Influence of cyclopropane fatty acids on heat, high pressure, acid and oxidative resistance in *Escherichia coli*

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Abstract

Heat and high pressure resistant strains of *Escherichia coli* are a challenge to food safety. This study investigated effects of cyclopropane fatty acids (CFAs) on stress tolerance in the heat- and pressure- resistant strain *E. coli* AW1.7 and the sensitive strain *E. coli* MG1655. The role of CFAs was explored by disruption of *cfa* coding for CFA synthase with an in-frame, unmarked deletion method. Both wild-type strains consumed all the unsaturated fatty acids ($C_{16:1}$ and $C_{18:1}$) that were mostly converted to CFAs and a low proportion to saturated fatty acid ($C_{16:0}$). Moreover, *E. coli* AW1.7 contained a higher proportion of membrane $C_{19:0}$ cyclopropane fatty acid than *E. coli* MG1655 (P<0.05). The Δ*cfa* mutant strains did not produce CFAs, and the corresponding substrates C16:1 and C18:1 accumulated in membrane lipids. The deletion of *cfa* did not alter resistance to H_2O_2 but increased the lethality of heat, high pressure and acid treatments in *E. coli* AW1.7, and *E. coli* MG1655. *E. coli* AW1.7 and its Δ*cfa* mutant were more resistant to pressure and heat but less resistant to acid stress than *E. coli* MG1655. Heat resistance of wild-type strains and their Δ*cfa* mutant was also assessed in beef patties grilled to an internal temperature of 71 °C. After treatment, cell counts of wild type strains were higher than those of the Δ*cfa* mutant strains. In conclusion, CFA synthesis in *E. coli* increases heat, high pressure and acid resistance, and increases heat resistance in food. This knowledge on mechanisms of stress resistance will facilitate the design of intervention methods for improved pathogen control in food production.

Keywords: *Escherichia coli*, heat resistance, pressure resistance, cyclopropane fatty acids, beef.

1. Introduction

Pathogenic strains of *Escherichia coli* are a major public health concern. Pathovars in the species *E. coli* include enterotoxigenic and enteropathogenic *Eschericha coli*, which are significant causes of childhood diarrhea, Shiga-toxin producing *E. coli* (STEC), which cause the hemolytic-uremic syndrome in humans, and *Shigella* spp., a human adapted pathovar which is a major cause of childhood diarrhea in the developing world (Croxen et al., 2013; Lanata et al., 2013; Niyogi 2005; Rasko et al., 2011). *Shigella* spp. and STEC have a low infectious dose, which necessitates elimination of even low levels of contamination. Control of *E. coli* in food, however, is challenged by its resistance to heat, pressure, acid, and oxidative stress. *E. coli* is relatively resistant to short-term exposure to oxidative stress or low pH (Foster, 2004; Storz and Imlay, 1999). *E. coli* also exhibit strain-specific resistance to heat (Juneja et al., 1997; Liu et al., 2015; Mercer et al., 2015; Smith et al., 2001). Strains of *E. coli* are among the most pressure resistant vegetative bacterial cells (Vanlint et al., 2012); some STEC resist application of 600 MPa (Liu et al., 2015, Gänzle and Liu, 2015).

Maintaining or establishing food safety by pathogen intervention technologies requires an improved understanding of the mechanism of stress resistance and cross-resistance to different environmental stressors. The cytoplasmic membrane is a main barrier of defence against environmental stresses. Bacterial survival depends on the ability to adjust lipid composition to acclimatize cells to different environments (Zhang and Rock, 2008). The synthesis of cyclopropane fatty acids (CFA) is a modification of the membrane phospholipids that occurs in early stationary phase (Wang and Cronan Jr, 1994; Zhang and Rock, 2008). CFA are formed by a soluble enzyme, CFA synthase, which transfers a methylene group from

S-adenosyl-L-methionine to *cis* double bonds of unsaturated fatty acids in membrane phospholipids (Cronan Jr et al., 1979; Huang et al., 2002). CFA in the cytoplasmic membrane protect bacterial cells against several environmental stressors including ethanol (Grandvalet et al., 2008; Teixeira et al., 2002), high osmotic pressure (Asakura et al., 2012; Guillot et al., 2000), low pH (Brown et al., 1997; Chang and Cronan Jr, 1999), and repeated freeze-thaw cycles (Grogan and Cronan Jr, 1986; Zavaglia et al., 2000); a high CFA content in membrane lipids also improves survival after freeze-drying (Muñoz-Rojas et al., 2006). The mutational disruption of *cfa* also increased the sensitivity of *E. coli* to pressure (Charoenwong et al., 2011). The heat resistant strain *E. coli* AW1.7 exhibits high levels of CFAs in the cytoplasmic membrane, which suggests a contribution to the exceptional heat resistance of this strain (Ruan et al., 2011). However, the role of CFAs in heat resistance of *E. coli* is poorly documented and its relevance to survival of *E. coli* during food processing remains unknown.

This study aimed to investigate the role of CFA on stress resistance in the heat- and pressure resistant beef isolate *E. coli* AW1.7 (Dlusskaya et al., 2011; Liu et al., 2012) and the reference strain *E. coli* MG1655. The role of CFA in stress resistance was evaluated by comparison of CFA levels in the cytoplasmic membrane, by disruption of *cfa* in both strains and characterization of the stress resistance of mutant strains, and by assessing their resistance to heat treatment in food.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are shown in Table 1. Strains *E. coli* AW1.7 and MG1655 are genome-sequenced and non-pathogenic strains of *E. coli*

(Mercer et al., 2015). *In silico* analysis of their serotypes (https://cge.cbs.dtu.dk/services/SerotypeFinder/, Joensen et al., 2015) predicted their serotypes as *E. coli* O128:H12 AW1.7 and *E. coli* O16:H48 K12 MG1655. *E. coli* DH5α served as a host for plasmids in the cloning procedures. *E. coli* strains were cultivated in Luria-Bertani (LB) medium (BD, Mississauga, CA) with agitation at 200 rpm and 37°C. Antibiotic-resistant *E. coli* carrying plasmid pUC19 or pKOV were selected by addition of 50 mg/l ampicillin or 34 mg/l chloramphenicol, respectively. Working cultures were activated by streaking strains from a -80 °C stock culture onto LB agar, followed by two successive 18h subcultures to obtain stationary phase cultures.

2.2. DNA manipulation

Genomic DNA was isolated with the Blood & Tissue Kit (Qiagen, Hilden, Germany). Primers were synthesized by Integrated DNA Technologies (SanDiego, USA). The Taq DNA polymerase was purchased from TaKaRa Bio (Shiga, Japan) and T4 DNA ligase and restriction enzymes from Thermo Scientific (Mississauga, CA). The Miniprep and DNA gel extraction kits were obtained from Qiagen. The DNA sequencing was conducted by Macrogen (Maryland, USA).

2.3. Construction of E. coli mutant strains

To disrupt the gene *cfa* from the genome of *E. coli* AW1.7 and MG1655 (Accession number LDYJ00000000 and NC_000913.3, respectively), an in-frame, unmarked deletion was performed by using the method of Link et al. (1997). The approximately 1000bp 5'-flanking regions (fragment A) and 1000bp 3'-flanking regions (fragment B) of the target genes *cfa* in *E. coli* AW1.7 and MG1655 were amplified by PCR with primers listed in table 2. The fragment A was digested with XbaI, SphI, and fragment

B was digested with SphI, HindIII. The resulting fragments were purified and sequentially ligated into vector pUC19 to generate pUC19/AB. The AB fragments in pUC19/AB was confirmed by sequencing, amplified with primers carrying an additional BamHI restriction site, and sub-cloned into the XbaI and BamHI restriction sites of the pKOV plasmid to create pKOV/Δ*cfa*-AW and pKOV/Δ*cfa*-MG. The resulting recombinant plasmids were transformed into electrocompetent *E. coli* at 12.5 kV/cm, 25 μF and 200 Ω. The cells were plated on chloramphenicol-LB plates and incubated at 43 °C to select for single-crossover mutants. Several colonies were isolated, serially diluted in LB broth, and plated on 5% (wt/vol) sucrose-LB plates at 30°C. Sucrose-resistant colonies were subsequently replica plated on chloramphenicol plates at 30 °C to identify the double-crossover mutants. Gene replacement was confirmed by PCR amplification and sequencing.

2.4. Membrane fatty acid composition

For total fatty acid extraction, cells grown to stationary phase were collected by centrifugation, washed twice with 10 mM phosphate-buffered saline (PBS) (pH 7.2), and lipids were extracted using a modified Bligh and Dyer (1959) procedure. Briefly, lipids in the fresh cell pellet were extracted twice with chloroform–methanol–water (1:2:0.8), followed by addition of chloroform-water (1:1) to allow partitioning water and organic phase. The lower chloroform phase was removed and evaporated to dryness under nitrogen. Lipids were converted to fatty acid methyl esters (FAME) by base-catalyzed transesterification. Fatty acid samples (less than 50mg) were saponified with 1 ml dry toluene, and methylated with 0.5 M sodium methoxide in 2 ml anhydrous methanol for 30 min at 50 °C. The reaction was terminated by addition of 0.1 ml glacial acetic acid and 5 ml water. FAME were extracted twice with 5 ml

hexane, and the solvent was removed under a stream of nitrogen. FAME were redissolved in 1 ml of hexane and analyzed with gas chromatography–mass spectrometry (GC-MS) (Model 7890A/5975C; Agilent Technologies, Santa Clara, CA, USA) fitted with a silica capillary column HP-5 ms (30 m length; 0.25 mm inner diameter; 0.25 μm film thickness). The splitless injection volume was 1 μl, and the temperature of the injector was 250 °C. Helium was used as the carrier gas with a flow rate of 4.4 ml/min. The oven temperature program was 50 °C held for 2 min, increased at a rate of 5 °C/min to 325 °C, and 325 °C held for 5 min. The mass spectrometer was performed with a full scan model, from 50 to 400 m/z. The temperature of mass source and mass quadrupole was 230 °C and 150 °C, respectively. FAME were identified by comparing their retention times with those of a standard mixture (463 NuChek Prep, Elysian, USA), and by comparing the mass spectra with reference spectra (NIST 05). Furthermore, compounds were quantified by comparing individual peak area with those in a standard mixture of known concentration. The fatty acid compositions were expressed as relative percentages (weight/weight).

2.5. Determination of stress resistance

2.5.1. Determination of heat resistance

Cells from stationary-phase cultures (18 h) were harvested by centrifugation and resuspended in an equal volume of LB broth, yielding a final cell concentration of about 10^8 CFU/ml. Three 200μl aliquots of cell suspensions were placed in 1.5ml Eppendorf tubes (Fisher Scientific, Toronto, CA) and heated in a water bath. The heat resistant strain *E. coli* AW1.7 was treated at 60 °C, while the heat sensitive strain *E. coli* MG1655 was treated at 57 °C to achieve a comparable reduction of cell counts.

Samples were submerged in ice water after 15 and 30 min treatments. To determine viable cell counts, appropriate dilutions were surface plated in duplicate on LB agar by using spiral plater (Don Whitley Scientific Limited, West Yorkshire, UK). Colonies were counted after the plates were incubated at 37°C for 24h. Survival was expressed as $\log(N_0/N)$ where N_0 designates cell counts of untreated cultures and N the cell counts of treated cultures. Heat resistance was determined in three independent experiments analyzed in duplicate.

2.5.2. Determination of pressure resistance

The pressure resistance of strains of *E. coli* was assessed with stationary-phase cells that were prepared as described above. Cell suspensions were packed into sterile 3 cm E3603 tubing (Fisher Scientific, Akron, OH, USA), heat sealed from both sides, and kept on ice before treatment. The samples were placed in a 2.2 ml pressure vessel (Micro-system, Unipress, Warsaw, Poland) filled with bis (2-ethylhexyl) sebacate (Sigma-Aldrich, Germany) as pressure-transmitting fluid. The pressure vessel was submerged in a water bath maintained at 20 °C. *E. coli* AW1.7 cells were treated at 500 MPa for 10 and 30 min, while MG1655 cells were treated for 5 and 10 min. The rates of compression and decompression were 277.8 MPa/min. The temperature in the pressure vessel was monitored by an internal thermocouple and the temperature changes during compression and decompression did not exceed 4.5 °C. The viable cell counts in untreated and pressure-treated samples were determined as described above. The pressure resistance was determined in three independent experiments analyzed in duplicate.

2.5.3. Determination of acid resistance

The acid tolerance of *E. coli* was carried out in LB broth adjusted to pH 2.5 with HCl

and sterilized by filtration. Stationary phase cells from 1ml of culture were harvested by centrifugation, and resuspended in the same volume of acidified LB broth to a cell density of approximately 10^8 CFU/ml. A 100µl aliquot of cell suspension was analyzed as untreated control. The samples were incubated in 37 °C and aliquots were removed in 1 h intervals transferred into saline. Subsequent serial dilutions were plated on LB agar. The acid resistance was determined in three independent experiments analyzed in duplicate.

2.5.4. Determination of resistance to oxidative stress

Oxidative stress resistance of *E. coli* was assessed by exposure to 50 mM hydrogen peroxide (Elkins et al., 1999). Stationary phase cells were harvested by centrifugation, washed in 10 mM phosphate-buffered saline (PBS) (pH 7.2), and diluted to a cell count of approximately 10^7 CFU/ml. H_2O_2 (Sigma-Aldrich, Germany) was added to the cell suspension at a final concentration of 50 mM and cell suspensions were incubated at 37 °C. Aliquots were withdrawn after 10 and 20 min treatments, serially diluted in PBS containing 0.2 % sodium thiosulfate (w/v) (Sigma-Aldrich, Germany) to neutralize the H_2O_2 before plating. The resistance to oxidative stress was determined in three independent experiments analyzed in duplicate.

2.6. Heat treatment in ground beef

The heat treatment of ground beef patties inoculated with *E. coli* AW1.7, MG1655 or their respective Δ*cfa* mutants was performed as described (Liu et al., 2015). In brief, lean ground beef with 4% or 10 % fat was obtained from a local processing plant on the day of processing and stored at -20 °C until use. Coliform counts of the ground beef were determined by plating on violet red bile agar. Coliform counts in ground beef were less than 250 CFU/g and coliforms were eliminated to counts below the

detection limit after grilling. Cells from 10 ml of 10^8 CFU/ml stationary-phase culture were mixed with 200 g ground beef in a sterile plastic bag and homogenized for 2 min with a Stomacher Lab-Blender. To determine cell counts before cooking, 20 grams of inoculated meat were removed and transferred into sterile plastic bag containing 20 ml of ice-chilled 0.1 % peptone water (BD, Mississauga, CA). The remaining meat was molded into a patty with a Single Hamburger Press (Weston Brand Pragotrade, Strongsville, OH, USA). The beef patties were grilled in a clam shell grill (Cuisinart 5-in-1 Griddler, Woodbridge, CA) to an internal temperature of 71±0.3 °C. The internal temperature was monitored by a digital thermometer (Barnant Company, Barrington, USA) inserted into the geometric center of the patty. The cooked beef patty was immediately placed into 200 ml ice-chilled 0.1% peptone water. Both uncooked control and cooked samples were stomached for 2 min separately, serially diluted in 0.1% peptone water, and surface plated on LB agar for colony enumeration. The heat resistance in ground beef was determined in three independent experiments analyzed in duplicate.

2.7. Statistical analysis

Data analysis was conducted with R 3.1.2 (www.r-project.org/). Significant differences in the membrane fatty acid composition were determined by one-way analysis (ANOVA); significant differences in the resistance of strains of *E. coli* to stress were determined by using T-test. Significance was assessed at a 5% probability of error ($P<0.05$); an error probability of 5 to 10% ($0.05 <P<0.1$) is reported as trend.

2.8. Nucleotide sequence accession numbers

The sequences of the truncated *cfa* gene in *E. coli* AW1.7 and MG1655 were deposited in GenBank with accession numbers KT591116 and KT591115

respectively.

3. Results

3.1. Construction of E. coli mutant strains

To investigate the potential role of CFAs on stress tolerance, the mutants deficient in CFA synthesis were generated with the suicide vector pKOV. The *cfa* of *E. coli* AW1.7 is 99% identical to *cfa* in *E. coli* K-12 MG1655. To compare the influence of *cfa* on stress tolerance between heat- and pressure-resistant strain *E. coli* AW1.7 and susceptible strain *E. coli* MG1655, *cfa* was inactivated in both strains with an in-frame, unmarked deletion method, which does not introduce polar effects on the downstream genes (Link et al., 1997). The mutations of *cfa* were confirmed by PCR amplification and sequencing.

3.2. Membrane fatty acid composition of wild-type and mutant strains

In *E. coli* wild-type cells, three main fatty acids were detected, $C_{16:0,}$ $C_{17:0}$cyclopropane and $C_{19:0}$cyclopropane fatty acids (Table 3), which represented about 85% of membrane fatty acids in *E. coli* AW1.7 and MG1655. Membranes from these cells also contained $C_{14:0}$, $C_{15:0}$ and $C_{17:0}$ fatty acids. The phenotype of *cfa* deletion mutants were confirmed by analysis of membrane fatty acids (Table 3). Disruption of *cfa* dramatically altered fatty acid profiles when compared to the corresponding wild-type strains. The major effect of *cfa* disruption was the absence of CFA and the accumulation of the corresponding substrates, $C_{16:1}$ and $C_{18:1}$ unsaturated fatty acids. Moreover, the abundance of the saturated $C_{16:0}$ was decreased in both Δ*cfa* mutant strains; this difference was significant (P<0.05) for the comparison of *E. coli* MG1655; a trend (P<0.1) was observed when comparing *E. coli* AW1.7 and the corresponding Δ*cfa* mutant.

The proportion of $C_{18:1}$ fatty acid- in both *cfa*-deficient mutants was approximately equivalent to the proportion of $C_{19:0}$ cyclopropane fatty acid- in the respective wild-type strains. Interestingly, the proportion of $C_{16:1}$ in mutant cells was approximately equal to the sum of its homologous $C_{17:0}$cyclopropane fatty acid plus the increased portion of $C_{16:0}$ in respective wild-type cells. The saturation index of lipids was 1.00 and 0.48 in stationary phase *E. coli* AW1.7 and AW1.7Δ*cfa*, and 1.00 and 0.53 in stationary phase *E. coli* MG1655 and MG1655Δ*cfa*, respectively. The increased level of unsaturated fatty acids in the two Δ*cfa* mutant strain reflects the accumulation of $C_{16:1}$ and $C_{18:1}$ unsaturated fatty acids.

The proportion of $C_{19:0}$cyclopropane fatty acid in *E. coli* AW1.7 was significantly higher when compared to *E. coli* MG1655 (P<0.05). Correspondingly, *E. coli* AW1.7Δ*cfa* had a higher proportion of $C_{18:1}$, the substrate of $C_{19:0}$ cyclopropane fatty acid, and a lower proportion of $C_{16:0}$ when compared to *E. coli* MG1655Δ*cfa* (P<0.05). These differences also resulted in a lower saturation index.

3.3. Stress resistance of E. coli cells

To examine whether differences in the composition of the cytoplasmic membrane between *E. coli* strains relates to difference in stress tolerance, survival under different stress conditions was quantified. Stress conditions were selected to reduce cell counts of the wild type strains by 1 – 6 $\log_{10}$(CFU/mL). During exposure to each stress condition, survival of wild-type strains and the corresponding Δ*cfa* mutant strains was monitored after several time points. To illustrate the difference in stress tolerance in a more concise overview, only two treatment times are displayed in Figure 1.

The parent strains were more heat resistant when compared to the corresponding Δ*cfa* mutants. Disruption of *cfa* increased the reduction of cell counts of *E. coli* AW1.7

after treatment at 60°C for 30 min by 1.1 $\log_{10}$(CFU/ml). Cell counts of *E. coli* MG1655 were additionally reduced by 0.9 $\log_{10}$(CFU/ml) after treatment of the corresponding Δ*cfa* mutant at 57 °C for 15 min (Figure 1). Likewise, *cfa* disruption increased the lethality of pressure treatment at 500 MPa by equal to or greater than one $\log_{10}$(CFU/ml) for various time points. Collectively, these data confirm the heat and pressure resistance of *E. coli* AW1.7 (Dlusskaya et al., 2011; Garcia-Hernandez et al., 2015) and demonstrate that CFA synthase contributes to the resistance to both stress conditions. In keeping with prior data on the role of CFAs in acid tolerance of *E. coli*, the survival of both Δ*cfa* mutant strains in acidified LB broth (pH 2.5) was significantly reduced (P<0.05) when compared to the survival of the respective wild-type strains (Figure 1). *E. coli* AW1.7 was less acid resistant than *E. coli* MG1655. Survival of the two wild-type strains and the respective Δ*cfa* mutant strains in presence of 50 mM H_2O_2 was similar (Figure 1).

3.4. Thermal inactivation of E. coli cells in ground beef

To validate the contribution of CFAs to heat resistance in food, beef patties with 4% or 10% fat were individually inoculated with *E. coli* AW1.7, MG1655 or their Δ*cfa* mutants were grilled to an internal temperature of 71 °C. The cell counts in uncooked control were approximately 10^7 CFU·g^{-1}. *E. coli* MG1655 was more resistant to treatment than the Δ*cfa* mutant strain (P<0.05). *E. coli* AW1.7 was more resistant to treatment in beef with 4% fat; similar trend was noted in beef with 10% fat (Fig. 2).

4. Discussion

Cyclopropane fatty acids are major components of membrane phospholipids in many bacteria including *E. coli* (Grogan and Cronan Jr, 1997). The conversion of unsaturated fatty acids by CFA synthase renders the membrane more stable to

environmental insults (Zhang and Rock, 2008). The contribution of CFAs to bacterial heat resistance, however, remains poorly documented and its contribution to survival during food processing has not been documented. In this study, two *cfa*-deficient mutant strains were generated. To explore the role of CFAs in stress tolerance, survival of wild-type and the respective Δ*cfa* mutant strains was compared after exposure to heat, high pressure, acid and oxidation, and after cooking of beef to a core temperature of 71°C.

Deletion of *cfa* substantially altered the profile of membrane phospholipids. Stationary phase cells of wild type strains contained high levels of CFAs but no unsaturated fatty acids. In contrast, membranes of *cfa* mutant strains contained high levels of unsaturated fatty acids. Membranes of exponentially growing cells of *E. coli* AW1.7 had a lower proportion of CFAs but a higher proportion of UFAs (Ruan et al., 2011) when compared to data obtained in this study for the same strain. The difference CFA levels in exponentially growing and stationary phase cells are attributable to the σ^S-mediated increased expression of CFA synthase during the stationary phase of growth (Pagán and Mackey, 2000; Wang and Cronan Jr, 1994).

Alteration of the composition of the cytoplasmic membrane is a primary mechanism of bacterial adaptation to environmental stress (Denich et al., 2003; Yoon et al., 2015). The current study demonstrated that the presence of CFAs increased resistance to heat, pressure, and acid in the heat- and pressure-resistant strain *E. coli* AW1.7 and susceptible strain *E. coli* MG1655. Substitution of saturated membrane lipids by CFAs increases the fluidity of lipid bilayers through disruption of lipid packing and increasing the lipid diffusion; however, substitution of unsaturated fatty acids by their analogous CFAs decreases the fluidity of the membrane (Poger and Mark, 2015).

The effect of CFAs on acid resistance of *E. coli* is well documented (Brown et al., 1997; Chang and Cronan Jr., 1999). The protective effect of CFAs relates to reduced proton permeability as well as an increased ability to extrude protons (Shabala and Ross, 2008). CFA synthesis in *E. coli* was proposed to increase resistance to oxidative stress (Grogan and Cronan Jr, 1997). The ability of *Mycobacterium tuberculosis* to convert mycolic acids to cyclopropane derivatives increased resistance to hydrogen peroxide (Yuan et al., 1995). However, an effect of CFA- formation on the oxidative stress resistance in *E. coli* has not been determined experimentally, and was not observed in this study. *E. coli* harbours multiple glutathione- and thioredoxin-dependent systems for reduction of peroxides (Carmel-Harel and Storz, 2000) which may mitigate H_2O_2-induced oxidation of unsaturated fatty acids under conditions used in this study.

Differences in pressure resistance between stationary-phase and exponentially growing cells of *E. coli* were attributed to σ^S mediated changes in the cytoplasmic membrane, including CFA formation (Pagán and Mackey, 2000). A comparison of the pressure resistance of *E. coli* BW2952 with mutant strains from the Keio collection demonstrated that the *cfa*, *rpoS*, and *trxB* deficient strains were most sensitive to pressure (Charoenwong et al., 2011). Our study extended knowledge on role of CFAs in pressure resistance to the heat- and pressure resistant food isolate *E. coli* AW1.7. The resistance of *E. coli* to pressure is linked to altered membrane properties as well as its resistance to oxidative stress (Malone et al., 2006; Gänzle and Liu, 2015). The contribution of CFAs to pressure resistance may thus relate to the reduced membrane fluidity but may also involve increased resistance of membrane lipids to oxidative attack.

A role of membrane properties on heat resistance of *E. coli* and *Salmonella* spp. was inferred from the increased resistance of heat-adapted cells (Katsui et al., 1981; Alvarez-Ordóñezet al., 2008). Ruan et al. (2011) noted an increased proportion of CFAs in the membrane of *E. coli* AW1.7 when compared to heat sensitive *E. coli*. The current study provided direct evidence for a role of *cfa* in heat resistance of *E. coli*. Moreover, this protective effect was confirmed in food. The effect of *cfa* deletion on heat resistance in LB broth and ground beef was comparable to the effect on pressure resistance. Although heat and pressure resistance in different strains of *E. coli* are not correlated (Liu et al., 2015), CFA synthesis is thus an important factor for the cross-resistance of *E. coli* to multiple stressors including heat and pressure. The accumulation of compatible solutes also account for cross-resistance of *E. coli* to heat and pressure (Pleitner et al., 2012; Vanlint et al., 2013).

The two strains of *E. coli* showed substantial differences with respect to their stress tolerance. *E. coli* AW1.7 was more resistant to heat and pressure but less resistant to acid stress than *E. coli* MG1655. CFA accumulation increased heat resistance in both strains, however, the difference in heat resistance between the two wild type strains is mainly attributable to a genomic island termed locus of heat resistance (LHR) in *E. coli* AW1.7 (Mercer et al., 2015). This 14 kbp genomic island encodes 16 putative proteins that are predicted to contribute to turnover of misfolded proteins, solute accumulation, and thiol homeostasis (Mercer et al., 2015).

In conclusion, our study demonstrates that the *cfa* gene in *E. coli* contributes to heat resistance in this species. Remarkably, cell counts of *E. coli* AW1.7 are reduced by less than 3 log(CFU/g) after cooking of meat according to Health Canada's recommendations for ground beef and poultry (Anonymous, 2014). This level of heat

resistance is exceptional but not unprecedented (Dlusskaya et al., 2011; Luchansky et al., 2013; Liu et al., 2015). Improved knowledge on mechanisms of stress resistance will facilitate the design of intervention methods for improved pathogen control in food production. This study therefore enables further investigations to control *E. coli*, including pathogenic strains of the species, by application of suitable pathogen intervention technologies in food processing.

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Figure legends.

Figure 1. Resistance of strains of *E. coli* to heat, pressure, acid, and oxidative stress. Panel A: *E. coli* AW1.7; Panel B: *E. coli* MG1655. White bars indicate wild-type strains; hatched bars indicate Δ*cfa* mutant strains. Asterisks indicate statistically significant differences between wild-type and respective Δ*cfa* mutant strains, * $P<0.1$, ** $P<0.05$. Data represent means ± standard deviations of three independent experiments with duplicate determinations of visible cell counts.

Figure 2. Survival of *E. coli* AW1.7 (black bars) and *E. coli* AW1.7Δ*cfa* (white bars) in beef patties cooked to an internal temperature of 71±0.3 °C. Asterisks indicate statistically significant difference between wild-type and Δ*cfa* mutant strains ($P<0.05$). Data represent means ± standard deviations of three independent experiments with duplicate determinations of viable cell counts.

Table 1. Bacterial strains and plasmids used in this study

Strains	Description	Reference or Source
E. coli AW1.7	heat- and pressure- resistant strain, isolated from beef-packing plant	(Aslam et al., 2004)
E. coli K-12 MG1655	heat- and pressure- sensitive reference strain	(Guyer et al., 1981)
E. coli DH5α	Cloning host for plasmids	New England Biolabs
E. coli AW1.7 Δ*cfa*	*E. coli* AW1.7 with truncated *cfa*	This study
E. coli MG1655 Δ*cfa*	*E. coli* MG1655 with truncated *cfa*	This study
Plasmids	**Description**	**Reference or Source**
pUC19	lacZα promoter; cloning vector used in *E. coli*; Amp^r	New England Biolabs
pUC19/A-AW	pUC19 plasmid with 1 kb fragment of *cfa* upstream region in *E. coli* AW1.7; Amp^r	This study
pUC19/B-AW	pUC19 plasmid with 1 kb fragment of *cfa* downstream region in *E. coli* AW1.7; Amp^r	This study
pUC19/AB-AW	pUC19 plasmid with 2 kb of *cfa* upstream and downstream fragment of *E. coli* AW1.7; Amp^r	This study
pUC19/A-MG	pUC19 plasmid with 1 kb fragment of *cfa* upstream region in *E. coli* MG1655; Amp^r	This study
pUC19/B-MG	pUC19 plasmid with 1 kb fragment of *cfa* downstream region in *E. coli* MG1655; Amp^r	This study
pUC19/AB-MG	pUC19 plasmid with 2 kb of *cfa* upstream and downstream fragment of *E. coli* MG1655; Amp^r	This study
pKOV	Temperature sensitive pSC101; Sac B; Cm^r	Link et al., 1997
pKOV/Δ*cfa*-AW	pKOV plasmid with 2 kb of *cfa* flanking fragment of *E. coli* AW1.7; resulting *cfa* deletion; Cm^r	This study
pKOV/Δ*cfa*-MG	pKOV plasmid with 2 kb of *cfa* flanking fragment of *E. coli* MG1655; resulting *cfa* deletion; Cm^r	This study

Amp^r: ampicillin-resistance gene; Cm^r: chloramphenicol -resistance gene.

Table 2. Primers used in this study

Primers (forward, F; reverse, R)	Sequence (5'-3')	Restriction site
AW-upstream A, F	5'-CTAGTCTAGACTGAATGGGCAGCAAAAAGAAGAAGCAGGG-3'	XbaI
AW-upstream A, R	5'-ACATGCATGCTGTGCAGGTGCTTTCCGCGCCCGTG-3'	SphI
AW-downstream B, F	5'-ACATGCATGCGGCTATACCGGCACGGCTAAGTAATTCG-3'	SphI
AW-downstream B, R1	5'-CCCAAGCTTTATCGCGGCAACGTGCTGATATACGCG-3'	HindIII
AW-downstream B, R2	5'-CGCGGATCCCCCAAGCTTTATCGCGGCAACGTGCTGATATACG-3'	BamHI
MG-upstream A, F	5'-CTAGTCTAGATATCGCGGCAACGTGCTGATATACGCAGCC-3'	XbaI
MG-upstream A, R	5'-ACATGCATGCGGCTATACCGGCACGGCTAAGTAATTC-3'	SphI
MG-downstream B, F	5'-ACATGCATGCTGTGCAGGTGCTTTCCGCGCCCGTG-3'	SphI
MG-downstream B, R1	5'-CCCAAGCTTCTGAATGGGCAGCAAAAAGAAGAAGCAGGG-3'	HindIII
MG-downstream B, R2	5'-CGCGGATCCCCCAAGCTTCTGAATGGGCAGCAAAAAGAAGAAGCAG-3'	BamHI

Table 3. Fatty acid composition of lipids extracted from *E. coli* AW1.7 and MG1655 and the cognate Δ*cfa* mutant strains.

Fatty acid	**% of total membrane fatty acids [1)]**			
	AW1.7	AW1.7Δ*cfa*	MG1655	MG1655Δ*cfa*
$C_{14:0}$	7.30±2.08[a2)]	6.07±0.29[a]	7.74±1.40[a]	6.12±0.78[a]
$C_{15:0}$	4.29±0.69[a]	3.50±0.14[a]	4.30±0.33[a,]	3.59±0.27[a]
$C_{16:1}$	ND[3)]	32.27+0.98[a]	ND	33.11±1.00[a]
$C_{16:0}$	38.84±0.94[bc]	34.63±0.90[c]	45.37±3.32[a]	39.57±0.74[b]
$C_{17:0}$cyclopropane	27.47±2.57[a]	ND	26.43±1.82[a]	ND
$C_{17:0}$	3.61±0.37[a]	4.12±0.29[a]	3.98±1.20[a]	4.12±0.83[a]
$C_{18:1}$	ND	19.41±0.23[a]	ND	13.50±0.7[b]
$C_{19:0}$cyclopropane	18.49±0.84[a]	ND	12.18±2.62[b]	ND
SI[4)]	1.00±0.00[a]	0.48±0.01[c]	1.00±0.00[a]	0.53±0.01[b]

[1)] % of total membrane fatty acids; values are expressed as mean ± standard deviation of three independent experiments.

[2)] Within a row, values with different superscripts are different at $P<0.05$.

[3)] ND, not detected.

[4)] SI, saturation index, sum of saturated fatty acids including CFAs /100%

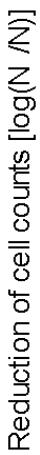

Figure 1. Resistance of strains of *E. coli* to heat, pressure, acid, and oxidative stress. Panel A: *E. coli* AW1.7; Panel B: *E. coli* MG1655. Black bars indicate wild-type strains; white bars indicate Δ*cfa* mutant strains. Asterisks indicate significant differences between wild-type and respective Δ*cfa* mutant strains (* $P < 0.05$). Data represent means ± standard deviations of three independent experiments with duplicate determinations of cell counts.

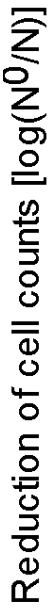

Figure 2. Survival of *E. coli* AW1.7 and *E. coli* MG1655 and their respective Δ*cfa* mutant strains (hatched bard) in beef patties cooked to an internal temperature of 71±0.3 °C. Ground beef contained 4% or 10% fat as indicated. Asterisks indicate a trend (*, $0.05<P<0.1$) or a significant difference between wild-type and the respective Δ*cfa* mutant strains (**, $P<0.05$). Data represent means ± standard deviations of three independent experiments with duplicate determinations of viable cell counts.