Quantitative proteomic analysis of *Rhodococcus ruber* responsive to organic solvents

Sufang Kuang, Xin Fan and Ren Peng

Department of Bioengineering, College of Life Science, Jiangxi Normal University, Nanchang, PR China

ABSTRACT

*Rhodococcus ruber* with organic tolerance has potential applications in biotransformation and bioremediation. To explore the possible organic tolerance mechanism, the response of *R. ruber* SD3 to toluene and phenol was investigated using a quantitative proteomics approach with isobaric tag for relative and absolute quantification (iTRAQ) and liquid chromatography-tandem mass spectrometry. A total of 362 and 488 differentially expressed proteins were identified in the toluene treatment group and the phenol treatment group as compared to the control group, respectively. Functional annotation and metabolic pathway enrichment showed that transporter, degradation pathway and two-component system were closely related to organic solvent tolerance of *R. ruber* SD3. The quantitative real-time polymerase chain reaction experiment indicated the mRNA levels of stress proteins with an increased expression of 3.23 times upon toluene stress as compared to the control. The expression of 4-nitrophenol 2-monoxygenase in the phenol treatment group was 243 times higher than the counterpart in the control group. The study revealed the possible tolerance mechanism of *R. ruber* SD3 to organic solvents stress and provided some potential targets for the engineering of *R. ruber* SD3 to improve its organic solvent tolerance.

ARTICLE HISTORY

Received 5 June 2018
Accepted 4 October 2018

KEYWORDS

Isobaric tag for relative and absolute quantification; quantitative real-time polymerase chain reaction; organic solvent tolerance

Contact

Ren Peng
renpeng@jxnu.edu.cn
Department of Bioengineering, College of Life Science, Jiangxi Normal University, Nanchang 330022, PR China

Supplemental data for this article can be accessed here.

© 2018 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
and manZ alone did not take effect [8]. To study the response of P. putida JUCT1 to organic solvents, Ni et al. [2] used 2D gel electrophoresis to compare the total protein content in the strain growing with or without cyclohexane. Three genes – mmsB, tsf and PSEENO851 – were identified, which may be closely related to the organic solvent tolerance [2]. Rhodococcus sp. is Gram-positive and has increasing usage in bioremediation and preparation of fine chemicals and pharmaceuticals [9,10]. However, there is limited information available about the organic solvent tolerance mechanisms of Rhodococcus sp.

Until now, proteomics has found wide utilisation in exploring the organic solvent tolerance mechanism of microbes. Two-dimensional gel electrophoresis was commonly applied in these studies [2,5]. Nevertheless, the isobaric tag for relative and absolute quantification (iTRAQ) technique is a novel method for proteomic research, which is more accurate for measuring protein abundance than two-dimensional gel electrophoresis [11]. The iTRAQ technique was successfully utilised to analyse the proteome of rice under cold and aluminium stress [11,12]. Szopinska et al. [13] made use of the iTRAQ technique to investigate the plasma membrane proteome of S. cerevisiae response to salt stress. The cellular responses of Fusarium oxysporum to crocidolite asbestos were also studied by using the iTRAQ technique [14]. To track the transformation of extracellular protein from waste activated sludge, the iTRAQ technique was successfully applied to analyse extracts of extracellular substance [15].

In a previous study, we isolated a novel strain Rhodococcus ruber SD3 with organic solvent tolerance [16]. Here, we employed a quantitative proteomics approach with the iTRAQ technique and liquid chromatography-tandem mass spectrometry to reveal possible tolerance mechanism of R. ruber SD3 under toluene and phenol stress. The quantitative real-time polymerase chain reaction experiments were applied for the further validation of some potential targets obtained by the quantitative proteomics results.

**Materials and methods**

**Bacterial growth and sampling conditions**

R. ruber SD3 was isolated from polluted sludge and deposited in the China Center for Type Culture Collection (CCTCC) with the accession number of M 2012035. The strain was grown in LB broth medium under 35 °C in a shaker (200 r/min) until the optical density at 600 nm reached 0.8–1.0. For the toluene treatment group and phenol treatment group, 1% of culture was then inoculated into 50 mL LB broth medium containing toluene and phenol, respectively. Toluene with 0.2%, 0.3% and 0.4% (v/v) and phenol with 0.08%, 0.10% and 0.12% (m/v) were added to study the effect of organic solvents on bacterial growth. In the non-treated control group, the LB broth medium did not contain any organic solvents. The cultivation was conducted under 35 °C with shaking (200 r/min) for 24 h. The cells were harvested by centrifugation, followed by washing (3×) with phosphate buffer (pH 7.4). Each treatment was replicated twice.

**Extraction of total proteins from R. ruber SD3**

The samples were ground with liquid nitrogen and then lysis buffer containing 8 mol/L urea, 1% (m/v) sodium dodecyl sulphate (SDS) and 1% (v/v) Halt™ protease inhibitor cocktail (100×) was added. The cell-free extract was obtained by centrifugation at 16000 g and 4 °C for 30 min. The supernatants were mixed well with ice-cold acetone (1:5, v/v) at −20 °C overnight and the precipitate was obtained by centrifugation at 12000 g and 4 °C for 30 min. The precipitate was washed with 90% ice-cold acetone and then air-dried, which was checked using 10% (w/v) SDS polyacrylamide gel electrophoresis and subsequently stained in Coomassie Brilliant Blue.

**Reductive alkylation and digestion**

One hundred micrograms of protein samples were re-suspended in lysis buffer to a final volume of 100 μL. Tris (2-carboxyethyl)-phosphine was added at a final concentration of 10 mmol/L and reductive reaction was performed at 37 °C for 60 min. Iodoacetamide was added at a final concentration of 40 mmol/L and alkylation reaction was allowed to take place in a dark place at room temperature for 40 min. Ice-cold acetonitrile (6:1, v/v) was added into the mixture, which was then kept at −20 °C for 4 h. The precipitate was obtained by centrifugation at 10000 g for 20 min, which was then fully dissolved with 100 μL of 100 mmol/L triethylamine borane (TEAB). Each aliquot was digested with trypsin (50:1 w/w) overnight at 37 °C.

**iTRAQ labelling**

After protein digestion, peptides were dried under vacuum and reconstituted with 400 mmol/L TEAB. The iTRAQ labelling of peptide samples was performed using iTRAQ reagent Multiplex kit according to the manufacturer’s protocol. Briefly, iTRAQ reagents
The peptide samples were reconstituted in RPLC buffer and were then fractionated using ACQUITY UPLC BEH C18 Column (1.7 μm, 2.1 mm × 150 mm) by Waters ACQUITY UPLC system at a flow rate of 200 μL/min. The 66-min gradient elution conditions are described in detail in Supplemental Table S1. Phase A (2% acetonitrile) and Phase B (80% acetonitrile) were adjusted to pH 10 with ammonium hydroxide. Fractions were collected and combined based on peak and retention time, which were concentrated to dryness using rotational vacuum concentration (Christ RVC 2-25, Germany).

Bioinformatics analysis

Proteins with a cut-off of 1.5-fold change between toluene treatment group or phenol treatment group and non-treated control group and a p value <.05 were regarded as significantly different proteins. Metabolic enrichment analysis of significantly different proteins was performed according to the KEGG Pathway Database. The KEGG pathways with a p value <.05 were regarded as enriched KEGG pathways. The reproducibility of the biological replicates was assessed by principal component analysis (PCA).

The mRNA levels of stress protein and 4-nitrophenol 2-monoxygenase by quantitative real-time polymerase chain reaction

Total RNA was extracted from R. ruber SD3 from the toluene (0.2% v/v) treatment group, phenol 0.08% (m/v) treatment group and the non-treated control group, using RNAprep Pure Cell/Bacteria Kit according to the manufacturer's protocol (Tiangen). The first cDNA strand was produced using All-in-One First Strand Synthesis Kit from FulenGen. The fluorescent dye method was applied for expression analysis of stress protein and 4-nitrophenol 2-monoxygenase. The 16S rRNA gene was used as an internal reference gene in the experiment. The primers are shown in Table 1. The reaction systems consisted of 2× SYBR Green Mix (5 μL), 10 μM forward primer (1 μL), 10 μM reverse primer (1 μL) and cDNA template (3 μL). The reaction was performed with a cycle for initial denaturation (95°C, 5 min), 40 cycles at 95°C (10 s) and 60°C (30 s) for amplification. All data were normalised to the 16S rRNA gene according to the $2^{- \Delta \Delta CT}$ method. All assays were performed in triplicate [17].
Results and discussion

Effect of toluene and phenol on the growth of R. ruber SD3

To determine a suitable concentration of toluene and phenol for comparative proteomic studies, the growth of R. ruber SD3 in the presence of toluene with 0%, 0.2%, 0.3% and 0.4% (v/v) and phenol with 0%, 0.08%, 0.10% and 0.12% (m/v) was studied, respectively. As shown in Figure 1, in the presence of 0.2% toluene, the growth of R. ruber SD3 was completely inhibited in the first 12 h, and then entered an exponential phase, which displayed good adaption to toluene. Therefore, the concentration (0.2% v/v) was selected for proteomic studies. Similarly, the results in Figure 2 indicated that 0.08% (m/v) phenol was appropriate for subsequent quantitative proteomics.

Extraction of total proteins from R. ruber SD3

We extracted total protein from R. ruber SD3 toluene treatment group, phenol treatment group and control group. All treatments were performed in duplicate from two independently grown cultures. The SDS-PAGE of total proteins from R. ruber SD3 shown in Figure 3 indicated visible change after toluene treatment and phenol treatment as compared to the control group, especially for the proteins with molecular weight above 35 kDa. For instance, there are thicker bands in the range of 35–40 kDa, 40–55 kDa and

![Figure 1. Effect of toluene on the growth of Rhodococcus ruber SD3. Note: The growth of Rhodococcus ruber SD3 was measured (OD600) every 12 h. All assays were performed in triplicates.](image)

![Figure 2. Effect of phenol on the growth of Rhodococcus ruber SD3. Note: The growth of Rhodococcus ruber SD3 was measured (OD600) every 12 h. All assays were performed in triplicates.](image)
55–70 kDa in lane 3 and lane 4 as compared to lane 5 and lane 6. However, another weaker band was also visible in the range of 35–40 kDa in lane 3 and lane 4.

Overview of proteomic data

iTRAQ was then employed to compare the proteomic patterns of *R. ruber* SD3 in response to organic solvents. A total number of 459,047 fragmentation spectra were obtained from the quantitative proteomic analysis, which were then filtered to remove any low-scoring spectra. The remaining 136,054 fragmentation spectra which finally met the confidence criteria for peptide identification were matched and grouped into 3295 unique proteins (Figure 4). Most of these unique proteins were identified with good peptide coverage: 497, 917, 801 and 367 proteins had peptide coverage of 11–20%, 21–40%, 41–60% and 61–80%, respectively. The repeatability of the biological replicates was evaluated by PCA. The results shown in Figure 5 indicated that the toluene treatment group (A1, A3), the phenol treatment group (B1, B3) and the control group (C1, C3) were clustered separately, demonstrating the analytical reproducibility.

According to KEGG database, the top 20 pathways except basic pathways involving these unique proteins are illustrated in Figure 6. Generally, the higher the bar, the more active the pathway in the sample is. Thus, fatty acid degradation was the most active pathway in *R. ruber* SD3. *R. ruber* SD3 also had a rather active benzoate degradation pathway, which accounted for the fact that it could thrive in the presence of toluene and phenol. After KEGG orthology annotation, the identified proteins were applied for BRITE classification based on KEGG metabolic pathways, in which they were involved. Figure 7 indicated that 117 proteins deal with xenobiotics degradation and metabolism, which may be related to the degradation of toluene and phenol.

**Figure 3.** SDS-PAGE of total protein extract from *Rhodococcus ruber* SD3. Note: Protein molecular weight marker (lane M), the toluene treatment group (lanes 1 and 2), phenol treatment group (lanes 3 and 4) and control group (lanes 5 and 6) were run in SDS-PAGE and stained in Coomassie Brilliant Blue.

**Differentially expressed proteins in the toluene treatment group and the control group**

Using a cut-off of 1.5-fold change and a *p* value of <.05, there were 362 proteins significantly differentially expressed proteins when comparing the toluene treatment group and the control group. Among them, 89 proteins were up-regulated and 273 proteins down-regulated by toluene treatment (Supplemental Table S3). Among these differentially expressed proteins, there were many uncharacterised ones, suggesting a complicated toluene-response mechanism.
Common stress response to toluene treatment

Proteomic analysis identified several proteins related to the common stress response. There was upregulation of 60 kDa chaperonin (A0A098BMC1), universal stress family protein (A0A098BHR9) and stress protein, member of the CspA-family (A0A098BRD6), under toluene treatment by 2.27-, 1.51- and 2.97-fold, respectively, as compared with the control. The 60 kDa chaperonin prevents proteins from misfolding and enhances the refolding and correct assembly of unfolded polypeptides produced under stress conditions. Ericsson et al. [18] reported increased expression of 60 kDa chaperonin in *Francisella tularensis* LVS in response to heat and hydrogen peroxide. Cold-shock proteins are small proteins with ~70 amino acid residues and ubiquitous in *Eubacteria* and *Archaea*. Under cold stress conditions, the expression of cold-shock proteins is induced to fulfil a wide array of cellular functions [19]. CspA functions through its RNA chaperoning role, which has a main impact on the transcription and translation process at low temperature [20]. Segura et al. reported that CspA works in the organic solvent tolerance of *P. putida* DOT-T1E by proteomic analysis and gene knockout [5].

It is well known that oxidative stress response is stimulated by an imbalance between the production of reactive oxygen species and antioxidant systems [21]. The antioxidant defence system in bacteria includes metal chelators, low-molecular-weight compounds, such as ascorbate and glutathione, antioxidant enzymes, DNA, protein and lipid repair system. It is reported that treatment of cells with organic solvents can give rise to oxidative stress response [22]. In this study, catalase (A0A098BMG0) was up-regulated
Figure 6. Top 20 pathways except basic pathways involving the identified unique proteins.

Figure 7. Brite classification based on KEGG metabolic pathways.
to deal with oxidative stress caused by toluene. RecA is a protein essential for the repair and maintenance of DNA. Increased production of RecA was beneficial for the repair of the damaged DNA. In a study by Nicholson et al. [23], the recA three gene operon in B. fragilis witnessed up-regulation during metronidazole treatment and exposure to reactive oxygen species.

**Membrane-bound proteins induced by toluene treatment**

Membrane-bound proteins such as transporter and permease for small molecules were essential for microbes to overcome solvent damage [24]. In this study, three transporters and two permeases were up-regulated under toluene treatment, including amino acid transporter (A0A098BGB5), benzoate transport protein (A0A098BGR9), vanillate transporter (A0A098BI04), γ-aminobutyric acid (GABA) permease (A0A098BMD5) and major facilitator family permease (A0A098BQN2). The up-regulation of these membrane-anchored proteins demonstrated their important effects in R. ruber SD3 under toluene stress. Amino acid transporters mediate the transfer of amino acids across plasma membranes. Shang et al. [25] reported that AroP, an aromatic amino acid and histidine transporter, may be helpful for assimilating extracellular amino acids to circumvent the high energy cost of amino acid biosynthesis in Corynebacterium glutamicum. However, in-depth reports on the relationship of amino acid transporters and organic solvents tolerance were not available. The up-regulation of amino acid transporter indicated that R. ruber SD3 must obtain more amino acids from the environment in the stress condition. Benzoate is the intermediate metabolite of toluene metabolism, which originates from toluene and subsequently converts to catechol in some microorganisms [26–28]. The study by Choudhary et al. [29] showed that benzoate transport is an inducible and active process in P. putida CSV86, which is in accordance with the higher expression of benzoate transport protein upon toluene stress in our study. GABA widely exists in prokaryotes and eukaryotes [30]. In plants, GABA accumulates in the cytosol in response to a variety of stresses [31]. GABA is also an important neurotransmitter in the brain of animals [32]. Nevertheless, to our knowledge, there are few reports on the relationship of GABA and microbes under stress conditions. Up-regulation of GABA permease revealed that GABA was probably involved in the response of R. ruber SD3 to toluene.

Interestingly, some membrane-bound proteins such as nitrate reductase molybdenum cofactor assembly chaperone (A0A098BLZ9), nitrate reductase 2 alpha subunit (A0A098BJ54), nitrate reductase 2 beta subunit (A0A098BKJ9), nitrate reductase gamma chain (A0A098BKK3), cytochrome-d-ubiquinol oxidase subunit 1 (A0A098BRM3) and cytochrome-d-ubiquinol oxidase subunit 2 (A0A098BQB1) were down-regulated in response to toluene treatment. A0A098BLZ9, A0A098BQB1, A0A098BKJ9 and A0A098BKK3 came down to the formation of nitrate reductase, which is responsible for generating the proton motive force across the cytoplasmatic membrane of the cells [33]. Cytochrome-d-ubiquinol oxidase is one of two oxidases in the aerobic respiratory chain, which oxidizes ubiquinol and reduces oxygen to water in a reaction which generates a transmembrane charge separation. The down-regulation of these proteins showed that the production of ATP by oxidative phosphorylation was inhibited under stress condition.
Metabolic pathways induced by toluene treatment

The mechanisms developed by microbes to deal with xenobiotics have been fixed and optimised by natural selection [34]. In *R. ruber* SD3, many enriched degradation pathways were differently regulated (Figure 8). They were limonene and pinene degradation, chloroalkane and chloroalkene degradation, nitrotoluene degradation, fluorobenzoate degradation, toluene degradation, naphthalene degradation and styrene degradation. This suggested the potential of *R. ruber* SD3 for degradation of all kinds of pollutants in the environment. Furthermore, up-regulation of polyhydroxyalkanoic acid synthase (Q53050) was observed. Polyhydroxyalkanoic acid (PHA) synthase is the key enzyme for the biosynthesis of PHA, which is regarded as an energy reserve [35]. Eggink et al. [36] reported that the presence of xenobiotics can give rise to an increase in the PHA content. The response is beneficial for survival and stress tolerance in changing environments [37].

Differentially expressed proteins in the phenol treatment group and the control group

There were also 488 significantly differentially expressed proteins in the phenol treatment group when compared to the non-treated control group. Among them, 72 proteins were up-regulated and 416 ones were down-regulated by phenol treatment (Supplemental Table S4).

Membrane-bound proteins induced by phenol treatment

Upon phenol treatment, there were three up-regulated transporters including iron ABC transporter (A0A098BJK0), Enterobactin exporter EntS (A0A098BQ14) and sugar ABC transporter periplasmic component-like protein (A0A098BWFS). Ferric iron is critical for the metabolic functions of many microorganisms. The iron ABC transporter is essential for bacteria to maintain the intracellular iron homeostasis [38]. Up-regulation of iron ABC transporter may meet the requirement of iron under phenol stress. Enterobactin exporter EntS is also responsible for iron transport [39]. These results indicated that the uptake of iron was especially important upon phenol stress. Furthermore, there was an uncharacterised protein (A0A098BH47) with the highest up-regulation of 5.25-fold, which is worth investigating in depth. Similarly, some membrane-bound proteins such as nitrate reductase molybdenum cofactor assembly chaperone (A0A098BLZ9), nitrate reductase 2 alpha subunit (A0A098BJ54), nitrate reductase 2 beta subunit (A0A098BKU9), nitrate reductase gamma chain (A0A098KK3) and cytochrome-d-ubiquinol oxidase subunit 1 (A0A098BRM3) were also down-regulated in response to phenol treatment.

Modification of cell membrane induced by phenol treatment

Previous studies have revealed that organic solvents generally have a marked effect on the bacterial cell membrane structure [40]. Crapoulet et al. [41] showed that proteins and enzymes involved in fatty acid biosynthesis played a critical role for membrane modification. The present study identified several up-regulated proteins including putative acyl-(acyl-carrier-protein) desaturase desA1 (A0A098BTY5), 3-oxoacid CoA-transferase subunit B (A0A098BI33) and acetyl-CoA acetyltransferase (A0A098BF5) related to fatty acid biosynthesis. The acyl-(acyl-carrier-protein) desaturase members have specificity for the acyl chain length of the particular substrate and produce double bonds between particular carbon atoms [42]. This protein is involved in drought and hypoxia stress signalling in *Arabidopsis* crown galls [43]. It has also been reported to defend against reactive oxygen species damage to plant cell structure [44]. In addition, the activity of 3-oxoacid-CoA transferase and acetyl-CoA acetyltransferase lead to the formation of acetoacetate, which serves as a precursor for short-chain acyl-CoAs and lipids in the cytosol. Therefore, we could speculate that the modification of the cell membrane by up-regulation of these proteins helped to withstand the toxicity of phenol.

Regulatory systems induced by phenol treatment

Three regulatory proteins were found with increased expression, including regulator of catechol degradative operon (A0A098BGU0), pca regulon regulatory protein (A0A098BJ00), tetR family transcriptional regulator (A0A098BRD2) and araC family transcriptional regulator (A0A098BRH7). It was reported that the gene cluster catRAcB was related to catechol degradation in phenol-degrading *Rhodococcus erythropolis* CCM2595. The genes *catA*, *catB*, *catC* and divergently transcribed *catR* were translated into catechol 1,2-dioxygenase, *cis,cis*-muconate cycloisomerase, muconolactone isomerase and an IclR-type transcriptional regulator, respectively [45]. Therefore, the up-regulation of the regulator of catechol degradative operon may activate the pathway of phenol degradation. It was previously
shown that the pca regulon regulatory protein was found to be required for induction of all the genes within the pca regulon (pcaBDC, pcaIJ and pcaF) in P. putida, which were required for complete degradation of p-hydroxybenzoate [46]. Thus, the increased expression of pca regulon regulatory protein in this study also played an important role in p-hydroxybenzoate degradation, which acts as a part of the pathway of phenol degradation. Boutrin et al. [47] reported that the TetR family transcriptional regulator is an NO responsive transcriptional regulator that may play an important role in the NO stress resistance regulatory network in Porphyromonas gingivalis with multiple pathways to facilitate the survival of the organism under stress conditions. Generally speaking, the AraC family transcriptional regulator has three main regulatory functions in common: carbon metabolism, stress response and pathogenesis. PheR, an AraC-type transcriptional regulator, was reported to activate the R. erythropolis CCM2595 pheA2 promoter, which is involved in the ortho-cleavage pathway of phenol [48]. Up to now, there are no reports available regarding these four regulatory proteins involved in the response of R. ruber to phenol stress. The observed improved expression of these regulatory proteins provided some clues to explain the organic solvents tolerance mechanisms of R. ruber SD3.

**Metabolic pathways induced by phenol treatment**

Except for one uncharacterised protein, 4-nitrophenol 2-monoxygenase component, 1,4-nitrophenol 2-monoxygenase component 2, catechol 1,2-dioxygenase and muconate cycloisomerase 1 were ranked as the top five up-regulated proteins with changing values of 4.41-, 4.19-, 4.01- and 3.77-fold, respectively. Phenol hydroxylase component 1, phenol hydroxylase component 2, catechol 1,2-dioxygenase and muconate cycloisomerase were responsible for the phenol metabolism [34,49]. Pathway enrichment analysis under phenol stress indicated that many enriched degradation pathways such as limonene and pinene degradation, geraniol degradation, benzoate degradation, toluene degradation, chlorocyclohexane and chlorobenzene degradation, fluorobenzoate degradation, nitrotoluene degradation, styrene degradation, chloroalkane and chloroalkene degradation, polycyclic aromatic hydrocarbon and naphthalene degradation were differently regulated (Figure 9).

Two-component systems are a basic stimulus–response coupling mechanism to allow organisms to sense and respond to changes in many different environmental conditions. Some proteins involved in two-component systems upon phenol treatment were differently regulated. One protein (citrate lyase subunit beta-like protein, A0A098BLM1) which belongs to the CitB family (citrate-CitB) was down-regulated. This indicated that the production of oxaloacetate from citrate was inhibited under phenol stress. However, Auger et al. [50] reported that the participation of citrate lyase was invoked in P. fluorescens under nitrosative stress. It suggested that different strains might have an opposite response upon different stress treatments.

**Expression analysis of stress protein and 4-nitrophenol 2-monoxygenase by quantitative real-time polymerase chain reaction**

To further validate the proteomics results, we performed expression analysis of stress protein (A0A098BRD6) and 4-nitrophenol 2-monoxygenase (A0A098BJ21) at the transcript level. The mRNA levels
of the former stress protein increased upon toluene stress (3.23 times higher than the control group) (Figure 10(a)). The expression of 4-nitrophenol 2-monooxygenase was strongly induced under phenol treatment: it was /C24123 times higher than in the control group (Figure 10(b)). Thus, the results from the quantitative real-time polymerase chain reaction were in accordance with the proteomics results for the expression of stress protein and 4-nitrophenol 2-monooxygenase. The up-regulation of stress protein and 4-nitrophenol 2-monooxygenase at the transcript and translation levels upon organic solvents stress suggests that these proteins may act as potential targets for the engineering of *R. ruber* SD3 to increase its organic solvent tolerance.

**Conclusions**

In this article, proteomics analysis of *R. ruber* SD3 under toluene and phenol stress was performed using the iTRAQ-LC-MS/MS approach. The analysis showed that the mechanism of *R. ruber* SD3 tolerance to organic solvents may involve common stress response, membrane-bound proteins, regulatory systems and metabolic pathways. Quantitative real-time polymerase chain reaction further verified the upregulation of stress protein and 4-nitrophenol 2-monooxygenase observed in the quantitative proteomics analysis. These results could provide clues for improvement of the organic solvent tolerance of *R. ruber* in the field of biocatalysis and bioremediation.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

The National Natural Science Foundation of China (31560018).

**References**

[1] Mezzina MP, Álvarez DS, Egobero DE, et al. A new player in the biorefineries field: phasin PhaP enhances tolerance to solvents and boosts ethanol and 1,3-propanediol synthesis in *Escherichia coli*. Appl Environ Microbiol. 2017;83:e00662–e00717.

[2] Ni Y, Song L, Qian X, et al. Proteomic analysis of *Pseudomonas putida* reveals an organic solvent tolerance-related gene mmsB. PLoS One. 2013;8:e55858. DOI: 10.1371/journal.pone.0055858

[3] Segura A, Molina L, Fillet S, et al. Solvent tolerance in Gram-negative bacteria. Curr Opin Biotechnol. 2012;23:415–421.

[4] Inoue A, Horikoshi K. A *Pseudomonas* thrives in high concentrations of toluene. Nature. 1989;338:264–266.

[5] Segura A, Godoy P, van Dillewijn P, et al. Proteomic analysis reveals the participation of energy- and stress-related proteins in the response of *Pseudomonas putida* DOT-T1E to toluene. J Bacteriol. 2005;187:5937–5945.

[6] Shimizu K, Hayashi S, Doukyu N, et al. Time-course data analysis of gene expression profiles reveals purr regulon concerns in organic solvent in *Escherichia coli*. J Biosci Bioeng. 2005;99:72–74.

[7] Shimizu K, Hayashi S, Kako T, et al. Discovery of glpC, an organic solvent tolerance-related gene in *Escherichia coli*, using gene expression profiles from DNA microarrays. Appl Environ Microbiol. 2005;71:1093–1096.

[8] Okochi M, Kurimoto M, Shimizu K, et al. Increase of organic solvent tolerance by overexpression of manXYZ in *Escherichia coli*. Appl Microbiol Biotechnol. 2007;73:1394–1399.

[9] Yam KC, Okamoto S, Roberts JN, et al. Adventures in *Rhodococcus* from steroids to explosives. Can J Microbiol. 2011;57:155–168.

**Figure 10.** Expression analysis of stress protein under toluene treatment (a) or 4-nitrophenol 2-monooxygenase under phenol treatment (b) as compared with control by quantitative real-time polymerase chain reaction.
[10] Maniyam MN, Ibrahim AL, Cass AEG. Enhanced cyanide biodegradation by immobilized crude extract of *Rhodococcus UKMP–5M*. Environ Technol. 2018; 1–13.

[11] Neilson KA, Mariani M, Haynes PA. Quantitative proteomic analysis of cold-responsive proteins in rice. Proteomics. 2011;11:1696–1706.

[12] Wang ZQ, Xu XY, Gong QQ, et al. Root proteome of rice studied by iTRAQ provides integrated insight into aluminum stress tolerance mechanisms in plants. J Proteomics. 2014;98:189–205.

[13] Szopinska A, Degand H, Hochstenbach JF, et al. Rapid response of the yeast plasma membrane proteome to salt stress. Mol Cell Proteomics. 2011;10:M111.009589. DOI:10.1074/mcp.M111.009589

[14] Chiapello M, Daghino S, Martino E, et al. Cellular response of *Fusarium oxyssporum* to crocidolite asbestos as revealed by a combined proteomic approach. J Proteome Res. 2010;9:3923–3931.

[15] Wu B, Su L, Song L, et al. Exploring the potential of iTRAQ proteomics for tracking the transformation of extracellular proteins from enzyme-disintegrated waste activated sludge. Bioresour Technol. 2017;225:75–83.

[16] Wang Q, Peng R, Chen W, et al. The study on screening of phenol-degrading microbes and their degradation performance. J Jiangxi Normal Univ. 2012;36:317–320.

[17] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^−ΔΔCT method. Methods. 2001;25:402–408.

[18] Ericsson M, Tärnvik A, Kuoppa K, et al. Increased synthesis of DnaK, GroEL, and GroES homologs by *Francisella tularensis* LVS in response to heat and hydrogen peroxide. Infect Immun. 1994;62:178–183.

[19] Rennella E, Sára T, Juen M, et al. RNA binding and chaperone activity of the E. coli cold-shock protein CspA. Nucleic Acids Res. 2017;45:4255–4268.

[20] Phadtare S, Severinov K. RNA remodeling and gene regulation by cold shock proteins. RNA Biol. 2010;7: 788–795.

[21] Schäfer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med. 2001;30:1191–1212.

[22] Du X, Takagi H. N-Acetyltransferase Mpr1 confers ethanol tolerance on *Saccharomyces cerevisiae* by reducing reactive oxygen species. Appl Microbiol Biotechnol. 2007;75:1343–1351.

[23] Nicholson SA, Smalley D, Smith CJ, et al. The reCa operon: a novel stress response gene cluster in *Bacteroides fragilis*. Res Microbiol. 2014;165:290–299.

[24] Qiao J, Wang J, Chen L, et al. Quantitative iTRAQ LC–MS/MS proteomics reveals metabolic responses to biofuel ethanol in cyanobacterial *Synechocystis* sp. PCC 6803. J Proteome Res. 2012;11:5286–5300.

[25] Shang X, Zhang Y, Zhang G, et al. Characterization and molecular mechanism of AroP as an aromatic amino acid and histidine transporter in *Corynebacterium glutamicum*. J Bacteriol. 2013;195:5334–5342.

[26] Tsipa A, Koutinas M, Pistikopoulos EN, et al. Transcriptional kinetics of the cross-talk between the ortho-cleavage and TOL pathways of toluene biodegradation in *Pseudomonas putida* mt-2. J Bacteriol. 2016;228:112–123.

[27] Martinková L, Uhnáková B, Pátěk M, et al. Biodegradation potential of the genus *Rhodococcus*. Environ Int. 2009;35:162–177.

[28] Pérez-Pantoja D, Donoso R, Aguilló L, et al. Genomic analysis of the potential for aromatic compounds biodegradation in Burkholderiales. Environ Int. 2012;14:1091–1117.

[29] Choudhary A, Purohit H, Phale PS. Benzoyl transporters in *Pseudomonas putida* CSV86. FEMS Microbiol Lett. 2017;364:fnx118.

[30] Kinnersley AM, Turano FJ. Gamma aminobutyric acid (GABA) and plant responses to stress. Crit Rev Plant Sci. 2000;19:479–509.

[31] Michaelis S, Fait A, Lagor K, et al. A mitochondrial GABA permease connects the GABA shunt and the TCA cycle, and is essential for normal carbon metabolism. Plant J. 2011;67:485–498.

[32] Gadea A, López-Colomé AM. Gial transporters for glutamate, glycine, and GABA: II. GABA transporters. J Neurosci Res. 2001;63:461–468.

[33] Coelho C, Romão MJ. Structural and mechanistic insights on nitrate reductases. Protein Sci. 2015;24:1901–1911.

[34] Diaz E, Jiménez Ji, Nogales J. Aerobic degradation of aromatic compounds. Curr Opin Biotechnol. 2013; 24: 431–442.

[35] Rehm BH, Steinbüchel A. Biochemical and genetic analysis of PHA synthases and other proteins required for PHA synthesis. Int J Biol Macromol. 1999;25:3–19.

[36] Eggink G, Waard PD, Huijberts GNM. The role of fatty acid biosynthesis and degradation in the supply of substrates for poly(3-hydroxyalkanoates) formation in *Pseudomonas putida*. FEMS Microbiol Rev. 1992;103:159–164.

[37] Kadowi D, Jurkevitch E, Okon Y, et al. Ecological and agricultural significance of bacterial polyhydroxyalkanoates. Crit Rev Microbiol. 2005;31:55–67.

[38] Gao X, Sun T, Wu L, et al. Overexpression of response regulator genes slr1037 and sll0039 improves tolerance of *Synechocystis* sp. PCC 6803 to 1-butanol. Bioresour Technol. 2017;245:1476–1483.

[39] Furrer JL, Sanders DN, Hook-Barnard IG, et al. Export of the siderophore enterobactin in *Escherichia coli*: involvement of a 43 kDa membrane exporter. Mol Microbiol. 2002;44:1225–1234.

[40] Gao L, Guo Z, Wang Y, et al. The two-operon-coded ABC transporter complex *FpxWX YZCDF* is required for *Pseudomonas aeruginosa* growth and virulence under iron-limiting conditions. J Membrane Biol. 2018;251:91–104.

[41] Crapoulet N, Babry P, Raoul D, et al. Global transcriptome analysis of *Tropheryma whipplei* in response to temperature stresses. J Bacteriol. 2006;188:5228–5239.

[42] Jin C, Li D, Gao C, et al. Conserved function of acyl–acyl carrier protein desaturase 5 on seed oil and oleic acid biosynthesis between *Arabidopsis thaliana* and *Brassica napus*. Front Plant Sci. 2017;8:1319. DOI: 10.3389/fpls.2017.01319
Klinkenberg J, Faist H, Saupe S, et al. Two fatty acid desaturases, stearoyl-acyl carrier protein Δ9-desaturase 6 and fatty acid desaturase 3, are involved in drought and hypoxia stress signaling in Arabidopsis crown galls. Plant Physiol. 2014;164:570–583.

Li J, Sun J, Yang Y, et al. Identification of hypoxic-responsive proteins in cucumber roots using a proteomic approach. Plant Physiol Biochem. 2012;51:74–80.

Veselý M, Knoppová M, Nesvera J, et al. Analysis of catRABC operon for catechol degradation from phenol-degrading Rhodococcus erythropolis. Appl Microbiol Biotechnol. 2007;76:159–168.

Romero-Steiner S, Parales RE, Harwood CS, et al. Characterization of the pcaR regulatory gene from Pseudomonas putida, which is required for the complete degradation of p-hydroxybenzoate. J Bacteriol. 1994;176:5771–5779.

Boutrin MC, Yu Y, Wang C, et al. A putative TetR regulator is involved in nitric oxide stress resistance in Porphyromonas gingivalis. Mol Oral Microbiol. 2016;31:340–353.

Szókől J, Rucká L, Šimčíková M, et al. Induction and carbon catabolite repression of phenol degradation genes in Rhodococcus erythropolis and Rhodococcus jostii. Appl Microbiol Biotechnol. 2014;98:8267–8279.

Omokoko B, Jäntges UK, Zimmermann M, et al. Isolation of the phe- operon from G. stearothermophilus comprising the phenol degradative meta-pathway genes and a novel transcriptional regulators. BMC Microbiol. 2008;8:197. DOI:10.1186/1471-2180-8-197

Auger C, Lemire J, Cecchini D, et al. The metabolic reprogramming evoked by nitrosative stress triggers the anaerobic utilization of citrate in Pseudomonas fluorescens. PLoS One. 2011;6:e28469. DOI:10.1371/journal.pone.0028469