Ddrl, a cAMP Receptor Protein Family Member, Acts as a Major Regulator for Adaptation of *Deinococcus radiodurans* to Various Stresses

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ABSTRACT The DNA damage response *ddrl* gene encodes a transcription regulator belonging to the cAMP receptor protein (CRP) family. Cells devoid of the Ddrl protein exhibit a pleiotropic phenotype, including growth defects and sensitivity to DNA-damaging agents and to oxidative stress. Here, we show that the absence of the Ddrl protein also confers sensitivity to heat shock treatment, and several genes involved in heat shock response were shown to be upregulated in a Ddrl-dependent manner. Interestingly, expression of the *Escherichia coli* CRP partially compensates for the absence of the Ddrl protein. Microscopic observations of $\Delta ddrl$ mutant cells revealed an increased proportion of two-tetrad and anucleated cells in the population compared to the wild-type strain, indicating that Ddrl is crucial for the completion of cell division and/or chromosome segregation. We show that Ddrl is also involved in the megaplasmid MP1 stability and in efficient plasmid transformation by facilitating the maintenance of the incoming plasmid in the cell. The *in silico* prediction of putative Ddrl binding sites in the *D. radiodurans* genome suggests that hundreds of genes, belonging to several functional groups, may be regulated by Ddrl. In addition, the Ddrl protein absolutely requires cAMP for *in vitro* binding to specific target sequences, and it acts as a dimer. All these data underline the major role of Ddrl in *D. radiodurans* physiology under normal and stress conditions by regulating, both directly and indirectly, a cohort of genes involved in various cellular processes, including central metabolism and specific responses to diverse harmful environments.

IMPORTANCE *Deinococcus radiodurans* has been extensively studied to elucidate the molecular mechanisms responsible for its exceptional ability to withstand lethal effects of various DNA-damaging agents. A complex network, including efficient DNA repair, protein protection against oxidation, and diverse metabolic pathways, plays a crucial role for its radioresistance. The regulatory networks orchestrating these various pathways are still missing. Our data provide new insights into the crucial contribution of the transcription factor Ddrl for the *D. radiodurans* ability to withstand harmful conditions, including UV radiation, mitomycin C treatment, heat shock, and oxidative stress. Finally, we highlight that Ddrl is also required for accurate cell division, for maintenance of plasmid replicons, and for central metabolism processes responsible for the overall cell physiology.

KEYWORDS *Deinococcus radiodurans*, CRP family, cAMP signaling, transcription regulator, heat shock response, plasmid maintenance

The molecular mechanisms contributing to the impressive capacity of *Deinococcus radiodurans* to withstand very harmful genotoxic stresses, including ionizing and UV radiation, desiccation, and reactive oxygen species (ROS), are now well documented (for recent reviews, see references 1–4). These include very efficient DNA repair mech-

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anisms encoded by *D. radiodurans* to reassemble an intact genome from hundreds of DNA fragments (5–7) and a compact structure of the nucleoid maintained after irradiation which may favor DNA double-strand break (DSB) repair by avoiding dispersion of DNA fragments (8, 9). *D. radiodurans* also possesses very efficient ROS detoxification pathways, including both enzymatic and nonenzymatic systems (for reviews, see references 2 and 10) and protects its genome by limiting iron import and concomitant Fenton reactions (11). In addition, the radiation-resistant *Deinococcus* species share an upregulation of specific DNA damage response (*ddr*) genes following irradiation or desiccation (12). Recently, a screening of a transposon insertion mutant library exposed to different genotoxic stresses has identified a combination of various metabolic and structural functions involved in the extreme radioresistance of *D. radiodurans* (13). Nevertheless, the regulatory networks orchestrating these various pathways remain to be elucidated.

Although *D. radiodurans* encodes two homologues of the *Escherichia coli* SOS response LexA repressor, LexA1 and LexA2, with self-protease activity stimulated by a mechanism requiring RecA, the physiological targets of these two potential repressors are still unidentified (14–16). Instead, another regulation pathway involving the IrrE metalloprotease and its cognate substrate, the DdrO repressor, has been shown to play a major regulatory role after exposure to ionizing radiation in both *Deinococcus deserti* (17) and *D. radiodurans* (18, 19). The IrrE metalloprotease from *D. deserti* cleaves the DdrO protein in response to gamma-ray exposure, allowing the induction of genes involved in DNA DSB repair (17). This pathway controls the radiation desiccation response (RDR) regulon, shared by several *Deinococcus* species (20, 21), and a regulatory RDR motif (RDRM) palindromic sequence was found upstream of the coding sequence of the RDR regulon members. In *D. radiodurans*, this regulon includes at least *recA*, *gyrA*, and *Deinococcus*-specific genes, like the *ddrB*, *pprA*, and *ddrC* genes. *ddrB* encodes an essential function for the single-strand annealing (SSA) repair mechanism (7, 22). All of these genes are repressed by DdrO in the absence of any genotoxic stress (18, 23, 24).

Another transcriptional regulator, DrRRA, crucial for the extreme resistance of *D. radiodurans* to gamma and UV irradiation, H₂O₂ exposure, and desiccation has been identified (25). It belongs to the response regulators of two-component systems, but its cognate histidine kinase is still unknown. Transcriptome analyses of the Δ *drRRA* mutant revealed impaired induction of numerous genes after gamma-ray exposure, including *recA*, *pprA*, and *uvr* (ultraviolet radiation) DNA repair genes; *kata* and *katE*, encoding catalases; *sodA* and *sodC*, encoding superoxide dismutases; and genes encoding thioredoxin and ferritin/Dps-like proteins (25).

The *ddrI* gene (*dr0997*) was initially identified by transcriptome analyses of *D. radiodurans* as induced 4-fold following gamma-ray exposure (3 kilogray [kGy]) and 11-fold after desiccation (12). We have recently demonstrated that inactivation of *ddrI* by transposon insertion sensitizes *D. radiodurans* to gamma and UV irradiation, mitomycin C (MMC), and H₂O₂ (13). Yang et al. confirmed that a deletion of the *ddrI* gene sensitizes cells to these harmful conditions, suggesting an important role of DdrI in the cellular response of *D. radiodurans* to DNA damage and oxidative stress (26). Moreover, the absence of DdrI results in slow bacterial growth (26). The transcription of *ddrI* is induced by the response regulator DrRRA following gamma irradiation, and purified DrRRA binds to the *ddrI* promoter region (25). The DdrI protein exhibits typical features of the transcription factors belonging to the cAMP receptor protein (CRP) family, including an effector cyclic nucleotide monophosphate (cNMP)-binding domain at the N-terminal end, and a helix-turn-helix (HTH) DNA-binding domain at the C-terminal end (26). In *E. coli*, the expression of several dozen genes is regulated by CRP, including many genes involved in central carbon metabolism and transport of various metabolites (27). In *D. radiodurans*, it has been shown that the transcription of 18 genes encoding proteins mainly involved in metabolic pathways or DNA repair mechanisms is directly regulated by the DdrI protein (26).

In this study, we showed that the *E. coli* CRP can partially restore resistance to

FIG 1 The $\Delta ddrI$ mutant displays severe growth defects partially complemented by the expression of the *E. coli* CRP. GY9613 (wild type [WT]), GY13630 ($\Delta ddrI$ mutant), GY13643 ($\Delta ddrI/ddrI^+$ complemented strain), and GY13644 ($\Delta ddrI/crp^+$ mutant) strains were grown at 30°C in TGY2X medium (supplemented with spectinomycin and 1 mM IPTG for the GY13644 strain). The A_{650} values of the cultures were measured and generation times calculated from 3 independent experiments.

DNA-damaging agents and oxidative stress and complements the growth defect of *D. radiodurans* cells devoid of the DdrI protein. The *in silico* prediction of potential DdrI binding sites in the *D. radiodurans* genome suggests that hundreds of genes, belonging to several functional groups, may be regulated by DdrI. We showed that DdrI is involved in the heat shock response, regulation of cell division and/or chromosome segregation, stability of the megaplasmid MP1, and efficient plasmid transformation by facilitating the maintenance of the incoming plasmid in the cell. Moreover, we demonstrate that DdrI absolutely requires cAMP for *in vitro* binding to its specific DNA target and acts as a dimer. All these data underline the important regulatory role of DdrI in the adaptation of *D. radiodurans* to various stresses.

RESULTS

The *ddrI* locus is transcribed as a monocistronic unit. The *ddrI* locus *dr0997* is located 178 bp upstream of a putative operon starting from *dr0998* and ending at *dr1000* (see Fig. S1 in the supplemental material), putatively encoding a member of the multiresistance and pH adaptation (MRP) family, a putative transcription regulator, and a 2'-5' RNA ligase, respectively. Whereas a stem-loop structure forming a Rho-independent transcription terminator was found downstream of *dr1000*, there was no evidence for the presence of such a transcription terminator in the intergenic region between *dr0997* and *dr0998*. To test if a *ddrI* deletion interferes with the expression of downstream genes, we assessed the presence of a putative operon from *dr0997* to *dr1000*. The reverse transcription-PCR (RT-PCR) products for each coding sequence, as well as fragments overlapping *dr0998* and *dr0999* or *dr0999* and *dr1000*, were amplified using cDNA synthesized from total *D. radiodurans* RNA as the template. No RT-PCR product was detected with one primer located in *dr0997* and the other in *dr0998*, whereas an internal fragment of *dr0997* was efficiently amplified (Fig. S1). Moreover, RT-PCR products beginning in *dr0998* and ending in *dr0999* or beginning in *dr0999* and ending in *dr1000* were amplified when *dr0997* was deleted (Fig. S1). Therefore, *dr0997* appeared to be transcribed as a monocistronic unit, upstream of the operon starting from *dr0998* and ending at *dr1000*.

Expression of the *E. coli* CRP partially complemented growth defects and sensitivity to various stresses of the *D. radiodurans ddrI* deletion mutant. As previously shown by Yang et al. (26), the $\Delta ddrI$ null mutant exhibited a dramatic growth deficiency fully complemented by expression of the DdrI protein (Fig. 1). Interestingly, we show here that *trans* expression of the *E. coli* CRP in cells devoid of the DdrI protein resulted in a partial restoration of growth, with cells exhibiting a doubling time of 195 (± 6) min compared to 304 (± 10) min for the $\Delta ddrI$ mutant strain and 120 (± 9) min for the wild-type parental R1 strain when cultivated under standard growth conditions (Fig. 1). We also show that heterologous expression of the *E. coli* CRP in the $\Delta ddrI$ mutant partially restored resistance to genotoxic agents, such as MMC and ultraviolet radiation

FIG 2 Expression of *E. coli* CRP in the $\Delta ddrI$ mutant partially restores resistance to UVC, mitomycin C (MMC), and Paraquat. (A) Sensitivity assays of *D. radiodurans* to UVC and mitomycin C (MMC). GY9613 (wild type), GY13630 ($\Delta ddrI$ mutant), GY13643 ($\Delta ddrI/ddrI^+$ complemented strain), and GY13644 ($\Delta ddrI/crp^+$ mutant) strains were grown to an A_{650} of 1, and appropriate dilutions (top) were spotted onto TGY agar plates exposed or not exposed to UVC rays (600 J/m²) or MMC (40 ng/ml). TGY agar plates were supplemented with spectinomycin and 1 mM IPTG for the GY13644 strain. (B) Sensitivity assays of *D. radiodurans* to oxidative stress generated by Paraquat. Exponential-phase cultures (A_{650} , 0.5) of GY9613, GY13630, GY13643, and GY13644 were spread on TGY plates or TGY agar plates supplemented with spectinomycin and 1 mM IPTG for strain GY13644, and 10 μ l of Paraquat (at the indicated concentrations, left) was spotted onto filter paper discs. After incubation at 30°C for 2 days, the diameters of the growth inhibition zones were measured.

(UVC) (Fig. 2A), and to oxidative stress, as shown by the diameters of the growth inhibition zones surrounding discs on which Paraquat (dimethyl-4,4'-bipyridinium dichloride) was spotted (Fig. 2B). These results strongly suggest that the *E. coli* CRP shared several common targets with the DdrI protein and regulated gene expression to restore efficient cell growth and resistance to harmful conditions.

In silico prediction of potential DdrI targets. The complementation experiments described above suggest a high similarity between the binding sites of *E. coli* CRP and the *D. radiodurans* DdrI protein. Potential DdrI sites were therefore predicted using CRP sites determined experimentally. The *E. coli* CRP box is characterized by the 5'-TGTG A-N₆-TCACA-3' consensus sequence (the most conserved bases are underlined) (27). The DNA motifs recognized by CRP homologs are well conserved among a variety of bacterial species, including the GlxR factor of *Corynebacterium glutamicum* (28), the CRP of *Mycobacterium tuberculosis* (CRP_{MT}) (29), and the CRP of *Haemophilus influenzae* (30) (Fig. S2). CRP and related regulators are able to recognize and bind to relatively divergent sites, reflected by the relatively low informational content of their consensus sequence.

The *in silico* prediction of potential DdrI targets in the *D. radiodurans* genome was performed using the Fast Investigation Tool for Bacterial and Archeal Regulons (FITBAR) Web tool (<http://archaea.u-psud.fr/fitbar/>) (31) dedicated to the identification of specific protein-DNA-binding sites on fully sequenced prokaryotic genomes. For this analysis, we used the "CRP-*E. coli*" matrix, which contains the set of 166 intergenic CRP target sites listed in RegulonDB (<http://regulondb.ccg.unam.mx/>), identified by genomic SELEX (27), and experimentally validated. The information carried by the compilation of these 166 CRP binding sites weighs only 7.29 bits, as calculated according to Schneider et al. (32).

A total of 176 sites located upstream of genes at distances <650 bp were found in intergenic regions (Table S1). These potential DdrI targets may regulate the expression of hundreds of genes, including monocistronic units or genes belonging to operons located downstream of these putative DdrI targets, and at least 68 divergent genes indicated in Table S1 (Hua and Hua [33]; GenBank accession numbers CP015081.1 to CP015081.4). These target genes are clustered into several functional categories, in-

cluding DNA replication and repair, responses to oxidative stress and heat shock, signal transduction, carbohydrate metabolism, and transport, cell wall biogenesis, and lipid metabolism (Table S1). A matrix based on the set of these 176 potential Ddrl sites was used in a second prediction to increase the informational content of the consensus sequence to 9.32 bits. With this additional information, 115 sites located upstream of genes at distances <650 bp were found in intergenic regions (Table S2). Among these 115 sites, 96 were among those present in Table S1, and 19 new sites were found when the 176 *D. radiodurans* binding sites were used to build the matrix.

We then defined a *D. radiodurans* consensus sequence [5'-TGTGA(N₆)TCACA-3'] (the palindromic sequence found in the consensus sequence is underlined) extrapolated by FITBAR from the 115 putative Ddrl binding sites described in Table S2 (Fig. 3A). To assess the biological significance of this consensus sequence, we tested the ability of Ddrl protein to bind to an 80-bp fragment containing, at position 33, the 5'-TGTGAA AAAACTCACA-3' sequence related to the Ddrl putative recognition sequence. For this purpose, we performed electrophoretic mobility shift assay (EMSA) analysis using a purified Ddrl protein containing an N-terminal His₆ tag, shown to be able to complement *in vivo* Δ *ddrl* mutant deficiencies (Fig. S3). We found that a band shift is observed using the His₆::Ddrl protein, even in the presence of a large amount of a poly(dI-dC) competitor, but only when cAMP was added, as commonly observed for many *E. coli* CRP homologs (Fig. 3B). We also tested the binding of the His₆::Ddrl protein to a variant of this consensus sequence (5'-TGTTAAAAAAACTTACA-3') (the nucleotides different from those found in the consensus sequence are indicated in bold), which is present in the promoter region of the *sodC* (*dr1546*) gene, listed in Tables S1 and S2, and showed that the His₆::Ddrl protein also bound to this variant (Fig. S4).

The putative Ddrl recognition sequence being palindromic, we tested the ability of the Ddrl protein to form dimers and any involvement of cAMP in the dimerization process. We observed that Ddrl was able to form dimers without the requirement of cAMP (Fig. 3C).

The Ddrl regulon includes genes involved in response to heat shock. The *ddrl* gene was previously found among the 25 most highly upregulated genes upon heat shock treatment, as determined by microarray analysis (34). We therefore chose to investigate the effect of a *ddrl* deletion on the sensitivity of *D. radiodurans* to heat shock treatment. We found that cell viability decreased 1,000-fold in the absence of Ddrl at 37°C, compared to wild-type cells grown under the same conditions, and cells devoid of Ddrl were unable to grow at 39°C (Fig. 4A). The thermosensitive phenotype of the Δ *ddrl* mutant was completely suppressed by the expression of a chromosomal ectopic *ddrl* gene and, to a lesser extent, by plasmid-based expression of the heterologous CRP from *E. coli* (Fig. 4A). Compared to the wild-type strain, the expression of the *ddrl* gene was shown to decrease in a *drRRA* mutant after DNA-damaging treatment (25). Therefore, the expression of Ddrl was studied by Western immunoblot analyses of cell extracts from the R1 or Δ *drRRA* mutant strain expressing the Ddrl-FLAG-tagged protein when bacteria were exposed to heat shock. We also verified the functionality of the Ddrl-FLAG protein to complement the *ddrl* deletion *in vivo* (Fig. S3). The expression of the Ddrl-FLAG protein was induced in response to heat shock (0.5 or 1 h at 48°C) in the wild-type background, whereas in the Δ *drRRA* mutant, the Ddrl-FLAG protein was undetectable at 30°C, as well as after heat shock treatment, indicating a DrRRA-dependent expression of the Ddrl protein (Fig. 4B). Thus, we tested the heat sensitivity of cells devoid of DrRRA and found that they also exhibited a thermosensitive phenotype at 39°C (Fig. 4A).

To test a possible Ddrl-dependent expression of heat shock genes, we compared the target genes of Ddrl predicted *in silico* (Tables S1 and S2) with the genes or proteins whose expression was found to be increased by heat shock (34, 35). We chose to study in wild-type and Ddrl-deficient cells at 30°C and after 5 min of incubation of the cells at 48°C, by quantitative reverse transcription-PCR (RT-qPCR), the expression of genes that appeared from both approaches. We also included *dr0004* in our study since many

FIG 3 DdrI needs cAMP for binding a DNA fragment containing the predicted DdrI binding consensus. (A) The sequence logo of the putative DdrI binding site was deduced from the second round of DdrI target predictions by using a matrix based on the 115 potential DdrI binding sites found in the *D. radiodurans* genome (Table S2). (B) Electrophoretic mobility shift assays performed with an 80-bp DNA fragment containing the predicted DdrI binding site and the recombinant His₆::DdrI protein (0.5 or 1 μM), in presence (+) or absence (–) of cAMP (200 μM). (C) DdrI protein (3 μg) was incubated with 0.05% glutaraldehyde in the presence or absence of cAMP at a final concentration of 200 μM. After migration, the gel was stained with Coomassie blue. M, molecular mass marker.

proteins of the Nudix family were found to be induced by heat shock treatment. We found that *dr0001* (*dnaN*), *dr0004* (*mutT*), *dr0219* (*ddrF*), *dr1571*, *dr1572* (*uvrD*₂), *dr1913* (*gyrA*), *dr1974* (*lon1*), *dr2381*, *drA0319*, and *drA0369* genes were induced by heat shock treatment in a DdrI-dependent manner (Fig. 5 and Table 1), suggesting that they may be direct targets of the DdrI regulatory protein (Tables S1 and S2). The *dr0219* coding sequence, not annotated by Hua and Hua (33), was found 43 bp downstream of a putative DdrI binding site (5'-TGTTATGTTATATACG-3') identified by FITBAR when the

FIG 4 The $\Delta ddrI$ mutant exhibits a highly temperature-sensitive phenotype and induction of DdrI in response to heat shock that is dependent on the response regulator DrRRA. (A) Serial dilutions of GY9613 (wild type), GY13630 ($\Delta ddrI$ mutant), GY13643 ($\Delta ddrI/ddrI^+$ complemented strain), GY13644 ($\Delta ddrI/crp^+$ mutant), and GY13681 ($\Delta drRRA$ mutant) bacteria were spotted on TGY agar plates, and the plates were incubated at 30°C, 37°C, or 39°C for 3 to 5 days. (B) Exponential-phase cultures (A_{650} 0.45) of GY13663 ($drRRA^+ ddrI$ -FLAG mutant) and GY13679 ($\Delta drRRA ddrI$ -FLAG mutant) bacteria grown at 30°C were transferred to 48°C for 0.5 or 1 h before Western blot analysis with anti-FLAG antibodies of their protein extracts (10 μ g). Time zero corresponds to protein extracts prepared just before transfer to 48°C.

search was performed on the genome sequence published by White et al. (36). In contrast, the *drA0143* gene predicted to be a DdrI target (Tables S1 and S2) was highly induced by heat shock treatment, but in a DdrI-independent manner (Fig. 5). However, in the $\Delta ddrI$ mutant, the *drA0143* gene was 2.5-fold less expressed than in the wild-type strain at 30°C and after heat shock. We did not observe any induction of *dr0950* and *dr0980* after heat shock treatment.

We also tested the expression of a panel of 6 genes (*dr0997* [*ddrI*], *dr0349* [*lon2*], *dr0561*, *dr1046* [*clpB*], and *dr1082* [*lrpA*, also called *hpf* or *raiA*], and *drA0199*) previously shown to be induced by heat shock a minimum of 5-fold in *D. radiodurans* (34), described as being not controlled by both Sig1 (an alternative sigma factor) and HspR (a negative regulator of the heat shock response) proteins (34, 35), and not found in Tables S1 and S2. As a control, we analyzed the expression of the *groEL* gene (*dr0607*), which is known to be induced by heat shock under the control of Sig1 and HspR (34, 35) and is not found in the list of putative genes controlled by DdrI (Tables S1 and S2). We found that *groEL* was induced by 3.5-fold after 5 min of incubation at 48°C, but in a DdrI-independent manner. We confirmed the upregulation of the *ddrI* gene after 5 min of incubation at 48°C (Fig. 5) (34), and we showed that *drA0199* was induced independently of the DdrI protein, whereas *dr0349*, *dr0561*, *dr1046*, and *dr1082* were induced by heat shock treatment in a DdrI-dependent manner (Fig. 5 and Table 1). The expression of these 4 genes may be indirectly regulated by the DdrI protein. The *dr0178* gene, belonging to a transposable element of the IS200/IS605 family, was also reported to be upregulated 5.9-fold after heat shock treatment (34). As previously shown, the standard R1 ATCC 13939 strain used in our study only contains one active copy of an IS200/IS605 family member, called ISDra2F, while the second copy is inactive (37). This is in contrast with the first published complete genome sequence of *D. radiodurans* (36), in which 7 complete ISDra2 copies and one partial ISDra2 copy were reported (38). The gene corresponding to *dr0178* in ISDra2F is *dr1651* (*tnpB*) (39, 40). Thus, we tested, by RT-qPCR, the effect of DdrI deficiency on heat shock induction of both *dr1651* (*tnpB*)

FIG 5 DdrI is important for transcriptional heat shock induction of several genes. GY9613 (wild type, dark-gray bars) and GY13630 ($\Delta ddrI$ mutant, light-gray bars) strains were grown to A_{650} of 0.5 at 30°C and transferred or not transferred to 48°C for 5 min before extraction of total RNA. Expression values at 48°C relative to 30°C were determined and were normalized to the expression values of reference genes *tuf1* (*dr0309*) and *recN* (*dr1477*), as described in Materials and Methods. Error bars represent the standard deviations of the results from triplicate qRT-PCRs from three or four biological replicates.

and *dr1652* (*tnpA*) genes belonging to the same operon and encoding a negative regulator of ISDra2 transposition and the ISDra2 transposase, respectively. We found that both ISDra2 genes were highly induced by heat shock treatment and that this induction required DdrI (Fig. 5).

TABLE 1 Main putative DdrI targets, sorted by their function in DNA metabolism or stress responses^a

Functional group and gene identified by Hua and Hua (33)	Gene identified by White et al. (36)	Function	FITBAR CRP ^b	FITBAR DdrI ^c	Heat shock ^d
Replication, recombination, and repair					
A2G07_00390	DR0001	DnaN, DNA polymerase III beta-subunit	X	X	X
A2G07_00380	—	DdrC, DNA damage response protein	X	X	
A2G07_00375	DR0004	MutT, Nudix family protein	X	X	X
A2G07_00040	DR0070	DdrB, DNA damage response protein	X	X	
A2G07_13010	DR0100	SSB, single-stranded DNA-binding protein	X	X	
—	DR0219	DdrF, DNA damage response protein			X
A2G07_11420	DR0423	DdrA, DNA damage response protein	X	X	
A2G07_05720	DR1572	UvrD ₂ , DNA helicase	X	X	X
A2G07_04095	DR1902	RecD, exodeoxyribonuclease V subunit	X		
A2G07_04045	DR1913	GyrA, DNA gyrase subunit A	X	X	X
A2G07_13525	DRA0001	ParA, chromosome-partitioning protein	X	X	
Stress response and chaperones					
A2G07_11785	DR0349	Lon2, ATP-dependent protease			X
A2G07_10695	DR0561	Periplasmic maltose-binding protein			X
A2G07_09290	DR0844	Thioredoxin domain-containing protein	X		
A2G07_08295	DR1046	ClpB, ATP-dependent protease			X
A2G07_08110	DR1082	RaiA, ribosome-associated translation inhibitor			X
A2G07_06675	DR1372	Water stress, hypersensitive response domain-containing protein	X	X	
A2G07_05840	DR1546	SodC, superoxide dismutase	X	X	
A2G07_05725	DR1571	Peptide ABC transporter	X	X	X
A2G07_03765	DR1974	Lon1, ATP-dependent protease	X		X
A2G07_01670	DR2381	Aldehyde dehydrogenase/enoyl-CoA hydratase	X	X	X
A2G07_14210	DRA0146	Catalase	X	X	
A2G07_14755	DRA0259	KatE2, catalase	X		
A2G07_13265	DRA0319	Urease beta/gamma-subunit	X	X	X
A2G07_13520	DRA0369	Hypothetical protein	X	X	X
Regulation of transcription and signal transduction mechanisms					
A2G07_09790	DR0743	DNA-binding response regulator	X	X	
A2G07_09785	DR0744	Two-component sensor histidine kinase	X		
A2G07_04605	DR1797	Transcription termination/antitermination protein NusA	X	X	
A2G07_04050	DR1912	Protein tyrosine phosphatase	X	X	
A2G07_00810	DR2556	Response regulator		X	
A2G07_00435	DR2629	PadR family transcriptional regulator	X	X	
A2G07_13430	DR0350	Histidine kinase	X		
A2G07_15105	DRB0044	Diguanylate cyclase	X	X	

^a—, not correctly annotated.^bGenes proposed by FITBAR to be regulated by DdrI using as a matrix the 166 CRP binding sites identified in the *E. coli* genome (Table S1).^cGenes proposed by FITBAR to be regulated by DdrI using as a matrix the 176 putative DdrI binding sites identified in the *D. radiodurans* genome (Table S2).^dGenes induced by heat shock treatment in a DdrI-dependent manner (Fig. 5).

Taken together, these results confirm the direct or indirect DdrI-dependent regulation of several key components of the heat shock regulon, in agreement with the thermosensitive phenotype of the $\Delta ddrI$ mutant.

DdrI is involved in completion of cell division, genome segregation, and stability of the MP1 megaplasmid in *D. radiodurans*. *D. radiodurans* cells depleted for DdrI were analyzed by fluorescence microscopy at three different growth states, exponential (A_{650} , 0.3), transitional (A_{650} , 1.5), and stationary (A_{650} , 3.5) phases. The absence of the DdrI protein in exponentially growing *D. radiodurans* cells led to 55.2% (544/986) of the cells belonging to tetrads versus 34.5% (252/730) for the R1 strain (Fig. 6 and S5). Surprisingly, in the absence of DdrI, structures containing 8 cells (two-tetrads), representing 2.4% of the exponentially growing cells (24/986), were observed,

FIG 6 DdrI is required for the regulation of cell division and/or genomic DNA segregation. (A) Fluorescence microscopy analyses of the cell shape of exponential (A_{650} 0.3), transitional (A_{650} 1.5) and stationary (A_{650} 3.5) phases cells from GY9613 (wild type) and GY13630 ($\Delta ddrI$ mutant) strains. The percentage of cells found in dyads (2 cells, blue), tetrads (4 cells, red) and two-tetrads (8 cells, green) are illustrated in the pie charts. Two-tetrads are indicated by green arrows. (B) Membranes were stained with FM4-64 (red) and nucleoids with DAPI (green). Anucleate cells are indicated by yellow arrows. n represents the total number of cells examined.

and their proportion increased dramatically at transitional- and stationary-growth phases to reach 36.7% (480/1,308) and 78.0% (1,336/1,712) of the cells, respectively. In contrast, the proportion of wild-type cells belonging to the two-tetrads was very low, these structures being undetectable during exponential growth, and only 2.6% (32/1,246) of them were present at stationary phase. Moreover, the proportion of $\Delta ddrI$ mutant cells belonging to dyads decreased rapidly during the cell cycle and dropped to 1.6% (28/1,712) at stationary phase (Fig. 6 and S5). These results suggest a putative role of the DdrI protein in the completion of *D. radiodurans* cell division.

FIG 7 The absence of the DdrI protein reduces the maintenance of the MP1 megaplasmid in *D. radiodurans*. (A) Putative DdrI binding sites located upstream of genes related to DNA replication and segregation of the MP1 megaplasmid are indicated. The DdrI binding sites potentially involved in transcriptional regulation of *drB0145* gene and of the divergently transcribed *drB0001*-*drB0002* operon are indicated by brackets. (B) Aliquots of GY13673 (*ddrI*⁺ Δ *drC0017* Ω *cat*), GY13674 (Δ *ddrI* Δ *drC0017* Ω *cat*), GY13696 (*ddrI*⁺ Δ *drB0121* Ω *cat*), and GY13697 (Δ *ddrI* Δ *drB0121* Ω *cat*) cells containing either CP1 Ω *cat* (left) or MP1 Ω *cat* (right) were grown for 10 generations in TGY2X liquid medium without selection pressure (see Materials and Methods). Appropriate dilutions were then plated on both TGY agar and TGY agar plates supplemented with chloramphenicol to determine the proportions of cells harboring either MP1 Ω *cat* or CP1 Ω *cat*. The proportions of chloramphenicol-resistant cells are the averages of the results from at least three independent experiments.

Interestingly, during exponential growth, 7.6% (15/197) of Δ *ddrI* mutant cells were found to be anucleated, compared to <0.5% (<1/202) for the R1 wild-type cells. This phenotype was even more pronounced during the transitional- and stationary-growth phases, where 15.4% (32/208) and 25% (53/212) of the Δ *ddrI* anucleate cells were observed, compared to <0.5% (<1/210) and 4% (8/201) for the R1 wild-type cells, respectively. These results suggest that DdrI may also be implicated in the regulation of genes involved in chromosome segregation. The *drB0145* gene encoding a putative plasmid replication initiator protein and the divergently transcribed operon encompassing the *drB0001* and *drB0002* genes annotated as encoding a ParA ATPase involved in chromosome partitioning and a protein of the ParB family, respectively, share two putative overlapping DdrI binding motifs (Fig. 7A and Tables S1 and S2). To compare the maintenance of the MP1 megaplasmid harboring these genes in wild-type and Δ *ddrI* mutant cells, we introduced a cassette expressing resistance to chloramphenicol into the *drB0121* gene located on the MP1 replicon and previously shown to be inactivated without any effect on growth (13). We found that the proportion of chloramphenicol-resistant (Cm^r) cells containing the MP1 megaplasmid diminished to 24% after 10 generations in the Δ *ddrI* mutant when cells are grown without selection pressure, whereas the megaplasmid was stably maintained in wild-type cells (Fig. 7B). In contrast, when the Cm^r cassette was introduced into the *drC0017* gene of the CP1 plasmid, the proportion of Cm^r cells after 10 generations did not decrease (Fig. 7B). These results indicate that DdrI participates in the maintenance of the MP1 megaplasmid.

FIG 8 Deletion of *ddrI* dramatically reduces p11559 plasmid transformation frequency in *D. radiodurans*. (A and B) GY9613 (wild type) and GY13630 ($\Delta ddrI$ mutant) competent cells were transformed with 300 ng of genomic DNA from the GY11733 strain (conferring rifampin resistance) (A) or 300 ng of p11559 plasmid DNA (conferring spectinomycin resistance) (B). Appropriate dilutions of the transformation mixtures were plated on TGY agar plates to determine the number of viable cells, and on TGY agar plates supplemented with rifampin or spectinomycin to determine the number of transformants. The transformation frequencies were calculated as the number of transformants divided by the number of viable cells in the transformation mixture. The values are the averages of the results from at least three independent experiments. (C) Aliquots of GY14142 (*ddrI*⁺), GY13670 ($\Delta ddrI$) and GY13644 ($\Delta ddrI$ *crp*⁺) cells harboring the p11559 plasmid were grown for 10 generations in TGY2X liquid medium without selection pressure. Then, appropriate dilutions were spread on both TGY agar and TGY agar plates supplemented with spectinomycin to calculate the proportions of spectinomycin-resistant cells harboring p11559. The values are the averages of the results from at least three independent experiments.

All the morphological defects observed in cells devoid of DdrI were increased in stationary phase. Thus, we analyzed by Western blotting the expression of the DdrI-FLAG protein in exponential, transitional, and stationary phases of growth, and we showed that the expression of DdrI was highly induced at stationary phase in a DrRRA-dependent manner (Fig. S6), suggesting an important role of DdrI at this growth phase.

DdrI is required for plasmid p11559 maintenance in *D. radiodurans*. During our strain constructions, we observed that transformation was less efficient in $\Delta ddrI$ mutant bacteria. Thus, we analyzed the effect of the absence of DdrI protein on DNA transformation in *D. radiodurans*. As shown in Fig. 8A, the deletion of *ddrI* moderately decreased chromosomal DNA transformation frequency (approximately 5-fold) but dramatically affected the plasmid DNA transformation efficiency. Indeed, the transformation frequency of the p11559 plasmid (41), conferring spectinomycin resistance, dropped 20,000-fold in cells devoid of DdrI compared to wild-type cells (Fig. 8B). These observations suggest the requirement of DdrI for efficient overall DNA transformation or for replication and/or maintenance of plasmid p11559 in *D. radiodurans*.

During the natural DNA transformation process in *D. radiodurans*, only one strand of incoming DNA is transported into the cytoplasm. Bouthier de la Tour et al. (7) previously demonstrated that the reconstitution of an intact plasmid by single-strand annealing (SSA) requires the DdrB protein, likely through its single-strand annealing activity. To

test whether DdrI regulates *ddrB* gene expression, we analyzed, by Western blotting, the cellular level of DdrB-FLAG-tagged protein expressed in R1 strain and in the $\Delta ddrI$ mutant. As shown in Fig. S7A, the absence of DdrI protein results in a significant increase in the DdrB-FLAG protein level. This result suggests that DdrI negatively regulates the expression of *ddrB*, in accordance with the location of its putative binding site downstream of the *ddrB* promoter and overlapping the RDRM recognized by the DdrO repressor (Fig. S7B) (18). Therefore, the strong reduction in plasmid DNA transformation efficiency in the $\Delta ddrI$ mutant may be related to a defect in stable plasmid maintenance rather than impairment of the SSA process. To test this hypothesis, we analyzed the maintenance of the p11559 plasmid in the $\Delta ddrI$ mutant (p11559) and R1 (p11559) strains grown for 10 generations without selection pressure. As shown in Fig. 8C, the spectinomycin-resistant (*Spc^r*) population of cells harboring plasmid p11559 diminished from 89.7% in the wild-type strain to approximately 10% in the $\Delta ddrI$ mutant after 10 generations. Interestingly, the induced plasmid-based production of *E. coli* CRP in the $\Delta ddrI$ mutant bacteria resulted in an intermediate 57% proportion of *Spc^r* cells after growth without any selection pressure for 10 generations. These results suggest that some putative DdrI target sites, located on plasmid p11559 or on the whole genome of *D. radiodurans*, may be involved in stable maintenance of plasmid p11559 and are shared with the *E. coli* CRP.

DISCUSSION

To date, approximately 100 genes have been annotated as encoding transcriptional regulators and transcription factors in the *D. radiodurans* genome, but only a few of them have been functionally studied. Cells devoid of the DdrI protein, a transcription regulator of the CRP family, display a pleiotropic phenotype including growth defects under laboratory standard conditions, sensitivity to genotoxic agents, oxidative stress (13, 26; this work), heat shock treatment, as well as defects in cell division and replicon stability.

We showed here that the expression of *E. coli* CRP (CRP_{Ec}) in a *D. radiodurans* $\Delta ddrI$ mutant partially restores growth and resistance to UVC, MMC, Paraquat, and heat shock, strongly suggesting that DdrI is a protein belonging to the CRP family, and that both the CRP_{Ec} and DdrI regulators can share the regulation of several common target genes. In agreement with this, the alignment of DNA-binding sites previously identified for several members of the CRP family revealed a consensus sequence among diverse bacterial species (see Fig. S2 in the supplemental material). These data led us to perform an *in silico* prediction of the potential DdrI targets in the *D. radiodurans* genome, using the FITBAR Web tool (31). A pseudopalindromic 5'-TGTGA(N₆)TCACA-3' consensus sequence was extrapolated from the 115 DdrI candidate target genes identified (Table S2 and Fig. 3A). This motif is in agreement with binding sites of the HTH regulator family members, which bind to DNA as dimers. EMSAs showed the important role played by cAMP for DNA binding on a DNA sequence containing this site (Fig. 3). These results suggest that DdrI likely undergoes a conformational change upon cAMP binding necessary for its DNA binding, as previously shown for *E. coli* CRP (42–44) and other CRP family members (45, 46), contrary to the SdrP factor of *Thermus thermophilus*, which acts independently of any added effector molecule (47). Although adenylate cyclases lack a specific signature, making them difficult to predict (48), DRA0006 was proposed to contain domains that are similar to those of adenylate cyclases (26). Moreover, *D. radiodurans* encodes several putative cAMP-binding proteins, and its cAMP intracellular concentration was shown to increase following gamma irradiation (49), suggesting a potential function of cAMP as a second messenger in *D. radiodurans*.

The 115 *in silico*-predicted DdrI target genes are involved in various cellular processes, including replication and DNA repair, response to oxidative stress and heat shock treatment, and signal transduction (Table 1), and several genes are similar to known CRP_{Ec} targets (particularly those involved in central carbon metabolism, transport of various metabolites, cell wall biogenesis, and lipid metabolism) (Table S2).

Surprisingly, several putative DdrI binding sites are distant from the beginning of the coding sequences (>250 bp). However, we showed that *dr2381* is strongly upregulated (56-fold) after heat shock treatment in a DdrI-dependent manner (Fig. 5), in spite of the distance of the putative DdrI binding site (604 bp) (Tables S1 and S2).

To better understand the sensitivity of the Δ *ddrI* mutant to heat shock treatment, we tested, by RT-qPCR, the expression levels of heat shock responsive genes. Here, we showed that *ddrI* was induced by heat shock treatment and that the expression of the DdrI protein was under the control of DrRRA, even under normal growth conditions. This control is direct since the DrRRA regulator binds to the promoter region of the *ddrI* gene (25). We thus propose that the heat shock stimulus activates a two-component system consisting of a not-yet-identified histidine kinase and its cognate response regulator DrRRA (25), which in turn induces the transcription of *ddrI*. After 5 min at 48°C, all the genes tested were upregulated in a DdrI-dependent manner except the *drA0199* gene, encoding a nodulation-related protein, and *drA0143*, encoding a 3-hydroxyacyl-coenzyme A (3-hydroxyacyl-CoA) dehydrogenase (Fig. 5). A direct DdrI control is expected for the genes exhibiting a predicted DdrI binding site in their upstream regulatory region (Tables S1 and S2), in contrast to the other genes tested, for which DdrI may act indirectly. However, the *in silico* prediction may not identify targets with less homology to the consensus sequence, and additional targets may exist *in vivo*, as suggested by the binding of DdrI shown by EMSAs, to sequences located upstream of different genes not found in Tables S1 and S2 (26). Among these sequences, some are located upstream of *dr0997* (*ddrI*), *dr1689* (*glgC*), *dr1819* (*uvrE*), *dr1921* (*sbcD*), and *dr2220* (*terB*) but contain 7-bp spacers and not 6 bp for CRP_{Ec} and the CRP from other bacterial species (Fig. S2). However, it is important to note that CRP and related regulators are able to recognize and bind to relatively divergent sites, which are reflected in the very low informational content of their consensus sequence, and in *E. coli*, genomic SELEX experiments also identified new CRP binding sites not predicted *in silico* (27).

We also showed that DdrI is involved in the upregulation of *tnpA* and *tnpB*, the two genes of the *ISDra2F* transposon, in response to heat shock treatment, presumably indirectly, since no predicted DdrI target sequence was found in *ISDra2*. The subterminal stem-loop necessary for *ISDra2* transposition (40) has been shown to act as a transcription terminator, avoiding any transcription readthrough in the IS. We previously showed that *ISDra2F* transposition is increased after exposure to ionizing radiation (37, 40) but did not result from an increase in TnpA expression during the 180-min postirradiation incubation (40). The consequences of the increased expression of the TnpA transposase and the TnpB negative regulator of transposition upon heat shock treatment, in the presence of DdrI, and to a lower extent in the absence of DdrI (Fig. 5), require further investigations.

We further showed that DdrI was induced in the stationary phase of growth in a DrRRA-dependent manner and that DdrI deficiency leads to a striking increase in two-tetrad-forming cell subpopulations during the cell cycle, compared to the wild-type strain, indicating that DdrI is important for the accurate completion of cell division, particularly during the stationary phase. Chou and Tan (50) previously showed that the addition of NaCl could induce 8-, 16-, and 32-cell unit formation and attributed this phenomenon to the failure of cells to septate after cell wall formation. Once a tetrad forms during active growth, adjacent pairs seem to separate by delamination. Thus, the DdrI protein might regulate some genes involved in this delamination process. DdrI deficiency also leads to a striking increase in anucleate cells during the cell cycle, compared to the wild-type strain, indicating that DdrI is important for genome segregation. The Par system has been shown in *Caulobacter crescentus* and *Bacillus subtilis* to be required for active chromosome segregation by assisting segregation of the replication origin (for a review, see references 51 and 52). In *D. radiodurans*, each genome replicon, except the CP1 plasmid, has its own Par proteins encoded by *parAB* operons. The deletion of *parB1* from chromosome I resulted in a significantly higher level of anucleation than that measured in the wild type (53), a phenotype similar to that observed for the Δ *ddrI* mutant. In addition, functional characterization of the partition-

ing proteins of *D. radiodurans* suggested the possibility of a role of ParA₂, encoded on chromosome II, in the regulation of cell division by nucleoid compaction at the vicinity of septum growth (54), and it was proposed that interaction of Par proteins with divisome proteins may play important roles in genome segregation (55). A predicted Ddrl target sequence was found 545 bp upstream of the promoter region of the *drA0001-drA0002* operon encoding the ParA₂ and ParB₂ chromosome-partitioning proteins (Tables S1 and S2), suggesting that the high anucleation levels observed in the Δ *ddrl* mutant may be partly related to a defect in completion of chromosome segregation due to downregulation of the *parA₂B₂* operon. In *D. radiodurans* lacking topoisomerase IV (Topo IV), its DNA gyrase (encoded by *gyrA* and *gyrB*) is expected to ensure the Topo IV-mediated functions in chromosome segregation, namely, the decatenase function required for partitioning the daughter chromosomes before cell division (56). The *gyrA* gene was shown to contain a putative Ddrl binding site 112 bp upstream of its coding region (Tables S1 and S2) and to be upregulated in a Ddrl-dependent manner upon heat shock treatment (Fig. 5). Therefore, the lack of Ddrl may also impair GyrA expression and thus accurate chromosome segregation.

We demonstrated that Ddrl is required for stable maintenance of the natural MP1 megaplasmid. Two putative Ddrl binding sites were identified upstream of the promoter region of *drB0145* (encoding a plasmid replication initiator protein) and the divergently transcribed operon encompassing the *drB0001* and *drB0002* genes encoding ParA₃ and ParB₃, respectively (Fig. 7). Therefore, the involvement of Ddrl in the stable maintenance of MP1 may be mediated through its positive control of both *drB0145* and *parA3B3* operon expression, as suggested above for the *parA2B2* operon expressed by chromosome II. Unfortunately, as chromosome II expresses essential genes, it was not possible to analyze the effect of Ddrl deficiency on its maintenance. In addition, Ddrl was shown to be required for stable maintenance of the p11559 plasmid (Fig. 8) but not for those of the natural CP1 plasmid. The reduction in p11559 stability may explain the partial complementation of the Δ *ddrl* mutant by CRP_{Ec} expressed from this plasmid, even if the experiments were performed under constant selection pressure. The p11559 plasmid (41) contains the basic replicon of the deinococcal SARK plasmid pUE10 (57), which is different from those of CP1, and both plasmids likely employ different mechanisms for their own replication, explaining their different stabilities in the absence of Ddrl.

In conclusion, we propose that Ddrl may regulate, directly or indirectly, a large variety of genes involved in DNA metabolism, DNA repair, response to oxidative stress or to heat shock, regulation of transcription, and signal transduction (Table 1), as well as many genes involved in ribosome structure and translation and in various metabolic pathways (Tables S1 and S2). We also showed that this regulator may be involved in cell division, chromosome segregation, and plasmid stability. The *ddrl* gene transcription levels are under the control of the DrRRA response regulator, but Ddrl itself putatively regulates the expression of other regulators (Tables 1, S1, and S2). Our results indicate that Ddrl is a major regulator in *D. radiodurans*, and further studies will better explain the cascade of regulatory pathways associated with the various phenotypes observed for a Δ *ddrl* mutant.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains used are listed in Table 2. *E. coli* strain DH5 α was the general cloning host, and strain SCS110 was used to propagate plasmids prior to their introduction into *D. radiodurans* via transformation (58). All *D. radiodurans* strains were derivatives of strain R1 ATCC 13939 and were grown at 30°C in TGY2X liquid medium or tryptone-glucose-yeast extract (TGY) plates (15). *E. coli* strains were grown at 37°C in lysogeny broth (LB). Media were supplemented with the appropriate antibiotics used at the following concentrations: 75 μ g/ml spectinomycin, 100 μ g/ml hygromycin, 6 μ g/ml kanamycin, 3.5 μ g/ml chloramphenicol, and 25 μ g/ml rifampin for *D. radiodurans* and 50 μ g/ml spectinomycin, 25 μ g/ml kanamycin, and 35 μ g/ml chloramphenicol for *E. coli*. The expression of *E. coli* CRP in *D. radiodurans* or His₆::Ddrl in Rosetta (DE3) was induced by adding 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) to the medium. The transformation of *D. radiodurans* with genomic DNA, PCR products, or plasmid DNA was performed as previously described (15).

TABLE 2 Bacterial strains and plasmids used

Strain or plasmid	Genotype or other relevant characteristics	Source or reference
Strains		
<i>E. coli</i>		
MG1655	K-12 F ⁻ λ^{-} <i>ilvG rfb-50 rph-1</i>	Laboratory stock
DH5 α	<i>supE44 ΔlacU(ϕ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Laboratory stock
SCS110	<i>endA dam dcm supE44 Δ(lac-proAB) (F'<i>traD36 proAB lacI^q</i>ΔM15)</i>	Laboratory stock
Rosetta (DE3)	F ⁻ <i>ompT hsdS20 gal dcm lacY1</i> (DE3)/pRARE ⁶ (Cm ^r)	
<i>D. radiodurans</i>		
GY9613	ATCC 13939, R1	64
GY11733	Rif ^r (<i>rpoB</i> Δ 9 Rif ^r) ^a	41
GY12830	<i>ddrB::spa-cat</i>	7
GY13109	Δ (ISDra2F) Ω tetA	40
GY13630	Δ ddrI Ω hph	This work
GY13641	Δ ddrI Ω hph Δ (ISDra2F) Ω tetA	This work
GY13643	Δ ddrI Ω hph Δ (ISDra2F) Ω tetA Ω P _{ddrI} ::ddrI-cat	This work
GY13644	Δ ddrI Ω hph/p14750	This work
GY13645	Δ ddrI Ω P _{ddrI} ::his6::ddrI-kan	This work
GY13653	Δ ddrI Ω hph <i>ddrB::spa-cat</i>	This work
GY13663	<i>ddrI::flag-cat</i>	This work
GY13670	Δ ddrI Ω hph/p11559	This work
GY13673	Δ drC0017 Ω cat	This work
GY13674	Δ ddrI Ω hph Δ drC0017 Ω cat	This work
GY13679	Δ drRRA Ω hph <i>ddrI::flag-cat</i>	This work
GY13681	Δ drRRA Ω hph	This work
GY13696	Δ drB0121 Ω cat	This work
GY13697	Δ ddrI Ω hph Δ drB0121 Ω cat	This work
GY14142	R1/p11559	This work
Plasmids		
p11086	Source of P _{out} ::kan cassette	Laboratory stock
p11559	Expression vector; P _{spac} -term116, P _{tufA} ::lacI, Spc ^r in <i>E. coli</i> and <i>D. radiodurans</i>	41
p12625	Source of P _{kat} ::hph cassette	Laboratory stock
p12723	Source of flag-cat cassette	This work
p14738	pET28a; P _{T7lac} ::His ₆ ::ddrI-termT7	This work
p14750	p11559; P _{spac} ::crp-term116	This work
pPS6	Source of P _{tufI} ::cat cassette	65
pET28a	pET expression system, P _{T7lac} N-terminal His ₆ tag	Novagen

^aRif^r, rifampin resistant.

Construction of *D. radiodurans* mutant and tester strains. GY13630 Δ ddrI, GY13681 Δ drRRA, GY13673 Δ drC0017, and GY13696 Δ drB0121 mutant strains were constructed by replacement of the corresponding locus with the appropriate antibiotic resistance cassette using the tripartite ligation method (41). The GY13674 Δ drC0017 Δ ddrI and GY13697 Δ drB0121 Δ ddrI double-mutant strains were constructed by transforming the Δ drC0017 Ω cat and Δ drB0121 Ω cat single mutants, respectively, with genomic DNA from the GY13630 Δ ddrI Ω hph strain. The Δ ddrI mutant, expressing an ectopic *ddrI* gene under the control of its own promoter, was constructed by replacement of the *tetA* gene in the GY13641 Δ ddrI Ω hph Δ ISDra2F Ω tetA strain, with a fragment encompassing the P_{ddrI}-*ddrI* and the Cm^r cassette. The *D. radiodurans* strain GY13663, expressing the DdrI protein fused to a FLAG tag at the C terminus (DdrI-FLAG), was constructed using the tripartite ligation method (41). The Δ ddrI mutant strain GY13653 expressing the DdrB-SPA protein and the Δ drRRA mutant strain GY13679 expressing the DdrI-FLAG protein were obtained by transforming the GY13630 Δ ddrI or the GY13681 Δ drRRA strain with genomic DNA from GY12830 and GY13663 expressing the appropriate tagged proteins, respectively. To assess the functionality of the His₆::DdrI-tagged protein, GY13645 expressing the His₆::DdrI-tagged protein was constructed by replacement of the *ddrI* locus with a fragment encompassing the *ddrI* coding sequence fused to a His₆ tag at the N terminus and the kanamycin resistance (Kan^r) cassette. The purity of the strains was systematically confirmed by diagnostic PCR and sequencing (see Fig. S8A and B for the genetic structure and the purity of the Δ ddrI mutant and Fig. S8C and D for those of Δ drC0017 Ω cat, Δ drC0017 Ω cat Δ ddrI, Δ drB0121 Ω cat, and Δ drB0121 Ω cat Δ ddrI mutants). The oligonucleotides used for strain construction and diagnostic PCR will be provided on request.

DNA manipulations. Plasmid DNA was extracted from *E. coli* using the NucleoSpin plasmid miniprep kit (Macherey-Nagel). *D. radiodurans* chromosomal DNA was isolated as described previously (59). PCRs were carried out with Phusion DNA polymerase (Thermo Scientific) to amplify fragments subsequently used for cloning, or with GoTaq Flexi DNA polymerase (Promega) for diagnostic PCR. PCR products were purified using the PCR cleanup kit (Macherey-Nagel).

Plasmid construction. The *crp* coding sequence was amplified by PCR using genomic DNA from *E. coli* strain MG1655 as the template. The PCR fragment was digested by NdeI and XhoI and ligated into the expression vector p11559, resulting in plasmid p14750 expressing *crp* under the control of the P_{spac} promoter inducible by IPTG.

The *ddrI* coding sequence was amplified by PCR using genomic DNA of the wild-type GY9613 strain as the template. The PCR fragment was digested by *NdeI* and *XhoI* and ligated into the expression vector pET-28a (Novagen). The resulting p14738 plasmid expresses the DdrI protein fused to a His₆ tag at the N terminus under the control of the T7 *lac* promoter inducible by IPTG.

His₆::DdrI-tagged protein purification. The p14738 plasmid, expressing the His₆::DdrI-tagged protein under the control of the T7 *lac* promoter, was used to transform *E. coli* Rosetta (DE3) cells. Transformants were grown in LB medium supplemented with the appropriate antibiotics at 37°C to an A₆₀₀ of 0.45, and the expression of the His₆::DdrI protein was induced by IPTG at a final concentration of 1 mM for 3 h. Cells were harvested (3,500 × *g*, 10 min, 4°C), resuspended in lysis buffer (20 mM Tris-HCl [pH 7.8], 800 mM NaCl, 5% glycerol, 10 mM imidazole, 0.03% Triton X-100) at a rate of 0.1 ml of lysis buffer for A₆₀₀ of 1, and disrupted using an ultrasonic cell disrupter (350 W, 3 × 2 min). The cleared lysate was recovered by centrifugation (13,000 × *g*, 1 h, 4°C) and mixed gently overnight at 4°C with 1 ml nickel-nitrilotriacetic acid (Ni-NTA) slurry equilibrated with lysis buffer (0.5 ml bed volume; Qiagen). The lysate–Ni-NTA mixture was then washed by 5 elution steps with 5 bed volumes of elution buffer (20 mM Tris-HCl [pH 7.8], 200 mM NaCl, 5% glycerol, and 0.03% Triton X-100) containing increasing concentrations of imidazole (40 mM, 60 mM, 100 mM, 150 mM, and 200 mM). The purity of the DdrI protein was verified by SDS-gel electrophoresis, and the protein fraction, eluted in the presence of 150 mM imidazole, was dialyzed on a PD10 column (GE Healthcare) with equilibration buffer (50 mM Tris-HCl [pH 7.8], 100 mM NaCl, and 30% glycerol), according to the manufacturer's protocol. Dithiothreitol (DTT) and EDTA were added at a final concentration of 1 mM, and the His₆::DdrI protein was stored at −80°C.

EMSA. A single-stranded Cy5-labeled oligonucleotide (10 μM), 5'-Cy5-CATCAGAAAATGTGTCTGGTGAAGCAGTTTGTGAAAAAAGCTCACAGCCCTGGGATAGAAAACAGCACTATCACAGCAT-3' (80 nt), containing a putative DdrI binding site (bold letters) at position 33, was incubated with the complementary single-stranded 80-nt oligonucleotide (10 μM) in 10 μl of annealing buffer (40 mM Tris-HCl [pH 7.8], 50 mM NaCl, 20 mM MgCl₂). The mixture was heated at 90°C for 10 min and cooled for 3 h at room temperature. Thirty femtomoles from the resulting double-stranded substrate was incubated for 20 min at 30°C with recombinant protein His₆::DdrI (0.5 or 1 μM) in 15 μl of binding buffer (10 mM Tris HCl [pH 7.8], 20 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1 mg/ml bovine serum albumin [BSA], 10% glycerol) in the presence or absence of 200 μM cAMP and poly(dI-dC) (13 or 33 μg/ml). The samples were loaded onto an 8% polyacrylamide (37.5/1 [wt/wt] acrylamide-bisacrylamide) gel in 0.25× Tris-borate-EDTA. After migration (20 V/cm, 3 h, 4°C), bands were visualized using a phosphorimager (Typhoon Trio imager; GE Healthcare).

Cross-linking of DdrI. Three micrograms of the DdrI protein was preincubated with 0 or 200 μM cAMP in 10 mM sodium phosphate buffer (pH 7) at 30°C for 20 min. Then, 0.05% glutaraldehyde was added or not added and the samples were incubated at 30°C for 30 min in a final volume of 20 μl. After incubation, 5 μl of 5× Laemmli buffer (312 mM Tris-HCl [pH 6.8], 50% glycerol, 250 mM DTT, 0.1% bromophenol blue) was added, and the samples were loaded onto a 15% SDS-polyacrylamide gel. After migration (14 V/cm, 2 h), the gel was stained using Coomassie blue.

Purification of total RNA and cDNA synthesis. Bacteria were grown to an A₆₅₀ of 0.5 at 30°C and transferred or not transferred to 48°C for 5 min. Then, bacteria (10 ml) were harvested and centrifuged (3,500 × *g*, 10 min at 4°C). The pellets were stored at −80°C for 2 h and total RNA were isolated using the FastRNA Pro Blue kit (MP Biomedicals, Irvine, CA) and the FastPrep-24 instrument, according to the manufacturer's protocols. The RNA concentration was measured using the DeNovix DS-11 spectrophotometer (SelectScience). One microgram of total RNA was then treated with DNase I amplification grade (Invitrogen), according to the manufacturer's instructions, except that 1 μl of DNase I was added every 5 min during the 15-min incubation. RNA purity and integrity were analyzed by 1.4% agarose gel electrophoresis. Subsequently, 250 ng of RNA was then transcribed into cDNA by reverse transcriptase using the Maxima first-strand cDNA synthesis kit (Thermo Scientific), according to the manufacturer's instructions, in a final volume of 200 μl. The reverse transcriptase negative control, containing all reagents for the reverse transcription reaction except the Maxima enzyme mix, was carried out to assess, by quantitative PCR (qPCR) experiments, genomic DNA contamination of the RNA samples.

qPCR analyses. qPCR assays were performed using 2 μl of cDNA as the template and the iTaq Universal SYBR Green Supermix (Bio-Rad), according to the manufacturer's instructions. For all genes analyzed, the primer pairs were designed using the IDT PrimerQuest tool (Integrated DNA Technologies) and will be provided upon request. The thermal cycling protocol used on the Bio-Rad CFX 96 real-time PCR detection system was 1st step, 95°C for 2 min; 2nd step, 39 cycles of 95°C for 5 s and 60°C for 30 s; and 3rd step, melt curve from 60°C to 95°C, with an increment of 0.5°C for 5 s. The amplification specificities were verified by examining the melt curve profile, and the amplification efficiencies were assayed by generating standard curves (using serial dilutions of the cDNA). All genes tested exhibited the recommended amplification efficiency of 90 to 110% and R² values of >0.98. qPCR assays performed using the reverse transcriptase negative control as the template confirmed the absence of genomic DNA contamination (data not shown). Four candidate reference genes, *tuf1* (*dr0309*), *recN* (*dr1477*), *amyE* (*dr1472*), and *hpi* (*dr2508*), were evaluated for their expression stability on all samples by geNorm analysis (60) (qbase+). The *tuf1* (*dr0309*) and *recN* (*dr1477*) genes were validated as reference genes. Expression values in wild-type and Δ*ddrI* mutant (GY13630) bacteria after 5 min at 48°C relative to 30°C were determined for candidate genes and were normalized to the expression values of *tuf1* and *recN* using the REST 2009 software (<http://www.gene-quantification.de/rest-2009.html>). RT-qPCRs were performed in triplicate from three or four biological replicates.

Western blot analysis of tagged proteins DdrI-FLAG or DdrB-SPA. Exponential-growth cultures (A₆₅₀ 0.45) of bacteria expressing the DdrI-FLAG protein were grown at 30°C and then transferred to

48°C, 20-ml aliquots taken at different times (0, 0.5, or 1 h) were centrifuged ($3,500 \times g$, 10 min, 4°C). The pellets were resuspended in 150 μ l of $1 \times$ SSC buffer ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and protein extracts were prepared as previously described (61). Protein concentrations were determined by Bradford assay (Bio-Rad protein assay dye reagent), and 10 μ g of proteins was loaded on 12% SDS-PAGE gels (stain-free precast gels; Bio-Rad) and transferred onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare). The Western blot analyses were performed as previously described (18) with a 1:5,000 dilution of anti-FLAG rabbit primary antibodies (Thermo Scientific). For an analysis of expression of DdrB-SPA protein, 20 ml of exponential-growth cultures (A_{650} , 0.5) of GY12830 and GY13653 expressing the DdrB-SPA protein was prepared, and 5 μ g of proteins was analyzed by Western blot experiments with a 1:5,000 dilution of anti-FLAG rabbit primary antibodies (Thermo Scientific), as described above.

Sensitivity assays to DNA-damaging agents UVC and mitomycin C. Bacteria were grown in TGY2X liquid medium to an A_{650} of 1 and then serially diluted in TGY2X and spotted on TGY agar plates supplemented or not supplemented with mitomycin C (40 ng/ml), exposed or not exposed, to UVC rays (600 J/m²) at a dose rate of 3.5 J/m²/s. TGY agar plates were supplemented with spectinomycin and 1 mM IPTG for strains harboring the p14750 plasmid to induce *E. coli* *crp* gene expression.

Sensitivity assay to oxidative stress generated by Paraquat. *D. radiodurans* strains were analyzed for their sensitivity to Paraquat-methyl viologen (Sigma-Aldrich) by using the disc inhibition assay, as follows. Exponential-phase cultures (1.5 ml; A_{650} , 0.5) were spread onto TGY plates (supplemented with spectinomycin and 1 mM IPTG for strain GY13644). Sterilized 6-mm-diameter filter paper discs (Dominique Dutscher) were placed on the agar surface. Then, 10 μ l of various concentrations (4, 6, 8, and 10 mM) of Paraquat (freshly diluted in sterile H₂O) was spotted onto each disc. After incubation at 30°C for 2 days, the diameters of the growth inhibition zones were measured.

Sensitivity to heat shock. Overnight cultures of bacteria were grown at 30°C, serially diluted in TGY2X, spotted on TGY agar plates, and then incubated at 30°C, 37°C, or 39°C for 3 to 5 days.

Fluorescence microscopy. Cells in exponential-growth (A_{650} , 0.3), transitional-growth (A_{650} , 1.5), and stationary-growth (A_{650} , 3.5) phases were fixed by adding toluene (3% final concentration) and kept at 4°C overnight. DNA and membranes were stained by incubation with 2 μ g/ml 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) and 6 μ g/ml FM4-64 (Life Technologies) for 5 min at room temperature. One microliter of the cell suspension (in 10 mM MgSO₄) was then immobilized onto 1% agarose-coated slides and observed by fluorescence microscopy on a wide-field Leica DM RXA microscope. Images were captured with a 5-MHz Micromax 1300Y charge-coupled-device (CCD) camera (Roper Instruments) with the appropriate filters and analyzed with the MetaMorph and ImageJ softwares.

In silico prediction of putative DdrI targets. The *in silico* prediction of potential DdrI targets in the *D. radiodurans* genome was performed using the Fast Investigation Tool for Bacterial and Archaeal Regulons (FITBAR) Web tool dedicated to statistically robust predictions of prokaryotic regulons (31). This analysis was performed on the sequenced *D. radiodurans* genome (33) by using the 166 binding sites recognized by the CRP of *E. coli* (27) as the matrix (see Table S1 in the supplemental material). A total of 478 putative DdrI binding sites were found in the *D. radiodurans* genome and sites located in intergenic regions at distances of <650 bp were listed (Table S1). The genome of *D. radiodurans* has a GC content of 67%, while that of *E. coli* is 50.8%. Thus, a matrix based on the set of 176 sequences among the potential DdrI intergenic targets was used in a second prediction to take this GC content discrepancy between *D. radiodurans* and *E. coli* into account and to increase the informational content of the consensus sequence; 269 sites were found in the *D. radiodurans* genome, with a majority (198 sites) located in intergenic regions, and sites located in intergenic regions at distances <650 bp were listed (Table S2). Position-specific scoring matrices (PSSM) were generated from the 166 CRP *E. coli* or the 176 putative DdrI binding sites (listed in Table S1) using log-odds PSSM (62). Chromosome scanning was concentrated on the intergenic regions, on both DNA strands, and false positives in the prediction were discerned by using compound importance sampling (CIS; described in reference 63) as a *P* value calculation algorithm. The list of putative DdrI binding sites (Tables S1 and S2) was sorted by position, and only predicted sites located from bp −650 to +3 of the downstream coding sequence were conserved.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00129-18>.

SUPPLEMENTAL FILE 1, PDF file, 9.6 MB.

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