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Reduction of the monounsaturated fatty acid content of *Escherichia coli* results in increased resistance to oxidative damage

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Reactive oxygen species (ROSs) affect several macromolecules and cellular components in eukaryotic and prokaryotic cells. In this work, the effect of various ROS-generating compounds on the *Escherichia coli* membrane was studied. Membrane fatty acid profiles, oxidative damage levels and bacterial resistance to these toxicants were determined. Studies included wild-type cells as well as a strain exhibiting a modified monounsaturated fatty acid (MUFA) profile (accomplished by overexpressing the *β*-hydroxyacyl acyl carrier protein dehydratase-encoding gene, *fabA*). Levels of membrane MUFAs and oxidative damage markers decreased slightly upon toxicant exposure with a concomitant increase in cell resistance to these ROS-generating compounds. A direct relationship between MUFAs and lipid peroxidation was observed. The lower the MUFA the lower the peroxide levels, suggesting that MUFAs are targets for membrane lipid oxidation.

INTRODUCTION

It is well established that reactive oxygen species (ROSs) are generated as byproducts during the normal functioning of the electron transport chain (Imlay, 2003). ROSs are highly reactive and can damage several cell macromolecules such as proteins, nucleic acids and lipids, among others (Ercal *et al.*, 2001). In addition, ROSs such as hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radical ($OH^{\bullet}$) can also be formed during cell exposure to redox-cycling toxicants like paraquat (Bus & Gibson, 1984) and metals (iron) or metalloid salts such as chromate and potassium tellurite (Ercal *et al.*, 2001; Bagchi *et al.*, 2002; Pérez *et al.*, 2007).

Although it has been largely thought that the *Escherichia coli* membrane fatty acids are virtually resistant to ROS-induced injury (Imlay, 2003), evidence for oxidative damage to these membrane components has become available recently. It has been shown that *E. coli* lacking the oxidative stress defence regulator *oxyR* exhibits increased levels of thiobarbituric acid-responsive substances (TBARS, a common lipid peroxidation marker) when exposed to *tert*-butyl-hydroperoxide (TBH) (Yoon *et al.*, 2002). This behaviour has also been observed in bacteria exposed to different oxidants such as hydrogen peroxide, potassium tellurite (K_2TeO_3), paraquat or TiO_2, among others (Maness *et al.*, 1999; Semchyshyn *et al.*, 2005; Pérez *et al.*, 2007, 2008).

We were interested in studying the *E. coli* response to oxidants, and we found that *E. coli* grown under conditions that resulted in changes to the membrane monounsaturated fatty acid (MUFA) ratio exhibited increased levels of TBARS, suggesting MUFA involvement in lipid peroxidation events. To further test this observation, we modified the MUFA ratio in the *E. coli* membrane by overexpressing *fabA*, encoding the *β*-hydroxyacyl acyl carrier protein dehydratase. This enzyme, involved in MUFA biosynthesis (Birge & Vagelos, 1972), is known to decrease the membrane MUFA levels when overproduced (Clark *et al.*, 1983). In this work, we found that overexpressing *fabA*, besides rendering *E. coli* more resistant to oxidative stress (particularly to membrane-oxidizing compounds), results in decreased lipid-derived peroxides, cell oxidative status and oxidized proteins levels.

METHODS

Plasmids and culture conditions. Plasmid p*fabA* was constructed by ligating the amplified *E. coli fabA* gene into the pBAD TOPO (Invitrogen) expression vector according to the manufacturer's specifications. Primers used to amplify *fabA* were 5′-ATGGTAGATAAACGCGAATCC-3′ (forward) and 5′-GAAGGCAGACGTATCCTGGA-3′ (reverse). The resulting p*fabA* was introduced into *E. coli* Top10 by electroporation. Correct *fabA* insertion was confirmed by *Nco*I digestion and PCR amplification. Then pBAD (control) and p*fabA*

Abbreviations: FOX, ferrous oxidation in the presence of xylenol orange; MUFA, monounsaturated fatty acid; ROS, reactive oxygen species; TBARS, thiobarbituric acid-responsive substances; TBH, *tert*-butyl-hydroperoxide.

A supplementary figure is available with the online version of this paper.

Table 1. Fatty acid profile (%) of *E. coli* BW25113 exposed to different toxicants

Fatty acids from *E. coli* carrying pBAD or p*fabA* plasmids were subjected to direct *trans*-esterification and analysed by gas chromatography. Results are expressed as the percentage of total fatty acids. C16 : 1, palmitoleic acid; C16, palmitic acid; C18 : 1, vaccenic acid; Δ% C16 : 1 + C18 : 1, variation of total MUFA content.

BW25113 pBAD	C16 : 1	C16	C18 : 1	C16 : 1 + C18 : 1	Δ% C16 : 1 + C18 : 1
Untreated	19.26 ± 0.81	39.70 ± 2.13	40.61 ± 0.83	59.86 ± 1.13	0
K_2TeO_3	18.76 ± 0.98	43.76 ± 2.91	37.11 ± 0.99	55.84 ± 0.85	−4.15 ± 0.89
H_2O_2	18.72 ± 0.77	42.19 ± 2.16	38.78 ± 0.93	57.50 ± 1.08	−2.82 ± 0.50
TBH	17.48 ± 0.73	43.48 ± 1.42	38.81 ± 0.65	56.30 ± 1.13	−4.10 ± 0.32
BW25113 p*fabA*					
Untreated	18.10 ± 0.31	63.89 ± 1.72	17.18 ± 0.83	35.28 ± 0.88	0
K_2TeO_3	16.05 ± 0.73	67.87 ± 1.71	15.08 ± 0.54	31.13 ± 0.67	−4.16 ± 1.51
H_2O_2	16.71 ± 1.20	67.72 ± 1.57	17.03 ± 1.12	33.77 ± 0.68	−1.51 ± 1.06
TBH	16.55 ± 0.54	67.05 ± 0.96	15.92 ± 1.08	32.47 ± 1.45	−2.81 ± 0.58

plasmids were introduced independently into *E. coli* BW25113. Cells were routinely grown in Vogel-Bonner (VB) minimal medium (Vogel & Bonner, 1956) containing 100 μg ampicillin ml^{-1} (Amp-VB).

MIC determination. Cells from overnight cultures were diluted 100-fold with fresh Amp-VB medium and grown to OD_{600} 0.3. Then, 10 μl was inoculated into 96-well plates containing serial dilutions of the toxicant in 150 μl VB medium. Plates were incubated for 48 h at 37 °C with shaking. ROS elicitors tested included the superoxide-generating compound tellurite (Pérez *et al.*, 2007), the lipid-peroxidating agent TBH (Masaki *et al.*, 1989) and hydrogen peroxide (Imlay, 2003).

Fatty acid profile determination. *E. coli* BW25113 cultures (20 ml) treated for 30 min with K_2TeO_3 (0.5 μM), H_2O_2 (300 μM) or TBH (50 μM) were sedimented and dehydrated by using a Speed Vac. Dehydrated cells were suspended in 500 μl methanolic HCl (5 %) and incubated at 80 °C for 10 min to form fatty acid methyl esters. The mixture was extracted with hexane (500 μl) and evaporated to a volume of 50 μl. An aliquot (5 μl) of this solution was injected in a diphenyl-dimethylsiloxane (5 : 95) column (PTE-5 Supelco) and fractionated by using a Perkin Elmer Autosystem gas chromatograph apparatus.

Protein carbonylation. Protein carbonyl group content was determined as described previously (Semchyshyn *et al.*, 2005). Briefly, nucleic acid-free cell extracts (100 μl) prepared from *E. coli* BW25113 exposed for 30 min to K_2TeO_3 (0.5 μM), H_2O_2 (300 μM) or TBH (50 μM) were mixed with 4 vols 10 mM 2,4-dinitrophenilhydrazine (2,4-DNHP; dissolved in 2 M HCl) and incubated for 1 h at room temperature with agitation. Proteins were precipitated with 1 vol 20 % trichloroacetic acid and sedimented at 10 000 ***g*** for 10 min. After washing three times with ethanol/ethyl acetate (1 : 1) the pellet was dissolved in 450 μl 6 M guanidine hydrochloride containing 50 mM dithiothreitol. Carbonyl group content was determined at 370 nm using a molar absorption coefficient of 22 000 M^{-1} cm^{-1} (Johnson, 1953).

Lipoperoxide content determination. Lipid peroxidation levels were assessed using the FOX (ferrous oxidation in the presence of xylenol orange) assay as described by Cha *et al.* (2004) with some modifications. Briefly, *E. coli* (50 mg fresh cell paste) was suspended in 500 μl Tris/HCl (pH 7.4) buffer, disrupted by sonication and centrifuged at 10 000 ***g*** for 10 min. Both protein extracts and pellets were sonicated again, this time in the presence of 1 % SDS and extracted twice with 1 vol methanol/chloroform (2 : 1, v/v). The organic phase was recovered, evaporated in a SpeedVac and suspended in 1 ml FOX reagent. After 1 h at room temperature, the lipoperoxide content was determined at 560 nm. Results were expressed as hydrogen peroxide equivalents.

Table 2. MIC (μM) of the indicated toxicants for *E. coli* carrying pBAD or p*fabA*

MICs were determined in VB minimal medium as described in Methods.

E. coli	K_2TeO_3	H_2O_2	TBH
BW25113 pBAD	1.25	600	125
BW25113 p*fabA*	2.50	2500	500

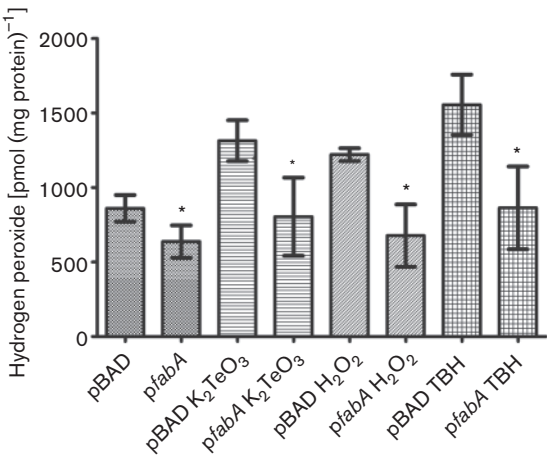

Fig. 1. Membrane peroxide assessment in stressed *E. coli*. Cells carrying pBAD or p*fabA* were exposed to the indicated toxicants for 30 min in minimal VB medium and used for determining lipid peroxides as described in Methods. Error bars represent SD (n=5). Asterisks indicate significant difference, $P<0.05$.

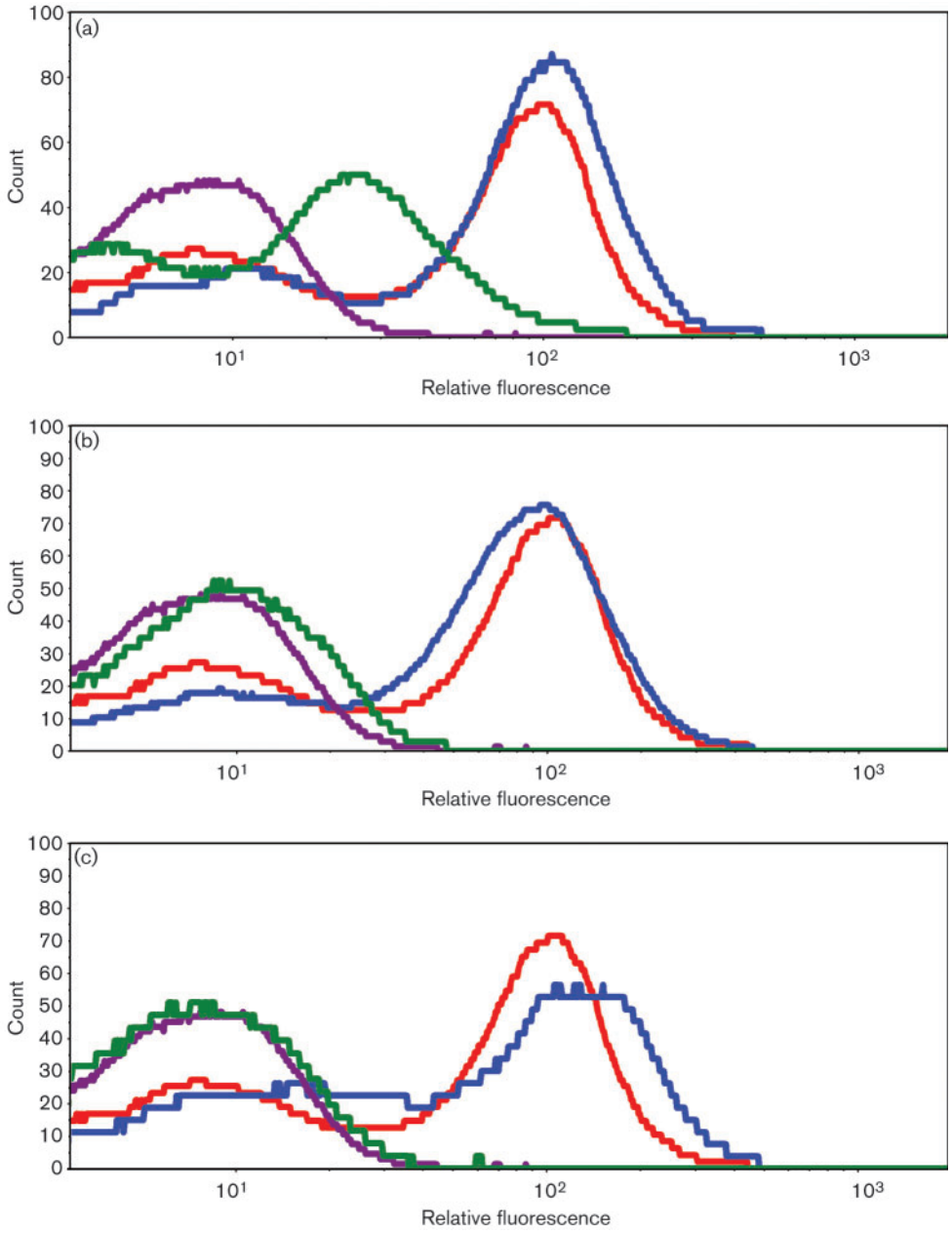

Fig. 2. Oxidative status assessment using the H_2DCFDA probe in toxicant-exposed *E. coli*. Flow cytometry analysis was carried out as described in Methods. *E. coli* carrying pBAD or p*fabA* was exposed to K_2TeO_3 (a), H_2O_2 (b) or TBH (c). A representative experiment is shown. Red, pBAD untreated; blue, pBAD treated; purple, p*fabA* untreated; green, p*fabA* treated.

Intracellular oxidative status. Intracellular ROS was assessed using the oxidation-sensitive probe 2′,7′-dihydrodichlorofluorescein diacetate (H_2DCFDA) (Royall & Ischiropoulos, 1993), which is activated by several intracellular oxidants including hydrogen peroxide and the hydroxyl radical, among others. H_2DCFDA has also been successfully used to detect lipid peroxides and peroxyl radicals (Aoyagi *et al.*, 1999). Cells grown to OD_{600} 0.3 were exposed for 30 min to potassium tellurite (0.5 μM), hydrogen peroxide (300 μM) or TBH (50 μM). Then cells were washed twice, diluted 50-fold with PBS buffer and treated for 30 min with H_2DCFDA. Cells were washed once more and analysed with a Becton Dickinson FacsCanto II cytometer (Argon laser, detection at 520 nm).

RESULTS AND DISCUSSION

Owing to the virtual unreactivity of MUFA with superoxide radicals (Bielski *et al.*, 1983), it has been generally accepted that the *E. coli* membrane is rather inert to ROS. However, this assumption is supported by studies performed in conditions far from those found in biological systems and does not consider that MUFAs can generate lipid peroxides and oxidation-derived aldehydes (Loidl-Stahlhofen *et al.*, 1995). Additional evidence shows that MUFAs can react with ROS to form toxic compounds in

eukaryotes (Borchman & Yappert, 1998; Rontani, 1998). In this context, our strategy to determine the effects of ROS-generating compounds on the *E. coli* membrane was to modify the membrane fatty acid composition. To accomplish this, the *E. coli fabA* gene was amplified, cloned and expressed in this bacterium.

To assess the effect of *fabA* expression on the lipid composition of the *E. coli* membrane, its fatty acid content was analysed by gas chromatography (GC). Higher amounts of palmitic (C16), palmitoleic (C16 : 1) and vaccenic (C18 : 1) acids were detected in membrane samples. Since it was found that <2 % of the cyclopropane comprised palmitoleic acid, it was not considered in the analysis depicted in Table 1. As expected, *fabA* expression resulted in augmented saturated fatty acid content (e.g. palmitic acid), as well as in decreased MUFA proportions (Table 1). Next, and to determine cell susceptibility to the toxicants, MICs of TeO_3^{2-}, H_2O_2 and TBH were determined for *E. coli* BW25113 carrying the plasmids pBAD (control) or p*fabA*. Upon *fabA* overexpression, cell resistance to tellurite and hydrogen peroxide or TBH increased by two- and fourfold, respectively (Table 2), suggesting an inverse relationship between MUFA content and resistance to oxidative stress. Such a relationship also suggests that MUFAs could be targets of oxidative damage that result in the generation of toxic derivatives such as aldehydes and lipid peroxides. No significant differences were observed in control and *fabA*-expressing cells regarding growth and resistance to Ni^{2+}, a metal whose toxicity does not involve direct generation of ROS (Macomber & Hausinger, 2011) (data not shown). In addition, the *E. coli* membrane fatty acid profile was analysed in cells exposed to oxidative stress elicitors. *E. coli* carrying pBAD or p*fabA* exhibited a slightly lower unsaturated fatty acid (C16 : 1 + C18 : 1) content when exposed to ROS-generating compounds (Table 1). In this context, it is interesting to note that the foremost effect was observed following tellurite treatment; MUFA levels decreased equally following toxicant treatment, irrespective of *fabA* expression.

Since the membrane MUFA content may be affected as a consequence of toxicant-induced lipid oxidation, membrane peroxidation was evaluated by using the FOX assay. Control *E. coli* had higher amounts of peroxide than *fabA*-expressing cells in all conditions (Fig. 1) apart from $NiCl_2$ treatment (not shown). Similar results were obtained regarding TBARS levels (Fig. S1, available with the online version of this paper). These results suggest a direct relationship between MUFA content and lipid peroxide generation. Monounsaturated fatty acids would be ROS targets, the oxidation of which would produce lipid peroxides in the same manner in which oleic acid (a MUFA) is oxidized to form hydroperoxides (Loidl-Stahlhofen *et al.*, 1995). Curiously, *fabA* expression results in significant lipid peroxidation even in the control condition; this most probably occurs because of the continuous ROS production during the normal functioning of the respiratory chain (Messner & Imlay, 1999). Thus, the lower the MUFA content, the lower the lipid peroxidation levels.

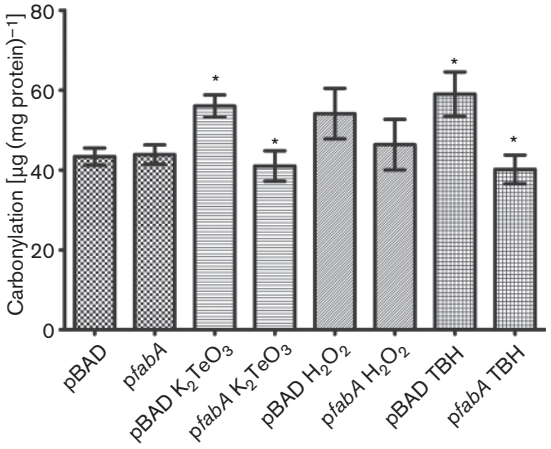

Fig. 3. Protein carbonylation in *E. coli* exposed to different toxicants. Cells carrying pBAD or p*fabA* were exposed to the indicated oxidative stress elicitors for 30 min in minimal media, and carbonyl groups were assessed using 2,4 DNPH as indicated in Methods. Error bars represent SD (n=5). Asterisks indicate significant difference, P<0.05.

Since lipid oxidation byproducts – as peroxides and/or reactive aldehydes – cause secondary oxidative damage to several macromolecules (Stadtman & Levine, 2000), the intracellular oxidative status of *E. coli* exposed to the toxicants tested was evaluated. In this context, intracellular levels of oxidant molecules were assessed by flow cytometry using the oxidation-sensitive probe H_2DCFDA. When exposed to the toxicants, pBAD-carrying cells showed little (tellurite and TBH) or no (H_2O_2) increase in probe activation compared with untreated controls. This low probe excitation may be explained because toxicant concentration was ¼ of the respective MICs (see Methods for details). However, *fabA*-expressing *E. coli* showed decreased probe activation in treated or untreated conditions, in agreement with lower intracellular ROS levels (Fig. 2). These observations support the lipid oxidation results, and suggest that any event resulting in decreased MUFA levels actually diminishes the available substrates – including lipid peroxides – that can react with the fluorescent probe (Rontani, 1998).

Another way by which oxidants damage cell components is through carbonylation events that affect proteins (Yan, 2009). This chemical modification is commonly used as a marker to assess protein oxidation. While exposure to oxidative damage elicitors increased protein carbonylation levels, *fabA* overexpression resulted in decreased protein oxidation in cells treated with potassium tellurite or TBH (Fig. 3). These results are consistent with those in which lipid peroxides were determined (Fig. 1) and suggest that both events could be membrane-related, since carbonylation

is caused by membrane-derived oxidized products such as TBARS (Esterbauer *et al.*, 1991).

In summary, our results suggest that the *E. coli* membrane composition is relevant in determining the oxidative status of the cell as well as in resistance to oxidative stress elicitors. In this context, MUFAs could react with ROS or ROS-generating compounds, thus producing oxidized membrane degradation byproducts (Loidl-Stahlhofen *et al.*, 1995). However, additional experiments with cells exhibiting altered membrane composition are needed to get a more complete picture of the relevance of membranes in *E. coli* facing oxidative stress.

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