Ultraviolet-B Radiation Stress-Induced Toxicity and Alterations in Proteome of *Deinococcus radiodurans*

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Keywords

Deinococcus radiodurans · Ultraviolet radiation · Reactive oxygen species · Protein · Gene expression

Abstract

Deinococcus radiodurans is a polyextremophilic bacterium capable to survive and grow at high doses of ionizing radiation. Besides resistance to ionizing radiation, the bacterium is also resistant to toxic chemicals and desiccation. This study deals with the effects of non-ionizing radiation (ultraviolet-B) on survival, alterations in proteomic profile, and gene expression in *D. radiodurans*. Exposure of culture to UV-B caused decrease in the percentage survival with increasing duration, complete killing occurred after 16 h. *D. radiodurans* also showed enhancement in the generation of reactive oxygen species and activities of antioxidative enzymes. Separation of proteins by 2-dimensional gel electrophoresis revealed major changes in number and abundance of different proteins. Twenty-eight differentially abundant protein spots were identified by MALDI-TOF MS/MS analysis and divided into 8 groups including unknown proteins. Gene expression of a few identified proteins was also analyzed employing qRT-PCR, which showed differential expression correspond-

ing to the respective proteins. In silico analysis of certain hypothetical proteins (HPs) suggested that these are novel and as yet not reported from *D. radiodurans* subjected to UV-B stress. These HPs may prove useful in future studies especially for assessing their significance in the adaptation and management of stress responses against UV-B stress.

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Introduction

UV-B radiation (280–315 nm) is the most cytotoxic wavelength of solar UV radiation and affects a number of structural and physiological processes in living organisms [Bancroft et al., 2007]. Several reports suggest an increase in the level of UV-B radiation reaching the Earth's surface mainly due to the depletion of the stratospheric ozone layer caused by the emission of anthropogenically generated pollutants [Crutzen, 1992; Madronich et al., 1998]. There is a consensus that gradual increase in UV-B radiation intensity may lead to severe climatic changes including the increase in global mean temperature, imbalances in natural ecosystems, and risks for the survival of living organisms including human beings [Madronich et al.,

1998; Häder et al., 2015]. Such a presumption is evident from the reports showing the damaging effects of UV-B radiation on growth, survival, pigmentation, nutrient uptake, enzyme activities, and various other metabolic processes in bacteria including O₂-evolving photosynthetic cyanobacteria [Singh et al., 2013; Häder et al., 2015; Kumar et al., 2016; Pérez et al., 2017]. Deleterious effects of UV-B are mediated mainly due to its absorption by the vital cellular constituents such as nucleic acids, proteins, and lipids. UV-B absorbed by the DNA results in the formation of cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidinone photoproducts. These photoproducts alter the DNA conformation, which in turn inhibits replication and transcription leading to cell death [Rastogi et al., 2010; Santos et al., 2012; Chudobova et al., 2015]. However, unlike several other bacteria, *Deinococcus radiodurans* can survive and grow under very high doses of ionizing radiation as well as other abiotic stresses due to the presence of very efficient and unique DNA repair mechanisms, which allows them to repair both single- and double-stranded DNA breaks [Cox et al., 2010; Devigne et al., 2019].

Like DNA, proteins are also the direct target of UV-B radiation. UV-B radiation effects on proteins are mediated either by modifying the structure and function of proteins, and/or inhibition of protein synthesis due to alterations in the DNA makeup [Shuryak and Brenner, 2012]. It also includes radiation-induced oxidation of proteins, specifically the active sites of enzymes needed for DNA replication and essential metabolic reactions. Additionally, protein oxidation and carbonylation can also contribute to radiation-induced cell death by reducing the fidelity of DNA repair [Cox et al., 2010; Shuryak and Brenner, 2012; Kurth et al., 2015]. In recent years, techniques of two-dimensional gel electrophoresis (2-DGE) and mass spectrometry have been widely used in proteome analysis particularly for the identification and understanding the changes in the protein abundance under varying growth conditions. Notably, the proteomic approach is most suited for the identification of stress-induced proteins and studying the post-translational modifications linked to physiological functions [Beranova-Giorgianni, 2003; Hecker and Volker, 2004].

Generally, bacteria are widely used as model organisms for studying the effects of UV radiation on various metabolic processes [Chudobova et al., 2015; Kumar et al., 2016; Pérez et al., 2017]. Among them, *D. radiodurans*, a gram-positive, red-pigmented, and non-photosynthetic bacterium has attracted much attention of researchers for studying the effects of UV radiations [de Groot et al.,

2005; Slade and Radman, 2011; Farci et al., 2018]. This extremophilic bacterium is one of the most radiation-resistant organisms known and can survive under long period of desiccation, cold and acidic conditions as well as at higher concentrations of toxic chemicals [Cox and Battista, 2005; de Groot et al., 2005]. However, the exact mechanism for extreme resistance of *Deinococcus* species is still not fully explored. It should be noted that the identification and characterization of unknown proteins may contribute to explore the mechanisms of resistance in *D. radiodurans*. Although the effects of ionizing radiations on various metabolic processes, especially DNA repairing mechanisms, have been widely studied in *D. radiodurans* [Battista, 1997; Slade and Radman, 2011; Yuan et al., 2012], only meager information is available for the effects of non-ionizing radiations, specifically UV-B [Gascón et al., 1995; Slade and Radman, 2011]. Virtually, no attempt has been made to examine the effects of UV-B radiation on proteomic profile of *D. radiodurans*. The main aim of the present investigation was to (a) study the proteomic changes and alterations in gene expression, if any, following exposure of cultures to UV-B radiation, and (b) identify important UV-B responsive proteins. The findings of the present study may offer new insights to understand how this bacterium can adapt and survive by modulating the expression of UV-B responsive proteins and respective genes.

Results

Effects of UV-B Radiation on Percent Survival of D. radiodurans

Before assessing the impact of UV-B radiation (2 W/m²) on protein profile, we tested its effect on survival of *D. radiodurans*. It is evident from the data shown in Figure 1a that the percentage survival of *D. radiodurans* decreased with increasing duration of UV-B radiation exposure lasting up to 18 h. However, complete killing occurred after continuous exposure to UV-B for 16 h. For better analysis of data, survival of bacterium was also plotted as semi-logarithmic graph (online suppl. Fig. S1; see www.karger.com/doi/10.1159/000512018 for all online suppl. material). Final growth yield of the bacterium showed gradual decrease with increasing duration of UV-B exposure, the yield being negligible at 18 h. That the prolonged exposure to UV-B indeed causes toxicity became evident from the fact that cells exposed to UV-B for 18 h failed to grow upon transfer to TGY medium even after 84 h of incubation (Fig. 1b).

Fig. 1. a Percent survival of *D. radiodurans* exposed to UV-B radiation (2 W/m²) for varying time periods. **b** Growth of UV-B-irradiated cultures upon transfer to normal TGY medium for toxicity test.

Effects of UV-B Radiation on Reactive Oxygen Species Generation and Antioxidative Enzyme Activity

Exposure of UV-B to the cultures of *D. radiodurans* showed gradual increase in reactive oxygen species (ROS) generation with increasing duration of exposure. The maximum increase in ROS production was attained at 6 h (4.64-fold), which was evident by an increase in DCF fluorescence (Fig. 2). However, after 6 h of exposure, ROS generation declined, which could be due to the loss of cell viability.

It is evident from the results that antioxidative enzyme activity was induced after increasing the duration of UV-B exposure. Catalase (CAT) enzyme showed 2.6-fold increased activity after 6 h of UV-B exposure to the culture (Fig. 3a). A 2.9-fold higher activity of superoxide dismutase (SOD) enzyme was noted after 6 h of continuous UV-B exposure (Fig. 3b). CAT and SOD showed continuous increase in the activities up to 6 h of UV-B exposure; however, thereafter enzyme activities were also found to be declined.

Fig. 2. ROS generation by *D. radiodurans* cultures exposed to UV-B radiation for 2, 4, 6, and 8 h. All the values are normalized against per mg of protein and represented as fold change.

Fig. 3. Activity of antioxidative enzymes in *D. radiodurans* following UV-B radiation exposure. **a** Catalase (CAT). **b** Superoxide dismutase (SOD). Results are based on three experiments conducted separately under identical conditions.

Does UV-B Cause Alterations in Whole Cell Protein Profile?

Knowing that UV-B exposure to *D. radiodurans* caused loss of survival and induced generation of ROS, our interest was aroused to test the changes, if any, in the whole cell protein profile. For testing this hypothesis, SDS-PAGE of protein extracted from control (untreated) and UV-B-irradiated cultures (2, 4, 6, and 8 h of exposure) were done. Analysis of gels showed changes both in intensity and number of protein bands; some protein bands were lost and intensity of a few bands increased/decreased in cultures exposed to UV-B in comparison to control cultures (online suppl. Fig. S2).

Once it became evident from the results of SDS-PAGE gel that UV-B exposure indeed causes alteration in whole cell protein profile, we performed 2-DGE to gain a better resolution of protein profile. It is evident from the 2-D gel images (Fig. 4a, b) that changes both in number and intensity/abundance of protein spots occur in *D. radiodurans* cultures exposed to UV-B radiation. Analysis of gels revealed 378 protein spots in control (untreated) and 365 spots in UV-B-treated cultures. For checking changes in abundance, a scatter plot was constructed (Fig. 5). Data of scatter plot indicate that a number of protein spots show higher and/or lower level of synthesis in cultures subjected to UV-B stress. Altogether, 133 proteins showed up-regulation, 129 down-regulation, and 116 normal formation (unchanged). Grouping of differentially formed proteins is presented in a Venn diagram (Fig. 6a). Data of differential formation of proteins attracted our attention to focus mainly on 28 UV-B respon-

sive protein spots showing more than 2-fold higher or lower level of synthesis in comparison to untreated control culture. Of the 28 differentially synthesized proteins, 23 belonged to up-regulated and 5 to down-regulated groups (Fig. 4c).

Identification and Characterization of Differentially Synthesized Proteins

Identification and characterization of the selected protein spots were made by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS/MS) analysis. For the functional studies, comparison of protein sequences was made with the secondary protein databases based on the information covering motifs, signature, and protein domains. A few proteins (unknown) having low Mascot scores were also selected in spite of non-availability of their structural and functional information in the database. Details of these proteins are presented in Table 1. Based on structural and physiological functions, 28 identified proteins could be placed in 8 groups. Percent involvement of all the 8 groups of proteins in different metabolic and structural events is presented in the form of a pie chart (Fig. 6b).

Characterization of Hypothetical Proteins

All six hypothetical proteins were characterized using different bioinformatical tools and their tentative functions were determined: gi 919195687-DUF45 super family metalloproteinase domain with Zn protease activity, gi 551078331-aldehyde dehydrogenase family protein, gi 653296043-CRISPR-associated protein Cas2, gi

Fig. 4. 2-D GE gel images stained by Colloidal Coomassie Blue G-250. **a** Protein spots of control culture. **b** UV-B-exposed culture protein spots. **c** Differentially abundant protein spots selected for MALDI-TOF MS/MS analysis. Arrows in red and green color on the lower panel of gel represent up- and down-regulated proteins, respectively.

746729045-membrane-bound protein predicted to be outside the membrane in the extracellular region with tetrapeptide repeat, gi 972322215-hydrophobic region of a signal peptide, and gi 746725089- membrane-bound protein predicted to be embedded in the membrane.

Changes in Gene Expression following UV-B Exposure

With a view to confirm the findings of differential synthesis of proteins, gene expression analysis was performed

employing quantitative real-time polymerase chain reaction (qRT-PCR) to test the level of mRNA transcript in cultures exposed to UV-B. The primers used showed amplification of all the genes of expected size on agarose gel. However, marked differences in the level of expression among all the genes were observed in control and cultures irradiated to UV-B. Evidently, there were marked differences in C_t values of each target gene from UV-B-irradiated cultures pointing to appreciable differences in the

Fig. 5. Scatter plot showing the abundance of different protein spots in the 2-D gels. Spots present over the blue line suggest more than 4-fold up-regulation, spots below the red line show more than 4-fold down-regulation, and spots closer to the green line show linear regression.

Fig. 6. a Changes in the whole cell proteomic profile is represented by a Venn diagram prepared by total spot count on 2-D gel by PDQuest™ software. **b** Pie chart showing the percent involvement of identified proteins in physiology and metabolism of *D. radiodurans*.

Table 1. Differentially expressed proteins identified by MALDI-TOF MS/MS in the cellular soluble fraction of *D. radiodurans* after UV-B exposure

Spot No.	Proteins	NCBI accession No.	Gene	Length, AA	Mass	Mascot score	Functional domain with InterProScan ID	Expression, fold change
<i>Carbohydrate metabolism</i>								
12	Aconitate hydratase	gi 499190815	<i>acnA</i>	906	97,788	66	Aconitase/iron-responsive element-binding protein 2 (IPR006249) Aconitase/3-isopropylmalate dehydratase large subunit, alpha/beta/alpha domain (IPR001030)	Up 3.83
16	Isocitrate dehydrogenase (NADP ⁺)	gi 942573296	<i>Idh</i>	418	46,103	103	Isocitrate dehydrogenase NADP-dependent, dimeric (IPR004439) Isopropylmalate dehydrogenase-like domain (IPR024084)	Up 2.65
38	Transaldolase	gi 746724470	<i>TalA</i>	321	35,403	143	Transaldolase/fructose-6-phosphate aldolase (IPR001585) Aldolase-type TIM barrel (IPR013785)	Down 3.16
40	Dihydrodipicolinate synthase	gi 746727668	<i>dapA</i>	305	32,599	114	DapA-like (IPR002220) Aldolase-type TIM barrel (IPR013785)	Up 2.28
<i>Energy metabolism</i>								
10	Oxidoreductase	gi 941866087	<i>Tas</i>	298	32,721	204	Aldo/keto reductase (IPR020471) NADP-dependent oxidoreductase domain (IPR023210)	Down 2.36
14	NADH dehydrogenase	gi 941866259	<i>ndh</i>	206	22,759	274	Nitroreductase-like domain (IPR000415)	Down 2.28
15	NADH oxidase	gi 821520846	<i>Pyr</i>		47,200	75	FAD/NAD(P)-binding domain (IPR023753)	Up 2.35
30	V-type ATP synthase alpha chain	gi 499189805	<i>atpA</i>	582	63,355	116	V-type ATP synthase catalytic alpha chain (IPR022878) ATPase, F1/V1/A1 complex, alpha/beta subunit, nucleotide-binding domain (IPR000194)	Up 3.28
35	ATP synthase subunit E	gi 746724271	<i>atpE</i>	192	20,736	197	ATPase, V1/A1 complex, subunit E (IPR002842) F-type ATP synthase subunit B-like, membrane domain (IPR028987)	Up 3.43
37	V-type ATP synthase subunit B	gi 517840881	<i>atpB</i>	469	51,331	65	V-type ATP synthase regulatory subunit B/beta (IPR022879) ATPase, F1/V1/A1 complex, alpha/beta subunit, nucleotide-binding domain (IPR000194)	Up 2.60
45	NAD(P)H:quinone oxidoreductase type IV	gi 746726655	<i>Nqo4</i>	206	21,685	63	Flavoprotein WrbA (IPR010089) Flavoprotein-like domain (IPR029039)	Up 2.01
<i>Fatty acid metabolism</i>								
5	Enoyl-ACP reductase	gi 499191060	<i>fabI</i>	264	28,531	74	Short-chain dehydrogenase/reductase SDR (IPR002347) NAD(P)-binding domain (IPR016040)	Up 3.20
11	Acyl-CoA dehydrogenase	gi 499191447	<i>acaD</i>	612	66,380	71	Acyl-CoA dehydrogenase, N-terminal, bacteria (IPR020953)	Up 3.17
<i>Nitrogen metabolism</i>								
17	2',3'-cyclic-nucleotide 2'-phosphodiesterase	gi 821522867	<i>cpdB</i>	620	67,231	65	5' Nucleotidase/apyrase (IPR006179) Calcineurin-like phosphoesterase domain, ApaH type (IPR004843)	Up 2.15
32	Nucleoside-diphosphate kinase	gi 746727793	<i>ndk</i>	138	14,784	106	Nucleoside diphosphate kinase (IPR001564) Nucleoside diphosphate kinase-like domain (IPR034907)	Up 2.20
39	MBL fold metallo-hydrolase	gi 746726660	<i>gloB</i>	285	31,640	77	Metallo-beta-lactamase domain (IPR001279)	Up 3.03

Table 1 (continued)

Spot No.	Proteins	NCBI accession No.	Gene	Length, AA	Mass	Mascot score	Functional domain with InterProScan ID	Expression, fold change
48	Purine-nucleoside phosphorylase	gi 746727399	<i>deoD</i>	236	26,008	86	Purine nucleoside phosphorylase DeoD-type (IPR004402) Nucleoside phosphorylase domain (IPR000845)	Up 2.60
<i>Transcription and translation</i>								
18	50S ribosomal protein L4	gi 941867231	<i>rplD</i>	203	21,800	62	Ribosomal protein L4/L1e (IPR002136) Ribosomal protein L4 domain (IPR023574)	Up 2.62
33	30S ribosomal protein S1	gi 746727179	<i>rpsA</i>	630	67,873	139	Nucleic acid-binding, OB-fold (IPR012340) RNA-binding domain, S1 (IPR022967)	Up 2.20
<i>Membrane proteins</i>								
27	S-layer protein	gi 821522997	<i>slpA</i>	391	39,329	94	S-layer homology domain (IPR001119)	Down 3.70
<i>Chaperones</i>								
13	Molecular chaperone DnaK	gi 499189237	<i>dnaK</i>	628	67,593	100	Heat shock protein 70 family (IPR013126) Heat shock protein 70kDa, peptide-binding domain (IPR029047)	Up 2.68
31	Molecular chaperone GroEL	gi 499189712	<i>groL</i>	548	57,647	180	Chaperonin Cpn60/TCP-1 family (IPR002423) GroEL-like equatorial domain (IPR027413)	Up 2.83
<i>Unknown proteins</i>								
2	Hypothetical protein	gi 919195687	<i>wp_052751188</i>	145	16,286	54	Metallopeptidase, catalytic domain (IPR024079)	Up 2.20
19	Hypothetical protein	gi 551078331	<i>wp_022803161</i>	104	11,896	56	Not predicted	Up 2.66
21	Hypothetical protein	gi 653296043	<i>wp_027480924</i>	89	10,023	44	Not predicted	Down 2.58
23	Hypothetical protein	gi 746729045	<i>wp_029483185</i>	176	18,522	335	Phobius non cytoplasmic domain – region of a membrane-bound protein predicted to be outside the membrane, in the extracellular region	Up 2.60
24	Hypothetical protein	gi 972322215	<i>wp_058979221</i>	356	38,409	42	Phobius signal peptide H region – hydrophobic region of a signal peptide	Up 3.36
36	Hypothetical protein	gi 746725089	<i>wp_039682412</i>	220	21,714	76	TMhelix – region of a membrane-bound protein predicted to be embedded in the membrane	Up 2.12

level of expression (Fig. 7). *atpE* and *dapA* showed more than 2-fold increase at 4 h, whereas *atpB* and *wp_039682412* showed more than 4-fold increase. Interestingly, *rpsA* and *dnaK* showed more than 8-fold increase in expression at 4 and 8 h, respectively. In fact, the expression of *dnaK* showed increasing trend till 8 h of UV-B irradiation. As expected, the gene (*slpA*) of down-regulated S-layer protein showed down-regulation at 2 h of UV-B exposure. On the other hand, virtually no change in the expression level of the reference gene (*16S rRNA*) was noticed irrespective of the increase in the time period. Altogether, results of gene expression level showed close correlation with the changes in abundance level of respective proteins (Table 1; Fig. 7).

Discussion

It has been well documented that UV-B radiation damages various cellular biomolecules and affects various metabolic processes of bacteria resulting in the loss of cell viability [Santos et al., 2012; Chudobova et al., 2015; Oliveira et al., 2016]. Findings of this study showed that *D. radiodurans* can survive up to 16 h of 2 W/m² UV-B treatment. Kumar et al. [2016] have reported that most of the bacteria in their study could not survive for more than 8 h following UV-B exposure. *D. radiodurans* is known to be the most radiation-resistant bacterium and can tolerate other oxidative stresses [Cox and Battista, 2005]. Such remarkable stress tolerance has been attributed to its highly efficient DNA repair system, pres-

Fig. 7. Expression of selected genes of *D. radiodurans* cultures exposed to UV-B radiation (2 W/m^2) for desired time periods and the values represent normalized fold change in expression level of selected genes.

ence of UV absorbing carotenoid pigment, and a strong protein protection system [Tian et al., 2007; Devigne et al., 2019]. In general, differential tolerance to UV radiation among bacteria could be due to the differences in the level of various defense mechanisms including the presence of certain photoprotective compounds [Battista, 1997; Slade and Radman, 2011]. Toxicity of UV-B after prolonged exposure (16 h) could be due to its deleterious effects on proteins, nucleic acid, and membrane integrity. Henceforth, damages caused to cellular constituents by UV-B lead to cell death [Santos et al., 2012; Oliveira et al., 2016]. Several researchers have also reported increase in the level of ROS generation following UV-B exposure that induces oxidative stress and affects DNA, proteins, lipids, etc. [Santos et al., 2012; Singh et al., 2013]. In this study, ROS generation increased in *D. radiodurans* with increasing duration of UV-B exposure (up to 6 h) and thereafter declined. The decrease in the level of ROS generation after prolonged exposure to UV-B could be due to the loss of cell viability or its neutralization by enzymatic/non-enzymatic modes. It is pertinent to mention that several antioxidative enzymes and free radical scavenging compounds are synthesized by the bacterium following oxidative stress to negate the detrimental effects [Singh et al., 2013]. Bacteria are endowed with various mechanisms to protect themselves from damaging effects of higher doses of solar UV radiations. The defense mechanisms include both enzymatic and non-enzymatic processes. Different antioxidative

enzymes and free radical scavenging compounds are synthesized by the bacterium to counteract the harmful effects of UV radiation. In our study, CAT and SOD enzymes showed increased synthesis/activity during UV-B stress, probably to overcome the impacts of UV-B-induced oxidative stress. Over-production of antioxidative enzymes and carotenoid pigments in *D. radiodurans* and their roles in protecting the ROS-mediated damaging effects have been also reported by different workers [Tian et al., 2007; Kim et al., 2013].

The major goal of this study relates to the identification of differentially formed proteins in *D. radiodurans* following UV-B exposure. To this effect, separation of proteins by SDS-PAGE showed significant changes in the intensity of different protein bands in UV-B-irradiated cultures as compared to control culture. However, it was not possible to explore the exact level of differences due to poor resolution of proteins by SDS-PAGE. To resolve this issue, 2-DGE was performed, which suggests that a few proteins are lost or degraded in the cultures exposed to UV-B radiation. Although the loss of proteins was marginal, a number of proteins showed up-/down-regulation. Significant changes in protein spots of UV-B-treated culture may be due to the adaptive mechanism of *D. radiodurans* to survive and grow under stress condition. To this effect, 28 different protein spots showing ≥ 2 -fold changes in formation were selected and identified by MALDI-TOF MS/MS analysis. These UV responsive proteins belonged to eight groups and may be playing impor-

tant roles in the protection of *D. radiodurans* against UV-B stress.

Differentially formed carbohydrate and energy metabolism proteins might play important roles in cell growth and energy storage during UV-B stress due to higher metabolic rate and energy demand [Babele et al., 2015; Kumar et al., 2016]. Aconitase hydratase, a TCA cycle enzyme involved in the isomerization of citrate to isocitrate by the formation of an intermediate *cis*-aconitate, showed up-regulation in this study. It has been reported that aconitase hydratase of *Escherichia coli* serves as a protective shield against oxidative stress allowing aerobic growth by acting as a sink for ROS and by changing the translation of the *sodA* gene transcript [Tang et al., 2002]. Formation of isocitrate dehydrogenase was significantly up-regulated during UV-B stress; this enzyme mainly generates NADPH; however, NADH dehydrogenase, a respiratory chain enzyme which supplies the bulk of intracellular NADH, was down-regulated. NADH dehydrogenase converts NAD from its reduced form (NADH) to its oxidized form (NAD⁺). Our findings are in agreement with an earlier report wherein increased NADPH synthesis and decreased NADH production were observed in *Pseudomonas fluorescens* during oxidative stress [Singh et al., 2007]. Kim et al. [2013] have also reported up-regulation of dihydrodipicolinate synthase in *Bacillus anthracis* during oxidative stress. This suggests that dihydrodipicolinate synthase might be playing a key role in stress tolerance, but the mechanism of action is as yet not well understood. Oxidoreductases consisting of a large class of enzymes catalyze the transfer of electrons from reductant to oxidant and play a vital role in driving metabolic reactions of different organisms. Changes in their abundance during oxidative stress have been reported from a few bacteria [Singh et al., 2007; Pérez et al., 2017]. In this study, down-regulation of oxidoreductase was observed during UV-B stress. Similarly, a study on H₂O₂ mediated oxidative stress responses in *B. anthracis* also showed down-regulation of oxidoreductase, which acts on the aldehyde or oxo group of donor substrates and uses NAD⁺ or NADP⁺ as cofactor [Kim et al., 2013]. Further study is needed to assign the exact function of oxidoreductases during UV-B stress, especially in bacteria.

Various ATP synthase enzymes involved in energy production and storage in the majority of organisms showed significant up-regulation in *D. radiodurans* after UV-B exposure. In view of extra energy demand during stress condition, these enzymes are probably up-regulated. Findings of this study are consistent with the report by Pérez et al. [2017], who observed up-regulation of ATP

synthase in *Rhodobacter* sp. upon exposure to UV radiation. Barbé et al. [1983] also reported a 2-fold increase in ATP in *E. coli* cells after 20 min of UV radiation exposure. Findings of the above studies allow us to conclude that ATP synthase enzymes play a key role in ATP supply during stress.

NADH oxidase and NAD(P)H:quinone oxidoreductase type IV showed up-regulation after UV-B irradiation. NADH oxidase catalyzes the production of superoxide by transferring electron, and NAD(P)H:quinone oxidoreductase type IV takes part in the formation of hydroquinone. To date, the control mechanisms for increased abundance of the above-mentioned enzymes and their role in UV-B stress is not known. However, overproduction of both enzymes might have a role in the induction of ROS generation due to photolytic reactions during UV-B stress. Enoyl-ACP reductase is a key enzyme of fatty acid biosynthesis and acyl-CoA dehydrogenase catalyzes the flavin-dependent oxidation of acyl-CoA thioesters with the transfer of reducing equivalents to flavoproteins. Both enzymes help in maintaining the membrane integrity during stress conditions [Ma et al., 2016]. Owing to the fact that UV-B radiation causes lipid peroxidation, up-regulation of both enzymes might be useful in protecting the integrity of membrane and thereby the survival of the bacterium under the oxidative stress caused by UV-B radiation. Similarly, 2,3'-cyclic-nucleotide 2'-phosphodiesterase, nucleoside-diphosphate kinase, and purine-nucleoside phosphorylase belonging to nitrogen metabolism group of proteins were up-regulated during UV-B stress. All of these enzymes are known to be involved in synthesis, repair, and degradation of structural units of DNA and RNA and may have an important role in the maintenance of genome integrity and stability under stress [Bender and Beavo, 2006; Yu et al., 2017]. Yu et al. [2017] have reported nucleoside-diphosphate kinase-mediated repairing of DNA damage caused by UV radiation and chemical exposure. MBL fold metallo-hydrolase, belonging to the class B beta-lactamase, was also up-regulated following UV-B exposure. This enzyme is known to catalyze the hydrolysis of a wide range of beta-lactam antibiotics and confers drug resistance to several bacteria. However, its role in protection against UV radiation is not known. Most probably, this enzyme is induced under different types of abiotic stresses. Detailed work is needed to assign its exact role in UV-B stress protection.

One of the identified membrane proteins, S-layer protein (SlpA), showed down-regulation following UV-B radiation. S-layer protein, a glycoprotein present on the cell

membrane of bacteria remains in direct contact with environmental exposures [Farci et al., 2018]. Rothfuss et al. [2006] reported that *D. radiodurans* mutant cells lacking SlpA protein caused degradation of Hpi protein coat and thus may have a significant role in the protection of the cell envelope. Two chaperone proteins were also up-regulated and identified as molecular chaperones DnaK and GroEL. The molecular chaperones are generally involved in the covalent folding or unfolding of the proteins. Another important function of chaperone proteins is to prevent the aggregation of newly synthesized proteins. Enhanced expression of chaperones during stress conditions seems vital for maintaining the proper folding of the newly synthesized proteins. Similar to our results, Yuan et al. [2012] using transcriptome analysis (cDNA sequencing) also reported up-regulation of DnaK and GroEL in *Deinococcus gobiensis* after UV irradiation.

One of the interesting findings of the present study relates to the identification of six proteins (Table 1), annotated as hypothetical proteins (HPs), which are new and as yet not reported from any species of *Deinococcus* subjected to UV-B radiation stress. Among these HPs, gi 653296043 was down-regulated and the remaining five proteins were up-regulated. Tentative functions of these proteins determined by using in silico approaches suggest their possible roles in *D. radiodurans* against mitigating the harmful effects of UV-B radiation. However, detailed structural and functional characterization of these HPs would be desirable to understand their exact roles in *D. radiodurans* against UV-B radiation stress.

To corroborate the findings of differential synthesis of proteins, transcript analysis of selected coding genes of *D. radiodurans* exposed to UV-B stress was performed by qRT-PCR. Results showed close correlation between expression levels of selected genes with respective proteins. Qiu et al. [2005] have studied the differential expression of genes following the exposure of UV-A, UV-B, and UV-C in *Shewanella oneidensis* MR-1. Our findings are in agreement with Qiu et al. [2005] and allow us to conclude that the up-regulation of proteins is due to increased expression of respective genes. It is pertinent to mention that the level of expression was differential among the selected genes, which might be due to the presence of hitherto unknown types of regulatory mechanisms operating in *D. radiodurans* during UV-B stress. Data from proteomic and transcriptomic analyses clearly suggest that resistance to UV-B radiation in *D. radiodurans* is partly due to the functioning of various molecular mechanisms including the changes in the abundance level of proteins.

In summary, 28 differentially abundant proteins were identified. Many of them are assumed to be involved in metabolic processes, suggesting an overall increased level of metabolism during stress conditions. Six HPs have been also identified, which are new and not reported till date from any other bacteria including the species of *Deinococcus* subjected to UV-B stress. Although this study is based on a limited number of proteins, identification of differentially abundant proteins may, nevertheless, prove useful in future studies, especially for assessing their role in the adaptation mechanism of *D. radiodurans* against UV-B radiation stress. Altogether, findings of this study suggest the efficacy of the proteomic approach as a tool in the analysis of stress response toward UV-B radiation.

Materials and Methods

Test Organism and Culture Conditions

D. radiodurans (MTCC-4913) was obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh (India). The bacterium was cultivated in TGY medium, which contained (g/L); Bacto tryptone 5.0, glucose 1.0, and yeast extract 3.0. It was grown at 30°C for 72 h in an incubator shaker with continuous shaking at 120 rpm (Orbitek LT, Scigenics Biotech. Pvt. Ltd., India). Unless otherwise stated, log phase cultures having an initial dry weight of approximately 0.15 mg/mL were used to perform all the experiments.

Mode of UV-B Treatment and Determination of Percent Survival

UV-B lamp (cat. No. 3-4408, Fotodyne Inc., USA), giving its main output at 312.67 nm, was used as the source of artificial UV-B radiation. Intensity of UV-B was determined by a Black-Ray Long Wave Ultraviolet Intensity Meter (UVP Inc., USA). All the experiments were performed at 2 W/m² intensity of UV-B radiation. UV-B lamps were covered with 0.13 mm thick cellulose acetate film to exclude UV-C radiation. To attain the desired level of UV-B intensity reaching the test cultures, the distance between the test samples and the UV-B lamp was adjusted as and when required. Percentage survival of *D. radiodurans* following exposure to UV-B radiation was determined by colony count method (CFU counting) as described previously [Kumar et al., 2016]. Cultures without exposure to UV-B served as control. For growth measurement, UV-B-irradiated cultures were transferred to liquid TGY medium for indicated time period and absorbance was recorded at 600 nm.

Measurement of ROS Production

Estimation of intracellular ROS production was made using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent dye as per the method of Santos et al. [2012] with slight modifications. The fluorescence of the samples was measured with a spectrofluorometer (Varian, Inc., USA) by setting excitation wavelength at 485 nm and the emission wavelength at 519 nm. Blank control (cell suspension without DCFH-DA) was used to correct the fluorescence intensity, which was normalized with the protein

content. The value of ROS production is expressed as per mg protein and protein content was measured by the Bradford method [Bradford, 1976].

Determination of Antioxidative Enzyme Activity

Antioxidative enzyme activity was determined following the exposure of *D. radiodurans* cultures to UV-B for varying time periods. Initially, crude extract was prepared in extraction buffer (50 mM potassium phosphate, 1 mM EDTA, 1% [w/v] polyvinyl pyrrolidone, and 0.5% [v/v] Triton X-100) and used as the source of enzyme. CAT and SOD enzyme activities were measured following the standard methods described previously by Singh et al. [2013]. CAT activity was determined by measuring the consumption of H₂O₂ at 240 nm for 3 min according to the method of Aebi [1984] and SOD activity was assayed by monitoring the inhibition of nitro-blue tetrazolium (NBT) reduction following the method of Giannopolitis and Ries [1977] using a double beam spectrophotometer (Shimadzu, 1800-UV, Shimadzu Corp., Japan). Data were analyzed and normalized with per mg of protein.

Protein Extraction

Whole cell protein was extracted according to the method of Kumar et al. [2016]. Accordingly, cells from control and UV-B-irradiated cultures (2, 4, 6, and 8 h) were harvested and the resulting pellets were suspended in 10 mM HEPES buffer (pH 7.2) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were disrupted by several cycles of freezing and thawing followed by mild sonication. The resulting suspension was centrifuged at 10,000 g for 10 min at 4°C to remove cell debris present, if any. If needed, 2-D Clean-Up kit (GE Healthcare, UK) was used to remove salts and insoluble impurities present in the supernatant. To the resulting clear supernatant, ice-chilled 10% trichloroacetic acid (TCA) was added and kept at -20°C for 2 h for precipitation of proteins. The latter were recovered by centrifugation at 14,000 g for 30 min at 4°C and the precipitate was washed thrice with chilled acetone (95% v/v). Pellets obtained were air-dried and dissolved in 500 µL rehydration buffer (7 M urea, 2 M thiourea, 2% [w/v] CHAPS, 0.3% [w/v] DTT, and 0.5% [v/v] IPG buffer). Insoluble impurities were removed by centrifugation and used for the separation of proteins by SDS-PAGE and 2-DGE.

Separation and Analysis of Proteomic Profile

For the separation of proteins by SDS-PAGE, 40 µg of protein dissolved in appropriate buffer from untreated control and UV-B-irradiated (2, 4, 6, and 8 h) cultures was run on a 12% SDS-PAGE. Electrophoresis was performed and gel was stained with Coomassie Brilliant Blue R-250. Image of the gel was recorded using the AlphaImager Gel Documentation System (Alpha Innotech, USA).

2-DGE was run with the protein samples of control and 4 h UV-B-irradiated cultures. The reason for the selection of protein sample from 4 h UV-B-treated culture was due to the fact that the bacterium showed about 45% survival at this time of exposure, which was near the LD₅₀ value. Immobililine DryStrip (pH 4–7, 13 cm; GE Healthcare Bio-Sciences AB, Sweden) was used to perform first dimension isoelectric focusing (IEF) following the steps described previously [Kumar et al., 2016]. Second dimension electrophoresis was performed on a 12% SDS-PAGE at 25 mA/gel for 5 h in a SE 600 Ruby multiple gel electrophoresis system (GE Healthcare, UK). For each protein sample, three gels were run under identical conditions. Colloidal Coomassie Blue G-250 was used to stain the gels according to the method of Candiano et al. [2004].

Background staining was thoroughly removed, and gels were scanned for the analysis of protein spots. Analysis for spot detection, quantification, background subtraction, and matching between control and treated gels was done by PDQuest™ 2-D gel analysis software version 8.0.1 (Bio-Rad Laboratories, USA). Spot quantification was done on the basis of spot volumes (intensity × mm²) following the method of Agrawal et al. [2014]. For the comparison of relative spot volume with respect to the UV-B-irradiated and untreated control samples, Student's *t* test was applied. *p* values <0.05 were considered statistically significant. A scatter plot was plotted to find differences in the abundance level of all the protein spots on the gel.

Identification of Differentially Formed Proteins

Identification of differentially synthesized proteins was made by MALDI-TOF MS/MS. Accordingly, desired protein spots were manually excised from the gel using sterile One Touch spot picker. Protein spots were digested by trypsin as per the method of Shevchenko et al. [2006] with slight modifications. Selected gel spots were destained using washing solution (5% acetonitrile [ACN] in 100 mM ammonium bicarbonate). Gel pieces were dehydrated with 100% ACN for 15 min and reduced by incubating in 50 µL of reduction solution (10 mM DTT in 100 mM ammonium bicarbonate) for 1 h at 55°C in the dark. Thereafter, alkylation of protein was performed in 50 mM iodoacetamide solution for 30 min in the dark at room temperature. Gel pieces were washed in 100 µL Milli-Q water and dehydrated in 100 µL of ACN. Dehydrated gel pieces were air-dried to remove the traces of ACN and incubated in 20 µL of digestion solution (20 ng/µL trypsin in 25 mM ammonium bicarbonate) at 37°C overnight. Peptides were extracted from the gel pieces and subjected to MALDI-TOF MS/MS (Autoflex Speed, Bruker Daltonics, Germany).

MALDI-TOF MS/MS analysis was performed at the Interdisciplinary School of Life Sciences, Banaras Hindu University, Varanasi. For the acquisition of mass spectra, 1 µL of each peptide sample was mixed with 1 µL of α -cyano-4-hydroxycinnamic acid and applied on the MALDI target plate (MTP 384 ground steel, Bruker Daltonics, Germany) and left at room temperature for co-crystallization. Mass spectra were captured in the *m/z* range of 800–3,500 Da in the positive ion mode and internally calibrated using trypsin autolytic products (*m/z*; 842.509, 1,045.564, and 2,211.104). MS/MS spectra were accumulated from 2,500 laser shots with the average mass tolerance of 100 ppm. Flex Analysis software version 3.3 (Bruker Daltonics, Germany) and BioTools software (Bruker Daltonics, Germany) were used for acquisition and processing of spectra. The proteins were identified by comparing peptide mass fingerprints at the NCBI database using the Mascot search engine (<http://www.matrixscience.com>). Search parameters allowed for oxidized methionines, carbamidomethylation of cysteines, and one missed cleavage site of trypsin with the mass accuracy of $\pm$ 30 ppm. Identification was based on the first ranking result, and Mascot scores of >65 indicated that the hits were significant.

In silico Analysis of HPs

Structural and functional characterizations of HPs were done with the help of Bioinformatics tools. HPs were analyzed by protein BLAST program to find the proteins of similar sequences and known functions available at NCBI database. InterProScan server 6.0 (<http://www.ebi.ac.uk/interpro/search/sequence-search>) and ProtParam tool (<http://web.expasy.org/protparam/>) were used for

Table 2. List of primers used for gene expression analysis in *D. radiodurans* under UV-B stress

Genes	Primer sequence (5'→3')	Amplicon size, bp	Annealing temperature, °C
<i>16S rRNA</i>	F: TACCGCGGTGAATACGTTCC R: CGCACCTTCCGGTACAGTTA	157	53
<i>dapA</i>	F: CCACCAGAATGTCCACCGAT R: TGCCGGTGATCCTGTACAAC	177	53
<i>atpE</i>	F: GTTGAGTTCGAGGTCGGCTG R: CTCGAAAACGAAGCGCAGAG	186	53
<i>atpB</i>	F: TGCCGGTCTGAATGAACTCC R: GCAAGGAAATGATCGGTCGC	150	53
<i>deoD</i>	F: CTGGGCAATCTGGTACGCC R: AGGACTACGGCTGCAAGAAC	194	53
<i>rpsA</i>	F: AAATCGGTCATGCCGGTGAT R: AGCGAAATGAGCTGGACCAA	212	53
<i>slpA</i>	F: GTCACGCGCTCCAGATCAC R: GAACGAGAACCTGACCCGTT	180	53
<i>Dank</i>	F: GCAGCGTAAGGAAAAGGTCTG R: ATCTTGCTGTCGTCTTCGCT	168	53
<i>wp_039682412</i>	F: GTGGTTCTCGTCCACACCTG R: GCCTGTTGGGAACGGCAGGC	158	53

F, forward; R, reverse.

the functional domain analysis and physico-chemical properties of proteins, respectively. Additionally, functional characterization of HPs was done using Conserved domain database (CDD; <https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>), ScanProsite (<https://prosite.expasy.org/scanprosite/>), and Pfam 31.0 (<https://pfam.xfam.org/>).

Gene Expression Analysis by qRT-PCR

Of the 28 identified proteins, 8 proteins showing up-/down-regulation (7 up- and 1 down-regulated) under UV-B stress were selected for studying the gene expression by qRT-PCR. These genes included hypothetical protein (wp_039682412), dihydrodipicolinate synthase (dapA), ATP synthase subunit E (atpE), ATP synthase subunit B (atpB), purine-nucleoside phosphorylase (deoD), 30S ribosomal protein S1 (rpsA), S-layer protein (slpA), and molecular chaperone DnaK (dnaK). *16S rRNA* gene was used as a bacterial reference gene for the normalization of the gene expression. Nucleotide sequences of the selected genes were obtained from NCBI database using tBLASTn program. Primers were designed by using Primer3 software (<http://primer3.ut.ee/>) and specificity was checked by Primer-BLAST. All the commercially synthesized primers were obtained from Integrated DNA Technologies Inc., USA (Table 2).

RNA isolation kit (Ambion Life Technologies, USA) was used for total RNA isolation from both UV-B-irradiated and control cultures. cDNA was synthesized using GoScript™ Reverse Transcription System (Promega Corporation, USA) following the man-

ufacturer's protocol. Quantity of cDNA was determined by Nano-drop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). qRT-PCR was performed in 20 µL final reaction volume on a MiniOpticon Real-Time PCR System (Bio-Rad Laboratories, USA) using 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, USA). Non-template control (NTC) was included in each run. Real-time amplification for each gene was done in triplicate maintaining identical conditions. Thermal cycles for the amplification were set as 95°C for 5 min, 40 cycles at 95°C for 15 s, 20 s at 53°C, and 30 s at 70°C. The melting curve analysis was performed for all the assays according to Kumar et al. [2016]. Data were analyzed using the CFX Manager Software (Bio-Rad, USA). The C_t values were automatically identified using the "Second Derivative Maximum Method." The transcript expression level was normalized with the reference gene (*16S rRNA*) relative to 0 h (control) using the $2^{-\Delta\Delta C_t}$ method [Livak and Schmittgen, 2001].

Statistical Analysis

Unless otherwise stated, the experiments were conducted in triplicate under identical conditions. Mean values and standard error were calculated from three replicates of each treatment. For confirming the significance of data, a one-way ANOVA (analysis of variance) was used based on Duncan's multiple range test (DMRT) at $p \leq 0.05$. GraphPad Prism 5.01 (GraphPad Software Inc., USA) software was used for the DMRT. Values reported are the mean $\pm$ SD ($n = 3$).

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Statement of Ethics

The authors have no ethical conflicts to disclose. This study does not include use of any animal/human samples. Therefore, institutional ethical approval was not required for this study. All authors have read the manuscript and agree with its content.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions

J.K. and A.K. designed the experiments. J.K. performed the experiments, analyzed data, and wrote the manuscript. P.G. helped in the experimentation. A.K. corrected and proofread the manuscript.

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