of the response of *E. coli* to a heat treatment, which caused a significant change of the transcription of 1422 genes, revealed a much weaker response to both individual constituents of essential oils (ICs).·Thus, inactivation by citral or carvacrol was not multitarget in nature.

1. Introduction

Bacterial inactivation with the purpose of guaranteeing commercial food safety and stability is generally achieved by heat treatments. Despite being the benchmark technology for microbial inactivation, heat treatments have a negative effect on food properties and quality; thus, alternatives are being studied. Individual constituents of essential oils (ICs) have been proposed for use in the food industry due to their natural origin: consumers increasingly prefer foods that resemble fresh products as much as possible, since the latter are appreciated for their enhanced natural appeal and perceived nutritional quality (Gorris and Smid, 2007). Because of the high concentration of ICs required to achieve significant microbial inactivation and the resulting undesirable changes in flavor, essential oils (EOs) and their ICs are normally utilized to inhibit microbial growth rather than to kill microbial cells. In addition, their combination, at small concentrations, with heat has been proposed to confer a synergistic lethal effect, thereby avoiding the nutritional and organoleptic changes that heat otherwise tends to produce (Ait-Ouazzou et al., 2011; Ait-Ouazzou et al., 2013a, 2013b; Espina et al., 2014; Espina et al., 2012).

Citral and carvacrol belong to the group of oxygenated monoterpenes. They are common constituents of many EOs, and they have been extensively studied as antimicrobial agents (Ait-Ouazzou et al., 2011; Ben Arfa et al., 2006; Burt, 2004). However, their mechanisms of microbial inactivation have not been elucidated in full. In general, the main action of these lipophilic compounds seems to be related to a direct interaction with the hydrophobic regions of membrane proteins and protein complexes (Sikkema et al., 1994). In this regard, Ait-Ouazzou et al. (2011) and Somolinos et al. (2010) detected sublethal injuries in bacterial envelopes as a consequence of a treatment with carvacrol or citral. Likewise, Ultee et al. (1998) and Somolinos et al. (2010) showed that carvacrol and citral increased cell membrane permeability. Previous research has not only described an ROS-dependent mechanism in the face of bactericidal antibiotics (Kohanski et al., 2007) and of the IC (+)-limonene (Chueca et al., 2014a), but also an ROS-dependent mechanism leading to *Escherichia coli* death after citral and carvacrol treatments (Chueca et al., 2014b). However, unlike the mechanisms of (+)-limonene and bactericidal antibiotics, citral and carvacrol-mediated bacterial death was independent of the Fenton reaction and the tricarboxylic acid (TCA) cycle. Nevertheless, in-depth studies

Abbreviations: IC, individual constituent of essential oils; EO, essential oil; PEF, pulsed electric fields; TSBYE, tryptic soy broth with yeast extract; CFU, colony-forming units; MIC, minimum inhibitory concentration; TSAYE, tryptic soy agar with yeast extract; SC, sodium chloride; BS, bile salts; GO, gene ontology; HSP, heat shock protein

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would be needed to better describe cell response to these ICs. It would be helpful to elucidate the molecular details of inactivation by antimicrobials not only to further understand bacterial resistance mechanisms, but also to improve the efficacy of preservation processes designed to provide safer food with an extended shelf life.

Recent techniques enable scientists to gain knowledge about global gene expression. Among them, transcriptomics based on DNA microarrays have brought about profound changes in the study of microbial physiology (Wecke and Mascher, 2011). Comparison of gene RNA transcripts obtained from bacterial cells before and after citral or carvacrol exposure would show sets of genes either up-regulated or down-regulated by the treatments, thereby possibly revealing more about cell resistance mechanisms and/or the regulatory networks that coordinate bacterial stress response (Jordan et al., 2008). This approach has been successfully used to describe the mode of action of many antibiotics (Wecke and Mascher, 2011) as well as the mechanisms of bacterial adaptation and inactivation in connection with food processing technologies such as heat (Guernec et al., 2013; Gunasekera et al., 2008), high hydrostatic pressure (Bowman et al., 2008), chlorine dioxide (Pleitner et al., 2014), pulsed electric fields (PEF) (Chueca et al., 2015) and the IC cinnamaldehyde (Visvalingam et al., 2013).

A recent study (Brauner et al., 2016) proposed a classification of bacterial survival strategies by applying three concepts: resistance, which allows a microorganism to grow in the constant presence of an antibiotic at lower concentrations; tolerance, which permits a microorganism to survive high antibiotic concentrations but only during a limited treatment duration; and persistence, similar to tolerance but effective over a longer duration of treatment. That threefold perspective can be further explored by separately evaluating the role of over-expressed genes detected in the microarray analysis and verifying if their contribution to the cell's defense and survival follows a resistance, a tolerance or a persistence strategy when ICs are applied.

The objective of this study was to investigate – via microarray hybridization – the global transcriptional pattern of *E. coli* MG1655 upon exposure to citral or carvacrol treatments. Furthermore, to improve our understanding of the mechanism of bacterial inactivation by citral and carvacrol, we also compared our results with *E. coli* transcriptomic response to lethal heat treatments. As a last step, we evaluated the role of over-expressed genes in microbial resistance and tolerance to citral and carvacrol.

2. Materials and methods

2.1. Micro-organisms and growth conditions

The strain used for the transcriptomic assays was *Escherichia coli* MG1655. The Keio collection (Baba et al., 2006) strains used in this study were parental strain BW25113 and single-gene deletion mutants Δ*arcA*, Δ*argC*, Δ*argG*, Δ*artJ*, Δ*inaA*, Δ*mdr*, Δ*ndh*, Δ*pspA*, Δ*pspB*, Δ*pspC*, Δ*pspD*, Δ*purC*, Δ*purM*, Δ*ygaV* and Δ*yjbO* for carvacrol resistance experiments; and Δ*purH*, Δ*pyrB*, Δ*pyrI*, Δ*sad/nusB*, Δ*sdhA*, Δ*yaaX*, Δ*ybiJ*, Δ*ybiM*, Δ*yejF*, Δ*yejG*, Δ*ygaV* and Δ*yneI* for citral treatments. Keio collection strains for post-transcriptional assays were supplied by the Japanese National Institute of Genetics. The cultures were maintained in a cryovial at − 80 °C.

Broth subcultures were prepared by inoculating, with one single colony from a plate, a test tube containing 5 mL of sterile Tryptic Soy Broth (Oxoid, Basingstoke, Hampshire, England) with 0.6% Yeast Extract added (Oxoid) (TSBYE). After inoculation, the tubes were incubated overnight at 37 °C. With those subcultures, 250-mL Erlenmeyer flasks containing 50 mL of TSBYE were inoculated to a final concentration of 10^4 colony-forming units (CFU)/mL. The flasks were incubated under agitation (130 rpm) (Selecta, mod. Rotabit, Barcelona, Spain) at 37 °C until the stationary growth phase was reached (24 h / 2×10^9 CFU/mL).

2.2. Bacterial treatments with citral and carvacrol

Citral (95%) and carvacrol (98%) were purchased from Sigma-Aldrich (Steinheim, Germany). Following the procedure described by Friedman et al. (2002), a vigorous shaking method was used to prepare antimicrobial compound suspensions. Prior to treatment, microorganisms were likewise centrifuged at 6000 × *g* for 5 min and resuspended to a final concentration of approximately 1×10^7 CFU/mL in McIlvaine citrate-phosphate buffer of pH 4.0 with the corresponding concentrations of citral and carvacrol added. Experiments began at room temperature (22 ± 2 °C). Control condition was stablished as bacterial cells held in the same pH 4.0 buffer for 10 min. These reference cells were not inactivated during the treatment. After treatment, 0.1 mL samples were used to count survivors.

For transcriptome evaluation, samples were treated with 50 and 100 ppm of citral and carvacrol, respectively. Immediately after treatment, 10 mL samples were used to extract RNA.

2.3. Minimum inhibitory concentration (MIC)

MIC against *E. coli* MG1655 was determined for carvacrol and citral by the tube dilution method with an initial concentration of 10^5 CFU/mL (Rota et al., 2004). The highest and lowest concentrations tested were 2500 and 50 ppm of ICs respectively. For ICs, we also prepared negative controls containing TSBYE plus 2500 ppm of ICs, and positive controls containing TSBYE with microorganisms at a final concentration of 10^5 CFU/mL. After 24 h incubation at 37 °C, the MIC was determined as the lowest concentration of each IC in the presence of which bacteria failed to grow, i.e. at which no visible changes could be detected in the broth medium (Clinical and Laboratory Standards Institute, 2012).

2.4. Heat treatments

For the transcriptomic assay, a heat treatment was carried out in an incubator (FX Incubator, mod. ZE/FX, Zeulab, Zaragoza, Spain) at 48 °C, with a thermocouple (Ahlborn, mod. Almemo 2450, Holzkirchen, Germany) to monitor temperature during treatment. Once temperature had stabilized, 50 μL of a diluted cell suspension was added to a sterile tube containing 450 μL of McIlvaine citrate-phosphate buffer of pH 4.0. The initial bacterial concentration was approximately 2×10^8 CFU/mL. Control condition was stablished as bacterial cells held in the same pH 4.0 buffer for 10 min. These reference cells were not inactivated during the treatment. After 10 min, 0.5 mL samples were immediately used to extract RNA, or 0.1 mL samples were used for purposes of survivor enumeration.

2.5. Counts of viable and sublethally injured cells

The bacterial cells' physiological response was investigated by counting viable cells. After respective treatments, samples were diluted in Phosphate Buffered Saline, pH 7.3 (PBS; Oxoid). Then 0.1 mL samples were pour-plated onto Tryptic Soy Agar (Oxoid) with 0.6% Yeast Extract added (Oxoid) (TSAYE). Treated samples were also plated on TSAYE with 3% (MG1655) or 4% (BW25113) of sodium chloride (Panreac, Barcelona, Spain) added (TSAYE-SC) and 0.1% (MG1655) or 0.2% (BW25113) of bile salts (Oxoid) added (TSAYE-BS), to evaluate cytoplasmic membrane damage and outer membrane damage, respectively (Mackey, 2000). These concentrations corresponded to the predetermined maximum non-inhibitory sodium chloride and bile salt concentrations for native cells (data not shown).

Plates were incubated at 37 °C for 24 h (TSBYE) or 48 h (TSBYE-SC and TSBYE-BS). Previous experiments showed that longer incubation times had no influence on survival counts. After plate incubation, the colonies were counted with an improved image analyzer automatic counter (Protos; Analytical Measuring Systems, Cambridge, United