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Original Article

Membrane fatty acid composition as a determinant of *Listeria monocytogenes* sensitivity to *trans*-cinnamaldehyde

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Abstract

trans-Cinnamaldehyde, the major compound of cinnamon essential oil, is a potentially interesting natural antimicrobial food preservative. Although a number of studies have addressed its mode of action, the factors that determine bacterial sensitivity or tolerance to *trans*-cinnamaldehyde are poorly understood. We report the detailed characterization of a *Listeria monocytogenes* Scott A *trans*-cinnamaldehyde hypersensitive mutant defective in IlvE, which catalyzes the reversible transamination of branched-chain amino acids to the corresponding short-chain α-ketoacids. This mutant showed an 8.4 fold extended lag phase during growth in sublethal concentrations (4 mM), and faster inactivation in lethal concentrations of *trans*-cinnamaldehyde (6 mM). *trans*-Cinnamaldehyde hypersensitivity could be corrected by genetic complementation with the *ilvE* gene and supplementation with branched-chain α-ketoacids. Whole-cell fatty acid analyses revealed an almost complete loss of anteiso branched-chain fatty acids (BCFAs), which was compensated by elevated levels of unbranched saturated fatty acids and iso-BCFAs. Sub-inhibitory concentrations of *trans*-cinnamaldehyde induced membrane fatty acid adaptations predicted to reduce membrane fluidity, possibly as a response to counteract the membrane fluidizing effect of *trans*-cinnamaldehyde. These results demonstrate the role of IlvE in BCFA production and the role of membrane composition as an important determinant of *trans*-cinnamaldehyde sensitivity in *L. monocytogenes*.

Keywords: Amino acids; Branched chain; Food preservatives; Oils; Volatile; Antimicrobial agents

1. Introduction

Minimally processed chilled foods are one of the strongest growing and innovative segments in the food industry since at least two decades. This expansion has stimulated research into mild food processing and preservation techniques that allow a maximal retention of the food fresh-like properties and at the same time provide a healthy image and a long shelf-life to the food. In this context, natural preservatives such as plant essential oils and their components are being studied to improve the safety and extend the microbiological shelf-life of foods without the use of traditional 'chemical' preservatives [1,2].

Dozens of essential oil compounds displaying a wide variety of properties like reactivity, water and fat solubility, volatility and smell and aroma have been documented to have antimicrobial activity. These compounds generally have a wide activity spectrum including both Gram-positive and Gram-negative bacteria, yeasts and molds, and this makes them potentially useful for food applications. On the other

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hand, many compounds can only be applied at concentrations that are not very effective because of their low water solubility and strong sensory impact. An attractive approach to overcome this problem is the use of hurdle technology, which is the combination of multiple gentle processing and preservation techniques to obtain maximal control of microbial safety and stability with a minimal impact on food quality [3]. Hurdle technology is most effective when it combines techniques that reinforce each other in a synergistic fashion. In the case of essential oil compounds, this can make them effective at lower concentrations and thus reduce their sensory impact. However, combined treatments can also have an additive or even an antagonistic effect, and it is currently not understood what determines the outcome of the interaction. This question can probably only be answered by acquiring a more detailed insight in the cellular or molecular mode of action of individual treatments. Studies on the mode of action of essential oil compounds are therefore essential as a basis for the development of new intelligent applications of hurdle technology using these compounds.

Our research group recently investigated the inactivation of different Gram-negative and Gram-positive food pathogens by the combination of high hydrostatic pressure and different small molecule antimicrobials, mostly essential oil compounds [4]. For less than half of the tested compounds this resulted in synergy with the high pressure treatment, the strongest synergy being observed with α, β-unsaturated aldehydes (*t*-cinnamaldehyde, *t*-2-hexenal, dimethylfumarate), isothiocyanates (allyl isothiocyanate, sulforaphane) and reuterin. These compounds are all electrophilic and show reactivity towards thiol groups [5,6], and it was therefore hypothesized that thiol reactivity might be the cause of synergy with high pressure treatment. In addition, since thiol-reactive compounds from plant essential oils like *t*-cinnamaldehyde, *t*-hexenal and isothiocyanates are hydrophobic, they are also predicted to partition into the bacterial cell membrane, thereby disturbing its structure and increasing its permeability [1]. Besides combinations with processes, the mutual combination of two or more essential oil compounds or the combination with other natural antimicrobials can also result in a synergistic effect. For example, the combination of *t*-cinnamaldehyde and carvacrol has been found to result in both a synergistic growth-inhibitory effect on a set of Gram-positive and Gram-negative food-borne bacteria, and a synergistic bactericidal effect on *Escherichia coli* and *Staphylococcus aureus* [7]. Also the combination of a nisin variant (nisin V) in the form of a fermentate with thymol, carvacrol and *t*-cinnamaldehyde elongated the lag phase of *Listeria monocytogenes* in a synergistic way [8].

Among the thiol-reactive compounds, *t*-cinnamaldehyde (t-CIN), the major constituent of cinnamon bark essential oil, is one of the most investigated both with regard to its mode of action and its potential food applications. Although the antimicrobial properties of t-CIN generally result from its overall electrophilic and lipophilic character, a number of studies have looked at the effects on bacterial cells in more detail. Based on the observation that t-CIN induced filamentation by inhibiting cell separation in *Bacillus cereus* [9], it was demonstrated that t-CIN binds with the *E. coli* cell division protein FtsZ with an affinity constant of $1.0 \pm 0.2\ \mu M^{-1}$ and inhibits its GTP-dependent polymerization [10]. Several phenylpropanoid compounds structurally related to t-CIN but lacking an aldehyde group (e.g. cinnamic acid, caffeic acid, chlorogenic acid) similarly inhibited FtsZ polymerization [11]. In addition, t-CIN also inhibited ATPase activity of isolated membranes from *E. coli* and *L. monocytogenes* [12], and this effect was suggested to be the cause of the reduced growth rate observed at sublethal concentrations. Genome-wide transcriptional profiles of *E. coli* O157:H7 after 2 h of exposure to a sublethal concentration of t-CIN showed elevated expression of oxidative stress-related genes and reduced expression of DNA, protein, O-antigen and fimbrial synthetic genes. Moreover *E. coli* O157:H7 was able to metabolize t-CIN to less toxic cinnamic alcohol after 4 h of exposure by using dehydrogenase/reductase enzymes [13]. Proteomic analysis of *Cronobacter sakazakii* exposed to a sub-inhibitory dose of t-CIN showed disrupting effects on the carbohydrate, amino acid and lipid metabolism, motility, invasion and attachment ability and cellular defenses against oxidative stress [14]. These omic studies indicate that t-CIN affects many essential bacterial processes and pathways, but most of these effects are likely to be secondary, and it is not possible to identify the primary target(s) of t-CIN based on these studies. A different, complementary approach that has not yet been applied to study t-CIN, but that may provide additional insights in its mode of action is the use of genetic analysis to identify genes that affect bacterial t-CIN sensitivity. Therefore, we have constructed and screened a transposon mutant library in *L. monocytogenes* and we report here the detailed characterization of a t-CIN sensitive mutant with a defect in the synthesis of branched-chain fatty acids. *L. monocytogenes* was chosen for this work because it is one of the most important pathogens in minimally processed chilled foods in view of its ability to grow at low temperature [15], its relatively high tolerance to elevated CO_2 concentrations [16] as used in modified atmosphere packaging, to salt and to preservatives like nitrite [17,18]. *L. monocytogenes* is widespread in the environment and a frequent contaminant in food production facilities. Although human listeriosis is rare, it has a high hospitalization and mortality rate, and is particularly dangerous for pregnant women and elderly people [19].

2. Materials and methods

2.1. Bacterial strains and growth conditions

L. monocytogenes Scott A, obtained from the International Life Sciences Institute North America (ILSI NA) *L. monocytogenes* strains collection [20], was used in this work as the wild-type (WT) strain. Cultures of both the WT and transposon mutants were routinely grown at 30 °C in Brain Heart Infusion (BHI, Oxoid, Hampshire, Great-Britain) broth or on BHI agar (BHI with 15 g/l agar). *E. coli* S17-1 λpir [21] was used as a donor for conjugational plasmid transfer to *L.*