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Hsp20, a Small Heat Shock Protein of *Deinococcus radiodurans*, Confers Tolerance to Hydrogen Peroxide in *Escherichia coli* S

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The present study shows that DR1114 (Hsp20), a small heat shock protein of the radiation-resistant bacterium *Deinococcus radiodurans*, enhances tolerance to hydrogen peroxide (H_2O_2) stress when expressed in *Escherichia coli*. A protein profile comparison showed that *E. coli* cells overexpressing *D. radiodurans* Hsp20 (EC-pHsp20) activated the redox state proteins, thus maintaining redox homeostasis. The cells also showed increased expression of pseudouridine (psi) synthases, which are important to the stability and proper functioning of structural RNA molecules. We found that the *D. radiodurans* mutant strain, which lacks a psi synthase (DR0896), was more sensitive to H_2O_2 stress than wild type. These suggest that an increased expression of proteins involved in the control of redox state homeostasis along with more stable ribosomal function may explain the improved tolerance of EC-pHsp20 to H_2O_2 stress.

Keywords: *Deinococcus radiodurans*, small heat shock protein (sHSP), Hsp20, H_2O_2 tolerance

Heat shock proteins (HSPs) are molecular chaperones that maintain cellular homeostasis. They bind to and assist in the proper folding of misfolded cellular proteins, thereby preventing protein aggregation [6, 10, 25]. These processes play an important role in protecting cells against various stresses, such as radiation, heat, cold, and oxidative stress [9]. Among the various HSPs that have been identifed, small HSPs (sHSPs) are proteins of low molecular mass (12–30 kDa) that are present in organisms from all domains of life [4, 17].

Deinococcus radiodurans is a Gram-positive coccus that is highly resistant to a variety of stresses, including gamma radiation (γ-radiation), UV light, desiccation, heat, hydrogen peroxide (H_2O_2), and other DNA-damaging agents [18]. *D. radiodurans* contains two sHSPs, Hsp17.7 (DR1691) and Hsp20.2 (DR1114). The two proteins differ in sequence, structure, and function. Hsp17 forms a dimer and unstable substrate complexes [2], whereas Hsp20 shows constitutive and stable expression, typical chaperone activity, and forms spherical homo-oligomers and stable substrate complexes. The expression of *hsp20* (*dr1114*) has been found to be up-regulated under the conditions of stress [2, 24].

Overexpression of sHSPs can improve the stress tolerance of sensitive hosts like *Escherichia coli*. For example, Hsp20 from the hyperthermophilic archaeon *Sulfolobus solfataricus* was found to enhance the thermotolerance of *E. coli* [17]. In addition, the overexpression of deinococcal genes such as *pprI*, which encodes a global regulator responsible for extreme radioresistance, and *pprA*, which encodes a radiation-induced DNA repair protein, in *E. coli* improved its tolerance to salt and oxidative stress [16, 20]. Here, we overexpressed the *D. radiodurans hsp20* gene in *E. coli* and investigated its effect on the stress tolerance.

The *D. radiodurans hsp20* gene was amplified by PCR using the primers hsp20F (5′-aca tat ggt ctc gaa tga gcg cag gtg gct caa c-3′) and hsp20R (5′-att tat ggt ctc agc gct tta ttc cgt ggc ggc ggt gt-3′). The amplified product was digested with *Bsa*I and then ligated into *Bsa*I-digested pASK-IBA3 (IBA GmbH, Goettingen, Germany). The ligated construct (pHsp20) was transformed into *E. coli* EPI300 cells (Epicentre Biotechnologies, Madison, WI, USA) and transformants were selected on LB (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) agar plates containing 100 μg/ml ampicillin. Positive clones were confirmed by sequencing. The

overexpression of *hsp20* was induced at an optical density (OD_{600}) of ~0.3 by anhydrotetracycline (AHT) at a final concentration of 200 ng/ml. SDS-PAGE analyses of the recombinant cells showed an overproduction of a 20 kDa protein (data not shown).

To investigate the effect of *D. radiodurans* Hsp20 on the stress tolerance, *E. coli* cells harboring pHsp20 (EC-pHsp20) were grown at 37°C to an OD_{600} of 0.4 and then incubated with AHT for an additional 2 h. The cells were then exposed to different stresses: (i) heat, incubation at 54°C for 30 min; (ii) mitomycin C (MMC), incubation with 0.1 μg/ml MMC for 1 h; (iii) γ-radiation, 50, 100, and 200 Gy; (iv) UVC, 600 J/m^2; and (v) H_2O_2, incubation with H_2O_2 (5–30 mM) for 1 h. After treatment, the cells were serially diluted and 10 μl of each dilution was spotted on LB-ampicillin (100 μg/ml) plates. Following incubation overnight at 37°C, the number of colony-forming units (CFU) was determined and the survival fraction was calculated after a comparison with empty vector cultures of *E. coli* (EC-pASK). Survival assays using heat-, MMC-, γ-radiation-, and UV-treated cells did not show any difference in sensitivity between EC-pASK and EC-pHsp20, but H_2O_2 stress resulted in improved tolerance in EC-pHsp20 (Fig. 1A). The difference in survival fraction between the two strains was more than 1 log cycle.

To determine the reason for the increased H_2O_2 tolerance of the cells, the protein profile of EC-pHsp20 was compared with that of EC-pASK, using liquid chromatography integrated with electrospray ionization mass spectrometry (LC/ESI-MS). Protein samples were separated by 12% SDS-PAGE followed by in-gel digestion [14]. The digested peptides were extracted, separated, and identified by an LC/ESI ion trap MS at the Korea Basic Science Institute (Daejeon, Korea). For protein identification, MS/MS spectra were searched by MASCOT 2.4.0 (Matrix Science, London, UK). The genomic sequence of *E. coli* K12 was used as the database for protein identification. For a quantitative analysis of the LC/ESI-MS data, the protein abundance index (PAI; the number of observed peptides per protein normalized by the theoretical number of peptides) was calculated [12, 21]. The exponentially modified PAI (emPAI) was then used to estimate the protein abundance in the protein mixture (expressed as mol%) [12]. In the present analysis, a total of 36 proteins were abundantly detected only in EC-pHsp20 (Table S1), and 58 proteins showed greater induction in EC-pHsp20 than in EC-pASK (Table S2). The proteins detected only in EC-pHsp20 and the most highly up-regulated proteins in EC-pHsp20 are shown in Table 1.

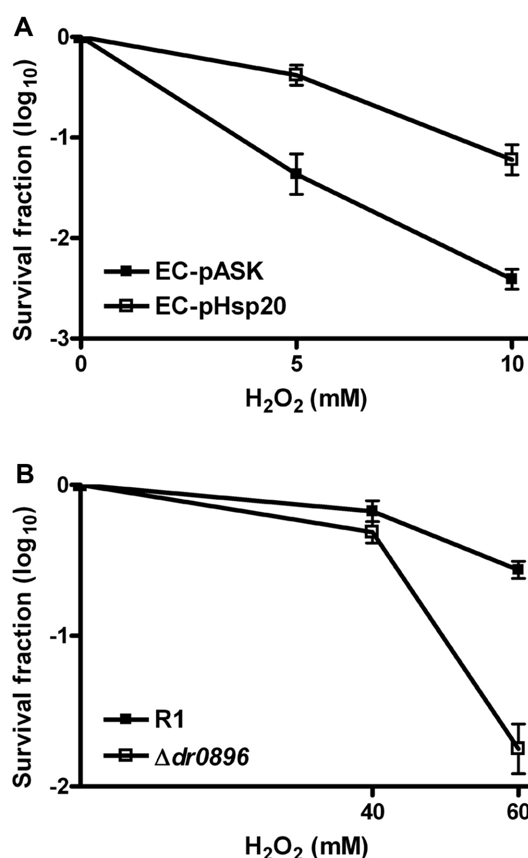

Fig. 1. Hydrogen peroxide (H_2O_2) stress tolerance assay.
(**A**) Exponential phase *E. coli* cells harboring the Hsp20 recombinant plasmid (EC-pHsp20) and empty vector (EC-pASK) and (**B**) *D. radiodurans* wild-type (R1) and deletion mutant (Δ*dr0896*) strains were treated with different concentrations of H_2O_2 for 1 h under shaking culture conditions. The cells were then plated on (**A**) LB and (**B**) TGY plates, followed by serial dilution to assess survival ability. Each assay was carried out in triplicates, and mean survival fraction was calculated and plotted in the figure.

Our LC/ESI-MS data indicated that proteins involved in the control of cellular redox status (GrxC, IscA, and Fdx), protein translation (ribosomal proteins), and rRNA/tRNA modification (RluC, TruB, RluB, RsuA, RsgA, and RlmE) were activated by Hsp20 overexpression. Glutaredoxins are involved in maintaining the redox state of proteins and in protecting them against oxidative damage [22]. IscA, an iron (Fe) chaperone, is part of the housekeeping Fe-S cluster assembly [7]. Fe-S clusters are the cellular redox centers and targets of oxidative stress, and *isc* gene clusters are involved in the biogenesis of Fe-S clusters [7]. Cells with higher levels of IscA protein might be more efficient in repairing and assembling damaged Fe-S proteins. Ferredoxins play an important role in a metabolism and are

Table 1. Proteins showing increased expression in EC-pHsp20.

Proteins detected only in EC-pHsp20			Proteins up-regulated in EC-pHsp20		
Name	Description	Mol%[a]	Name	Description	Fold change[b]
ZapB	Cell division protein	0.063	RluB	Ribosomal large subunit pseudouridine synthase B	5.740
GrxC	Glutaredoxin-3	0.060	SdaB	L-Serine dehydratase 2	4.615
IscA	Iron-binding protein IscA	0.033	RsgA	Putative ribosome biogenesis GTPase	4.559
Fdx	2Fe-2S ferredoxin	0.030	RsuA	Ribosomal small subunit pseudouridine synthase A	4.411
Cnu	OriC-binding nucleoid-associated protein	0.029	RhlB	ATP-dependent RNA helicase rhlB	4.387
RluC	Ribosomal large subunit pseudouridine synthase	0.028	FdoG	Formate dehydrogenase-O major subunit	3.815
Pth	Peptidyl-tRNA hydrolase	0.025	AroE	Shikimate dehydrogenase	3.633
PdxY	Pyridoxamine kinase	0.024	RlmE	Ribosomal RNA large subunit methyltransferase	3.406
YhiR	Uncharacterized protein yhiR	0.023	HemC	Porphobilinogen deaminase	3.282
Tdk	Thymidine kinase	0.023	Tmk	Thymidylate kinase	3.207
TruB	tRNA pseudouridine synthase	0.022	GlpQ	Glycerophosphoryl diester phosphodiesterase	3.027

[a]emPAI-based mole percentage (mol% > 0.2) [12].

[b]Fold change is the ratio of the mol% from EC-pHsp20 to the mol% from EC-pASK (fold change > 3.0).

involved in electron transfer processes [5]. Oxidative stress down-regulates ferredoxin, and its overexpression can counteract this effect [8]. Collectively, the up-regulation of redox homeostasis genes may contribute to the increased tolerance of EC-pHsp20 to oxidative stress.

Bepperling *et al*. [2] recently reported that many different proteins can act as potential substrates for Hsp20 in *Deinococcus*, including proteins involved in translation, such as ribosomal proteins and elongation factors. In this study, we found that pseudouridine (psi) synthases (RluB, RluC, RsuA, and TruB) were among the proteins that were strongly induced or highly abundant in EC-pHsp20 (Table 1). These synthases are involved in the isomerization of uridine to pseudouridine in structural RNA [26]. The exact role of psi synthases is still not completely understood, but they have been suggested to be required for the proper assembly and functioning of ribosomes [19]. Among the different psi synthases, only TruB has been intensively studied. The mutant of a *truB* homolog in *Saccharomyces cerevisiae* showed no alteration in phenotype [1], whereas in *Pseudomonas aeruginosa* sensitivity to high temperature and a decreased salt stress tolerance were observed [23]. An *E. coli truB* mutant exhibited growth defect and the accumulation of outer membrane proteins, which affected the activity of the alternative sigma factor RpoE. This, in turn, affected the heat stress regulon, resulting in a heat stress-sensitive cell population [15]. Thus, the psi synthase function is essential for the stability of structural RNA [19], but to the best of our knowledge, its role in oxidative stress tolerance has not been reported.

To determine the role of psi synthases, we searched for psi synthases in *D. radiodurans*. *D. radiodurans* encodes seven psi synthases. Three (DRA1789, DR0961, and DR1684) belong to the RluA family, whereas DR0896, DR2290, DR1323, and DR1991 belong to the RsuA, TruA, TruB, and TruD families, respectively [3, 13, 19]. Because RluB was the most strongly up-regulated protein in EC-pHsp20 (Table 1), we investigated the effect of RluB on H_2O_2 tolerance in *D. radiodurans*. The homolog of *rluB* in *D. radiodurans* is *dr0896* [3]. The deletion of *D. radiodurans dr0896* was achieved by deletion mutagenesis, as described by Im *et al*. [11]. In brief, the upstream and downstream regions of *dr0896* were PCR-amplified using the primers UpF/UpR (5′-tat act cga gta aag gtg cgt ccg ttg gtc-3′/5′- tct aga tat cag ctc ctc ggc ggc gc-3′) and DnF/DnR (5′-ttc atc tag agc acc cgc aca aga ccc-3′/5′- tat act gca ggc tga cct cgc gct tca a-3′) and then cloned into the *Xho*I/*Eco*RV and *Xba*I/*Pst*I sites of pKatAPH3 [11], respectively. The resulting plasmid was transformed into wild-type *D. radiodurans*, and the *dr0896* deletion was confirmed by diagnostic PCR using the primers DiaF/DiaR (5′-tgc tcg cct ggg gcc tgt tt-3′/5′- ctc acc ttc cct gcc gaa aaa gc-3′) and by nucleotide sequencing. Cells grown to the log phase (OD_{600} = ~1.0) in TGY medium (1% tryptone, 0.5% yeast extract, and 0.1% glucose) were adjusted to ~10^7 CFU/ml and then exposed to 40 or 60 mM of H_2O_2. The cells were then spotted on

TGY-kanamycin (50 μg/ml) agar plates and incubated at 30°C for 2−3 days. As compared with the wild-type control culture, the *dr0896* mutant was found to be sensitive to H_2O_2 with more than 1 log cycle difference (Fig. 1B). This suggests that DR0896, the deinococcal psi synthase, contributes to cellular tolerance to oxidative stress. Taken together, Hsp20 (DR1114) from *D. radiodurans,* when overexpressed in *E. coli*, activates redox state proteins and increases psi synthase expression, thereby promoting efficient redox homeostasis and stable translational machinery, leading to improved tolerance to H_2O_2-generated oxidative stress.

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