LIÈGE université
Gembloux
Agro-Bio Tech

Bacterial adaptation to temperature stress: molecular responses in two Gram-positive species from distinct ecological niches

Dong XUE

Promoteur: Prof. Marc ONGENA

Co-promoteur: Prof. Jin WANG

2020

COMMUNAUTÉ FRANÇAISE DE BELGIQUE
UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

Bacterial adaptation to temperature stress: molecular responses in two Gram-positive species from distinct ecological niches

Dong XUE

Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

Promoteurs: Prof. Marc Ongena & Prof. Jin Wang

Année civile: 2020

Résumé

Dong XUE (2020) ***Adaptation bactérienne au stress thermique : réponses moléculaires chez deux espèces Gram-positives de niches écologiques distinctes*** (thèse de doctorat). Gembloux, Belgique, Liège Université, Gembloux Agro-Bio Tech, 133 p., 14 Tableaux, 44 Figures.

Résumé : Les micro-organismes sont souvent affectés par divers facteurs environnementaux. Ces facteurs environnementaux affectent leurs fonctions physiologiques et biochimiques. Parmi ces facteurs environnementaux, la température joue un rôle important dans les activités physiologiques normales des micro-organismes. Pour s'adapter à différents environnements de température, les bactéries ont développé de nombreux mécanismes adaptatifs pour coordonner une gamme de changements dans l'expression génique et l'activité protéines. Dans cette étude, nous avons étudié les mécanismes d'adaptation de deux bactéries Gram-positives à différentes températures. Les principaux résultats de cette thèse sont les suivants :

(1) *Deinococcus radiodurans* est une bactérie Gram-positive, pigmentée de rose et à haut G + C. La réponse thermique de *D. radiodurans* est considérée comme un système de régulation classique induit par le stress qui se caractérise par une reprogrammation transcriptionnelle étendue. Dans cette partie, nous avons étudié les gènes fonctionnels clés impliqués dans le stress thermique qui a été exprimé et accumulé dans les cellules après un traitement thermique à 48 °C pendant 2 heures (R48). Considérant que la dégradation des protéines est un bioprocessus chronophage, nous avons prédit que pour maintenir l'homéostasie cellulaire, l'expression des protéines fonctionnelles clés serait considérablement diminuée dans les cellules qui s'étaient partiellement rétablies du stress thermique (RH) par rapport à leur expression dans les cellules cultivées sous température optimale (R30). La transcriptomique comparative a identifié 15 gènes qui étaient significativement régulés à la baisse dans l'RH par rapport à R30, dont sept avaient été précédemment caractérisés comme étant des protéines de choc thermique. Parmi ces gènes, trois gènes hypothétiques (*dr_0127*, *dr_1083* et *dr_1325*) sont très susceptibles d'être impliqués en réponse au stress thermique. L'analyse de survie des souches mutantes dépourvues de *dr_0127*, *dr_1083* et *dr_1325* a montré une réduction de la tolérance à la chaleur par rapport à la souche de type sauvage. Ces résultats suggèrent que *dr_0127*, *dr_1083* et *dr_1325* pourraient jouer un rôle dans la réponse au stress thermique.

Sur la base de nos données RNA-seq et des rapports précédents, nous avons identifié deux nouveaux ARN non codants (ncRNA) inductibles par la chaleur chez *D. radiodurans*, nommés *DnrH* et *dsr11*. L'analyse de la tolérance à la chaleur a montré que la suppression de *DnrH* inhibait significativement la viabilité en réponse à des conditions de température élevée. Des analyses phénotypiques et qRT-PCR comparatives d'un mutant *DnrH* (Δ*DnrH*) et de type sauvage (WT) ont suggéré que *DnrH* est potentiellement impliqué dans la

régulation de l'expression du gène Hsp20 lié au choc thermique. La thermophorèse à l'échelle microscopique et la complémentation génétique ont montré qu'une séquence de 28 nucléotides (nt) dans la structure tige-boucle des paires *DnrH* (143-170 nt) interagit avec son homologue dans la région codante de mRNA *Hsp20* (91-117 nt) via une séquence de 22 nt Région. *In vivo*, la mutation de la région des 22 nt dans le génome de *D. radiodurans* a entraîné une réduction de la tolérance à la chaleur similaire à celle observée chez le mutant *DnrH*. Nos résultats montrent que la *DnrH* influence positivement la tolérance à la chaleur en augmentant la transcription de mRNA de *Hsp20*, démontrant, pour la première fois, un ncRNA qui contrôle directement l'expression d'un gène de résistance au stress thermique. De manière similaire à *DnrH*, nous avons caractérisé un autre ncRNA *dsr11*. Notre résultat a montré que le niveau de transcription de *dsr11* était augmenté de 4,2 fois sous stress thermique par analyse qRT-PCR. Un essai de tolérance à la chaleur a montré que la suppression de *dsr11* inhibait significativement la viabilité dans des conditions de température élevée. Pour évaluer l'influence de *dsr11* sur le transcriptome de *D. radioduans*, 157 gènes ont été trouvés différentiellement exprimés dans le mutant knock-out par l'expérience RNA-Seq. En combinant RNA-Seq et analyse bioinformatique, nous avons constaté que *dr_0457* (protéine de transport de biopolymère) était probablement la cible directe de *dsr11*. D'autres résultats de thermophorèse à l'échelle microscopique ont démontré que le *dsr11* peut se lier directement au mRNA de *dr_0457*. Nos résultats ont indiqué que *dsr11* peut améliorer la tolérance au stress thermique de *D. radiodurans* en se liant au mRNA de *dr_0457*.

(2) *Bacillus velezensis* GA1 est une bactérie modèle Gram-positive qui vit naturellement en association avec les plantes et possède des propriétés de biocontrôle permettant de protéger son hôte végétal contre les phytopathogènes. Dans ce travail, nous avons évalué l'impact d'une température basse qui correspond à celle des sols sur cette bactérie. Une température basse a un impact négatif sur le taux de croissance, reflétant la réduction générale de l'activité métabolique des cellules cultivées à basse température. Les cultures *in vitro* ont révélé que la productivité de certains métabolites liés au biocontrôle changeait sensiblement lorsque la température était abaissée. Nous avons observé qu'après plusieurs cycles de culture à 15 et 18 ° C, la croissance de GA1 est devenue plus rapide qu'auparavant. Nous avons testé GA1 à 18 ° C sur tomate, les résultats ont montré que la formation de biofilm sur les racines était un peu plus lente qu'à 22 ° C, réduisant ainsi la population de GA1 sur ces tissus.

En résumé, cette étude a analysé en profondeur les voies d'adaptation et les mécanismes développés par deux bactéries à Gram-positive en réponse à différentes conditions de stress températures. Notre étude fournira une base théorique pour des applications futures dans l'industrie et l'agriculture.

Mots-clés : *Deinococcus radiodurans*, stress thermique, non codant RNA, *dnrH*, *dsr11*, *Bacillus velezensis* GA1, PGPR, stress froid

Abstract

Dong XUE (2020) ***Bacterial adaptation to temperature stress: molecular responses in two Gram-positive species from distinct ecological niches*** (PhD thesis). Gembloux, Belgium, University of Liège, Gembloux Agro-Bio Tech, 133 p., 14 Tables, 44 Figures.

Abstract: Microorganisms are often affected by various environmental factors. These environmental conditions have an effect on their physiological and biochemical functions. Among these environmental factors, temperature plays an important role in the normal physiological behaviors of microorganisms. To adapt to different temperature environments, bacteria have evolved a variety of adaptive mechanisms to coordinate a range of gene expression and protein activity changes. In this study, we investigated the adaptation mechanisms of two Gram-positive bacteria at different temperatures. The main results of this thesis are as follows:

(1) *Deinococcus radiodurans* is a gram-positive, pink-pigmented, and high G+C bacterium. The heat response of *D. radiodurans* is considered to be a classical stress-induced regulatory system characterized by extensive transcriptional reprogramming. In this part, we investigated the key functional genes involved in heat stress that were expressed and accumulated in cells following heat treatment at 48°C for 2 hours (R48). Considering that protein degradation is a time-consuming process, we predicted that in order to maintain cellular homeostasis, the expression of the key functional proteins would be significantly decreased in cells that had partly recovered from heat stress (RH) relative to their expression in cells grown under optimal temperature (R30). Comparative transcriptomics identified fifteen genes that were significantly downregulated in RH relative to R30, seven of which were previously characterized as heat shock proteins. Among these candidates, three genes (*dr_0127*, *dr_1083*, and *dr_1325*) are more likely involved in response to heat stress as survival analysis of mutant strains lacking *dr_0127*, *dr_1325*, and *dr_1083* showed a reduction in heat tolerance compared to the wild-type strain.

Based on our RNA-seq results and previous studies, we identified two novel heat-inducible ncRNAs in *D. radiodurans*, named *DnrH* and *dsr11*. Heat tolerance analysis showed that deleting *DnrH* significantly inhibited viability in response to high temperature conditions. Comparative phenotypic and qRT-PCR analyses of a *DnrH* mutant (Δ*DnrH*) and wild-type (WT) suggested that *DnrH* is potentially involved in regulating the expression of the heat shock-related gene *Hsp20*. Microscale thermophoresis and genetic complementation showed that a 28-nucleotide (nt) sequence in the stem-loop structure of *DnrH* (143–170 nt) pairs with its counterpart in the coding region of *Hsp20* mRNA (91–117 nt) via a 22 nt region. *In vivo*, mutation of the 22-nt region in the *D. radiodurans* genome led to a reduction in heat tolerance similar to that observed in the *DnrH*-mutant. Our

results show that *DnrH* positively influences heat tolerance by increasing the transcription of *Hsp20* mRNA, demonstrating, for the first time that a ncRNA may directly controls the expression of a heat stress-resistance gene. Similar to *dnrH*, we characterized another ncRNA, *dsr11*. Our result showed that the transcription level of *dsr11* was upregulated 4.2-fold under heat stress by qRT-PCR analysis. Heat tolerance assays showed that deleting *dsr11* significantly inhibited the viability under high temperature stress conditions. To assess the influence of *dsr11* on the *D. radioduans* transcriptome, 157 genes were found differentially expressed in the knock-out mutant by RNA-Seq experiment. Combined RNA-Seq and bioinformatic analysis, we found that *dr_0457* (biopolymer transport protein) was likely to be the direct targets of *dsr11*. Further microscale thermophoresis results demonstrated that *dsr11* can directly bind to the mRNA of *dr_0457*. Our results indicated that *dsr11* can enhance the tolerance to heat stress of *D. radiodurans* by binding to *dr_0457* mRNA.

(2) *Bacillus velezensis* GA1 is a Gram-positive bacterium living in association with plant roots and which may provide some protective effects against phytopathogens (biocontrol). In this work, we evaluated the impact on GA1 of temperatures typical of soils and lower than the optimal one commonly used under laboratory conditions. Cold temperature negatively impacted the cell growth rate of GA1, reflecting a general reduction in the metabolic activity. *In vitro* cultures revealed that production of some metabolites involved in biocontrol changed markedly when the temperature was lowered. We observed that after several rounds of culture on RE liquid medium at 15 and 18°C the growth of GA1 became faster than before suggesting that the bacterium may somehow adapt to cold conditions. We also tested the behavior of GA1 at 18°C on tomato plants, and showed that biofilm formation on root was a bit slower than at 22°C which correlated with reduced populations as revealed by flow cytometry measurements.

In summary, this study deeply analyzed the adaptation pathways and mechanisms of two gram-positive bacteria in response to different temperatures. Our work will provide a theoretical basis for future applications in industry and agriculture.

Keywords: *Deinococcus radiodurans*, heat stress, noncoding RNA, *dnrH*, *dsr11*, *Bacillus velezensis* GA1, PGPR, cold stress

Acknowledgments

This PhD dissertation is the results of much collaboration with both scientific colleagues but also with nonscientific actors. I would like to give my thanks to everyone who contributed and help to make this doctoral dissertation finally happen. I would also like to express my gratitude especially to:

Firstly, I would like to express my sincere gratitude to my supervisor Prof. Marc Ongena, who has a really good sense of humor and always gives me understandings for my life and study. You always shared new ideas and discussed with me about my research interests. You have helped me a lot in technical research and writing this thesis. Without you, I could not finish this fruitful study. The word thanks cannot express my gratitude feeling.

I would also like to thank my co-supervisor Prof. Jin Wang for her guidance and suggestion during my thesis. She always shared her busy schedule to meet and have a discussion with me for the method and data analysis.

I would also like thank to Prof. Min Lin, who is rich in precious ideas, kind guidance and give me constructional advices in my study and cultivating my personal way of working in scientific search.

My sincere thanks and appreciation to the rest of my thesis committee: Prof. Philippe Jacques, Prof. Frank Delvigne, Prof. Marc Hanikenne, Prof. Yuquan Xu, and Dr. Anthony Arias Arguelles for contributing their valuable time to read my manuscript, and their insightful comments, suggestions to make my thesis manuscript better and also for the hard questions which incented me to widen my research from various perspectives.

Thanks to every member in molecular microbiology research team for their concern and help during my PhD study, thanks to Prof. Ming Chen, Prof. Wei Zhang, Prof. Shuzhen Ping, Prof. Wei Lu, Prof. Yongliang Yan, Dr. Zhengfu Zhou, Dr. Yuhua Zhan, and Dr. Xiubing Ke.

I would also thank Prof. Marc Ongena, Prof. Frank Delvigne, Prof. Patrick Fickers and Prof. Philippe Jacque who are executors of the lab and MiPI team. Thank all the members from MiPI lab. Thank you all for welcoming me, for your support, for your kindness and your friendship.

Thank my classmates for taking care of me in Gembloux, especially to Tie Cheng Bai, Xuewei Zhou, Minmin Li, Lin Li, and Wei Fang, for taking care of me during my operation in Belgium.

I would like to give thanks to my family. Thank you, mam and dad, for giving me the rights to choose what I want in my life. During the past five years, there were some struggling times for me, thank you all for all the warmness and supports you have offered. You are the most important reasons that I could finish this dissertation and get my PhD degree. Thank you for being there for me all the time. I also would like to thank my girlfriend Feng Li, thanks her for understanding and supporting me during my PhD study.

Additionally, I would like thank to the Key Research and Development Projects, the Ministry of Agriculture Transgenic Program, and the National Natural Science Foundation of China support this research. I wish to thank China Scholarship Council (CSC) for the financial support to study in Belgium.

Finally, I would like to thank sincerely all the people who helped me to carry it out successfully.

List of Tables

List of Figures

List of Abbreviations

Amp	Ampicillin
cDNA	Complementary DNA
Cm	Chloromycetin
DNA	Deoxyribonucleic
dNTP	Deoxy-ribonucleoside-triphosphate
EDTA	Ethylene dinitrilotetracetic acid
IGR	Intergenic region
Km	Kamacycine
LB	Luria-Bertani
mRNA	Messenger RNA
MST	Microscale thermophoresis
ncRNA	Noncoding RNA
OD	Optical density
ORF	Opening read frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
qRT-PCR	Quantity real-time PCR
RNA	Ribonucleic acid
RBS	Ribosome binding site
rpm	Rounds per minute
RT-PCR	Reverse-transcription PCR
SD	Shine-Dalgarno sequence
SDS	Sodium dodecyl sulfate
TEMED	N, N, N', N'-Tetramethylethylenediamine
Tris	Tris (hytdroxymethyl) aminomethane
tRNA	Transfer RNA
TSS	Transcription start site
UTR	Untranslated regions
UV	ultraviolet
WT	Wild type
X-gal	5-bromo-4-cholro-3-indoly-β-D-galactose
RNA-Seq	RNA sequencing

OGT	Optimal growth temperature
TEM	Transmission electron microscopy
PI	Propidium iodide
RSG	Redox sensor green
RIN	RNA integrity
DEGs	Differentially expressed genes
FPKMs	Fragments per kilobase of transcript per million mapped reads
GO	Gene ontology
KEGG	Kyoto Encyclopedia of gene and genomes
Spec	spectinomycin
SD	Standard deviations
PGPR	Plant growth-promoting rhizobacteria
FCM	Flow cytometer

1

Chapter I: General Introduction

1.1. The effect of temperature on the physiology in prokaryotes

Prokaryotes have high physiological diversity and wide ecological distribution (Whitman et al., 1998). Prokaryotes are the main components in the biosphere and play an important role in the biogeochemical cycle. The prokaryotes living environment often changes, such as temperature, pH, oxygen concentration, pressure, and nutrients (Knoll et al., 1989). Temperature is one of the key environmental factors in microbial life, it can determine whether organisms can survive. Most microorganisms have an optimal growth temperature (OGT) ranging from 24 °C to 50 °C, which called mesophile (Bell et al., 2002). Besides, there are also psychrophile with OGT<24°C, thermophile (50°C<OGT<80°C), and hyperthermophile (OGT>80°C) (Chandrayan et al., 2014; Galtier et al., 1997; Musto et al., 2006; Nakashima et al., 2003; Ohtani et al., 2010) (Table 1-1).

Table 1-1. The four types of prokaryotes according to OGT

Type	OGT
Psychrophile	OGT<24°C
Mesophile	24°C<OGT<50°C
Thermophile	50°C<OGT<80°C
Hyperthermophile	OGT>80°C

The low temperature will cause a decrease in enzyme activity in microbial cells. The speed of various biochemical reactions, cell metabolism, and the fluidity of biomass membranes will be slow down (Hébraud et al., 1999; Shivaji et al., 2010). However, when the temperature is further lowered below the freezing point, the water in the cells will condense into ice crystals, causing irreversible mechanical damage to the biomass membrane such as the cell membrane, eventually leading to the death of the cells (Wilson et al., 2010). The high temperature will cause irreversible denaturation of protein in microorganisms. In particular, it affects normal cell physiological activities, such as affecting the enzymes involved in the tricarboxylic acid cycle (Ventura et al., 2006). Besides, high temperatures can lead to the destruction of the plasma membrane structure and to the damage of nucleic acid molecules, which leads to the death of the cells (Russell, 2003; Teixeira et al., 1997). When microorganisms in a non-fatal high-temperature environment, their growth will slow down or stagnate for a short period, and the level of cellular metabolism will decrease. The cells exhibit a heat shock response to cope with the effects of high temperature pressure on the cells. In the long-term heat stress process, bacteria can finally get the adaptability to heat stress through a series of stress responses (Gayán et al., 2016; Van der Veen et al., 2007).

1.2. Research progress on heat stress response in bacteria

Severe high-temperature stress can cause cell damage and cell death, while sub-lethal high-temperature stress can induce various cellular heat shock response (Suo et al., 2012). Heat shock response can protect cells from heat damage, help cells restore to normal physiological activities, and enhance the heat tolerance of bacteria. The thermal denaturation of proteins and enzymes is critical for cell viability. To adapt to high-temperature, microorganisms have developed a variety of adaptive mechanisms to protect cells from high-temperature stress, such as heat shock gene regulation mechanisms, and the stringent response at the global regulatory level (J.-s. Li et al., 2011; Murata et al., 2011). These components are discussed below:

1.2.1. Regulated by the sigma factor

In *E. coli*, sig32 is an RNA polymerase subunit that is encoded by the *rpoH* gene. In general, the expression level of the *rpoH* gene is very low, mainly because the molecular chaperone system (like DnaK and GroE-type) accelerates the degradation of sig32 by protease (FtsH) (Arsène et al., 2000; Guisbert et al., 2004; Yura et al., 1999). As the external temperature rises, the expression of *rpoH* increases dramatically over time, and enhances the synthesis of sig32. The increase in the level of sig32 promotes the synthesis of molecular chaperone (DnaK/J; GroEL/S) and protease (FtsH). Chaperones and proteases further bind the misfolded proteins to help them fold correctly or degrade the misfolded proteins. In the adaptation phase that follows the induction phase, sig32 is inactivated when excess heat shock proteins (HSPs) are present (Guisbert et al., 2008) (Figure 1-1).

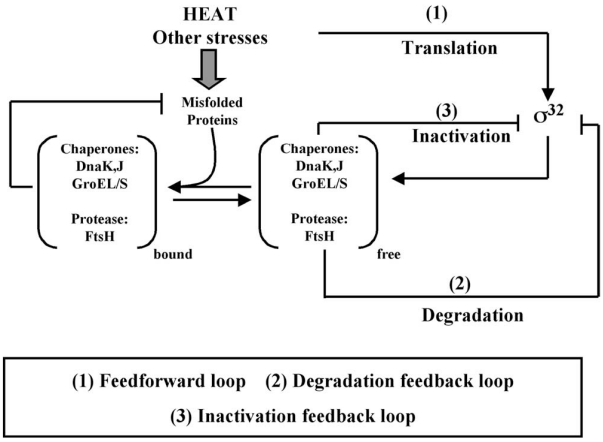


Figure 1-1. Wiring diagram of sig32 regulation.

There are three primary modes of regulation as follows: (1) excess free DnaK/J and GroEL/S chaperones directly bind to and inactivate sig32; (2) the FtsH protease degrades

sig32, with chaperones participating in this process; and (3) temperature directly controls the rate of sig32 translation. Misfolded proteins titrate chaperones from these regulatory roles, allowing active sig32 to increase the synthesis of chaperones and proteases during conditions where they are required (Guisbert et al., 2008).

1.2.2. Regulated by the CIRCE/HrcA regulatory system

In the Gram-positive bacterium *Bacillus subtilis*, the expression of some chaperone genes (*dnaK*, *groE*) is regulated by the *HrcA* gene (Zuber et al., 1994). The *HrcA* gene-encoded protein products affect the transcription of these genes by binding to a conserved sequence (CIRCE). A related regulatory mechanism, HAIR/HspR, is also present in *Streptomyces albus*, and genes such as the *dnaK* operon and *clpB* are regulated by that regulatory system (Narberhaus, 1999) (Figure 1-2).

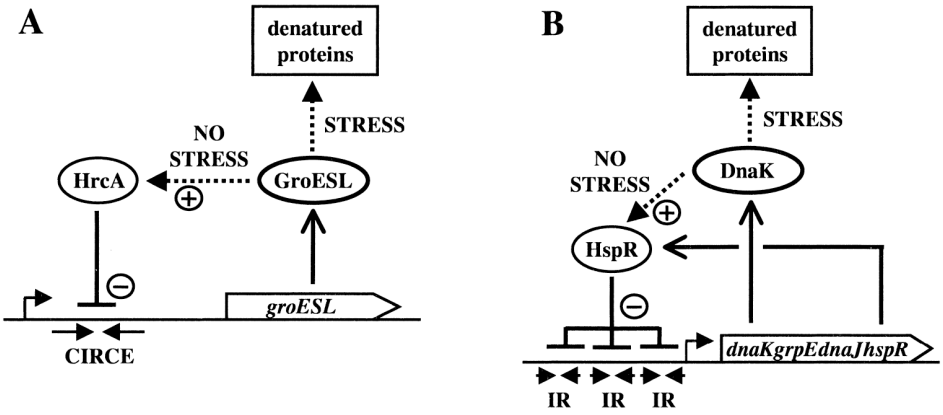


Figure 1-2. Titration models for homeostatic control of chaperone expression. A. GroEL as a cellular thermometer in HrcA-controlled systems. Dotted arrows indicate protein-protein interactions; (+) symbolizes activation and (-) repression. B. DnaK titration in an HspR-controlled system (Narberhaus, 1999).

1.2.3. Regulated by RNA sensors

Bacteria use complex strategies to coordinate the expression of temperature-dependent genes. For these genes, riboswitches monitor the metabolic state of a cell by binding metabolites with high specificity and affinity. Riboswitches are located in the 5' -UTR of genes involved in the biosynthesis, uptake or degradation of small metabolites and provide feedback control to these pathways. Their complex architecture consists in a receptor region (aptamer) and an output region (expression platform). The receptor region is characterized by the consensus sequence specifying the substrate molecule. A conformational switch is triggered by the binding of small molecules, which changes gene expression through one of three possible mechanisms: premature transcription

termination; translation initiation, or mRNA processing (Mironov et al., 2002; Nahvi et al., 2002; Winkler et al., 2004) (Figure 1-3A). In comparison to highly specialized metabolic binding riboswitches, RNA thermometers respond to a fairly global physical signal (i.e. the intracellular temperature), which is a significant parameter under constant vigilance. A well-known feature of structured nucleic acid molecules is that they melt as the temperature rises. All currently known *cis*- and *trans*-acting molecular thermometers achieve translational regulation by sequestration of the ribosome-binding region (Narberhaus et al., 2006) (Figure 1-3B).

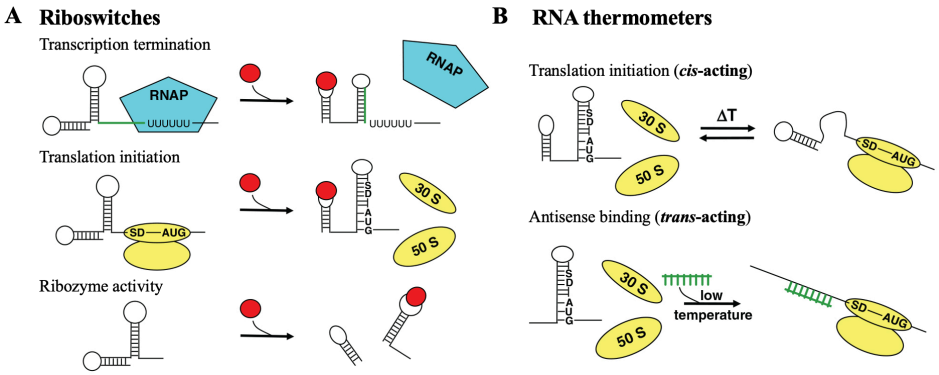


Figure 1-3. Schematic illustration of regulatory principles of riboswitches (A) and RNA thermometers (B).
The processes that are controlled are indicated in each case. Red circles represent metabolites. RNAP, RNA polymerase; SD, Shine–Dalgarno sequence; 30S and 50S, ribosomal subunits, Δ*T*, temperature change. AUG and UUUUUU; ribonucleotide sequences (Narberhaus et al., 2006).

1.2.4. DNA topology affects the gene expression

In thermophilic and ambient species, temperature causes change in the topology of DNA, which primarily affects supercoiling (López-García et al., 2000). The transcriptional efficiency of genes is very sensitive to changes in the topology of DNA (Pruss et al., 1989). Therefore, an increase of the environment temperature in which the bacteria is exposed can lead to changes in the topology of the DNA and thus influence the gene expression (Figure 1-4).

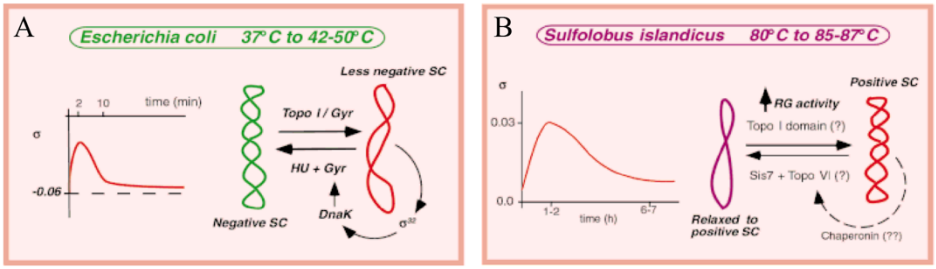


Figure 1-4. Effects of heat shock on plasmid topology in *Escherichia coli* and *Sulfolobus islandicus*.
On the left of each panel is shown the variation of plasmid specific linking difference ($\sigma=\Delta Lk/Lk_0$) with the time of exposure to the shock temperature. The right part of each panel corresponds to the schematic interpretation of the respective topological changes and the proteins involved in their regulation. Hypothetical regulators are followed by (?). SC, supercoiling; Topo, topoisomerase; Gyr, gyrase; RG, reverse gyrase (López-García et al., 2000).

1.3. Key technologies of transcriptomics and application in bacterial functional genomics research

1.3.1. RNA-Seq technology

The effects of temperature on bacteria and the response to temperature changes are reflected in multiple levels from gene to phenotypic. The transcriptional level is an important part of this. A transcriptome is a set of transcript data in a cell at a particular developmental stage or physiological condition. In recent years, a new generation of high-throughput sequencing technology has developed rapidly. This is followed by high-resolution deep sequencing of transcripts across the genome, using high-throughput quantitative assays to sequence various types of transcripts called RNA-seq (Z. Wang et al., 2009). RNA-seq does not require the gene probes compared to the traditional chip hybridization platforms. RNA samples from any source can be tested, while high-throughput RNA sequencing can complete gene annotation and quantify genes across all subtypes. It has currently become the most powerful tool for a comprehensive analysis of the complexity of the transcriptome (Marioni et al., 2008).

RNA-seq has been successfully applied to the detection of gene expression levels, as well as the definition of gene boundaries, the study of transcriptional differences, the functional studies of noncoding small RNAs, and the discovery of new transcripts. As one of the most valuable high-technologies in the 21st century, transcriptomics can discover new genes that may cause disease or disease-related genes by comparing intracellular expression differences, in addition to making breakthroughs in basic research. It has been successfully

applied to cell regulatory networks and biochemical metabolic pathway studies (Licatalosi et al., 2010), as well as gene function and environmental signaling responses (Livny et al., 2014).

1.3.2. Transcriptomics application in the global regulation of bacterial stress response

Gene expression levels can be compared across the genome, and differentially expressed genes can be mapped to metabolic pathways to elucidate the regulatory mechanisms under specific bacterial environments. The bacteria's living environment is constantly changing. To adapt to the changing environment, bacteria use their complex regulatory networks to respond to their physiology and phenotype (De Groot et al., 2014). Transcriptomics has been the necessary method to study the gene regulation of bacteria. Its characteristics are more in-depth than the traditional bacterial stress reaction research methods and can reveal the physiological mechanism of bacteria adapting to diverse environments.

Jozefczuk et al. used transcriptomics to analyze the response mechanisms of *E. coli* under high pressure, low temperature, high temperature, salt, and acid stress, indicating that the adaptation of bacteria to stress conditions may have a similar response mechanism. These transcriptomics studies are important for revealing the physiological mechanisms by which bacteria adapt to stressful environments (Dahlsten et al., 2014; Jozefczuk et al., 2010).

1.4. Research progress on *Deinococcus radiodurans*

1.4.1. Introduction on D. radiodurans

D. radiodurans is a gram-positive, nonsporulating, nonmotile, nonpathogenic high G+C, pink-pigmented, and aerobic bacterium existing in the form of diads or tetrads. *D. radiodurans* was first isolated from gamma-radiated canned meat. *D. radiodurans* has attracted more attention because of its unprecedented resistance to ionizing radiation (Daly, 2009; Hua et al., 2016; Omelchenko et al., 2005). *D. radiodurans* was originally classified into *Micrococcus* based on morphology, and named *M. radiodurans* (Moseley, 1967). However, 16S rRNA analysis indicated that it forms a unique phylogenetic group of bacteria; hence, it was included in a new genus, the Deinococcus (Brooks et al., 1981), which is closely related to the genus Thermus of heat-resistant bacteria (Hensel et al., 1986; Omelchenko et al., 2005; Weisburg et al., 1989). *D. radiodurans* can often be observed in the form of a double or quadruplex under a microscope and the individual cells have an average diameter of 0.5 - 3.5 μm, an average of about 1 μm (Work et al., 1968). It is red due to carotenoid production, and gram-stain is positive but has a double-layered cell membrane (Tian et al., 2009). The optimal growth temperature was 30 °C, and cell doubling takes approximately 100 min in TGY liquid medium (0.5% tryptone, 0.1% glucose, 0.15% yeast extract) (He, 2009).

In 1999, White et al. have completed the whole genome sequencing of *D. radiodurans* (White et al., 1999). The genome of *D. radiodurans* is 3.28M in size, with an average GC content of 66.6% and protein-coding region accounted for 90.0%. *D. radiodurans* genome is composed of four replicons: two chromosomes (chromosome I: 2,648,638bp, and chromosome II: 412,348bp), a megaplasmid (177,466bp), and a small plasmid (45,704bp). The genome of *D. radiodurans* encodes 3,187 proteins. About 75% of the *D. radiodurans* proteins have homologs in other prokaryotes, and 24% of the proteins are unique. The function of excavating these genes has great significance for revealing the mechanism of extreme resistance in *D. radiodurans*.

1.4.2. The extreme resistance of D. radiodurans

1.4.2.1. Ionizing radiation resistance

The most striking thing is that *D. radiodurans* can tolerate high doses of ionizing radiation, the exponentially grown cells can endure gamma-ray irradiation over 15 kGy , which is equivalent to more than 250 times of *E. coli* radiation resistance, more than 3,000 times the dose of human tolerance (Daly, 2009). *D. radiodurans* can still grow normally under 60 Gy/h continuous irradiation without affecting its growth rate and expression of the exogenous genes (Lange et al., 1998). The effects of irradiation on cells include genomic damage that can cause more than 200 DNA double-strand breaks (DSBs) into the genome of the bacterium. However, it can repair 100 to 200 DSBs per chromosome within 12 to 24 hours and accurately reconstruct the genome but under the same conditions, other bacteria can generally only repair several DSBs (Slade et al., 2011). Its superior radiation resistance is thus mainly due to the efficient and accurate DNA repair ability. As initially stated by Daly et al. (Daly et al., 1996), and more recently reinforced by Radman et al. (Slade et al., 2009; Zahradka et al., 2006), the recombinant repair of DSBs in *D. radiodurans* proceeds via two homologous recombination processes (Slade et al., 2009; Zahradka et al., 2006) (Figure 1-5). The first step is a mechanism known as extended synthesis-dependent single-strand DNA annealing (ESDSA). The second phase involves RecA protein-mediated double-strand break repair. The fragmented DNA is recessed in a 5'- to -3' direction, releasing single-stranded 3'overhangs, which, via RecA- and RadA-mediated strand invasion, prime synthesis on overlapping fragments through a migrating D loop. DNA synthesis is initiated and elongated by polymerase, with polymerase filling up gaps arising from the excision repair of damaged bases. Two non-contiguous fragments are linked by the converging elongations on a third "bridging" fragment. Newly synthesized single strands dissociate from the template and anneal to complementary single-stranded extensions, forming dsDNA intermediates. The flaps are removed, and the gaps are filled. Long linear intermediates are joined into circular chromosomes by RecA-dependent crossovers.

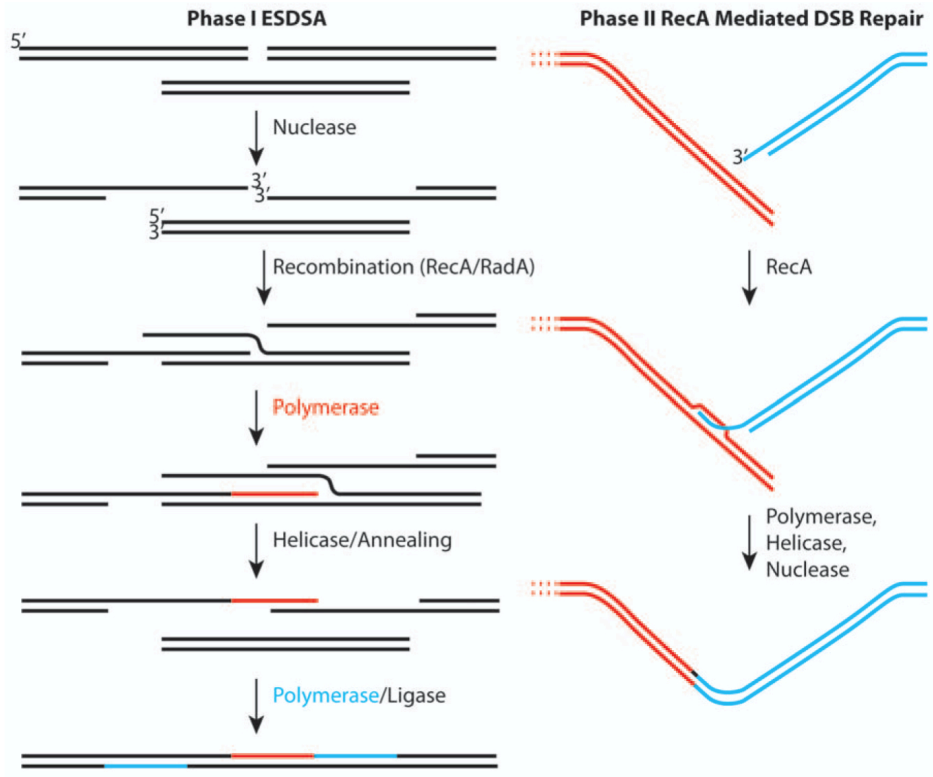


Figure 1-5. Two-step of genome reconstitution in *D. radiodurans* shattered by ionizing radiation.
The first step, ESDSA is dominated by nuclease and DNA polymerase functions. The second step is a more conventional RecA-mediated double-strand break repair process focused on the final splicing of large chromosomal segments (Cox et al., 2010).

1.4.2.2. Oxidative resistance

D. radiodurans is also highly tolerant to a wide range of oxidative stresses. Reactive oxygen species (ROS) induced oxidative stress is deleterious for all species. Oxidative stress results from the formation of ROS, the three primary groups of which are hydroxyl radicals, superoxide radicals, and hydrogen peroxide (D'Autréaux et al., 2007). It is well known that ROS can be generated endogenously by metabolism or formed by environmental factors such as exposure to ionizing radiation (Battista et al., 1999), ultraviolet (Battista, 1997), desiccation (Mattimore et al., 1996), and hydrogen peroxide (Slade et al., 2011). To adapt the oxygen-rich environment of earth and remove the dangerous ROS, aerobic species have evolved a range of ROS scavenging antioxidants to cope with oxidative stress. Noticeably, *D. radiodurans* provide an unprecedented antioxidant system to defend itself from oxidative damage relative to other species. It has been reported that the catalase activity of *D. radiodurans* was 127

times and 32 times greater than that of *E. coli* during the exponential and stationary phases, respectively (P. Wang et al., 1995). *D. radiodurans* genome encodes two KatE-type catalases, DR1998 (KatE1), and DRA0259 (KatE2), and one eukaryotic-type catalase, DRA0146 (Slade et al., 2011).

1.4.2.3. Desiccation resistance

D. radiodurans is also highly resistant to prolonged desiccation stress (Figure 1-6). Desiccation is defined as water content below 0.1 g H_2O g^{-1} dry mass. *D. radiodurans* can survive for 6 weeks in a dry environment with a relative humidity of less than 5% and do not affect the growth ability (Mattimore et al., 1996). This exceptionally high survival efficiency is powerful for the bacteria that cannot produce spores. The dehydration process causes DNA damage and fragmentation. Both radiation- and desiccation-resistant bacteria have a high Mn/Fe ratio, and their proteins are less susceptible to protein oxidation than are those of sensitive bacteria (Fredrickson et al., 2008). Recently identified manganese complexes acting as the most efficient *D. radiodurans* ROS scavengers are expected to protect proteins against desiccation-induced damage (Daly et al., 2010). *D. radiodurans* is unique among bacteria in possessing as many as four homologs of plant desiccation resistance-associated proteins (Makarova et al., 2001), which may help the cells against the desiccation stress.

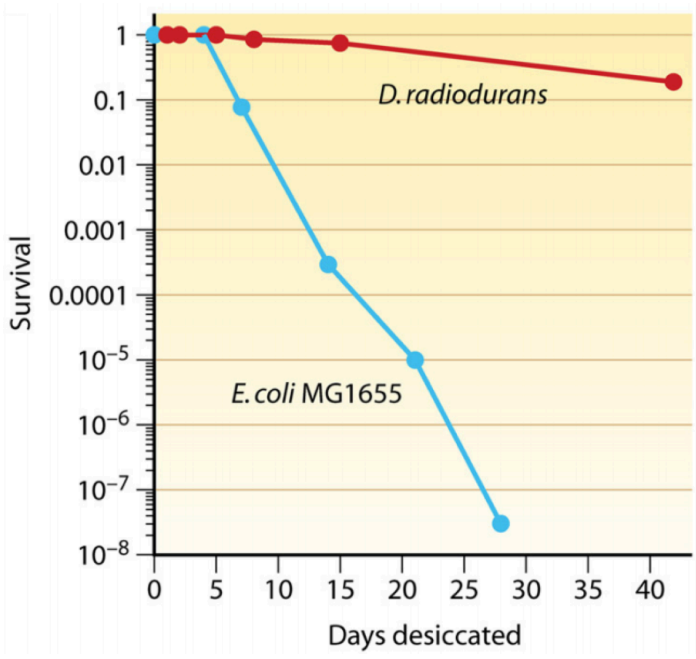


Figure 1-6. Extreme resistance of *D. radiodurans* to desiccation (Slade et al., 2011).

1.4.2.4. Heat stress resistance

D. radiodurans also has strong heat stress tolerance. Studies have shown that

D. radiodurans cells treated with 100 °C for 10 min under dry heat conditions still has more than 90% survival (Bauermeister et al., 2012). Under heat shock conditions, more than 130 genes were induced, including the common molecular chaperones (groESL, dnaKJ, and clpB) (Schmid et al., 2005a, 2005b). Recent studies have shown that the presence of the small heat shock protein two-component system HSP17.7/HSP20.2 in *D. radiodurans* plays an important role in preventing protein aggregation under heat stress (Bepperling et al., 2012). More recently, Meyer et al. show that *ddrI* is also involved in heat stress (Meyer et al., 2018).

1.5. Research progress on noncoding RNA in bacteria

1.5.1. Definition of noncoding RNA in bacteria

Noncoding RNAs (ncRNAs) cannot be translated into proteins but are capable of functioning at the transcriptional level, and 10 - 30% of genes in the whole genome are regulated by ncRNA in bacteria (Wassarman et al., 1999). Normally, their size is 40 - 500 nucleotides (nt). The first ncRNA was found in *E. coli* 40 years ago (Griffin, 1971), but its function was not determined at that time. Recent studies have shown that ncRNAs control different cellular activities in bacteria, such as acid resistance (Opdyke et al., 2004), sugar metabolism (Vanderpool et al., 2004), environmental stress response (Eyraud et al., 2014; Zhang et al., 2019), and bacterial virulence (Mann et al., 2012). Most of the ncRNAs are influenced by environmental factors and have independent transcriptional targets (Gottesman, 2005; Storz et al., 2005) (Figure 1-7). ncRNAs exert their function by binding to the target mRNA through complementary base pairing, thereby affecting the translation and/or stability of the target mRNA (Valverde et al., 2008). An ncRNA may have multiple mRNA targets, and one target mRNA may be regulated by multiple ncRNAs, such as *oxyS* and *dsrA* affects *rpoS* expression in *E. coli* (Majdalani et al., 2001). In recent years, the research on the function and regulation mechanism of bacterial ncRNAs has become one of the research hotspots.

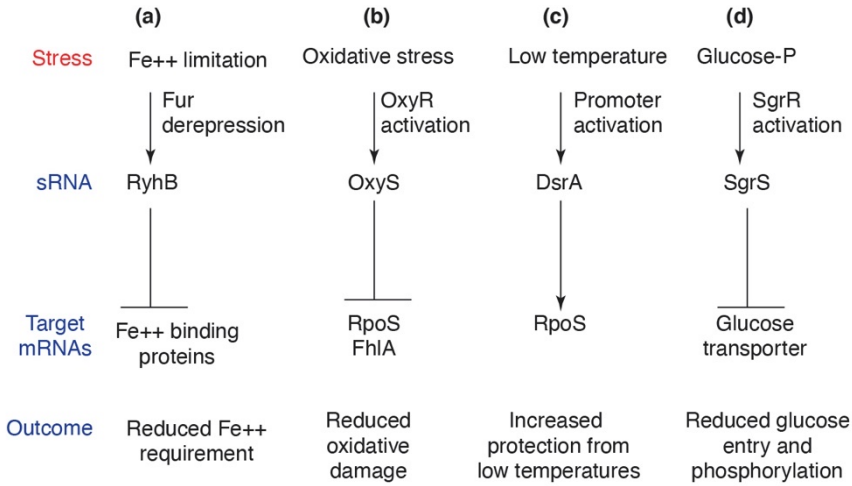


Figure 1-7. ncRNAs in global regulation cascades.
Various stress conditions and changes in growth lead to induction of global responses, generally by affecting the activity and/or synthesis of a transcriptional regulator. Depicted here are several of the conditions, regulators, and resulting small RNAs and their targets that have been studied in *Escherichia coli* (Gottesman, 2005).

With the development of biotechnology detection methods for finding ncRNAs, which includes RNA labeling and staining, functional gene screening, protein co-purification, shotgun cloning, microarray detection, and bioinformatics (Ahmed et al., 2018). By these identification methods, more and more ncRNAs have been found in different bacteria in the past decade, and researchers have begun to study the functions of these ncRNAs. By studying how these ncRNAs exert their regulatory functions at the molecular level, it will help to understand the relationship between their structure and function and improve the prediction of ncRNA.

Normally, ncRNAs have been divided into two groups (*cis*-encoded ncRNAs and *trans*-encoded ncRNAs) according to functional base-pairing with their target (Wagner et al., 2015). The *cis*-encoded ncRNA is fully complementary to a single target mRNA to form a complete complex, while *trans*-encoded ncRNAs exert their regulatory roles through imperfect base-pairing with target mRNA to modulate their stability and/or translation (Figure 1-8) (W. Li et al., 2012). *Trans*-encoded ncRNAs are located in the intergenic regions of the genome and the genetic manipulation and functional studies are relatively easier than *cis*-encoded ncRNA, which has been the best characterized and most extensively studied. Therefore, the current research in bacteria is more in-depth on the function and regulation mechanism of *trans*-encoded ncRNA. The following figure provides a description of the regulatory mechanism of *trans*-encoded ncRNA.

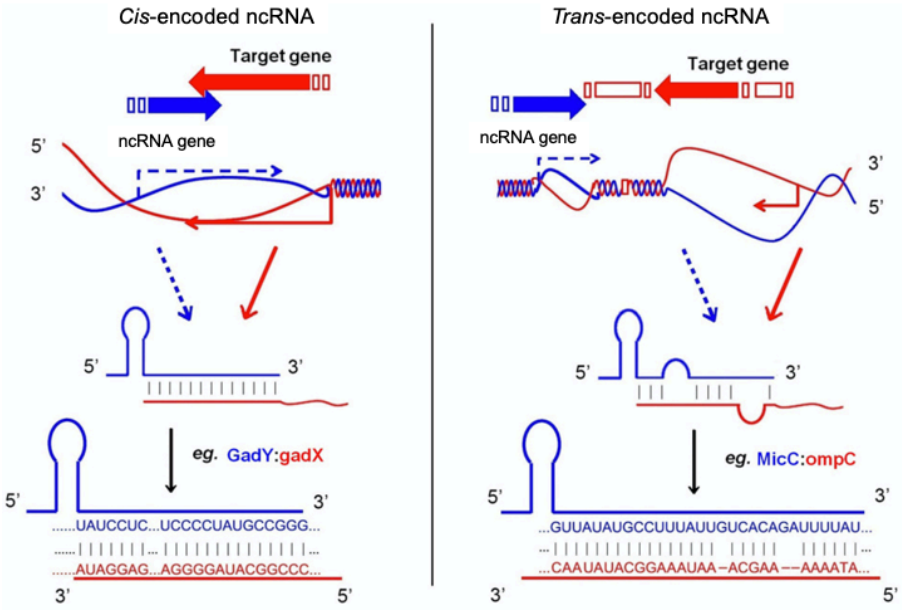


Figure 1-8. Overview of *cis*- and *trans*-encoded ncRNAs (W. Li et al., 2012).

1.5.2. Molecular mechanism of bacterial ncRNA

1.5.2.1. Interaction with target mRNA

Typically, the majority of bacterial *trans*-encoded ncRNAs act by imperfect base-pairing with target mRNA (Vogel et al., 2005), resulting in inhibition or activation of translation of the target mRNA. If the binding site covers or is close to the region adjacent to the SD (Shine-Dalgarno) sequence, or if the translation initiation codon is in the translation initiation region (TIR) of the target mRNA, it may result in blocking the ribosome binding site and inhibit the translation. If the inhibited secondary structure is melted, it leads to the exposure of the ribosome binding site and of the translation initiation site of mRNA and active the translation (Repoila et al., 2009) (Figure 1-9).

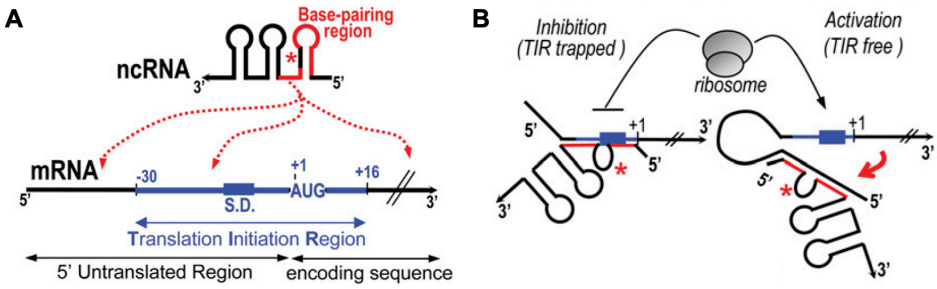


Figure 1-9. Basic mechanisms of the ncRNA/mRNA interaction.
A. mRNA regions targeted by a ncRNA; B. general mechanisms of translation regulation (Repoila et al., 2009).

1.5.2.2. Interaction with target proteins

mRNA is not the only target for ncRNAs. Previous studies have shown that three ncRNAs in *E. coli* and homologous genes in other bacteria can interact with cellular proteins to regulate their activity (Brantl, 2009). For example, 6S RNA (a highly abundant noncoding RNA) is highly conserved in prokaryotes and accumulates during the stationary phase. 6S RNA regulates transcription through interaction with σ70 polymerase in *E. coli* (Trotochaud et al., 2004, 2005). *csrB* and *csrC* RNAs have a binding site for the global post-transcriptional regulatory protein CsrA, which binds to regulatory proteins, attenuates the regulation of CsrA protein, and firmly controls the activity of this protein. This promotes or inhibits the expression of certain genes regulated by the CsrA protein, which in turn affects cell-related metabolism, such as cell membrane formation, movement, and acetate metabolism (Romeo, 1998; Weilbacher et al., 2003) (Figure 1-10).

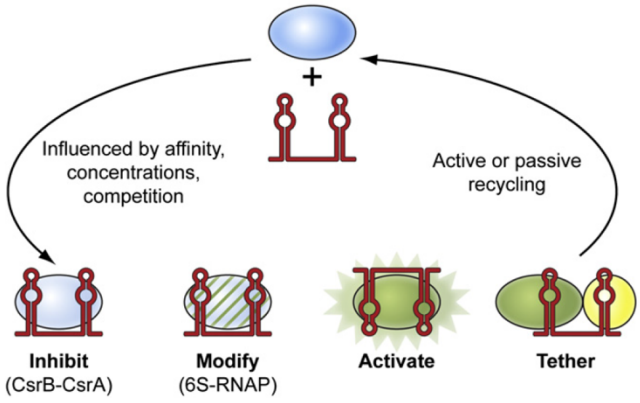


Figure 1-10. General properties of ncRNAs that modulate protein activity.
Bacterial ncRNA binding to proteins has been demonstrated to inhibit and/or modify

protein activities (Storz et al., 2011).

1.5.3. Study progress of ncRNA in D. radiodurans

ncRNAs also play an important physiological role in *D. radiodurans*. At present, there are few reports on ncRNAs in *D. radiodurans*. Only in 2015, Tsai et al reported that there were 199 ncRNA candidates (Tsai et al., 2015), but there is no in-depth study on their actual function. With the improvement of biotechnology, more and more unknown ncRNAs have been identified and verified. It has become a frontier and hot spot in the field to identify their target, mode of action and physiological significance.

1.6. Biocontrol effect of plant growth-promoting rhizobacteria for plant protection

1.6.1. Plant growth-promoting rhizobacteria

Plant growth-promoting rhizobacteria (PGPR) colonize the rhizosphere of many plant species and can promote plant growth by a wide range of mechanisms (Bhattacharyya et al., 2012). The main functions are: (1) to provide nutrition for crops (2) to stimulate plant growth (3) to inhibit the activity of phytopathogenic bacteria (4) to improve soil structure (5) to accumulate inorganic matter. PGPR often fixed around the root system of the plant to promote plant growth. At present, it is more popular to use biological methods to increase plant nutrition and crop yield (Dey et al., 2004; Shoebitz et al., 2009). There are several pathways by which PGPR promotes plant growth. Generally, PGPR can affect plants through two ways, both direct and indirect. PGPR directly promotes plant growth refers to the fact that PGPR can synthesize certain substances (such as auxin) that have a direct impact on plant growth and development and/or change the form of certain ineffective elements in the soil to make it effective and facilitate plant absorption (such as nitrogen fixation, phosphorus solubilization, etc.) (Dobbelaere et al., 2003; Rodríguez et al., 2006). Indirect effects refer to inhibiting or minimizing the detrimental effects of certain plant pathogens on plant growth and yield.

1.6.2. The role of PGPR in plants under abiotic stresses

Plant–microbes symbiosis is affected by environmental changes in the soil, which mainly include water, soil temperature, salinity, nutrient deficiencies, alkalinity and acidity (Hungria et al., 2000; Meena et al., 2014; Meena et al., 2015). Low soil temperature is an important abiotic stress factor, pose a major limitation on plant growth and development. It affects the symbiosis of rhizobacteria and plants by regulating the rate of physico-chemical reactions (Sardesai et al., 2001). Exposure to low temperatures is one of the most damaging environmental factors affecting plants (Nagarajan et al., 2009). Recently, the use of beneficial microorganisms such as PGPR has emerged as a potential new approach to reduce abiotic stress-induced

damage (Yang et al., 2009). As a result, intimate associations between bacteria and host plants can be formed without harming the plant (Compant et al., 2005; Gray et al., 2005). PGPR colonizes the plant rhizosphere and has a beneficial impact on their growth and responses to stress (Compant et al., 2008; Yang et al., 2009).

1.6.3. Bacillus velezensis GA1

Many PGPR strains have been described, the main species of which include *Bacillus* and *Pseudomonas*. Indeed, nowadays, the market of PGPRs is dominated by *Bacilli* due to their adaptability to the agro-industrial world (i.e. endospore-forming ability), some of which are proven to be effective PGPRs. The operational group 'amyloliquefaciens', along with the species *B. subtilis* and *B. pumilus*, gather most of the available strains (Borriss, 2015). The dominant position of *Bacillus* products underlines the importance of this genus in the PGPR sector.

Our study focuses on one specific PGPR: *Bacillus velezensis* GA1 (GA1), which has become a typical bacterium for our team to study plant-bacterial interactions. GA1 is a Gram-positive, aerobic, spore-forming, motile, rod-shaped bacteria. GA1 is often encountered as *B. amyloliquefaciens* GA1 (Arguelles-Arias et al., 2009). However, some controversy has occurred regarding the taxonomy of *B. velezensis*, *B. amyloliquefaciens subsp* plantarum, *B. oryzicola* and *B. methylotrophicus*. As a result, the above-mentioned four species constitute only one species, *Bacillus velezensis*, and that this species belongs to the 'operational' group 'amyloliquefaciens'. *Bacillus sp.* FZB42 is a closely related strain that belongs to the same species as GA1, is now classified as velezensis rather than amyloliquefaciens subsp plantarum. The same logic should be applied to *Bacillus sp.* GA1 and it should be called *B. velezensis* GA1 (Dunlap et al., 2016; Fan et al., 2017). It is worth noting that this operational group gathers most of the best PGPR Bacilli (Fan et al., 2017) and that it has undergone an evolutionary adaptation to plant associated habitat (Belbahri et al., 2017). Thus, GA1 is a good representative of the bacillus PGPRs.

GA1 can produce large amounts of Bioactive Secondary Metabolites (BSMs) (Arguelles-Arias et al., 2009). The BSMs produced by GA1 include non-ribosomal peptides (NRPs), polyketides (PKs), Lipopeptides (CLPs), siderophores and ribosomally synthesized peptides. Because of the large number of BSM, GA1 has an antimicrobial activity toward a large group of pathogens and thus has great potential as a biocontrol agent.

1.7. Objectives

Temperature is one of the stressful environments that can give pressure to biological evolution. The research on the response process of prokaryotic to heat or cold can help us better understand its stress adaptation mechanism, find important biomacromolecules with thermal/cold stability, and find the physiological effects and response methods caused by thermal/cold adaptation

evolution. The results can be applied to evolutionary research, genetic engineering, food engineering, fermentation engineering, enzyme engineering, and new biomaterials.

D. radiodurans is capable of living in a variety of extreme environments, with ionizing radiation resistance and a variety of abiotic stress (such as cold, heat, etc.) resistance, is an ideal strain to study the adaptation mechanism of extreme microbial abiotic stress. In recent years, there are many studies on the molecular mechanism of *D. radiodurans* anti-radiation, but little research on the response mechanism to heat stress. We used the Illumina sequencing platform for transcriptome analysis of wild-type strains under normal growth, heat stress treatment, and recovery after heat stress. The heat stress reaction process of *D. radiodurans* was initially explored, and some novel heat stress response proteins were discovered. It provided theoretical support for further understanding of the adaptation and response mechanism of bacteria in extreme environments.

In recent years, the study of ncRNA in *D. radiodurans* has been limited only to the discovery and identification of ncRNA. However, little research has been done on its mechanisms involved in various activities in cells. In the complex regulatory network of the heat stress response, in addition to the important regulatory role of key proteins, whether there is a more direct regulatory ncRNA involved in the entire regulatory process still needs further research. Based on our and previous report, we found two ncRNAs that are highly expressed under heat stress and carried out in-depth research on their molecular mechanisms involved in response to heat stress, which laid a theoretical foundation for further understanding of the mechanism of ncRNA involvement in heat stress. This will help to fully understand the importance of ncRNA in microbial heat stress response mechanisms at a new level.

PGPR is a group of bacteria that can enhance plant growth and yield during agricultural production. The environment during crop production is complex. Temperature is a vital factor during plant growth. The impact of temperature on biological control has occasionally been addressed in previous works. However, the effect of low temperature on the expression of biocontrol traits by those beneficial rhizobacteria. Our objective is to investigate the effect of temperature on several *B. velezensis* GA1 (a PGPR) traits, such as growth rate, motility, biofilm formation, and root colonization ability. We also gave special emphasis to finding out how *B. velezensis* GA1 metabolite production can be modulated at low temperatures *in vitro* and planta.

1.8. Overview of the chapters

In this chapter, we present a review of the bibliography on temperature adaptation in bacteria. Information is presented on the mechanisms of temperature tolerance in bacteria and the scope for further research in this area. We also provided a brief description of the context and the aims of this work.

The second chapter presents our research on investigated key functional genes

involved in heat resistance that were expressed and accumulated in cells following heat treatment at 48 °C for 2 hours and recovered from heat stress for 2 hours. In this work, we tried to provide deeper insights into the transcriptional regulation of the heat response in *D. radiodurans*.

In chapter three, we reported the identification and characterization of a novel heat-inducible ncRNA, called *DnrH*. Our data indicate that *DnrH* is positively influencing heat resistance by increasing the transcription level of *Hsp20* mRNA, demonstrating for the first instance of an ncRNA that specifically regulates the expression of a heat stress resistance gene, which provides new insights into the response mechanism of heat stress in *D. radiodurans*.

In Chapter four, we showed that the transcription level of another novel ncRNA *dsr11* was upregulated 4.2-fold under heat stress by qRT-PCR analysis. Heat tolerance test revealed that the deletion of *dsr11* significantly inhibited the viability under high-temperature conditions. Combined RNA-seq and bioinformatic analysis, we observed that the *dr_0457* (encoding biopolymer transport protein) was most probably the direct target of *dsr11*. Further microscale thermophoresis results demonstrated that *dsr11* can be specifically bound to the mRNA of *dr_0457*. Our results indicated that *dsr11* can enhance the tolerance to heat stress of *D. radiodurans* by binding to *dr_0457* mRNA.

In Chapter five, we evaluated the adaptation of *B. velezensis* GA1 to low temperatures and the relationship between GA1 and tomato roots at low temperature. Understanding how temperature affects protocooperation between plants and GA1 may reduce the abiotic stress damage in plant. However, little is known about the effects of temperature on biocontrol mechanisms and effectiveness by GA1. Cold temperature negatively influenced the cell growth rate of GA1, reflecting the general reduction in the metabolic activity of cells cultivated at low temperatures. *In vitro* cultures showed that metabolic productivity changed markedly when the temperature was lowered. We observed that after third rounds of cultivation on RE liquid medium at 18°C, the growth of GA1 became faster than before. We tested GA1 at 18°C on tomato roots, the results showed that the biofilm formation on the root hair was a little slower than at 22°C, and FCM analysis showed that at low temperature clearly resulted in reduced GA1 populations on the roots.

The discussion part, chapter six, proposes the view of the information offered in the five main chapters. In addition, this section incorporates possible fascinating studies in the future and sheds some new light on the temperature adaptation in gram-positive bacteria.

References

Ahmed, W., Hafeez, M. A., & Mahmood, S. 2018. Identification and functional characterization of bacterial small non-coding RNAs and their target: A review. Gene Reports, 10, 167-176.

Arguelles-Arias, A., Ongena, M., Halimi, B., Lara, Y., Brans, A., Joris, B., et al. 2009. *Bacillus amyloliquefaciens* GA1 as a source of potent antibiotics and other secondary metabolites for biocontrol of plant pathogens. Microbial cell factories, 8(1), 63.

Arsène, F., Tomoyasu, T., & Bukau, B. 2000. The heat shock response of *Escherichia coli*. International journal of food microbiology, 55(1-3), 3-9.

Battista, J. R. 1997. Against all odds: the survival strategies of *Deinococcus radiodurans*. Annual review of microbiology, 51(1), 203-224.

Battista, J. R., Earl, A. M., & Park, M.-J. 1999. Why is *Deinococcus radiodurans* so resistant to ionizing radiation? Trends in microbiology, 7(9), 362-365.

Bauermeister, A., Hahn, C., Rettberg, P., Reitz, G., & Moeller, R. 2012. Roles of DNA repair and membrane integrity in heat resistance of *Deinococcus radiodurans*. Archives of microbiology, 194(11), 959-966.

Belbahri, L., Chenari Bouket, A., Rekik, I., Alenezi, F. N., Vallat, A., Luptakova, L., et al. 2017. Comparative genomics of *Bacillus amyloliquefaciens* strains reveals a core genome with traits for habitat adaptation and a secondary metabolites rich accessory genome. Frontiers in microbiology, 8, 1438.

Bell, G. S., Russell, R. J., Connaris, H., Hough, D. W., Danson, M. J., & Taylor, G. L. 2002. Stepwise adaptations of citrate synthase to survival at life's extremes: from psychrophile to hyperthermophile. European journal of biochemistry, 269(24), 6250-6260.

Bepperling, A., Alte, F., Kriehuber, T., Braun, N., Weinkauf, S., Groll, M., et al. 2012. Alternative bacterial two-component small heat shock protein systems. Proceedings of the National Academy of Sciences, 109(50), 20407-20412.

Bhattacharyya, P. N., & Jha, D. K. 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World Journal of Microbiology and Biotechnology, 28(4), 1327-1350.

Borriss, R. 2015. Bacillus, a plant beneficial bacterium. In B. Lugtenberg (Ed.), Principles of Plant-Microbe Interactions. Microbes for Sustainable Agriculture. (pp. 379-391): Berlin: Springer).

Brantl, S. 2009. Bacterial chromosome-encoded small regulatory RNAs. Future microbiology, 4(1), 85-103.

Brooks, B., & Murray, R. 1981. Nomenclature for "*Micrococcus radiodurans*" and other radiation-resistant cocci: *Deinococcaceae* fam. nov. and *Deinococcus* gen. nov., including five species. International Journal of Systematic and Evolutionary

Microbiology, 31(3), 353-360.

Chandrayan, S. K., Prakash, S., Ahmed, S., & Guptasarma, P. 2014. Hyperthermophile protein behavior: partially-structured conformations of *Pyrococcus furiosus* rubredoxin monomers generated through forced cold-denaturation and refolding. PloS one, 9(3), e80014.

Compant, S., Duffy, B., Nowak, J., Clément, C., & Barka, E. A. 2005. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Applied and environmental microbiology, 71(9), 4951-4959.

Compant, S., Nowak, J., Coenye, T., Clément, C., & Ait Barka, E. 2008. Diversity and occurrence of *Burkholderia spp*. in the natural environment. FEMS microbiology reviews, 32(4), 607-626.

Cox, M. M., Keck, J. L., & Battista, J. R. 2010. Rising from the Ashes: DNA Repair in *Deinococcus radiodurans*. PLoS Genet, 6(1), e1000815.

D'Autréaux, B., & Toledano, M. B. 2007. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. Nature reviews Molecular cell biology, 8(10), 813-824.

Dahlsten, E., Isokallio, M., Somervuo, P., Lindström, M., & Korkeala, H. 2014. Transcriptomic analysis of (group I) *Clostridium botulinum* ATCC 3502 cold shock response. PloS one, 9(2), e89958.

Daly, M. J. 2009. A new perspective on radiation resistance based on *Deinococcus radiodurans*. Nature Reviews Microbiology, 7(3), 237-245.

Daly, M. J., Gaidamakova, E. K., Matrosova, V. Y., Kiang, J. G., Fukumoto, R., Lee, D.-Y., et al. 2010. Small-molecule antioxidant proteome-shields in *Deinococcus radiodurans*. PloS one, 5(9), e12570.

Daly, M. J., & Minton, K. W. 1996. An alternative pathway of recombination of chromosomal fragments precedes recA-dependent recombination in the radioresistant bacterium *Deinococcus radiodurans*. Journal of bacteriology, 178(15), 4461-4471.

De Groot, A., Roche, D., Fernandez, B., Ludanyi, M., Cruveiller, S., Pignol, D., et al. 2014. RNA sequencing and proteogenomics reveal the importance of leaderless mRNAs in the radiation-tolerant bacterium *Deinococcus deserti*. Genome biology and evolution, 6(4), 932-948.

Dey, R., Pal, K., Bhatt, D., & Chauhan, S. 2004. Growth promotion and yield enhancement of peanut (*Arachis hypogaea* L.) by application of plant

growth-promoting rhizobacteria. Microbiological research, 159(4), 371-394.

Dobbelaere, S., Vanderleyden, J., & Okon, Y. 2003. Plant growth-promoting effects of diazotrophs in the rhizosphere. Critical reviews in plant sciences, 22(2), 107-149.

Dunlap, C. A., Kim, S.-J., Kwon, S.-W., & Rooney, A. P. 2016. *Bacillus velezensis* is not a later heterotypic synonym of *Bacillus amyloliquefaciens*; *Bacillus methylotrophicus*, *Bacillus amyloliquefaciens* subsp. plantarum and '*Bacillus oryzicola*'are later heterotypic synonyms of *Bacillus velezensis* based on phylogenomics. International Journal of Systematic and Evolutionary Microbiology, 66(3), 1212-1217.

Eyraud, A., Tattevin, P., Chabelskaya, S., & Felden, B. 2014. A small RNA controls a protein regulator involved in antibiotic resistance in *Staphylococcus aureus*. Nucleic acids research, 42(8), 4892-4905.

Fan, B., Blom, J., Klenk, H.-P., & Borriss, R. 2017. *Bacillus amyloliquefaciens*, *Bacillus velezensis*, and *Bacillus siamensis* form an "operational group *B. amyloliquefaciens*" within the *B. subtilis* species complex. Frontiers in microbiology, 8, 22.

Fredrickson, J. K., Shu-mei, W. L., Gaidamakova, E. K., Matrosova, V. Y., Zhai, M., Sulloway, H. M., et al. 2008. Protein oxidation: key to bacterial desiccation resistance? The ISME journal, 2(4), 393-403.

Galtier, N., & Lobry, J. 1997. Relationships between genomic G+ C content, RNA secondary structures, and optimal growth temperature in prokaryotes. Journal of molecular evolution, 44(6), 632-636.

Gayán, E., Cambré, A., Michiels, C. W., & Aertsen, A. 2016. Stress-induced evolution of heat resistance and resuscitation speed in *Escherichia coli* O157: H7 ATCC 43888. Applied and environmental microbiology, 82(22), 6656-6663.

Gottesman, S. 2005. Micros for microbes: non-coding regulatory RNAs in bacteria. TRENDS in Genetics, 21(7), 399-404.

Gray, E., & Smith, D. 2005. Intracellular and extracellular PGPR: commonalities and distinctions in the plant–bacterium signaling processes. Soil Biology and Biochemistry, 37(3), 395-412.

Griffin, B. E. 1971. Separation of 32P-labelled ribonucleic acid components. The use of polyethylenimine-cellulose (TLC) as a second dimension in separating oligoribonucleotides of '4.5 S'and 5 S from *E. coli*. FEBS letters, 15(3), 165-168.

Guisbert, E., Herman, C., Lu, C. Z., & Gross, C. A. 2004. A chaperone network controls the heat shock response in *E. coli*. Genes & development, 18(22), 2812-2821.

Guisbert, E., Yura, T., Rhodius, V. A., & Gross, C. A. 2008. Convergence of molecular, modeling, and systems approaches for an understanding of the *Escherichia coli* heat shock response. Microbiology and Molecular Biology Reviews, 72(3), 545-554.

He, Y. 2009. High cell density production of *Deinococcus radiodurans* under optimized conditions. Journal of industrial microbiology & biotechnology, 36(4), 539-546.

Hébraud, M., & Potier, P. 1999. Cold shock response and low temperature adaptation in psychrotrophic bacteria. Journal of molecular microbiology and biotechnology, 1(2), 211-219.

Hensel, R., Demharter, W., Kandler, O., Kroppenstedt, R. M., & Stackebrandt, E. 1986. Chemotaxonomic and molecular-genetic studies of the genus Thermus: evidence for a phylogenetic relationship of *Thermus aquaticus* and Thermus ruber to the genus Deinococcus. International Journal of Systematic and Evolutionary Microbiology, 36(3), 444-453.

Hua, X., & Hua, Y. 2016. Improved complete genome sequence of the extremely radioresistant bacterium *Deinococcus radiodurans* R1 obtained using PacBio single-molecule sequencing. Genome announcements, 4(5), e00886-16.

Hungria, M., & Vargas, M. A. 2000. Environmental factors affecting N2 fixation in grain legumes in the tropics, with an emphasis on Brazil. Field crops research, 65(2-3), 151-164.

Jozefczuk, S., Klie, S., Catchpole, G., Szymanski, J., Cuadros-Inostroza, A., Steinhauser, D., et al. 2010. Metabolomic and transcriptomic stress response of *Escherichia coli*. Molecular systems biology, 6(1), 364.

Knoll, A. H., & Bauld, J. 1989. The evolution of ecological tolerance in prokaryotes. Earth and Environmental Science Transactions of The Royal Society of Edinburgh, 80(3-4), 209-223.

Lange, C. C., Wackett, L. P., Minton, K. W., & Daly, M. J. 1998. Engineering a recombinant *Deinococcus radiodurans* for organopollutant degradation in radioactive mixed waste environments. Nature biotechnology, 16(10), 929-933.

Li, J.-s., Bi, Y.-t., Dong, C., Yang, J.-f., & Liang, W.-d. 2011. Transcriptome analysis of adaptive heat shock response of *Streptococcus thermophilus*. PloS one, 6(10), e25777.

Li, W., Ying, X., Lu, Q., & Chen, L. 2012. Predicting sRNAs and their targets in bacteria. Genomics, proteomics & bioinformatics, 10(5), 276-284.

Licatalosi, D. D., & Darnell, R. B. 2010. RNA processing and its regulation: global insights into biological networks. Nature reviews genetics, 11(1), 75-87.

Livny, J., Zhou, X., Mandlik, A., Hubbard, T., Davis, B. M., & Waldor, M. K. 2014. Comparative RNA-Seq based dissection of the regulatory networks and environmental stimuli underlying *Vibrio parahaemolyticus* gene expression during infection. Nucleic acids research, 42(19), 12212-12223.

López-García, P., & Forterre, P. 2000. DNA topology and the thermal stress response, a tale from mesophiles and hyperthermophiles. Bioessays, 22(8), 738-746.

Majdalani, N., Chen, S., Murrow, J., St John, K., & Gottesman, S. 2001. Regulation of RpoS by a novel small RNA: the characterization of RprA. Molecular microbiology, 39(5), 1382-1394.

Makarova, K. S., Aravind, L., Wolf, Y. I., Tatusov, R. L., Minton, K. W., Koonin, E. V., et al. 2001. Genome of the extremely radiation-resistant bacterium *Deinococcus radiodurans* viewed from the perspective of comparative genomics. Microbiology and Molecular Biology Reviews, 65(1), 44-79.

Mann, B., van Opijnen, T., Wang, J., Obert, C., Wang, Y.-D., Carter, R., et al. 2012. Control of virulence by small RNAs in *Streptococcus pneumoniae*. PLoS Pathog, 8(7), e1002788.

Marioni, J. C., Mason, C. E., Mane, S. M., Stephens, M., & Gilad, Y. 2008. RNA-seq: an assessment of technical reproducibility and comparison with gene expression arrays. Genome research, 18(9), 1509-1517.

Mattimore, V., & Battista, J. R. 1996. Radioresistance of *Deinococcus radiodurans*: functions necessary to survive ionizing radiation are also necessary to survive prolonged desiccation. Journal of bacteriology, 178(3), 633-637.

Meena, V. S., Maurya, B., & Verma, J. P. 2014. Does a rhizospheric microorganism enhance K+ availability in agricultural soils? Microbiological research, 169(5-6), 337-347.

Meena, V. S., Verma, J. P., & Meena, S. K. 2015. Towards the current scenario of nutrient use efficiency in crop species. Journal of Cleaner Production, 102, 556-667.

Meyer, L., Coste, G., Sommer, S., Oberto, J., Confalonieri, F., Servant, P., et al. 2018. DdrI, a cAMP receptor protein family member, acts as a major regulator for adaptation of *Deinococcus radiodurans* to various stresses. Journal of

bacteriology, 200(13), e00129-18.

Mironov, A. S., Gusarov, I., Rafikov, R., Lopez, L. E., Shatalin, K., Kreneva, R. A., et al. 2002. Sensing small molecules by nascent RNA: a mechanism to control transcription in bacteria. Cell, 111(5), 747-756.

Moseley, B. 1967. The isolation and some properties of radiation-sensitive mutants of *Micrococcus radiodurans*. Microbiology, 49(2), 293-300.

Murata, M., Fujimoto, H., Nishimura, K., Charoensuk, K., Nagamitsu, H., Raina, S., et al. 2011. Molecular strategy for survival at a critical high temperature in *Eschierichia coli*. PloS one, 6(6), e20063.

Musto, H., Naya, H., Zavala, A., Romero, H., Alvarez-Valín, F., & Bernardi, G. 2006. Genomic GC level, optimal growth temperature, and genome size in prokaryotes. Biochemical and biophysical research communications, 347(1), 1-3.

Nagarajan, S., & Nagarajan, S. 2009. Abiotic tolerance and crop improvement. In Abiotic stress adaptation in plants (pp. 1-11): Springer.

Nahvi, A., Sudarsan, N., Ebert, M. S., Zou, X., Brown, K. L., & Breaker, R. R. 2002. Genetic control by a metabolite binding mRNA. Chemistry & biology, 9(9), 1043-1049.

Nakashima, H., Fukuchi, S., & Nishikawa, K. 2003. Compositional changes in RNA, DNA and proteins for bacterial adaptation to higher and lower temperatures. Journal of biochemistry, 133(4), 507-513.

Narberhaus, F. 1999. Negative regulation of bacterial heat shock genes. Molecular microbiology, 31(1), 1-8.

Narberhaus, F., Waldminghaus, T., & Chowdhury, S. 2006. RNA thermometers. FEMS microbiology reviews, 30(1), 3-16.

Ohtani, N., Tomita, M., & Itaya, M. 2010. An extreme thermophile, Thermus thermophilus, is a polyploid bacterium. Journal of bacteriology, 192(20), 5499-5505.

Omelchenko, M. V., Wolf, Y. I., Gaidamakova, E. K., Matrosova, V. Y., Vasilenko, A., Zhai, M., et al. 2005. Comparative genomics of *Thermus thermophilus* and *Deinococcus radiodurans*: divergent routes of adaptation to thermophily and radiation resistance. BMC evolutionary biology, 5(1), 57.

Opdyke, J. A., Kang, J.-G., & Storz, G. 2004. GadY, a small-RNA regulator of acid response genes in *Escherichia coli*. Journal of bacteriology, 186(20), 6698-6705.

Pruss, G. J., & Drlica, K. 1989. DNA supercoiling and prokaryotic transcription. Cell, 56(4), 521-523.

Repoila, F., & Darfeuille, F. 2009. Small regulatory non-coding RNAs in bacteria: physiology and mechanistic aspects. Biology of the Cell, 101(2), 117-131.

Rodríguez, H., Fraga, R., Gonzalez, T., & Bashan, Y. 2006. Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria. Plant and soil, 287(1-2), 15-21.

Romeo, T. 1998. Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule *CsrB*. Molecular microbiology, 29(6), 1321-1330.

Russell, A. 2003. Lethal effects of heat on bacterial physiology and structure. Science progress, 86(1-2), 115-137.

Sardesai, N., & Babu, C. R. 2001. Cold stress induced high molecular weight membrane polypeptides are responsible for cold tolerance in *Rhizobium* DDSS69. Microbiological research, 156(3), 279-284.

Schmid, A. K., Howell, H. A., Battista, J. R., Peterson, S. N., & Lidstrom, M. E. 2005a. Global transcriptional and proteomic analysis of the Sig1 heat shock regulon of *Deinococcus radiodurans*. Journal of bacteriology, 187(10), 3339-3351.

Schmid, A. K., Howell, H. A., Battista, J. R., Peterson, S. N., & Lidstrom, M. E. 2005b. HspR is a global negative regulator of heat shock gene expression in *Deinococcus radiodurans*. Molecular microbiology, 55(5), 1579-1590.

Shivaji, S., & Prakash, J. S. 2010. How do bacteria sense and respond to low temperature? Archives of microbiology, 192(2), 85-95.

Shoebitz, M., Ribaudo, C. M., Pardo, M. A., Cantore, M. L., Ciampi, L., & Cura, J. A. 2009. Plant growth promoting properties of a strain of *Enterobacter ludwigii* isolated from *Lolium perenne* rhizosphere. Soil Biology and Biochemistry, 41(9), 1768-1774.

Slade, D., Lindner, A. B., Paul, G., & Radman, M. 2009. Recombination and replication in DNA repair of heavily irradiated *Deinococcus radiodurans*. Cell, 136(6), 1044-1055.

Slade, D., & Radman, M. 2011. Oxidative stress resistance in *Deinococcus radiodurans*. Microbiology and Molecular Biology Reviews, 75(1), 133-191.

Storz, G., Altuvia, S., & Wassarman, K. M. 2005. An abundance of RNA regulators. Annu. Rev. Biochem., 74, 199-217.

Storz, G., Vogel, J., & Wassarman, K. M. 2011. Regulation by small RNAs in bacteria: expanding frontiers. Molecular cell, 43(6), 880-891.

Suo, B., Shi, C., & Shi, X. 2012. Inactivation and occurrence of sublethal injury of *Salmonella Typhimurium* under mild heat stress in broth. Journal für Verbraucherschutz und Lebensmittelsicherheit, 7(2), 125-131.

Teixeira, P., Castro, H., Mohácsi-Farkas, C., & Kirby, R. 1997. Identification of sites of injury in *Lactobacillus bulgaricus* during heat stress. Journal of Applied Microbiology, 83(2), 219-226.

Tian, B., Sun, Z., Shen, S., Wang, H., Jiao, J., Wang, L., et al. 2009. Effects of carotenoids from *Deinococcus radiodurans* on protein oxidation. Letters in applied microbiology, 49(6), 689-694.

Trotochaud, A. E., & Wassarman, K. M. 2004. 6S RNA function enhances long-term cell survival. Journal of bacteriology, 186(15), 4978-4985.

Trotochaud, A. E., & Wassarman, K. M. 2005. A highly conserved 6S RNA structure is required for regulation of transcription. Nature structural & molecular biology, 12(4), 313-319.

Tsai, C.-H., Liao, R., Chou, B., & Contreras, L. M. 2015. Transcriptional analysis of *Deinococcus radiodurans* reveals novel small RNAs that are differentially expressed under ionizing radiation. Applied and environmental microbiology, 81(5), 1754-1764.

Valverde, C., Livny, J., Schlüter, J.-P., Reinkensmeier, J., Becker, A., & Parisi, G. 2008. Prediction of *Sinorhizobium meliloti* sRNA genes and experimental detection in strain 2011. BMC genomics, 9(1), 416.

Van der Veen, S., Hain, T., Wouters, J. A., Hossain, H., de Vos, W. M., Abee, T., et al. 2007. The heat-shock response of *Listeria monocytogenes* comprises genes involved in heat shock, cell division, cell wall synthesis, and the SOS response. Microbiology, 153(10), 3593-3607.

Vanderpool, C. K., & Gottesman, S. 2004. Involvement of a novel transcriptional activator and small RNA in post-transcriptional regulation of the glucose phosphoenolpyruvate phosphotransferase system. Molecular microbiology, 54(4), 1076-1089.

Ventura, M., Canchaya, C., Zhang, Z., Bernini, V., Fitzgerald, G. F., & Van Sinderen, D. 2006. How high G+ C Gram-positive bacteria and in particular bifidobacteria cope with heat stress: protein players and regulators. FEMS microbiology reviews, 30(5), 734-759.

Vogel, J., & Sharma, C. M. 2005. How to find small non-coding RNAs in bacteria. Biological chemistry, 386(12), 1219-1238.

Wagner, E. G. H., & Romby, P. 2015. Small RNAs in bacteria and archaea: who they are, what they do, and how they do it. In Advances in genetics (pp. 133-208): Elsevier.

Wang, P., & Schellhorn, H. E. 1995. Induction of resistance to hydrogen peroxide and radiation in *Deinococcus radiodurans*. Canadian journal of microbiology, 41(2), 170-176.

Wang, Z., Gerstein, M., & Snyder, M. 2009. RNA-Seq: a revolutionary tool for transcriptomics. Nature reviews genetics, 10(1), 57-63.

Wassarman, K. M., Zhang, A., & Storz, G. 1999. Small RNAs in *Escherichia coli*. Trends in microbiology, 7(1), 37-45.

Weilbacher, T., Suzuki, K., Dubey, A. K., Wang, X., Gudapaty, S., Morozov, I., et al. 2003. A novel sRNA component of the carbon storage regulatory system of *Escherichia coli*. Molecular microbiology, 48(3), 657-670.

Weisburg, W. G., Giovannoni, S. J., & Woese, C. R. 1989. The Deinococcus-Thermus phylum and the effect of rRNA composition on phylogenetic tree construction. Systematic and Applied Microbiology, 11(2), 128-134.

White, O., Eisen, J. A., Heidelberg, J. F., Hickey, E. K., Peterson, J. D., Dodson, R. J., et al. 1999. Genome sequence of the radioresistant bacterium *Deinococcus radiodurans* R1. Science, 286(5444), 1571-1577.

Whitman, W. B., Coleman, D. C., & Wiebe, W. J. 1998. Prokaryotes: the unseen majority. Proceedings of the National Academy of Sciences, 95(12), 6578-6583.

Wilson, S. L., & Walker, V. K. 2010. Selection of low-temperature resistance in bacteria and potential applications. Environmental technology, 31(8-9), 943-956.

Winkler, W. C., Nahvi, A., Roth, A., Collins, J. A., & Breaker, R. R. 2004. Control of gene expression by a natural metabolite-responsive ribozyme. Nature, 428(6980), 281-286.

Work, E., & Griffiths, H. 1968. Morphology and chemistry of cell walls of *Micrococcus radiodurans*. Journal of bacteriology, 95(2), 641-657.

Yang, J., Kloepper, J. W., & Ryu, C.-M. 2009. Rhizosphere bacteria help plants tolerate abiotic stress. Trends in plant science, 14(1), 1-4.

Yura, T., & Nakahigashi, K. 1999. Regulation of the heat-shock response. Current opinion in microbiology, 2(2), 153-158.

Zahradka, K., Slade, D., Bailone, A., Sommer, S., Averbeck, D., Petranovic, M., et al. 2006. Reassembly of shattered chromosomes in *Deinococcus radiodurans*.

Nature, 443(7111), 569-573.

Zhang, H., Zhan, Y., Yan, Y., Liu, Y., Hu, G., Wang, S., et al. 2019. The *Pseudomonas stutzeri*-specific regulatory noncoding RNA NfiS targets katB mRNA encoding a catalase essential for optimal oxidative resistance and nitrogenase activity. Journal of bacteriology, 201(19), e00334-00319.

Zuber, U., & Schumann, W. 1994. CIRCE, a novel heat shock element involved in regulation of heat shock operon dnaK of *Bacillus subtilis*. Journal of bacteriology, 176(5), 1359-1363.

2

Chapter II: RNA-Seq-based comparative transcriptome analysis highlights new features of the heat-stress response in the extremophilic bacterium *Deinococcus radiodurans*

From **Xue, D**., Liu, W., Chen, Y., Liu, Y., Han, J., Geng, X., et al. 2019. RNA-seq-based comparative transcriptome analysis highlights new features of the heat-stress response in the extremophilic bacterium *Deinococcus radiodurans*. International journal of molecular sciences, 20(22), 5603.

2.1. Foreword

In this work, we performed an RNA-seq study in order to identify some new features in the response to heat stress of *D. radiodurans*. During the previous literature reports have shown that when *D. radiodurans* is exposed to 48°C for up to 2 hours, cell mortality is low and significant lethality only occurred later on. Proteins involved in heat stress tolerance/adaptation are thus assumed to accumulated during that timeframe of two hours post shift of temperature to 48°C and we decided to focus on genes specifically encoding those proteins and further investigate their expression upon recovery once cells have returned to normal temperature. Considering that it takes time for accumulated proteins to be degraded, we indeed hypothesized that some proteins retaining key roles in this process still remain in cells after recovery treatment for two hours. Genes encoding these key proteins are hypothesized to be expressed at distinct levels in different treatment groups, which has been verified in the performed transcriptomics analysis. We assume that the use of this strategy would provide a deeper understanding of *D. radiodurans* heat stress adaptation mechanism. Based on this principle, we set a control group at 30 ˚C (R30), a 48 ˚C treatment group (R48), and a recovery treatment group at 30 ˚C after heat treatment (RH). After growth status and electron microscope examination in these three groups, a transcriptome study was conducted. Via transcriptome sequences, we discovered new heat stress properties and discovered several new heat-related genes. By constructing mutants of these genes, the mechanisms involved in heat stress resistance have been investigated. This chapter was published in the "International Journal of Molecular Sciences" in 2019.

2.2. Abstract

Deinococcus radiodurans is best known for its extraordinary resistance to diverse environmental stress factors, such as ionizing radiation, ultraviolet (UV) irradiation, desiccation, oxidation, and high temperatures. The heat response of this bacterium is considered to be due to a classical, stress-induced regulatory system that is characterized by extensive transcriptional reprogramming. In this study, we investigated the key functional genes involved in heat stress that were expressed and accumulated in cells following heat treatment at 48 °C for 2 h (R48). Considering that protein degradation is a time-consuming bioprocess, we predicted that to maintain cellular homeostasis, the expression of the key functional genes would be decreased in cells that had partly recovered from heat stress (RH) relative to their expression in cells grown under optimal temperature (R30). Comparative transcriptomics identified 15 genes that were downregulated (eight-fold) in RH relative to R30, seven of which had previously been characterized to be heat shock genes. Among these genes, we hypothesis that three hypothetical genes (*dr_0127*, *dr_1083*, and *dr_1325*) are likely to be involved in response to heat stress. Survival analysis of mutant strains lacking

dr_0127, *dr_1325*, and *dr_1083* showed a reduction in heat tolerance compared to the wild-type strain. These results suggest that *dr_0127*, *dr_1083*, and *dr_1325* might play roles in the heat stress response. Overall, the results of this study provide deeper insights into the transcriptional regulation of the heat response in *D. radiodurans*.

Keywords

Deinococcus radiodurans; recovery; RNA-Seq; heat stress; novel heat-related gene

2.3. Introduction

Deinococcus radiodurans (*D. radiodurans*) is a nonmotile, pink-pigmented, Gram-positive bacterium belonging to the Deinococcus-Thermus phylum. This bacterium was first isolated from gamma-irradiated canned meat and is best known for its extraordinary resistance to ionizing and ultraviolet (UV) irradiation (Battista, 1997; White et al., 1999). Previous studies have reported that this species presents a rapid response and adaptation to a wide variety of extreme environments and stresses, such as desiccation (Mattimore et al., 1996), hydrogen peroxide (P. Wang et al., 1995), heat (Bauermeister et al., 2011), and other abiotic stresses. The rapid adaption of *D. radiodurans* is accompanied by a powerful DNA repair ability and extreme stress resistance. Hence, this species has become an ideal model organism for studying bacterial tolerance mechanisms under various extreme stress conditions (Blasius et al., 2008; Slade et al., 2011).

One of the earliest studies conducted on heat stress in *D. radiodurans* suggested that proteins synthesized de novo during the incubation interval were involved in either the appearance of thermotolerance or in the recovery from injury induced by heating at 52 °C for 30 min (Harada et al., 1988). In a later work, sigma factors (*sig1* and *sig2*) were identified as being involved in the active response of *D. radiodurans* to high-temperature stress. The *sig1* gene was identified as essential for the induction of the heat shock proteins GroESL and DnaKJ, whereas a *sig2* mutant exhibited only modest deficiencies in DnaKJ production (Schmid, Howell, et al., 2005a; Schmid et al., 2002). The global negative regulator encoded by *dr_0934* (HspR) binds to HspR-associated inverted repeat (HAIR) sites in close proximity to promoter regions, thereby directly inhibiting the expression of regulated genes encoding chaperone proteins and protease (Schmid, Howell, et al., 2005b). 2D-PAGE and global whole-cell Fourier transform ion cyclotron resonance mass spectrometric (FTICR-MS) proteomics have been widely employed to identify the molecular mechanisms underlying the heat tolerance of *D. radiodurans* at the proteomics level. The majority of the highly-induced heat shock proteins were identified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) (Airo et al., 2004; Schmid, Lipton, et al., 2005). The results of a more recent report investigating the DNA repair capacity and membrane integrity of *D. radiodurans* under dry and wet heat suggest that DNA damage repair (e.g., DNA double-strand breaks by *recA* and *pprA*) is essential after treatment with wet or dry heat (Bauermeister et al., 2012). Two small heat shock proteins (sHsps) identified in *Escherichia coli*, IbpA and IbpB, were analyzed in *D. radiodurans*, with subsequent analyses showing that these two proteins were very different in their quaternary structures and chaperone properties and were considered to represent a second type of bacterial two-component sHsp system (Bepperling et al., 2012). Recently, DdrI (encoded by *dr_0997*) has been reported to be involved in the heat shock response (Meyer et al., 2018).

Heat treatment has been reported to result in protein unfolding and aggregation in microbes (Lesley et al., 2002; Lim et al., 2010; Ventura et al., 2006). Accordingly, microorganisms have evolved complex molecular mechanisms to mitigate heat stress (Arsène et al., 2000; Chastanet et al., 2003; Lesley et al., 2002; Lim et al., 2010). Previous studies have shown that bacteria display both common and unique changes in their gene expression profiles in response to temperature fluctuations in their surrounding microenvironments (Anderson et al., 2006; Gunasekera et al., 2008; Wick et al., 2004). Most heat stress-induced genes encode molecular chaperones or proteases that either protect proteins/enzymes from misfolding or accelerate the degradation of damaged proteins. These processes lead to changes in the cell wall/membrane composition, protein synthesis rates, energy metabolism, and other biological processes, thereby maintaining internal cell homeostasis (Chastanet et al., 2003; Chowdhury Paul et al., 2012; Lim et al., 2010; Ma et al., 2015; Ventura et al., 2006; Ye et al., 2012). The use of RNA sequencing (RNA-Seq) allowed Chan et al. (2016) and Gomide et al. (2018) to identify many unanticipated genes associated with heat tolerance in *Pseudomonas aeruginosa* and *Corynebacterium pseudotuberculosis* following heat shock (Chan et al., 2016; Gomide et al., 2018).

Although much effort has been put into elucidating the molecular mechanisms underlying the response of *D. radiodurans* to heat stress, gene expression in *D. radiodurans* during heat stress is complex and not fully understood. However, due to the rapid development of sequencing technologies and bioinformatic analysis, many novel functional genes have been identified in *D. radiodurans* under a variety of stresses (Liu et al., 2003; Tanaka et al., 2004; Tsai et al., 2015). RNA-Seq has been successfully used to determine the deep transcriptional profiles of a complete genome in specific environments (López-Leal et al., 2014; J. Wang et al., 2012; Z. Wang et al., 2009). In this study, we utilized an RNA-Seq-based technique to examine the changes in the transcriptome of *D. radiodurans* in response to heat stress. Under heat stress, heat-related proteins are highly expressed to cope with cell metabolism and protein changes. When the external environment is restored to conditions that suitable for growth, a large amount of heat stress-related proteins will accumulate. Considering that degradation of these protein is a time-consuming bioprocess, we predicted that to maintain cellular homeostasis, the expression of key functional proteins would be significantly decreased in cells (RH) that had partly recovered from heat stress relative to their expression in cells (R30) grown under control conditions. Through this analysis, we identified many of the classical heat shock genes, the expressions of which were significantly increased in response to elevated temperature. In addition, our results show that three hypothetical genes (*dr_0127*, *dr_1083*, and *dr_1325*) might play important roles in the heat stress adaptation through mechanisms that require future study. The results of this study provide insight into the transcriptional regulation of the heat tolerance in *D. radiodurans*.

2.4. Materials and Methods

2.4.1. Strain and growth conditions

D. radiodurans was obtained from the China General Microbiological Culture Collection Center (CGMCC 1.633, Beijing, China). *D. radiodurans* was cultured at 30 °C in TGY medium (1% tryptone, 0.5% yeast extract, and 0.1% glucose) with shaking at 220 rpm/min.

2.4.2. Heat stress treatment of D. radiodurans and recovery conditions

Bacterial cells were pre-cultured in TGY to an OD_{600} = 2, were harvested by centrifugation at 7000× *g* for 3 min, washed twice in sterile phosphate-buffered saline (PBS, 0.02% KH_2PO_4, 0.29% $Na_2HPO_4 \cdot 12H_2O$, 0.8% NaCl, 0.02% KCl, pH 7.5), and resuspended in fresh TGY broth to the same cell density. For heat stress, cells were incubated at 48 °C for 2 h. They were then collected by centrifugation and resuspended to the same OD in TGY pre-conditioned at 30 °C. Bacteria were transferred to a second incubator pre-set at 30 °C and grown as a recovery culture for 0.5, 1, 1.5, or 2 h. A total of 100 µL was collected for dilution and plating onto solid TGY medium to calculate the number of colony-forming units (CFU). Cells that did not receive the heat treatment (2 h of growth at 30 °C) served as controls.

For transcriptomics, cells were treated at 48 °C for 2 h and then divided into two samples (1 and 2). Cells from sample 1 that were harvested by centrifugation at 12,000× *g* for 3 min and stored at –80 °C served as heat-treated group (R48). Cells from sample 2 that were centrifuged at 7000× *g* for 3 min, washed twice in PBS, and transferred to fresh TGY medium for recovery growth at 30 °C for 2 h served as the recovery group (RH). The restored cells were harvested by centrifugation at 12,000× *g* for 3 min and stored at −80 °C. Untreated *D. radiodurans* bacteria were used as the control (R30). All assays were performed in three biological replicates.

2.4.3. Transmission electron microscopy (TEM) and fluorescence assay

For TEM analysis, cells grown to OD_{600} = 2.0 were washed twice with PBS. The cells in the three treatment groups were collected and fixed overnight with 2.5% glutaraldehyde at 4 °C and then embedded in 2% agarose after centrifugation at 3000× *g*. Thin sections of the samples were stained with uranyl acetate for 15 min and observed using a Hitachi H-7650 transmission electron microscope (Hitach, Tokyo, Japan).

Cell viability after heat treatment was studied by staining with specific fluorochromes followed by epifluorescence microscopy. Cells were stained using a BacLight™ RedoxSensor™ Green Vitality Kit (ThermoFisher, MA, USA) containing PI and RSG. *D. radiodurans* cells were washed with PBS. This kit is convenient and easy-to-use for monitoring the viability of bacterial populations as

a function of cell membrane integrity. Cells with a compromised membrane, which are considered dead or dying, stain red (PI), whereas cells with an intact membrane stain green (RSG). A fluorescence assay was performed using a 100× oil immersion lens with a *Nikon* T*i*2 inverted fluorescence microscope and processed with the NIS-Elements (*Nikon*, Tokyo, Japan).

2.4.4. Total RNA extraction, complementary DNA (cDNA) library preparation, and sequencing

Total cellular RNA was extracted from *D. radiodurans* using TRIzol reagent (Invitrogen, Thermo Fisher, MA, USA), Lysing Matrix Tubes (MP Bio, CA, USA), and the PureLink RNA Mini Kit (Invitrogen, Thermo Fisher, MA, USA) following the manufacturer's instructions. RNA purity was assessed using a NanoDrop® spectrophotometer (Thermo Fisher, MA, USA). The RNA concentration was measured using a Qubit® RNA Assay Kit and a Qubit® 3.0 Fluorometer (Life Technologies, CA, USA). RNA integrity (RIN) was assessed using an RNA Nano 6000 Assay Kit and the Bioanalyzer 2100 system (Agilent Technologies, CA, USA), RIN > 9.5.

A total of 1 μg of high-quality RNA per sample was used as the input material for library preparation. Sequencing libraries were generated using a VAHTS Total RNA-Seq Library Prep Kit for Illumina® (Vazyme, NR603), following the manufacturer's recommendations. Following purification, the RNA was fragmented into small pieces using divalent cations under an elevated temperature. The cleaved RNA fragments were copied into first-strand cDNA using reverse transcriptase and random primers. Strand specificity was achieved by replacing dTTP with dUTP in 2nd strand marking buffer, followed by second-strand cDNA synthesis using DNA Polymerase I and Rnase H. Then, the cDNA fragments were end-repaired with the addition of a single 'A' base at the 3′-end of each strand and subsequently ligated to special sequencing adapters (Vazyme, N803). PCR was performed, and the products were purified. The library concentration was measured using a Qubit® RNA Assay Kit in Qubit® 3.0 for preliminary quantification. The sizes of the inserted fragments were assessed using the Agilent Bioanalyzer 2100 system, and high-quality insert fragments were accurately amplified using qPCR with the StepOne Plus Real-Time PCR system (ABI, USA). Clustering of the index-coded samples was performed on the cBot Cluster Generation System (Illumina, USA) according to the manufacturer's instructions. Then the well-prepared library was sequenced using the Illumina HiSeq X Ten platform with a 150-bp paired-end module. All samples were sequenced three times.

2.4.5. Assembly and functional enrichment analyses of differentially expressed genes (DEGs)

Clean reads with an average length of 150 bp were achieved after removing contaminated, poly-N and low-quality sequences from the raw reads. On average, 30.6 million transcripts were mapped to the reference genome with sample sizes ranging between 28.3 and 40.2 million reads (Supplementary Table

2-3). Pearson correlation analysis showed that the overall expression levels among the three biological replicates of each group were highly similar (R^2 > 0.92; Supplementary Figures 2-6 and 2-7), indicating that the RNA-Seq data were suitable for pairwise statistical comparisons**.** The reference genome and gene model annotation files of *D. radiodurans* were directly collected from the genome website (https://www.ncbi.nlm.nih.gov/genome/1020?genome_assembly_id=300483). The reference genome index was built using Bowtie2 (v2.2.9) (Langmead et al., 2012), and paired-end clean reads were aligned to the reference genome using TopHat (v2.1.1) (Kim et al., 2013). The mapped reads of each sample were assembled using Cufflinks (v2.2.1) (Trapnell et al., 2012) with a reference-based approach. Cufflinks uses a probabilistic model to simultaneously assemble and quantify the expression levels of a minimal set of isoforms, which provides a maximum likelihood explanation of the expression data for a given locus. Cuffdiff (v1.3.0) (Trapnell et al., 2012) was used to calculate the FPKMs of the coding genes in each sample. The gene FPKMs were computed by summing the FPKMs of the transcripts in each gene group. Cuffdiff (v2.2.1) (Trapnell et al., 2012) provides statistical routines for determining differential expression in a digital transcript or gene expression dataset using a model based on a negative binomial distribution. Genes with corrected *p*-values less than 0.05 and absolute $\log_2$ values (fold changes) >1.5 were considered significant DEGs.

2.4.6. Gene Ontology (GO) and Kyoto Encyclopedia of Gene and Genomes (KEGG) enrichment analysis

GO is a standardized system for classifying gene functionality and provides a dynamically-updated controlled vocabulary for fully characterizing gene properties and products in organisms. GO analysis is classified into three domains: biological processes, cellular components, and molecular functions of gene products. GO enrichment analysis of the DEGs was performed with the Perl module (GO::TermFinder) (Boyle et al., 2004). GO terms with a corrected *p*-values less than 0.05 were considered to be significantly enriched among the DEGs. In vivo, different genes coordinate with each other to perform their biological functions, and pathway-based analysis is helpful to understand the biological functions of gene interactions in various pathways. KEGG is a major public database containing manually-drawn pathway maps representing knowledge of molecular interactions and reaction networks. R functions (phyper and *q*-value) were used to test for the statistical enrichment of the DEGs among the KEGG pathways. KEGG pathways with corrected *p*-values less than 0.05 were considered to be significantly enriched for the DEGs.

2.4.7. Quantitative real-time PCR (qRT-PCR) validation

We randomly selected several genes with or without detectably differential expression for subsequent qRT-PCR analysis to verify the quality of the sequencing data. We used the same RNA as that used for RNA sequencing to do the qRT-PCR validation. In addition, we also collect the treated samples in three

biological replicates for qRT-PCR validation. Total RNA was extracted as described for the cDNA library preparation, and RNA was used for cDNA synthesis using a PrimeScript^{TM}RT reagent kit with gDNA Eraser (TaKaRa) as described in the manufacturer's protocol. Subsequently, qRT-PCR was performed using ChamQ SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., China) on a 7500 Fast Real-time PCR System (Applied Biosystems, USA). The primers are listed in Supplementary Table 2-4. The 16S rRNA gene was used as the endogenous reference control to normalize differences in total RNA quantity, and relative gene expression was quantified by the $2^{-\Delta\Delta CT}$ method. Three biological replicates for each condition were conducted.

2.4.8. Construction of gene deletion mutant strains and heat stress phenotype assays

Mutant strains lacking *dr_0127*, *dr_1325*, and *dr_1083* were constructed by fusion PCR recombination of a spectinomycin resistance cassette into the genome as previously described (Sheng et al., 2005). Briefly, fusion PCR products for the *dr_0127*, *dr_1325*, and *dr_1083* deletions were constructed in two steps. In the first step, three different PCRs were used to generate fragments complementary to the spectinomycin-resistance gene from the plasmid pKatAAD2 (896 bp) and the upstream and downstream regions (500 bp each) of the *dr_0127*, *dr_1325*, and *dr_1083* sequences using the appropriate primer pairs (Supplementary Table 2-4). In the second step, the upstream, spectinomycin-resistance gene and downstream fragments were annealed at their overlapping regions and PCR amplified as a single fragment using the outer primers (1896 bp). The resulting PCR fragment was directly transformed into *D. radiodurans*. Colonies resistant to spectinomycin (340 μg/mL) were selected, and these mutants were subsequently verified by PCR and DNA sequencing, and named Δ *dr_0127*, Δ *dr_1325*, and Δ *dr_1083*.

Cells were grown in TGY medium with the appropriate antibiotics to OD_{600} = 2 at 30 °C and were then shifted to 48 °C for 4 h. Subsequently, 100 μL of the cell suspension was aliquoted into 900 μL of PBS, after which 10-fold serial dilutions were made for all the strains, and 8 μL of each dilution was spotted onto TGY agar plates. These plates were incubated at 30 °C for 3 days before colony growth was observed and calculated. All assays were performed in triplicate.

2.4.9. Statistical analysis

All experiments were repeated at least three times with identical or similar results. The mean values from the individual experiments were expressed as averages ± standard deviations (SD). A p-value < 0.05 was considered to be significant. R studio and GraphPad Prism 7.0 software were used for the analysis.

2.5. Results

2.5.1. Cell growth state and viability of D. radiodurans under heat stress

In order to investigate the mechanism of adaptation to heat stress of *D. radiodurans*, we used a series of recovery times (0, 0.5, 1, 1.5, and 2 h) for growth at 30 °C following incubation at 48 °C. This series allowed us to determine the time necessary for the cells to return to their initial growth state after the heat stress treatment. We found that after 2 h of cell recovery, the growth viability according to the cell number was essentially the same as that of R30 subjected to continuous culture for 2 h (Figure 2-1).

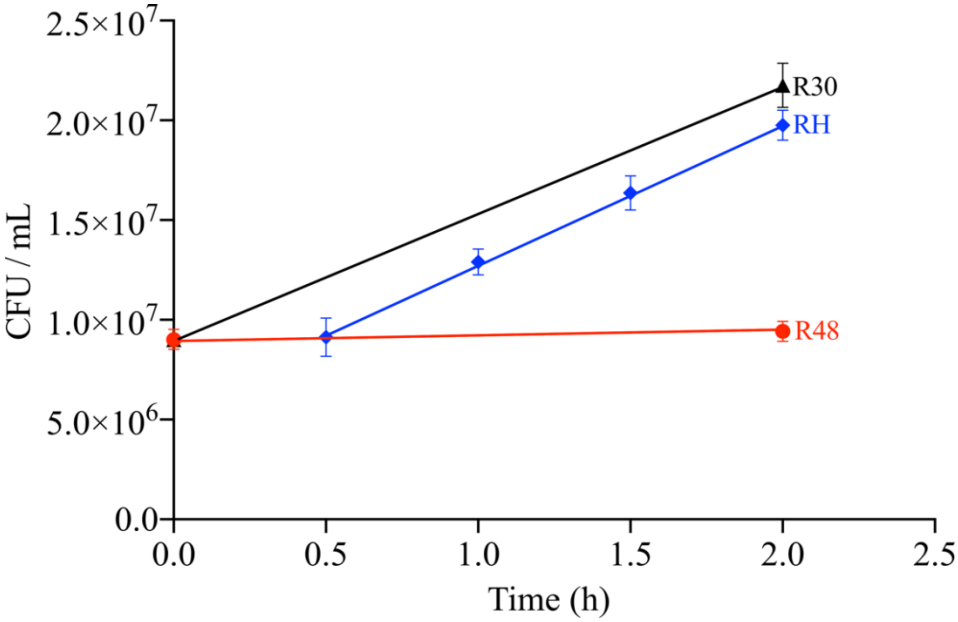


Figure 2-1. Correlation analysis of the cell viability and the different treatments using three biological replicates.
The triangle symbol with the black line represents the untreated group (R30), the circles with the red line represent the heat treatment group (R48), and the diamond dots with the blue line represent the recovery treatment group at various incubation periods (RH). Error bars represent the SD calculated from three sets of independent experiments.

Because heat stress may affect the integrity of *D. radiodurans*, we used transmission electron microscopy (TEM) to observe the morphology of *D. radiodurans* cells. As shown in Figure 2-2A1,B1, the ultrastructure of cells incubated at 48 °C (R48) was different compared to R30, and R48 exhibited some visible damages in some parts of the envelope. After a further culture at 30 °C for 2 h, the cell envelope remained slightly damaged (Figure 2-2C1), but

to a lower extent that before recovery, indicating that *D. radiodurans* cells underwent some efficient repair mechanisms. These results show that heat stress may affect the structure of the bacterial cell wall but it is rather limited, having no significant effect on cell death, as revealed by fluorescence microscopy (Figure 2-2A2,B2,C2) showing that almost all cells remained alive.

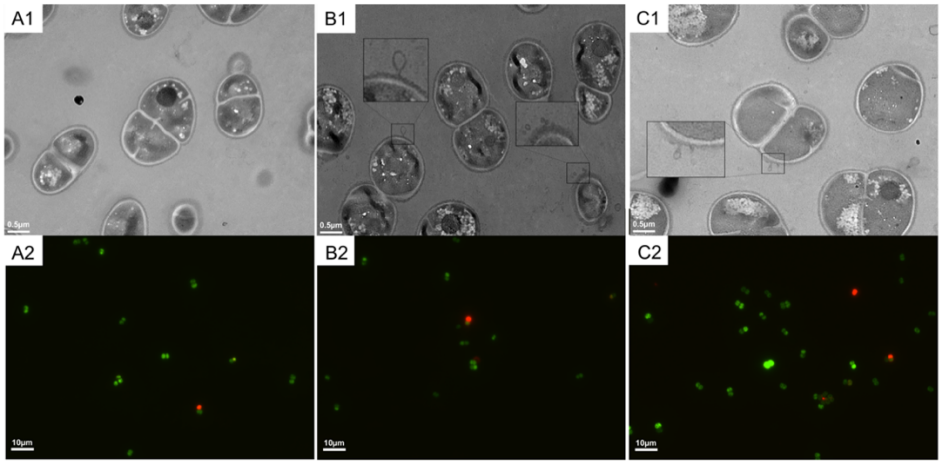


Figure 2-2. TEM and fluorescence images of the heat-induced lesions on *D. radiodurans* cells following exposure to 48 °C for 2 h.
(**A1**,**B1**,**C1**) represent the TEM results; (**A2**,**B2**,**C2**) represent the fluorescence analysis results. (**A1**,**A2**) *D. radiodurans* cells at 30 °C (control samples), (**B1**,**B2**) *D. radiodurans* cells at 48 °C (heat-treated samples), and (**C1**,**C2**) *D. radiodurans* cells recovered at 30 °C after the heat treatment (recovery samples). The inset diagram (**B1**,**C1**) show an amplified region of the cell envelope. Living cells were stained by Redox Sensor Green (RSG) (green), and dead cells were stained by propidium iodide (PI) (red). The scale bars indicate the corresponding lengths.

2.5.2. Qualitative and quantitative analyses of the DEGs in the three groups under heat stress

The phenotypic results presented above showed that the growth state of heat-treated cells almost reached that of the untreated cells after recovery for 2 h. We expected that a transcriptional analysis of these samples would provide a deeper understanding of the mechanisms responsible for adaptation of *D. radiodurans* to high temperature. Several DEGs representing different functional categories were selected for quantitative real-time PCR (qRT-PCR) analysis (Supplementary Figure 2-1). The expression profiles of the selected genes showed the same tendencies as those detected by RNA-Seq, indicating the good quality of the sequencing data.

DEG analysis after fragments per kilobase of transcript per million mapped reads (FPKM) normalization yielded 818 and 162 genes with significantly

different expression levels (FDR-corrected $p < 0.05$, ≥ 1.5 $\log_2$-fold change) in the pairwise comparisons of R48 versus R30 and RH versus R30 (Figure 2-3 A,B; Supplementary Table 2-1). Among these genes, 316 were upregulated and 502 were downregulated (R48 versus R30) and 28 were upregulated and 134 were downregulated (RH versus R30). As shown in the Venn diagram (Figure 2-3C), 124 and 23 DEGs were unique to the pairwise comparisons of R48 versus R30 and RH versus R30. While investigating the gene expression of *D. radiodurans*, three replicates of R30, R48, and RH were used to create three distinct clusters via principal component analysis of the samples (Figure 2-3D). These data indicate that the DEGs have a very consistent and unique expression profile.

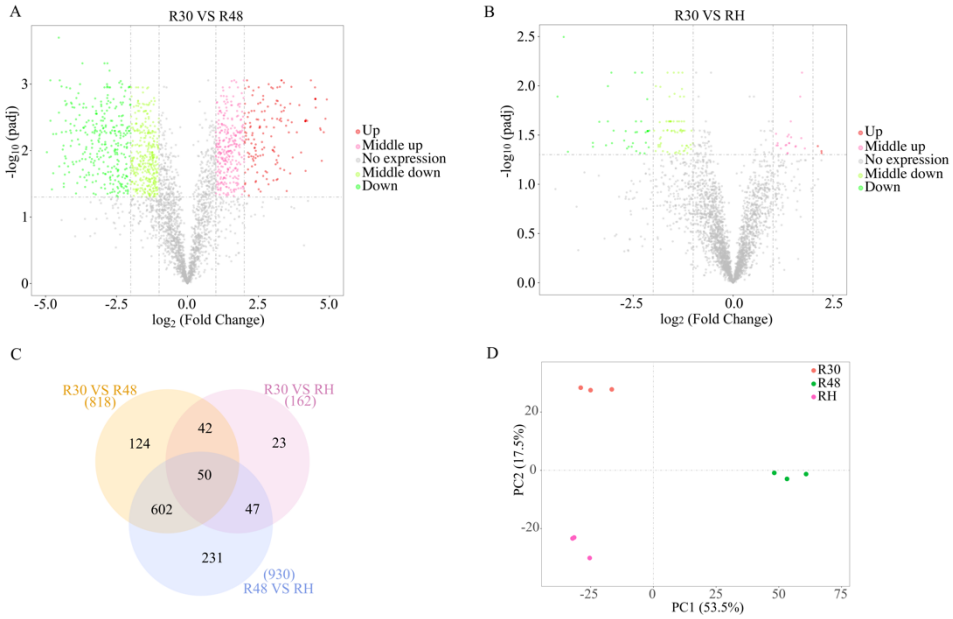


Figure 2-3. Differential expression levels among the three treatment groups. Each point represents a unigene. The x-axis represents the $\log_{10}$ values of the normalized expression level (FPKM) of the unigenes in each group. The red and green points indicate significant changes in the absolute value of $\log_2$ (the FPKM ratio in two groups) ≥ 1.5 and FDR ≤ 0.05, respectively; i.e., the red points indicate upregulated unigenes, and the green points indicate downregulated unigenes in the two groups, with the differential expression levels presented along the X-axis. The gray points indicate nonsignificant, differentially expressed unigenes. (**A**) R48 versus R30; (**B**) RH versus R30. (**C**) Venn diagram showing significant gene expression changes in response to heat. (**D**) Principal component analysis based on the FPKM expression values among the three treatment groups.

Regarding these significantly DEGs in response to heat stress, Table 2-1 presents a list of the top 30 most significantly up and downregulated-genes (R48 versus R30). Interestingly, we identified highly expressed genes

(*dr_A0075-A0087*) with unknown functions. These genes are transcribed in the same transcription direction, and we hypothesized that they may be in the same operon and work together to cope with heat stress. In addition, some heat-induced genes that were reported in previous studies were also identified in our study (Table 2-1). We also identified many previously unreported genes that may be involved in this heat stress adaptation and wanted to further investigate the roles of these genes in the heat shock response.

Table 2-1. The top 30 most upregulated and downregulated genes when exposed to heat stress (R48 versus R30).

Gene ID	Log2 (Fold Change)	*p* Value	Function Description	References
DR_A0081	13.0548	0.02115134	Hypothetical protein	
DR_A0078	9.3231	0.01952321	Hypothetical protein	
DR_2307	8.5243	1.16×10^{-5}	Multidrug-efflux transporter, putative	(Schmid, Howell, et al., 2005a)
DR_0518	8.3853	0.00804687	Hypothetical protein	
DR_0524	8.1827	0.00604411	Hypothetical protein	
DR_A0082	7.8581	0.02528407	Hypothetical protein	
DR_A0079	7.745	0.0212175	Hypothetical protein	
DR_A0080	7.6759	0.0183724	Hypothetical protein	
DR_A0083	7.4529	0.02027402	Hypothetical protein	
DR_A0086	7.3763	0.01788636	Hypothetical protein	
DR_0519	7.3405	0.00011836	Hypothetical protein	
DR_A0101	7.2857	0.0161855	Hypothetical protein	
DR_A0077	7.1326	0.01621666	Hypothetical protein	
DR_A0075	6.9708	0.04170709	Transposase, putative	
DR_A0087	6.5568	0.01613077	Hypothetical protein	
DR_B0072	6.0839	5.39×10^{-6}	Salicylate monooxygenase-related protein	
DR_B0074	6.0164	7.87×10^{-7}	1-Phosphofructokinase	
DR_B0073	5.8393	1.1×10^{-5}	PTS system, fructose-specific IIBC component	
DR_A0085	5.833	0.02305785	Hypothetical protein	
DR_A0211	5.5236	4.38×10^{-6}	Transcriptional regulator, GntR family	

DR_C0023	5.1883	2.24×10^{-5}	Hypothetical protein	
DR_A0076	5.1069	0.02545438	ATP-dependent target DNA activator	
DR_B0141	5.0516	9×10^{-5}	HicB-related protein	
DR_B0142	4.9077	5.11×10^{-5}	Hypothetical protein	
DR_0422	4.8592	0.00024562	Trans-aconitate 2-methyltransferase	
DR_A0182	4.799	0.00068635	Hypothetical protein	(Schmid, Lipton, et al., 2005)
DR_0516	4.7289	0.00382339	Hypothetical protein	
DR_2374	4.6897	0.0005564	Ribonucleoside-diphosphate reductase-related protein	(Schmid, Lipton, et al., 2005)
DR_0532	4.6735	0.00051046	Hypothetical protein	
DR_0423	4.5152	0.00011999	Hypothetical protein	
DR_A0364	−4.3096	5.93×10^{-5}	Oxidoreductase, short-chain dehydrogenase/reductase family	(Schmid, Lipton, et al., 2005)
DR_0201	−4.321	0.00785386	Hypothetical protein	
DR_0392	−4.3342	0.00434331	Hypothetical protein	(Schmid, Howell, et al., 2005a)
DR_0334	−4.3572	0.01241591	Lipase, putative	
DR_B0038	−4.3573	0.01463299	Hypothetical protein	
DR_A0352	−4.3662	0.00155047	Methyl-accepting chemotaxis protein	
DR_2240	−4.3942	0.00195473	Hypothetical protein	
DR_2527	−4.4273	0.00068251	Hypothetical protein	(Schmid, Howell, et al., 2005a)
DR_A0233	−4.4361	1.05×10^{-5}	Oxidoreductase, iron-sulfur subunit	(Schmid, Lipton, et al., 2005)
DR_1987	−4.4998	3.18×10^{-6}	Hypothetical protein	
DR_A0231	−4.5169	0.00510755	Oxidoreductase	(Schmid, Lipton, et al., 2005)
DR_1778	−4.5277	0.00016505	3-Isopropylmalate dehydratase, large subunit	
DR_2263	−4.5338	6.47×10^{-8}	DNA-binding stress response protein, Dps family	(Schmid, Howell, et al., 2005a)
DR_2563	−4.6247	0.00080366	Hypothetical protein	

DR_1712	−4.7284	0.00739225	Extracellular solute-binding protein, family 5	(Schmid, Lipton, et al., 2005)
DR_A0232	−4.7289	0.00081206	Oxidoreductase	
DR_1277	−4.7754	0.01673083	ABC transporter, periplasmic substrate-binding protein,	
DR_1711	−4.8264	0.00028517	N-Acyl-L-amino acid amidohydrolase	
DR_1067	−4.8305	5.2×10^{-6}	Hypothetical protein	
DR_0791	−4.9522	0.00226901	Chloride peroxidase, putative	
DR_0644	−5.4271	0.00078552	Hypothetical protein	
DR_2560	−5.4566	0.0002672	Hypothetical protein	
DR_1315	−5.4743	0.00327873	Hypothetical protein	(Schmid, Howell, et al., 2005a; Schmid, Lipton, et al., 2005)
DR_0105	−5.4893	1.54×10^{-6}	Hypothetical protein	
DR_1066	−5.6713	0.02719424	Hypothetical protein	
DR_1483	−5.8009	0.00071264	Hypothetical protein	
DR_1790	−5.8218	1.62×10^{-6}	Yellow-related protein	
DR_0465	−5.8342	0.00052829	Conserved hypothetical	
DR_1314	−6.1749	0.00281391	Conserved hypothetical protein	(Schmid, Howell, et al., 2005a)
DR_0891	−6.4635	0.00113242	DNA-binding response	

Note: The previously reported genes involved in heat stress are indicated in bold fonts.

2.5.3. GO and KEGG analyses of the DEGs

To determine the functions of the DEGs, all of the DEGs were annotated to terms in the GO database. The most dominant subcategories were the “oxidation-reduction process,” “cell,” “peptidase activity,” “cell part,” and “oxidoreductase activity” (Supplementary Figure 2-2; Supplementary Table 2-2). The top 20 most significantly enriched KEGG pathways identified via KEGG analysis are shown in Supplementary Figure 2-3. Several key molecular mechanisms are represented, including nitrogen metabolism, pyruvate metabolism, glycolysis, ribosome, oxidative phosphorylation, and propanoate metabolism. These results reveal that cells utilize numerous repair processes in response to high temperature.

2.5.4. Analysis of heat-related genes with over eight-fold higher expressions in response to heat stress

Comparisons among R30, R48, and RH revealed a total of 257 significantly

regulated genes that exhibited at least an eight-fold difference in expression (Figure 2-4A). To obtain an overview of the functions that are altered during heat treatment and the recovery stage, 100 response genes (excluding hypothetical genes) were annotated with KEGG functional groups. Figure 2-4B presents the distributions within each category. A total of 19 genes were assigned to "Replication and repair." The expressions of genes associated with "carbohydrate metabolism and transport" and "amino acid metabolism and transport" were greatly reduced at high temperature.

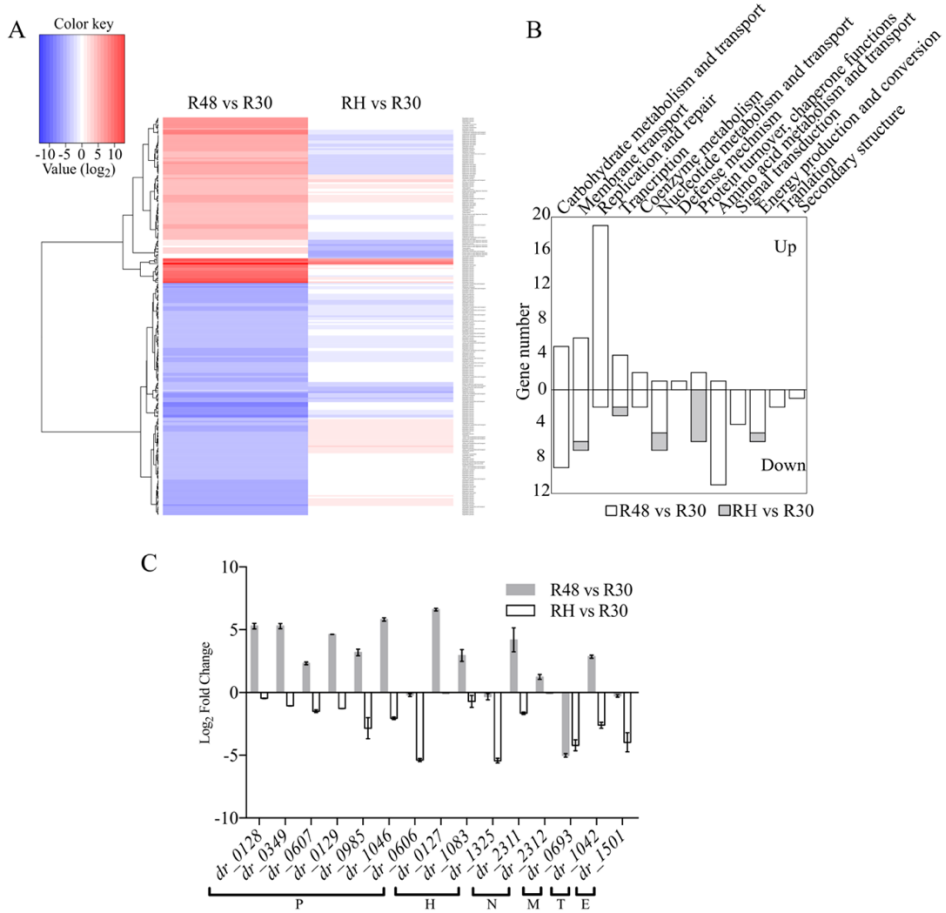


Figure 2-4. Heat-related genes with over eight-fold higher expressions in response to heat stress.

(**A**) Heatmap of the log2-fold changes (LFCs) among R30, R48, and RH. Included are 257 genes with fold changes >8. The dendrogram represents the hierarchical clustering of the LFCs. (**B**) Likert chart of the KEGG functional categorizations of the differentially expressed genes between R48 versus R30 and RH versus R30. (**C**) qRT-PCR analysis of the high differentially expressed genes; P: protein turnover and chaperone functions, H: hypothetical protein, N: nucleotide metabolism and transport, M: membrane transport, T: transcription, E: energy production and conversion.

2.5.5. Function analysis of the novel, potentially heat-related genes

A total of 16 genes were differentially expressed (over eight-fold) in RH versus R30. Of these 16 genes, only one was upregulated, whereas 15 were downregulated (Table 2-2). These 15 genes showed at least an eight-fold downregulation in RH and were divided into the following categories: protein turnover and chaperone function (seven genes), hypothetical proteins (three genes), nucleotide metabolism and transport function (two genes), membrane transport (one gene), transcription (one gene), and energy production and conversion (one gene) (Figure 2-4C).

Table 2-2. Descriptions of the 16 genes with altered expression in cells recovered from heat stress compared with non-stressed cells (RH versus R30).

Gene ID	Gene name	Functions	Induction ratio Log2 (RH vs R30)	*p* value
DR_0128	grpE	Protein turnover and chaperone function	-3.1812	0.00160996
DR_0349	Lon	Protein turnover and chaperone function	-3.5087	0.00188947
DR_0607	groEL	Protein turnover and chaperone function	-3.5102	0.00128474
DR_0129	dnaK	Protein turnover and chaperone function	-3.9169	0.04542787
DR_0985		Protein turnover and chaperone function	-4.1346	0.00249103
DR_1046	clpB	Protein turnover and chaperone function	-4.4467	0.01832036
DR_0606	groES	Protein turnover and chaperone function	-3.3489	0.00035202
DR_0127		Hypothetical protein	-3.0322	0.00135957
DR_1083		Hypothetical protein	-3.1333	4.23E-05
DR_1325		Hypothetical protein	-3.3623	0.00886153
DR_2311		Nucleotide metabolism and transport function	-4.3949	9.48E-05
DR_2312		Nucleotide metabolism and transport function	-4.2404	1.03E-06
DR_1501		Energy production and conversion	-3.1352	0.00033852
DR_0693		Membrane transport	-3.7209	0.00754623
DR_1042	PadR	Transcription	-3.0467	1.60E-05

Three hypothetical genes (*dr_1325*, *dr_0127*, and *dr_1083*) were selected for further study. According to BLASTp, these three genes encode, respectively, for a predicted LysM peptidoglycan-binding protein (DR_1325), a predicted DNA-binding protein (DR_0127), and a hypothetical protein (DR_1083). Two of these genes, *dr_1325* and *dr_0127*, may be, respectively, involved in adaptation to heat stress through cell wall biogenesis and a DNA-binding protein, although their specific functions remain to be elucidated.

In order to further investigate the roles of those three genes *dr_0127*, *dr_1325*, and *dr_1083*, significantly upregulated in response to heat stress, disruption mutant strains were constructed and the insertion of the spectinomycin gene into each gene was confirmed by genomic PCR and sequencing (Supplementary Figure 2-4). To examine whether the *dr_0127* gene deletion had an effect on the expression of its flanking genes, we compared the expressions of *dr_0126*, *dr_0128*, and *dr_0129* in *D. radiodurans* wild type and Δ *dr_0127* by qRT-PCR. Figure 2-5A shows that the deletion of *dr_0127* caused a sharp decrease in the expression of *dr_0126*, increased the expression of *dr_0128* two-fold, and had no effect on *dr_0129* expression. At 48 °C, the deletion of *dr_0127* resulted in a decrease in the expression levels of these three genes. These results suggested that this deletion somehow affected the expression of the flanking genes under both normal and heat stress conditions, possibly because these genes work together in response to heat stress. In addition, the strain with the deletion of *dr_0127* showed no significant growth difference compared to the wild type under standard conditions (Figure 2-5B). A test for growth potential on TGY plate assays at a high temperature (48 °C) showed that the three mutant strains were more susceptible than the *D. radiodurans* wild-type, strongly suggesting that *dr_0127*, *dr_1083*, and *dr_1325* play key roles in the tolerance to heat stress (Figure 2-5C). Particular attention was devoted to *dr_0127* because the cognate mutant was the most severely impacted in terms of tolerance to heat shock (Figure 2-5C) and because it was the most differentially up-regulated upon heat stress (Figure 2-4C) among the 15 genes tested.

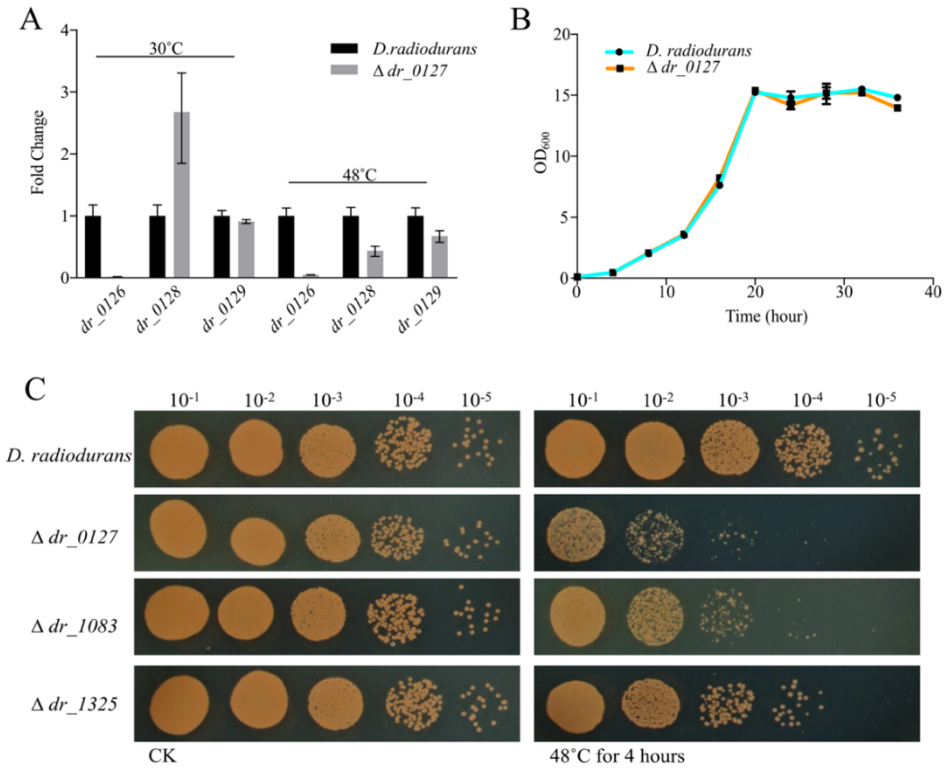


Figure 2-5. The effect of the *dr_0127* deletion on the expression of its flanking genes and survival phenotype plate assay upon heat stress.
(**A**) The effect of the *dr_0127* deletion on the expression of its flanking genes (*dr_0126*, *dr_0128*, and *dr_0129*) under normal growth and heat stress conditions. The relative levels of transcripts are presented as the mean values ± standard deviations, calculated from three sets of independent experiments and normalized to levels in the wild-type strain. (**B**) Growth curves in TGY broth of wild type and Δ *dr_0127*. The error bars represent the standard deviations of the measurements of three biological replicates. (**C**) Serial, 10-fold dilutions of OD-standardized *D. radiodurans* and three mutants (Δ*dr_0127*, Δ*dr_1083*, and Δ*dr_1325*) spotted on TGY plates after exposure to 48 °C. CK, untreated culture control. All experiments were performed three times.

2.6. Discussion

In this study, we used RNA-Seq to investigate the mechanism of adaptation of *D. radiodurans* to heat stress. A sublethal high temperature is the best way to study heat stress genes (Lindquist, 1986). Previous data have shown that 48 °C is the optimal temperature to study heat stress in *D. radiodurans* (Schmid et al., 2002). After the heat treatment, a large number of genes were differentially expressed to allow *D. radiodurans* to adapt to the external environment. Fluorescence electron microscopy showed that the heat treatment for 2 h did not

cause the death of *D. radiodurans* (Figure 2-2). This is consistent with previously reported results showing that almost all of the cells remained alive after 2 h of heat treatment (Schmid, Howell, et al., 2005a). To analyze heat-related genes in detail, we performed recovery growth after the heat treatment. Our data show that after 2 h of recovery at 30 °C, the cells recovered normal life activities (Figure 2-1). Interestingly, the growth rate of *D. radiodurans* is approximately 2 h for one generation, a duration equivalent to our recovery time. We hypothesize that when *D. radiodurans* cells undergo self-repair after sublethal stress, it may take one generation for them to recover to prestress levels. The transcriptome data showed that most of the gene expression in RH was equivalent to that in R30, which confirmed that the 2 h of recovery allowed the intracellular homeostasis of the previous stage to be restored.

The cell wall is crucial for cellular function, especially for protecting cell physiological activity, as it separates the internal environment from the external environment. However, TEM showed that the *D. radiodurans* cell wall was slightly damaged after treatment at 48 °C treatment for 2 h (Figure 2-2B1). Although most of the cells remained alive (Figure 2-2B2), the high temperature damaged the integrity of the cell wall. Similarly, Stéphane Guyot et.al (2010) and Bożena Bruhn-Olszewska et al. (2018) reported that high temperatures can destroy the integrity of the cell wall in *E. coli* (Bruhn-Olszewska et al., 2018; Guyot et al., 2010). In this study, the TEM data showed that after the cells had recovered at 30 °C for 2 h, their cell walls showed partial repair (Figure 2-2C1). Complete cell wall repair would likely require additional time after the heat stress.

Chaperones and proteases function by preventing protein aggregation and facilitating the degradation of improperly folded proteins under heat stress conditions (Baneyx et al., 2004; Parsell et al., 1993). In the present study, heat-induced genes (including *groEL*, *lon1*, *lon2*, *dnaK*, and *hsp20*) were highly expressed in R48, with these genes have been well characterized in many organisms (Arsène et al., 2000; Schumann, 2016). In *E. coli*, the aggregation of abnormal proteins stimulates the transcription of Lon protease to degrade damaged proteins (Kuroda et al., 2001). There are two Lon homologs in *D. radiodurans* (DR_0349 and DR_1974), and the deletion of these factors was observed to increase cell sensitivity to puromycin, indicating a reduced capacity to degrade abnormal proteins (Servant et al., 2007). There are two sHSP members (IbpA and IbpB) in *E. coli*. These proteins were reported to be involved in the aggregation of proteins after heat stress (Kuczynska-Wisnik et al., 2002; Matuszewska et al., 2005). Bepperling et al. reported that *D. radiodurans* also harbors IbpA (Hsp20) and IbpB (DR_1691), which can work in parallel and independently of each other to combat protein aggregation during stress (Bepperling et al., 2012). In addition, *hsp20* was reported to confer tolerance to hydrogen peroxide (Singh et al., 2014). These previous results and those of our transcriptomic analysis suggest that these proteases and chaperones likely play important roles in *D. radiodurans* during heat stress. Further analyses are needed to clarify the roles of chaperones and proteases in the heat tolerance of *D. radiodurans*.

The heat shock response of *D. radiodurans* illustrates the extensive diversity of gene regulation in this species. We identified genes with more than eight-fold differential expression between groups, with 15 genes in RH being downregulated by more than eight-fold relative to the expression observed in R30 (Figure 2-4). A subsequent investigation revealed that almost all these genes are involved in heat stress and that most are involved in protein turnover and chaperone function. Moreover, three of these genes are hypothetical genes (*dr_0127*, *dr_1083*, and *dr_1325*), among which one (*dr_0127*) was substantially upregulated in R48 (Figure 4). Annotation revealed that DR_0127 was predicted to be a DNA-binding protein. Genomic DNA packaging is mediated by a set of DNA-binding proteins that have major impacts on gene transcription and DNA replication (Azam et al., 1999; Browning et al., 2010; Dillon et al., 2010). The nucleoid of a bacterium is organized by DNA-binding proteins, especially under stress conditions; these proteins also protect the bacterial genome and regulate transcription to promote survival under stress conditions. Through alignment analyses, we observed that DR_0127 is highly conserved in other *Deinococcus* species, such as *D. wulumuqiensis*, *D. gobiensis*, *D. soli*, and *D. actinosclerus*. More interestingly, the upstream and downstream genes encode DnaJ, GrpE, and DnaK. The qRT-PCR data showed that the deletion of *dr_0127* drastically decreased the expression of the downstream gene *dr_0126* (which encodes a DNA binding protein). Through the genome analysis, we predicted that these genes may share the same operon. In addition, we believe that the reduction in tolerance to heat stress may not only be due to the absence of *dr_0127* but also due to the dramatic downregulation of *dr_0126* expression. In future work, we will further clarify the exact relationship between *dr_0126* and *dr_0127* in response to heat stress by performing insertional mutagenesis. The deletion of *dr_0127* resulted in decreased expression of *dr_0126* (*dnaJ*), *dr_0128* (*grpE*), and *dr_0129 (dnaK)* relative to the wild-type strain under heat stress. DR_1027 is predicted to be a genus-specific DNA binding protein that we hypothesize may work together with DnaJ, GrpE, and DnaK in response to heat stress, although their mechanisms of action require further study. DR_1325 is predicted to be a protein containing a LysM peptidoglycan-binding domain, which may be involved in cell wall biogenesis. In addition, our phenotype experiments showed that deletion of DR_1325 resulted in sensitivity to heat stress compared to the wild type strain. *dr_1325* appears to be the first gene of the operon (*dr_1325-27*), and the deletion of *dr_1325* also caused the upregulation of *dr_1326* and *dr_1327* (Supplementary Figure 2-5). The phenotype of the mutant strain resulting from the lack of the *dr_1325* may be caused by the changes in the transcription of the affected genes. Our results also show that heat stress damages the cell wall of *D. radiodurans* (Figure 2-2). We speculate that DR_1325 may regulate the response of the heat stress mechanism by participating in cell wall repair. That idea requires experimental support from further studies.

Taken together, the results of this study reveal a highly complex gene-expression response to heat stress in *D. radiodurans* that involves numerous key genes related to various cell processes. Chief among these genes

are those encoding chaperones, heat shock proteins, proteases, and posttranscriptional regulatory proteins (Figure 2-6). Our study also revealed novel, potentially important features exhibited by *D. radiodurans* in response to heat stress, such as the substantial recovery of RH cells compared with R30 cells, confirming that *D. radiodurans* also has a strong self-repair ability under heat stress. By analyzing the transcriptome data of the three groups, some new heat-related genes were discovered and verified, which expand the heat stress-related gene family in *D. radiodurans*. Further metabolome analyses and gene knockout experiments, particularly those targeting the novel genes, will enhance our understanding of the molecular mechanisms underlying heat stress in this species.

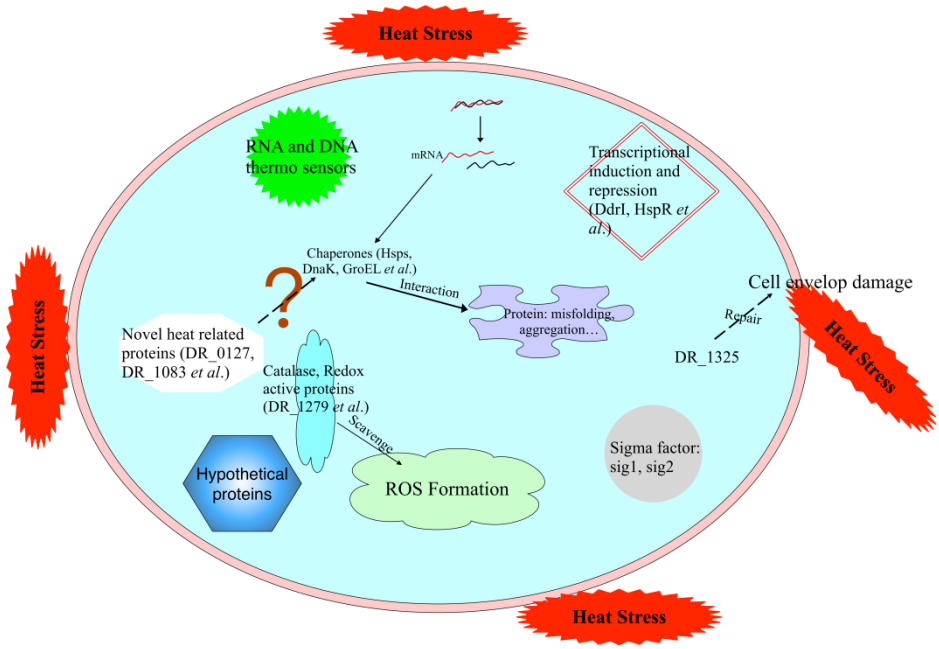
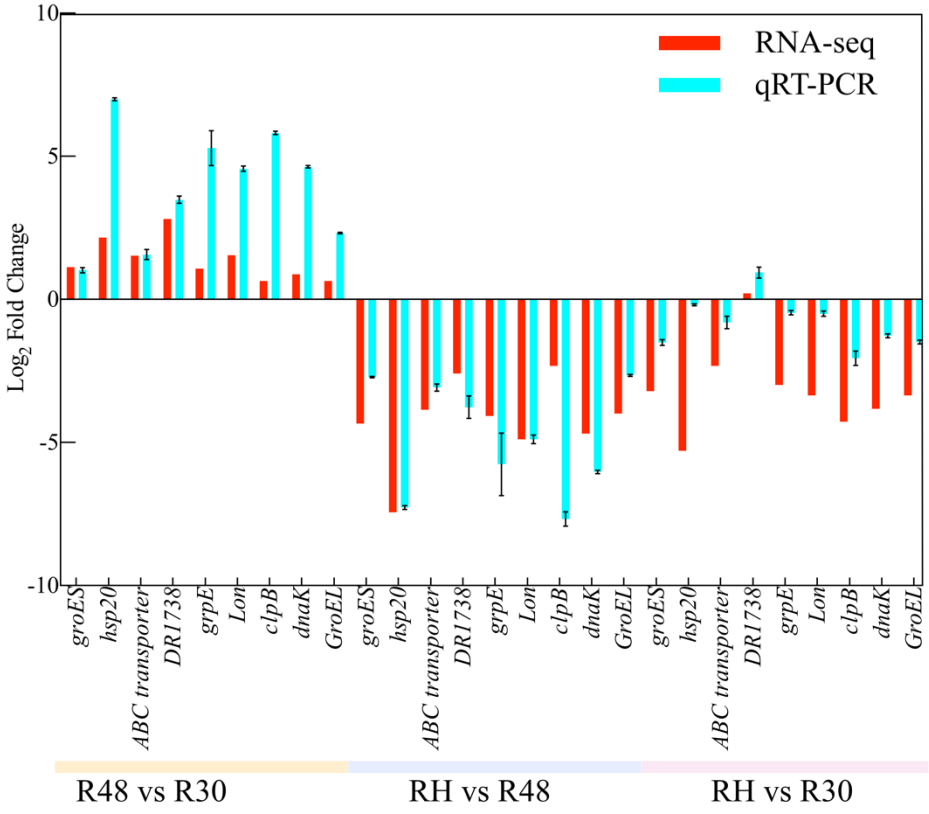


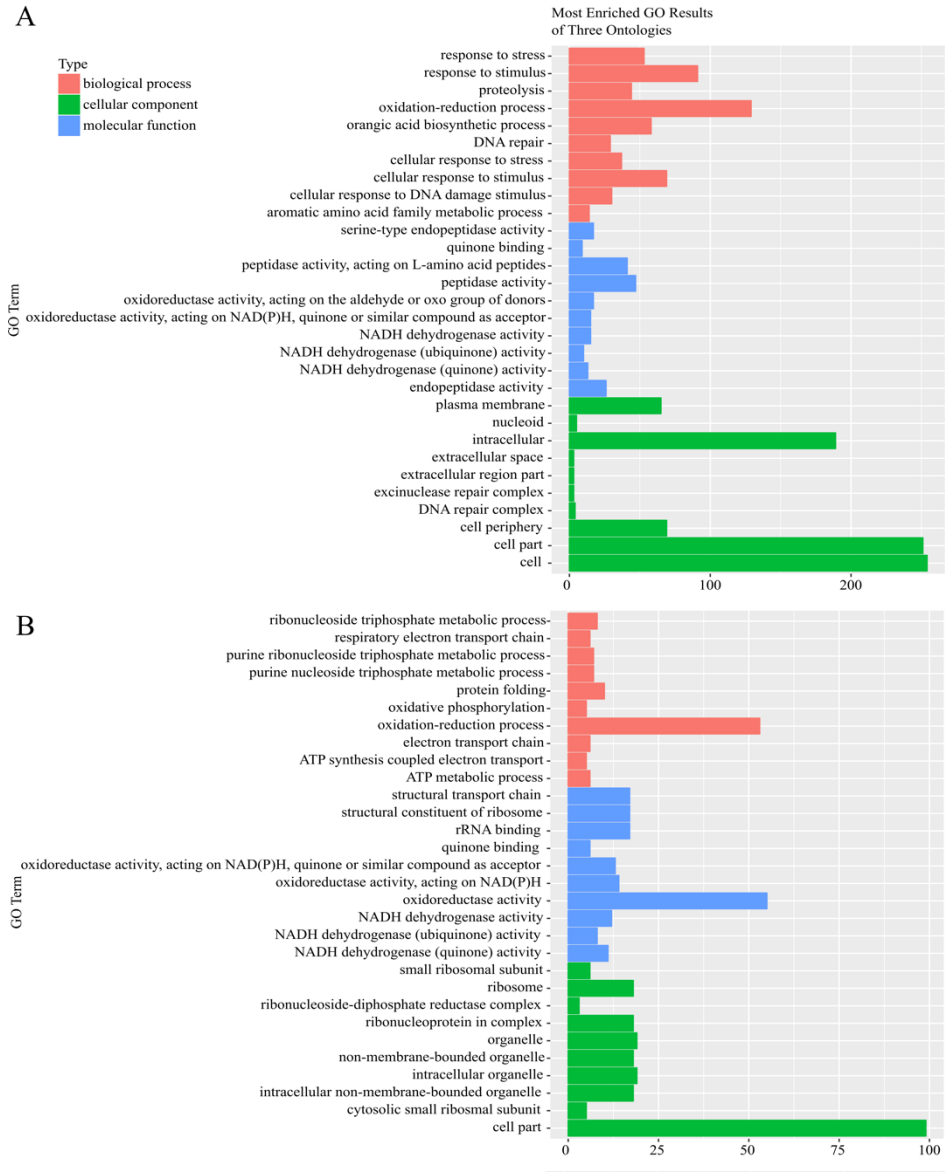
Figure 2-6. Molecular response of *D. radiodurans* under high temperature conditions. Chaperones, RNA and DNA thermosensors, transcription induction and repression, sigma factors, catalase and redox active proteins, and hypothetical proteins regulate the molecular mechanism of the heat stress response in cells. Chaperones mediate the correct folding of other polypeptides, such as hsp20 and DnaK; RNA and DNA thermosensors sense the temperature change; transcription induction and repression is involved in heat stress regulation, such as by HspR; sigma factors (sig1 and sig 2) that control heat-shock regulons have evolved to respond to protein misfolding, and catalase and redox active proteins scavenge the ROS caused by high temperature. Solid arrows and dashed lines represent the known and unknown mechanisms, respectively.

2.7. Supplementary Materials

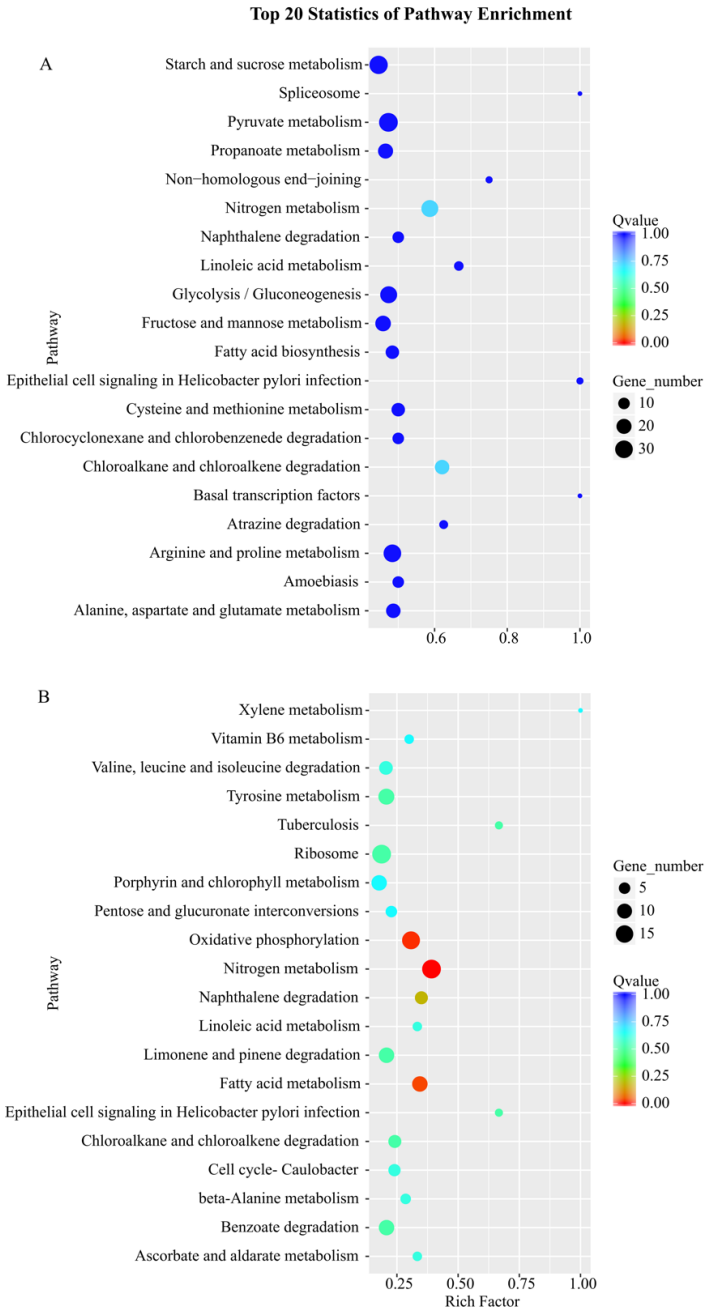
Supplementary materials are list below, which can be found at https://www.mdpi.com/1422-0067/20/22/5603/s1.



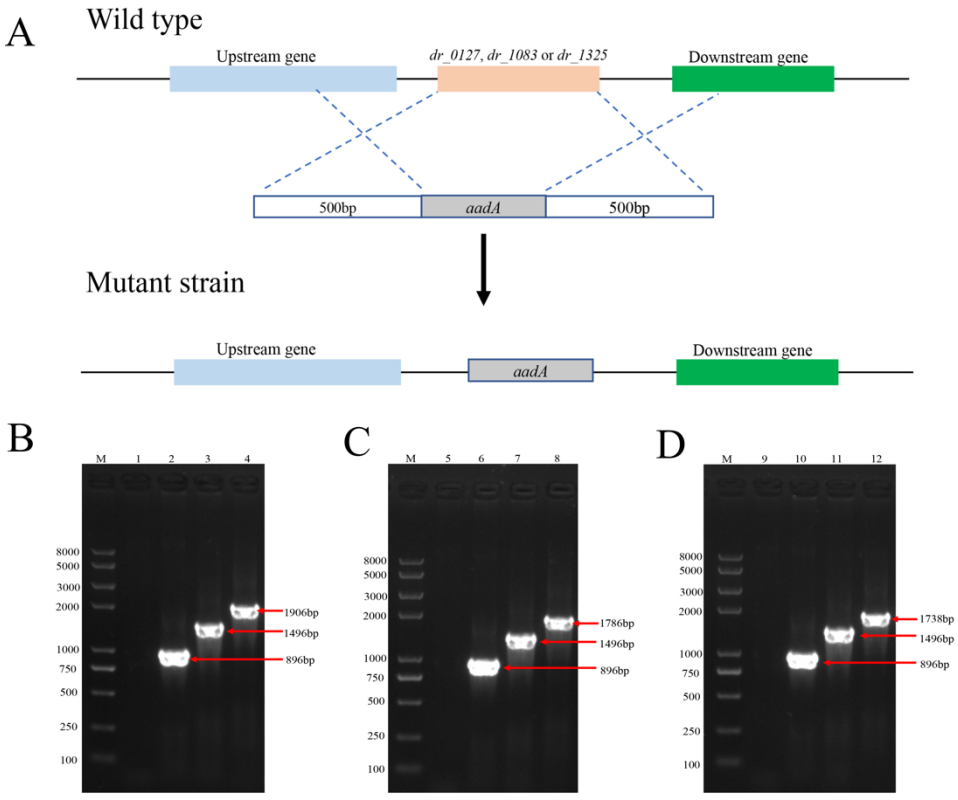
Supplementary Figure 2-1. Validation of the RNA-Seq data using qRT-PCR. Black bars represent the RNA-Seq data, and white bars represent mean $\log_2$ values (fold changes). Data are presented as the mean ± SD values of triplicate reactions for each gene transcript.



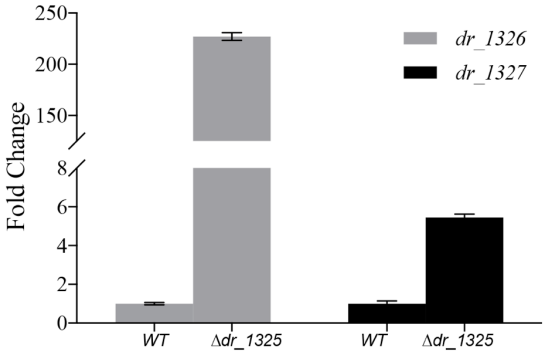
Supplementary Figure 2-2. Gene ontology (GO) enrichment analysis of differentially expressed genes in *D. radiodurans* with and without heat stress.
The 30 most enriched GO terms are shown. (A: R48 vs R30, B: RH vs R30)



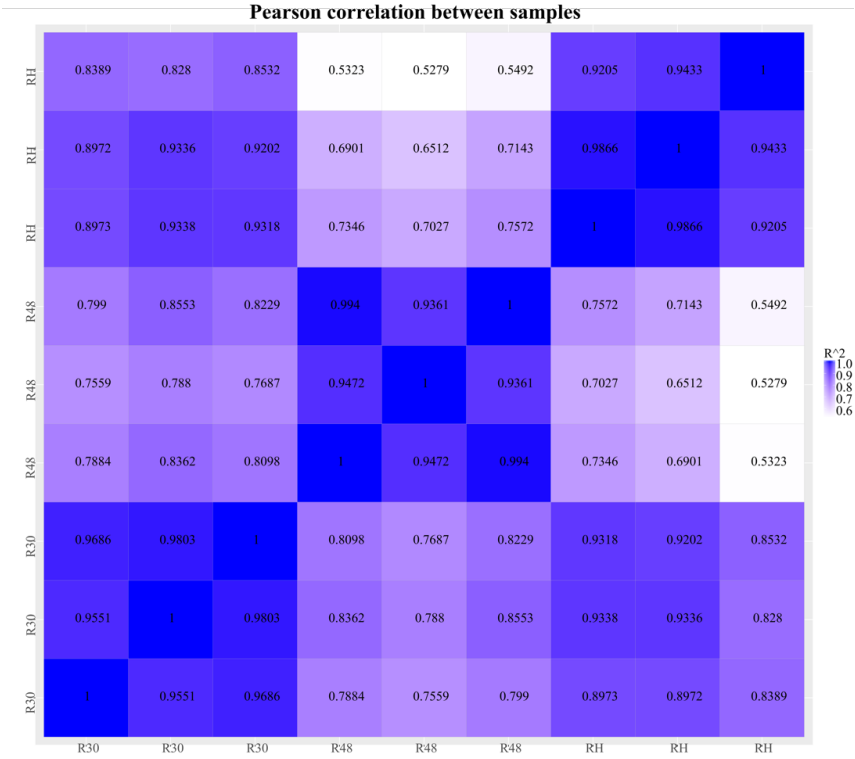
Supplementary Figure 2-3. Top 20 KEGG biological pathway classification histograms for annotated unigenes. (A: R48 vs R30, B: RH vs R30)



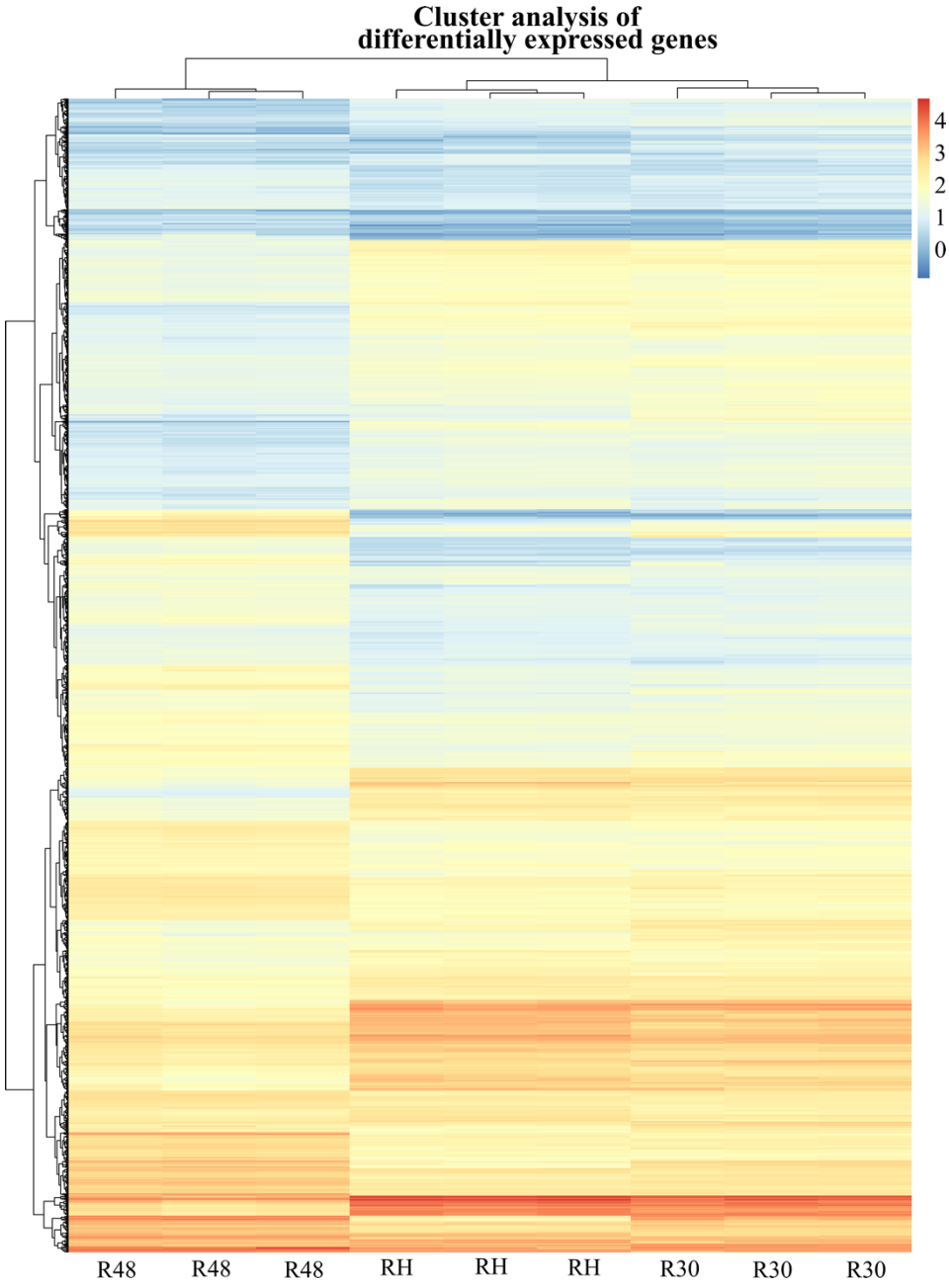
Supplementary Figure 2-4. Construction and verification of the three mutants. (A) Schematic representation of the mutant generated by replacing the *dr_0127*, *dr_1325*, or *dr_1083* region with the spectinomycin resistance gene *aadA*. (B) PCR verification of the Δdr_1325 mutant. Lane M: Trans2K PlusII DNA marker; lanes 1: PCR products amplified from *D. radiodurans* WT using *aadA* primers; lanes 2: PCR products amplified from the Δdr_1325 mutant using *aadA* primers; lanes 3: PCR products amplified from the Δ*dr_1325* mutant using upstream and downstream gene primers; and lanes 4: PCR products amplified from *D. radiodurans* WT using upstream and downstream gene primers. (C) PCR verification of the Δ*dr_0127* mutant. Lane M: Trans2K PlusII DNA marker; lanes 5: PCR products amplified from *D. radiodurans* WT using *aadA* primers; lanes 6: PCR products amplified from the Δ*dr_0127* mutant using *aadA* primers; lanes 7: PCR products amplified from the Δ*dr_0127* mutant using upstream and downstream gene primers; and lanes 8: PCR products amplified from *D. radiodurans* WT using upstream and downstream gene primers. (D) PCR verification of the Δ*dr_1083* mutant. Lane M: Trans2K PlusII DNA marker; lanes 9: PCR products amplified from *D. radiodurans* WT using *aadA* primers; lanes 10: PCR products amplified from the Δ*dr_1083* mutant using *aadA* primers; lanes 11: PCR products amplified from the Δ*dr_1083* mutant using upstream and downstream gene primers; and lanes 12: PCR products amplified from *D. radiodurans* WT using upstream and downstream gene primers.



Supplementary Figure 2-5. Effect of the *dr_1325* deletion on the expression of its downstream genes (*dr1326* and *dr1327*) under normal growth conditions. The relative levels of the transcripts are presented as the mean values ± standard deviation, calculated from three sets of independent experiments and normalized to levels in the WT strain.



Supplementary Figure 2-6. Pearson correlation analysis among 9 samples



Supplementary Figure 2-7. Cluster analysis of differentially expressed genes in 9 samples under heat stress

Supplementary Table 2-1. The significantly different gene expression in three groups.

Supplementary Table 2-2. GO annotation of the DEGs

Supplementary Table 2-3. Summary of the control and heat stress transcriptome sequencing data.

Name	Read length	Raw reads	Clean reads	Raw_Q20	Clean_Q20	Total mapped reads
R30-1	150 bp	40243726	35593566	96.47%	96.34%	82.3%
R30-2	150 bp	36659576	32317906	96.75%	96.64%	84.9%
R30-3	150 bp	36686154	32648744	96.13%	95.98%	85.2%
R48-1	150 bp	30941592	27268952	96.45%	96.29%	83.9%
R48-2	150 bp	28375830	25064318	96.34%	96.16%	80.7%
R48-3	150 bp	29987026	26644728	96.25%	96.09%	85.3%
RH-1	150 bp	37883232	32560522	96.25%	96.11%	88.0%
RH-2	150 bp	39150736	34891584	96.47%	96.36%	89.0%
RH-3	150 bp	32387858	28419606	96.22%	96.08%	82.4%

Supplementary Table 2-4. List of primers used for this study

Gene name	Primers (5' to 3')	Purpose
DR_0606 (groES)	F: GCGACCGCGTTCTGGTTG R: GTACTTGGCGAAGTACACG	qRT-PCR
DR_1114 (hsp20)	F: GGCCTCGAACTGACCTTGGACATTC R: AAAGGTGCCGTAGGCGCGCTCGACA	qRT-PCR
DR_1655	F: CTGCGCCACCTGCTGCACG R: CGTTCACGATGAGCTTGGC	qRT-PCR
DR_1738	F: GAGTGTGCCGTCCCAGGTG R: GAGGTCAGACAGTGCCGTGTC	qRT-PCR
DR_0128 (grpE)	F: GCCGGACACCGAAGCCGAC R: CCAGCTCGTCCACGCGCTCGAGC	qRT-PCR
DR_0127	F: GCACGACGAGTACGGGTG R: CGTGAAGTCCAGCGCCTGC	qRT-PCR
DR_0349	F: CGACGGCACCTTGCAGATGC R: CTTGGAGTTGATGGTCTGC	qRT-PCR
DR_1046	F: GAACCCTGCTCGACAACGAC	qRT-PCR

(ClpB)	R: GCGAGCGTGTCGGCCTTCTG	
DR_0129	F: GTCAGGCTGCGCTCAAC	qRT-PCR
(dnaK)	R: CGTCGCTCACCAGCTTGCGC	
DR_0607	F: GAGCTGGAGGACAAGCTG	qRT-PCR
(groEL)	R: GCCACGGCCTTGTCGATGC	
DR_1083	F: TGGCTTTGCTGCTGTTGCTG	qRT-PCR
	R: TGGCGGTGCCCGTGACCGTC	
DR_0985	F: TGAAAGCGACGGCAAACTCG	qRT-PCR
	R: AGGTAACGGCTCACGTCCTC	
DR_1042	F: CCTCAAATCTGGCACGGTG	qRT-PCR
	R: CAATCCAGTCGGCCTTGCTC	
DR_1501	F: ACGCTGATGTGCGCGCTTGC	qRT-PCR
	R: AGCCCTCGTCGTTGAGCTG	
DR_1325	F: TCCTGACCACCCTGATCTTG	qRT-PCR
	R: TCGGGCGAGGCGAGGTTGTTG	
DR_2311	F: TCTGGACATTGGCCTGAC	qRT-PCR
	R: AGCACGCGGGCGGCCTCG	
DR_2312	F: CGACGAGAGTCACGAGTTGC	qRT-PCR
	R: TGAGAGGTCAGGGACGACG	
DR_0693	F: TTCTTTTACGGCGGCCTGAC	qRT-PCR
	R: CGAACATGGCCTGAAACGAG	
DR0126	F: AGGACTACTACGACGTGCTCG	qRT-PCR
(dnaJ)	R: TGGCCGTACTGGTCGTACAG	
	F: ATTCCTGGTGTAGCGGTG	qRT-PCR
DR_r06 (16S)	R: CATCGTTTAGGGTGTGGAC	
DR_0127-up	F: TGGAACCCGCCGCCCATGCCG	*DR_0127*
	R:CAAGGGCCCTCGGTCTCCATGCGACCACACAAG	mutant
	GAAAGGAGGTTTATG	construction
DR_0127-	F:CCTCCTTTCCTTGTGTGGTCGCATGGAGACCGAG	
middle	GGCCCTTGACATTG	
	R:GCTGATTATGTTCATGTTGATTTATTTGCCGACT	
	ACCTTGGTGATCTC	
DR_0127-	F:ACCAAGGTAGTCGGCAAATAAATCAACATGAAC	
down	ATAATCAGCCAGAGAG	

	R: ATTCAGGTCGTTGCTGGTGACGAAG	
DR_1325-up	F: CGTCTTCATGGGAGCTTGCTGTG R:CAAGGGCCCTCGGTCTCCATGCGGGCTGTCTCTC AGGGGAAGGCACTC	*DR_1325* mutant construction
DR_1325-middle	F:CTTCCCCTGAGAGACAGCCCGCATGGAGACCGA GGGCCCTTGACATTG R:CCCGCTCCGCGCTCAGTGCGCTTATTTGCCGACT ACCTTGGTGATCTC	
DR_1325-down	F:ACCAAGGTAGTCGGCAAATAAGCGCACTGAGCG CGGAGCGGGTCCGAAG R: GTGCTCACCGCGTCCTGGCCGAG	
DR_1083-up	F: CGCCGAGGCCGAGGTCAATGC R:CAAGGGCCCTCGGTCTCCATGGTTGCACCCTAA AACCCCCGACACGGA	*DR_1083* mutant construction
DR_1083-middle	F:TCGGGGGTTTTAGGGTGCAACCATGGAGACCGA GGGCCCTTGACATTG R:GTTCGCATGTCCTCATCAGGCTTATTTGCCGACT ACCTTGGTGATCTC	
DR_1083-down	F:ACCAAGGTAGTCGGCAAATAAGCCTGATGAGGA CATGCGAACGCTCGT R: TCCAGGGGCGGGCGGCGTACATG	

References

Airo, A., Chan, S. L., Martinez, Z., Platt, M. O., & Trent, J. D. 2004. Heat shock and cold shock in *Deinococcus radiodurans*. Cell biochemistry and biophysics, 40(3), 277-288.

Anderson, K. L., Roberts, C., Disz, T., Vonstein, V., Hwang, K., Overbeek, R., et al. 2006. Characterization of the *Staphylococcus aureus* heat shock, cold shock, stringent, and SOS responses and their effects on log-phase mRNA turnover. Journal of bacteriology, 188(19), 6739-6756.

Arsène, F., Tomoyasu, T., & Bukau, B. 2000. The heat shock response of *Escherichia coli*. International journal of food microbiology, 55(1-3), 3-9.

Azam, T. A., & Ishihama, A. 1999. Twelve species of the nucleoid-associated protein from *Escherichia coli* sequence recognition specificity and DNA binding

affinity. Journal of Biological Chemistry, 274(46), 33105-33113.

Baneyx, F., & Mujacic, M. 2004. Recombinant protein folding and misfolding in *Escherichia coli*. Nature biotechnology, 22(11), 1399-1408.

Battista, J. R. 1997. Against all odds: the survival strategies of *Deinococcus radiodurans*. Annual review of microbiology, 51(1), 203-224.

Bauermeister, A., Hahn, C., Rettberg, P., Reitz, G., & Moeller, R. 2012. Roles of DNA repair and membrane integrity in heat resistance of *Deinococcus radiodurans*. Archives of microbiology, 194(11), 959-966.

Bauermeister, A., Moeller, R., Reitz, G., Sommer, S., & Rettberg, P. 2011. Effect of relative humidity on *Deinococcus radiodurans*' resistance to prolonged desiccation, heat, ionizing, germicidal, and environmentally relevant UV radiation. Microbial ecology, 61(3), 715-722.

Bepperling, A., Alte, F., Kriehuber, T., Braun, N., Weinkauf, S., Groll, M., et al. 2012. Alternative bacterial two-component small heat shock protein systems. Proceedings of the National Academy of Sciences, 109(50), 20407-20412.

Blasius, M., Hübscher, U., & Sommer, S. 2008. *Deinococcus radiodurans*: what belongs to the survival kit? Critical reviews in biochemistry and molecular biology, 43(3), 221-238.

Boyle, E. I., Weng, S., Gollub, J., Jin, H., Botstein, D., Cherry, J. M., et al. 2004. GO:: TermFinder—open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. Bioinformatics, 20(18), 3710-3715.

Browning, D. F., Grainger, D. C., & Busby, S. J. 2010. Effects of nucleoid-associated proteins on bacterial chromosome structure and gene expression. Current opinion in microbiology, 13(6), 773-780.

Bruhn-Olszewska, B., Szczepaniak, P., Matuszewska, E., Kuczyńska-Wiśnik, D., Stojowska-Swędrzyńska, K., Algara, M. M., et al. 2018. Physiologically distinct subpopulations formed in *Escherichia coli* cultures in response to heat shock. Microbiological research, 209, 33-42.

Chan, K.-G., Priya, K., Chang, C.-Y., Rahman, A. Y. A., Tee, K. K., & Yin, W.-F. 2016. Transcriptome analysis of *Pseudomonas aeruginosa* PAO1 grown at both body and elevated temperatures. PeerJ, 4, e2223.

Chastanet, A., Fert, J., & Msadek, T. 2003. Comparative genomics reveal novel heat shock regulatory mechanisms in *Staphylococcus aureus* and other Gram-positive bacteria. Molecular microbiology, 47(4), 1061-1073.

Chowdhury Paul, S., Jain, P., Mitra, J., Dutta, S., Bhattacharya, P., Bal, B., et al. 2012. Induction of Cr (VI) reduction activity in an Anoxybacillus strain under heat stress: a biochemical and proteomic study. FEMS microbiology letters, 331(1), 70-80.

Dillon, S. C., & Dorman, C. J. 2010. Bacterial nucleoid-associated proteins, nucleoid structure and gene expression. Nature Reviews Microbiology, 8(3), 185-195.

Gomide, A. C. P., de Sá, P. G., Cavalcante, A. L. Q., de Jesus Sousa, T., Gomes, L. G. R., Ramos, R. T. J., et al. 2018. Heat shock stress: Profile of differential expression in *Corynebacterium pseudotuberculosis biovar Equi*. Gene, 645, 124-130.

Gunasekera, T. S., Csonka, L. N., & Paliy, O. 2008. Genome-wide transcriptional responses of *Escherichia col*i K-12 to continuous osmotic and heat stresses. Journal of bacteriology, 190(10), 3712-3720.

Guyot, S., Pottier, L., Ferret, E., Gal, L., & Gervais, P. 2010. Physiological responses of *Escherichia coli* exposed to different heat-stress kinetics. Archives of microbiology, 192(8), 651-661.

Harada, K., & Oda, S. 1988. Induction of thermotolerance by split-dose hyperthermia at 52 ˚C in *Deinococcus radiodurans*. Agricultural and biological chemistry, 52(10), 2391-2396.

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., & Salzberg, S. L. 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome biology, 14(4), R36.

Kuczynska-Wisnik, D., Kçdzierska, S., Matuszewska, E., Lund, P., Taylor, A., Lipinska, B., et al. 2002. The *Escherichia coli* small heat-shock proteins IbpA and IbpB prevent the aggregation of endogenous proteins denatured in vivo during extreme heat shock. Microbiology, 148(6), 1757-1765.

Kuroda, A., Nomura, K., Ohtomo, R., Kato, J., Ikeda, T., Takiguchi, N., et al. 2001. Role of inorganic polyphosphate in promoting ribosomal protein degradation by the Lon protease in *E. coli*. Science, 293(5530), 705-708.

Langmead, B., & Salzberg, S. L. 2012. Fast gapped-read alignment with Bowtie 2. Nature methods, 9(4), 357.

Lesley, S. A., Graziano, J., Cho, C. Y., Knuth, M. W., & Klock, H. E. 2002. Gene expression response to misfolded protein as a screen for soluble recombinant protein. Protein engineering, 15(2), 153-160.

Lim, B., & Gross, C. A. 2010. Cellular response to heat shock and cold shock. Bacterial stress responses, 91-114.

Lindquist, S. 1986. The heat-shock response. Annual review of biochemistry, 55(1), 1151-1191.

Liu, Y., Zhou, J., Omelchenko, M. V., Beliaev, A. S., Venkateswaran, A., Stair, J., et al. 2003. Transcriptome dynamics of *Deinococcus radiodurans* recovering from ionizing radiation. Proceedings of the National Academy of Sciences, 100(7), 4191-4196.

López-Leal, G., Tabche, M. L., Castillo-Ramírez, S., Mendoza-Vargas, A., Ramírez-Romero, M. A., & Dávila, G. 2014. RNA-Seq analysis of the multipartite genome of *Rhizobium etli* CE3 shows different replicon contributions under heat and saline shock. BMC genomics, 15(1), 1-15.

Ma, D. K., Li, Z., Lu, A. Y., Sun, F., Chen, S., Rothe, M., et al. 2015. Acyl-CoA dehydrogenase drives heat adaptation by sequestering fatty acids. Cell, 161(5), 1152-1163.

Mattimore, V., & Battista, J. R. 1996. Radioresistance of *Deinococcus radiodurans*: functions necessary to survive ionizing radiation are also necessary to survive prolonged desiccation. Journal of bacteriology, 178(3), 633-637.

Matuszewska, M., Kuczyńska-Wiśnik, D., Laskowska, E., & Liberek, K. 2005. The small heat shock protein IbpA of *Escherichia coli* cooperates with IbpB in stabilization of thermally aggregated proteins in a disaggregation competent state. Journal of Biological Chemistry, 280(13), 12292-12298.

Meyer, L., Coste, G., Sommer, S., Oberto, J., Confalonieri, F., Servant, P., et al. 2018. DdrI, a cAMP receptor protein family member, acts as a major regulator for adaptation of *Deinococcus radiodurans* to various stresses. Journal of bacteriology, 200(13), e00129-18.

Parsell, D., & Lindquist, S. 1993. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annual review of genetics, 27, 437-497.

Schmid, A. K., Howell, H. A., Battista, J. R., Peterson, S. N., & Lidstrom, M. E. 2005a. Global transcriptional and proteomic analysis of the Sig1 heat shock regulon of *Deinococcus radiodurans*. Journal of bacteriology, 187(10), 3339-3351.

Schmid, A. K., Howell, H. A., Battista, J. R., Peterson, S. N., & Lidstrom, M. E. 2005b. HspR is a global negative regulator of heat shock gene expression in *Deinococcus radiodurans*. Molecular microbiology, 55(5), 1579-1590.

Schmid, A. K., & Lidstrom, M. E. 2002. Involvement of two putative alternative sigma factors in stress response of the radioresistant bacterium *Deinococcus*

radiodurans. Journal of bacteriology, 184(22), 6182-6189.

Schmid, A. K., Lipton, M. S., Mottaz, H., Monroe, M. E., Smith, R. D., & Lidstrom, M. E. 2005. Global whole-cell FTICR mass spectrometric proteomics analysis of the heat shock response in the radioresistant bacterium *Deinococcus radiodurans*. Journal of proteome research, 4(3), 709-718.

Schumann, W. 2016. Regulation of bacterial heat shock stimulons. Cell Stress and Chaperones, 21(6), 959-968.

Servant, P., Jolivet, E., Bentchikou, E., Mennecier, S., Bailone, A., & Sommer, S. 2007. The ClpPX protease is required for radioresistance and regulates cell division after γ-irradiation in *Deinococcus radiodurans*. Molecular microbiology, 66(5), 1231-1239.

Sheng, D., Gao, G., Tian, B., Xu, Z., Zheng, Z., & Hua, Y. 2005. RecX is involved in antioxidant mechanisms of the radioresistant bacterium *Deinococcus radiodurans*. FEMS microbiology letters, 244(2), 251-257.

Singh, H., Appukuttan, D., & Lim, S. 2014. Hsp20, a small heat shock protein of *Deinococcus radiodurans*, confers tolerance to hydrogen peroxide in *Escherichia coli*. J. Microbiol. Biotechnol, 24(8), 1118-1122.

Slade, D., & Radman, M. 2011. Oxidative stress resistance in *Deinococcus radiodurans*. Microbiology and Molecular Biology Reviews, 75(1), 133-191.

Tanaka, M., Earl, A. M., Howell, H. A., Park, M.-J., Eisen, J. A., Peterson, S. N., et al. 2004. Analysis of *Deinococcus radiodurans*'s transcriptional response to ionizing radiation and desiccation reveals novel proteins that contribute to extreme radioresistance. Genetics, 168(1), 21-33.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., et al. 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nature protocols, 7(3), 562-578.

Tsai, C.-H., Liao, R., Chou, B., & Contreras, L. M. 2015. Transcriptional analysis of *Deinococcus radiodurans* reveals novel small RNAs that are differentially expressed under ionizing radiation. Applied and environmental microbiology, 81(5), 1754-1764.

Ventura, M., Canchaya, C., Zhang, Z., Bernini, V., Fitzgerald, G. F., & Van Sinderen, D. 2006. How high G+ C Gram-positive bacteria and in particular bifidobacteria cope with heat stress: protein players and regulators. FEMS microbiology reviews, 30(5), 734-759.

Wang, J., Chen, L., Huang, S., Liu, J., Ren, X., Tian, X., et al. 2012. RNA-seq based

identification and mutant validation of gene targets related to ethanol resistance in cyanobacterial *Synechocystis sp.* PCC 6803. Biotechnology for biofuels, 5(1), 89.

Wang, P., & Schellhorn, H. E. 1995. Induction of resistance to hydrogen peroxide and radiation in *Deinococcus radiodurans*. Canadian journal of microbiology, 41(2), 170-176.

Wang, Z., Gerstein, M., & Snyder, M. 2009. RNA-Seq: a revolutionary tool for transcriptomics. Nature reviews genetics, 10(1), 57-63.

White, O., Eisen, J. A., Heidelberg, J. F., Hickey, E. K., Peterson, J. D., Dodson, R. J., et al. 1999. Genome sequence of the radioresistant bacterium *Deinococcus radiodurans* R1. Science, 286(5444), 1571-1577.

Wick, L. M., & Egli, T. 2004. Molecular components of physiological stress responses in *Escherichia coli*. In Physiological Stress Responses in Bioprocesses (pp. 1-45): Springer.

Ye, Y., Zhang, L., Hao, F., Zhang, J., Wang, Y., & Tang, H. 2012. Global metabolomic responses of *Escherichia coli* to heat stress. Journal of proteome research, 11(4), 2559-2566.

3

Chapter III: Targeting *Hsp20* using the novel small noncoding RNA *DnrH* regulates heat tolerance in *Deinococcus radiodurans*

From **Xue, D**., Chen, Y., Li, J., Han, J., Liu, Y., Jiang, S., et al. 2019. Targeting *Hsp20* using the novel small noncoding RNA *DnrH* regulates heat tolerance in *Deinococcus radiodurans*. Frontiers in microbiology, 10, 2354.

3.1. Foreword

In the previous chapter, we used RNA-seq to explore new features in *D. radiodurans* under high temperature conditions. Based on our RNA-seq results, we performed the analysis of ncRNA in *D. radiodurans* genome under high temperature treatment. It is well known that bacterial ncRNA plays an important role in response to environmental stress. We also identified a number of ncRNAs in the genome that could be related to heat stress (data not shown). The specific function heat-related ncRNAs are worthy of our further study. In this chapter, based on previous reports and our study, we reported a novel heat-induced ncRNA. Our goal was to try to explain the regulated role of this novel ncRNA during heat stress tolerance. This chapter was published in the "Frontiers in Microbiology" in 2019.

3.2. Abstract

Small noncoding RNAs (ncRNAs) are a class of regulatory molecules, which remain understudied in bacteria. In the extremophilic bacterium *Deinococcus radiodurans*, although hundreds of ncRNAs have been identified, few have been characterized in detail. In this study, we report the identification and characterization of a novel heat-inducible ncRNA named *DnrH*. Heat tolerance analysis showed that deleting *DnrH* significantly inhibited viability in response to high temperature conditions. Comparative phenotypic and qRT-PCR analyses of a *DnrH* mutant (Δ*DnrH*) and wild-type (WT) *D. radiodurans* suggested that *DnrH* is potentially involved in regulating the expression of the heat shock-related gene *Hsp20*. Microscale thermophoresis and genetic complementation showed that a 28-nucleotide (nt) sequence in the stem-loop structure of *DnrH* (143–170 nt) pairs with its counterpart in the coding region of *Hsp20* mRNA (91–117 nt) via a 22 nt region. *In vivo*, mutation of the 22-nt region in the *D. radiodurans* genome led to a reduction in heat tolerance similar to that observed in the *DnrH*-mutant. Our results show that *DnrH* positively influences heat tolerance by increasing the transcription of *Hsp20* mRNA, demonstrating, for the first time, an ncRNA that directly controls the expression of a heat stress-resistance gene. This work provides new insight into the heat stress response mechanism of *D. radiodurans* as well as other extremophiles that express similar Hsp20 proteins.

Keywords

small noncoding RNA, *Deinococcus radiodurans*, heat stress, *DnrH*, *Hsp20* mRNA

3.3. Introduction

Small noncoding RNAs (ncRNAs) play critical roles in gene expression at the posttranscriptional level and are recognized as key transcriptional regulators in bacteria (Acebo et al., 2012; Arnvig et al., 2011; Liu et al., 2010). Generally, ncRNAs remain untranslated with typical lengths ranging from approximately 50 to 500 nucleotides (nt) (Karen M Wassarman, 2002). Over the past decades, many ncRNAs have been identified both in gram-positive and gram-negative bacteria (Acebo et al., 2012; Arnvig et al., 2011; Del Val et al., 2007; Landt et al., 2008; Mandin et al., 2007; Karen Montzka Wassarman et al., 1999) and these have been divided into four groups as follows: *cis*-encoded, *trans*-encoded, protein-binding, and CRISPR ncRNAs. *Trans*-encoded ncRNAs are the best characterized and most extensively studied (Wagner et al., 2015) and exert their regulatory functions through imperfect base-pairing to modulate target mRNA stability and/or translation (Richards et al., 2011; Storz et al., 2011). Nonetheless, a wide range of physiological functions may be regulated by ncRNAs, many of which are related to environmental changes, such as iron limitation, acidity, osmotic shock, envelops stress, temperature, and nutrient stress (Barreto et al., 2016; Boysen et al., 2010; Fantappiè et al., 2011; Fröhlich et al., 2012; Jin et al., 2009; Lybecker et al., 2007; Patel et al., 2009).

Deinococcus radiodurans (*D. radiodurans*) is a model species best known for its extraordinary resistance to diverse environmental stress factors, such as ionizing radiation, ultraviolet irradiation, desiccation, oxidation, and temperature (Bauermeister et al., 2011; Blasius et al., 2008; Daly, 2009; Patel et al., 2009; Timmins et al., 2016; Wang et al., 2008). Because of its genetic operability and extreme resistance, *D. radiodurans* thus represents a leading model to study various extreme stress environments. Temperature fluxes have been reported to be a vital factor for bacterial life, affecting metabolism, and protein unfolding and aggregation in microbes and leading to a higher risk of cell death (Lesley et al., 2002; Ventura et al., 2006). Studying the regulatory mechanisms involved in temperature stress responses of *D. radiodurans* may provide new insights into the stress response of other unrelated species. Multiple genes associated with heat tolerance have been identified in *D. radiodurans* and the first study of heat stress in *D. radiodurans* was published in 1988. Results from this study suggested that proteins synthesized *de novo* during incubation at different temperature intervals (exposure to 52 °C for 30 min, with immediate subsequent transfer to 30 °C or 42 °C for various intervals) are involved either in the thermotolerance phenomenon itself or the recovery from injury (Harada et al., 1988). In the year following the aforementioned study, various heat-inducible proteins were discovered (generally, by proteomics analyses), including Hsp20, GroEL, DnaK, SodA, Csp, and Protease I (Airo et al., 2004; Schmid, Lipton, et al., 2005). Sig1 was determined to be essential for induction of the heat shock proteins groESL and dnaKJ (Schmid, Howell, et al., 2005a; Schmid et al., 2002), whereas *hspR* binds HAIR sites in close proximity to promoter regions, thereby

directly inhibiting the expression of regulated genes encoding chaperone proteins and proteases (Schmid, Howell, et al., 2005b). Recently, an analysis of two small heat shock proteins (sHSPs; IbpA and IbpB) showed that in *D. radiodurans*, these proteins are very different in term of their quaternary structures and chaperone properties and were considered to represent a second type of bacterial two-component sHsp systems (Bepperling et al., 2012). More recently, DdrI (encoded by DR_0997) was reported to enhance heat tolerance in *D. radiodurans* (Meyer et al., 2018).

Using a genome-wide RNA sequencing approach, Tsai *et al*. (Tsai et al., 2015) identified 41 ncRNA candidates; however, functional characterizations of these molecules are still lacking. Considering that ncRNAs often act as regulators in response to various stresses, it is plausible that they could play important roles in the extremophilic properties of *D. radiodurans*. In this study, we describe the function and targets of a novel ncRNA that is potentially involved in heat tolerance in *D. radiodurans*. This ncRNA was designated *DnrH* (***D****. radiodurans* **n**cRNA **r**esponse to **h**eat stress) and its expression was found to be upregulated during heat stress. Further characterization and target identification showed that *DnrH* functions by directly binding *Hsp20* mRNA, which provides evidence of a novel posttranscriptional regulatory mechanism underlying the heat stress response of *D. radiodurans*.

3.4. Materials and Methods

3.4.1. Bacterial strains, plasmids, primers, and culture conditions

All strains and plasmids used in this study are described in Table 3-1. *D. radiodurans* was obtained from China General Microbiological Culture Collection Center (CGMCC 1.633, Beijing, China). *D. radiodurans* and derivatives were routinely cultured in TGY broth (1% tryptone, 0.5% yeast extract, and 0.1% glucose) or on TGY plates supplemented with agar (1.5%) at 30 °C. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or on LB plates supplemented with agar (1.5%) at 37°C. When required, ampicillin, kanamycin, spectinomycin, and chloromycetin were added to final concentrations of 50, 20, 340, and 3.4 μg/mL, respectively.

Table 3-1. Strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Source
Deinococcus radiodurans	WT	Lab stock
Δ*DnrH*	*D. radiodurans* DnrH-deletion mutation, kanamycin	This study

Com-DnrH	*D. radiodurans* DnrH-deletion mutation containing the complementation plasmid pRADZ3-DnrH, kanamycin and chloromycetin	This study
Δ*Hsp20*	*D. radiodurans* Hsp20-deletion mutation, spectinomycin	This study
cHsp20	*D. radiodurans* containing the complementation plasmid pRADZ3-Hsp20, spectinomycin and chloromycetin	This study
cDnrH-mut	*D. radiodurans* DnrH-deletion mutation containing the complementation plasmid pRADZ3-DnrH-mut, kanamycin and chloromycetin	This study
Escherichia coli		
Trans 109	Host for plasmid sub-cloning	TransGen
Trans 109 Z3	As trans109 with pRADZ3	This study
Trans 109 Z3-DnrH	As trans109 with Z3-DnrH	This study
Trans 109 Z3-Hsp20	As trans109 with Z3-Hsp20	This study
Trans 109 Z3-DnrH-mut	As trans109 with Z3-DnrH-mut	This study
Plasmid		
pRADZ3	Shuttle plasmid between *E. coli* and *D. radiodurans*, ampicillin in *E. coli* chloromycetin in *D. radiodurans*	Lab stock

3.4.2. RNA isolation

Cells cultured to OD_{600}= 2 were harvested by centrifugation at 7000 ×*g* for 3 min, washed twice in sterile phosphate-buffered saline (PBS; 0.02% KH_2PO_4, 0.29% $Na_2HPO_4 \cdot 12H_2O$, 0.8% NaCl, 0.02% KCl, pH 7.5), and resuspended in TGY broth to a final OD_{600}= 2 (10^7 cells/mL). Then, cells were treated at 48 °C. These cells were harvested by centrifugation at 12,000 ×g for 3 min and stored at −80 °C. The untreated cells (30 °C) were also harvested and stored in the same manner. Total cells from *D. radiodurans* were prepared using TRIzol reagent (Invitrogen, Thermo Fisher, MA, USA) with Lysing Matrix Tubes (MP Bio, CA, USA) and total cellular RNA was extracted with the PureLink RNA Mini Kit (Invitrogen, Thermo Fisher, MA, USA) following the manufacturer's instructions. RNA purity was assessed using absorbance readings (260nm/280nm) with a NanoDrop® spectrophotometer (Thermo Fisher, MA, USA).

3.4.3. 5'RACE

The transcriptional start site of *DnrH* was determined using the 5′ RACE kit (Roche, Mannheim, Germany) following the manufacturer's instructions. Briefly, an initial strand of cDNA was generated using a sequence-specific primer (i.e., GSP1) to the *DnrH* gene. The first strand cDNA was purified, and the 3′ end of

the cDNA was tailed with dATP by the recombinant terminal transferase. The amplification of dA-tailed cDNA was performed using the sequence-specific primer GSP2 and the anchor primer provided by the 5′ RACE system. The sequence-specific primer GSP3 was using for the second amplification round. Primers GSP1, GSP2, and GSP3, specifically for the *DnrH* gene and tested here are listed in Supplementary Table 3-1. The 5′ RACE products were cloned into the pJET1.2/blunt vector (Thermo Scientific, MA, USA) and sequenced to map the 5′ end of the transcript.

3.4.4. Construction of the mutant and complementary strains

The *DnrH*-mutant strain was generated by replacing the target gene with a kanamycin resistance cassette via fusion PCR recombination, as previously described (Sheng et al., 2005). Briefly, fusion PCR products for the *DnrH* deletions were constructed in two steps. In the first step, PCR was used to generate fragments complementary to the kanamycin-resistance gene from the plasmid pKatAPH3 (920 bp) and the upstream (515 bp) and downstream (529 bp) regions of the *DnrH* sequence using the appropriate primer pairs (Supplementary Table 3-1). In the second step, the upstream, kanamycin-resistance gene, and downstream fragments were annealed at their overlapping regions and PCR amplified the product as a single fragment using the outer primers (1964 bp). The resulting PCR fragment was directly transformed into *D. radiodurans* and colonies resistant to kanamycin (20 μg/mL) were selected. The mutant was subsequently verified by PCR and DNA sequencing. The successfully constructed mutant was named Δ*DnrH*.

The pRADZ3 vector is generally used for complementation experiments with *D. radiodurans*. This plasmid was digested with HindIII/BamHI, and the *DnrH* gene was ligated into linear pRADZ3 to generate the complementation plasmid Z3-DnrH. The complementary strain was constructed by transforming Z3-DnrH into Δ*DnrH* and was selected with 20 μg/mL kanamycin and 3.4 μg/mL chloramphenicol. This strain was confirmed by PCR and Sanger sequencing. This successfully constructed *DnrH*-complemented strain was named *cDnrH*. Similarly, the Hsp20 mutant, complementary strain, and specific point mutants (introduced by site-directed mutagenesis) were constructed using the same method with specific primers (Supplementary Table 3-1).

3.4.5. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using the PureLink RNA Mini Kit (Invitrogen, Thermo Fisher, MA, USA) following the manufacturer's instructions. Then, cDNA synthesis was performed using the PrimeScript^{TM}RT reagent kit with gDNA Eraser (TaKaRa) as described in the manufacturer's protocol. Subsequently, qRT-PCR was performed using ChamQ SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., China) with an AB7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The primers are listed in Supplementary Table 3-1. The 16S (*DR_r06*) rRNA gene was used as the

endogenous reference control to normalize for differences in total RNA quantity, and the relative gene expression was quantified by the $2^{-\Delta\Delta CT}$ method. Three biological replicates for each condition were conducted.

3.4.6. Bacterial growth curve and heat stress survival assays

D. radiodurans wild-type (WT), mutant (Δ*DnrH*), and complementary (*cDnrH*) strains were grown in the shake (220 rpm) TGY cultures in triplicate at 30 °C. The OD_{600} of each sample was measured every 4 h.

WT, Δ*DnrH*, *cDnrH*, and all the other mutant derivatives were cultured in TGY broth with appropriate antibiotics to OD_{600} = 2 at 30 °C and were then shifting to 48 °C for 4 h. Subsequently, 100 μL of the cell suspension was aliquoted into 900 μL of PBS, after which 10-fold serial dilutions were made for all strains, and 8 μL of each dilution was spotted onto TGY agar plates. These plates were incubated at 30 °C for 3 days before colony growth was observed and calculated. The survival rate was expressed as the percentage of the number of colonies in the treated samples compared to those in untreated controls. All assays were performed in triplicate.

3.4.7. Northern blot

The northern blot method was used as previously described (Zhan et al., 2016). Briefly, total RNA was isolated from WT and derivatives grown under heat stress and normal conditions. Next, 30-nt single-strand DNA probes were synthesized (Supplementary Table 3-1), and the 5′ end of the synthesized product was labeled with digoxigenin (Sangon Biotech, Shanghai, China). RNA samples (10 μg) were separated on 8% denaturing gels using 3.2 mL UreaGel 29:1 Concentrate (National Diagnostics, Atlanta, USA), 5.8 mL UreaGel Diluent (National Diagnostics, Atlanta, USA), 1 mL UreaGel Buffer (National Diagnostics, Atlanta, USA), 4 μL TEMED (Thermo Fisher, MA, USA), and 40 μL 10% ammonium persulfate (Sigma-Aldrich, MO, USA). The gel was transferred to a nylon membrane using a semi-dry transfer cell and then incubated at 60 °C for 1–2 h with freshly prepared cross-linking EDC reagent. The membrane was then hybridized with the suitable digoxigenin labeled probe overnight at 37 °C and washed with a low stringent buffer and high stringent buffer. Next, the membrane was incubated in a blocking buffer for 3 h at room temperature. Then, the membrane was placed in the blocking buffer with a DIG antibody solution prepared by mixing a DIG antibody solution with the blocking buffer at a ratio of 1:15,000 for 30 min. The membrane was washed in DIG washing buffer four times for 15 min each. The membrane was incubated in a detection buffer for 5 min and incubated in CSPD solution in the dark for 15min. Band intensity was analyzed using an Amersham imager 600 RGB (GE, Healthcare, USA).

3.4.8. Microscale thermophoresis (MST) measurements

MST experiments were performed according to a previous report

(Jerabek-Willemsen et al., 2011). A set of 30-nt ncRNA oligonucleotides, containing wild-type (wt) or mutated (mut) base-pairing regions of *DnrH* or complementary regions of *Hsp20* mRNA were synthesized by GenePharma (GenePharma, Shanghai, China), as listed in Supplementary Table 3-2. The wt and mut *DnrH* probe molecules were labeled with 6-carboxyfluorescein. 4 μL of sample containing 200 nM labeled probe and increasing concentrations of a non-labeled competitor (from 18.3 nM to 600 μM) were loaded on standard treated glass capillaries (Monolith NT.115 Series Capillaries, Cat#MO-K002) and measurements were carried out using a Monolith NT.115 instrument (NanoTemper Technologies, Germany) at room temperature in diethylpyrocarbonate water with 40% excitation power and medium MST-Power. The dissociation constants (K_d) were calculated as previously described (Lippok et al., 2012). Data analyses were performed using Nanotemper Analysis software (NanoTemper Technologies, Germany).

3.5. Results

3.5.1. Experimental identification and transcriptional start sites of DnrH

DnrH was identified based on our Illumina RNA sequencing (NCBI database Sequence Read Archive, Accession number: SUB5875813) and a previous report (Valverde et al., 2008). *DnrH* was significantly upregulated (6.54-fold) under heat stress and was selected to elucidate its physiological role in response to this pressure (Figure 3-1A). We therefore, speculate that *DnrH* may positively regulated the heat tolerance of *D. radiodurans*.

The transcriptional start site of *DnrH* was then determined by 5′RACE analysis. The results showed that *DnrH* starts at position 2615872 in chromosome 1, extending 294 nt (Figure 3-1B, C). It was located in a 490bp intergenic region between *DR_2606* (encoding a putative primosomal protein N') and *DR_2607* (encoding a MoaE–MoaD fusion protein) (Figure 3-1BC). The promoter regions (–35 and –10 boxes) were predicted by BPROM (Salamov et al., 2011) 222 and 191 nt upstream of the transcription start site. Since it is conserved in other Deinococci (Figure 3-2A), it is also speculated that the region must serve the same purpose throughout the genus. Secondary structure alignment revealed that *DnrH* contains multiple stem-loop structures (Figure 3-2B).

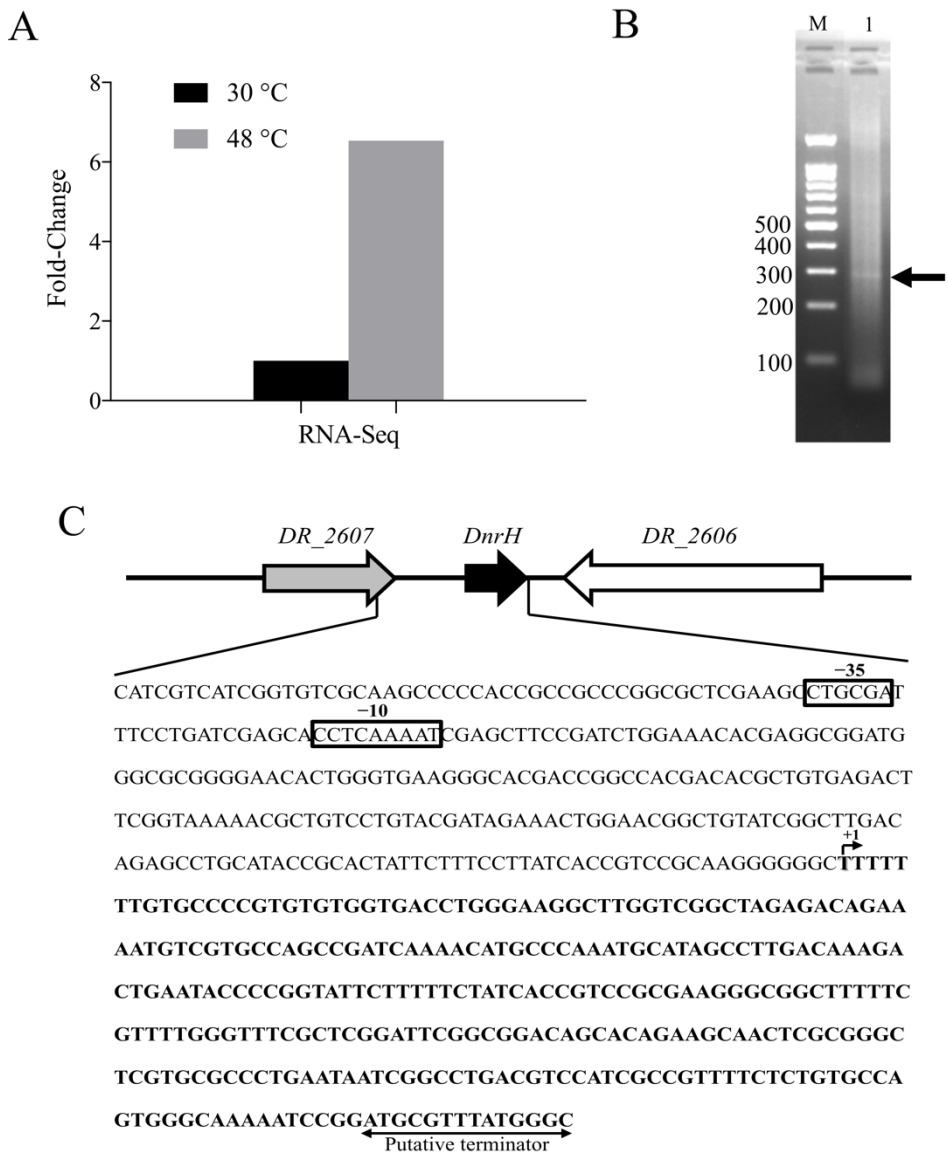


Figure 3-1. Locus features and the expression of *DnrH*.
(**A**) The expression of *DnrH* at 30 and 48 °C based on RNA-Seq. **(B**) PCR results of 5′ RACE. The bands are indicated by arrows. Lane M: 100-bp DNA Ladder (Transgen Biotech), cropped from Supplementary Figure 3-1 lane M; Lane 1: 5′ RACE of *DnrH*, cropped from Supplementary Figure 3-1 lane 3. (**C**) Physical map and nucleotide sequence of the *DnrH* region of *Deinococcus radiodurans*. Promoter elements (−35 and −10 box) are depicted in solid boxes. $^{+1}$, transcription start site mapped by 5′ RACE;

arrowheads, putative transcriptional terminator.

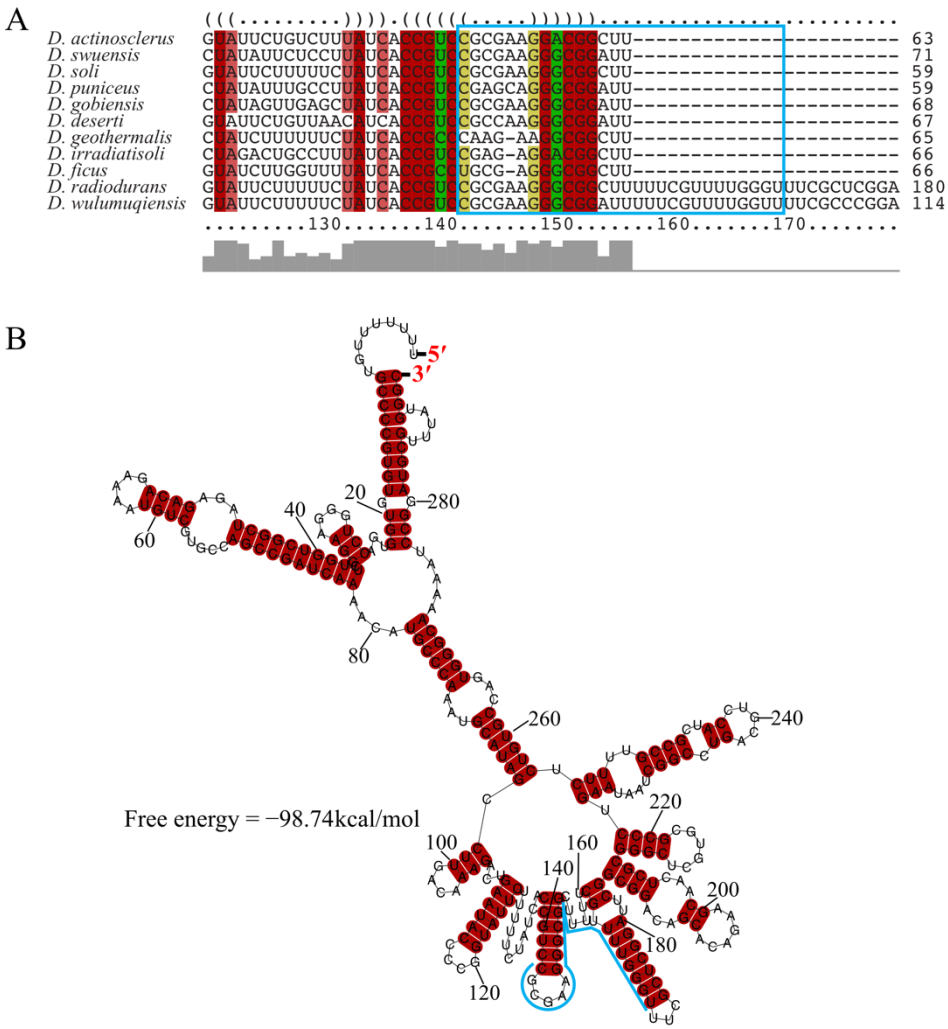


Figure 3-2. Conservation of *DnrH* sequence and structure.
(**A**) Multi-sequence alignment of *DnrH* homologs performed with LocARNA (Will et al., 2012). The colors indicate the structural conservation, for which red indicates 100% of the structural sequence is identical, brown indicates that one nucleotide is changed, and green indicates that two nucleotides are changed. (**B**) Secondary structure prediction performed with RNAalifold (Bernhart et al., 2008). The potential interaction region of *DnrH* is indicated in blue.

3.5.2. DnrH is a novel factor involved in the heat stress response

To further investigate the effect of *DnrH* on heat tolerance in *D. radiodurans*, the *DnrH*-knockout strain Δ*DnrH* and a plasmid-based complementation strain *cDnrH* were constructed. The results of northern blot and qRT-PCR assay showed the significant upregulation of DnrH in heat stress response (Figure 3-3A). The effect of this mutation on the growth potential of the bacterium was examined (Figure 3-3B). The growth of the mutant did not show any significant difference compared to that of the WT, and the complementation strain showed a slight decrease in growth during the stationary period compared to that in Δ*DnrH* and the WT. This decrease maybe caused by the insert plasmid (Figure 3-3B). In order to further determine the role of *DnrH* in *D. radiodurans* under heat stress, the Δ*DnrH*, *cDnrH* and WT strains were exposed to high temperature 48 °C for 4 h. The relative survival ratios of different strains under heat stress were determined by the recovered colony forming units on TGY plates and showed a higher sensitivity of Δ*DnrH* than the WT under heat stress while *cDnrH* exhibited no significant difference (Figure 3-3C). These results strongly suggest that *DnrH* act as an important positive regulator in the response to heat stress.

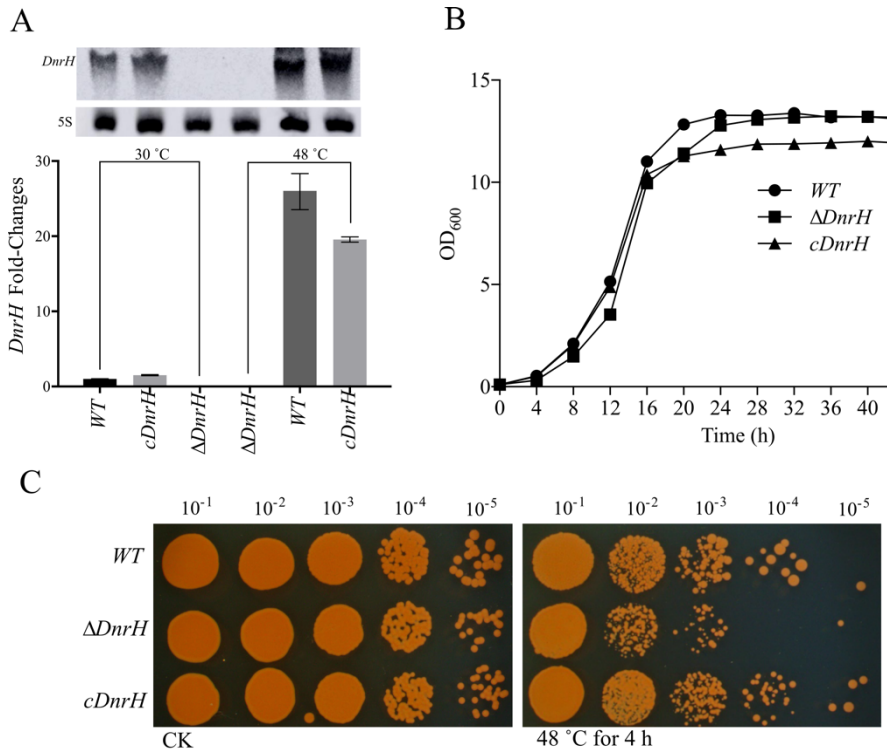


Figure 3-3. Transcriptional and functional analysis of *DnrH* ncRNA in *Deinococcus radiodurans*.
(**A**) *DnrH* transcription under heat stress conditions in the WT, Δ*DnrH* (mutant), and *cDnrH* (complemented) strains. Total RNA was extracted, and the expression of *DnrH* was measured by qRT-PCR. (Inset) RNA northern blot assay using RNA extracted from the same strains under the same conditions and hybridized with the *DnrH*-specific probe. Measurements were normalized to the WT values, and fold differences are plotted. (**B**) Growth curves of WT, Δ*DnrH*, and *cDnrH* in TGY broth. The error bars represent the calculated standard deviation of the measurements of three biological replicates. (**C**) Serial 10-fold dilutions of OD-standardized cultures were spotted on TGY plates after exposure to 48 °C.

3.5.3. Hsp20 as target of DnrH in response to heat stress

To identify the potential targets of *DnrH*, we used qRT-PCR analyses to evaluate the transcriptional levels of heat-related genes in *D. radiodurans* (see Supplementary Figure 3-2). The highly expressed heat-induced genes were selected for further measurements of their relative expression levels in the presence and absence of *DnrH* under heat stress. As shown in Figure 3-4A, the transcriptional level of *Hsp20* in Δ*DnrH* was significantly decreased compared to that in the WT, whereas its expression in *cDnrH* was consistent with that in the WT strain. This phenomenon was also observed by northern blotting (Figure 3-4B).

Hsp20 is described as a molecular chaperone that can prevent the aggregation of denatured proteins during abiotic stresses (Muthusamy et al., 2017). We constructed and tested the *Hsp20*-knockout mutant (Δ*hsp20*) and complementary strain (*chsp20*) and data showed that Δ*hsp20* was more sensitive to heat stress at 48 °C than the WT strain, whereas *chsp20* was essentially identical to the WT with respect to this property (Figure 3-4C). These results indicate that the loss of *hsp20* gene markedly affects heat stress tolerance in *D. radiodurans*.

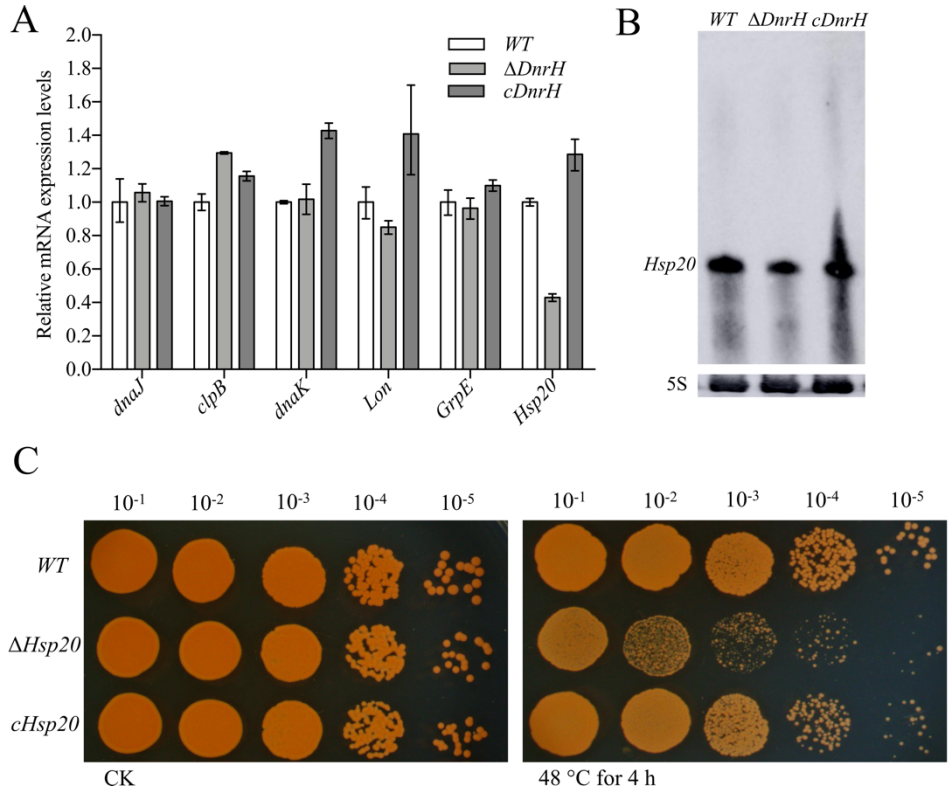


Figure 3-4. Effect of the presence or absence of *DnrH* on heat-related genes expression and phenotypic analysis of *Hsp20* during heat stress.
(**A**) Expression of the most upregulated heat-related genes comparing Δ*DnrH* (mutant) and WT strains. Relative levels of transcripts are presented as the mean values ± SD, calculated from three sets of independent experiments, and normalized to levels in the WT strain. (**B**) RNA northern blot assay using RNA extracted from the same strains under the same conditions and hybridized with the *Hsp20*-specific probe. (**C**) Serial 10-fold dilutions of OD-standardized cultures were spotted on TGY plates after exposure to 48 °C.

3.5.4. DnrH increases heat tolerance in D. radiodurans through regulation of Hsp20 mRNA

Combining qRT-PCR, bioinformatics analysis, and heat phenotype assay experiments, we preliminarily concluded that *Hsp20* is the target of *DnrH* during the regulation of heat stress tolerance. According to the structure predicted by RNAalifold (see Supplementary Figure 3-3), *Hsp20* can form a stable secondary structure with −202.77 kcal/mol free energy. In addition, the computational RNA predictive interaction online tool IntaRNA (Mann et al., 2017) revealed that *DnrH* (91-117bp) can bind *Hsp20* (143-170bp) with an interaction energy of −14.2573 kcal/mol. The binding sites of the *Hsp20* mRNA

are located on a typical stem-loop structure in *DnrH* (Figure 3-5A). To further validate this, a set of 30-nt ncRNA oligonucleotides containing the wt or mut sequences were synthesized and the interaction between fluorescently-labeled *DnrH-wt* or *DnrH-mut* probes and the nonlabelled competitor molecule on *Hsp20* mRNA was assayed using MST, which allows for the sensitive measurement of molecular interactions in solution. Results indicated that *DnrH-wt* binds *Hsp20* mRNA at low micromolar concentrations in the titrant, exhibiting a dissociation constant (K_d) of 28.34 ± 4.7 μM, which suggests a relatively strong interaction. In contrast, the mutant derivative (*DnrH-mut*) harboring substitutions in all complementary bases displayed a complete defect in binding to *Hsp20* mRNA (Figure 3-5B).

In order to validate this putative *Hsp20* binding sites onto *DnrH*, mutated *DnrH* was cloned into the pRAZ3 plasmid to obtain a recombinant vector (pRAZ3-DnrH-mut). pRAZ3-DnrH-mut was transformed into the *DnrH*-mutant Δ*DnrH* to construct a complementary strain (*cDnrH-mut*). Heat stress experiments showed that the complementary strain *cDnrH-mut* could not rescue the heat-resistance phenotype (Figure 3-5C). This result was in agreement with the fact that *DnrH-mut* lost its ability to bind *Hsp20* mRNA. Therefore, we concluded that it is highly likely that *DnrH* enhances heat tolerance in *D. radiodurans* based on a regulatory affect mediated by its 22-nt base-pair complementarity with *Hsp20* mRNA molecules.

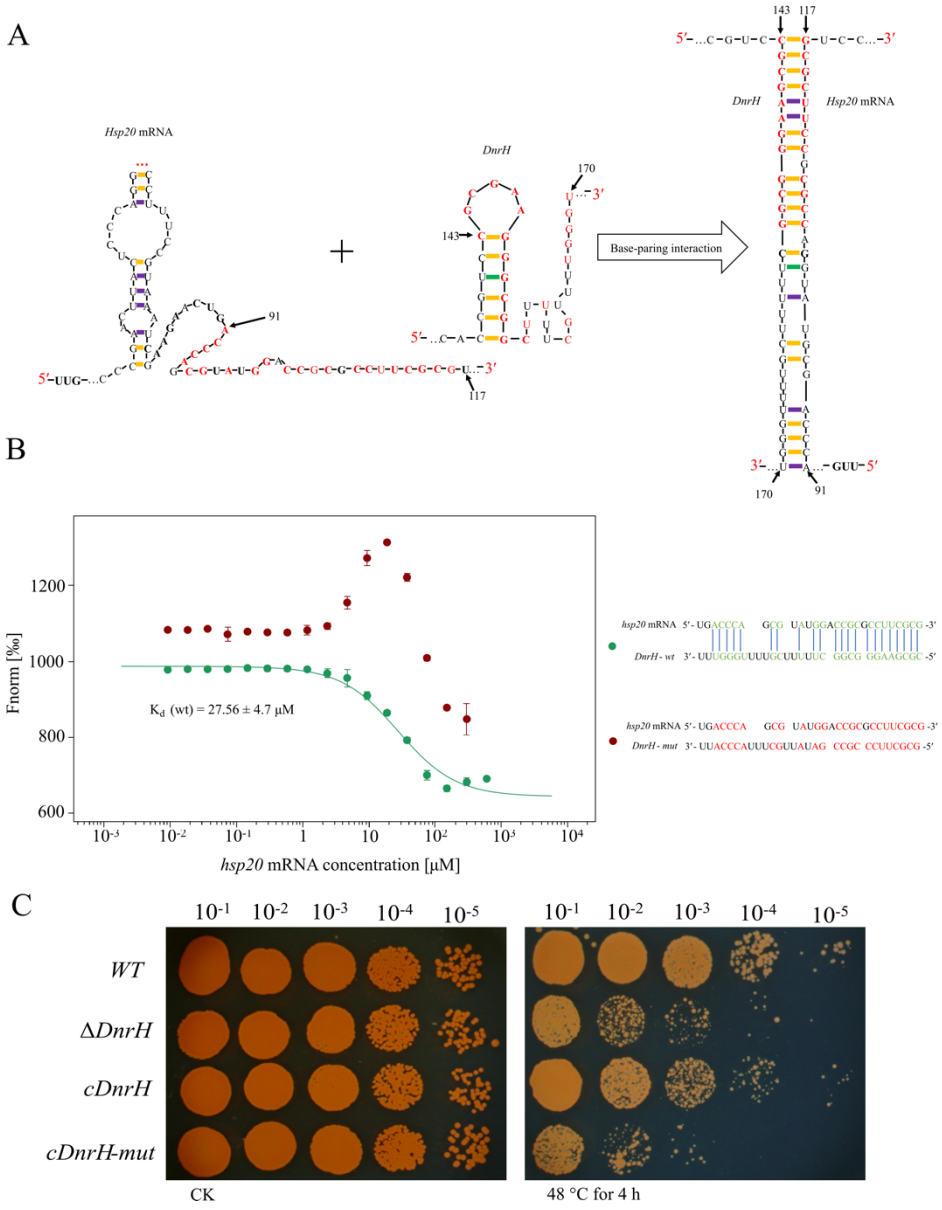


Figure 3-5. Interaction between *DnrH* and *Hsp20* mRNA *in vitro* and *in vivo*. (**A**) Schematic of the interaction between *DnrH* and *Hsp20* mRNA based on IntaRNA. Exemplary interaction between *Hsp20* and *DnrH*. The region of the interaction is depicted in red bold letters. (**B**) Interactions between FAM-labeled 5′ upstream region of *DnrH-wt* (green, Kd (wt)), *DnrH-mut* (red) and *Hsp20* mRNA. All interactions were measured using Monolith NT.115 and K_d-values were calculated based on at least three independent

replicates using the Nanotemper MO. Affinity Analysis Software. (C) Serial 10-fold dilutions of OD-standardized cultures were spotted on TGY plates after exposure to 48 °C.

3.6. Discussion

The heat response of *D. radiodurans* is considered a classical stress-induced regulatory system that is characterized by extensive transcriptional reprogramming. Most current research has led to the discovery and characterization of new heat-related proteins, such as sigma factor, groESL, HspR and DdrI (Airo et al., 2004; Meyer et al., 2018; Schmid, Howell, et al., 2005a, 2005b). Although much effort has been made to elucidate the molecular mechanisms underlying the tolerance of *D. radiodurans* to heat stress, gene expression reprogramming is complex and not yet fully understood. Many ncRNAs have been reported to be associated with bacterial responses to various enviromental stresses (Citartan et al., 2016; Delihas et al., 2001; Majdalani et al., 2001; Massé et al., 2002). While there has been a rapid increase in the identification of bacterial ncRNAs over the last few years, the identification of mRNA targets and the study of ncRNAs' functions have progressed more slowly. So far, to our knowledge, no ncRNAs with a regulatory role in heat tolerance in extreme bacteria have been reported. Our work shows that *DnrH* could be the first ncRNA with direct roles in regulating heat tolerance in the model extremophilic bacterium *D. radiodurans*. As revealed by qRT-PCR and northern blot analysis, *DnrH* is expressed at a higher level under heat stress, and accordingly, the survival of Δ*DnrH* was reduced in response to heat stress conditions as compared to that with the WT strain.

To fully understand the function of *DnrH* in response to heat stress, the regulatory mechanisms of such factors must be identified. ncRNAs usually regulate other genes at the post-transcriptional level by directly or indirectly base-pairing with the target gene mRNA (Storz et al., 2011; Wagner et al., 2015). We identified as target the heat shock protein Hsp20 reported to assist in the refolding and hydrolysis of abnormal proteins (Bepperling et al., 2012). Moreover, previous studies have reported that the expression of *Hsp20* is increased following exposure to various stresses, including temperature changes (Schmid, Howell, et al., 2005b; Singh et al., 2014). Here we show that the expression of *Hsp20* is significantly down-regulated in the absence of *DnrH* and provide strong indications for *Hsp20* mRNA being a direct target of *DnrH*. Further, results of MST and genetic complementation suggested that *Hsp20* mRNA is a direct target of *DnrH*. The location of this interaction indicated that *DnrH* asserts its positive regulatory effect on *Hsp20* expression by assisting in ribosome binding.

sHsps can be considered guardians of proteins, especially upon exposure to sudden protein toxic stresses that lead to the accumulation and aggregation of denatured forms. *Hsp20* belongs to the large spherical homo-oligomeric sHsps, which are constitutively active and form stable substrate complexes (Haslbeck et

al., 2005). Our data confirmed that *Hsp20* contribute to heat tolerance (Figure 3-4C), which is consistent with previously reported results (Airo et al., 2004). Further, *in vivo* transcriptional analysis indicated that *Hsp20* transcripts accumulate to high levels during heat stress, again confirming the role of *Hsp20* in high temperature adaptation. A working model, which integrates *DnrH* and *Hsp20* mRNA in response to heat stress is presented in Figure 3-6. The precise molecular mechanisms underlying the modulation of *DnrH* activities are not yet known and require further investigation. Considering that *Hsp20* functions as a chaperon protein, regulation by *DnrH* might be a two-step process in which *DnrH* regulates *Hsp20* and then affects the transcription of downstream genes. This hypothesis also requires further research.

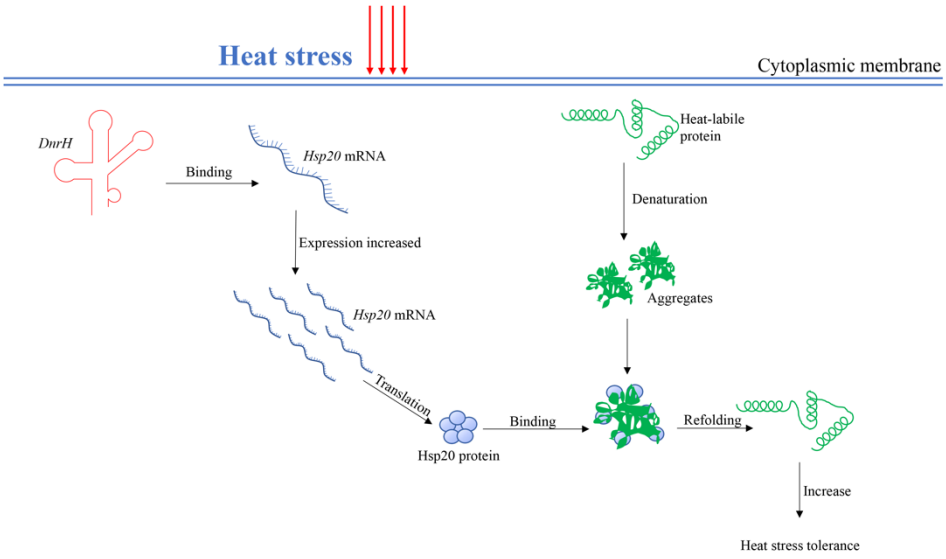
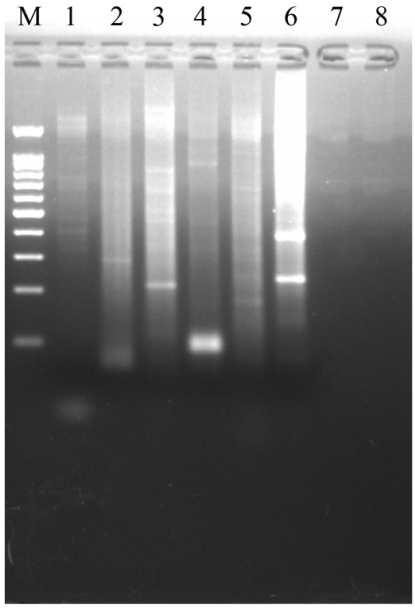


Figure 3-6. A proposed working model for the *DnrH*-mediated regulatory network in heat stress response in *Deinococcus radiodurans*.

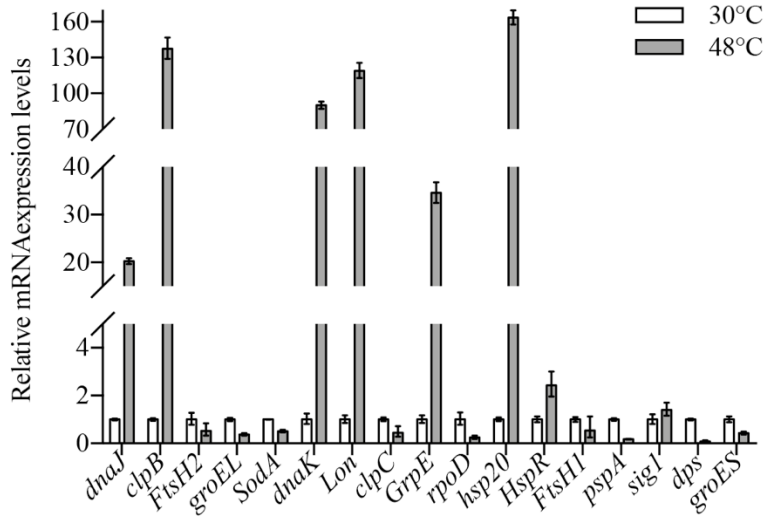
DnrH act as a riboregulator efficiently binds *Hsp20* mRNA to increase heat stress tolerance. This regulation integrates adaptation to heat stress with other cellular metabolic processes help to protect cells against heat stress damage. For more details, see the results or discussion.

3.7. Supplementary Materials

The supplementary materials for this article are list below, which can be found online at https://www.frontiersin.org/articles/10.3389/fmicb.2019.02354/full#supplementary-material.

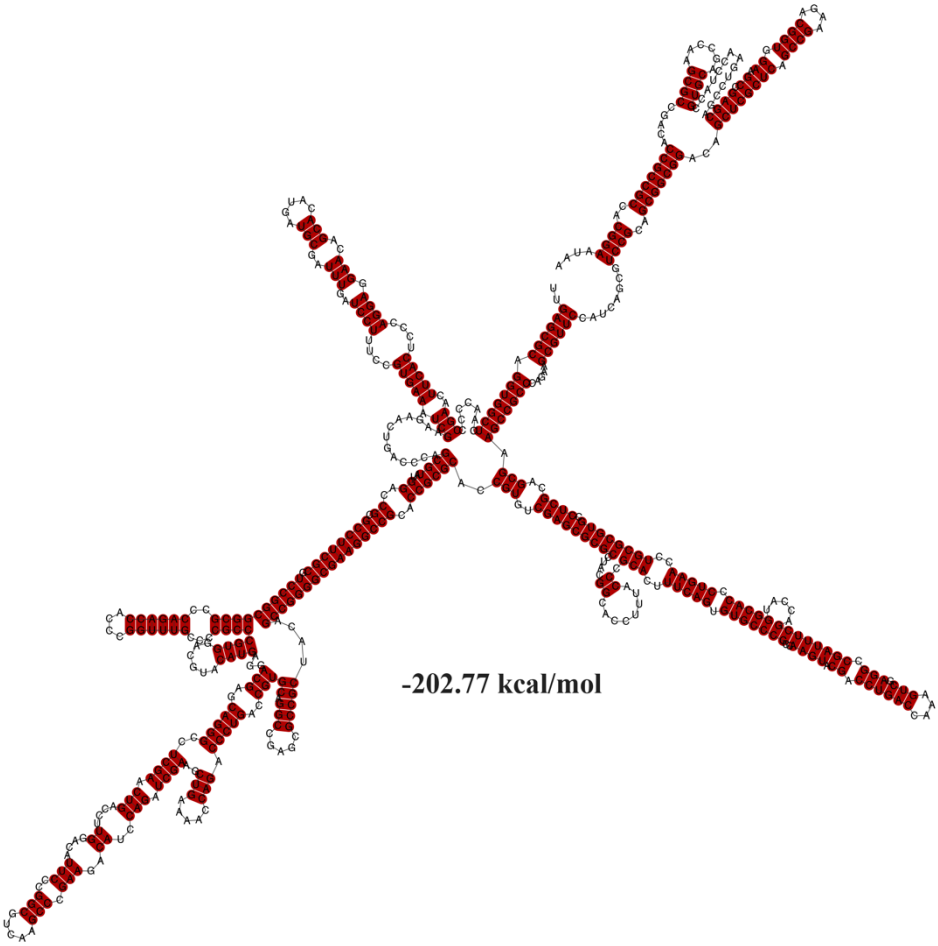


Supplementary Figure 3-1. The original gels of 5'RACE results. PCR results of 5'-RACE. Lane M: 100bp DNA Ladder (Transgen Biotech); Lane 3: 5'RACE of *DnrH*; Remaining lanes are 5'-RACE results of other ncRNAs those have been done at the same time.



Supplementary Figure 3-2. Expression of heat regulatory genes in wildtype *D.*

radiodurans under heat stress.



Supplementary Figure 3-3. Secondary structure of *Hsp20* prediction performed with RNAalifold.

Supplementary Table 3-1. Premiers used in this study

Primers	Sequence (5' – 3')	Purpose
GSP1	GCCCATAAACGCATCC	5' RACE
GSP2	TCCGGATTTTTGCCCACTGGCACAGA	
GSP3	TTTGCCCACTGGCACAGAGAAAACG	

16S	F: ATTCCTGGTGTAGCGGTG	qRT-PCR
	R: CATCGTTTAGGGTGTGGAC	
DnrH	F: GCGAGTTGCTTCTGTGCTGTC	
	R: GCCTTGACAAAGACTGAATACC	
dnaJ(DR0126)	F: AGGACTACTACGACGTGCTCG	
	R: TGGCCGTACTGGTCGTACAG	
clpB(DR1046)	F: GAACCCTGCTCGACAACGAC	
	R: GCGAGCGTGTCGGCCTTCTG	
FtsH-1(DR0583)	F: AGTTGCTCGTCGAGATGGACG	
	R: AGGTCCACCGACACGTCGAGTG	
groEL(DR0607)	F: GAGCTGGAGGACAAGCTG	
	R: GCCACGGCCTTGTCGATGC	
SodA(DR1279)	F: GCTGGGCGTGGCTGGTCGTC	
	R: GCTTCGAGACTTCGTCCCAGTTC	
dnaK(DR0129)	F: GTCAGGCTGCGCTCAAC	
	R: CGTCGCTCACCAGCTTGCGC	
Lon(DR1974)	F: ACGCACCACGCCACCTGGAC	
	R: TCTGCTCGCGCAGGTAGTACTC	
clpC(DR1117)	F: ACCAAGTACCGCGGTGAATTCG	
	R: TGAGGATGTTGGCTGCGTCG	
ropD(DR0916)	F: TCTCTATCGCCAAGAAGTAC	
	R: TGACGAATCCACCACGTC	
hsp20(DR1114)	F: GGCCTCGAACTGACCTTGGACATTC	
	R: AAAGGTGCCGTAGGCGCGCTCGACA	
HspR(DR0934)	F: TGTCTACGTCATCTCGGTG	
	R: TGCAGGTCGTCGAGCTGGTG	
FtsH-2(DR1020)	F: ATTAAGCTCACCTTCGCGGAC	
	R: ACGAAGTCGGAGCCGGAGATG	
pspA(DR1473)	F: ACCACAAGGACCTCGCCAAG	
	R: TGAAGCCTGACACGCGGTCG	
sig1(DR0180)	F: GTGCAGGCCGCCGAGAGCGAG	
	R: TGCACGTCCGACACGTCGGCCAG	
dps(DR2263)	F: CAAGAGCGAGGCAGCCAGCAAG	
	R: TGATGGTGGTCGCCAGGTTGCGTTG	

groES(DR0606)	F: GCGACCGCGTTCTGGTTG	
	R: GTACTTGGCGAAGTACACG	
5S-dig	F: ACACCCCCGTGCCCATAGCACTGTGGAAC	Northern blot
	R: AAACCCCCGCGCTGACCGACTTTTCCG	
hsp20-dig	F: GGCCTCGAACTGACCTTGGACATTCCCGG	
	R: CACCGTGTCGAGCGCGCCTACGGCACCTTT	
DnrH-dig	F: CGCATCCGGATTTTTGCCCACTGGCACAGA	
	R: CGTTTTGGGTTTCGCTCGGATTCGGCGGAC	
Hsp20-U	F: AGCGTTCAACCTGCGCCTTCTGCGTG	hsp20 mutant construction
	R:CAAGGGCCCTCGGTCTCCATGGTAGTCCTATATTAAAACTTGAGTGC	
Hsp20-M	F:CAAGTTTTAATATAGGACTACCATGGAGACCGAGGGCCCTTGACATTG	
	R:GGTAAAGATGGGTTTACGGGTTATTTGCCGACTACCTTGGTGATCTCG	
Hsp20-D	F:CACCAAGGTAGTCGGCAAATAACCCGTAAACCCATCTTTACCTTTTC	
	R: AGGTCGATGGCGCCGTCGGCGTTCAC	
Hsp20-C	F:ACACTGGCGGCCGTTACTAGTCTGCTCGGGTCGCGCGGCTGG	hsp20 complementary strain construct
	R:TGCCTGCAGGTCGAATCGGATCCTTATTCCGTGGCGGCGGTGTC	
DnrH-mut-com-1	F:CCGAATTCCAGCACACTGGCGGCCGTTACTAGTTACATCGACTACGAAGCC	DnrH site-directed mutagenesis
	R:AATGGGTAAAGCAATATCGGCGGGAAGCGCGACGGTGATAGAAAAAGAATACCG	
DnrH-mut-com-2	F:GCGCTTCCCGCCGATATTGCTTTACCCATTCGCTCGGATTCGGCGGACAGCACAG	
	R:GCTTGCATGCCTGCAGGTCGAATCGGATCCCCCGCGCTCGACCTGAGCGGCAGC	
Z3	F: CTCGCGAGGCCTCGAGATCT	Complementary strain checking
	R: TTGGGTTCTCAGTTTGCTGA	
DnrH-U	F: CCACTGAGCCTCAGCGAGGCCGACGCTTTC	DnrH mutant construction
	R:GCAGTTTCATTTGATGCTCGATGAGTTTTTCTAA	

	GCCCCCCTTGCGGACGGTGAT	
DnrH-M	F:CACTATTCTTTCCTTATCACCGTCCGCAAGGGGG GCTTAGAAAAACTCATCGAGCATC	
	R:GATAAAGAAAGAATAGTGCGGTATACGCGCGTT GTCAACATGGAGACCGAGGGCCCTTG	
DnrH-D	F:GAATCATTCTCAATGTCAAGGGCCCTCGGTCTCC ATGTTGACAACGCGCGTATACCGC	
	R:GCTGCTGCGTCAGCTCGCCGAGTGGCAC	
DnrH-C	F:CCGAATTCCAGCACACTGGCGGCCGTTACTAGTT ACATCGACTACGAAGCC	DnrH complementary strain construct
	R:GCATGCCTGCAGGTCGAATCGGATCCCCCGCGCT CGACCTGAGCGG	

Supplementary Table 3-2. Synthesized ssRNA oligonucleotide derivatives for MST

Name	Sequence (5' – 3')	Relevant characteristics
DnrH-wt（5'FAM）	CGCGAAGGGCGGCUUUUUCGUUUUGGGUUU	WT, interaction with *Hsp20-wt*
DnrH-mut（5'FAM）	GCGCUUCCCGCCGAUAUUGCUUUACCCAUU	Dismatch mutation, no interaction with *Hsp20-wt*
Hsp20-wt	CUGACCCAGCGUAUGGACCGCGCCUUCGCG	WT, interaction with *DnrH-wt*

Reference

Acebo, P., Martin-Galiano, A. J., Navarro, S., Zaballos, Á., & Amblar, M. 2012. Identification of 88 regulatory small RNAs in the TIGR4 strain of the human pathogen *Streptococcus pneumoniae*. RNA, 18(3), 530-546.

Airo, A., Chan, S. L., Martinez, Z., Platt, M. O., & Trent, J. D. 2004. Heat shock and cold shock in *Deinococcus radiodurans*. Cell biochemistry and biophysics, 40(3), 277-288.

Arnvig, K. B., Comas, I., Thomson, N. R., Houghton, J., Boshoff, H. I., Croucher, N. J., et al. 2011. Sequence-based analysis uncovers an abundance of non-coding RNA in the total transcriptome of *Mycobacterium tuberculosis*. PLoS Pathog,

7(11), e1002342.

Barreto, B., Rogers, E., Xia, J., Frisch, R. L., Richters, M., Fitzgerald, D. M., et al. 2016. The small RNA GcvB promotes mutagenic break repair by opposing the membrane stress response. Journal of bacteriology, 198(24), 3296-3308.

Bauermeister, A., Moeller, R., Reitz, G., Sommer, S., & Rettberg, P. 2011. Effect of relative humidity on *Deinococcus radiodurans*' resistance to prolonged desiccation, heat, ionizing, germicidal, and environmentally relevant UV radiation. Microbial ecology, 61(3), 715-722.

Bepperling, A., Alte, F., Kriehuber, T., Braun, N., Weinkauf, S., Groll, M., et al. 2012. Alternative bacterial two-component small heat shock protein systems. Proceedings of the National Academy of Sciences, 109(50), 20407-20412.

Bernhart, S. H., Hofacker, I. L., Will, S., Gruber, A. R., & Stadler, P. F. 2008. RNAalifold: improved consensus structure prediction for RNA alignments. BMC bioinformatics, 9(1), 474.

Blasius, M., Hübscher, U., & Sommer, S. 2008. *Deinococcus radiodurans*: what belongs to the survival kit? Critical reviews in biochemistry and molecular biology, 43(3), 221-238.

Boysen, A., Møller-Jensen, J., Kallipolitis, B., Valentin-Hansen, P., & Overgaard, M. 2010. Translational regulation of gene expression by an anaerobically induced small non-coding RNA in *Escherichia coli*. Journal of Biological Chemistry, 285(14), 10690-10702.

Citartan, M., Raabe, C. A., Hoe, C. H., Rozhdestvensky, T. S., & Tang, T. H. 2016. Bacterial sRNAs: regulation in Stress. Stress and Environmental Regulation of Gene Expression and Adaptation in Bacteria, 108-114.

Daly, M. J. 2009. A new perspective on radiation resistance based on *Deinococcus radiodurans*. Nature Reviews Microbiology, 7(3), 237-245.

Del Val, C., Rivas, E., Torres-Quesada, O., Toro, N., & Jiménez-Zurdo, J. I. 2007. Identification of differentially expressed small non-coding RNAs in the legume endosymbiont *Sinorhizobium meliloti* by comparative genomics. Molecular microbiology, 66(5), 1080-1091.

Delihas, N., & Forst, S. 2001. MicF: an antisense RNA gene involved in response of *Escherichia coli* to global stress factors. Journal of molecular biology, 313(1), 1-12.

Fantappiè, L., Oriente, F., Muzzi, A., Serruto, D., Scarlato, V., & Delany, I. 2011. A novel Hfq-dependent sRNA that is under FNR control and is synthesized in

oxygen limitation in *Neisseria meningitidis*. Molecular microbiology, 80(2), 507-523.

Fröhlich, K. S., Papenfort, K., Berger, A. A., & Vogel, J. 2012. A conserved RpoS-dependent small RNA controls the synthesis of major porin OmpD. Nucleic acids research, 40(8), 3623-3640.

Harada, K., & Oda, S. 1988. Induction of thermotolerance by split-dose hyperthermia at 52 ˚C in *Deinococcus radiodurans*. Agricultural and biological chemistry, 52(10), 2391-2396.

Haslbeck, M., Miess, A., Stromer, T., Walter, S., & Buchner, J. 2005. Disassembling protein aggregates in the yeast cytosol The cooperation of Hsp26 with Ssa1 and Hsp104. Journal of Biological Chemistry, 280(25), 23861-23868.

Jerabek-Willemsen, M., Wienken, C. J., Braun, D., Baaske, P., & Duhr, S. 2011. Molecular interaction studies using microscale thermophoresis. Assay and drug development technologies, 9(4), 342-353.

Jin, Y., Watt, R. M., Danchin, A., & Huang, J.-d. 2009. Small noncoding RNA GcvB is a novel regulator of acid resistance in *Escherichia coli*. BMC genomics, 10(1), 165.

Landt, S. G., Abeliuk, E., McGrath, P. T., Lesley, J. A., McAdams, H. H., & Shapiro, L. 2008. Small non-coding RNAs in *Caulobacter crescentus*. Molecular microbiology, 68(3), 600-614.

Lesley, S. A., Graziano, J., Cho, C. Y., Knuth, M. W., & Klock, H. E. 2002. Gene expression response to misfolded protein as a screen for soluble recombinant protein. Protein engineering, 15(2), 153-160.

Lippok, S., Seidel, S. A., Duhr, S., Uhland, K., Holthoff, H.-P., Jenne, D., et al. 2012. Direct detection of antibody concentration and affinity in human serum using microscale thermophoresis. Analytical chemistry, 84(8), 3523-3530.

Liu, J. M., & Camilli, A. 2010. A broadening world of bacterial small RNAs. Current opinion in microbiology, 13(1), 18-23.

Lybecker, M. C., & Samuels, D. S. 2007. Temperature-induced regulation of RpoS by a small RNA in *Borrelia burgdorferi*. Molecular microbiology, 64(4), 1075-1089.

Majdalani, N., Chen, S., Murrow, J., St John, K., & Gottesman, S. 2001. Regulation of RpoS by a novel small RNA: the characterization of RprA. Molecular microbiology, 39(5), 1382-1394.

Mandin, P., Repoila, F., Vergassola, M., Geissmann, T., & Cossart, P. 2007. Identification of new noncoding RNAs in Listeria monocytogenes and

prediction of mRNA targets. Nucleic acids research, 35(3), 962-974.

Mann, M., Wright, P. R., & Backofen, R. 2017. IntaRNA 2.0: enhanced and customizable prediction of RNA–RNA interactions. Nucleic acids research, 45(W1), W435-W439.

Massé, E., & Gottesman, S. 2002. A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. Proceedings of the National Academy of Sciences, 99(7), 4620-4625.

Meyer, L., Coste, G., Sommer, S., Oberto, J., Confalonieri, F., Servant, P., et al. 2018. DdrI, a cAMP receptor protein family member, acts as a major regulator for adaptation of *Deinococcus radiodurans* to various stresses. Journal of bacteriology, 200(13), e00129-18.

Muthusamy, S. K., Dalal, M., Chinnusamy, V., & Bansal, K. C. 2017. Genome-wide identification and analysis of biotic and abiotic stress regulation of small heat shock protein (HSP20) family genes in bread wheat. Journal of plant physiology, 211, 100-113.

Patel, B. A., Moreau, M., Widom, J., Chen, H., Yin, L., Hua, Y., et al. 2009. Endogenous nitric oxide regulates the recovery of the radiation-resistant bacterium *Deinococcus radiodurans* from exposure to UV light. Proceedings of the National Academy of Sciences, 106(43), 18183-18188.

Richards, G. R., & Vanderpool, C. K. 2011. Molecular call and response: the physiology of bacterial small RNAs. Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms, 1809(10), 525-531.

Salamov, V. S. A., & Solovyevand, A. 2011. Automatic annotation of microbial genomes and metagenomic sequences. Metagenomics and its applications in agriculture, biomedicine and environmental studies. Hauppauge: Nova Science Publishers, 61-78.

Schmid, A. K., Howell, H. A., Battista, J. R., Peterson, S. N., & Lidstrom, M. E. 2005a. Global transcriptional and proteomic analysis of the Sig1 heat shock regulon of *Deinococcus radiodurans*. Journal of bacteriology, 187(10), 3339-3351.

Schmid, A. K., Howell, H. A., Battista, J. R., Peterson, S. N., & Lidstrom, M. E. 2005b. HspR is a global negative regulator of heat shock gene expression in *Deinococcus radiodurans*. Molecular microbiology, 55(5), 1579-1590.

Schmid, A. K., & Lidstrom, M. E. 2002. Involvement of two putative alternative sigma factors in stress response of the radioresistant bacterium *Deinococcus radiodurans*. Journal of bacteriology, 184(22), 6182-6189.

Schmid, A. K., Lipton, M. S., Mottaz, H., Monroe, M. E., Smith, R. D., & Lidstrom, M. E. 2005. Global whole-cell FTICR mass spectrometric proteomics analysis of the heat shock response in the radioresistant bacterium *Deinococcus radiodurans*. Journal of proteome research, 4(3), 709-718.

Sheng, D., Gao, G., Tian, B., Xu, Z., Zheng, Z., & Hua, Y. 2005. RecX is involved in antioxidant mechanisms of the radioresistant bacterium *Deinococcus radiodurans*. FEMS microbiology letters, 244(2), 251-257.

Singh, H., Appukuttan, D., & Lim, S. 2014. Hsp20, a small heat shock protein of *Deinococcus radiodurans*, confers tolerance to hydrogen peroxide in *Escherichia coli*. J. Microbiol. Biotechnol, 24(8), 1118-1122.

Storz, G., Vogel, J., & Wassarman, K. M. 2011. Regulation by small RNAs in bacteria: expanding frontiers. Molecular cell, 43(6), 880-891.

Timmins, J., & Moe, E. 2016. A decade of biochemical and structural studies of the DNA repair machinery of *Deinococcus radiodurans*: major findings, functional and mechanistic insight and challenges. Computational and structural biotechnology journal, 14, 168-176.

Tsai, C.-H., Liao, R., Chou, B., & Contreras, L. M. 2015. Transcriptional analysis of *Deinococcus radiodurans* reveals novel small RNAs that are differentially expressed under ionizing radiation. Applied and environmental microbiology, 81(5), 1754-1764.

Valverde, C., Livny, J., Schlüter, J.-P., Reinkensmeier, J., Becker, A., & Parisi, G. 2008. Prediction of *Sinorhizobium meliloti* sRNA genes and experimental detection in strain 2011. BMC genomics, 9(1), 416.

Ventura, M., Canchaya, C., Zhang, Z., Bernini, V., Fitzgerald, G. F., & Van Sinderen, D. 2006. How high G+ C Gram-positive bacteria and in particular bifidobacteria cope with heat stress: protein players and regulators. FEMS microbiology reviews, 30(5), 734-759.

Wagner, E. G. H., & Romby, P. 2015. Small RNAs in bacteria and archaea: who they are, what they do, and how they do it. In Advances in genetics (pp. 133-208): Elsevier.

Wang, L., Xu, G., Chen, H., Zhao, Y., Xu, N., Tian, B., et al. 2008. DrRRA: a novel response regulator essential for the extreme radioresistance of *Deinococcus radiodurans*. Molecular microbiology, 67(6), 1211-1222.

Wassarman, K. M. 2002. Small RNAs in bacteria: diverse regulators of gene expression in response to environmental changes. Cell, 109(2), 141-144.

Wassarman, K. M., Zhang, A., & Storz, G. 1999. Small RNAs in *Escherichia coli*. Trends in microbiology, 7(1), 37-45.

Will, S., Joshi, T., Hofacker, I. L., Stadler, P. F., & Backofen, R. 2012. LocARNA-P: accurate boundary prediction and improved detection of structural RNAs. RNA, 18(5), 900-914.

Zhan, Y., Yan, Y., Deng, Z., Chen, M., Lu, W., Lu, C., et al. 2016. The novel regulatory ncRNA, NfiS, optimizes nitrogen fixation via base pairing with the nitrogenase gene nifK mRNA in *Pseudomonas stutzeri* A1501. Proceedings of the National Academy of Sciences, 113(30), E4348-E4356.

4

Chapter IV: A novel noncoding RNA *dsr11* involved in heat stress tolerance in *Deinococcus radiodurans*

From **Xue, D**., Chen, Y., Li, J., Han, J., Zhou, Z., Zhang, W., et al. 2020. A novel noncoding RNA *dsr11* involved in heat stress tolerance in *Deinococcus radiodurans*. Biomolecules, 10(1), 22.

4.1. Foreword

In chapter three, we described in detail a new ncRNA (*DnrH*) involved in the regulation of heat stress. This study provides a new insight into the heat stress response mechanism of *D. radiodurans*. In this chapter we report the identification of another novel ncRNA participating in heat stress tolerance. We identified ncRNA *dsr11* induced by heat stress. This research further extends our knowledge on ncRNA involvement in *D. radiodurans* heat stress regulation, which can provide a basis for future research and application. This chapter has been published in "Biomolecules" in 2020.

4.2. Abstract

Deinococcus radiodurans is an extremely resistant bacteria that has evolved masterful strategies to enable survival under various environmental stress conditions. Heat stress is a major environmental stress factor that can cause denaturation of proteins, membrane disruption, and oxidative stress. Previous studies have examined the mechanisms of the heat stress response by analyzing changes in protein levels; however, little is known about the role of small noncoding RNAs (ncRNAs), which are known to play important regulatory functions in bacteria during the various environmental stress response. The ncRNA *dsr11* of *D. radiodurans* was previously identified by RNA-seq and Northern blot. In this study, we showed that the transcription level of *dsr11* was up-regulated 4.2-fold under heat stress by qRT-PCR analysis. Heat tolerance assay showed that deleting *dsr11* significantly inhibited the viability under high temperature conditions. To assess the influence of *dsr11* on the *D. radiodurans* transcriptome, 157 genes were found differentially expressed in the knock-out mutant by RNA-seq experiment. Combining RNA-seq and in silico analysis, we found that *dr_0457* (biopolymer transport protein) was likely to be the direct target of *dsr11*. Further microscale thermophoresis results demonstrated that *dsr11* can directly bind to the mRNA of *dr_0457*. Our results indicated that *dsr11* can enhance the tolerance to heat stress of *D. radiodurans* by binding to *dr_0457* mRNA. Overall, these results extend our understanding of ncRNA regulation and provide new insights into the heat stress response in *D. radiodurans*.

Keywords

Deinococcus radiodurans, noncoding RNA, *dsr11*, heat stress, *dr_0457*

4.3. Introduction

Deinococcus radiodurans (*D. radiodurans*) is a gram-positive, pink-pigmented, high G+C bacterium that belongs to the Deinicoccus-thermus phylum. It was isolated from gamma-radiated canned meat and is best known for its extraordinary resistance to ionizing irradiation (Daly, 2009; Hua et al., 2016; Omelchenko et al., 2005). However, previous studies also reported that this bacterium presents a rapid response and adaptation to a wide variety of extreme environmental stresses, such as ultraviolet irradiation, desiccation, hydrogen peroxide, and temperature (Airo et al., 2004; Mattimore et al., 1996; Meyer et al., 2018; Patel et al., 2009; Slade et al., 2011). Due to its powerful DNA repair ability and extreme stress resistance, this bacterium has become a model for studying bacterial tolerance mechanisms under various environmental stress conditions. To adapt to rapid changes in environmental conditions, many genes associated with stress tolerance have been identified in *D. radiodurans* (Blasius et al., 2008). Although much effort has been carried out to elucidate the molecular mechanisms conferring high resistance capability in response to a various environment in *D. radiodurans*, the complex post-transcriptional regulation and gene expression still requires deeper investigation.

Small noncoding RNAs (ncRNAs) are ubiquitously found in bacteria (50 - 500 nt), which have been divided into two groups (*cis*-encoded ncRNAs and *trans*-encoded ncRNAs) based on the location of their targets (Gottesman, 2005; Waters et al., 2009). They usually function by repressing or activating gene expression via base-pairing with target mRNAs to modulate translational activation and mRNA stabilization (Storz et al., 2011). Bacterial ncRNAs have been reported to act as a vital regulator in response to various environmental stresses, such as, virulence, pH, oxidative, antibiotic, and temperature (Eyraud et al., 2014; Lybecker et al., 2007; Mann et al., 2012; Opdyke et al., 2004; Zhang et al., 2019). Thanks to the development of biocomputational and experimental methods, a large number of ncRNAs have been found in prokaryote (Sharma et al., 2009). In extremophilic bacteria and archaea such as *Methanococcus jannaschii*, *Bacillus halodurans*, and *Haloferax volcanii*, a large amount of ncRNA was found (Gelsinger et al., 2018; Klein et al., 2002; Puerta-Fernandez et al., 2006). In particular, many of these ncRNAs are involved in the regulation of various stresses. For example, hundreds of differentially expressed ncRNAs in response to oxidative stress were identified in *H. volcanii* (Gelsinger et al., 2018). In *H. volcanii*, sRNA132 is important for rapid adaptation to phosphate starvation (Kliemt et al., 2019). Recently, Tsai et al. (Tsai et al., 2015) identified 41 ncRNA candidates in *D. radiodurans* by genome-wide RNA sequencing approach but the functional roles of these ncRNAs are still poorly understood.

This study aimed at providing new insights into the environmental adaption of *D. radiodurans*. We have characterized *dsr11* as novel ncRNA but conserved among Deinococci and which is highly induced upon exposure to high

temperature. We combined various experimental and in *silico* approaches in order to identify the potential targets of *dsr11* and the potential regulatory pathway leading to heat tolerance in that bacterium.

4.4. Materials and Methods

4.4.1. Strain and growth conditions

D. radiodurans was obtained from China General Microbiological Culture Collection Center (CGMCC 1.633, Beijing, China). *D. radiodurans* and derivatives were routinely cultured in TGY broth (1% tryptone, 0.5% yeast extract, and 0.1% glucose) or on TGY plates supplemented with agar (1.5%) at 30 °C. When required, ampicillin and kanamycin were added to final concentrations of 50 and 20 μg/mL, respectively.

4.4.2. RNA extraction

Total cells from *D. radiodurans* were prepared using TRIzol reagent (Invitrogen, Thermo Fisher, MA, USA) with Lysing Matrix Tubes (MP Bio, CA, USA) and total cellular RNA was extracted with the PureLink RNA Mini Kit (Invitrogen, Thermo Fisher, MA, USA) following the manufacturer's instructions. RNA purity was assessed using absorbance readings (260nm/280nm) with a NanoDrop® spectrophotometer (Thermo Fisher, MA, USA).

4.4.3. Construction of gene deletion mutant strain

A mutant strain lacking *dsr11* was constructed by fusion PCR recombination of a kanamycin resistance cassette into the genome as previously described (Sheng et al., 2005). Briefly, fusion PCR products for dsr11 deletion was constructed in two steps. In the first step, a pair of specific premiers were used to generate fragment complementary to the kanamycin-resistance gene from the plasmid pKatAPH3 (920 bp) and the upstream (414 bp) and downstream regions (417 bp) of dsr11 sequences using the appropriate primer pairs (Supplementary Table 4-1). In the second step, the upstream, kanamycin-resistance gene and downstream fragments were annealed at their overlapping regions and PCR amplified as a single fragment using the outer primers (1751 bp). The resulting PCR fragment was directly transformed into *D. radiodurans*. Colonies resistant to kanamycin (20 μg/mL) were selected, and these mutants were subsequently verified by PCR and DNA sequencing and named Δ*Dsr11*.

4.4.4. Quantitative real-time PCR (qRT-PCR)

cDNA synthesis by using a PrimeScript^{TM}RT reagent kit with gDNA Eraser (TaKaRa) as described in the manufacturer's protocol. Subsequently, qRT-PCR was performed using ChamQ SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., China) on a 7500 Fast Real-time PCR System (Applied Biosystems, USA). The primers are listed in Supplementary Table 4-1. The 16S rRNA gene was

used as the endogenous reference control to normalize differences in total RNA quantity, and relative gene expression was quantified by the $2^{-\Delta\Delta CT}$ method. Three biological replicates for each condition were conducted.

4.4.5. Bacterial Growth curve and heat stress tolerance assays

D. radiodurans wild-type (WT) and mutant (Δ*Dsr11*) strains were grown in shacked (220 rpm) TGY cultures in triplicate at 30 °C. The OD_{600} of each sample was measured every 4 h. WT and Δ*Dsr11* were cultured in TGY broth with appropriate antibiotics to OD_{600} = 2 at 30 °C and were then shifting to 48 °C for 4 h. Subsequently, 100 μL of the cell suspension was aliquoted into 900 μL of PBS, after which 10-fold serial dilutions were made for all strains, and 8 μL of each dilution was spotted onto TGY agar plates. These plates were incubated at 30 °C for 3 days before colony growth was observed and calculated. All assays were performed in triplicate.

4.4.6. RNA-seq and data analysis

WT and Δ*Dsr11* were cultured in TGY broth with appropriate antibiotics to OD_{600} = 2 at 30 °C. Then, the cells were harvested by centrifugation at 12,000 ×g for 3 min and stored at -80°C for RNA extraction. For each strain, total RNA was extracted from at least three independent biological replicates as described in RNA isolation section. A total of 1 μg of high-quality RNA per sample was used as the input material for library preparation. Sequencing libraries were generated using a VAHTS Total RNA-Seq Library Prep Kit for Illumina® (Vazyme, NR603) following the manufacturer's recommendations. Then the well-prepared library was sequenced using the Illumina HiSeq X Ten platform with a 150-bp paired-end module. Raw reads were filtered by removing reads containing adapter, poly-N and low-quality read for subsequent analysis. The resulting sequences were then mapped onto the *D. radiodurans* reference genome (NC_001263.1, NC_001264.1, NC_000958.1, and NC_000959.1) using TopHat (v2.1.1) (Kim et al., 2013). The mapped reads of each sample were assembled using Cufflinks (v2.2.1) (Trapnell et al., 2012) with a reference-based approach. Cuffdiff (v2.2.1) (Trapnell et al., 2012) provides statistical routines for determining differential expression in a digital transcript or gene expression dataset using a model based on a negative binomial distribution. Genes with corrected *p* values less than 0.05 and absolute $\log_2$ values (Fold changes) > 1 were considered significant differentially expressed genes (DEGs). All samples were sequenced three times. Gene Ontology (GO) enrichment analysis of the DEGs was performed with the Perl module (GO::TermFinder) (Boyle et al., 2004). GO terms with a corrected p value less than 0.05 were considered to be significantly enriched among the DEGs. Kyoto Encyclopedia of Gene and Genomes (KEGG) is a major public database containing manually drawn pathway maps representing knowledge of molecular interactions and reaction networks. R functions (phyper and q-value) were used to test for the statistical enrichment of the DEGs among the KEGG pathways. KEGG pathways with

corrected p-values less than 0.05 were considered to be significantly enriched for the DEGs.

4.4.7. Bioinformatics analysis

The secondary structure prediction performed with RNAalifold (Bernhart et al., 2008) based on the lowest folding energy. The sequence conservation of *dsr11* by using BLASTN. All sequenced bacterial genomes were compared with parameter values set to the default, and sequences with a nucleotide identity > 60% and coverage value > 80% considered as being conserved.

The target genes of *dsr11* were predicted by using Web-based program TargetRNA2 (Kery et al., 2014). The hybridization between the ncRNA transcript sequence and the sequence comprising 75 nucleotides (nt) upstream until 75 nt downstream of the start codon of each annotated gene was screened in the genome of *D. radiodurans*. To consider an interaction as positive, we used the corresponding $p < 0.05$ and synonym energy < -8 kcal/mol was taken as the threshold. Finally, the common predictions of TargetRNA2 and RNA-seq analysis confirmed as the target candidates.

4.4.8. Microscale thermophoresis (MST) analysis

MST technique was performed to quantify the interactions between ncRNAs and the target mRNAs in vitro (Beckert et al., 2015; Buddeweg et al., 2018; Jerabek-Willemsen et al., 2014). The 5′FAM-labeled mRNAs of the target gene *dr_0457* were synthesized by the BGI company (BGI, Beijing, China). The ssRNAs oligonucleotides containing wild-type (*wt*) or mutated (*mut*) base-pairing regions of *dsr11* were synthesized by the BGI company (BGI, Beijing, China), as listed in Supplementary Table 4-2. The *wt* and *mut dsr11* probe molecules were labeled with 5′FAM. Four microliters of a sample containing 200 nM labeled mRNA and increasing concentrations of a non-labeled competitor (from 18.3 nM to 600 mM) were loaded on standard treated glass capillaries (Monolith NT.115 Series Capillaries, Cat#MO-K002). Thermophoresis was carried out using a Monolith NT.115 instrument (NanoTemper Technologies, Munich, Germany) at 25 °C in diethylpyrocarbonate water with 40% excitation power and medium MST-Power. The dissociation constants (K_d) were calculated as previously described (Lippok et al., 2012). Data analyses were performed using MO. Affinity Analysis software (NanoTemper Technologies, Munich, Germany).

4.5. Results and discussion

4.5.1. Characterization of the novel ncRNA dsr11

In a previous study, 41 ncRNAs were reported in *D. radiodurans* under normal conditions (Tsai et al., 2015). Here we further functionally analyzed these ncRNAs under different abiotic stresses (data not shown). Interestingly, we

found a novel ncRNA named *dsr11* which was 4.2-fold up-regulated upon growth at 48 ˚C (Figure 4-1A). This ncRNA was thus selected for further investigation during the heat stress response. Since the expression of *dsr11* was enhanced under heat stress, we hypothesize that *dsr11* might positively regulated heat tolerance in *D. radiodurans*.

According to the previous results of RNA-seq and deep RACE, the sequence of *dsr11* was 126 nt long. In general, ncRNAs form secondary structures, which contribute to their stability (Gottesman, 2005). The secondary structure of *dsr11* was predicted by RNAalifold (Bernhart et al., 2008), and the four stem-loop structures with a free energy −45.74 kcal/mol may facilitate the high stability of *dsr11* (Figure 4-1B). It has been reported that ncRNAs can have conserved sequences and structures in homologs (Peer et al., 2011). We used BlastN to examined *dsr11* sequence conservation and compared all sequenced bacterial genomes with parameter values set to the default. The results indicate that there are no homologous sequences in other species and that *dsr11* is highly conserved in *D. radiodurans*. We hypothesize that *dsr11* may play an important role in *D. radiodurans*, especially under heat stress.

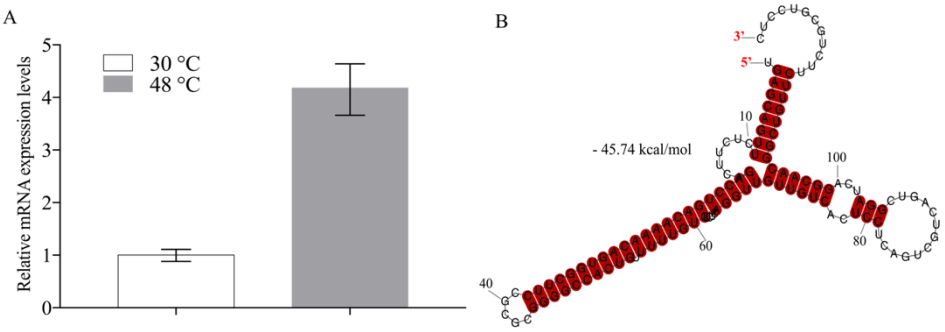


Figure 4-1. The expression of *dsr11* under heat stress and secondary structure of *dsr11*. (A) The expression of *dsr11* under heat stress in *D. radiodurans* WT by qRT-PCR. (B) *dsr11* consisted of four stem-loop structures with free energy –45.74 kcal/mol.

4.5.2. dsr11 is required for heat tolerance of D. radiodurans

dsr11 was located in the intergenic region between *dr_2376* (encoding a TetR family transcriptional regulator) and *dr_2377* (encoding a hypothetical protein) (Figure 4-2A). To further investigate the influence of *dsr11* on *D. radiodurans*, the knockout gene of *dsr11* was constructed successfully and named Δ*Dsr11* (Figure 4-2A). It was important to verify whether the deletion had a polar effect on the flanking genes, thus we checked the expression by qRT-PCR of the flanking genes (*dr_2376* and *dr_2377*) and *dsr11* in WT and Δ*Dsr11*. As shown in Figure 4-2B, a significant difference of expression was observed only in the case of *dsr11* (expected expression could not be detected in the mutant), while there is no difference of *dr_2376* and *dr_2377* between WT and Δ*Dsr11*. The

effect of *dsr11* on *D. radiodurans* growth at optimum growth temperature was then examined and there was no significant difference between the WT and Δ*Dsr11*(Figure 4-2C).

To determine the role of *dsr11* in *D. radiodurans* under heat stress, the WT and Δ*Dsr11* strains were exposed to high temperature conditions at 48˚C for 4 h. As shown in Figure 4-2D, the TGY plate assays showed that the mutant strain Δ*Dsr11* was more susceptible than the WT strain upon growth at 48 ˚C confirming that *dsr11* serves an important role in the adaptation to heat stress.

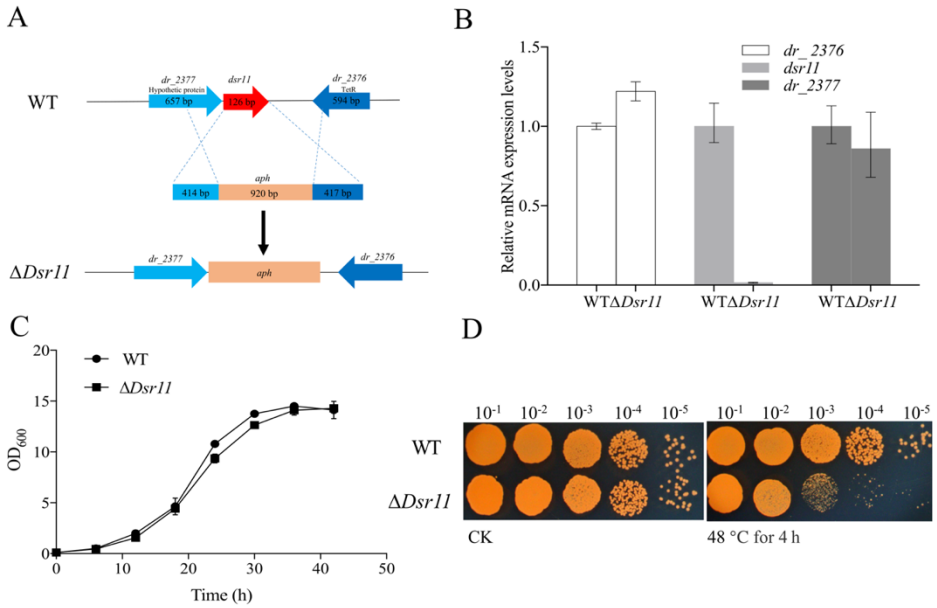


Figure 4-2. Transcriptional and functional analysis of *dsr11* in *Deinococcus radiodurans.* (A) The genomic location of *dsr11* and construction of the *dsr11* mutant. The *dsr11* is shaded in red. Schematic representation of the mutant generated by replacing the *dsr11* region with the kanamycin resistance gene *aph***.** (B) Relative expression of *dsr11*, the upstream gene (*dr_2376*) and the downstream gene (*dr_2377*) in Δ*Dsr11* compared to the WT under normal conditions. The relative levels of the transcripts are presented as the mean values ± standard deviation, calculated from three sets of independent experiments and normalized to levels in the WT strain. (C) Growth curves in TGY broth of WT and Δ*Dsr11*. The error bars represent the calculated standard deviation of the measurements of three biological replicates. (D) Viability of *dsr11* knockout cells after exposure to 48 ˚C. CK, untreated culture control. All experiments were performed three times.

4.5.3. RNA-Seq analysis sheds new light on the mechanisms of dsr11-mediated heat tolerance

ncRNAs are usually involved in post-transcriptional regulation in various

environmental stresses. Thus, to expand the panel of how *dsr11* involved in heat stress tolerance, and to have a better view of the effect of *dsr11* activity on the global transcription, an RNA sequencing transcriptome analysis was performed comparing Δ*Dsr11* to WT strain. We observed 157 genes (Supplementary Table 4-3 and the most representative genes listed in Table 4-1) differentially expressed in the *dsr11* deletion mutant compared to the WT strain, including 121 genes that were up-regulated and 36 genes were down-regulated (Figure 4-3A). Among the up-regulated and down-regulated genes in the *dsr11* deletion mutant we identified genes involved in heat stress response (*csp*, *dnaJ*, *grpE*, *clpB*, and *hsp20*). The genome of *D. radiodurans* contains 3,079 protein-coding genes (Hua et al., 2016) and forty-one ncRNAs were also identified (Tsai et al., 2015). Deletion of *dsr11* altered the expression of about 5.1% of all genes. These results indicating that *dsr11* directly or indirectly regulate these factors.

Table 4-1. Selection of the most representative genes differentially expressed in *D. radiodurans dsr11* deletion mutant with Log_2 (FC) >1 or Log_2 (FC) < 1.

Gene name	Log_2(FC)*	*p* value	Description
Down-regulated in Δ*Dsr11* strain			
dr_0828	-1,9173	1,17E-05	Isocitrate lyase
dr_2464	-1,38547	4,06E-05	Peptidyl-prolyl cis-trans isomerase, FKBP-type
dr_1325	-1,2897	4,02E-05	Endopeptidase-related protein
dr_0815	-1,1394	4,23E-06	Transcriptional regulator, GntR family
dr_A0367	-1,12989	0,0013	UDP-galactopyranose mutase
dr_0907†	-1,08722	1,73E-05	Cold shock protein, CSD family
Up-regulated in Δ*Dsr11* strain			
dr_0128†	1,00099	1,29E-05	GrpE protein
dr_0166	1,03439	4,26E-06	Acyl-CoA-binding protein
dr_0651	1.10117	3,56E-05	Arginase
dr_1359	1,13075	3,08E-06	Outer membrane protein
dr_0139	1,1902	7,89E-06	GTP-binding protein HflX
dr_1424†	1,23589	9,26E-06	DnaJ protein
dr_1046†	1,24258	7,63E-06	ATP-dependent Clp protease, ATP-binding subunit ClpB
dr_2328	1,25488	4,53E-05	Sensor histidine kinase
dr_0440	1,30399	9,42E-05	Holliday junction resolvase
dr_0942	1,31442	0,0075	Tryptophan synthase, alpha subunit
dr_1114†	1,51777	1,07E-05	Heat shock protein, HSP20 family
dr_1473	1,64409	2,04E-05	Phage shock protein A
dr_1016	1.70629	1,85E-05	trmE; tRNA modification GTPase TrmE
dr_1994	4.24133	4,23E-05	Hypothetical protein

*Log_2 (FC) = log_2 of fold change calculated as the ratio between gene expression of WT vs Δ*Dsr11*. †Genes involved in heat stress tolerance regulation.

The function of the DEGs was determined by GO enrichment analysis, which

has been assigned as “membrane”, “membrane part”, “nucleotide binding”, “purine nucleotide binding”, “response to temperature stimulus”, and “response to heat” (Figure 4-3A). Knockout of the *dsr11* affects the expression of genes involved in cell membranes. Previous reports have shown that heat stress can lead to cell membrane disruption in *D. radiodurans* (Bauermeister et al., 2012). We hypothesize that the loss of *dsr11* may lead to changes in cell membrane function, thus affecting heat stress tolerance. In addition, both nucleotide binding and purine nucleotide binding are affected, which may also lead to sensitivity to heat stress. Through KEGG analysis, different pathways were significantly affected by *dsr11* knockout, including mismatch repair, homologous recombination, DNA replication, ABC transporters, arginine and proline metabolism (Figure 4-3B). This result suggests that genes involved in these pathways may interact with *dsr11* to regulate the heat tolerance of *D. radiodurans*, which still requires further validation.

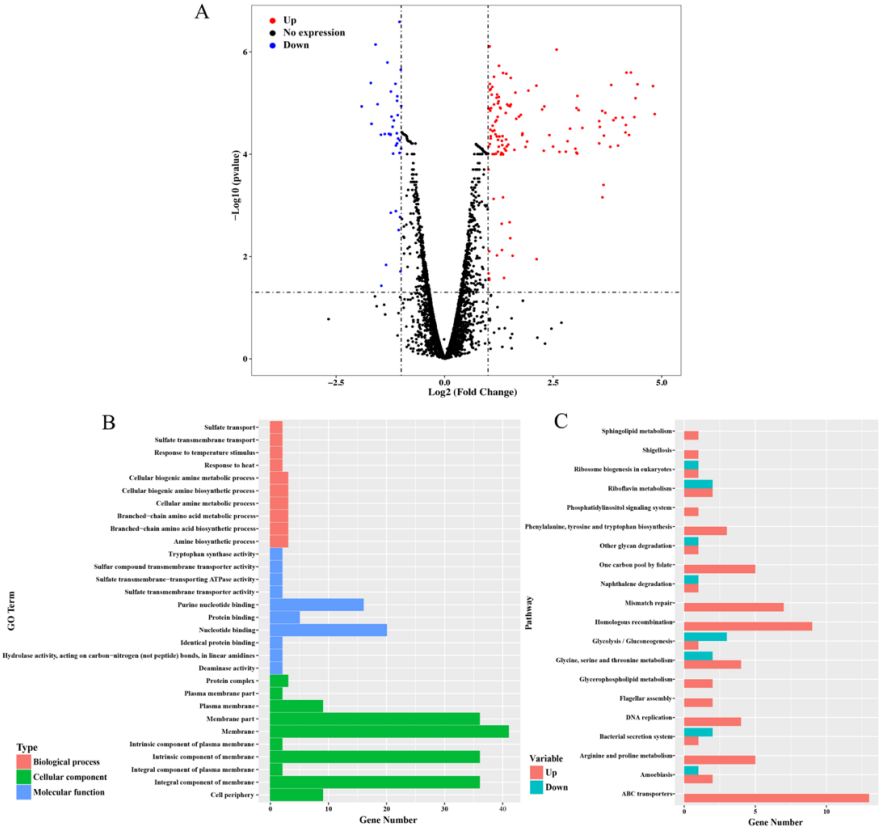


Figure 4-3. RNA-Seq analysis between WT and Δ*Dsr11*.

(A) Volcano map of the differential expression genes Δ*Dsr11* versus WT. Each point represents a unigene. The red and blue points indicate significant changes in the absolute value of log2 (Fold Change) ≥ 1 and FDR ≤ 0.05, respectively; i.e., the red points indicate up-regulated unigenes, and the blue points indicate down-regulated unigenes in the two

groups, with the differential expression levels presented along the X-axis. The black points indicate nonsignificant, differentially expressed unigenes (B) GO terms with significant enrichment analysis of differentially expressed genes in Δ*Dsr11* compared to WT. (C) Top 20 KEGG biological pathway classification histograms for annotated unigenes.

4.5.4. Identification of the dsr11 targets

In bacteria, ncRNAs are commonly known to regulate target mRNAs through sequence-specific base pairing (Waters et al., 2009). Base pairing resulting in target activation can involve ncRNA interactions with the 5′ untranslated region (UTR), the coding sequence, or the 3′ UTR of the target mRNAs, in many respects, functionally analogous to eukaryotic miRNAs (Waters et al., 2009). The region of potential base pairing between ncRNAs and target mRNAs typically encompasses 10–25nt (Kawamoto et al., 2006). The majority of these ncRNAs frequently repress or activate stress response pathways required for adaptation to changing environments. Bacterial ncRNAs activate translation or enhance the stability of one or more mRNA targets (Gottesman, 2005; Prévost et al., 2007). Therefore, the identification and validation of putative ncRNA targets are essential to understand the roles of these regulators in bacteria. We used the webservers TargetRNA2 (Kery et al., 2014) to predict the potential targets of *dsr11*, and a total of 26 targets were identified according to the parameters used (Supplementary Table 4-4).

We combined this in silico target prediction and the results from RNA-seq analysis and identified one gene common in these two approaches (Figure 4-4). We selected this gene *dr_0457* (biopolymer transport protein ExbD) as the target candidate for experimental validation. The binding sites between this predicted target and *dsr11* were analyzed (Figure 4-5). Biopolymer transporter ExbD plays an essential role in the iron uptake for bacteria survival and participated in oxidative stress (Da Silva Neto et al., 2009; Xie et al., 2009). Mukhopadhyay et al. reported that ExbD may have an important role in heat stress response in *Chlamydia pneumoniae* (Mukhopadhyay et al., 2006). We hypothesize that *dsr11* enhances heat tolerance by imperfect base-pairing to *dr_0457*, thereby affecting the transcription and translation of *dr_0457*.

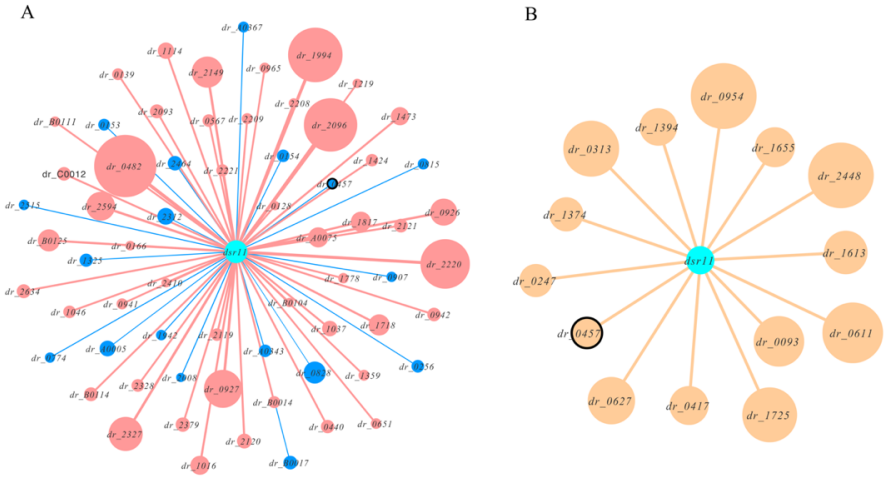


Figure 4-4. Network plots of *dsr11* and targets interaction by RNA-Seq and *in silico* prediction. All hypothetical genes were eliminated. (A)The effect of *dsr11* knockout on gene expression by RNA-Seq. Red nodes represent up-regulated genes and blue nodes represents down-regulated genes. The size of the node represents the expression. The common genes as *in silico* predicted are marked with black circles. (B) The predicted targets of *dsr11* by TargetRNA2. The size of the node represents the binding strength based on the thermodynamic energy of hybridization. The common genes as RNA-Seq analysis are marked with black circles.

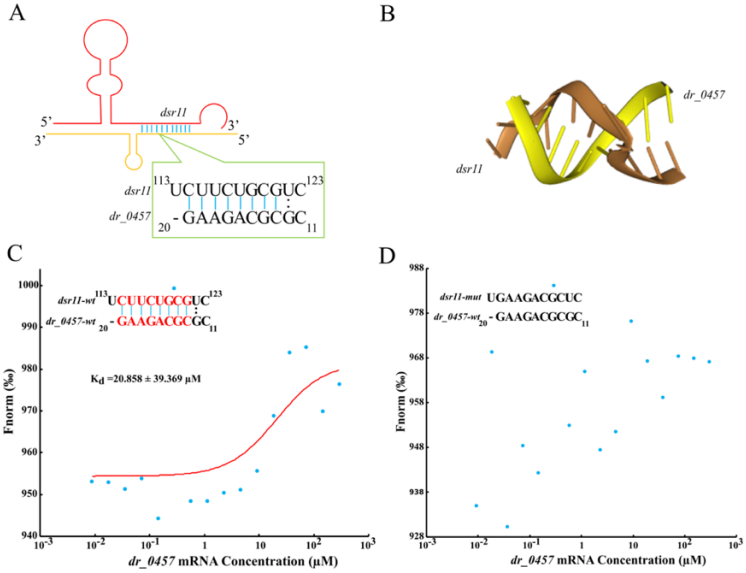


Figure 4-5. Predicted interaction sites of *dsr11* and target gene and determination of the binding affinity of *dsr11* to target mRNAs by microscale thermophoresis (MST).

Red bases mean complementary bases in the binding sequence, the red curve is the fitted combination curve, and the Kd (dissociation equilibrium constant) value is the binding constant of *dsr11* and their targets. (**A**) Schematization of the interaction between *dsr11* and *dr_0457* mRNA based on TargetRNA2. (**B**) The tri-dimensional interaction model was predicted by HNADOCK (He et al., 2019). (**C**) is the binding of *dr_0457* mRNA with *dsr11-wt*. (**D**) is the binding of *dr_0457* mRNA with *dsr11-mut*.

To further validate whether *dsr11* binds directly to the target gene, MST was applied to identify the binding strength between fluorescently labeled *dsr11-wt* or *dsr11-mut* probes and the non-labeled competitor molecule on target genes *dr_0457* mRNA. Results indicated that *dsr11-wt* binds *dr_0457* mRNA at low micromolar concentrations in the titrant, exhibiting a dissociation constant (Kd) of 20.858 ± 39.369 nM, which suggests a relatively strong interaction (Figure 4-5C). In contrast, the mutant derivative (*dsr11*-mut) harboring substitutions in all complementary bases displayed a complete defect in binding to *dr_0457* mRNA (Figure 4-5D), suggesting that *dsr11* binds to the target gene through different bases. The binding assay showed that *dsr11* can regulate target gene in different cellular pathways, indicating that *dsr11* is required for adaptation to extreme environments and probably has specific regulatory functions in *D. radiodurans*.

4.6. Conclusions

In this study, we identified and characterized a novel ncRNA *dsr11* that contributes to heat tolerance in *D. radiodurans*. It is highly conserved in *D. radiodurans* and no homologous to other bacteria. Phenotypic characterization of the mutant strain Δ*Dsr11* revealed that it was more susceptible than the WT to heat stress. Furthermore, RNA-seq analysis indicated that *dsr11* deletion affected the transcriptional expression of genes related to heat stress response, metabolism, and mismatch repair in *D. radiodurans*. The combination of in silico prediction and RNA-seq analysis allowed us to identify the biopolymer transport protein gene *dr_0457* as direct targets of *dsr11*. Our findings elucidate a potential regulatory mechanism of ncRNA *dsr11*. However, the other possible mechanisms of *dsr11* interaction with target mRNA involved in heat stress tolerance remain to be fully understood.

4.7. Supplementary Materials

The supplementary materials for this article are list below, which can be found online at https://www.mdpi.com/2218-273X/10/1/22/s1.

Supplementary Table 4-1. Premiers used in this study

Primers	Sequence (5' – 3')	Purpose
16S	F: ATTCCTGGTGTAGCGGTG	qRT-PCR
	R: CATCGTTTAGGGTGTGGAC	
Dsr11	F: GAGGACGCAGAAGAACAGC	
	R: GAGCAGTTCTCTTCAGACCTGAC	
DR_2376	F: GTTCGACGAGGCGCTGTTC	
	R: GGCTCGGCGGAAAGGTGCTTC	
DR_2377	F: GAATGGACCAGAGCCGCCTGATGAG	
	R: GTCTTTGCCGGTCGCGGCCTTGTAG	
Dsr11-U	F: GCAACCTGATTCTGTCTGCCGTG	Dsr11 mutant construction
	R: GCTCGGTCTCCATGCTCTGCCCCCACTTCGATAAC	
Dsr11-M	F: GTGGGGGCAGAGCATGGAGACCGAGGGCCCTTG	
	R: GCAGTTCTCTTCTTAGAAAAACTCATCGAGCATC	
Dsr11-D	F: CGAGTTTTTCTAAGAAGAGAACTGCTCAGGCGC	
	R: CGCGCAGCAGGTGTACGACTAC	

Supplementary Table 4-2. Synthesized ssRNA oligonucleotide derivatives for MST

Name	Sequence (5' – 3')	Relevant characteristics
dsr11-wt（5'FAM）	UCUUCUGCGUC	interaction with and dr_0457-wt
dsr11-mut（5'FAM）	UGAAGACGCUC	interaction with and dr_0457-wt
dr0457-wt	CGCGCAGAAG	interaction with dsr11-wt

Supplementary Table 4-3. List of genes differentially expressed in the *dsr11* deletion mutant compared to the WT strain.

Supplementary Table 4-4. The possible targets of *dsr11* predicted by TargetRNA2

Rank	Gene ID	Description	Energy	Pvalue
1	DR_0954	Succinate dehydrogenase, cytochrome subunit	-15.14	0.001
2	DR_2448	MerR family transcriptional regulator	-15.1	0.001
3	DR_0089	Hypothetical protein	-14.69	0.001
4	DR_0611	LuxA-like protein	-14.08	0.002
5	DR_0135	Hypothetical protein	-13.89	0.002

6	DR_2163 DR_0313	Hypothetical protein	-13.38	0.003
7	(rplW)	50S ribosomal protein L23	-13.1	0.003
8	DR_1725	WD repeat-containing protein	-12.88	0.004
9	DR_2546	Hypothetical protein	-12.41	0.006
10	DR_1322	Hypothetical protein	-12.1	0.007
11	DR_0093	Phytoene dehydrogenase	-11.99	0.008
12	DR_0627	UDP-N-acetylmuramate--alanine ligase	-11.33	0.011
13	DR_0437	Hypothetical protein	-10.81	0.015
14	DR_1613	ArsR family transcriptional regulator	-10.55	0.018
15	DR_1655	ABC transporter periplasmic substrate-Binding protein	-9.81	0.026
16	DR_0491	Hypothetical protein	-9.63	0.028
17	DR_0417	ppGpp-regulated growth inhibitor ChpA/MazF	-9.61	0.028
18	DR_2541	Hypothetical protein	-9.58	0.029
19	DR_2066	Hypothetical protein	-9.36	0.032
20	DR_1394	Phosphoribosylaminoimidazole synthetase	-9.31	0.032
21	DR_0375	Hypothetical protein	-8.91	0.039
22	DR_0803	Hypothetical protein	-8.8	0.040
23	DR_1374	DNA topoisomerase I	-8.6	0.044
24	DR_2232	Hypothetical protein	-8.44	0.047
25	DR_0247 (acpS)	4'-phosphopantetheinyl transferase	-8.39	0.048
26	DR_0457	Biopolymer transport protein	-8.3	0.050

Reference

Airo, A., Chan, S. L., Martinez, Z., Platt, M. O., & Trent, J. D. 2004. Heat shock and cold shock in *Deinococcus radiodurans*. Cell biochemistry and biophysics, 40(3), 277-288.

Bauermeister, A., Hahn, C., Rettberg, P., Reitz, G., & Moeller, R. 2012. Roles of DNA repair and membrane integrity in heat resistance of *Deinococcus radiodurans*. Archives of microbiology, 194(11), 959-966.

Beckert, B., Kedrov, A., Sohmen, D., Kempf, G., Wild, K., Sinning, I., et al. 2015. Translational arrest by a prokaryotic signal recognition particle is mediated by

RNA interactions. Nature structural & molecular biology, 22(10), 767-773.

Bernhart, S. H., Hofacker, I. L., Will, S., Gruber, A. R., & Stadler, P. F. 2008. RNAalifold: improved consensus structure prediction for RNA alignments. BMC bioinformatics, 9(1), 474.

Blasius, M., Hübscher, U., & Sommer, S. 2008. *Deinococcus radiodurans*: what belongs to the survival kit? Critical reviews in biochemistry and molecular biology, 43(3), 221-238.

Boyle, E. I., Weng, S., Gollub, J., Jin, H., Botstein, D., Cherry, J. M., et al. 2004. GO:: TermFinder—open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. Bioinformatics, 20(18), 3710-3715.

Buddeweg, A., Sharma, K., Urlaub, H., & Schmitz, R. A. 2018. sRNA41 affects ribosome binding sites within polycistronic mRNAs in *Methanosarcina mazei* Gö1. Molecular microbiology, 107(5), 595-609.

Da Silva Neto, J. F., Braz, V. S., Italiani, V. C., & Marques, M. V. 2009. Fur controls iron homeostasis and oxidative stress defense in the oligotrophic alpha-proteobacterium *Caulobacter crescentus*. Nucleic acids research, 37(14), 4812-4825.

Daly, M. J. 2009. A new perspective on radiation resistance based on *Deinococcus radiodurans*. Nature Reviews Microbiology, 7(3), 237-245.

Eyraud, A., Tattevin, P., Chabelskaya, S., & Felden, B. 2014. A small RNA controls a protein regulator involved in antibiotic resistance in *Staphylococcus aureus*. Nucleic acids research, 42(8), 4892-4905.

Gelsinger, D. R., & DiRuggiero, J. 2018. Transcriptional landscape and regulatory roles of small noncoding RNAs in the oxidative stress response of the haloarchaeon *Haloferax volcanii*. Journal of bacteriology, 200(9), e00779-17.

Gottesman, S. 2005. Micros for microbes: non-coding regulatory RNAs in bacteria. TRENDS in Genetics, 21(7), 399-404.

He, J., Wang, J., Tao, H., Xiao, Y., & Huang, S.-Y. 2019. HNADOCK: a nucleic acid docking server for modeling RNA/DNA–RNA/DNA 3D complex structures. Nucleic acids research, 47(W1), W35-W42.

Hua, X., & Hua, Y. 2016. Improved complete genome sequence of the extremely radioresistant bacterium *Deinococcus radiodurans* R1 obtained using PacBio single-molecule sequencing. Genome announcements, 4(5), e00886-16.

Jerabek-Willemsen, M., André, T., Wanner, R., Roth, H. M., Duhr, S., Baaske, P., et al.

2014. MicroScale Thermophoresis: Interaction analysis and beyond. Journal of Molecular Structure, 1077, 101-113.

Kawamoto, H., Koide, Y., Morita, T., & Aiba, H. 2006. Base-pairing requirement for RNA silencing by a bacterial small RNA and acceleration of duplex formation by Hfq. Molecular microbiology, 61(4), 1013-1022.

Kery, M. B., Feldman, M., Livny, J., & Tjaden, B. 2014. TargetRNA2: identifying targets of small regulatory RNAs in bacteria. Nucleic acids research, 42(W1), W124-W129.

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., & Salzberg, S. L. 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome biology, 14(4), R36.

Klein, R. J., Misulovin, Z., & Eddy, S. R. 2002. Noncoding RNA genes identified in AT-rich hyperthermophiles. Proceedings of the National Academy of Sciences, 99(11), 7542-7547.

Kliemt, J., Jaschinski, K., & Soppa, J. 2019. A haloarchaeal small regulatory RNA (sRNA) is essential for rapid adaptation to phosphate starvation conditions. Frontiers in microbiology, 10, 1219.

Lippok, S., Seidel, S. A., Duhr, S., Uhland, K., Holthoff, H.-P., Jenne, D., et al. 2012. Direct detection of antibody concentration and affinity in human serum using microscale thermophoresis. Analytical chemistry, 84(8), 3523-3530.

Lybecker, M. C., & Samuels, D. S. 2007. Temperature-induced regulation of RpoS by a small RNA in *Borrelia burgdorferi*. Molecular microbiology, 64(4), 1075-1089.

Mann, B., van Opijnen, T., Wang, J., Obert, C., Wang, Y.-D., Carter, R., et al. 2012. Control of virulence by small RNAs in *Streptococcus pneumoniae*. PLoS Pathog, 8(7), e1002788.

Mattimore, V., & Battista, J. R. 1996. Radioresistance of *Deinococcus radiodurans*: functions necessary to survive ionizing radiation are also necessary to survive prolonged desiccation. Journal of bacteriology, 178(3), 633-637.

Meyer, L., Coste, G., Sommer, S., Oberto, J., Confalonieri, F., Servant, P., et al. 2018. DdrI, a cAMP receptor protein family member, acts as a major regulator for adaptation of *Deinococcus radiodurans* to various stresses. Journal of bacteriology, 200(13), e00129-18.

Mukhopadhyay, S., Miller, R. D., Sullivan, E. D., Theodoropoulos, C., Mathews, S. A., Timms, P., et al. 2006. Protein expression profiles of *Chlamydia pneumoniae* in models of persistence versus those of heat shock stress response. Infection and

immunity, 74(7), 3853-3863.

Omelchenko, M. V., Wolf, Y. I., Gaidamakova, E. K., Matrosova, V. Y., Vasilenko, A., Zhai, M., et al. 2005. Comparative genomics of *Thermus thermophilus* and *Deinococcus radiodurans*: divergent routes of adaptation to thermophily and radiation resistance. BMC evolutionary biology, 5(1), 57.

Opdyke, J. A., Kang, J.-G., & Storz, G. 2004. GadY, a small-RNA regulator of acid response genes in *Escherichia coli*. Journal of bacteriology, 186(20), 6698-6705.

Patel, B. A., Moreau, M., Widom, J., Chen, H., Yin, L., Hua, Y., et al. 2009. Endogenous nitric oxide regulates the recovery of the radiation-resistant bacterium *Deinococcus radiodurans* from exposure to UV light. Proceedings of the National Academy of Sciences, 106(43), 18183-18188.

Peer, A., & Margalit, H. 2011. Accessibility and evolutionary conservation mark bacterial small-RNA target-binding regions. Journal of bacteriology, 193(7), 1690-1701.

Prévost, K., Salvail, H., Desnoyers, G., Jacques, J. F., Phaneuf, É., & Massé, E. 2007. The small RNA RyhB activates the translation of shiA mRNA encoding a permease of shikimate, a compound involved in siderophore synthesis. Molecular microbiology, 64(5), 1260-1273.

Puerta-Fernandez, E., Barrick, J. E., Roth, A., & Breaker, R. R. 2006. Identification of a large noncoding RNA in extremophilic eubacteria. Proceedings of the National Academy of Sciences, 103(51), 19490-19495.

Sharma, C. M., & Vogel, J. 2009. Experimental approaches for the discovery and characterization of regulatory small RNA. Current opinion in microbiology, 12(5), 536-546.

Sheng, D., Gao, G., Tian, B., Xu, Z., Zheng, Z., & Hua, Y. 2005. RecX is involved in antioxidant mechanisms of the radioresistant bacterium *Deinococcus radiodurans*. FEMS microbiology letters, 244(2), 251-257.

Slade, D., & Radman, M. 2011. Oxidative stress resistance in *Deinococcus radiodurans*. Microbiology and Molecular Biology Reviews, 75(1), 133-191.

Storz, G., Vogel, J., & Wassarman, K. M. 2011. Regulation by small RNAs in bacteria: expanding frontiers. Molecular cell, 43(6), 880-891.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., et al. 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nature protocols, 7(3), 562-578.

Tsai, C.-H., Liao, R., Chou, B., & Contreras, L. M. 2015. Transcriptional analysis of *Deinococcus radiodurans* reveals novel small RNAs that are differentially expressed under ionizing radiation. Applied and environmental microbiology, 81(5), 1754-1764.

Waters, L. S., & Storz, G. 2009. Regulatory RNAs in bacteria. Cell, 136(4), 615-628.

Xie, Q., Jin, H., Luo, R., Wan, Y., Chu, J., Zhou, H., et al. 2009. Transcriptional responses of *Haemophilus parasuis* to iron-restriction stress *in vitro*. Biometals, 22(6), 907.

Zhang, H., Zhan, Y., Yan, Y., Liu, Y., Hu, G., Wang, S., et al. 2019. The *Pseudomonas stutzeri*-specific regulatory noncoding RNA NfiS targets katB mRNA encoding a catalase essential for optimal oxidative resistance and nitrogenase activity. Journal of bacteriology, 201(19), e00334-00319.

5

Chapter V: Adaptation to cold stress in *Bacillus velezensis* GA1 and interaction with tomato at low temperature

5.1. Foreword

In this chapter, we investigated the effect of cold on another gram-positive bacterium, *Bacillus velezensis* studied as biocontrol agent of plant diseases in the MiPI laboratory in Belgium. *B. velezensis* is a model organism that studied in MiPI lab. The laboratory works on the plant diseases biocontrol using Plant-Growth Promoting Rhizobacteria. *Bacillus sp.* is one of the most promising species as biocontrol agent notably due to their potential to produce many bioactive secondary metabolites involved in antimicrobial activities (cyclic lipopetides, polyketides, etc.). The research also focuses on the influence of abiotic stress on this metabolite production and on the interaction with plants. More specifically, these rhizobacteria live in soil associated to roots where temperature is in average lower than in the atmosphere during the growing season. Therefore, understanding how low-temperature stress may impact the physiology and metabolite production by *B. velezensis* and proto-cooperation established with the host plants. In this chapter, we give an overview of the preliminary results obtained in that respect which are yet unpublished and still need further study and confirmation. The data summarized in this chapter provide interesting additional information on the influence of low-temperature on *B. velezensis* GA1.

5.2. Introduction

Crop plants of commercial importance are severely limited by a number of environmental factors such as drought, salt and diseases s and the growth of crop plant growth would be adversely affected, leading to a worldwide reduction in agricultural production (Boyer, 1982; Qin et al., 2011; Tuteja, 2007). Low temperature is one of the key determinants of agricultural production. In order to generate adaptive responses to low temperature perception, plants require transducer signals to enable the expression of appropriate genes that combat the diverse stresses that impose on living cells at low temperature. The effects of low temperature range from morphological to molecular levels and are evident at all phenological stages of plant growth. Cold stress is a direct result of low-temperature effects on cellular macromolecules that cause a slowdown of metabolism, solidification of cell membranes and loss of membrane functions (Arun-Chinnappa et al., 2017). In the recent year, many efforts have been made to reduce the effect of cold stress on growth and productivity.

Plant beneficial bacteria are often termed plant growth-promoting rhizobacteria (PGPR). PGPR are typically colonizing the rhizosphere of many plant species and confer beneficial effects, such as enhance plant growth, reduced susceptibility to diseases caused by plant pathogens, and enhance abiotic stress resistance (Bhattacharyya et al., 2012; Ongena et al., 2005; Zubair et al., 2019). For example, *Bacillus subtilis* can induce plant resistance to stress and release various growth-enhancing plant hormones. Similar findings have

been documented by Saleh *et al.* in artichoke and Woitke *et al.* in tomato to alleviate the toxic effect of salinity (Woitke et al., 2004). Similar findings have been documented by Saleh *et al.* in artichoke and Woitke *et al.* in tomato to alleviate the toxic effect of salinity (Saleh et al., 2005; Woitke et al., 2004). *Serratia proteamaculans* can increase the nodulation and growth of soybean under a low temperature stress (Dashti et al., 2000).

Recently, the use of PGPR has emerged as a potential new solution to reduce abiotic stress-induced damage. In this context, the first aim of this work was to investigate the effect of low temperature on *B. velezensis* GA1 traits, such as growth rate, motility, and root colonization ability. Also, we wanted to evaluate the effect of low temperature on the ability of *B. velezensis* GA1 to colonize tomato with the prospect of reducing the harmful consequences of low temperature by the use of PGPR treatment.

5.3. Material and methods

5.3.1. Bacteria strain and plant used in this study

Bacillus velezensis GA1 (Arguelles-Arias et al., 2009) was used in this study, which was stocked in MiPI lab. GA1 was routinely cultured in RE broth (Nihorimbere et al., 2012) (1/2 of all medium (0.685g of KH_3PO_4, 21g of MOPS, 0.5g of $MgSO_4.7H_2O$, 0.5g of KCl, 1.0g of yeast extract and 100 µL of the trace solution (120mg of $Fe_2(SO_4)_3$, 40mg of $MnSO_4$, 160mg of $CuSO_4$ and 400mg Na_2MoO_4 per 10ml) and 1/2 of tobacco medium (2.0g of glucose, 3.4g of fructose, 0.4g of maltose, 0.6g of ribose, 4.0g of citrate, 4.0g of oxalate, 3.0g of succinate, 1.0g of malate, 10g of fumarate, 1.0g of casamino acids, 2g of $(NH_4)_2SO_4$ per liter, pH 7)) or on RE plates supplemented with agar (1.5%) at 30 °C. The tomato seeds used in this study is *Lycopersicum esculentum* (Moneymaker). *Lycopersicum esculentum* was cultured in Hoagland medium (26 g of agar per liter, pH adjusted to 7).

5.3.2. Time-frame study for cold-adaptive

For RE solid medium experiment, cells were pre-cultured in RE to stationary phase, were harvested by centrifugation at 5000× *g* for 5 min, washed twice in sterile phosphate-buffered saline (PBS, 0.02% KH_2PO_4, 0.29% $Na_2HPO_4 \cdot 12H_2O$, 0.8% NaCl, 0.02% KCl, pH 7), and resuspended in PBS and adjust to OD_{600} =0.1. Eight microliters of the suspension were spotted on RE agar plates and were incubated at 22 ˚C, 18 ˚C, and 15 ˚C for up to 96 h (Round 1). The colonies grown to 96h were scraped from the plate, washed twice with PBS, and resuspended in PBS adjust to OD_{600} = 0.1. Eight microliters of the suspension were spotted on RE agar plates and were kept at 22 ˚C, 18 ˚C, and 15 ˚C for up to 96 h (Round 2). Round 3 is the same as round 2 (Figure 5-1A).

For RE liquid medium experiment, bacterial cells were pre-cultured in RE to stationary phase, were harvested by centrifugation at 5000× g for 5 min, washed twice in sterile PBS. Adjust the OD_{600} to 0.1 in fresh RE liquid medium in the

flask under agitation (120 rpm) and the OD_{600} of each sample was measured every 6 h (Round 1). Collect the round 1 bacteria in stationary phase, centrifuge and wash twice with PBS. Adjust the OD_{600} to 0.1 in fresh RE liquid medium in the flask under agitation (120 rpm) and the OD_{600} of each sample was measured every 6 h (Round 2). The other round is the same as round 2 (Figure 5-1B).

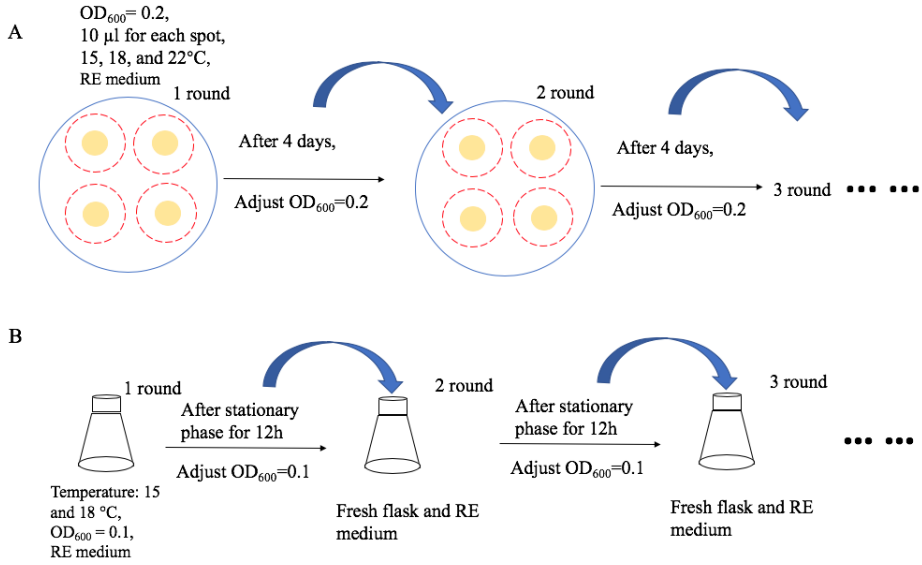


Figure 5-1. The workflow diagram in solid RE medium (A) and liquid RE medium (B).

5.3.3. Preparing tomato seeds for germination

Tomato seeds were surface sterilized with 75% ethanol for 2 min and in 4.5% sodium hypochlorite for 15 min and rinsed with sterile water. Then, they were put in Petri dish containing filter paper wetted with Hoagland solution and then left for germination for 4 days at 22 °C. After germination, they were used for the experiments of rhizosphere colonization.

5.3.4. Bacterial colonization on tomato rhizosphere

Sterilized tomato seeds were pregerminated for 4 days on solid Hoagland medium at 22 °C and 60 % humidity under 16 hours of light and 8 hours of darkness, circadian cycle. Four-day-old seedlings were inoculated with 10 µL of bacterial suspension (at the top of the root tip). For each strain, this suspension was obtained by centrifuging an overnight culture (RE medium, 30 °C), washing the cells with $MgSO_4$ 10 mM and resuspending them to OD_{600} = 0.5. After inoculation, plantlets were then incubated in 15˚C, 18˚C, and 22˚C for 2, 4, 6, 8, and 10 days. The colonization was evaluated through flow cytometry (FCM) analysis. For UPLC-MS analysis, plated samples were obtained from the total rhizosphere including the plantlet roots, the 2 mm of agar surrounding them.

5.3.5. LC-MS analysis

Secondary metabolites analyses were performed by using UPLC–MS with UPLC (Acquity H-class, Waters) coupled to a single quadrupole mass spectrometer (SQD mass analyzer, Waters) using an C18 column (Acquity UPLC BEH C18 2.1 mm × 50 mm, 1.7 µm). Elution was performed at 40°C with a constant flow rate of 0.6 mL/min using a gradient of Acetonitrile (solvent B) and water (solvent A) both acidified with 0.1% formic acid as follows: gradient from 30% to 95% during 2.43 min before maintaining 95% B for 2.67min before going back to initial conditions at 5.2 min during 1.8 minutes before next injection. Compounds were detected in electrospray positive ion mode by setting SQD parameters as follows: cone voltage: 60V; source temperature 130°C; desolvation temperature 400°C, and nitrogen flow: 1000 L.h-1 with mass range from m/z 100 to 2048.

5.3.6. Flow cytometry (FCM) analysis

FCM analyses are realized directly after sampling to avoid community evolution. They are carried out on BD Accuri C6 flow cytometer (BD Biosciences, NJ, USA) equipped with 20 mW Solid State Blue Laser (488 nm) and 14.7 mW Diode Red Laser (640 nm). Plant inoculated samples were diluted in pH 7 PBS to fit into the desired range of 500–2500 events/µL for cytometry analysis. Cells were stained using a BacLightTM RedoxSensorTM Green Vitality Kit (ThermoFisher, MA, USA) containing Propidium iodide (PI) and Redox Sensor Green (RSG) (Sigma Aldrich Fluka, Saint-Louis Missouri USA). 1 mL of cell suspension is supplemented with 10 µL of stain solution and incubated 30 minutes at room temperature. Forward light scatter (FSC), Side light scatter (SSC) are collected with a 488/10 bandpass filter and green fluorescence (FL1 channel) is collected with photomultiplier tubes using533/30 bandpass filter. For each sample run, data for 40,000 events are collected with a flow rate of 14 ul/min. Raw data were extracted as fcs files and loaded into FlowJo analysis software.

5.4. Results and discussion

5.4.1 Bacterial growth and time-frame study in a solid medium under low temperature

To investigate whether low temperature affects the growth of GA1, we performed aerobic cultures of GA1 at 15˚C, 18˚C, and 22˚C in RE solid medium (a mimicking soil nutrition medium). Cells can grow and spread well in 72h at 22˚C. At 18˚C, the growth rate of cells become slow. When it reached 96 hours, the colony started to spread out. At 15˚C, the cells still can grow, but the growth rate becomes very slow. At 96h, the colony did not spread outward, and it may need longer incubation time (Figure 5-2). Concurrently, to further study the growth adaptability of GA1 to low temperature, several rounds of growth were carried out on GA1 at different temperatures. As shown in Figure 5-2, there is no significant changes under low

temperatures (15°C and 18°C) after several rounds culture. To our surprise, after two rounds of culture the colony morphology of GA1 has become smaller at 22˚C. We hypothesize that after two rounds culture changes its metabolites production causing its creep on the solid plate, resulting in the colony morphology. At the same time, genes related to metabolites may also be affected under low temperature. Regarding this part, further analyses are necessary for these samples by UPLC-MS analysis and RNA analysis.

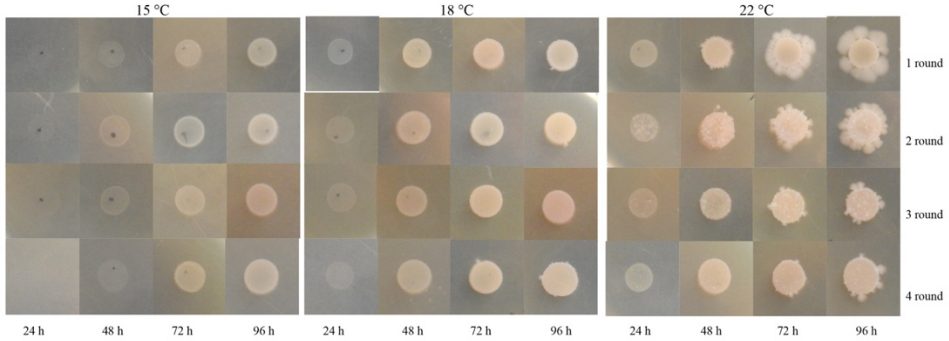


Figure 5-2. Growth of GA1 on four rounds of continuous culture on RE solid medium at three temperatures (15°C, 18°C, and 22°C).

5.4.2. Bacterial growth and time-frame study in liquid medium under low temperature

With a similar idea as for the solid medium, we have conducted liquid culture assays. GA1 can grow at all three temperatures, but the low temperature can slow down the growth rate. We observed that it would take 48 hours to the stationary phase at 18˚C, while it would take 72 hours to reach the stationary phase at 15˚C (Figure 5-3). To further validate the adaptation of GA1 at low temperature in liquid medium, we carried out several rounds of cultivation. The results show that it only takes 42 hours to reach the stationary phase after two rounds of cultivation at 18˚C, while it takes 66 hours to reach the stationary phase after four rounds of growth at 15˚C (Figure 5-3). In these liquid cultures, we observed that the bacteria can adapt well to the cold environment. We hypothesis that after several rounds of cultivation, low temperature stimulated the expression of cold-related genes and adapted to cold stress, thereby increasing the growth rate.

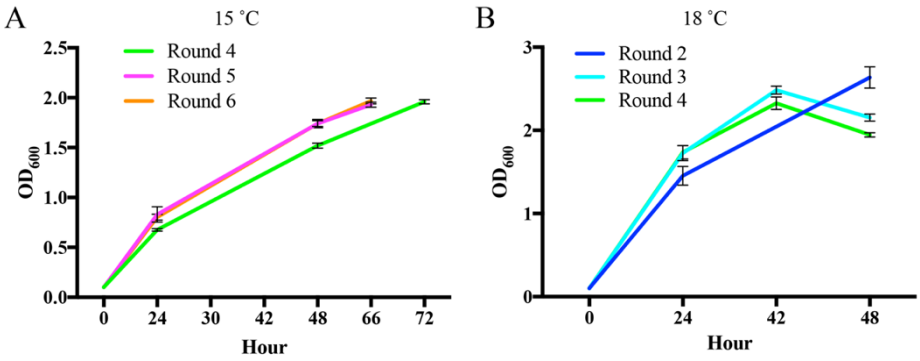


Figure 5-3. The adaptation of GA1 after several rounds at lower temperatures (15 °C and 18 °C).

We also studied metabolite production by GA1 grown at low temperatures. We collected the supernatant and performed metabolite analysis. Interestingly, UPLC-MS analysis of supernatant extracts showed that GA1 growth at low temperatures (15°C and 18˚C) resulted in a much higher production of the surfactin lipopeptide than at 22 °C (Figure 5-4A). Very similar results were obtained with another plant-associated *B. amyloliquefaciens* S499 for surfactin production (Pertot et al., 2013). Further investigation revealed that relative production of surfactin by GA1 was clearly decrease after several round cultivation at low temperature (compare the 7th round to 1st round cultivation at 15˚C) (Figure 5-4B), making it possible to counter-balance the negative effect on its motility observed *in vitro* in 22˚C (Figure 5-2).

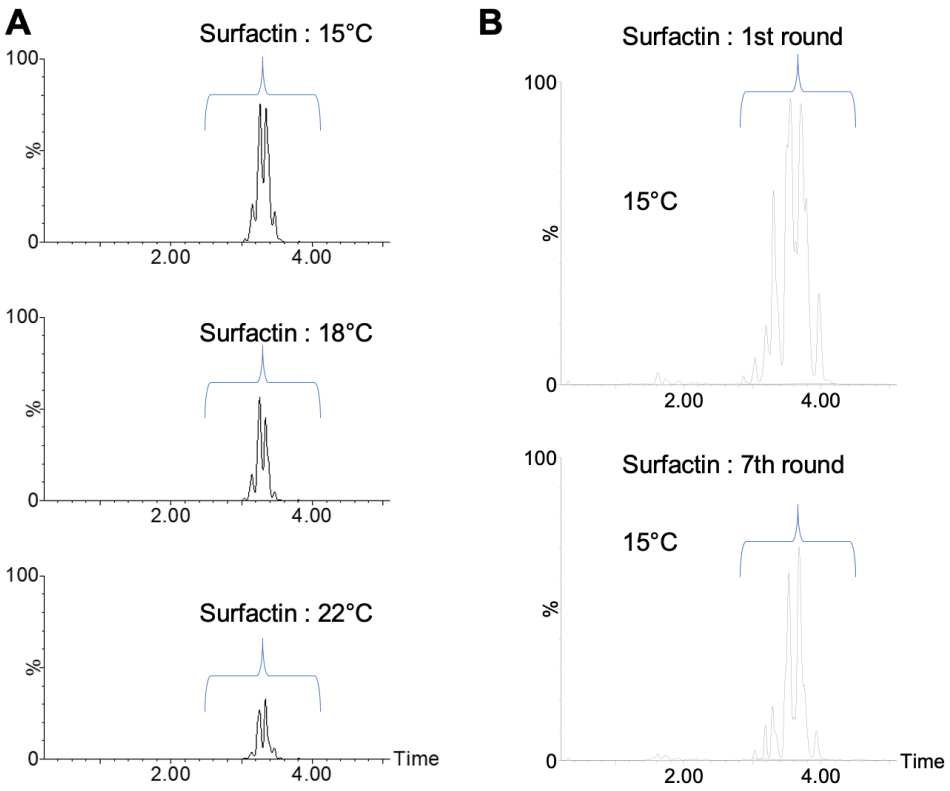


Figure 5-4. Analysis the surfactin production of the supernatant of GA1 in RE liquid medium at different temperatures and rounds

5.4.3. Influence of cold temperature on colonization of tomato roots

Based on the adaptation of *B. velezensis* GA1 to low temperature, we then focused on understanding how temperature directly affect *B. velezensis* GA1 physiology in regard to the basic features of rhizosphere fitness. These experiments were performed on tomato as model plant commonly used in the lab. The ability to form biofilm-related structures on roots correlates with an efficient colonization by rhizobacteria. We inoculated GA1 at root tips of the tomatoes and observed that at 15˚C, GA1 did not colonize the root hairs well after 3 days of cultivation, while at 18˚C and 22˚C GA1 had colonized on the root hair on the first day (form biofilm structure). And the formation is quicker at 22˚C (Figure 5-5).

We collected the cells in the roots and performed FCM experiments to examine the population of GA1 cells in the roots of tomatoes by PI and RSG staining. However, lowering the temperature clearly resulted in reduced GA1

populations on the roots (Figure 5-6). We found that the bacteria are classified into three subgroups. Among them, Q2 quadrants are cells with relatively good activity, and Q1 quadrants are cells with relatively low activity. Q3 represents living cells. We observed that as the time of culture grew, the number of Q3 cells increased and formed a good colonization with the rhizosphere. But under low temperature conditions, these all happen more slowly. In the further study, a fluorescence microscope will be used to observe the morphology of bacterial cells. Simultaneous study of metabolites and transcription levels is expected to further investigate the relationship between GA1 and tomato rhizosphere under low temperature.

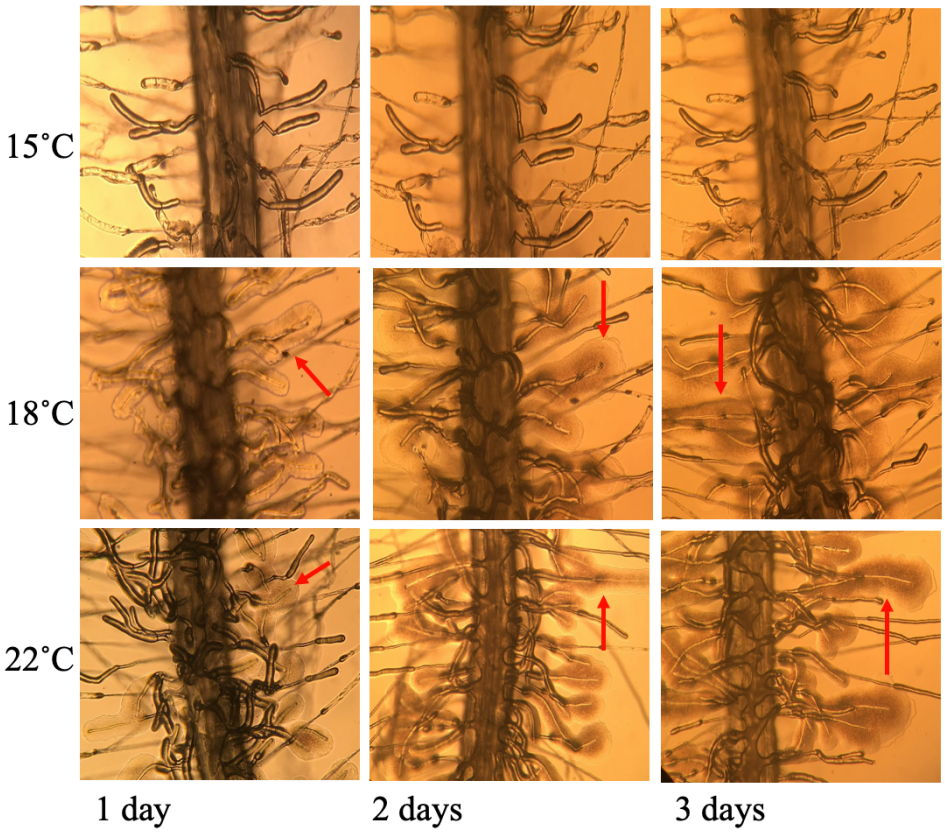


Figure 5-5. *B. velezensis* GA1 colonization on tomato roots

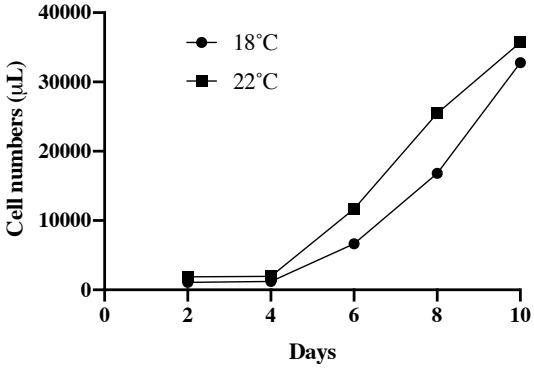


Figure 5-6. Root colonization by GA1 on tomato root exposed to different temperatures by flow cytometry.

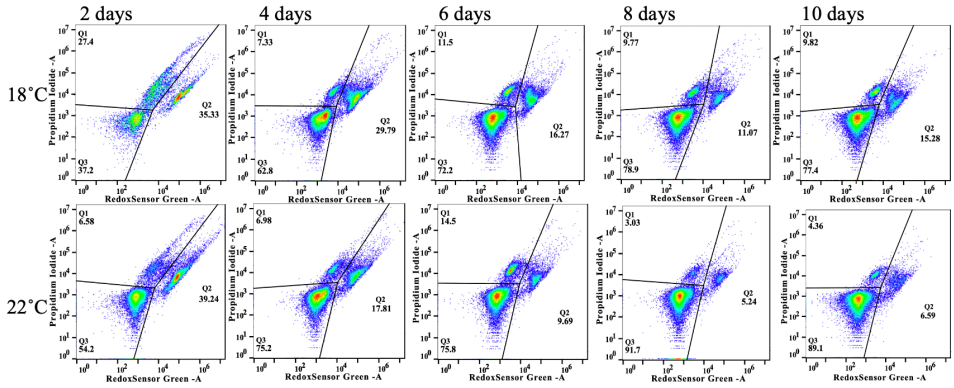


Figure 5-7. The different types of GA1 colonized on tomato root at different time periods by flow cytometry

In summary, in this study we have tried to explore the potential features of *Bacillus velezensis* GA1 in order to detect and study the expression of the genes which could impart cold tolerance in these microbes enabling them to perform their metabolic and physiological functions efficiently under cold stress. Due to the potential of these bacteria to alleviate cold stress and promote plant growth by modulating metabolites at low temperature, it is highly desirable to further investigate GA1 to use it in the form of different bio-formulations and develop bio-fertilizers for better agricultural production in extreme environments.

References

Arguelles-Arias, A., Ongena, M., Halimi, B., Lara, Y., Brans, A., Joris, B., et al. 2009. *Bacillus amyloliquefaciens* GA1 as a source of potent antibiotics and other secondary metabolites for biocontrol of plant pathogens. Microbial cell factories, 8(1), 63.

Arun-Chinnappa, K. S., Ranawake, L., & Seneweera, S. 2017. Impacts and management of temperature and water stress in crop plants. In Abiotic stress management for resilient agriculture (pp. 221-233): Springer.

Bhattacharyya, P. N., & Jha, D. K. 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World Journal of Microbiology and Biotechnology, 28(4), 1327-1350.

Boyer, J. S. 1982. Plant productivity and environment. Science, 218(4571), 443-448.

Dashti, N., Prithiviraj, B., Zhou, X., Hynes, R. K., & Smith, D. L. 2000. Combined effects of plant growth-promoting rhizobacteria and genistein on nitrogen fixation in soybean at suboptimal root zone temperatures. Journal of plant nutrition, 23(5), 593-604.

Nihorimbere, V., Cawoy, H., Seyer, A., Brunelle, A., Thonart, P., & Ongena, M. 2012. Impact of rhizosphere factors on cyclic lipopeptide signature from the plant beneficial strain *Bacillus amyloliquefaciens* S499. FEMS microbiology ecology, 79(1), 176-191.

Ongena, M., Jacques, P., Touré, Y., Destain, J., Jabrane, A., & Thonart, P. 2005. Involvement of fengycin-type lipopeptides in the multifaceted biocontrol potential of *Bacillus subtilis*. Applied microbiology and biotechnology, 69(1), 29.

Pertot, I., Puopolo, G., Hosni, T., Pedrotti, L., Jourdan, E., & Ongena, M. 2013. Limited impact of abiotic stress on surfactin production in planta and on disease resistance induced by *Bacillus amyloliquefaciens* S499 in tomato and bean. FEMS microbiology ecology, 86(3), 505-519.

Qin, F., Shinozaki, K., & Yamaguchi-Shinozaki, K. 2011. Achievements and challenges in understanding plant abiotic stress responses and tolerance. Plant and Cell Physiology, 52(9), 1569-1582.

Saleh, S., Heuberger, H., & Schnitzler, W. 2005. Alleviation of salinity effect on artichoke productivity by *Bacillus subtilis* FZB24, supplemental Ca and micronutrients. J Appl Bot Food Qual, 79(1), 24-32.

Tuteja, N. 2007. Abscisic acid and abiotic stress signaling. Plant signaling & behavior, 2(3), 135-138.

Woitke, M., Junge, H., & Schnitzler, W. 2004. *Bacillus subtilis* as growth promotor in hydroponically grown tomatoes under saline conditions. VII International Symposium on Protected Cultivation in Mild Winter Climates: Production, Pest Management and Global Competition 659 (pp. 363-369).

Zubair, M., Hanif, A., Farzand, A., Sheikh, T. M. M., Khan, A. R., Suleman, M., et al. 2019. Genetic screening and expression analysis of psychrophilic *Bacillus* spp. reveal their potential to alleviate cold stress and modulate phytohormones in wheat. Microorganisms, 7(9), 337.

6

Chapter VI: General discussion and perspectives

General discussion and perspectives

Microorganisms are often affected by various environmental factors. This affects their physiological and biochemical functions. Among these environmental factors, temperature plays an important role in the normal physiological activities of microorganisms, and the temperature is a limiting factor affecting the survival of organisms. Therefore, studying the adaptive pathways and mechanisms of microorganisms at different temperatures and clarifying their adaptive effects can provide a theoretical basis for future applications in industry and agriculture.

In chapter 2, we describe the use of an RNA-Seq-based technique to examine the changes in the transcriptome of *D. radiodurans* in response to heat stress. Previous studies have shown that when *D. radiodurans* is exposed to 48°C for up to 2 hours, cell mortality is low and significant lethality only occurred later on (Harada et al., 1988; Schmid et al., 2005a, 2005b; Schmid et al., 2002). It correlates quite well with our data in Figure 2-1 and Figure 2-2 showing very low loss of viability for 48°C heat-treated cells. From these results, we conclude that 48°C is an optimal temperature for *D. radiodurans* under our experimental conditions. Proteins involved in heat stress tolerance/adaptation are thus assumed to accumulate during that timeframe of two hours post-shift of temperature to 48°C and we hope to focus on genes specifically encoding those proteins and further investigate their expression upon recovery once cells are again grown back to normal temperature. Considering that it takes time for accumulated proteins to be degraded, we indeed hypothesized that some proteins retaining key roles in this process remains in cells after recovery treatment for two hours. Genes encoding these key proteins are hypothesized to be expressed at distinct levels in different treatment groups, which has been verified in the performed transcriptomics analysis. We think the use of this strategy will provide a deeper understanding of the mechanism of the heat stress adaptation of *D. radiodurans*.

Upon integration of the data from RNA-seq analysis, we have identified 134 genes whose expression increases and 28 whose expression decreases (RH vsR30). Among these genes, 15 genes were differentially dowregulated over eight-fold). All of these genes are reported participated in heat stess response except three hypothetical genes (*dr_1325*, *dr_0127* and *dr_1083*). According to BLASTp, these three genes encode respectively for a predicted LysM peptidoglycan-binding protein, a predicted DNA-binding protein, and a hypothetical protein. The major structural component of bacterial cells is the cell wall, which is crucial for cellular function, especially for protecting cell physiological activity, as it separates the internal environment from the external environment. DR_1325 is predicted to be a protein containing a LysM peptidoglycan-binding domain, which may be involved in cell wall biogenesis (Mesnage et al., 2014). We speculate that DR_1325 may regulate the response of the heat stress mechanism by participating in cell wall repair, which requires

experimental support from further studies. That idea still requires experimental support from further studies In our study, TEM showed that the *D. radiodurans* cell wall was slightly damaged after treatment at 48 °C treatment for 2 h (Figure 2-2B1). Although most of the cells remained alive (Figure 2-2B2), the high temperature damaged the integrity of the cell wall. Similarly, Stéphane Guyot et.al (2010) and Bożena Bruhn-Olszewska et al. (2018) reported that high temperatures can destroy the integrity of the cell wall in *E. coli* (Bruhn-Olszewska et al., 2018; Guyot et al., 2010). Besides, the TEM data showed that after the cells had recovered at 30 °C for 2 h, their cell walls showed partial repair (Figure 2-2C1). Complete cell wall repair would likely require additional time after the heat stress.

Moreover, *dr_0127* was substantially upregulated in R48 (Figure 4). Annotation revealed that DR_0127 was predicted to be a DNA-binding protein. Genomic DNA packaging is mediated by a set of DNA-binding proteins that have major impacts on gene transcription and DNA replication (Azam et al., 1999; Browning et al., 2010; Dillon et al., 2010). The nucleoid of a bacterium is organized by DNA-binding proteins, especially under stress conditions; these proteins also protect the bacterial genome and regulate transcription to promote survival under stress conditions. Through alignment analyses, we observed that DR_0127 is highly conserved in other *Deinococcus* species, such as *D. wulumuqiensis*, *D. gobiensis*, *D. soli*, and *D. actinosclerus*. More interestingly, the upstream and downstream genes encode DnaJ, GrpE, and DnaK. The qRT-PCR data showed that deletion of *dr_0127* drastically decreased the expression of the downstream gene *dr_0126* (which encodes a DNA binding protein). Through the genome analysis, we predicted that these genes may share the same operon. In addition, we believe that the reduction in tolerance to heat stress may not only be due to the absence of *dr_0127* but also due to the dramatic downregulation of *dr_0126* expression. In future work, we will further clarify the exact relationship between *dr_0126* and *dr_0127* in response to heat stress by performing insertional mutagenesis. Deletion of *dr_0127* resulted in decreased expression of *dr_0126* (*dnaJ*), *dr_0128* (*grpE*), and *dr_0129 (dnaK)* relative to the wild-type strain under heat stress. DR_1027 is predicted to be a genus-specific DNA binding protein that we hypothesize may work together with DnaJ, GrpE, and DnaK in response to heat stress, although their mechanisms of action require further study.

High temperature can cause the denaturation of double-stranded DNA and alter the secondary structure of RNA, which can affect cell function (Klein et al., 2002). Our results revealed that the DNA repair genes *dr_A0346* (*pprA*), *dr_1696* (*mutL*), *dr_1477* (*recN*), *dr_2340* (*recA*), *dr_1902* (*recD*), *dr_1289* (*recQ*), *dr_1922* (*sbcC*), *dr_1921* (*sbcD*), and *dr_2275* (*uvrB*) were upregulated in response to heat stress. Previous reports have indicated that these genes play important roles in DNA repair after exposure to UV, ionizing radiation, and hydrogen peroxide (Blasius et al., 2008). Our results indicate that these genes are also involved in the DNA repair mechanism in response to heat stress. We

postulate that under heat stress, replication of DNA is upregulated and the probability of a DNA mismatch is increased; thus, these DNA repair genes ensure the stability of DNA replication. The increase in DNA content helps to maintain genomic stability, fidelity, and integrity under heat stress. Intriguingly, we found that the expression levels of *dr_B0107*, *dr_B0108*, *dr_B0109*, and *dr_B0110* were highly increased (RH). These four genes constitute the *nrdIEF* operon (*dr_B0107-0110*), which encodes the ribonucleotide reductase essential for DNA synthesis (Härtig et al., 2006). The *nrdIEF* operon has previously been reported to be involved in salt stress (Im et al., 2013). We speculate that these genes are also involved in DNA repair after heat stress. At the same time, *dr_1042-1046* was significantly downregulated in RH vs R30, with *dr_1042* and *dr_1046* encoding a PadR transcriptional regulator and a heat stress-related protein, respectively. DR_1043-1045 are proteins with unknown functions, and we believe that the *dr_1043-1045* genes may play important roles under heat stress. We also observed that *PaaABCD* (*dr_2383-2386*) was downregulated in RH vs R30, with these genes being involved in diverse cellular processes, including cellular respiration and DNA replication and repair. Also, DR_2381 and DR_2382 may potentially function by accelerating the degradation of short-lived proteins. We hypothesized that *PaaABCD* and *dr_2381-2382* work together to regulate the heat stress response. Besides, we found that an LEA homolog, *dr_B0118*, was inhibited by the heat stress treatment, but its expression level was significantly increased during the recovery phase; thus, we speculate that this gene might be involved in cell repair. A previous study reported that this gene is involved in desiccation tolerance (Battista et al., 2001), and our transcriptome data suggest that *dr_B0118* participates in the heat stress response, which possibility warrants further study.

At present, due to the important regulatory role of bacterial ncRNA in bacterial life and environmental stress, it has received more and more attention. ncRNAs are widely found in many species, they do not encode proteins and are highly conserved in evolution. It plays a regulatory role directly at the RNA level of many organisms such as archaea, bacteria, and eukaryotes. For example, it plays an important role in the structure of chromosomes, gene expression and closure, protein transport and stability regulation, and cell cycle and individual development. According to available data, bacterial ncRNA is widespread in a variety of bacteria. It is estimated that there may be hundreds of ncRNA genes in each bacterial genome. More than 1,000 ncRNAs have been found in some common bacterial genomes, and a large number of ncRNAs have not yet been discovered. The discovery of new bacterial ncRNAs, the study of their functions, is important for us to understand the activities of bacteria and the role of bacteria and the environment. Among various experimental and bioinformatics methods for predicting bacterial ncRNA, bioinformatics methods predict bacterial ncRNA as part of the ncRNA functional research strategy due to the fast predictive and low cost of bioinformatics methods. At present, bioinformatics predicts that bacterial ncRNA methods can be divided into three major categories based on comparative genomics, transcription-based signal finding,

and machine-based learning. These methods have good effects in their respective evaluation systems, but in the prediction results, various methods cannot predict all known ncRNAs, and the coincidence between the various prediction results is less than 10%. The above shortcomings indicate that people's understanding of bacterial ncRNA is still limited, and it has not touched its essential characteristics. It does not have a comprehensive, systematic and profound understanding of ncRNA. With the development of technology, RNA-seq has become a powerful tool for discovering ncRNA. The research history of *D. radiodurans* has been more than half a century. It has been studied from different angles and levels such as its morphology, adaptation of environmental change, different resistance mechanisms during the genome and cell level. Although related studies have shown that ncRNA plays an important role in the biotic or abiotic environmental stress response of microorganisms. However, to the best of our knowledge, the role of ncRNAs in *D. radiodurans* has rarely been reported under heat stress conditions before. There are still many ncRNAs in *D. radiodurans*, and the role of these ncRNAs in the mechanism of heat stress is still unclear.

Although much effort has been made to elucidate the molecular mechanisms underlying the tolerance of *D. radiodurans* to heat stress, gene expression reprogramming is complex and not yet fully understood. Many ncRNAs have been reported to be associated with bacterial responses to various environmental stresses (Citartan et al., 2016; Delihas et al., 2001; Majdalani et al., 2001; Massé et al., 2002). While there has been a rapid increase in the identification of bacterial ncRNAs over the last few years, the identification of mRNA targets and the study of ncRNAs' functions have progressed more slowly. So far, to our knowledge, no ncRNAs with a regulatory role in heat tolerance in extreme bacteria have been reported. In chapter three, Δ*DnrH* has been used to analyze the involvement of the ncRNA *DnrH* on high temperature response compared to *D. radiodurans* wild-type strain. Our results show that the absence of *DnrH* significantly inhibited viability in response to high temperature. These evidences suggest a positive contribution of *DnrH* on the heat stress response. ncRNAs usually regulate other genes at the post-transcriptional level by directly or indirectly base-pairing with the target gene mRNA (Storz et al., 2011; Wagner et al., 2015). Comparative phenotypic and qRT-PCR analyses of Δ*DnrH* and wild-type (WT) suggested that *Dnr*H is potentially involved in regulating the expression of the heat shock-related gene *Hsp20*. *Hsp20* is described as a molecular chaperone that can prevent the aggregation of denatured proteins and assist in the refolding and hydrolysis of abnormal proteins during abiotic stresses (Bepperling et al., 2012; Muthusamy et al., 2017). Moreover, previous studies have reported that the expression of *Hsp20* is increased following exposure to various stresses, including temperature changes (Schmid et al., 2005b; Singh et al., 2014). Here we show that the expression of *Hsp20* is significantly down-regulated in the absence of *DnrH* and provide strong indications for *Hsp20* mRNA being a direct target of *DnrH*. To investigate the interaction *DnrH-hsp20* mRNA we employed different approaches: i)

bioinformatics analysis, ii) *in vitro* microscale thermophoresis, iii) *in vivo* fusions-PCR experiment based on the mutant binding site. Further, results of MST and genetic complementation suggested that *Hsp20* mRNA is a direct target of *DnrH*. Further, *in vivo* transcriptional analysis indicated that *Hsp20* transcripts accumulate to high levels during heat stress, again confirming the role of *Hsp20* in high temperature adaptation. The precise molecular mechanisms underlying the modulation of *DnrH* activities are not yet known and require further investigation. Considering that *Hsp20* functions as a chaperon protein, regulation by *DnrH* might be a two-step process in which *DnrH* regulates *Hsp20* and then affects the transcription of downstream genes. This hypothesis also requires further research. Similar to *dnrH*, in chapter four we characterized another ncRNA associate to heat tolerance, named *dsr11*. In addition, the deletion of *dsr11* resulted in WT more sensitive to heat stress treatment. We combined the target prediction and the RNA-Seq analysis and found that *trmE* (coding for tRNA modification GTPase) and *dr_0651* (coding for arginase) may be the target mRNA of *dsr11*. As shown in Figure 4-4, *dsr11* interacts with *dr_0457* mRNA through base-pairing. In conclusion, *dsr11* enhances heat tolerance by imperfect base-pairing to *dr_0457*, and affecting the transcription and translation of *dr_0457*. On the whole, the panel of results collected by this study suggests an interesting role of both *dnrH* and *dsr11* in *D. radiodurans* heat stress tolerance. These two ncRNAs can be used to study the biological function of *D. radiodurans* and provide a basis for further study of the molecular mechanism of heat stress resistance. It enriches and perfects the existing theory of extreme environmental resistance mechanisms and lays a theoretical foundation for its applications in medicine, biology, agriculture and military.

In Chapter five, our test found that the colony was smaller at 15 and 18˚C compared to 22˚C in solid medium. Low temperatures indeed extend the lag time required for the production of metabolic and physiological changes associated with swarmer cells. The reduced growth rate under cold may explain the negative impact of low temperature on GA1 swarming as surface migration starts more quickly at a high cell density. In the study of Budde et al. (Budde et al., 2006), microarray analysis showed some decreased expression of several genes involved in chemotaxis and motility in chill-stressed B. subtilis cells, indicating that the basic capacity to move could also be affected under cold conditions. That said our results on swarming at 15–22 °C are consistent with previous studies, suggesting that Bacillus colonies forming at higher temperatures expanding to larger diameters (Julkowska et al., 2004). Looking more closely at the direct influence of temperature on GA1, in vitro cultures showed that the productivity of surfactin improved dramatically when the temperature was reduced (Figure 5-4). Non-Ribosomal Peptide Synthetases (NRPS, genes coding for surfactin) machinery have not been reported as upregulated in the microarray study of cold adapted Bacillus cells performed by Budde et al. (Budde et al., 2006). However, the cell growth rate of B. velezensis was negatively affected by cold, suggesting a general decrease in the metabolic

activity of cells cultivated at low temperature (Brigulla et al., 2003; Budde et al., 2006; Weber et al., 2003). As a consequence, such slow growth rate may partially explain the high surfactin productivity in the rhizosphere at 15 °C, especially whether this indirect impact of low temperature on cell growth is combined with the nutrient restriction that obviously occurs in this environment (Bais et al., 2006; Lugtenberg et al., 2001). In addition, we inoculate GA1 to the tomato rhizosphere to study its relationship with plants. Low temperatures have been shown to influence the number of cells colonized in the rhizosphere and the evolution of cell status, but more study is also needed on how low temperature influence the effect of GA1 on plants. The main focus of future studies is on association with tomatoes, such as: (1) on colonization rate and dynamics; (2) on phenotypic heterogeneity in root-associated populations (biofilm); (3) on the biocontrol-related metabolome (quantitative and qualitative); (4) on the biological functions of this metabolome as antibacterial, antifungal, reactive oxygen species (ROS) elicitation.

In conclusion, this study deeply analyzed the adaptation pathways and mechanisms of *D. radiodurans* to high temperature environment, and clarified the important role of ncRNA under high temperature stress. At the same time, preliminary research was conducted on the adaptation mechanism of *B. velezensis* GA1 at low temperature and the interaction between PGPR and tomato plants. These studies will provide a theoretical basis for future applications in industry and agriculture.

References

Azam, T. A., & Ishihama, A. 1999. Twelve species of the nucleoid-associated protein from *Escherichia coli* sequence recognition specificity and DNA binding affinity. Journal of Biological Chemistry, 274(46), 33105-33113.

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., & Vivanco, J. M. 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. Annu. Rev. Plant Biol., 57, 233-266.

Battista, J. R., Park, M.-J., & McLemore, A. E. 2001. Inactivation of two homologues of proteins presumed to be involved in the desiccation tolerance of plants sensitizes *Deinococcus radiodurans* R1 to desiccation. Cryobiology, 43(2), 133-139.

Bepperling, A., Alte, F., Kriehuber, T., Braun, N., Weinkauf, S., Groll, M., et al. 2012. Alternative bacterial two-component small heat shock protein systems. Proceedings of the National Academy of Sciences, 109(50), 20407-20412.

Blasius, M., Hübscher, U., & Sommer, S. 2008. *Deinococcus radiodurans*: what belongs to the survival kit? Critical reviews in biochemistry and molecular biology,

43(3), 221-238.

Brigulla, M., Hoffmann, T., Krisp, A., Völker, A., Bremer, E., & Völker, U. 2003. Chill induction of the SigB-dependent general stress response in *Bacillus subtilis* and its contribution to low-temperature adaptation. Journal of bacteriology, 185(15), 4305-4314.

Browning, D. F., Grainger, D. C., & Busby, S. J. 2010. Effects of nucleoid-associated proteins on bacterial chromosome structure and gene expression. Current opinion in microbiology, 13(6), 773-780.

Bruhn-Olszewska, B., Szczepaniak, P., Matuszewska, E., Kuczyńska-Wiśnik, D., Stojowska-Swędrzyńska, K., Algara, M. M., et al. 2018. Physiologically distinct subpopulations formed in *Escherichia coli* cultures in response to heat shock. Microbiological research, 209, 33-42.

Budde, I., Steil, L., Scharf, C., Völker, U., & Bremer, E. 2006. Adaptation of *Bacillus subtilis* to growth at low temperature: a combined transcriptomic and proteomic appraisal. Microbiology, 152(3), 831-853.

Citartan, M., Raabe, C. A., Hoe, C. H., Rozhdestvensky, T. S., & Tang, T. H. 2016. Bacterial sRNAs: regulation in Stress. Stress and Environmental Regulation of Gene Expression and Adaptation in Bacteria, 108-114.

Delihas, N., & Forst, S. 2001. MicF: an antisense RNA gene involved in response of *Escherichia coli* to global stress factors. Journal of molecular biology, 313(1), 1-12.

Dillon, S. C., & Dorman, C. J. 2010. Bacterial nucleoid-associated proteins, nucleoid structure and gene expression. Nature Reviews Microbiology, 8(3), 185-195.

Guyot, S., Pottier, L., Ferret, E., Gal, L., & Gervais, P. 2010. Physiological responses of *Escherichia coli* exposed to different heat-stress kinetics. Archives of microbiology, 192(8), 651-661.

Harada, K., & Oda, S. 1988. Induction of thermotolerance by split-dose hyperthermia at 52 ˚C in *Deinococcus radiodurans*. Agricultural and biological chemistry, 52(10), 2391-2396.

Härtig, E., Hartmann, A., Schätzle, M., Albertini, A. M., & Jahn, D. 2006. The *Bacillus subtilis* nrdEF genes, encoding a class Ib ribonucleotide reductase, are essential for aerobic and anaerobic growth. Applied and environmental microbiology, 72(8), 5260-5265.

Im, S., Joe, M., Kim, D., Park, D.-H., & Lim, S. 2013. Transcriptome analysis of salt-stressed *Deinococcus radiodurans* and characterization of salt-sensitive

mutants. Research in microbiology, 164(9), 923-932.

Julkowska, D., Obuchowski, M., Holland, I. B., & Séror, S. J. 2004. Branched swarming patterns on a synthetic medium formed by wild-type *Bacillus subtilis* strain 3610: detection of different cellular morphologies and constellations of cells as the complex architecture develops. Microbiology, 150(6), 1839-1849.

Klein, R. J., Misulovin, Z., & Eddy, S. R. 2002. Noncoding RNA genes identified in AT-rich hyperthermophiles. Proceedings of the National Academy of Sciences, 99(11), 7542-7547.

Lugtenberg, B. J., Dekkers, L., & Bloemberg, G. V. 2001. Molecular determinants of rhizosphere colonization by *Pseudomonas*. Annual review of phytopathology, 39(1), 461-490.

Majdalani, N., Chen, S., Murrow, J., St John, K., & Gottesman, S. 2001. Regulation of RpoS by a novel small RNA: the characterization of RprA. Molecular microbiology, 39(5), 1382-1394.

Massé, E., & Gottesman, S. 2002. A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. Proceedings of the National Academy of Sciences, 99(7), 4620-4625.

Mesnage, S., Dellarole, M., Baxter, N. J., Rouget, J.-B., Dimitrov, J. D., Wang, N., et al. 2014. Molecular basis for bacterial peptidoglycan recognition by LysM domains. Nature communications, 5(1), 1-11.

Muthusamy, S. K., Dalal, M., Chinnusamy, V., & Bansal, K. C. 2017. Genome-wide identification and analysis of biotic and abiotic stress regulation of small heat shock protein (HSP20) family genes in bread wheat. Journal of plant physiology, 211, 100-113.

Schmid, A. K., Howell, H. A., Battista, J. R., Peterson, S. N., & Lidstrom, M. E. 2005a. Global transcriptional and proteomic analysis of the Sig1 heat shock regulon of *Deinococcus radiodurans*. Journal of bacteriology, 187(10), 3339-3351.

Schmid, A. K., Howell, H. A., Battista, J. R., Peterson, S. N., & Lidstrom, M. E. 2005b. HspR is a global negative regulator of heat shock gene expression in *Deinococcus radiodurans*. Molecular microbiology, 55(5), 1579-1590.

Schmid, A. K., & Lidstrom, M. E. 2002. Involvement of two putative alternative sigma factors in stress response of the radioresistant bacterium *Deinococcus radiodurans*. Journal of bacteriology, 184(22), 6182-6189.

Singh, H., Appukuttan, D., & Lim, S. 2014. Hsp20, a small heat shock protein of *Deinococcus radiodurans*, confers tolerance to hydrogen peroxide in

Escherichia coli. J. Microbiol. Biotechnol, 24(8), 1118-1122.

Storz, G., Vogel, J., & Wassarman, K. M. 2011. Regulation by small RNAs in bacteria: expanding frontiers. Molecular cell, 43(6), 880-891.

Wagner, E. G. H., & Romby, P. 2015. Small RNAs in bacteria and archaea: who they are, what they do, and how they do it. In Advances in genetics (pp. 133-208): Elsevier.

Weber, M. H., & Marahiel, M. A. 2003. Bacterial cold shock responses. Science progress, 86(1-2), 9-75.

Appendix – Publications

1. **Xue, D**., Chen, Y., Li, J., Han, J., Liu, Y., Jiang, S., et al. 2019. Targeting *Hsp20* using the novel small noncoding RNA *DnrH* regulates heat tolerance in *Deinococcus radiodurans*. Frontiers in microbiology, 10, 2354.

2. **Xue, D**., Chen, Y., Li, J., Han, J., Zhou, Z., Zhang, W., et al. 2020. A novel noncoding RNA *dsr11* involved in heat stress tolerance in *Deinococcus radiodurans*. Biomolecules, 10(1), 22.

3. **Xue, D**., Liu, W., Chen, Y., Liu, Y., Han, J., Geng, X., et al. 2019. RNA-seq-based comparative transcriptome analysis highlights new features of the heat-stress response in the extremophilic bacterium *Deinococcus radiodurans*. International journal of molecular sciences, 20(22), 5603.