J. Microbiol. Biotechnol. (2011), **21**(6), 574–581
doi: 10.4014/jmb.1012.12031
First published online 14 April 2011

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Proteomic Analysis of Global Changes in Protein Expression During Exposure of Gamma Radiation in *Bacillus* sp. HKG 112 Isolated from Saline Soil

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Received: December 27, 2010 / Revised: March 14, 2011 / Accepted: March 15, 2011

A Gram-positive bacterium was isolated from the saline soils of Jangpura (U.P.), India, and showed high-level of radiation-resistant property and survived upto 12.5 kGy dose of gamma radiation. The 16S rDNA sequence of this strain was examined, identified as *Bacillus* sp. strain HKG 112, and was submitted to the NCBI GenBank (Accession No. GQ925432). The mechanism of radiation resistance and gene level expression were examined by proteomic analysis of whole-cell extract. Two proteins, 38 kDa and 86.5 kDa excised from SDS–PAGE, which showed more significant changes after radiation exposure, were identified by MALDI–TOF as being flagellin and S-layer protein, respectively. Twenty selected 2-DE protein spots from the crude extracts of *Bacillus* sp. HKG 112, excised from 2-DE, were identified by liquid chromatography mass spectrometry (LC–MS) out of which 16 spots showed significant changes after radiation exposure and might be responsible for the radiation resistance property. Our results suggest that the different responses of some genes under radiation for the expression of radiation-dependent proteins could contribute to a physiological advantage and would be a significant initial step towards a full-system understanding of the radiation stress protection mechanisms of bacteria in different environments.

Keywords: *Bacillus* sp., 16S rRNA gene sequencing, gamma irradiation, 2-D gel electrophoresis, MALDI–TOF/TOF, LC–MS/MS

Extreme ionizing-radiation resistance has been observed in several members of the archaeal and bacterial domains isolated from diverse environments [1, 22] including saw dust [10], sewage [11], soils [16], and marine thermal springs [12]. A whole-body exposure of just 10 Gy is lethal to most vertebrate animals, including humans, and most bacteria cannot survive on exposure to more than 200 Gy [21]. Bacteria belonging to the family Deinococcaceae are the most radiation-resistant organisms known [2, 7, 15, 17]. The bacteria found in stressed habitats like salt, desiccation, and non-ionizing radiation like ultraviolet (UV) radiation show more resistant to gamma radiation [20]. During the last few years, proteomics has been established as a powerful tool for understanding various biological problems in several organisms, and we want to find out the properties responsible for radiation resistant among bacterial populations using proteomic analysis. From previous data, it is clear that some structural changes or loss of the S-layer protein of the bacterial strains of *B. cereus* increased the radiation sensitivity of the vegetative cells [13]. S-layer, a crystalline surface protein layer, is a common structure present in both Gram-positive and Gram-negative bacteria [4]. Electron microscopy, SDS–PAGE, Western blot, and fluorescent antibody staining indicated that the higher resistance to radiation of the strains from plate cultures was associated with the presence of the S-layer on the cell surface. An S-layer-containing bacterial cell was 2.6 times as resistant to radiation as the two reference strains without an S-layer [13].

In the present study, we isolated one gamma radiation-resistant bacterial colony from sodic soil sample, with a temperature ranging from 30°C to 37°C (optimum 37°C) and performed morphological, 16S genotypic analysis [9], and proteomics evaluations [24] of the selected sample accordingly. We found some overexpressed proteins like S-layer and flagellin proteins along with some other proteins that might play a key role in the radiation resistance property.

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Therefore, this bacterium will act as a model system for understanding of the molecular mechanisms of the radiation resistance property in prokaryotes as well as in eukaryotes and for finding the ways to protect people from atomic radiation.

Materials and Methods

Sample Collection, Bacterial Strain, and Culture Conditions

Soil samples from the district Jungpura (U. P.), India were collected randomly, from different local areas of the Shahpura jungle, in sterilized poly bags and brought to the laboratory for further analysis. The pour plate method using nutrient agar was used for isolation of strains. For this purpose, standard dilution plating procedure was used to recover bacteria from soil samples. Individual colonies were picked up on the basis of visual morphology and color, and streaked onto fresh nutrient agar (NA) plates for purification. This process was repeated several times until pure isolates were obtained. The growth rates were determined by measuring the turbidity at 600 nm of liquid cultures. The temperature range for growth was determined on nutrient broth (NB) medium at a temperature between 25°C to 50°C, and the pH range was determined at 37°C between pH 5.0 to pH 13.0. All growth experiments were performed in triplicates. The pure culture strains were maintained on nutrient agar plates and stored at 4°C. They were also stored as nutrient broth containing 20% (v/v) glycerol at −80°C.

Bacterial Identification

After cultivation of strains on nutrients agar plates, the cell morphology was examined by phase-contrast microscopy through Gram staining. Microbiological properties of the isolated strains were determined according to the methods described in *Bergey's Manual of Determinative Bacteriology* [3]. Genomic DNA was extracted and purified (by enzymatic method) according to Sambrook and Russell [19] and its purity was assessed as the OD_{260}/OD_{280}. The 16S rDNA gene was selectively amplified with the 16S partial PCR forward (5'-CAGCAGCCGCGGTAATAC-3') and reverse (5'-TACGGCTACCT TGTTACG-3') primers. A thermal cycler (G-storm) was used for amplification and programmed as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 1 min (denaturation), 59°C for 50 s (annealing), 72°C for 1 min (extension), and a final extension of 5 min to allow for extension of any incomplete products. The amplification products were purified by using gel extraction kits (Qiagen, Germany) and then DNA sequencing on both strands were directly performed by The Centre of Genomic Application (TCGA), New Delhi (India).

Gamma Irradiation

The protocols used to evaluate radiation resistance were as described in previous studies [18, 23]. Mid-log-phase culture ($OD_{600\ nm}$=0.3) was divided into 10 ml aliquots, placed in 15 ml falcon tubes, and exposed to gamma (γ) irradiation at various dose levels of 5 kGy, 7.5 kGy, 10 kGy, 12.5 kGy, and 15 kGy using a ^{60}Co gamma chamber (Gamma Cell 5000; Bhabha Radiation Isotope Technology, Bombay) installed at the Institute of Nuclear Medicine and Allied Sciences, Defence Research and Development Organization, Delhi, India. The dose rate delivered was 1.67 kGy/h. All the irradiated strains and unirradiated control was transferred into fresh nutrient broth medium and incubated on a rotary shaker at 200 rpm for 24 h at 37°C. Bacterial growth was observed by measuring the turbidity at 600 nm of liquid cultures (Nutrient broth, HiMedia), and the viability of irradiated cells was evaluated using an unirradiated suspension of strain under the similar condition.

Preparation of Sample

Bacterial cell pellets (both irradiated strains and unirradiated control) were washed with 1× PBS buffer (pH 7.2) for 2 to 3 times and resuspended in 400 µl of sterile sonication buffer with 10 µl of PIC (protease inhibitor cocktail) and then sonicated at 4°C. After sonication, cell debris were removed by centrifugation at maximum speed (13,000 rpm) for 30 min at 4°C and the clear supernatants were then stored at −80°C. The protein concentrations were measured by Bradford assay [6].

Preparation of SDS–PAGE

The whole-cell protein profiles of the *Bacillus* sp. was examined by SDS–PAGE (10% polyacrylamide). Thirty µg of proteins of each sample along with prestained protein molecular marker (Fermentas, USA) were loaded on the gel and run on a mini gel electrophoresis at 100 V for 2 h and stained by the silver staining procedure [5]. The differentially expressed protein bands (Fig. 2) were subjected to MALDI–TOF/TOF analysis.

Two-Dimensional Gel Electrophoresis (2-DE)

The concentrated and dialyzed whole-cell protein extracts were cleaned-up using a 2-D clean-up kit (Amersham Biosciences) as recommended by the supplier. Protein samples (100 µg) were mixed separately with denaturing buffer [8 M urea, 2% CHAPS (w/v), 50 mM Tris, 65 mM DTT, 0.5% (v/v) ampholine]. The samples were then loaded into the strip holder and the 7 cm immobilized pH gradient (IPG) strip with a linear pH range 3–10 (ReadyStrip, BioRad, Munich, Germany) was placed into the strip holder and transferred into a Protean IEF Cell (BioRad, Munich, Germany). IEF was carried out using a multistep protocol (14 h for rehydration, 3 h at 500 V, 2.5 h at 4,500 V, and finally to reach at 4,000 V) [8]. All steps were carried out at 20°C. After IEF, the gel strips were subjected to a two-step equilibration for 10 min. The first step was with an equilibration buffer containing 8 M urea, 50 mM Tris-HCl (pH 8.8), 2% (w/v) sodium dodecyl sulfate (SDS), 1% DTT, 20% glycerol, and then in the same buffer containing 2% (w/v) iodoacetamide for another 10 min. After equilibration, the strip was loaded onto a vertical 12.5% polyacrylamide gel for SDS–PAGE. The electrophoresis was carried out at constant current (mA) in the buffer consisting of 250 mM glycine, 25 mM Tris, and 0.1% SDS. The gels were silver stained using a method described by Blum *et al*. [5].

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI–TOF/TOF) Analysis

The differentially expressed protein bands excised from silver stained SDS–PAGE were send to TCGA, New Delhi, where protein identification (peptide mass fingerprinting) and sequencing were done by MALDI–TOF MS/MS. Digested mixture and peptides was subjected to MALDI–TOF analysis for peptide mass fingerprinting and protein sequencing by using a Bruker ultraflex MALDI–TOF/TOF. Specially designed MASCOT software by Bruker (Clinprot) was used for comparing protein profiles of before and after radiation exposure to determining their relative abundance.

Liquid Chromatography–Mass Spectrometry (LC–MS) Analysis
Protein identification and sequencing were carried out by using two-dimensional liquid chromatography ESI–MS (Agilent 1100 series 2-D Nano LC–MS). The protein contents in a sample were separated by 2-D gel electrophoresis, stained, and each observed protein spots was quantified by its staining intensity. Selected 20 spots were excised from the 2-D gel, which corresponds to different proteins, isolated and subjected to in-gel tryptic (enzymatic) digestion for further mass spectrometry analysis. Tryptic-digested proteins were subjected to column followed by reverse-phase separation. Peptides get ionized in the liquid phase in the electrospray ionizer and enter the ion trap, get fragmented (MS/MS), and detection occurs. The data were analyzed by spectrum mill software (Agilent).

RESULTS AND DISCUSSION

Isolation and Identification of Strain

We have isolated many bacterial strains from sodic soil of Jungpura (U. P.), India, which were γ-irradiated upto 15 kGy. Following the irradiation, only one of them remained alive, which led to the isolation of the most radio-resistant strain from these colonies. It was found to be Gram-positive, obligate aerobic, rod shaped, motile, spore forming, and morphologically appeared as a white colony on culture plate and was able to grow in salt-containing nutrient media of different concentrations (up to 9.5%). The growth temperatures of this strain was in the range of 30°C to 48°C (optimum 37°C) and pH range of about 7.0 to 13.0 with an optimum pH of about 9.0 (data not shown). In order to confirm the identity, a partial 16S rDNA sequence of 781 bp was determined and sequence analyzed by BLAST at NCBI. Thus the combination of morphological, physiological, "biochemical" and 16S rRNA gene sequencing data suggests that this strain was a *Bacillus* species. The obtained 16S rDNA sequence of *Bacillus* sp. HKG-112 has been deposited in GenBank under Accession No. GQ925432 (http://www.ncbi.nlm.nih.gov).

Survival of Bacteria at Different Doses of Gamma Radiation

The survival curves for resistance to gamma-radiation of the *Bacillus* sp. strain HKG-112 are shown in Fig. 1. The shoulder doses of radiation (the dose required before the number of CFU declines) of the strain were 5.0 kGy, which is comparable to the shoulder dose of *D. radiodurans*, but the thermophilic species *D. murrayi* exhibited stronger resistance to gamma-radiation (shoulder doses, 7.5 kGy) [7]. The required dose to reduce the number of viable units to 50% (the mean dose required to inactive a single CFU of the irradiated population) is approximately 10 kGy. Our data clearly indicate that the isolated novel extremophilic bacteria *Bacillus* sp. HKG-112 is highly resistant to gamma radiation.

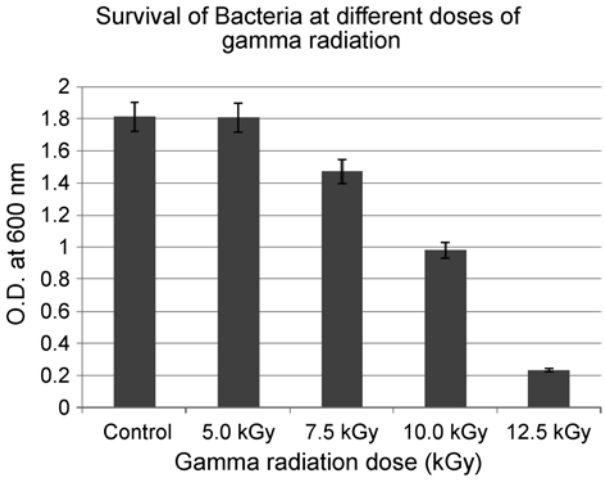

Fig. 1. Survival curve of *Bacillus* sp. HKG-112 (unirradiated control and irradiated strains) at different doses of gamma radiation, by measuring the turbidity at 600 nm.

Proteomics Analysis

In order to analyze the obtained protein profile, we examined the protein production level in this strain followed by γ-radiation, in comparison with unirradiated controls, using polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 2) and two-dimensional gel electrophoresis (2-DE) (Fig. 3A and 3B). The protein patterns of strains that were acutely irradiated at room temperature by ^{60}Co γ-rays at 12.5 kGy significantly changed. Although some proteins were increased in density, some protein spots were decreased in density, but our focus was primarily on those spots that were induced as these proteins are the most likely to be directly involved in the DNA repair mechanism and radio-resistant property in microorganisms.

Protein Identification and Sequencing by MALDI–TOF/TOF and 2-D Nano LC–MS/MS

The 20 different 2-DE protein spots from the crude extracts of *Bacillus* sp. HKG 112, which showed at least one significant effect, were excised and identified by liquid chromatography mass spectrometry analysis. Our results revealed that there were 10 regulatory proteins (spots 5, 8, 9, 10, 11, 15, 16, 18, 19, and 20), which increased or appeared when cells were acutely irradiated (Table 1). These proteins may participate in the processes of recovery from radiation challenge. To obtain functional information for the proteins annotated either as unknown or hypothetical proteins, we searched databases with Mascot (http://www.matrixscience.com) for their homologs using their protein sequences as queries. All spots shared more than 50% positive identity with their homologs at the amino acid level, suggesting that they may be involved in the similar functions. The identified proteins were classified into several functional

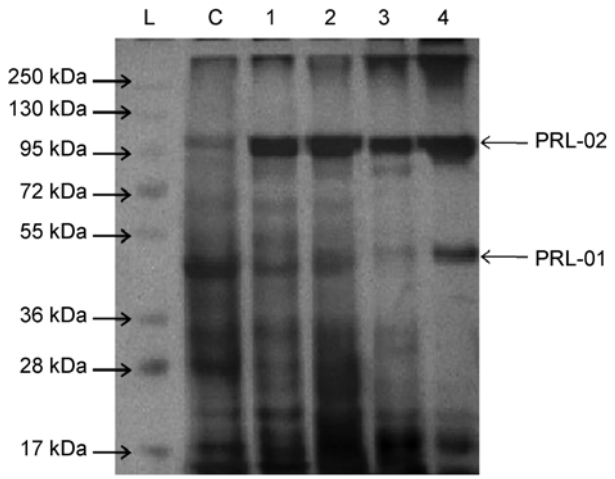

Fig. 2. Polyacrylamide gel electrophoresis (SDS–PAGE) showing the effect of different doses of gamma radiation on whole-cell protein profiling of *Bacillus* sp. strain HKG 112.
L: Prestained molecular mass standard; C: Before radiation treatment (Control); 1: 5 kGy of gamma radiation dose; 2: 7.5 kGy of gamma radiation dose; 3: 10 kGy of gamma radiation dose; 4: 12.5 kGy of gamma radiation dose.

categories. All 16 identified protein spots that showed significant changes after gamma irradiation were subjected to functional annotation in accordance with the Uniprot database (http://www.uniprot.org) and Kognitor (http://www.ncbi.nlm.nih.gov/COG/grace/kognitor). It was observed that several proteins not belonging to any particular functional category may be involved in various important functions in bacterial survival. The majority of the 16 proteins identified in the present study belonged to biosynthesis, metabolism, and energy production and conservation. Most of these proteins have not been reported to be relevant to extreme radioresistance. In all organisms, proteolysis assures vital functions in the regulation of many metabolic processes, maintains cell homeostasis, and keeps optimal metabolic activities by removing non-functional proteins. This later function becomes essential during environmental stresses where the occurrence of damaged proteins increases. In bacteria, ATP-dependent Clp proteases (spot number 9) are involved in many processes ranging from developmental changes (sporulation, development of competence) to abiotic stress tolerance (heat shock, salt and oxidative stresses, glucose and oxygen starvation, pH changes). Pyruvate dehydrogenase beta-subunit (spot number 5) is an E1 subunit of the pyruvate dehydrogenase complex, and was found to be increased in the irradiated strain. This complex is a critical link between glycolysis and the tricarboxylic acid cycle, catalyzing the oxidative decarboxylation of pyruvate in the formation of acetyl-CoA in mitochondria. The increase in E1 subunit could be due to its phosphorylation, which, however, remains to be investigated by phosphoprotein staining or Western blot analysis. Another major protein was identified as the elongation factor EF-Tu (spot number 11), known to synthesize ppGpp during stress and effectively regulate coupling between transcription and translation.

The two proteins PRL-01 and PRL-02, which were identified by the MALDI–TOF method as flagellin protein and S-layer protein (Fig. 4A and 4B), showed major changes in gene expression level. The Mascot search results suggest that both proteins have stress-tolerant properties. It was observed that the flagellin induces expression of genes with direct antibacterial activity and activates heat-shock protein expression, which better equip these cells to survive a bacterial challenge [14] that may result from irradiation.

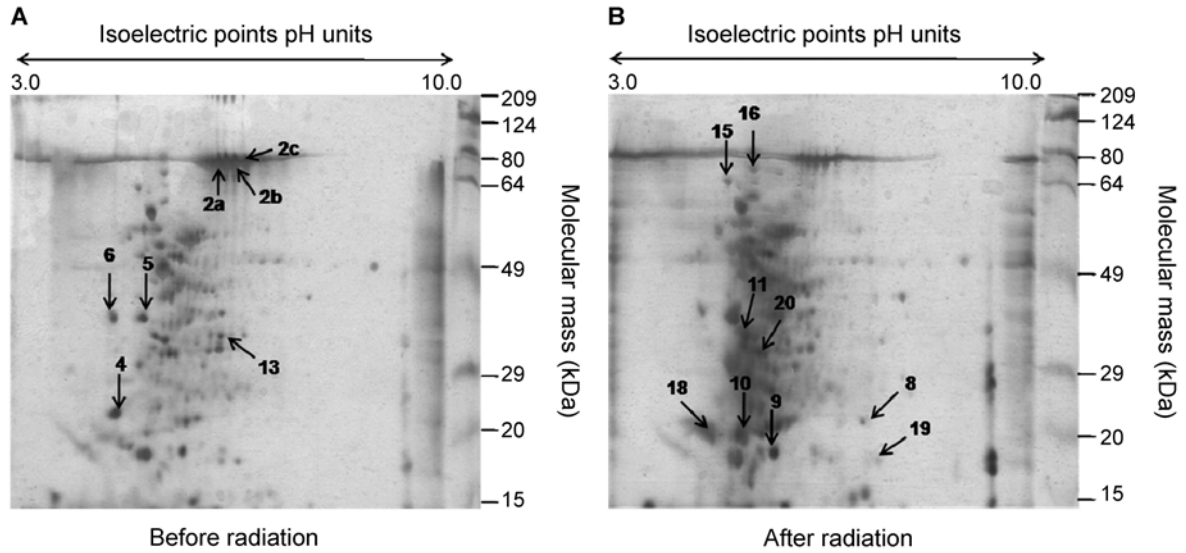

Fig. 3. Typical image of 2-D gel electrophoresis analysis of whole-cell protein extracts from *Bacillus* sp. HKG 112, (**A**) before radiation treatment (Control), and (**B**) after exposing to 12.5 kGy of gamma radiation.

Table 1. Liquid chromatography–mass spectrometry (LC–MS) analysis of sixteen excised two-dimensional gel electrophoresis protein spots from whole-cell protein extracts of 12.5 kGy gamma-irradiated *Bacillus* sp. HKG 112.

Spot no.	Accession No.	Name	Peptide match	Sequence coverage (%)	MASCOT score	Theoretical mass/ pI	Observed mass/ pI	Appeared signals	Biological functions
2 (a-c)	YP_082393	S-layer protein sap precursor	36	30%	1304	86.55/6.89	82.0/6.6	Decreased	Stress-tolerant properties
4	NP_843760	Spore coat-associated protein	8	17%	152	21.69/4.62	22.0/4.7	Decreased	Proteolytic activity
5	YP_002447538	Pyruvate dehydrogenase complex E1 component beta subunit	13	32%	601	35.19/4.75	40.0/4.9	Induced	Oxidation–reduction process
6	NP_834231	Electron transfer flavoprotein (beta subunit)	6	17%	196	28.18/4.33	39.0/4.4	Decreased	Electron carrier activity
8	YP_001377018	Uracil phosphoribosyltransferase	4	10%	78	23.02/6.09	24.0/6.5	Induced	Nucleoside metabolic process
9	NP_834816	ATP-dependent Clp protease proteolytic subunit	16	34%	459	21.46/5.26	19.0/5.3	Induced	Proteolysis
10	NP_978580	Azoreductase	7	19%	241	22.97/4.95	22.0/5.0	Induced	Oxidation–reduction process
11	NP_830009	Elongation factor Tu	7	10%	288	43.03/4.93	37.0/5.0	Induced	Protein biosynthesis
13	ZP_03109327	NLP/P60 family protein	3	12%	107	37.16/6.26	35.0/6.25	Decreased	Not known
15	NP_976486	Glucosamine--fructose-6-phosphate aminotransferase	6	8%	247	66.05/5.01	65.0/4.8	Induced	Carbohydrate biosynthetic process
16	ZP_03103295	5-Methyl tetrahydro pteroyltriglutamate--homocysteine methyl transferase	2	2%	101	87.51/5.26	78.0/5.2	Induced	Not known
18	ZP_04076298	Hypothetical protein bthur0013_66980	1	6%	72	21.89/4.83	21.0/4.6	Induced	Not known
19	NP_831290	GTP cyclohydrolase I	4	16%	170	21.12/6.36	18.0/6.9	Induced	Tetrahydrofolate biosynthetic process
20	NP_834552	Naphthoate synthase	2	8%	48	29.81/5.31	31.0/5.3	Induced	Lyase

Spot No.=spot numbers correspond to silver stained 2-DE gels. MASCOT score=Probability-based Mowse score by homology-based MASCOT search (all $p<0.05$). Theoretical mass and observed mass in kDa. pI=isoelectric point. Peptide matches=number of matched peptides by MS/MS analysis. Appeared signals=appearance of 2-D gel spots after exposing to gamma irradiation. Biological functions =predicted biological function from http://www.uniprot.org.

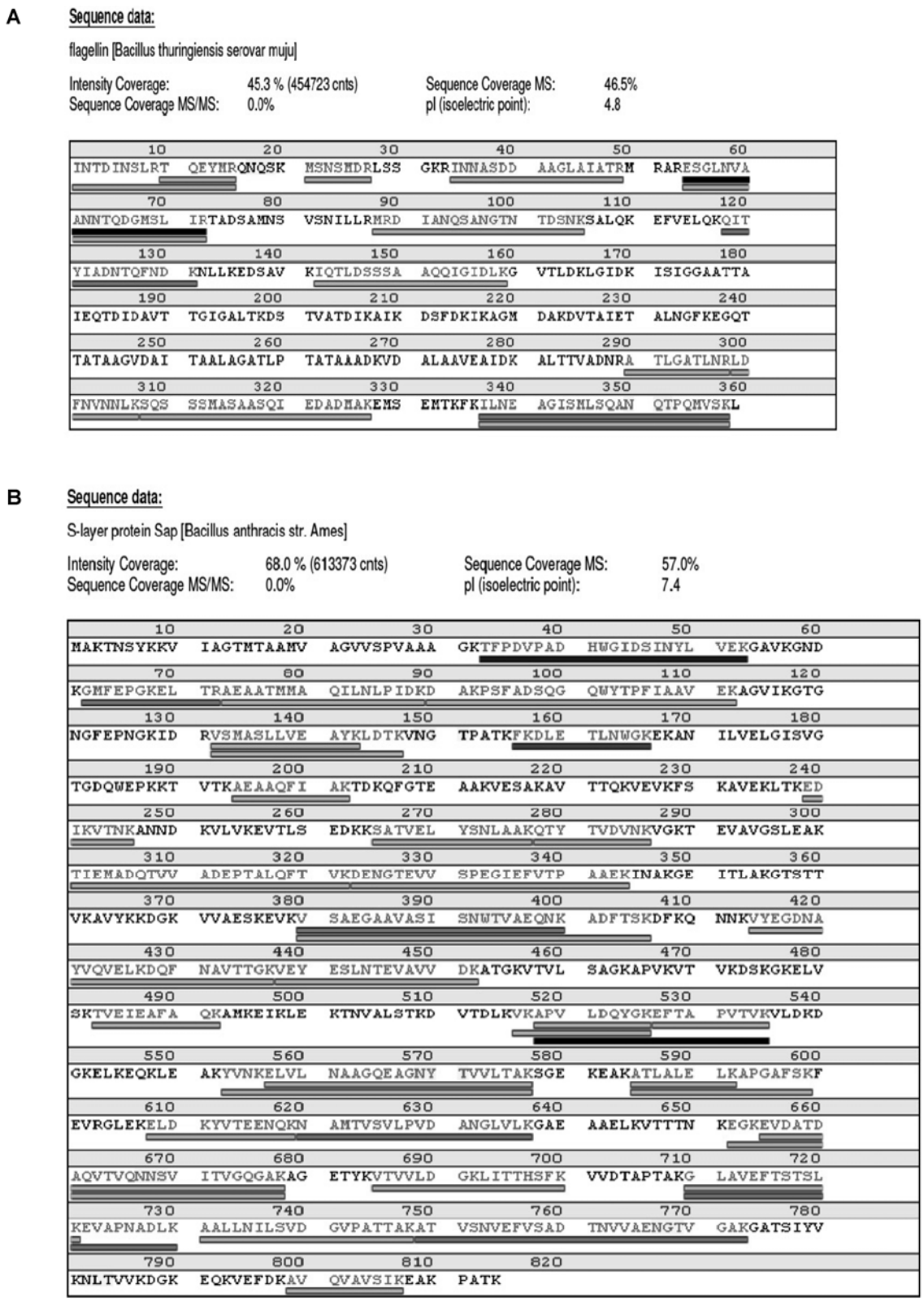

Fig. 4. Matched peptides after MASCOT search analysis of MALDI–TOF data of (**A**) PRL-01: overexpressed protein of 38 kDa excised from SDS–PAGE after 12.5 kGy gamma radiation dose, and (**B**) PRL-02: overexpressed protein of 86.5 kDa excised from SDS–PAGE after 12.5 kGy gamma radiation doses.

Bar regions under peptides in the full-length sequence of protein show the presence of matched peptides generated by tryptic digestion.

The bone marrow isolated from flagellin-treated mice had increased ability to protect against radiation, supporting this possibility. Understanding the mechanisms by which flagellin exerts its protective effects, which will likely have different correlates of protection against different challenges, remains a key challenge in this area. In the case of S-layer protein, it may be possible that the S-layer protein absorbing the free radicals induced by gamma radiation and reducing the radiation sensitivity of the bacterial cells plays a key role in the DNA protection mechanism in *Bacillus* sp. HKG 112. Our prediction and experimental evidence supports the opinion that overexpressed proteins are more important than other unaffected proteins during irradiation. Another important issue is that a number of "hypothetical genes" that might be unexplored genes for resisting irradiation are helpful for further understanding the radiation-resistant mechanisms and provide important clues to identifying new radiation-resistant genes and proteins. We indeed identified one such protein (spot no. 18) without any known experimental or putative functional elucidation. The characterization of this hypothetical protein of unknown function may reveal its possible role in radiation resistance property in bacteria. Individuals exposed to chronic or acute doses of radiation could potentially benefit from treatments that deliver purified protein complexes into their cells. This new model of radiation toxicity opens up novel avenues for radioprotection in diverse settings. Thus, analyzing these data in combination with some other additional experiments may reveal the mechanisms that help living cells survive in high levels of radiation and can provide a foundation for the future research.

Acknowledgments

We are grateful to the director of IGIB for providing infrastructural facilities and financial assistance (OLP-6101) to carry out this work, and R. Pathak is thankful to the University Grants Commission, India, for providing a Junior Research Fellowship.

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