Characterization of the Action of Selected Essential Oil Components on Gram-Negative Bacteria

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Carvacrol, (+)-carvone, thymol, and *trans*-cinnamaldehyde were tested for their inhibitory activity against *Escherichia coli* O157:H7 and *Salmonella typhimurium*. In addition, their toxicity to *Photobacterium leiognathi* was determined, utilizing a bioluminescence assay. Their effects on the cell surface were investigated by measuring the uptake of 1-*N*-phenylnaphthylamine (NPN), by measuring their sensitization of bacterial suspensions toward detergents and lysozyme, and by analyzing material released from cells upon treatment by these agents. Carvacrol, thymol, and *trans*-cinnamaldehyde inhibited *E. coli* and *S. typhimurium* at 1−3 mM, whereas (+)-carvone was less inhibitory. *trans*-Cinnamaldehyde was the most inhibitory component toward *P. leiognathi*. Carvacrol and thymol disintegrated the outer membrane and released outer membrane-associated material from the cells to the external medium; such release by (+)-carvone or *trans*-cinnamaldehyde was negligible. Of the tested components, carvacrol and thymol decreased the intracellular ATP pool of *E. coli* and also inreased extracellular ATP, indicating disruptive action on the cytoplasmic membrane.

Keywords: *Essential oils; carvacrol; carvone; cinnamaldehyde; thymol; antimicrobial activity; outer membrane; lipopolysaccharide*

INTRODUCTION

Plant-derived essential oils have long served as flavoring agents in foods and beverages, and due to their versatile content of antimicrobial compounds, they possess potential as natural agents for food preservation (Conner, 1993). The antimicrobial activity of essential oils is assigned to a number of small terpenoid and phenolic compounds, which also in pure form have been shown to exhibit antibacterial or antifungal activity (Karapinar and Aktug, 1987; Didry et al., 1993; Conner, 1993; Juven et al., 1994; Oosterhaven et al., 1996; Smid et al., 1996). Specific mechanisms of bactericidal action of essential oil constituents such as thymol and carvacrol remain, however, poorly characterized. The antibacterial properties of these compounds are evidently associated with their lipophilic character, leading to accumulation in membranes and to subsequent membrane-associated events such as energy depletion (Conner, 1993; Sikkema et al., 1995).

Essential oils and their components are known to be active against a wide variety of microorganisms, including Gram-negative bacteria (Deans and Ritchie, 1987; Conner, 1993; Sivropoulou et al., 1996). All Gram-negative bacteria possess an outer membrane (OM), which provides the bacterium with a hydrophilic surface, due to the presence of lipopolysaccharide (LPS) molecules (Nikaido, 1996). Small hydrophilic solutes are able to pass the OM through abundant porin proteins providing hydrophilic transmembrane channels, whereas the OM serves as a penetration barrier toward macromolecules and to hydrophobic compounds, and it is for this reason that Gram-negative bacteria are relatively resistant to hydrophobic antibiotics and toxic drugs (Nikaido and Vaara, 1985; Nikaido, 1996). The OM is, however, not totally impermeable to hydrophobic molecules, some of which can slowly traverse through porins (Plésiat and Nikaido, 1992; Nikaido, 1996). In general, bypassing the OM is a prerequisite for any solute to exert bactericidal activity toward Gram-negative bacteria. It should therefore be worthwhile to evaluate antimicrobial compounds present in plants for their activity on Gram-negative bacteria in a number of real food systems. There are indications that also microorganisms, for example, certain lactic acid bacteria, produce small molecular mass antibacterial and antifungal compounds (Helander et al., 1997b) that should be very useful in food preservation. It was therefore of interest to investigate the mode of action of selected essential oil components on Gram-negative bacteria, focusing on the effects on the OM.

MATERIALS AND METHODS

Chemicals. Carvacrol, (+)-carvone, *trans*-cinnamaldehyde, and thymol (referred to below as essential oil components) were obtained from Fluka. Stock solutions (1 mM) of these were prepared in aqueous 85% ethanol. The maximum solubility of these compounds in water at 20 °C was determined as 10 mM for carvacrol and (+)-carvone, 13 mM for *trans*-cinnamaldehyde, and 7 mM for thymol. Chicken egg white lysozyme (EC 3.2.1.17), *n*-heptadecanoic acid methyl ester, 1-*N*-phenylnaphthylamine (NPN), and SDS were purchased from Sigma-Aldrich, and Triton X-100 was from BDH.

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Proteinase K (EC 3.4.21.64) was from Merck and EDTA from Riedel-de-Haen AG. Silicon oils AR200 and AR20 were from Wacker Chemicals.

Bacteria. *Escherichia coli* ATCC 35150 (O157:H7) and *Salmonella typhimurium* ATCC 13311 were grown in LB broth (per liter, 10 g of Difco tryptone, 5 g of Difco yeast extract, and 5 g of NaCl, pH 7.0) at 37 °C with shaking (200 revolutions/min). Growth was monitored spectrophotometrically at 630 nm. *Photobacterium leiognathi* ATCC 33469 was cultivated at 25 °C with shaking in a medium containing, per liter, 30 g of NaCl, 3.9 g of K_2HPO_4, 2.1 g of KH_2PO_4, 5 g of NH_4Cl, 1 g of $MgSO_4$ heptahydrate, 0.75 g of KCl, 1 g of $CaCO_3$, 5 g of Difco yeast extract, 5 g of Difco tryptone, 3 mL of glycerol, and 50 mL of 1 M Tris-HCl, pH 7.5.

Growth Inhibition Tests. Bacteria were grown to OD_{630} of 0.2 and subsequently diluted at 1/100 into LB broth (2 mL) supplemented with 0.2, 0.6, 2, 6, and 10 μmol of the particular essential oil component (corresponding to end concentrations of 0.1, 0.3, 1, 3, and 10 mM, respectively) and then cultivated for 20 h. The OD_{630} values of the cultures were measured, and the lowest concentration that completely inhibited bacterial growth was taken as the minimal inhibitory concentration (MIC).

Photobacter Toxicity Test. This method was a modification of the DIN 38412 method originally designed for ecotoxicological examination of water samples, with *P. leiognathi* as the test organism. From a culture grown for 24 h was made a 1% (v/v) dilution into 2% (w/v) NaCl. After 15 min at 25 °C, the bioluminescence was measured (BioOrbit 1253 Luminometer, BioOrbit, Turku, Finland), and the test components appropriately diluted in 2% (w/v) NaCl were added in amounts ranging from 0.0125 to 0.5 μmol. The bioluminescence was again measured after 15 min of incubation. The values obtained were corrected for the decrease in bioluminescence observed in untreated control tubes, and the results are expressed as inhibition percentages calculated from duplicate measurements of bioluminescence.

Uptake of NPN. The uptake of the hydrophobic fluorescent probe NPN by bacterial cells in buffer suspension was determined fluorometrically as described recently (Helander et al., 1997a). Briefly, cultures were grown to OD_{630} of 0.5 and then washed with and suspended in 5 mM HEPES buffer, pH 7.2. Samples of this suspension supplemented with 10 μM NPN were monitored with a Shimadzu RF-5000 spectrophotometer at 420 nm (excitation wavelength, 350 nm; slit widths, 5 nm), and fluorescence levels (in arbitrary units) were recorded before and after addition of desired concentrations of test substances. The stable fluorescence level, typically reached after 30 s from addition of the test substance, was taken as the result value. In testing the effect of $MgCl_2$ to the carvacrol- or thymol-induced fluoresecence, this salt was added to the buffer before NPN. Increased fluorescence indicates uptake of NPN by the bacterial membrane(s), as the quantum yield of NPN is greatly enhanced in lipid versus aqueous environment (Loh et al., 1984).

Bacteriolysis. The effect of essential oil components on detergent- or lysozyme-induced bacteriolysis was assayed on microtiter plates as described recently (Helander et al., 1997a). The bacteria were first cultivated and washed as above and then suspended in a similar volume of 10 mM HEPES containing 50 mM NaCl, pH 7.2. The suspension was divided into two portions, and to one was added the essential oil component at the indicated concentration; the other served as control. The suspensions were incubated at room temperature for 10 min, centrifuged, and resuspended in the buffer without essential oil components. From such suspensions, aliquots (10^8 cells in 100 μL) were pipetted into microtiter wells, which already contained the indicated concentrations of lysozyme, Triton X-100, or SDS. Cell lysis was monitored spectrophotometrically at 405 nm by a Multiskan MCC/340 spectrophotometer, Labsystems; results are expressed in percentages based on absorbances of component-pretreated versus untreated cell suspensions at 4 min after addition of detergent or lysozyme. Thus, 100% equals no lysis; lower percentages indicate lytic action. Each determination was performed in quadruplicate. At least three independent lysis tests were done.

Lipopolysaccharide and Protein Release. The possible release of OM components [see Vaara (1992)] was investigated by analyses of cell-free supernatants after treatment with the essential oil components. The bacteria were grown to OD_{630} of 0.5, and the cultures were divided in 1-mL portions into microcentrifuge tubes. After centrifugation at room temperature (12000 revolutions/min, 2 min, Eppendorf microfuge), the cells were suspended in 10 mM Tris-HCl buffer, pH 7.2, and centrifuged again as above. The deposited cells were again suspended into the Tris buffer, to which the test substance (essential oil components or EDTA) was added, and the suspensions were kept at 37 °C for 10 min. The control samples were suspended in Tris buffer without additions. After centrifugation, 0.9 mL of the cell-free supernatant was removed and freeze-dried. For each test substance, two parallel vials were prepared so that the total volume of recovered supernatant was 1.8 mL. The freeze-dried supernatants were dissolved in 100 μL of SDS−PAGE sample buffer (Novex) and heated at 100 °C for 10 min. Each sample was then divided in two equal portions, to one of which 10 μL of proteinase K solution (2.5 mg/mL) was added. Subsequently, the vials were kept at 60 °C for 1 h. The samples were analyzed by SDS−PAGE in Novex precast 12% acrylamide gels; 10 μL of lysate was applied to the gel. The gels with proteinase K-treated samples were stained with silver (Silver Xpress staining kit, Novex), as were the gels of the untreated samples, which were alternatively stained for protein by the Novex Colloidal Coomassie stain.

Lipid (including phospholipid and LPS) released from the bacterial cells by the above treatments was assayed on the basis of fatty acids found in the cell-free supernatants after treatment of cultures with essential oil components. The bacteria were grown to OD_{630} of 0.6, washed with 10 mM Tris-HCl, pH 7.2 (centrifugation at 3000*g*, 10 min, 25 °C), and resuspended in the same buffer, which was supplemented with essential oil components (final concentration = 2 mM) or EDTA (1 mM). Control samples were suspended in buffer only. After the suspensions (10 mL) had been incubated at 37 °C for 10 min, they were centrifuged at 11000 rpm (1 min, Eppendorf microfuge, 25 °C), whereupon clear supernatants (total volume = 9.1 mL) were carefully removed and freeze-dried. After addition of internal fatty acid standard (*n*-heptadecanoic acid methyl ester, 105 μg), the samples were processed by saponification and methylation as described by Moore et al. (1994). It is recognized that this procedure leads to an underestimation of 3-hydroxytetradecanoic acid, which is partially amide-linked in the lipid A component of the LPS (Zähringer et al., 1994) and therefore converted to the corresponding methyl ester only by a ~65% yield (Helander and Haikara, 1995). The resulting fatty acid methyl esters were analyzed by capillary gas chromatography (GC), the instrumentation and conditions of which were described recently (Helander et al., 1997a).

Determination of Intra- and Extracellular ATP. To study the possible effect of essential oils on the permeability of the cytoplasmic membrane for small solutes, internal and external ATP pools of *E. coli* were determined in the presence and absence of carvacrol, thymol, (+)-carvone, and *trans*-cinnamaldehyde. Cells of *E. coli* ATCC 35150 were harvested at the exponential growth phase (OD_{660} of 0.8), washed twice in 5 mM HEPES buffer, pH 7.2, and concentrated (~50-fold). Washed cells were subsequently diluted in HEPES buffer to a density of ~0.2 mg of protein/mL at room temperature. Glucose was added at time zero to a final concentration of 0.5% (w/v). After 5 min of incubation, essential oil components (carvone, carvacrol, thymol, or *trans*-cinnamaldehyde) were added at a final concentration of 2 mM. In control experiments, no essential oil component was added. The intracellular and extracellular ATP concentrations were determined at regular time intervals by separating the cells from the external medium by silicon oil centrifugation (Ten Brink et al., 1985). Briefly, samples (200 μL) from a cell suspension were transferred to microcentrifugation tubes containing 200

Table 1. Minimal Inhibitory Concentrations (MIC) of Essential Oil Components for *E. coli* O157:H7 and *S. typhimurium*

component	MIC (mM)	
	E. coli	*S. typhimurium*
carvacrol	3	1
(+)-carvone	10	10
trans-cinnamaldehyde	3	3
thymol	3	1

μL of a 2:1 mixture of silicon oil AR200 (ρ = 1.05 g/mL) and silicon oil AR20 (ρ = 0.96 g/mL) on top of a layer of 100 μL of 10% (w/v) trichloroacetic acid with 2 mM EDTA. The cells were spun through the silicon oil (5 min, 12000*g*), and samples (5 μL) of both aqueous layers were taken to determine the ATP content using the firefly luciferase assay (Lundin and Thore, 1975). Luminescence was recorded using a BioOrbit 1250 luminometer (BioOrbit, Turku, Finland).

RESULTS

Effect of Essential Oil Components on Bacterial Growth. Each of the four essential oil components inhibited the growth of *E. coli* and *S. typhimurium*. The minimal inhibitory concentrations listed in Table 1 reveal that *E. coli* was equally inhibited by thymol, carvacrol, and *trans*-cinnamaldehyde, whereas (+)-carvone was considerably less inhibitory. A similar pattern was obtained for *S. typhimurium*, which was slightly more sensitive than *E. coli* toward carvacrol and thymol.

Toxicity of Essential Oil Components to *P. leiognathi*. The toxicity test based on the inhibition of bioluminescence of the Gram-negative *Photobacterium* revealed inhibitory activity for each of the components, as shown in Figure 1. *trans*-Cinnamaldehyde was most inhibitory, followed (in the order of toxicity) by carvacrol, thymol, and (+)-carvone.

Effect of Essential Oil Components on the Uptake of NPN. Thymol and carvacrol brought about increased NPN uptake for both *E. coli* and *S. typhimurium*, as opposed to (+)-carvone and *trans*-cinnamaldehyde, which exhibited no effect on NPN uptake for either species. None of the components yielded any fluorescence in tests without bacterial cells. As shown in Table 2, there was a large variation in the NPN uptake values with *S. typhimurium*. This strain tended to take relatively high amounts of NPN also in the absence of permeabilizing components, indicating a less stable OM than that of the *E. coli* strain. Magnesium ions (supplied as $MgCl_2$) in concentrations equimolar to those of carvacrol or thymol did not affect the NPN uptake induced by the latter substances (Table 2).

Sensitization of Bacteria to Lytic Effects. Since the increased uptake of NPN observed for carvacrol and thymol indicated cell envelope-permeating activity, further experiments were performed to measure sensitization of the bacteria to detergent- or lysozyme-induced bacteriolysis. The test species were subjected to treatments with the essential oil components and, subsequently, to the detergents SDS or Triton X-100 or to lysozyme. The lytic effects for each of the test bacteria are shown in Tables 3 and 4. SDS alone had a slight lytic effect on *E. coli* but a strong effect on *S. typhimurium*. Tested at a concentration of 1%, Triton X-100 alone slightly lysed both *E. coli* and *S. typhimurium*. The exposure to carvacrol sensitized *E. coli* to SDS and *S. typhimurium* to Triton X-100, whereas no sensitization to lysozyme was observed. There was

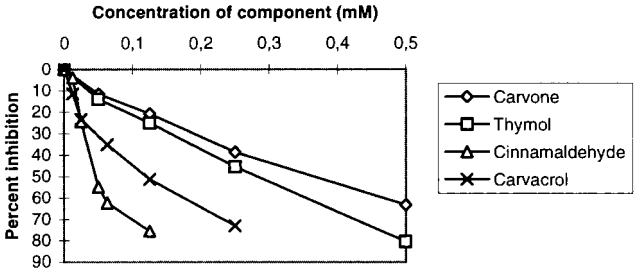

Figure 1. Inhibition of bioluminescence of *P. leiognathi* by essential oil components.

Table 2. Values for NPN Uptake Induced by Essential Oil Components in Suspensions of *E. coli* O157:H7 and *S. typhimurium*

component	concn (mM)	$MgCl_2$ (mM)	fluoresecence increase[a] (rel fluorescence units ± SD)	
			E. coli	*S. typhimurium*
thymol	0.5	−	22 ± 3.5	24 ± 11
	1	−	44 ± 2.5	36 ± 11
	2	−	90 ± 5.3	44 ± 18
	2	2	91 ± 3.5	57 ± 13
carvacrol	0.5	−	15 ± 2.1	23 ± 17
	1	−	38 ± 2.1	30 ± 24
	2	−	83 ± 4	45 ± 26
	2	2	88 ± 3.2	42 ± 9
(+)-carvone	2	−	3 ± 3	3 ± 3
	10	−	0	0
trans-cinnamaldehyde	2	−	0	0

[a] Total fluorescence values in the presence of the test component subtracted from the background value obtained after NPN addition. Each determination was done in triplicate.

marked sensitization by thymol toward SDS, whereas *S. typhimurium* was sensitized by thymol to a small extent toward Triton X-100. (+)-Carvone had a small sensitizing effect toward SDS in *E. coli*, whereas *trans*-cinnamaldehyde exhibited no sensitization for any combination or species.

Table 5 shows that for a sensitizing effect of thymol toward SDS and Triton X-100 in *S. typhimurium*, a thymol concentration of at least 2 mM was required and that the effect was slightly but certainly not totally reversed by $MgCl_2$.

Release of LPS and Protein. Shown in Figure 2 is a silver-stained gel in which proteinase K-treated samples of cell-free supernatants from suspensions treated with the essential oil components or with EDTA were electrophoresed. In contrast to the supernatant of untreated cells, the EDTA supernatant revealed a prominent ladder pattern typical of smooth-type LPS. Supernatants of carvacrol- and thymol-treated suspensions revealed ladder patterns that stained with intensity similar to that of the EDTA supernatant material. On the basis of the staining intensity, (+)-carvone and *trans*-cinnamaldehyde supernatants contained much less LPS. The observed LPS patterns were all qualitatively similar. After silver staining, the samples that were not treated with proteinase K revealed a number of protein bands in patterns that did not differ between essential oil or EDTA supernatants; again, thymol and carvacrol yielded strongest staining (not shown). In the gel stained with Coomassie blue, there was only one prominent band present in supernatant samples of cells treated with EDTA, thymol, and carvacrol. The estimated molecular mass of this protein was 37 kDa, and thus it is most likely to be one of the main porin proteins of the *E. coli* OM (Nikaido and Vaara, 1985).

Table 3. Lytic Activity of Lysozyme, Triton X-100, and SDS against *E. coli* O157:H7 with (+) or without (−) Pretreatment with Essential Oil Components (2 mM)

test substance	concn	percentage (± SD) remaining turbidity after 4 min in the lytic substance							
		carvacrol		thymol		(+)-carvone		*trans*-cinnamaldehyde	
		−	+	−	+	−	+	−	+
lysozyme	10 μg/mL	101 ± 4	98 ± 1	101 ± 3	101 ± 3	99 ± 2	100 ± 1	100 ± 4	100 ± 2
Triton X-100	0.1%	104 ± 3	101 ± 1	101 ± 3	103 ± 2	100 ± 2	102 ± 1	100 ± 3	101 ± 2
Triton X-100	1%	95 ± 4	92 ± 1	94 ± 3	93 ± 3	93 ± 3	94 ± 2	93 ± 4	94 ± 4
SDS	0.1%	101 ± 1	56 ± 17	102 ± 2	42 ± 6	99 ± 1	94 ± 4	100 ± 3	99 ± 3
SDS	1%	96 ± 4	47 ± 16	93 ± 2	34 ± 6	93 ± 3	85 ± 4	94 ± 4	93 ± 4

Table 4. Lytic Activity of Lysozyme, Triton X-100, and SDS against *S. typhimurium* with (+) or without (−) Pretreatment with Essential Oil Components (2 mM)

test substance	concn	percentage (± SD) remaining turbidity after 4 min in the lytic substance							
		carvacrol		thymol		(+)-carvone		*trans*-cinnamaldehyde	
		−	+	−	+	−	+	−	+
lysozyme	10 μg/mL	99 ± 1	97 ± 1	98 ± 1	97 ± 2	98 ± 5	97 ± 4	98 ± 4	96 ± 1
Triton X-100	0.1%	101 ± 1	88 ± 5	101 ± 2	87 ± 5	102 ± 2	100 ± 2	102 ± 3	98 ± 2
Triton X-100	1%	92 ± 1	80 ± 5	92 ± 1	77 ± 6	95 ± 3	91 ± 2	94 ± 3	91 ± 1
SDS	0.1%	68 ± 14	37 ± 20	62 ± 14	28 ± 18	57 ± 15	74 ± 13	57 ± 15	70 ± 12
SDS	1%	33 ± 9	27 ± 16	29 ± 7	19 ± 11	26 ± 1	42 ± 1	26 ± 2	36 ± 2

Table 5. Effect of Thymol Concentration and of $MgCl_2$ on Thymol-Induced Sensitization of *S. typhimurium*

test substance	concn	percentage (± SD) remaining turbidity after 4 min in the lytic substance							
		0.1 mM thymol		1 mM thymol		2 mM thymol		2 mM thymol + 2 mM $MgCl_2$	
		−	+	−	+	−	+	−	+
lysozyme	10 μg/mL	98 ± 1	100 ± 5	100 ± 3	99 ± 3	98 ± 1	97 ± 2	99 ± 2	99 ± 2
Triton X-100	0.1%	101 ± 1	101 ± 2	102 ± 1	97 ± 5	101 ± 2	87 ± 5	101 ± 3	98 ± 1
Triton X-100	1%	92 ± 1	92 ± 3	93 ± 3	88 ± 4	92 ± 1	77 ± 6	92 ± 2	88 ± 3
SDS	0.1%	65 ± 7	76 ± 6	62 ± 1	54 ± 6	62 ± 14	28 ± 18	58 ± 2	38 ± 16
SDS	1%	29 ± 7	36 ± 12	28 ± 6	28 ± 6	29 ± 7	19 ± 11	22 ± 2	27 ± 11

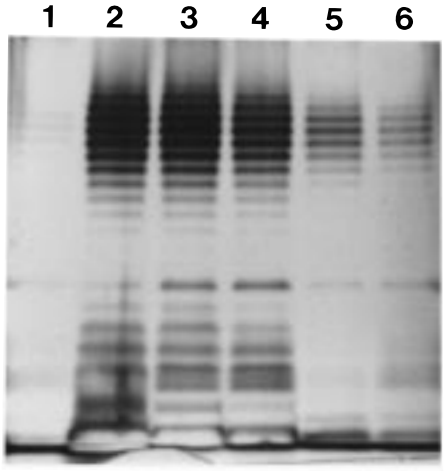

Figure 2. SDS−PAGE of proteinase K-treated cell-free supernatants of *E. coli* O157:H7 exposed to essential oil components (2 mM) or EDTA (1 mM). An equal volume (10 μL) of each sample was electrophoresed, whereupon the gel was stained with silver. Lanes: 1, untreated (control); 2, EDTA supernatant; 3, thymol supernatant; 4, carvacrol aupernatant; 5, (+)-carvone supernatant; 6, *trans*-cinnamaldehyde supernatant.

Analysis of Released Lipid. Analysis of fatty acids of the cell-free supernatants of *E. coli* treated with the essential oil components revealed typical cellular fatty acids (Table 6). There was considerable liberation of lipid by 1 mM EDTA, as indicated by the nearly 5-fold higher amount of fatty acid in the EDTA supernatant as compared to the control sample. Also, thymol and carvacrol supernatants contained much lipid, whereas (+)-carvone had liberated slightly more lipid than found in the control sample. The lipid content of the *trans*-cinnamaldehyde supernatant was similar to the control. Notably, the proportion of LPS-specific fatty acids was nearly 2 times higher in the thymol and carvacrol supernatants than in the control sample, and this proportion was also higher in the EDTA supernatant.

Effect of Essential Oil Components on ATP Pools. Addition of glucose to washed cells of *E. coli* did not change the size of the internal ATP pool (Figure 3A), showing that harvesting and washing had no significant effect on the bioenergetic status of the cells. Addition of carvacrol resulted in a gradual decrease of the internal ATP pool from 3.1 to <0.3 nmol/mg of protein in 10 min (Figure 3A). In the same time period, a small but significant increase of external ATP was observed to a level corresponding to 0.75 nmol of ATP/mg of protein (Figure 3B). Also, thymol decreased the intracellular ATP pool with the appearance of extracellular ATP (Figure 3C,D), although the changes were less prominent than those observed for carvacrol. The amount of ATP appearing outside cannot entirely be explained by the decrease of the intracellular pool, indicating that also increased ATP turnover occurs, either inside or outside the cells.

Addition of (+)-carvone (Figure 3A) or *trans*-cinnamaldehyde (Figure 3C) did not have a notable effect on the size of the internal ATP pool during the incubation period. Furthermore, exposure of the cells to (+)-carvone (Figure 3B) or *trans*-cinnamaldehyde (Figure 3D) did not induce the appearance of extracellular ATP. These results show that of all tested oil components carvacrol and thymol increased the permeability of the membrane for ATP.

DISCUSSION

With respect to bactericidal activity and membrane-disintegrating properties, the essential oil components

Table 6. Fatty Acid Analysis of Cell-Free Supernatants of *E. coli* Cultures Treated with EDTA or with the Indicated Essential Oil Components for 10 min at 37 °C in Tris Buffer

	μg of fatty acid in 9.1 mL of culture supernatant treated with					
fatty acid	nothing (control)	EDTA (1 mM)	thymol (2 mM)	carvacrol (2 mM)	(+)-carvone (2 mM)	*trans*-cinnamaldehyde (2 mM)
12:0	0.5	2.2	1.9	1.9	0.6	0.4
14:0	0.5	4.2	2.5	2.7	1.0	0.4
14:0(3-OH)	0.7	7.0	4.9	4.9	1.0	0.5
16:1	1.2	4.6	2.6	2.3	1.6	0.6
16:0	2.0	6.5	3.7	3.3	2.8	1.1
18:1	0.9	3.6	1.8	1.4	1.3	0.5
sum of all fatty acids	5.8	28.1	17.4	16.5	8.3	3.5
sum LPS-specific[a] fatty acids	1.7	13.4	9.3	9.5	2.6	1.3
percentage proportion of LPS-specific fatty acids	29	48	53	58	31	26

[a] LPS-specific fatty acids: 12:0; 14:0; 14:0(3-OH).

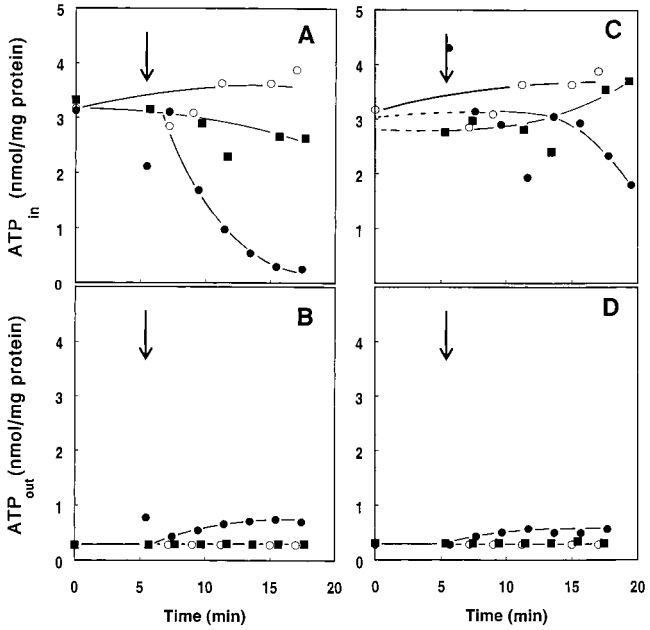

Figure 3. Effect of the essential oil components (each at 2 mM) on intracellular (A, C) and extracellular (B, D) ATP pools of *E. coli* ATCC 35150. Panels A and B show ATP levels after addition of carvacrol (solid circles) or (+)-carvone (solid squares) at 5 min (indicated by arrow). Panels C and D show ATP levels after addition of thymol (solid circles) and *trans*-cinnamaldehyde (solid squares). As a control, the ATP pools were monitored in the absence of essential oil components (open circles). The data come from one set of experiments with duplicate measurements (±0.07 nmol).

investigated in this study fall into different categories. Thymol and carvacrol both have prominent OM-disintegrating properties, as indicated by their enhancing effect on NPN uptake and LPS release, as well as sensitization to detergents. These compounds also inhibited bacterial growth at concentrations similar to those required for OM disintegration and increased the permeability of the cytoplasmic membrane to ATP. *trans*-Cinnamaldehyde was inhibitory at a similar concentration as thymol and carvacrol for the growth of the enteric bacteria and exhibited high toxicity in the photobacter test, yet it exhibited neither OM-disintegrating activity nor depletion of intracellular ATP. (+)-Carvone was ineffective on the OM and exhibited low activity both in growth inhibition and in photobacter tests and did not affect the cellular ATP pool.

The MIC values presented here for carvacrol, thymol, and *trans*-cinnamaldehyde agree with previously reported values for *E. coli* and *S. typhimurium* (Karapinar and Aktug, 1987; Didry et al., 1993). Although the enhanced NPN uptake was already observed at somewhat lower concentrations than the MICs, thymol or carvacrol did not directly act as OM-permeabilizing agents such as EDTA or polyethylenimine, which disintegrate the OM at concentrations that do not affect bacterial survival or growth (Vaara, 1992; Helander et al., 1997a). Furthermore, the observation that $MgCl_2$ had no major effect on the OM-disintegrating ability of carvacrol or thymol suggests mechanisms of action other than chelation of divalent cations from the OM (EDTA) or intercalation into the OM with replacement of stabilizing cations and concomitant disturbances of interactions between OM constituents (polyethylenimine). Of the compounds studied, *trans*-cinnamaldehyde is exceptional because, without exerting disintegrative effects on the OM, it strongly inhibited enterobacterial growth and the bioluminescence of *P. leiognathi*. The conclusion can thus be drawn that *trans*-cinnamaldehyde and partly also thymol and carvacrol gain access to the periplasm and to the deeper parts of the cell; indeed, the OM-traversing porin proteins have been shown to allow the penetration of lipophilic probes at significant rates (Plésiat and Nikaido, 1992; Nikaido, 1996). It will be of interest to measure the antibacterial action of these components on a porin-deficient Gram-negative bacterial mutant; such an experiment would be especially helpful in attempts to reveal the target of action of *trans*-cinnamaldehyde. Why exposure to *trans*-cinnamaldehyde does not result in the disintegration of OM, whereas carvacrol and thymol cause significant liberation of OM components, might be related to the phenolic character of carvacrol and thymol; phenols are known for their membrane-disturbing activities (Keweloh et al., 1990; Sikkema et al., 1995). This property of phenol is actually widely utilized in extraction of LPS from bacteria (Helander et al., 1985). A plausible reason underlying the inability of (+)-carvone to inhibit growth or to affect the OM could be that it is pumped out from the periplasm at a rate exceeding its penetration rate (Nikaido, 1994). (+)-Carvone, on the other hand, possesses a strong antifungal activity (Oosterhaven et al., 1996; Smid et al., 1996). The same is true for *trans*-cinnamaldehyde. In a study with *Saccharomyces cerevisiae* it was demonstrated that *trans*-cinnamaldehyde (at 5 mM) caused a (partial) collapse of the integrity of the cytoplasmic membrane, leading to excessive leakage of metabolites and enzymes from the cell and finally loss of viability (Smid et al., 1996).

Beside shedding light on the differential toxicity of essential oil components on Gram-negative bacteria, our

data further validate the importance of low molecular mass lipophilic compounds. Despite their limited solubility in water, these compounds are able to penetrate Gram-negative bacteria and may thus be able to influence the proliferation of certain pathogenic or spoilage bacteria in food-related environments.

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LITERATURE CITED

Conner, D. E. Naturally occurring compounds. In *Antimicrobials in Foods*; Davidson, P. M., Branen, A. L., Eds.; Dekker: New York, 1993; pp 441−468.

Deans, S. G.; Ritchie, G. Antibacterial properties of plant essential oils. *Int. J. Food Microbiol*. **1987**, *5*, 165−180.

Didry, N.; Dubreuil, L.; Pinkas, M. Activité antibactérienne du thymol, du carvacrol et de l'aldéhyde cinnamique seuls ou associes (Antibacterial activity of thymol, carvacrol, and cinnamaldehyde singly or in combinations). *Pharmazie* **1993**, *48*, 301−304.

Helander, I. M. Isolation and electrophoretic analysis of bacterial lipopolysaccharides. In *Enterobacterial Surface Antigens: Methods for Molecular Characterization*; Korhonen, T. K., Mäkelä, P. H., Dawes, E. A., Eds.; Elsevier: Amsterdam, 1985; pp 263−274.

Helander, I. M.; Haikara, A. Cellular fatty acids and alkenyl residues in *Megasphaera* and *Pectinatus* species: contrasting profiles and detection of beer spoilage. *Microbiology* **1995**, *141*, 1131−1137.

Helander, I. M.; Alakomi, H.-L.; Latva-Kala, K.; Koski, P. Polyethyleneimine is an effective permeabilizer of Gram-negative bacteria. *Microbiology* **1997a**, *143*, 3193−3199.

Helander, I. M.; von Wright, A.; Mattila-Sandholm, T.-M. Potential of lactic acid bacteria and novel antimicrobials against Gram-negative bacteria. *Trends Food Sci. Technol*. **1997b**, *8*, 146−150.

Juven, B. J.; Kanner, J.; Schved, F.; Weisslowicz, H. Factors that interact with the antibacterial action of thyme essential oil and its active constituents. *J. Appl. Bacteriol*. **1994**, *76*, 626−631.

Karapinar, M.; Aktug, S. E. Inhibition of foodborne pathogens by thymol, eugenol, menthol and anethole. *Int. J. Food Microbiol*. **1987**, *4*, 161−166.

Keweloh, H.; Weyrauch, G.; Rehm, H.-J. Phenol-induced membrane changes in free and immobilized *Escherichia coli*. *Appl. Microbiol. Biotechnol*. **1990**, *33*, 66−71.

Loh, B.; Grant, C.; Hancock, R. E. W. Use of the fluorescent probe 1-*N*-phenylnaphthylamine to study the interactions of aminoglycoside antibiotics with the outer membrane of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother*. **1984**, *26*, 546−551.

Lundin, A.; Thore, A. Analytical information obtainable by evaluation of the time course of firefly bioluminescence in the assay of ATP. *Anal. Biochem*. **1975**, *66*, 47−63.

Moore, L. V. H.; Bourne, D. M.; Moore, W. E. C. Comparative distribution and taxonomic value of cellular fatty acids in thirty-three genera of anaerobic Gram-negative bacilli. *Int. J. Syst. Bacteriol*. **1994**, *44*, 338−347.

Nikaido, H. Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* **1994**, *264*, 382−388.

Nikaido, H. Outer membrane. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*; Neidhardt, F. C., Ed.; ASM Press: Washington, DC, 1996; Vol. I., pp 29−47.

Nikaido, H.; Vaara, M. Molecular basis of bacterial outer membrane permeability. *Microbiol. Rev*. **1985**, *49*, 1−32.

Oosterhaven, K.; Chambel Leitao, A.; Gorris, L. G. M.; Smid, E. J. Comparative study on the action of S-(+)-carvone, *in situ*, on the potato storage fungi *Fusarium solani* var. *coeruleum* and *F. sulphureum*. *J. Appl. Bacteriol*. **1996**, *80*, 535−539.

Plésiat, P.; Nikaido, H. Outer membranes of gram-negative bacteria are permeable to steroid probes. *Mol. Microbiol*. **1992**, *6*, 1323−1333.

Sikkema, J.; de Bont, J. A. M.; Poolman, B. Mechanism of membrane toxicity of hydrocarbons. *Microbiol. Rev*. **1995**, *59*, 201−222.

Sivropoulou, A.; Papanikolaou, E.; Nikolaou, C.; Kokkini, S.; Lanaras, T.; Arsenakis, M. Antimicrobial and cytotoxic activities of *Origanum* essential oils. *J. Agric. Food Chem*. **1996**, *44*, 1202−1205.

Smid, E. J.; Koeken, J. P. G.; Gorris, L. G. M. Fungicidal and fungistatic action of the secondary plant metabolites cinnamaldehyde and carvone. In *Modern Fungicides and Antimicrobial Compounds*; Lyr, H., Russell, P. E., Sisler, H. D., Eds.; Intercept: Andover, U.K., 1996; pp 173−180.

Ten Brink, B.; Otto, R.; Hansen, U. P.; Konings, W. N. Energy recycling by lactate efflux in growing and non-growing cells of *Streptococcus cremoris*. *J. Bacteriol*. **1985**, *162*, 383−390.

Vaara, M. Agents that increase the permeability of the outer membrane. *Microbiol. Rev*. **1992**, *56*, 395−411.

Zähringer, U.; Lindner, B.; Rietschel, E. Th. Molecular structure of lipid A, the endotoxic center of bacterial lipopolysaccharides. *Adv. Carbohydr. Chem. Biochem*. **1994**, *50*, 211−276.

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