Environmental Pollution 220 (2017) 532–539

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Influence of perfluorooctanoic acid on proteomic expression and cell membrane fatty acid of *Escherichia coli*☆

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ARTICLE INFO

Article history:
Received 22 June 2016
Received in revised form
30 August 2016
Accepted 30 September 2016
Available online 11 October 2016

Keywords:
Resistance mechanism
PFOA
Escherichia coli
iTRAQ
Proteomic analysis

ABSTRACT

Perfluorooctanoic acid (PFOA) has received an increasing attention in the agricultural and food industries due to its risk to human health. To facilitate the development of novel biomarkers of *Escherichia coli* against PFOA through multi-omics technologies, and to reveal the resistance mechanism of *E. coli* against PFOA at protein levels, the interactions among pollutant stress, protein expression and cell metabolism was investigated by using iTRAQ-based quantitative proteomic analysis. The results revealed that the 63 up-regulated proteins mainly involved in tricarboxylic acid cycle, glyoxylate and dicarboxylate metabolism and fatty acid biosynthesis, whereas, the 69 down-regulated proteins related to oxidative phosphorylation, pyruvate metabolism and the cell cycle-caulobacter pathway, were also associated with the increase of membrane permeability, excessive expenditure of ATP, disruption of fatty acid biosynthesis under PFOA stress. The results provide novel insights into the influence mechanisms of PFOA on fatty acid and protein networks.

1. Introduction

Perfluorooctanoic acid (PFOA), an emerging persistent organic pollutant, is highly toxic to wildlife and human, even in cord blood and breast milk (Apelberg et al., 2007; Avanasi et al., 2016). And it is polluting the global environment as it is used worldwide due to its high performance properties including the extremely chemical and thermal stabilities, and high surface activity (Lindim et al., 2015; Mak et al., 2009). Recent toxicological researches have indicated that PFOA is correlated with multiple toxicities, including hepatotoxicity, carcinogenicity, immunotoxicity, and developmental effects (Gorrochategui et al., 2016; Hemat et al., 2010; Smits and Nain, 2013; Tian et al., 2012). These findings have resulted in an increasing worldwide public concern regarding the environmental health risks associated with PFOA.

Fatty acids, which consist of the membrane phospholipid whose homeostasis is directly related to bacterial survival, are researched to acclimatize the bacterial cells to different environment. The ability of bacteria to modify their membrane composition in response to environmental changes, such as in temperature, osmolarity, salinity and pH, was determined previously in the studies of bacterial lipid metabolism (de Mendoza, 2014; Sharpe et al., 2010). Meanwhile, these phospholipid acyl chains determine the viscosity of the membrane, which in turn influences many crucial membrane associated functions, such as the passive permeability of hydrophobic molecules, active transport and protein-protein interactions. The adjustments in fatty acid composition that maintains the biophysical properties of membranes are referred to as homoviscous adaptation, and are interpreted as a mechanism that modifies the permeability of the phospholipid bilayer to minimize energy expenditure and optimize growth. This paper will discuss the primary metabolic and biochemical processes that underlie these adjustments and are responsible for membrane phospholipid homeostasis in bacteria.

However, it's difficult to know how the fatty acid alteration triggers phospholipid homeostasis, while the relevant chemical analysis is not enough to provide useful insight into the harm risk posed by the pollutants. On the other hand, it is possible to detect some molecular signatures related to the biomarkers by transcriptomics and proteomics when associated with the exposures to toxic stresses, which provides a great advantage for toxicity assessment. Proteins are important functional molecules in cellular

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http://dx.doi.org/10.1016/j.envpol.2016.09.097
0269-7491/

processes; investigating the different expression of proteins induced by xenobiotics can provide novel insight into the cellular mechanism under pollutant stress, and enable development of biomarkers for both exposure effect and susceptibility that can be used in health risk assessment (Colquhoun et al., 2009; Huang et al., 2013). This can be achieved by analyzing the proteome of the exposed *E. coli* by proteomic technologies.

Labeling-based mass spectrometry quantification technologies, including isotope-coded affinity tags, stable isotope labeling by amino acids in cell culture, and isobaric tag for relative and absolute quantification (iTRAQ) (Picotti et al., 2013) have been recently used to quantitatively detect the changes of protein expression in different organisms. The iTRAQ-based technology has attracted the most attention for use in quantitative proteomic applications due to its high sensitivity. A wide range of studies have been conducted utilizing iTRAQ-based quantitative proteomic technology (Kambiranda et al., 2014; Lü et al., 2014). Here an iTRAQ-based proteomic analysis technique was utilized to determine the changes of fatty acids in global protein expression of *E. coli* in response to the treatment of PFOA; in doing so, an insight into the interactions among fatty acid synthesis, proteomic expression and phospholipid homeostasis under PFOA stress would be revealed. Database for Annotation, Visualization and Integrated Discovery (DAVID) gene functional analytical tools (http://david.abcc.ncifcrf.gov/) including Gene Ontology (GO) annotation and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed to analyze the key pathways and subcategories of the proteins that were shown to have differential expression (Srivastava et al., 2012). The results will provide fundamental information regarding the cellular mechanism of PFOA on fatty acid synthesis and protein networks.

2. Materials and methods

2.1. Strain and chemicals

E. coli ATCC 8739 was purchased from microbial culture collection center in Guangdong Province, China. PFOA (Potassium salt, purity >98%) was purchased from Sigma Aldrich (St. Louis, MO, USA). The concentrations of beef extract, peptone and NaCl in the culture medium were 3, 10 and 5 g L^{-1}, respectively. The mineral salt medium (MSM) for PFOA resistance contained (in gL^{-1}) 4 $K_2HPO_4 \cdot 12H_2O$, 2 KH_2PO_4, 20 $(NH_4)_2SO_4$, 2 KCl and 1 $MgSO_4$.

2.2. Microbial culture and PFOA treatment

The methods of *E. coli* culture and PFOA treatment were provided in Text S1.

2.3. Extraction and determination of fatty acids

Analytical details on extraction and determination of fatty acids were provided in Text S2.

2.4. Extraction, quantification and digestion of cellular proteins

Cultures of *E. coli*, at an optical density of 0.6 at 600 nm, were incubated either with or without PFOA at the dose of 1.0 mg L^{-1} for 2 days and then harvested by centrifugation at 6000*g* for 10 min at 4 °C. Subsequently, the total proteins of the control and treated groups were extracted as previously described (Guo et al., 2014). Following the digestion with trypsin, vacuum centrifugation was performed to dry the peptides. The concentrations of protein and tryptic peptides were measured using the Bradford method (Bradford, 1976). Additionally, three independent experiments have been performed for protein assay.

2.5. Protein labeling and SCX fractionation

The detailed description of the protein labeling and SCX fractionation was supplied in Text S3.

2.6. LC-ESI-MS/MS analysis based on triple TOF 5600

The samples were resolved with solution including 2% (v/v) ACN, 0.1% (v/v) formic acid, centrifuged at 12,000*g* for 20 min, and detected by an AB Sciex Triple TOF 5600 mass spectrometer (AB SCIEX, Framingham, MA, USA) equipped with a Nanospray III source (AB SCIEX). Detailed of the LC-ESI-MS/MS analysis based on Triple TOF 5600 was summarized in Text S4.

For iTRAQ quantification, the peptide was automatically selected by the Pro GroupTM algorithm to calculate the reporter peak area, error factor and *p* value. A reverse database search strategy was adopted to estimate the FDR for peptide identification. A strict unused confidence score of >1.3, which corresponds to at least a peptide confidence level of 95%, was used as the qualification criterion. Identified proteins with at least two matched peptides higher than 95% confidence and an FDR value $\leq$ 1% were used to perform protein quantification. Subsequently, lists of proteins with at least a 1.2-fold increase or decrease were finalized for the three biological replicates (Chow et al., 2014).

2.7. Protein functional analyses

Up-regulated and down-regulated proteins in the 115/114 (pre-exposure MSM group to pre-exposure culture medium group), 116/114 (post-exposure MSM with PFOA group to pre-exposure culture medium group), and 116/115 (post-exposure MSM with PFOA group to pre-exposure MSM group) were subjected to functional pathway analyses using the DAVID gene functional analytical tools (http://david.abcc.ncifcrf.gov/), including GO, which are used in the functional annotation of the proteins, and KEGG pathway enrichment analysis, which was used to determine the functional subcategories and metabolic pathways for the differentially expressed proteins (Kanehisa et al., 2016). For the DAVID analysis, an enhanced score of $\geq$1.3, set as the threshold, was considered significant. (Huang et al., 2009).

2.8. Statistical analysis

Data are expressed as the mean ± standard deviation. Details are presented in Text S5.

3. Results and discussion

3.1. Fatty acid synthesis under PFOA stress

Bacteria have evolved mechanisms to regulate the formation of new fatty acids and modify the structure of existing ones, and these allow bacteria to adjust membrane viscosity to match environmental requirements (Mironov et al., 2012; Sen et al., 2015). The de novo fatty acid biosynthetic pathway is a major focal point for the regulatory events that control membrane homeostasis (Parsons and Rock, 2013). Lauric acid (C12:0), myristic acid (C14:0),pentadecanoic acid (C15:0), palmitoleic acid (C16:1ω9c), palmitic acid (C16:0), heptadecanoic acid (C17:0), elaidic acid (C18:1ω9t) and stearic acid (C18:0) had been detected in *E. coli* under PFOA stress. Among of them, palmitoleic acid was identified as the specific fatty acid, which could be the potential biomarker under PFOA stress. Besides, there were significant differences among contents of these

total fatty acids, unsaturated fatty acids (UFA) and specific fatty acids between the control and treated groups during the experiment process ($p > 0.05$). Meanwhile, the treated group with 1.0 mg L^{-1} PFOA for 2 days showed a more significant alteration of fatty acids compared with the control group (Fig. 1A, B and C). To gain insights of mechanism on fatty acid alteration to membrane homeostasis's effect, the alterations in the related protein expression of *E. coli* induced by PFOA for 2 days were investigated.

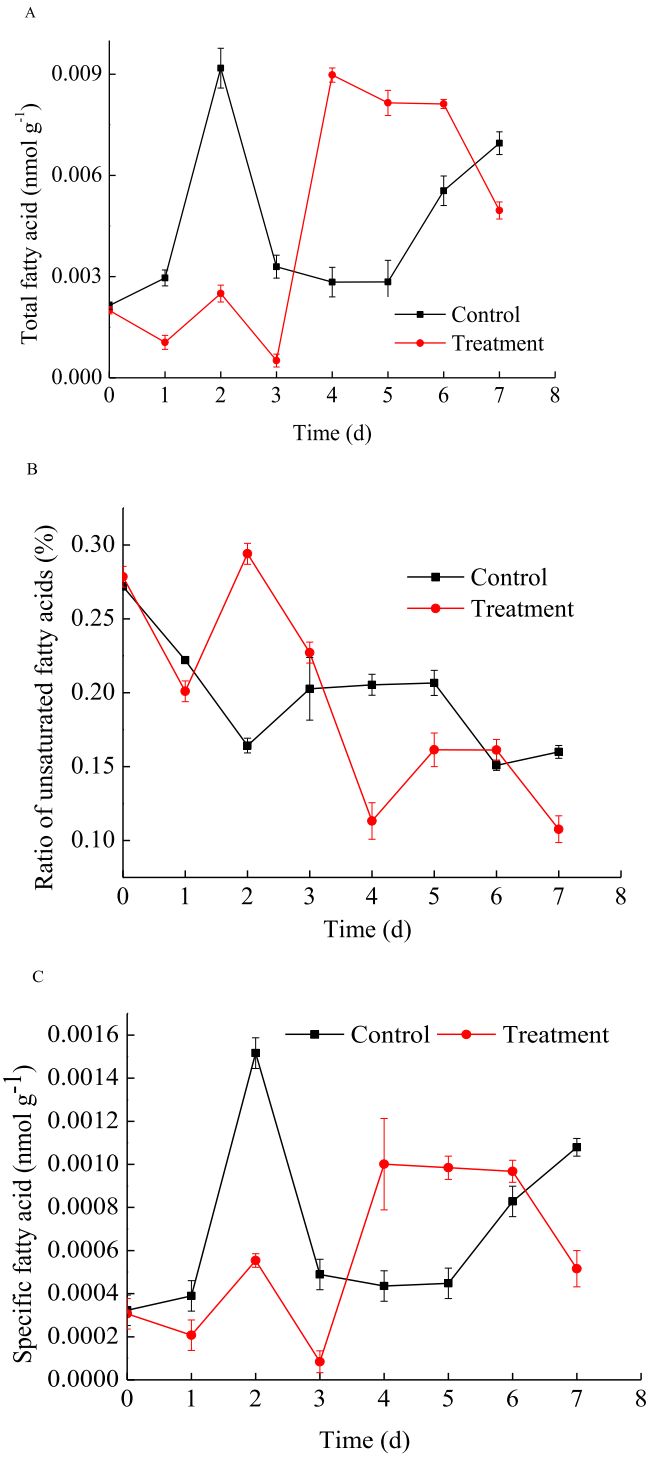

Fig. 1. Fatty acid concentrations of *E. coli* during the exposure process to PFOA, (A) Content of total fatty acids, (B) ratio of content of unsaturated fatty acid to total fatty acids, and (C) content of specific acids, the results expressed with nmol g^{-1}.

3.2. Identification and GO analysis of proteins with altered expression induced by PFOA

The MS/MS spectra of the total proteins in *E. coli* cells were processed using the Mascot software and resulted in the identification of 368 proteins (Table S1), which were quantified for the three iTRAQ-labeled samples. iTRAQ ratios were obtained by the intensities of 115/114, 116/114, and protein ratios contributed by at least two peptide ratios. The expression levels of 132 proteins (116/115) were shown to be significantly different (>1.2-fold change, $p < 0.05$) between the treated and untreated cells. Among these proteins, 63 were up-regulated and 69 were down-regulated in the treated cells as compared to the untreated cells (Table S2). GO enrichment analysis was performed to classify the cellular components, molecular functions, and biological processes that these proteins are involved in (Fig. 2A, B and C). The cellular component analysis revealed that most of the proteins with altered expression associated with external encapsulating structure, proton-transporting ATP synthase complex, coupling factor F(0), large ribosomal subunit, the periplasmic space, pertidoglycan-based cell wall. Among the 132 differentially expressed proteins, 102 of them have notable molecular functions, mainly involving the binding of different factors (such as cofactors, pyridoxal phosphate, tetrapyrrole, sulfur cluster, Mg^{2+}, Fe^{3+} and Zn^{2+}), enzymatic activity (including fatty acid synthase, metallopeptidase, ATPase, carbon-nitrogen lyase, oxidoreductase, transferase, ligase and peroxidase activities), catalytic activity, and acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor, or acting on peroxide as accepter. The biological process analysis indicated that 109 of the 132 proteins play an important role in 144 different biological processes, with a significant enrichment level of them acting in varieties of metabolic, biosynthetic and cellular processes.

3.3. Pathway analysis

The DAVID analysis was conducted on all differentially expressed proteins in the post-exposure MSM with PFOA group to pre-exposure MSM group to reveal their pathways. The results (Table S3) indicate that the 132 differentially expressed proteins which reached at significant enrichment level among their distribution of pathways in *E. coli* (p value ≤ 0.05) are mainly involved in tricarboxylic acid cycle, oxidative phosphorylation, ABC transporters, Ribosome, glycolysis/gluconeogenesis, bacterial chemotaxis, fatty acid biosynthesis, and glyoxylate, dicarboxylate, propanoate, methane, pyruvate, tryptophan, and pyrimidine metabolism, respectively. These pathways above chiefly belong to metabolism, translation and membrane transport pathways. Furthermore, to better explain which pathways involved in the membrane phospholipids homeostasis are governed by up- or down-regulated proteins, the KEGG pathway analysis was used. The results are shown in Fig. 3 that among all the related pathways, only ribosome, ABC transporters, TCA cycle, and the propanoate, tryptophan, pyrimidine, glyoxylate and dicarboxylate metabolism were significant pathways involved in up-regulated proteins, especially ABC transporters (with enhanced scores of 2.68) and propanoate metabolism (with enhanced scores of 3.55).

There were also nine pathways identified among down-regulated proteins in the post-exposure MSM with PFOA group to pre-exposure MSM group based on the identified proteins (Fig. 3). Only the oxidative phosphorylation, glycine, serine and threonine metabolism, and bacterial chemotaxis were the unique pathways among down-regulated proteins; besides, the oxidative phosphorylation, and glycine, serine and threonine metabolism were significant pathways among down-regulated proteins (with enhanced scores of 4.26 and 1.92). Altogether, the oxidative

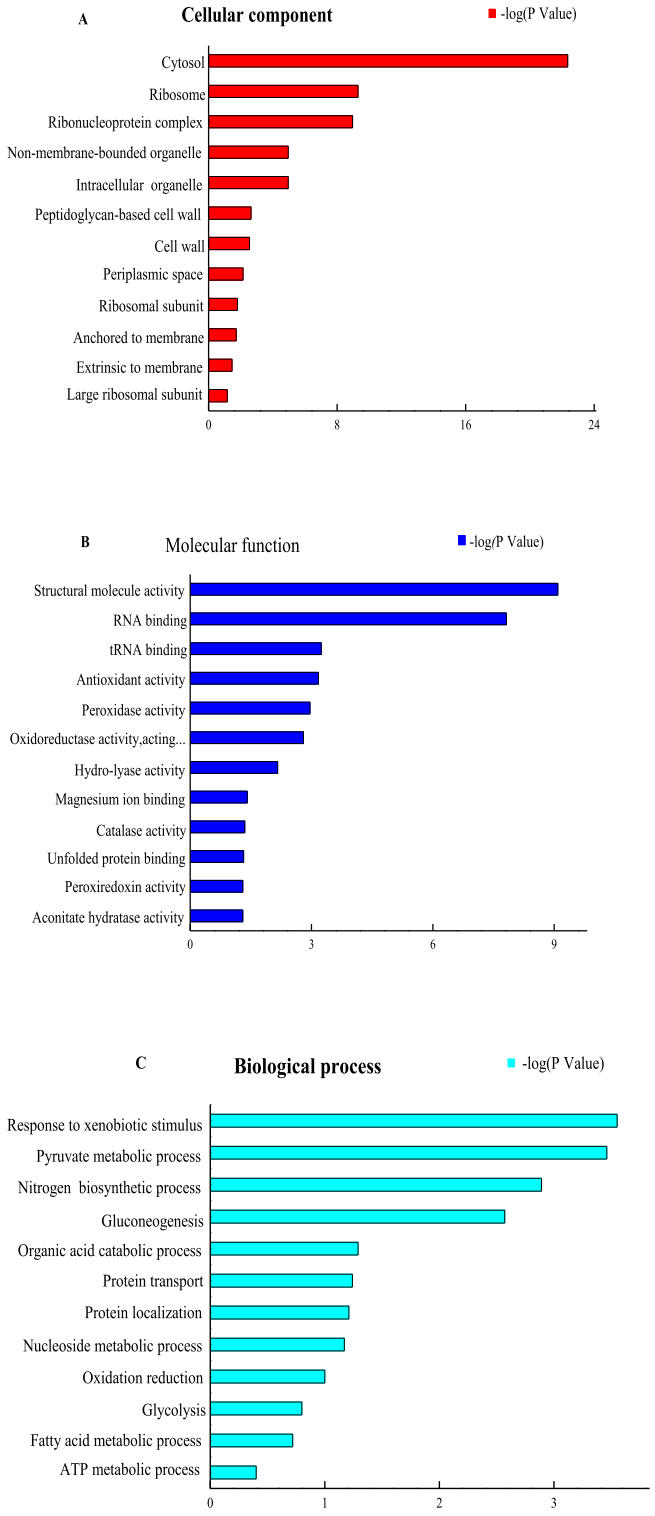

Fig. 2. GO enrichment analysis of the differentially expressed proteins. (A) The 12 components for cellular components, (B) molecular function, and (C) biological process of the differentially expressed proteins are presented along with their respective enrichment score represented as a *P* value.

phosphorylation, bacterial chemotaxis, pyrimidine, propanoate and tryptophan metabolism, and glycine, serine and threonine metabolism were the important pathways in response to the stress of PFOA at 1.0 mg L^{-1}, which were only found either in up-regulated proteins or in down-regulated ones. What's more, the ribosome, glyoxylate and dicarboxylate metabolism, ABC transporters and methane metabolism were also significant pathways, and were found distributed in the pathways enriched both by up- and down-regulation proteins, the corresponding differentially expressed proteins of which were decreased in down-regulated proteins and increased in up-regulated proteins. However, TCA cycle, pyruvate metabolism and methane metabolism distributed at up- and down-regulated proteins pathways were decreased in up-regulated proteins and increased in down-regulated proteins.

3.4. Roles of up-regulated proteins

Among the 63 up-regulated proteins in response to PFOA stress, most of them, including succinyl-CoA ligase [ADP-forming] subunit beta, 3-oxoacyl-[acyl-carrier-protein] reductase and 3-oxoacyl-[acyl-carrier-protein] synthase 1, aconitatehydratase 1, citrate synthase, periplasmic oligopeptide-binding protein, catalase HPII, D-ribose-binding periplasmic protein, putative osmoprotectant uptake system substrate-binding protein osmF, tryptophanase, uridinephosphorylase, keto-acid formate acetyltransferase, UTP-glucose-1-phosphate uridylyltransferase, oligopeptide transport ATP-binding protein oppD and oppF, are related to genetic information processing, environmental information processing, and energy, lipid and carbohydrate metabolism pathways.

Among them, 3-oxoacyl-[acyl-carrier-protein] reductase and 3-oxoacyl-[acyl-carrier-protein] synthase 1 are important enzymes in *E. coli*, playing an irreplaceable role in the type II fatty acid biosynthetic pathway (Zhang and Rock, 2008). In *E. coli*, 3-oxoacyl-[acyl-carrier-protein] synthase 1 is expressed in FabB isoform, which has a specific role in UFA formation (Feng and Cronan, 2009). In addition, FabB is an elongation condensing enzyme, which is involving in initiating a new round of chain elongation during the fatty acid biosynthetic process and has the unique catalytic property of elongating *cis* 3-decenoyl-ACP. However, UFA ratio depends on the ratio between the use of *cis* 3-decenoyl-ACP by FabB and the utilization of *trans*2-decenoyl-ACP by FabI. Up-regulation of 3-oxoacyl-[acyl-carrier-protein] synthase 1 positively catalyzes the UPA synthesis, which is consistent with the current finding that treatment of PFOA results in the increasing of UFA in *E. coli* (Fig. 1B). Meanwhile, the *cis*-UFA introduces a pronounced kink in the chain, which disrupts the order of the bilayer and results in lower transition temperatures and higher permeability (van Meer et al., 2008). Regarding 3-oxoacyl-[acyl-carrier-protein] reductase, it is only a single isoform of FabG in bacteria, which suggests that FabG could be a target for broad spectrum antibiotics. Besides, FabG is associated with catalysis of the NADPH-dependent reduction of beta-ketoacyl-ACP substrates to beta-hydroxyacyl-ACP products and the first reductive step in the elongation cycle of fatty acid biosynthesis. Up-regulation of this reductase promoted the formation of precursor of fatty acids and membrane phospholipids, which matches the current finding (Fig. 1A).

Succinyl-CoA ligase [ADP-forming] subunit beta belongs to the succinate/malate CoA ligase beta subunit family and contains 1 ATP-grasp domain, which possesses many functions, including succinate-CoA ligase activity; succinate-CoA ligase (ADP-forming) activity; and necleotide, Mg^{2+}, Mn^{2+}, nucleoside, purine nucleoside, adenyl nucleotide, and transition metal ion binding. Additionally, it is capable of generating precursor metabolites and energy, when it catalyzes the reaction related to propanoate and TCA cycle (Fig. 3). Furthermore, what its enzyme regulation is to exhibit two interesting properties: “substrate synergism”, in which the enzyme is most active for the catalysis of its partial reactions only when all the substrate binding sites are occupied, and “catalytic cooperativity” between alternating active sites in the tetramer, whereby the interaction of substrates (particularly ATP) at one site

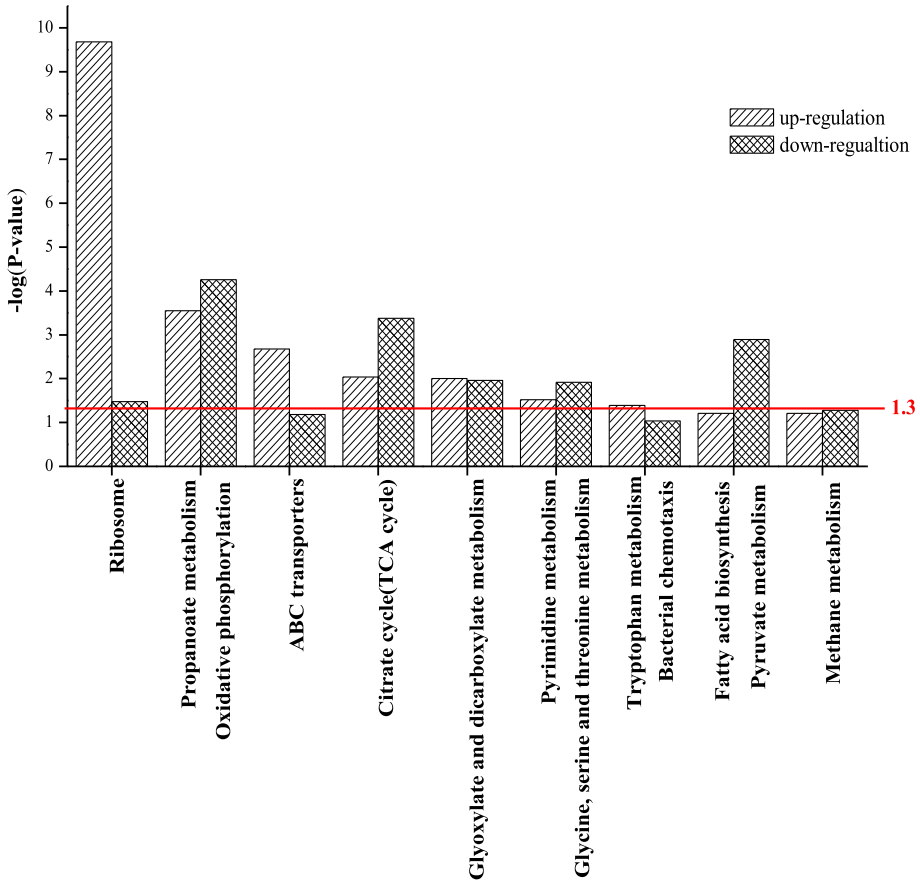

Fig. 3. Pathways associated with up- and down-regulation of proteins responses in the post-exposure MSM with PFOA group to pre-exposure MSM group were determined using a DAVID analysis. An enhanced score [-log(*p* value)] of ≥1.3 threshold (red line) was considered significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

is needed to promote catalysis at the other (Braesen et al., 2008). During aerobic metabolism it functions in the TCA cycle, coupling the hydrolysis of succinyl-CoA to the synthesis of ATP and thus represents an important site of substrate-level phosphorylation. It can also exhibit functions for anabolic purposes, and this may be particularly important for providing succinyl-CoA during anaerobic growth when the oxidative route from 2-oxoglutarate is severely repressed (Shikata et al., 2007). Succinyl-CoA synthetase of *E. coli* catalyzes its reaction via three steps that involve phosphoryl enzyme and enzyme-bound succinyl phosphate as intermediates. The beta-subunit contains the attachment sites for succinate. The complete active site is probably located in the region of alpha-beta contact. Up-regulation of succinyl-CoA ligase [ADP-forming] subunit beta leads to the excessively expenditure of ATP. However, almost all of the metabolic energy that is used to produce membrane lipids is expended in the formation of fatty acids, and therefore their production must be precisely controlled to support membrane biogenesis (Zhang and Rock, 2008), which exactly explains the alteration of fatty acid (Fig. 1A, B and C).

3.5. Roles of down-regulated proteins

Among the 69 down-regulated differentially expressed proteins, several, including ATP synthase subunits b and c, ATP-dependent Clp protease ATP-binding subunit clpX, catalase-peroxidase 1, D-galactose-binding periplasmic protein, lysine-tRNA ligase, oligopeptidaseA, phosphoenol pyruvate carboxy kinase [ATP] have been shown to have catalytic activities or macromolecular degradation activities. ATP synthase subunits b and c are both present in cell wall and plasma membrane. The two proteins have been observed to have monovalent inorganic cation transmembrane transporter activity, hydrogen ion transporting ATP synthase activity, and rotational mechanism (Nakanishi-Matsui et al., 2016). The primary pathway the two proteins involved in is oxidative phosphorylation (Table S3), which produces reactive oxygen species such as superoxide and hydrogen peroxide, and makes the free radical spreading to damage cells, cause lesions and lead to aging. The current finding that the damage of cell membrane and the increases of cell permeability lead to increasing synthesis of unsaturated fatty acid in response to PFOA stress is in conformity with the cellular metabolism pathway related to down-regulation proteins.

ATP-dependent Clp protease ATP-binding subunit clpX, which belongs to the clpX chaperone family, can perform chaperone functions in the absence of clpP inducing by heat shock (Skinner and Trempy, 2001). In *E. coli*, there are two types of Clp proteases, including the ClpP and ClpQ (or HslV) proteolytic subunits. Among them, the ClpP subunit, which serves as the proteolytic core, is capable of interacting with either ClpX or ClpA (Gribun et al., 2005). It has been reported about the cell cycle-caulobacter pathway, the ClpP subunit associates with ClpX, referring to as the ClpP-ClpX protease system, which is an autolyzed system that governs normal cell division and proliferation (Deepa et al., 2016). Down-regulation of ClpX leads to a decreased ability to depredate proteins, and it also inhibits the effectiveness of the ClpP proteolytic core. An autolysis system in bacterial cells has been reported that, under normal circumstances, is involved in the regulation of cell

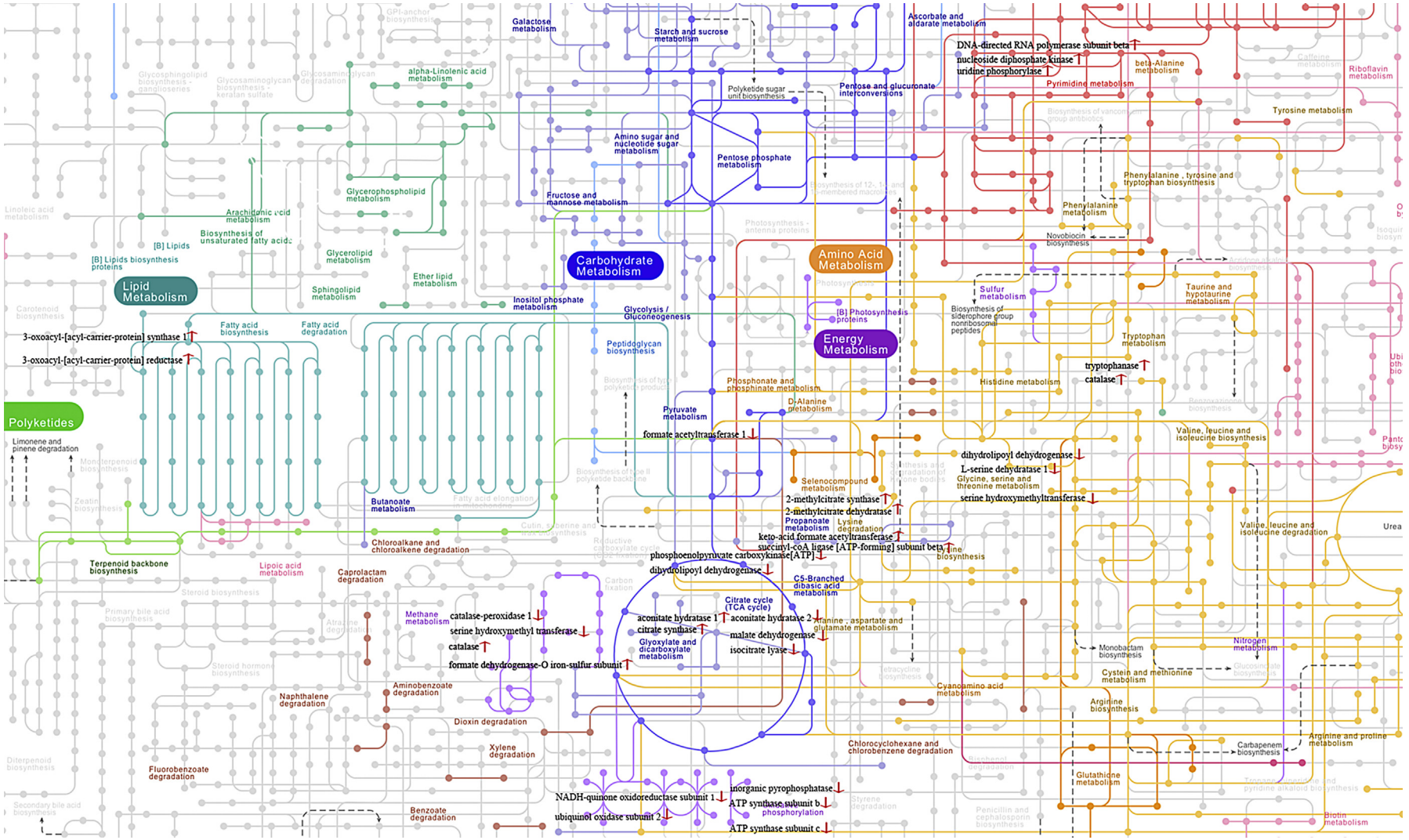

Fig. 4. The details of the whole metabolic pathways involved in all up-regulation and down-regulation proteins are displayed."↑" means the protein in some pathway is up-regulated, "↓" means the protein in some pathway is down-regulated.

division, the elimination of unwanted cell wall components and the control of generation process after damage occurs from external factors. The autolysis system consists of enzymes with muramidase activity, *N*-acetylglucosaminidase activity, amidase activity and peptidase activity (Huber et al., 2016). Under normal conditions, these autolytic enzymes are inhibited by the endogenous lipids, such as phospholipids and lipoteichoic acid. When an external stimulus disrupts the balance between these autolytic enzymes and the inhibitory lipids, the autolytic enzymes are activated, resulting in the autolysis and disruption of biosynthetic process of fatty acids of *E. coli* cells. The decreasing tendency of total fatty content observed for the *E. coli* cells that had been treated by PFOA confirmed the process of autolysis and disruption of biosynthesis of fatty acid (Fig. 1A).

D-galactose-binding periplasmic protein (GBP) related to ABC transporters and bacterial chemotaxis pathways (Fig. 3) possesses calcium binding and metal ion binding activities, and plays a role in the chemotaxis towards the galactose and glucose by interacting with the trg chemoreceptor (Piszczek et al., 2004). Additionally, this protein which locates in outer membrane-bounded periplasmic space is involved in the active transport of two sugars. Meanwhile, chemotaxis is the process by which cells sense chemical gradients in their environment and then move towards more favorable conditions. In chemotaxis, events at the receptors control autophosphorylation of the CheA histidine kinase, and the phosphohistidine is the substrate for the response regulator CheY, which catalyzes the transfer of the phosphoryl group to a conserved aspartate (Barak and Eisenbach, 2004). The resulting CheY-P can interact with the switch mechanism in the motor. This interaction causes a change in behavior. Hence, down-regulation of GBP slows down the speed of transportation of harmful metabolites and resulted in the harm to cell membrane, which leads to the promotion of UFA to resist PFOA stress (Fig. 1B). Besides, the down-regulated GBP probably does damage to the interaction of CheY-P and changes the mechanism in the motor, finally leads to the change of cell behavior and increases permeability of cellular membrane and inhibits the activities of some enzymes which can biosynthesize the fatty acid treated by PFOA (Fig. 1).

Finally, the details of the whole metabolic pathways involved in all these expressed differentially proteins are summarily displayed in Fig. 4. Upon treatment of *E. coli* with PFOA, there is a comprehensive global protein response characterized by the up-regulation of major catalytic enzymes involved in carbohydrate, lipid and nucleotide metabolism, and the down-regulation of proteins with energy metabolism activity and membrane transport activity. The inhibitions of the membrane transport pathway and the oxidative phosphorylation pathway were associated with the observed alteration of fatty acids. The promotion of the TCA cycle pathway and the fatty acid biosynthesis pathway potentially increased the permeability of the cellular membrane, resulting in the autolysis of the cellular structures (Green et al., 2014).

4. Conclusions

In response to treatment with PFOA, 132 of the 368 identified proteins in *E. coli* showed alterations in their expression, including 69 down-regulated and 63 up-regulated proteins. The up-regulated proteins mainly involved in tricarboxylic acid cycle, glyoxylate and dicarboxylate metabolism and fatty acid biosynthesis, the down-regulated proteins involved in oxidative phosphorylation, pyruvate metabolism and the cell cycle-caulobacter pathway, were also associated with the increase of membrane permeability, excessively expenditure of ATP, disruption of fatty acid biosynthesis. Generally, these interactions of all the pathways together triggered the membrane homeostasis to resist PFOA stress in *E. coli*.

Acknowledgements

The authors would like to thank the National Natural Science Foundation of China (Nos. 21377047, 21577049), and the Science and Technology Project of Guangdong Province (No. 2014A020216013) for their financial support.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.09.097.

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