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RecX is Involved In the Switch between DNA Damage Response and Normal Metabolism in *D. radiodurans*

Duohong Sheng[1,*], Jiandong Jao[2], Mingfeng Li[2], Peng Xu[1] and Junbing Zhang[1]

[1]State Key Laboratory of Microbial Technology, Shandong University, 250100 Jinan, China; and [2]Institute of Nuclear-Agricultural Sciences, Zhejiang University, 310029 Hangzhou, China

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Apart from inhibiting RecA activity through protein–protein interactions, *Deinococcus radiodurans* RecX inhibits the expression of RecA and two other anti-oxidant proteins. To identify the repertoire of proteins regulated by RecX, comparative proteomic studies were undertaken on a wild-type strain (R1) and *recX* null mutant (RecX$^-$). Two-dimensional electrophoresis followed by MALDI-TOF identification revealed 35 differentially expressed proteins, including 12 up-regulated and 23 down-regulated proteins in the mutant. The 12 up-regulated proteins are DNA repair proteins, stress response proteins, and metabolism-related proteins. Most of these have been previously characterized as ionizing radiation-induced proteins. The 23 down-regulated proteins are mainly involved in cellular metabolism, and some of these are key enzymes in the metabolic pathway. Thus, RecX is suggested to be involved in the switch between DNA damage response and normal metabolism in *D. radiodurans*.

Key words: *Deinococcus radiodurans*, DNA repair, RecX, two-dimensional electrophoresis.

Abbreviations: ACN, acetonitrile; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulphonate; 2DE, two-dimensional electrophoresis; DTT, dithiothreitol; IR, ionizing radiation; MALDI, matrix-assisted laser desorption ionization mass spectroscopy; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

RecX was first described in 1993 as a bacterial RecA regulatory protein in *Pseudomonas aeruginosa* (*1*). Since then, RecX homologues have been found in many species from each of the major bacterial lineages. In *Escherichia coli* and many other bacteria, *recX* is usually found downstream of *recA* and is cotranscribed with *recA* (*2–4*). Biochemical assays have established that RecX inhibits both RecA recombinase and coprotease activities through direct interaction with the RecA protein or its filaments and acts as an anti-recombinase to quell inappropriate recombinational repair during normal DNA metabolism (*4–6*). However, the *Deinococcus radiodurans recX* homologue (DR1310) is located far from *recA* (DR2340), and the RecX protein shows dual negative regulation of RecA function—it not only directly inhibits RecA activity at the protein level but also inhibits RecA induction at the transcriptional level (*7*). In addition, RecX was found to down-regulate the expression of catalase (CAT) and superoxide dismutase (SOD) and was involved in the anti-oxidant mechanisms of *D. radiodurans* (*8*).

To identify the repertoire of proteins whose expressions are affected by RecX, comparative proteomic studies were undertaken. By comparing the two-dimensional electrophoresis (2DE) gel of the wild-type strain and *recX* mutant, we found that RecX could inhibit the expression of DNA damage-induced proteins, including the known DNA repair proteins RecA, SSB and PprA, and facilitate the expression of some important proteins involved in normal metabolism in *D. radiodurans*.

*To whom correspondence should be addressed. Tel: +13-658612032, Fax: 086-531-88362928, E-mail: dhsheng@sdu.edu.cn

MATERIALS AND METHODS

Strains and Growth Conditions—D. radiodurans wild-type strain R1 and the *recX* mutant RecX$^-$ *(8)* were used in this study and grown in TGY broth [0.8% Bacto Tryptone, 0.4% Bacto Yeast Extract and 0.1% glucose (pH 7.0)] at 32°C with aeration. Cells were harvested in the mid-exponential phase by centrifugation at 8000*g* for 10 min and stored at −80°C.

Sample Preparation for 2DE—Deep-frozen cells were directly disrupted in lysis buffer {8 M urea, 4% w/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulphonate (CHAPS), 1% w/v dithiothreitol (DTT), 2% v/v immobilized pH gradient (IPG) buffer (pH 3–10), and 40 mM Tris base} by 2/5 intermittent sonication for 9 min. Cell debris was removed by centrifugation (25,000*g* for 20 min), and the clear supernatant was aliquoted and stored at −80°C until further analysis. Protein concentrations were measured by the Bradford assay.

2DE—2DE was performed according to the instructions provided in the handbook from GE Healthcare (Uppsala, Sweden). The sample was electrofocused as follows: 50 μA per strip at 20°C followed by rehydration at 30 V for 12 h, 500 V for 1 h (step and hold), 1,000 V for

1 h (step and hold) and 8,000 V (step and hold) until the total volt-hours reached 64.0 kVh. After isoelectric focusing, the strips were equilibrated twice with gentle shaking for 15 min in the sodium dodecyl sulfate (SDS) equilibration buffer. The first step was performed in an 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 that was modified by replacing DTT with 2.5% w/v iodoacetamide. After equilibration, the strips were loaded onto a 12.5% SDS gel and run at 1500 mW until the dye front reached the bottom of the gel.

MALDI-TOF Mass Spectrometric Analysis and Database Search—Chemical destaining and enzymatic digestion of protein spots were performed as previously described (*9*, *10*). Mass spectrometric (MS) analysis was performed as described by Gharahdaghi *et al*. (*11*) with some modifications. Peptide mixtures (1 μl) were mixed with an equal volume of 10 mg/ml α-cyano-4--hydroxycinnamic acid (Sigma) saturated with 50% acetonitrile (ACN) in 0.05% trifluoroacetic acid (TFA) and analysed in a matrix-assisted laser desorption ionization (MALDI) MS using a delayed ion extraction and ion mirror reflector mass spectrometer (Applied Biosystems, CA, USA). The instrument was set in the reflector mode with 160-ns delay extraction time, positive polarity, 60–65% grid voltage, and 20,000 V accelerating voltage. Laser shots (200 per spectrum) were used to acquire the spectra in the mass range 1,000–4,000 Da. External calibration was carried out using the Peptide Mass Standard Kit (PerSeptive Biosystems, MA, USA), and the matrix and autolytic peaks of trypsin served as internal standards for mass calibration.

The mass spectra were interpreted using the Mascot peptide mass fingerprint program available on the Mascot web site (http://www.matrixscience.com).

Immunoblot Analysis—The samples were separated on 2DE gel and the desirable proteins were detected with RecA antibody (rat IgG, laboratory stock) using a standard immunoblot protocol (*12*).

RNA Preparation and RT–PCR Analysis—*Deinococcus* cells were resuspended in TE buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA) and pre-incubated with 20 μg/ml lysozyme at 30°C for 10 min. Total RNA from the cells was prepared using an RNA extraction kit according to the manufacturer's instructions (Shenergy Biocolor, Shanghai, China), and the amounts were quantified spectrophotometrically. Reverse transcription was carried out with random primers using the First-Strand cDNA Synthesis Kit (Shenergy Biocolor, Shanghai, China), and PCR was subsequently performed according to a standard protocol (*12*).

RESULTS

The Absence of RecX Results in Changes in the Protein Expression Pattern—Total proteins from R1 and RecX$^-$ were prepared and separated on 2DE gel according to the method described in MATERIALS AND METHODS section. In the pH range 4–7, 462 well-resolved proteins were detected in the R1 gel, and 405 proteins were detected in the RecX$^-$ gel (Fig. 1). In the mutant, more proteins were down-regulated than up-regulated, suggesting that RecX promotes more genes expression besides its inhibition on *recA* induction.

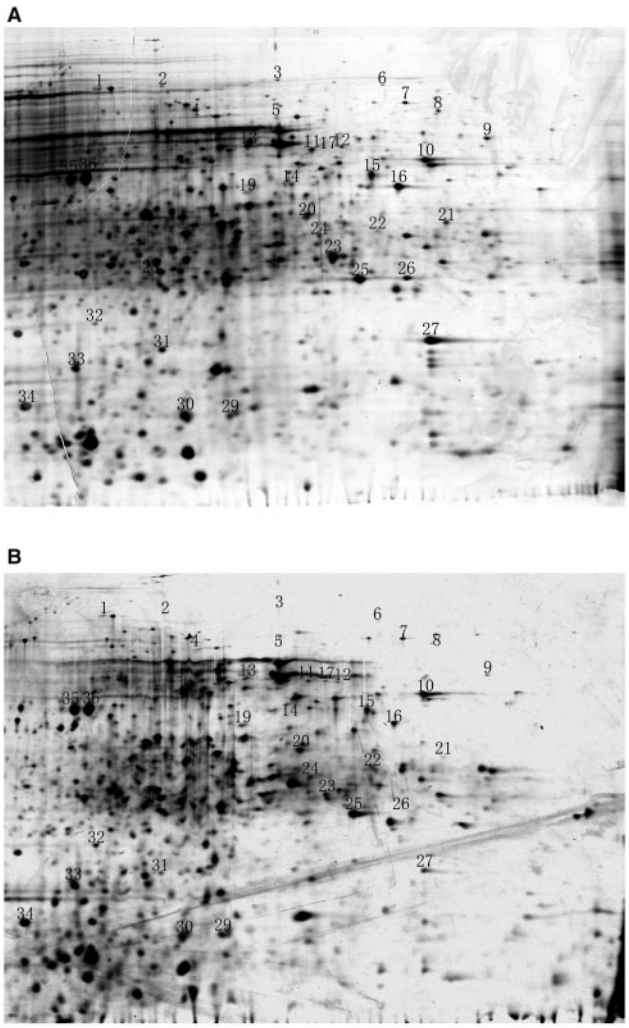

Fig. 1. **2DE PAGE analysis of cellular proteins of R1 (A) and RecX$^-$ (B).** The extracted proteins were separated by isoelectric focusing in the pH range 4–7 in the first dimension and by 12.5% vertical SDS-PAGE in the second dimension. The resolved proteins were visualized by silver staining. The analyzed spots were numbered in the gels, and the numbers in the two gels were matched.

Under normal conditions, the expression of *D. radiodurans* RecA is low and therefore difficult to detect in a 2DE gel. However, immunological analysis facilitated the detection of RecA (Fig. 2) in both gels, and the absence of RecX was found to enhance *recA* expression, which is in agreement with previously reported results (*7*). To further study the proteins regulated by RecX, protein spots whose expression obviously differed between the two gels were excised from the gels and subjected to peptide mass fingerprinting analysis to assign their putative functions. Thirty-five proteins, of which 12 were up-regulated and 23 were down-regulated, were identified successfully (Table 1). These proteins mainly belong to three functional classes—DNA

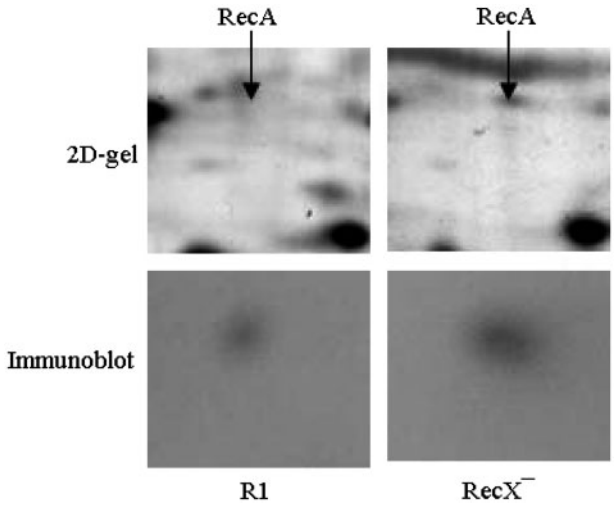

Fig. 2. **Immunoblot analysis of RecA.** According the theoretical pI/MW of RecA (5.45/38145), all the proteins between PH 5-6 and molecular weight 25000–66000 were transferred to a nitrocellulose membrane, and anti-RecA antibody (rat IgG, laboratory stock) was used to locate and quantify RecA in 2DE gel.

recombination and repair, metabolism and environmental stress response.

To independently confirm the validity of the proteomic results, the transcription of the identified proteins was analysed using RT–PCR. Changes in the transcript abundance of the 35 genes are shown in Fig. 3. Although changes in the magnitude of transcription were slightly different from the changes in the protein intensity in the 2D gel, the 12 up-regulated proteins showed up-regulated transcriptional activities, while the 23 down-regulated proteins showed down-regulated transcriptional activities in the mutant. Therefore, RecX regulation of these proteins occurs at the transcriptional level.

RecX Inaction Enhances the Expression of IR Damage-induced Protein—Among the 12 up-regulated spots, 8 (4, 17, 19, 20, 24, 25, 29, and 33) were previously reported as being ionizing radiation (IR)-induced proteins (*13*, *14*). Spot 4 (GroEL, DR0607) is a heat-shock protein. Spots 17, 19 and 20 are the known DNA repair proteins RecA, SSB and PprA (*13–15*). Spot 24 is enoyl–acyl carrier protein reductase (FabI, DR1967), which belongs to the oxidoreductase family and catalyses an essential step in fatty acid biosynthesis (*16*). Although its role in the DNA damage reaction remains unclear, FabI is induced by IR (*15*). Spots 25 and 33 were identified as superoxide dismutase (SOD, DR1279) and ferredoxin (DR2075), respectively, which are important IR-induced proteins involved in the anti-oxidant mechanisms of *D. radiodurans* (*8*, *13*, *17*). Scavenging of reactive oxygen species (ROS) is important in *D. radiodurans* radioresistance, because the lethal effect of IR is known to be enhanced in the presence of oxygen by the generation of oxygen radicals and hydrogen peroxide that damage the cell membrane, proteins and nucleic acids (*8*, *15*). RecX is involved in ROS scavenging and acts by inhibiting the induction of anti-oxidant enzymes. Thus, the up-regulated expression of SOD and ferredoxin in the mutant is in agreement with previously reported results (*8*, *13*). In addition, bacterioferritin comigratory protein (BCP, DR0846) is also under the control of RecX. In *E. coli*, BCP was shown to be an oxygen stress-induced protein that could reduce hydrogen peroxide and other organic peroxides as substrates, and it plays an important role in protecting cells against oxygen-induced oxidative stress (*18*, *19*). Expression of these IR-related proteins is enhanced in the mutant, suggesting that RecX is involved in the negative regulation of IR damage induction.

Two proteins that are up-regulated in the mutant (DR0278 and DR1467) are involved in the metabolism of carbohydrates and lipids, respectively, while a third up-regulated protein (DR1185) is involved in the environmental stress response (*15*); however, at present, it is unclear whether these are involved in the IR damage reaction.

RecX is Also Involved in the Cellular Metabolism of Amino Acids, Carbohydrates, Lipids and Inorganic Ions and in Energy production—Of the 23 proteins that were identified as being down-regulated, 20 are involved in the metabolism of amino acids, carbohydrates, lipids and inorganic ions and in energy production. Some of these, such as citrate synthase (DR0757), pyruvate dehydrogenase complex (DR2370), glyceraldehyde 3-phosphate dehydrogenase (DR1343), fructose-bisphosphate aldolase (DR1589), ketol-acid reductoisomerase (DR1519) and acetyl-CoA acetyltransferase (DR2480), are key enzymes in metabolic pathways.

The expression of five metabolism-related proteins was increased in the mutant. Of these, three (DR0846, DR1967 and DR2075) are radiation-induced proteins, which were described above, and two, i.e. phosphoglycerate mutase (DR0278) and oxidoreductase (DR1467), are involved in the metabolism of carbohydrates and lipids, respectively. Sequence blast analysis showed that DR0278 has 4 homologues (DR0278, DR1079, DR1393 and DR2227), while DR1467 has 12 homologues in *D. radiodurans*; however, the functions of these homologues are unknown at present. They may belong to different metabolic pathways (*15*).

Therefore, it is suggested that RecX plays an important role in the regulation of multiple metabolic pathways, especially in facilitating the primary metabolism of amino acids, carbohydrates, lipids and inorganic ions and in energy production in *D. radiodurans*, showing its positive regulation on cellular normal metabolism.

RecX Regulation In Environmental Information Processing—Four of the identified proteins were found to be involved in environmental stress response. Three of these were up-regulated in the absence of RecX. DR1279 and DR0607 encode SOD and GroEL, respectively, as mentioned earlier. DR1185 is a surface layer (S-layer)-like array-related protein. S-layers composed of proteins or glycoproteins are a common surface structure in prokaryotes (*20*). The role of S-layers in extreme resistance has not yet been elucidated, but it has been proposed that these participate in the response to radiation damage (*21*). As an S-layer component, DR1185 is

Table 1. **Protein spots whose expression levels are altered in the mutant.**

Spot number	Identified protein	Gene number	Theoretical pI/MW	Coverage (%)	Fold change	Functional category
Proteins that were up-regulated in the mutant						
17	RecA protein	DR2340	5.45/38,145	26.8	2.7	DNA repair
19	Single-stranded DNA-binding protein	DR0099	6.30/15,684	63.1	4.0	DNA repair
20	DNA damage repair protein	DRA0346	5.54/32,210	49.3	5.7	DNA repair
28	Phosphoglycerate mutase	DR0278	6.43/22,171	26.5	3.1	Carbohydrate metabolism
22	Oxidoreductase	DR1467	5.63/27,400	26.5	2.3	Lipid metabolism
24	Enoyl–acyl carrier protein reductase	DR1967	5.33/28,492	37.2	2.7	Lipid metabolism
32	Bacterioferritin comigratory protein	DR0846	4.95/19,057	35.5	2.7	Inorganic ion metabolism
33	Ferredoxin	DR2075	4.83/12,692	27.3	2.8	Energy production
4	GroEL protein	DR0607	5.03/57,777	39.1	1.2	Stress response
25	Superoxide dismutase (sodA), Mn family	DR1279	5.55/23,464	41.0	2.9	Stress response
34	S-layer-like array-related protein	DR1185	9.33/36,707	20.5	2.7	Stress response
29	Hypothetical protein	DR2373	5.35/15,374	46.9	2.6	Unknown
Proteins that were down-regulated in the mutant						
1	Serine protease, subtilase family	DRA0283	4.90/76,708	37.9	−3.4	Amino acid metabolism
12	Ketol-acid reductoisomerase	DR1519	5.65/39,860	23.3	−2.8	Amino acid metabolism
35	Proline iminopeptidase-related protein	DR0654	4.46/34,906	27.3	−1.7	Amino acid metabolism
36	Peptidase-related protein	DR0478	5.03/57,742	17.5	−2.3	Amino acid metabolism
2	Aconitate hydratase, B subunit	DR1720	4.94/97,941	54.7	−6.5	Carbohydrate transport and metabolism
5	β-Lactamase-like protein	DRA0304	5.65/53,019	21.4	−4.1	Carbohydrate metabolism
7	Pyruvate dehydrogenase complex	DR2370	5.58/49,182	28.8	−1.8	Carbohydrate metabolism
9	Fumarate hydratase	DR2627	6.07/49,876	16.5	−3.3	Carbohydrate metabolism
10	Glyceraldehyde 3-phosphate dehydrogenase	DR1343	5.65/35,712	29.1	−2.7	Carbohydrate metabolism
11	Citrate synthase	DR0757	5.86/45,523	51.8	−3.1	Carbohydrate metabolism
15	Succinyl-CoA synthetase, α subunit	DR1248	5.47/30,883	32.5	−1.8	Carbohydrate metabolism
16	Fructose-bisphosphate aldolase	DR1589	5.48/32,421	25.5	−3.6	Carbohydrate metabolism
3	Acyl-CoA dehydrogenase	DR2361	5.24/66,194	19.5	−3.0	Lipid metabolism
6	3-Hydroxyacyl-CoA dehydrogenase	DR2477	5.81/84,739	31.5	−2.1	Lipid metabolism
13	Acetyl-CoA acetyltransferase	DR2480	5.15/40,858	25.6	−2.9	Lipid metabolism
8	Toxic anion resistance protein	DR1127	5.91/46,055	29.0	−4.1	Inorganic ion metabolism
30	Tellurium resistance protein	DR2220	5.25/16,618	37.5	−2.5	Inorganic ion metabolism
14	Potassium channel, β subunit, putative	DR2317	5.33/35,134	59.9	−3.0	Energy production
21	Cytochrome complex iron–sulphur subunit	DR0435	4.92/18,265	61.6	−2.8	Energy production
26	V-type ATP synthase, E subunit	DR0697	5.68/20,139	41.2	−6.0	Energy production
23	Phage shock protein A	DR1473	5.51/24,591	37.3	−3.6	Stress response
31	Transcription elongation factor GreA	DR1970	4.94/18,053	16.5	−5.1	Transcription
27	Hypothetical protein	DR1768	7.93/15,164	32.0	−3.3	Unknown

MALDI-TOF MS measurement and database search led to the identification of these spots. Classification of the proteins is based on NCBI's clusters of orthologous groups (COGs). The percentage coverage is the amount of the protein sequence covered by the matched peptides. Statistical significance of replicates was accepted when $P \leq 0.05$.

important in the maintenance of cell envelope integrity in response to specific environmental conditions (*15*, *20*, *22*).

Another stress response-related protein, phage shock protein A (PspA, DR1473), is down-regulated in the mutant. PspA is encoded by the first gene of the *pspABCDE* operon, the expression of which can be transiently induced by several membrane-altering stresses (phage, heat, ethanol and osmotic shock). PspA was found to negatively regulate the expression of the *pspABCDE* operon (*23*, *24*). RecX promoted PspA expression; therefore, it inhibited the expression of other phage shock proteins in *D. radiodurans* to a certain extent.

Environmental stress causes damage to macromolecules (in particular, DNA and proteins). Cells have evolved elaborate systems (environmental stress response) to monitor the features of their environment and rapidly mount defence systems against environmental stress. Here, RecX regulating on the induction of stress response proteins suggests that the protein is involved in environmental information processing.

DISCUSSION—Deinococcus radiodurans is well-known for its remarkable resistance to severe damage caused by IR, desiccation, UV radiation and oxidizing agents. It is a popular model organism for DNA repair studies (*15*, *25*). Following DNA damage, there are changes in the cellular abundance of proteins, with an increase in the synthesis of some DNA repair-related proteins, including RecA, PprA, SSB and SOD (*13*, *14*). However, at present, the mechanism of DNA repair gene induction is unclear. Two LexAs are found in *D. radiodurans*, but neither of these is involved in the DNA

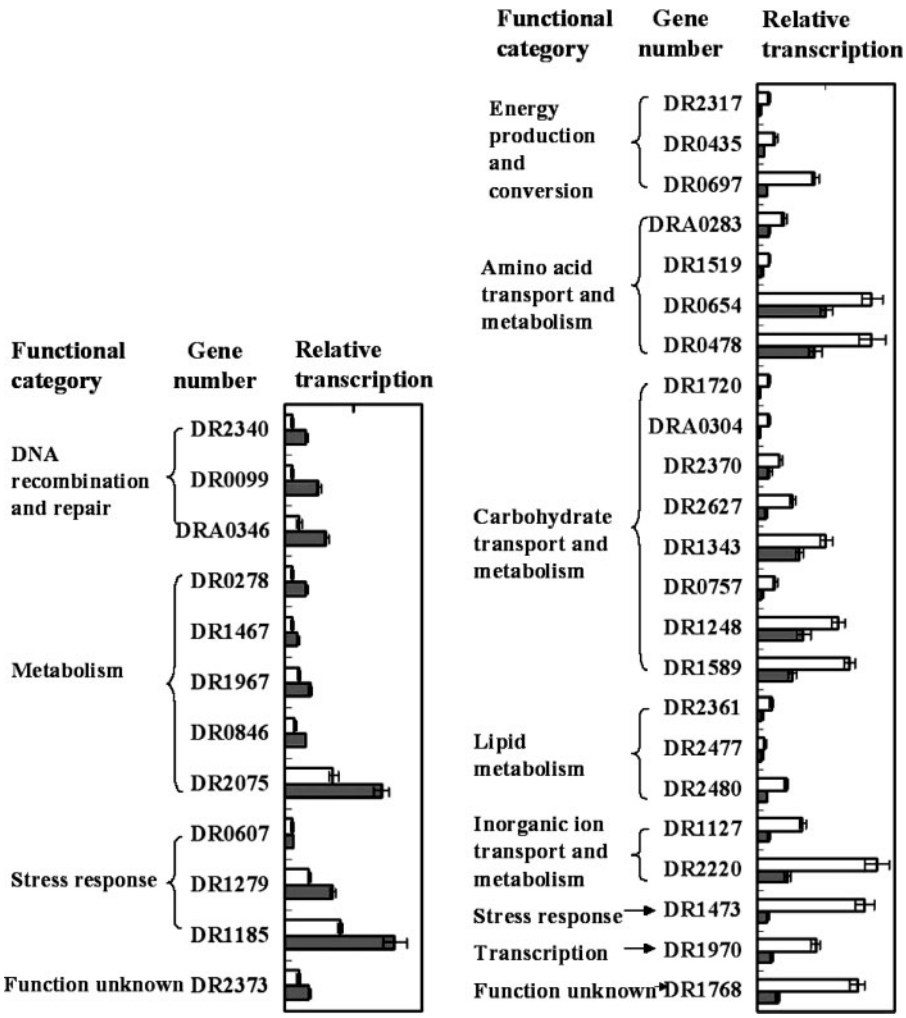

Fig. 3. **RT-PCR analysis of the transcriptional abundance of the genes of the 35 identified proteins in R1 (white) and RecX⁻ (grey).** Quantification of the intensity of the PCR product. The values are the means ± SD obtained from four individual experiments.

damage induction reaction (*5*). In this study, the absence of RecX resulted in increased expression of these DNA damage-induced proteins; thus, RecX participates in inhibiting the DNA damage induction reaction. Although the regulation mode is still under investigation, RecX was found to interact with DNA, and its N-terminus sequence that is not present in *E. coli* RecX might be involved in the interaction; this may be helpful for understanding RecX inhibition of protein induction.

In addition to inhibiting the radiation damage repair protein, RecX is also involved in the regulation of cellular metabolism and environmental stress information processing and behaves as a pleiotropic regulator. In contrast to most prokaryotes, *D. radiodurans* is believed to have a DNA damage checkpoint that regulates the switch between DNA damage response and cell cycle progression (*15*). RecX exerts its effects on the switch by facilitating normal cellular metabolism and arresting the IR damage-induced response and other stress responses; therefore, it may be involved in the checkpoint reaction.

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CONFLICT OF INTEREST

None declared.

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