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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.

1. Introduction

Pathogenic strains of *Escherichia coli* are a major public health concern. Pathovars in the species *E. coli* include enterotoxigenic and enteropathogenic *E. 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 defense 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, 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 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, 1999), and repeated freeze–thaw cycles (Grogan and Cronan, 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

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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 18 h 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 1000 bp 5′-flanking regions (fragment A) and 1000 bp 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 50 mg) 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

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		
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.