Comparative proteomics analysis of a novel γ-radiation-resistant bacterium wild-type *Bacillus megaterium* strain WHO DQ973298 recovering from 5 KGy γ-irradiation

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Abstract
In order to examine radiation-induced proteins in an extremely radio-resistant bacterium, it became possible to perform comparative proteomic analysis on radio-resistance *Bacillus megaterium* WHO as a wild-type strain. Variation in cellular proteins profiles of the *Bacillus megaterium* WHO after 5 KGy γ-irradiation were analyzed by two-dimensional poly acryl amide gel electrophoresis and silver staining. Although many spots were decreased in density, our primary focus was on the induced spots. The expression level of 48 protein spots showed significant increase under radiation stress. Of these spots, 45 were identified with MALDI TOF-TOF (peptide mass fingerprinting using matrix-assisted laser desorption/ionization time of flight mass spectrometry) after tryptic in-gel digestion. These proteins exhibited various interesting cellular functions including: (i) transcription (ii) translation (iii) signal transduction (iv) carbohydrate transport and metabolism (v) energy production and conversion (vi) nucleotide transport and metabolism (vii) posttranslational modification, protein turnover and chaperones (viii) DNA replication, recombination and repair (ix) bacterial general stress response and (iix) different and some still unknown functions. The appearance of four spots (24, 27, 30 and 36) in response to γ-irradiation was the distinct result of present study. These proteins appear to mediate processes related to ionizing radiation resistance and clearly demonstrate that *Bacillus megaterium* WHO, significantly has mechanisms contribute to the ionizing radiation resistance.
Keywords**:** *Bacillus megaterium;* γ-irradiation; proteomics; Two-dimensional gel electrophoresis

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INTRODUCTION

Proteins play an important role in regulating the biological systems and they are common diagnostic and repair. The study of proteome may lead to discovery of new proteins. Classical proteomics, or what is now referred as "expression profiling", is a process in which total cellular or tissue proteins are separated on 2-D gels and the visible protein spots are identified by peptide mass fingerprinting.

The 2-DE method was optimized for the separation of intracellular proteins of wild-type *Bacillus megaterium* strain WHO DQ973298. This strain is a radio-resistance bacterium that we selected it from Abe-Siah hot spring in the city of Ramsar, the North part of Iran with high level of radiation (up to 4Gy). The strain WHO is a white colony, Gram positive and not similar to species of the *Deinococcus* and *Rubrobacter* (Yazdani *et al*., 2009). Well-aerated exponential phase cultures of the WHO strain will survive 5KGy of gamma radiation without loss of viability and survivors are routinely recovered from cultures exposed to as much as 20 KGy (Yazdani *et al*., 2009).

Ionizing radiation induces a variety of potentially harmful types of DNA damage, but of these the DNA double strand break is considered the most lethal as a pair of double-strand breaks cause deletion of a segment in cells and thus the loss of essential genetic information (Dean, 1969). Although all living organisms have DNA repair mechanisms, only a few dou-

ble-strand breaks DSBs can be repaired in most species. *D. radiodurans* is capable of repairing the fragmented genome during post-irradiation incubation. Genome sequence analysis of *D. radiodurans* has revealed that the genome encodes almost all the major prokaryotic proteins involved in DNA repair. However, the molecular mechanisms underlying its radiation resistance remain unclear and little is known about how an organism responds environmental stressors (White *et al*., 1999). Previous studies of stress response systems in *D. radiodurans* demonstrated that the organism mounts a regulated protective response against DNA damage induced by UV light (Earl *et al*., 2002), γ-irradiation (Battista *et al*., 1997), and oxidative stress (Markillie *et al*., 1999; Sweet., 1976). In addition, *D. radiodurans* also protects itself in a regulated manner against heat and cold shock (Schmid *et al*., 2002; Allen *et al*., 1992). The secret of understanding the radio-resistance bacteria will presumably be found among these proteins with unknown functions (Tanaka *et al*., 2004).

Our previous results (Yazdani *et al*., 2009) clearly indicated that the isolated novel mesophilic bacteria *B. megaterium* WHO is highly resistant to radiation in such a way that one percent of the cell population has survived after exposure to 22 KGy γ-radiation, while the required dose for inactivation of *B. megaterium* is approximately 5.9 KGy. *B. megaterium* WHO also shows stronger resistance to gamma-radiation than *B. megaterium* (English *et al*., 1986). The genome of *B. megaterium* has not yet been sequenced and to our knowledge no information on proteomic analysis of *B. megaterium* has been published. In this work, for the first time we report variation in cellular proteins profile in wild-type *Bacillus megaterium* strain WHO DQ973298 following γ-irradiation. To better understand of the repair and metabolic responses of radiation conditions, we carried out proteomic analysis using two-dimensional gel electrophoresis in combination with mass spectrometric techniques (2-DE/ MALDI-TOF MS) based on PMF after tryptic in -gel digestion for protein separation and characterization. At present, the most straightforward and real high throughput mass spectrometric method of protein identification is peptide mass fingerprinting. This approach relies on stringent matching of measured peptide masses with computer- generated masses. Alternatively, if a protein of interest is not present in a database, peptide sequences deduced from tandem mass spectra can be employed for protein identification via sequence similarity searching. In this way a putative function can also be assigned to an unknown protein. Because of the rapid growth of completed genome sequences, it is possible to identify proteins of an unsequenced organism via sequence similarity searching, especially when sequences of organisms within the same kingdom or related species already exist in any of the public-accessible protein sequence databases (Habermann *et al*., 2004; Liska, *et al*., 2003; Shevchenko *et al*., 2003; Shevchenko *et al*., 2002). On the other hand the biochemical details of ionizing radiation resistance are poorly understood, but it is clear that proteins needed for cell survival are synthesized in cultures exposed to ionizing radiation (Zhang *et al*., 2005). In agreement with this, comparative proteomic analysis was performed to study cellular protein expression changes related to defined gamma radiation conditions for *B. megaterium* strain.

MATERIALS AND METHODS

Cell culture and radiation treatment: A wild-type *Bacillus megaterium* strain WHO DQ973298, was transferred into fresh TGY medium (1%Tryptone, 0.1% Glucose, and 0.5%Yeast extract), and grown by shaking at 30ºC to early stationary phase. Bacterial growth was assessed by measuring turbidity (OD_{600}) or by determining colony forming unit (CFUs) on TGY agar plates. Cells were then harvested by centrifuging, washed twice with 10 mM phosphate buffer (pH 7.0) and resuspended in fresh buffer. Suspensions were acutely irradiated at room temperature by ^{60}Co γ-rays at 5 KGy. Unirradiated controls were placed in the same conditions for the same duration. Irradiated and control cells were centrifuged at 5000 ×*g* for 3 min, transferred to fresh TGY broth at a starting cell turbidity (OD_{600}) of 0.5 and incubated at 30ºC for 60 min with shaking. Subsequently, cells were rewashed twice with 10 mM phosphate buffer, harvested by centrifuging and storing at -80ºC before the sample preparation. The experiment was repeated three times and the observed variation was less than 10%.

Sample preparation: Deep-frozen cells were directly disrupted into lysis buffer (8 M Urea, 4% (w/v) CHAPS, 1% (w/v) DTT, 2% (v/v) IPG buffer pH 3-10 L, 40 mM Tris base buffer) at 0°C by 2/5 intermittent sonication for 9 min. Cell debris was removed by centrifugation (25000 ×*g* for 20 min), and the clear supernatants were then stored in aliquots at -80ºC until analyzed (Yan *et al*., 2000). The protein concentrations

were measured by Bradford assay using bovine serum albumin (BSA) as the standard.

Two-dimensional electrophoresis (2-DE): 2-DE was generally performed using the isoelectric focusing Cell (BioRad,USA). Briefly, sample containing lysis buffer was diluted with rehydration solution (8 M Urea, 2% (w/v) CHAPS, 20 mM (w/v) DTT, 0.5% (v/v) IPG buffer pH 3-10 L, 0.002% (w/v) bromophenol blue) to 450 µl. Immobiline Dry Strip gels (pH 4-7,17 cm; Bio Rad). Then it was rehydrated with 300 µl of mixture solutions in 17 cm strip holders and electro focused with the Bio Rad IPGphor. Six gels were run at once, of which three gels were unirradiated controls and three were radiation-treated samples. About 80 µg of protein were loaded using in-gel rehydration. The used protocol was as follows: 50 µA per strip at 20°C; (i) rehydration for 12 h; (ii) 250 V for 20 min (step and hold); (iii) 10000 V for 2.5 h (step and hold); and (iv) 10000 V (step and hold) was applied until the total V-h reached 50.0 kV-h. After IEF, the strips were equilibrated twice with gentle shaking for 15 min in SDS equilibration buffer. The first step was performed in a equilibration solution containing 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT, 50 mM Tris-HCl buffer, pH 8.8 and 0.002% (w/v) bromophenol blue. The second step was performed in a solution modified by the replacement of DTT with 2.5% (w/v) iodoacetamide for a further 15 min. When the equilibration was finished, the strips were loaded onto vertical SDS PAGE (12% T constant), without stacking gels, using an electrophoresis protein II xi Cell (Bio-Rad, USA). The standard Laemmli buffer system was used and the running conditions were 16 mA/gel for 30 min and 24 mA/gel for approximately 5 h until the bromophenol blue front was 1cm above the bottom of the gel.

Silver staining, image acquisition and data analysis: The gels were stained by silver nitrate (Yan *et al*., 2000). The silver stained gels were scanned at resolution of 200 dpi using the Densitometer GS-800 scanner (BioRad,USA). The scanned gels saved as TiF images for subsequent analysis and were analyzed with Melanie (version 4) software supplied by the manufacturer (Gene Bio, Geneva. Switzerland). Three 2-D gels per cell were run and percentage volume of each spot was estimated and analyzed. The three irradiated and unirradiated cells were analyzed by one-way analyses of variance. Six gels were divided into two matches, three for unirradiated controls and three for radiation-treated samples. Image spots were initially detected, background subtracted and total spot volume were normalized and matched by manual assistance. Three gel images of unirradiated controls were averaged and set as the reference gel for comparison. Protein spots separated by 2-DE gels were quantities in terms of their relative volume (each spot volume/total spot volume×100). The average %volume of each spot was applied as input data. The statistical significance differences between expression profiles of WHO with or without γ-irradiation was evaluated using the Student's *t*-test ($P < 0.05$), and all statistical calculations in our test were done with Microsoft Excel. Only those spots whose expression level changed were greater than 2-fold, were considered significant, selected and excised manually from silver stained gels and placed in 96 well v-shape polypropylene plates and dried completely. The Ettan Spot Handling workstation (GE Healthcare, UK) was used for automatic in gel digestion of samples. Each gel plug was soaked in 100 µl of washing solution (50% MeOH, 50 mM NH_4HCO_3) to re-swell and then washed two more times in the same solution. The gels plugs were farther washed twice in 75% ACN, before being dried to completion. Samples were then re-hydrated by addition of freshly prepared trypsin solution (0.5 µg modified procine trypsin in 25 µl 20 Mm NH_4HCO_3) and incubated for 4 h at 37ºC. Peptides were extracted from the gel plugs by washing twice in 100 µl of 50% ACN, 0.1%TFA and transferred into a solution of a fresh 96 well plate, where samples were dried.

MALDI-TOF/TOF and database searching: Tryptic peptides were resuspended in 3 µl of 50%ACN, 0.1%Trifluoroacetic acid (TFA), 0.3 µl of resuspended. Tryptic peptides were spotted onto a steel Applied Bio Systems 96 sample MALDI target plate and mixed (while wet) with 0.3 µl of a 90% saturated α-cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN, 0.1% TFA. The dried samples were analyzed using a MALDI-TOF/TOF MS (4700 Proteomic analyzers Applied Biosysthem.UK), performing MS analysis and subsequent MS/MS analysis on up to 10 precursor peptides. Each sample was internally calibrated by reference to specific autolytic fragments of trypsin. The PMF MS/MS information were automatically searched against the NCBI non-redundant database using the Mascot search engine (Matrix Science, UK). Mass tolerance settings of 1.2 Da for parent ion and 0.5 Da for fragment ions were applied. Search settings allowed one missed cleavage with trypsin and two

modifications (Carboxamidomethyllation of Cysteine and oxidation of Methionine). Statistically confidence limits of 95% were applied for protein identification.

RESULTS

Methods used for the comparative proteomic of *B. megaterium* are the characterization of protein expression changes by 2-DE and the identification of proteins of interest by MS. This is aimed at finding protein network of *B. megaterium*, especially those involved in the metabolism and repairs, as well as the identification of metabolic pathways and cellular processes closely related to the DNA repair. Figure 1 shows a typical image of 2-DE separation of soluble proteins of *B. megaterium* in the pH range of 4-7. When 80 μg of a protein samples were applied, about 1000-1060 protein spots could be detected on different gels after silver staining. The spots matching rates between individual gels were between 58% to 75%. Figures 1 A and B show a representative pattern of two-dimensional gel electrophoresis of soluble proteins prepared from unirradiated cells and irradiated cells at 5 KGy in *Bacillus megaterium* WHO DQ 973298, which led to drastic changes in cellular proteins. As indicated in Figures 1A and B following irradiation, although intensity of many spots were decreased, our focus was primarily on those spots that were induced. These proteins are the most likely to be directly involved in DNA repair. Proteins whose expression levels significantly changed in the *Bacillus megaterium* WHO DQ 973298 following γ-irradiations are listed in Table 1. Molecular weight and pI of these proteins are also shown in Table 1.

Genome sequence information of *B. megaterium* is not available yet. However, 76 protein sequences of *B. megaterium* could be found in the Swiss-Prot, annotated protein database, up to now. Another 215 protein sequences exist in the protein database TrEMBL, a computer-annotated supplement of Swiss-Prot available at http://www.expasy.org/sprot/. The most of these proteins are hypothetical proteins and almost no enzymes of the central carbon metabolism or amino acid and protein biosynthesis has been presented for them.

On the other hand, genome sequencing of several microorganisms from the genus *Bacillus*, i.e. *B. subtilis*, *B. halodurans*, *Oceanobacillus iheyensis*, *B. anthracis*, *B. cereus*, and recently *B. licheniformis*, have been accomplished. These *Bacillus* species show highly conserved orthologous genes, including those

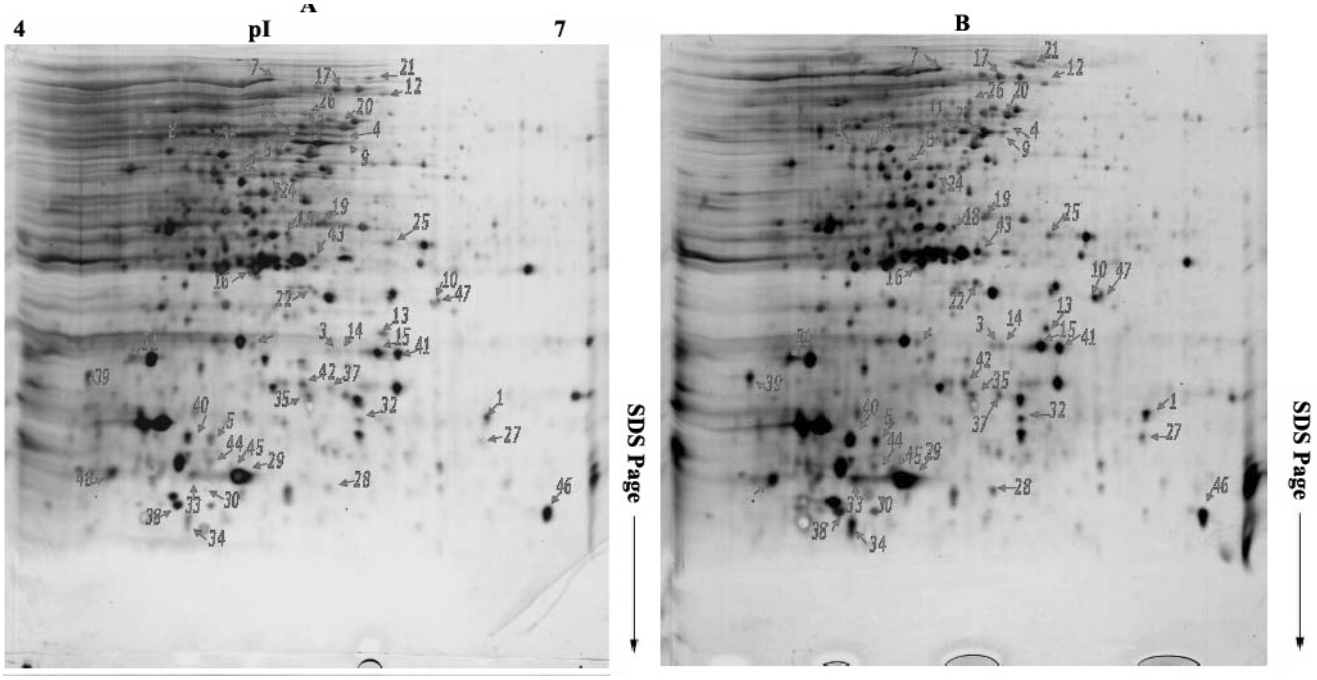

Figure 1. Two-dimensional electrophoresis (2-DE). Cells were analyzed by 2-DE and visualized by Silver-staining. First dimension, IPG 4-7, linear; separation distance, 17 cm. About 80 μg of protein was loaded using in-gel rehydration. Second dimension vertical SDS-PAGE (12% T constant). A: *Bacillus megaterium strain* WHO unirradiated; B: *Bacillus megaterium strain* WHO treated with ^{60}Co γ-rays. Numbers represent increased spots in irradiated cell.

Table 1. Proteins expression levels changed in WHO following γ-irradiation.

Spot No.[C)]	Identified protein,[a)] Function	Mass (Da)	*pI*	Accession No.	Exp. Level[b)]	Score[d)]
Transcription						
1	Transcriptional regulator, MarR family	17791.3	6.65	gi|126653975	↑	50
2	Transcriptional regulator-like	39076.2	5.03	gi|86741301	↑	45
3	Transcriptional regulator, TetR family	24889.2	5.85	gi|91782309	↑	57
Translation						
4	glycyl-tRNA synthetase	67355.3	6.07	gi|42525539	↑	54
5	Ribosomal protein L7/L12	12536.8	4.81	gi|89203738	↑	55
6	Histidyl-tRNA synthetase	48547.7	5.76	gi|81428473	↑	56
7	alanyl-tRNA synthetase	96081.5	5.48	gi|87122506	↑	49
8	Elongation factor EF-1α	58308.1	4.64	gi|116057527	↑	47
Signal transduction						
9	Response regulator transcription regulator protein	64008.3	6.04	gi|17544911	↑	47
Nucleotide transport and metabolism						
10	uracilphosphoribosyl transferase	28889	6.67	gi|7546185	↑	48
11	phosphoribosylaminoimidazolesuccinocarboxamide synthase	62402.7	5.66	gi|15793925	↑	44
Energy production and conversion						
12	sugar-specific enzyme III	82723.5	6.32	gi|16764665	↑	45
13	lyase, Aconitate hydratase	26928.1	6.3	gi|118059340	↑	46
14	Citrate synthase	24677.7	5.98	gi|62720393	↑	55
15	Citrate synthase	26278.3	6.3	gi|52313086	↑	56
16	pyruvate dehydrogenase (E1 beta subunit)	35452.3	5.10	gi|16078523	↑	55
Replication, recombination and repair						
17	hypothetical protein	84864.2	5.98	gi|116516401	↑	56
18	DNA polymerase III, delta subunit	37875.4	5.66	gi|115423568	↑	49
19	Formamidopyrimidine-DNA glycolase	38308.4	5.70	gi|69285890	↑	41
20	Similar to M-phase phosphoprotein 1	67062.5	5.98	gi|91091196	↑	51

(continued on next page)

Spot No.[C)]	Identified protein,[a)] Function	Mass (Da)	*pI*	Accession No.	Exp. Level[b)]	Score[d)]
21	putative deoxyribonuclease	101269.5	6.01	gi|110666929	↑	53
22	gyrase A	23999.5	5.70	gi|28916594	↑	43
23	DNA repair protein RecN	62381	5.71	gi|77919920	↑	48
24	Site-specific recombinases	49677.9	5.24	gi|46200914	+	50
25	RepA	35599.8	6.03	gi|20800435	↑	62
Carbohydrate transport and metabolism						
26	carbon-monoxide dehydrogenase	75031.1	5.79	gi|125975283	↑	50
27	Glycolate oxidase subunit GlcE	15402.2	6.45	gi|10802719	+	50
Post-translational modification, protein turnover, chaperones						
28	Cold shock protein	7388.7	5.98	gi|145688872	↑	45
29	Chaperone protein DnaK	6788.7	5.16	gi|145688872	↑	54
30	Chaperone protein DnaK	7389.9	4.90	gi|118474258	↑	45
31	hypothetical protein	26515.9	4.54	gi|15604457	↑	49
Bacterial general stress response protein						
32	Rubrerythrin	17048.2	6.14	gi|119477870	↑	48
33	CsbD	7096.6	4.45	gi|91978538	↑	50
34	AMP-activated protein kinase alpha2	4374.1	4.74	gi|62183749	↑	43
35	Peptidase S14, ClpP	21491.1	5.76	gi|89205016	↑	47
36	ClpX, ATPase regulatory subunit	46282.9	5.76	gi|89090946	↑	75
37	similar to ubiquitin specific protease	20952.9	5.97	gi|115894535	↑	55
Different						
38	small acid soluble protein	5826.9	4.64	gi|115354247	↑	169
39	putative ubiquinone biosynthesis protein	22810.7	4.31	gi|71906287	↑	47
40	Apa G	14316.2	4.75	gi|145963301	↑	54
41	OmpA	25421.5	6.51	gi|29345476	↑	55
42	Conserved hypothetical protein	22783.6	5.70	gi|119474083	↑	36
43	hypothetical protein Cpha 266_2183	37811.3	5.72	gi|119357966	↑	44
44	hypothetical protein Mbur_2168	9058.9	4.9	gi|91774095	↑	45
45	hypothetical protein TT_P0143 None	9531.9	5.03	gi|46255214	↑	42
Replication, recombination and repair						

[a]Protein function classification is based on NCBI's clusters of orthologous groups. [b]The pluses and upward arrows represent those spots which were newly appeared or increased respectively in WHO following γ-irradiation. [c]Spot number corresponds to the spot numbering in Figure 1. [d]Database search was based on MASCOT, a significance threshold of P<0.05 was represented by a score of 40.

for central carbon metabolism and for amino acid and protein biosynthesis. In addition, the genome sequence of *B. subtilis*, as a model system of gram-positive bactria, has been well annotated. Therefore, it is conceivable that the sequence information of these *Bacilli* can help the identification of unknown proteins of *B. megaterium* through sequence similarity searching (Wang *et al*., 2005).

It is worthy to mention that some proteins appeared as isoforms on the 2-D gels, namely as several spots having similar molecular weights (Mw) but different isoelectric points (pI). According to the categorization used in the KEGG PATHWAY database (http://www.genome.jp/kegg/metab olism.html), most identified proteins in this study can be classified into the following functional categories based on their functions or, at least, putative functions assigned. A list of identified proteins and their functions, *M*r and p*I* are shown in Table 1.The presented results in this study showed that at least the expression level of 48 kinds of proteins are increased by γ- irradiation at a high dose which the cells of *Bacillus megaterium* WHO DQ 973298 survived.

DISCUSSION

Of the total of 48 spots, 45 proteins were identified by PMF. These proteins displayed various cellular functions, including (i) transcription; (ii) translation; (iii) signal transduction (iv) carbohydrate transport and metabolism; (v) energy production and conversion; (vi) nucleotide transport and metabolism; (vii) post-translational modification, protein turnover, chaperones; (viii) DNA replication, recombination and repair; (ix) bacterial general stress response protein, and (iix) unknown protein spots.Four spots (22, 27, 29, and 45) appeared in response to γ-irradiation in *Bacillus megaterium* WHO DQ 973298. These proteins may participate in the process of recovery from radiation challenge. To survive exposure to ionizing radiation, an organism must avoid consequence of DNA double-strand breaks either by passively preventing break formation or by repairing the breaks in a manner that prevents the loss of information (Allen *et al*., 1992). Before DNA repair process starts , cell division should be stopped by the Tig, GroEL and Dna K (Spots 29, 30) that are the co-translational and post-translational chaperones .These proteins may facilitate rapid recovery of the depleted proteome during post-irradiation recovery. Dna K is also known to regulate cell division in bacteria (Joshi *et al*., 2004) and its up regulation along with DNA damage may affect the growth arrest. This may produce proteins that regulate the cell cycle, providing sufficient time for the cell to complete genome reassembly before potentially deleterious attempts are made to replicate damaged genome (Battista. 2000). Since cell division is arrested; DNA repair machinery is started.

Spot 24 shown in Table 1,is a site specific recombinase protein. It catalyzes site-specific recombination of DNA molecules by a connected, four-strand cleavage and rejoining mechanism. Previous observation suggested that PprA modifies the process of homologous recombination in a way that enhances the cell's capacity *of D. radiodurans* to deal with ionizing -radiation-induced DNA damage. The nature of this enhancement is still unknown. *D. radiodurans* appears to have the ability to control DNA degradation post irradiation by synthesizing proteins that prevent extensive digestion of the genome. Perhaps PprA is one of these proteins (Hua *et al*., 2003; Earl *et al*., 2002), but any mechanisms that improves the efficiency of recombination repair would also improve cell survival. Spot 23, RecN is thought, to play a role in DSB repair and genetic recombination even in the absence of RecA with RecN binding of DSBs and protecting these regions from nuclease degradation before RecA-dependent repair. A eukaryote-type DNA repair pathway by non-homologous end-joining (NHEJ) was discovered in *Bacillus subtilis* and *Mycobacterium tuberculosis* (Weller *et al*., 2002). NHEJ may offer a particular advantage to bacterial species that spend at least part of their life cycle either as spores, like *B. subtilis*, or in stationary phase, like *M. tuberculosis*, where homologous recombination may not be possible (MacNeill. 2005; Earl *et al*., 2002).

According to the Table 1, spot 38 is the small acid soluble protein that plays a major role in spore resistance to UV light as well as a minor role in spore heat resistance (Setlow, *et al*., 1995). While radiation undoubtedly kills spores by DNA damage, it seems that, DNA of *Bacillus megaterium* WHO is extremely well protected against many different types of damage through the saturation of the spore chromosome with a group of DNA-binding proteins, acid-soluble spore proteins (SASP). Consequently, *Bacillus megaterium* WHO DQ 973298 might contain an as-yet undiscovered *NHEJ* pathway for the repair of radiation-induced DNA DSBs.

Spot 22, Protein gyrase, *gyrA* also induced in response to ionizing radiation. DNA gyrase forms neg-

ative super coils in the DNA helix to alleviate excess positive super coiling caused by transcription, DNA replication, and repair processes. In *Escherichia coli*, treatments that reduce negative super coiling increase expression of *gyrA* and *gyrB* (Menzel *et al*., 1983), and the introduction DNA double-strand breaks will trigger their expression and degraded DNA is used again by uracil phosphoribosyl transferase (spot 10) as nucleotides to DNA repair pathway and DNA replication in salvage pathway.

Although cell division was stopped, synthesis of chaperones and TCA cycle enzymes are necessary for restoration minimal metabolic machinery following DNA repair, before growing resumes. Synthesis of the key TCA cycle enzymes (spots 13, 14, 15) and pyruvate dehydrogenase (spot 16) also would help to restore the energy statues of irradiated cells poised for recovery. Importing biosynthetic precursors and differentially regulating TCA cycle activity following ionizing radiation could provide biosynthetic intermediates needed for recovery without generating high levels of reactive oxidative stress (ROS). The prevailing hypothesis that most metabolically induced ROS are generated from within the respiratory chain has been expanded to include sources outside the respiratory chain .The glyoxylate cycle bypasses the steps in the citric acid cycle where carbon is lost in the form of CO_2. The two initial steps of the glyoxylate cycle are identical to those in the citric acid cycle. To begin a turn of the citric acid cycle, acetyl-CoA donates its acetyl group to the four-carbon compound oxaloacetate to form the six-carbon citrate. Citrate is then transformed into isocitrate.. In the next step, catalyzed by the first glyoxylate cycle enzyme, isocitrate lyase (spot 13), isocitrate undergoes cleavage into succinate and glyoxylate. Consequently *Bacillus megaterium* WHO DQ 973298 represses its TCA intermediate. In contrast the glyoxylate cycle bypass induced and could provide some biosynthetic intermediates needed for recovery without generating free radicals (spots 13, 14, 15 and 27).

Rubrerythin (spot 32) is thought to reduce hydrogen peroxide as part of an oxidative stress protection system related to shown ionizing radiation to enhance the production of ROS in a variety of cells. Recent evidence suggested that ROS play an important role in the action of ionizing radiation (Debabrota *et al*., 2005). Although the damage caused by gamma irradiation was mechanistically differ from that produced by hydrogen peroxide or by the superoxide-generating redox-cycling drug paraquat (Robinson *et al*., 2011).

Following irradiation some proteins were degraded. The protease may selectively degrade the damaged proteins. Spot 33 (Csb D) and spots 35 and 36 (CLp P and CLp X, respectively). ClpXP is a protease involved in DNA damage repair, stationary-phase gene expression, protein quality control. Spot 9 is a response regulator transcription protein. It is originally thought to be unique to bacteria. We presume that a checkpoint system in *Bacillus megaterium* WHO exists, as it was reported in *D. radiodurans* that reconstructs its chromosomes via a complicated signal transduction pathway, which promotes the expression of a number of DNA repair genes to overcome cellular genotoxic stress via complex pathway (Zhang *et al*., 2005). It seems that spot 8 identified as the elongation factor has a role in protein synthesis; it is known that EF-Tu stimulates higher rRNA synthesis in vitro by two to three times (Joshi *et al*., 2004). Therefore, we proposed that apart from EF-Tu role in protein synthesis and regulate coupling between transcription and translation may contribute to restoration of protein profile with re-synthesized up regulated protein and as a result a few proteins escaped degradation and their level was maintained throughout the protein recycling.

Spot 28 is a cold shock protein. This response results in up regulation of stress-response genes involved in cellular adaptation to cold stress and in down regulation of many other genes for the time of adaptation (Max *et al*., 2006). Subsequently it is fair to assume that there is similar mechanism for adaptation both for cold and irradiation stress.

In conclusion, the recovery phase includes many processes such as DNA replication, repair, recombination, metabolism, and many encoding uncharacterized proteins. The *Bacillus megaterium* WHO by a complex network which both DNA repair and metabolic functions play critical role, overcomes this radiation stress. Components of this network include a DNA gyrase, site directed recombinase, RecN (Fig. 2) and metabolic pathway switching that could prevent additional genomic damage elicited by metabolism-induced free radicals. There are still several proteins with unknown roles. These new proteins perhaps participate in the repair of DNA lesion. Such bacterium demonstrated the presence of different DNA repair mechanism and altered protein profile to overcome the genotoxic insult. Following irradiation of cultures of *B. megaterium WHO*, DNA replication ceases due to DNA damage. It is proposed that these processes are sensitive to the generation of the intracellular signals. DNA replication resumes only after repairing of DNA dam-

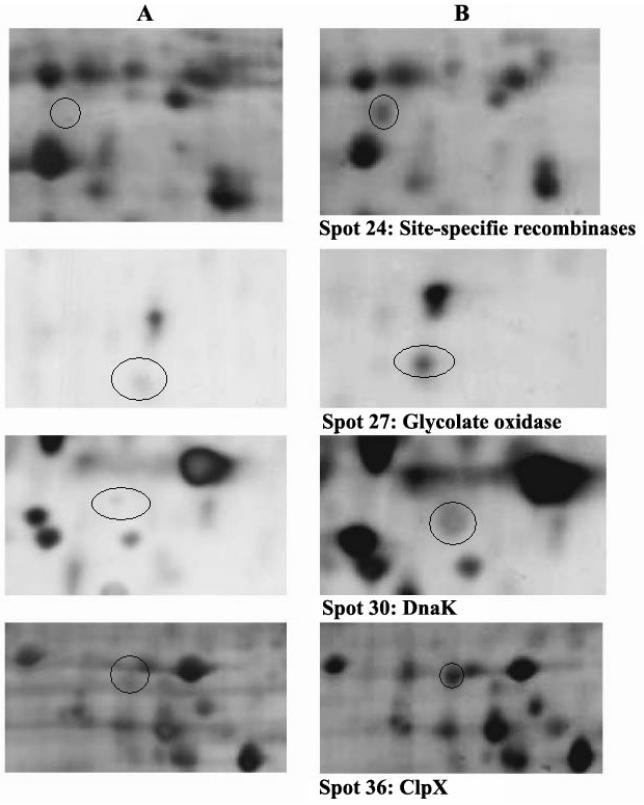

Figure 2. Zoomed visualization of appearance of four spots (24, 27, 30 and 36) in response to γ-irradiation (spots: 24, 27, 30, and 36 respectively). A: *Bacillus megaterium strain* WHO non-irradiated; B: *Bacillus megaterium strain* WHO treated with ^{60}Co g-rays. Circles represent appeared spots in irradiated cell.

age has been completed, but there is also data to support that chromosomal DNA degradation is essential part of DNA repair (Battista *et al*., 1999). Our results supported the hypothesis that there are some universal mechanisms for stress adaptation to different environmental signals.. Prokaryotes are remarkably diverse, occupying every conceivable environmental niche, so *Bacillus megaterium* WHO like *D. radiodurans* tolerance to high dose γ-irradiations, but this is not a general pathway for all species. In summary our study supports the previous observations (Zhang *et al*., 2005), and provides new evidence that this bacterium will serve as a model system for understanding of molecular mechanisms of radiation resistance in prokaryotes.

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References

Allen SP, Polanzzi JO, Gierse JK, Easton AM (1992). Two novel heat shock genes encoding proteins produced in response to heterologous protein expression in *Escherichia* coli. *J Bacterial*. 174: 6938-6947.

Battista JR (1997). Against all odds: the survival strategies of Deinococcus radiodurans. *Annu. Rev. Microbiol*. 51: 203-224.

Battista JR, Earl AM, Park MJ (1999). Why is Deinococcus radiodurans so resistant to ionizing radiation? *T Microbiol*. 9:362-365.

Battista JR (2000). Radiation resistance: the fragments that remain. *Curr Bio*. 10: 204-205.

Dean CJ, Ormerod MG, Serianni RW, Alexander P (1969). DNA strand breakage in cells irradiated with x-rays. *Nature* 222: 1042-1044.

Debabrota G., Marina V, Omelchenko EK, Gaidamakova VY, Matrosova VY, Vasilenko A, Venkateswaran A, Zhai M,Kostandarithes HM, Brim H, Makarova KS, Wackett LP,Fredrickson JK, Daly MJ (2005). How radiation kills cells: Survival of Deinococcus radiodurans and Shewanella oneidensis under oxidative stress. *FEMS Mic. Rev*. 29: 361-375.

Earl AM, Rankin SK, Kim KP, Lamendola ON, Battista JR (2002).Genetic evidence that the uvsE gene product of Deinococcus radiodurans R1 is a UV damage endonuclease. *J Bacteriol*. 194: 1003-1009.

Earl AM, Mohundro MM, Battista JR, Mian IS (2002). The IrrE protein of Deinococcus radiodurans R1 is a novel regulator of recA expression. *J Bacterial*. 184: 6216-6224.

English JD, VARY PS (1986). Isolation of recombination-defective and UV-sensitive mutants of *Bacillus megaterium*. *J Bacteriol*. 165: 155-160.

Habermann B, Oegema J, Sunyaev S, Shevchenko A (2004). The power and the limitations of cross-species protein identification by mass spectrometry-driven sequence similarity searches. *Mol Cell Proteomics*. 3: 238-249.

Hua YJ, Narumi I, Gao GJ, Tian B (2003). PprI: a general switch responsible for Extreme radioresistance of Deinococcus radiodurans. *Biochem Biophys Res Commun*. 306: 354-360.

Joshi B, Schmid R, Altendorf K, Apte SK (2004). Protein recycling is a major component in *Deinococcus radiodurans* strain R1. *Biochem Biophys Res Commun*. 320: 1112-1117.

Liska AJ, Shevchenko A (2003). Expanding the organismal scope of proteomics:cross-species protein identification by mass spectrometryand its implications. *Proteomics* 3: 19-28.

MacNeill SA (2005). Genome Stability: A Self-Sufficient DNA Repair Machine. *Cur Bio*. 15: R21-R23.

Markillie LM, Varnum SM, kradecky P, Wong KK (1999). Targeted mutagenesis by duplication insertion in the radioresistant bacterium Deinococcus radiodurans: radiation sensitivities of catalase (katA) and superoxide dismutase (sodA) mutants. *J Bacteriol*. 181:666-669.

Max KE, Zeeb M, Bienert R, Balbach J, Heinemann U (2006). T-rich DNA single strands bind to a preformed site on the bacterial cold shock protein Bs-Csp. *B J Mol Biol*. 360: 702-714.

Menzel R, Gellert M (1983). Regulation of the genes for *E. coli* DNA gyrase: homeostatic control of DNA supercoiling. *Cell* 34:105-113.

Robinson CK, Webb K, Kaur A, Jaruga P, Dizdaroglu M, Baliga NS, Place A, DiRuggiero J (2011). A major role for nonenzymatic antioxidant processes in the radioresistance of Halobacterium salinarum. *J Bacteriol*. 193: 1653-1666.

Schmid AK, Lidstrom ME (2002). Involvement of two putative alternative sigma factors in stress response of the radioresistant bacterium Deinococcus radiodurans. *J Bacteriol*. 84: 6182-6189.

Setlow B, Setlow P (1995). Small, Acid-Soluble Proteins Bound to DNA Protect *Bacillus subtilis* Spores from Killing by Dry Heat. *A E Microbiology*. 61: 2787-2790.

Shevchenko A, Chernushevic I, Shevchenko A, Wilm M, Mann M (2002). "Denovo" sequencin of peptides recovered from in-gel digested proteins by nanoelectrospray tandem mass spectrometry. *Mol Biotechnol*. 20: 107-118.

Shevchenko A, Sunyaev S, Liska A, BorkP, Shevchenko A (2003). Nanoelectrospray tandem mass spectrometry and sequence similarity searching for identification of proteins from organisms with unknown genomes. *Methods Mol Biol*. 211: 221-234.

Sweet DM, Moseley BE (1976). The resistance of Micrococcus radiodurans to killing and mutation by agents which damage DNA. *Mutat Res*. 34: 175-186.

Tanaka M, Earl AM, Howel HA, Park MJ, Eisen JA, Peterson SN,Battista JR (2004). Analysis of Deinococcus radiodurans's Transcriptional Response to Ionizing Radiation and Desiccation Reveals Novel Proteins That Contribute to Extreme Radioresistance. *Genetics* 168: 21-33.

Wang W, Hollmann R, Fürch T, Nimtz M, Malten M, (2005). Proteome analysis of a recombinant Bacillus megaterium strain during heterologous production of a glucosyltransferase. *Proteome Sci*. 3: 1-16.

Weller GR, Kysela B, Roy R, Tonkin LM, Scanlan E, Della M, Devine SK, Day JP, Wilkinson A, d'Adda di Fagagna F, Devine KM, Bowater RP, Jeggo PA, Jackson SP, Doherty AJ (2002). Identification of a DNA nonhomologous end-joining complex in bacteria. *Science* 297: 1686-1689.

White O, Eisen JA, Heidelberg JF, Hickey EK, Peterson JD, Dodson RJ, Haft DH, Gwinn ML, Nelson WC, Richardson DL, Moffat KS, Qin H, Jiang L, Pamphile W, Crosby M,Shen M, Vamathevan JJ, Lam P, McDonald L, Utterback T, Zalewski C, Makarova KS, Aravind L, Daly MJ, MintonKW, Fleischmann RD, Ketchum KA, Nelson KE, Salzberg S, Smith HO, Venter JC, Fraser CM (1999). Genome sequence of the radioresistant bacterium Deinococcus radiodurans R1. *Science* 286: 1571-1577.

Yan JX, Wait R, Berkelman T, Harry RA, Westbrook JA, WheelerCH, Dunn MJ (2000). A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electrospray ionization-mass spectrometry. *Electrophoresis* 21: 3666-3672.

Yazdani M, Naderi-Manesha H, Khajeh K, Soudi MR, AsghariSM, Sharifzadeh M (2009). Isolation and characterization of a novel γ-radiation- resistant bacterium from hot spring in Iran. *J B Microbiology*. 49: 119-127.

Zhang C, Wei J, Zheng Z, Ying N, Sheng D, Hua Y (2005). Proteomic analysis of Deinococcus radiodurans recovering from gamma-irradiation. *Proteomics* 5: 138-143.