Molecular Microbiology (2005) **55**(5), 1579–1590 doi:10.1111/j.1365-2958.2005.04494.x

HspR is a global negative regulator of heat shock gene expression in *Deinococcus radiodurans*

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Summary

The HspR protein functions as a negative regulator of chaperone and protease gene expression in a diversity of bacteria. Here we have identified, cloned and deleted the *Deinococcus radiodurans* HspR homologue, DR0934. Δ*hspR* mutants exhibit moderate growth defects when shifted to mild heat shock temperatures, but are severely impaired for survival at 48°C. Using quantitative reverse transcription polymerase chain reaction and global transcriptional analysis, we have identified 14 genes that are derepressed in the absence of stress in the Δ*hspR* background, 11 of which encode predicted chaperones and proteases, including *dnaKJgrpE*, *ftsH*, *lonB*, *hsp20* and *clpB*. Promoter mapping indicated that the transcription of these genes initiates from a promoter bearing a σ^{70}-type consensus, and that putative HspR binding sites (HAIR) were present in the 5′-untranslated regions. Electrophoretic mobility shift assays indicated that HspR binds to these promoters at the HAIR site *in vitro*. These results strongly suggest that DR0934 encodes the HspR-like global negative regulator of *D. radiodurans* that directly represses chaperone and protease gene expression by binding to the HAIR site in close proximity to promoter regions.

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Introduction

The heat shock response is well conserved throughout evolution. Misfolded, aggregated and partially degraded proteins accumulate upon temperature elevation, and in response, cells transiently increase the production of protective factors called heat shock proteins that function as proteases and chaperones in alleviating the adverse effects of aberrant proteins (reviewed in Gross, 1996; Wickner *et al*., 1999). The regulation of heat shock gene induction is complex and diverse among the prokaryotes, and involves positive and negative regulatory mechanisms. In *Escherichia coli*, σ^{32}-associated RNA polymerase triggers the induction of more than 30 genes encoding heat shock protective factors in response to elevated temperature (Yura *et al*., 2000). In addition, at extremely high temperatures, the extracytoplasmic function (ECF) sigma factor σ^{E} governs the transcription of *rpoH* as well as periplasmic stress protective genes (Raina *et al*., 1995; Rouviére *et al*., 1995; Dartigalongue *et al*., 2001). Similarly, *Bacillus subtilis* σ^{B} governs the heat shock transcription of more than 100 genes in addition to general stress protective genes (Helmann *et al*., 2001; Petersohn *et al*., 2001; Price *et al*., 2001).

Proteases add an additional level of regulation to the *E. coli* heat shock regulatory system. For example, AAA-type metalloproteases FtsH and Lon act in concert with the DnaKJgrpE chaperone machinery to control stability and activity of σ^{32} at the post-translational level, allowing for a more rapid response to elevated temperature (Yura *et al*., 2000; Jenal and Hengge-Aronis, 2003). *Caulobacter crescentus* FtsH also plays a similar role in the regulation of σ^{32} in response to the stress of high salt, heat shock and antibiotics (Fischer *et al*., 2002). Recent evidence suggests that the *B. subtilis* FtsH protease may also play a role in the regulated proteolysis of σ^{W}, as expression of σ^{W} regulon genes is upregulated in the absence of stress in *B. subtilis* cells lacking FtsH (Zellmeier *et al*., 2003). In *B. subtilis*, σ^{W} is an ECF sigma factor whose regulon is induced in response to alkaline shock (Wiegert *et al*., 2001), mutations in anti-microbial biosynthesis genes and sugar isomerases (Turner and Helmann, 2000), and σ^{W} mutants are unable to grow in the presence of cell wall biosynthesis inhibitors (Huang *et al*., 1999).

In contrast to the positive heat shock regulatory mechanisms, negative regulatory systems are more wide-

spread in the prokaryotes, typically involving a repressor protein binding to *cis*-encoded regulatory sites in the 5′-untranslated regions (UTR) of genes coding for chaperones and proteases. For example, HrcA represses *groESL* and *dnaKJE* by binding to the CIRCE (controlling inverted repeat of chaperone expression) element in many Gram-positive bacteria, including *B. subtilis* (Narberhaus, 1999). In the *Streptomycetes* and *Mycobacteria*, however, an alternative protein, HspR, represses the *dnaK* operon in addition to *clpB*, *lon* and its own gene by binding at the HAIR (HspR-associated inverted repeat) site within the promoter regions (reviewed in Grandvalet *et al*., 1999; Servant and Mazodier, 2001; Stewart *et al*., 2002; Bucca *et al*., 2003). The *clp* protease genes *clpC*, *clpE* and *clpP*, encoding members of the Hsp100 protein superfamily with diverse functions (Schirmer *et al*., 1996), are controlled by the CtsR system in *B. subtilis* (Derre *et al*., 1999a,b); and *hsp18*, encoding a small heat shock protein, is governed by its own repressor protein RheA in *Streptomyces albus* (Servant *et al*., 1999). Interestingly, heat shock proteins themselves can add an additional level of regulation to these systems, with DnaK autoregulating its own promoter in cooperation with HspR in *Streptomyces coelicolor* (Bucca *et al*., 2000; 2003).

Recent evidence suggests that in many bacteria, dual negative regulatory proteins, and in some cases positive and negative regulatory proteins, simultaneously control heat shock gene expression at the same promoter, rendering these systems more complicated than originally appreciated. HrcA is most commonly involved in dual regulation, partnering with CtsR in *Staphylococcus aureus* to control *dnaK* and *groESL* operons during heat shock (Chastanet *et al*., 2003), with HspR to govern *groESL* and *hrcAdnaKgrpE* operon expression in *Helicobacter pylori* (Spohn *et al*., 2004), and with the heat shock sigma factor σ^H in *C. crescentus* and *Agrobacterium tumefaciens* to activate *groESL* operon transcription (Baldini *et al*., 1998; Nakahigashi *et al*., 1999). Recent evidence suggests that three factors can control a single operon, as is the case in *Corynebacterium glutamicum*, where ClgR, HspR and the ECF-type sigma factor σ^H work together to tightly control the *clpP1P2* operon (Engels *et al*., 2004). In addition, some sigma factors, such as the ECF-type factors σ^W and σ^X in *B. subtilis*, control partially overlapping regulons by recognizing similar but not identical promoter sequences (Huang *et al*., 1998).

Deinococcus radiodurans is a highly radioresistant, non-pathogenic bacterium that can survive an acute dose of up to 5 Mrad of γ-irradiation and up to 1000 J m^{-2} of UV irradiation without mutation (Sweet and Moseley, 1976; Battista, 1997). *D. radiodurans* contains 16S rRNA sequences divergent from that of other known bacteria, with *Thermus* as the only closely related genus (Battista, 1997). The sequenced and annotated genome of *D. radiodurans* contains four high %G+C genetic elements (White *et al*., 1999), which are present in 4–10 copies, depending on the growth phase (Battista, 1997).

Recent studies of the *D. radiodurans* heat shock response, spurred by the paucity of information about the response to stresses other than radiation, have indicated that the ECF-type sigma factor Sig1 appears to control the heat shock induction of more than 40 genes, including *groESL* and *dnaKJgrpE* operons (Schmid and Lidstrom, 2002; A. K. Schmid *et al*., submitted). However, our data also suggest that heat shock gene transcription in *D. radiodurans* may require an additional regulator, as *sig1*-deficient cells can still partially induce *hsp20* and the *dnaKJgrpE* operon transcription upon temperature upshift (A. K. Schmid *et al*., submitted). We therefore undertook the current study, in which we report the identification of an *hspR* homologue in *D. radiodurans* that encodes a global negative regulator of heat shock gene transcription. In addition, we demonstrate that HspR directly regulates the transcription of *dnaK*, *clpB*, *lon*, *hsp20* and *ftsH* by binding to the HAIR site in the 5′-UTR of these genes.

Results

Identification of D. radiodurans *HspR homologue, DR0934*

In preliminary searches, no homologues of the known heat shock negative regulators HrcA or HspR could be recognized in the annotated *D. radiodurans* genome. However, when the *D. radiodurans* genome was searched with the HspR sequences of *S. coelicolor* or *H. pylori*, six MerR-type regulators were detected, all of which showed 30–50% identity to HspR (data not shown). Using CLUSTALW, we determined that, compared with the other five HspR candidates of *D. radiodurans*, DR0934 classes most closely with the characterized HspR sequences from other organisms, exhibiting strong sequence similarity in the N-terminal helix–turn–helix motif, and showing the expected weak conservation in the C-terminal co-activator binding domain (Fig. 1). Interestingly, compared with the HspR sequences of other organisms, the DR0934 sequence contains a long insert within its coiled-coil domain, which has been shown to be important for oligomerization of HspR in *H. pylori* (Spohn *et al*., 2004). Nonetheless, DR0934 was chosen as the HspR candidate in subsequent analysis.

Mutagenesis of DR0934 *and determination of its role in heat shock protection*

To determine the physiological role of *DR0934* in the heat shock response, we constructed a *D. radiodurans* strain with an in-frame deletion of the chromosomal copy of *DR0934*, designated *ΔDR0934*, and tested its heat shock

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Sco       1 --MDGRRRNPYELTEDTPVYVISVAAQLSGLHPQTLRQYDRLGLVSPDRTAGRGRRYSAR
Sal       1 MDRDGRRRNPYELTEDTPVYVISVAAQLSGLHPQTLRQYDRLGLVSPDRTAG-GRRYSAR
Mle       1 -----MAKNPKDGESRT--FLISVAAELAGMHAQTLRTYDRLGLVSPRRTSGGGRRYSLH
DR0934    1 MLVVCSTSLMGSDAKHRPVYVISVAAELVDMHPQTLRLYERKGLIRPGRSSGKTRLYSER
Hpy       1 -----------MCDYDEPLYLISVVAKILGVHPQTLRQYEKEGLIEPSRTDGKMRLYSQR

Sco      59 DIELLRQVQQLSQDEGINLAGIKRIIELENQVAELQAR----------------------
Sal      60 DIELLRQVQQLSQNEGINLAGIKRIIELENQVTALQQR----------------------
Mle      54 DVELLRQVQHLSQDEGVNLAGIKRIIELTSQVEALQSR----------------------
DR0934   61 DIEHLREIRRLTQELGVNXAGVEEVMRLQHQLDDLQQEFEAEIERIEDELREQARPRALP
Hpy      50 DMDKIKTILRLTRDMGVNLAGVDIILRLKEKLDELDNLN---------------------

Sco      97 ------------------------------------------------------------
Sal      98 ------------------------------------------------------------
Mle      92 ------------------------------------------------------------
DR0934  121 APDAPPDPRDRPVYVISIAAELVDMHPQTLRLYERKQLIHPGRSSGKTRLYSERDIEHLR
Hpy      89 ------------------------------------------------------------

Sco      97 -----AAELAAALDGAATAMRQR----EAAVHASYRRDLVPYQEVQQTSALVVWR-----
Sal      98 -----VAELSAAVDGAAAQMRQREAQVEAQVHASYRRDLVRYEDVRHTSALVVWR-----
Mle      92 -----LQEMAEELAVLRANQRRE------------------VAVVPKSTALVVWK-----
DR0934  181 EIRRLTQELGVNLAGVEEIMRLR--HELDASRAHLEGN-VRRLQDDLSERMTTWRTLPAP
Hpy      89 ------KELQDALHKHSKNTKTP---------T------KNLNTPTNFYELILFKK----

Sco     143 -----PSRRGQSSD------
Sal     148 -----PGRPAGE--------
Mle     124 -----PRR------------
DR0934  238 ESEAGPEEDAAAADAGEDQN
Hpy         --------------------
```

Fig. 1. CLUSTALW alignment of the DR0934 amino acid sequence with those of characterized HspR from other organisms. Similar residues are shaded in grey, identical residues in black, and dashed lines indicate gaps in the alignment. Numbers to the left of each sequence denote the amino acid positions relative to the sequence start. Sequences were obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), and accession numbers are as follows: *Streptomyces coelicolor* (Sco), P40183; *Streptomyces albus* (Sal), AAB48078; *Mycobacterium leprae* (Mle), NP_302610; *Helicobacter pylori* (Hpy), NP_207815.

growth and survival compared with wild type. At the normal growth temperature of 30°C, wild-type *D. radiodurans* exhibited a typical doubling time of 2.3 ± 0.05 h, whereas Δ*DR0934* was slightly impaired in its growth with a rate of 2.7 ± 0.1 h. Similarly, after a mid-exponential phase shift to mild heat shock conditions at 40°C or 42°C, the Δ*DR0934* doubling time of 2.0 ± 0.13 h was slightly slower than the 1.68 ± 0.06 h doubling time of wild type. However, Δ*DR0934* was severely impaired for its viability when shifted to heat shock at 48°C during mid-exponential phase, losing up to seven orders of magnitude viability over the course of the 8 h experiment (Fig. 2). In contrast, wild-type cultures lost only two orders of magnitude viability (Fig. 2). Taken together, these results suggest that *DR0934* encodes an HspR-like factor that is essential for heat shock protection in *D. radiodurans*.

Expression of the heat shock genes dnaK *and* hsp20 *in the* ΔDR0934 *mutant background*

In organisms such as *S. coelicolor* and *S. albus*, HspR functions as a negative regulator of *dnaK* induction during heat shock (Bucca *et al*., 1995; Grandvalet *et al*., 1997). To test whether DR0934 performs a similar function in *D. radiodurans*, we conducted quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR) experiments to measure the heat shock expression of *dnaK* and *hsp20* in the Δ*DR0934* background. cDNA synthesized from mutant and wild-type cultures heat shocked for 5 min at 48°C or left untreated at 30°C was used as a template for real-time thermocycling reactions using SYBR green as the fluorescence chemistry (see *Experi-*

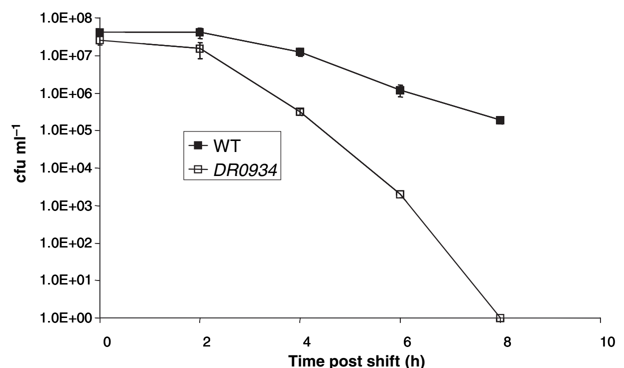

Fig. 2. DR0934 protects *D. radiodurans* against heat shock. *D. radiodurans* wild type (filled squares) and Δ*DR0934* (open squares) cultures were grown to mid-logarithmic phase at 30°C and split into two aliquots, one of which was maintained at 30°C as a control (data not shown), the other of which was shifted to heat shock at 48°C for 8 h. Every 2 h, aliquots were removed from each culture, serially diluted and spotted in 5 μl of drops in triplicate onto TGY plates to assess survival. Error bars represent ± standard deviations from the averages of three biological replicate experiments. Data point symbols obscure error bars where error is small.

mental procedures). Data were quantified relative to the *hpi* housekeeping gene, which has been previously shown to be unaltered in its expression under heat shock (Peters *et al.*, 1987; A. K. Schmid *et al.*, submitted). Expression values of 1 represent equivalent expression levels to that of *hpi*. As shown in Fig. 3A, *dnaK* transcription is present at a moderate level of about 1 unit of gene expression at 30°C in the wild-type background, and highly induced up to 18 units of relative expression upon heat shock. Furthermore, *hsp20* expression increases almost 100-fold upon temperature upshift in the wild type (Fig. 3B). In contrast, *ΔDR0934* mutant cells exhibit a high level of *dnaK* and *hsp20* gene expression at 30°C that is approximately equivalent to the wild-type level under heat shock (Fig. 3). Interestingly, upon heat shock at 48°C, the expression of *dnaK* and *hsp20* appears to be hyperinduced in the *ΔDR0934* strain compared with wild type, increasing approximately 1.5-fold for *dnaK* (Fig. 3A) and about 10-fold for *hsp20* (Fig. 3B). Taken together, these results strongly suggest that *DR0934* encodes an HspR-like negative regulator of *dnaK* and *hsp20* heat shock expression. We have therefore designated DR0934 as HspR, and will henceforth refer to it as such.

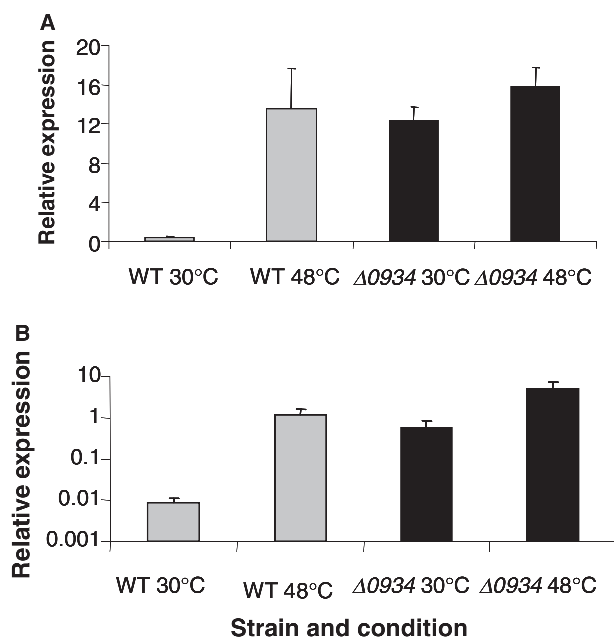

Fig. 3. Heat shock genes are overexpressed in the *ΔDR0934* background at 30°C. Quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR) was performed on RNA from heat shocked and untreated wild type (grey bars) and *ΔDR0934* (black bars) using primers annealing to 100–200 bp internal gene regions of *dnaK* (A) and *hsp20* (B). Reactions lacking reverse transcriptase confirmed the absence of contaminating genomic DNA (data not shown). Data were quantified using *hpi* as a reference housekeeping gene, and relative expression values of 1 represent equivalent transcription levels to that of *hpi*. Averages of three biological replicate experiments ± standard deviations are shown.

Global transcriptional analysis of heat shock gene expression in ΔDR0934 *mutant cells*

To determine whether *D. radiodurans hspR* encodes the negative regulator of a larger heat shock regulon, we compared the global transcriptional profiles of *D. radiodurans* wild-type and *ΔhspR* cells in response to heat shock. Using the whole-genome polymerase chain reaction (PCR) array of *D. radiodurans* that contains 3180 unique open reading frames (ORFs), each spotted in triplicate (Tanaka *et al.*, 2004), we conducted three biological replicate competitive hybridizations comparing the transcriptomes of: (i) heat shocked with untreated wild-type cells, (ii) heat shocked with untreated *ΔhspR* cells and (iii) untreated wild-type cells with untreated *ΔhspR* cells. Using a clustering program in which *hsp20* and *dnaK* were employed as templates, data were mined for genes exhibiting high induction ratios (>2.5-fold) in the wild-type strain, little change in the mutant strain at 48°C compared with 30°C (less than fivefold), and overproduction in *ΔhspR* cells compared with wild type (Fig. 4A). As shown in Fig. 4, we identified 14 genes by this analysis, all but three of which are predicted to encode heat shock and protease functions. The three genes of other functions included *DR1738* and *DR0194*, predicted to encode hypothetical proteins, and *DR1655*, a predicted ABC transporter. Interestingly, *DR0194* bears 45% identity to a putative zinc-metalloprotease of *B. subtilis*, while *DR1738* has no homologues in the database.

Genes identified with predicted heat shock functions included *clpB*, *lonB* and the *dnaKJgrpE* and *groESL* operons, which are all known to be under HspR control in other organisms (reviewed in Grandvalet *et al.*, 1999; Servant and Mazodier, 2001; Stewart *et al.*, 2002; Bucca *et al.*, 2003). All of these genes bore strong resemblance to those of other organisms: *clpB* of *D. radiodurans* exhibiting 64% homology to that of *Thermus thermophilus* and *lonB* bearing 46% homology to *lon* protease of *E. coli* (http://www.tigr.org). Surprisingly, we observed that *ftsH*, bearing 49% homology to *E. coli ftsH*, was also overexpressed in the *ΔhspR* strain, suggesting that it is also negatively controlled in an HspR-dependent manner under heat shock conditions in *D. radiodurans*. In *E. coli*, σ^{32} controls FtsH heat shock induction, and this is the only other organism for which the regulator of *ftsH* heat shock expression is known (Yura *et al.*, 2000). Interestingly, the two other *ftsH* copies (DR0583, DR1020) and the other *lon* homologue (DR1974) predicted in the annotated *D. radiodurans* genome were unaffected by heat shock in the wild-type strain (data not shown).

Transcription start site mapping and validation of microarray data by primer extension analysis

In other bacteria such as *S. coelicolor*, HspR has been

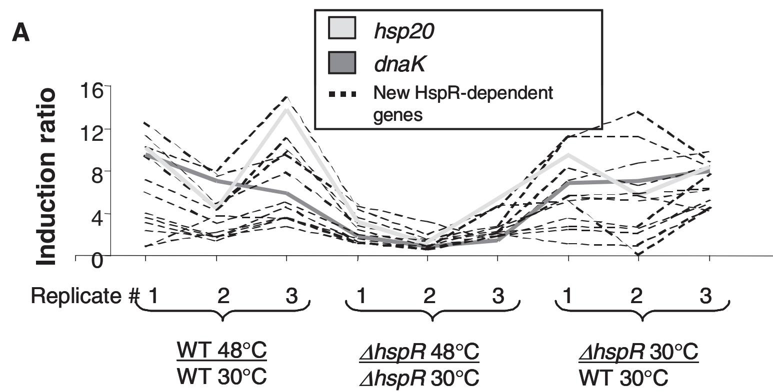

Fig. 4. Global transcriptional analysis of the HspR regulon.
A. Pavlidis Template Matching (PTM) clustering analysis of microarray data from comparisons of heat shocked and untreated wild-type and *ΔhspR* cells. Numbers on the *x*-axis denote the biological replicate data set, and labels below the axis indicate the hybridization experiment from which expression ratios are derived, with Cy5-labelled samples indicated in the numerator, and Cy3-labelled samples in the denominator.
B. Average expression ratios resulting from PTM analysis of microarray data. Genes are grouped into categories according to the functional annotation at the left (http://www.tigr.org; White *et al*., 1999).

B

Common name/ORF number	WT 48°C vs. 30°C	Δ*hspR* 48°C vs. 30°C	WT 30°C vs. *DhspR* 30°C
	Heat shock functions		
dnaJ/DR0126	7.9	2.69	10
DR0127	11.06	4.9	11.01
grpE/DR0128	9.22	3.11	8.2
dnaK/DR0129	7.49	1.4	6.6
lon/DR0349	7.49	1.76	6.11
groES/DR0606	2.36	1.26	3.57
groEL/DR0607	3.16	1.5	3.5
clpB/DR1046	7.99	2.23	7.18
hsp20DR1114	11.99	2.18	7.72
Signal peptidase/*DR1737*	2.27	1.37	5.97
ftsH/DRA0290	2.98	1.02	4.86
	Other functions		
ABC transporter/*DR1655*	2.59	1.25	4.44
DR1738	3.51	1.69	4.66
DR0194	4.77	1.84	2.29

shown to repress gene transcription from σ^{70} vegetative-type promoters by binding directly to a 7 bp inverted repeat sequence called HAIR (HspR-associated inverted repeat) with the consensus CTTGAGT-N7-ACTCAAG (Grandvalet *et al*., 1999). We therefore searched the sequences in the 5′-UTR of the 14 putative HspR regulon genes of *D. radiodurans*, and detected potential HAIR sequences upstream of all but three of the genes (Fig. 5A). These three genes, which included the putative signal peptidase *DR1737*, hypothetical gene *DR1738* and ABC transporter *DR1655*, organized into two potential transcriptional units, are candidates for indirect control by HspR.

To determine the location of the potential HAIR sites relative to the transcription start site, we performed primer extension analysis on three of these genes for which start sites are not yet known, *lonB*, *ftsH* and *DR0194*. The resultant promoter sequences and relative positions of the putative HAIR sites are aligned with the previously mapped *groESL*, *dnaKJE* and *hsp20* promoters in Fig. 5A (Meima *et al*., 2001; A. K. Schmid *et al*., submitted). In most cases, transcription appears to initiate from a canonical σ^{70} consensus promoter, identical to *E. coli* σ^{70} in the –35 regions with the sequence TTGACA/T, but more divergent in the –10 regions, as was expected from previous promoter mapping studies in *D. radiodurans* (Meima *et al*., 2001; A. K. Schmid *et al*., submitted). The *lonB* and *ftsH* promoters, in contrast, exhibited virtually no sequence similarity to the σ^{70} consensus of *E. coli*; however, the *lonB* –35 region showed some conservation with the previously mapped *DR1314* –35 promoter region (AACAAA) (Schmid *et al*., 2004), diverging from it at two nucleotide positions (Fig. 5A).

For each of the mapped promoters, the putative HAIR sequences were located either upstream of the –35/–10 regions, as was the case for the *dnaK*, *ftsH* and *groES* promoters, or overlapping the –35/–10 regions, as for *hsp20*, *lonB* and *DR0194*. It was not possible to map the *clpB* promoter because of technical issues, but an obvious σ^{70}-type promoter was identified, and a potential HAIR sequence very similar to the consensus was found overlapping the predicted –35/–10 regions (Fig. 5A). Interest-

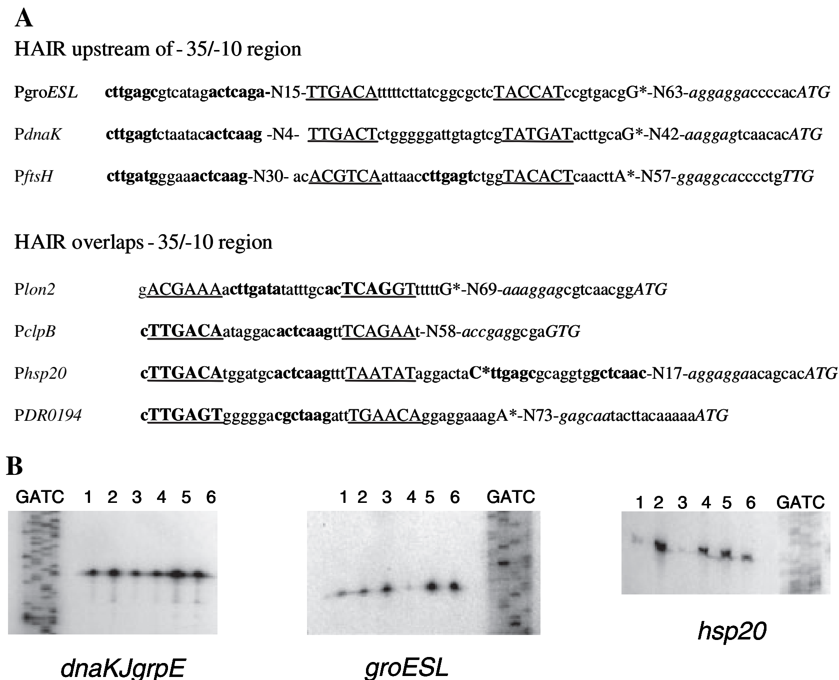

Fig. 5. Mapping of HspR-repressed genes by primer extension.

A. Promoter alignments. Predicted HspR binding sites (HAIR sites) are in bold, and –35/–10 promoter regions are in underlined uppercase letters. Transcription start site nucleotides are shown in capital letters and marked with an asterisk (*). Predicted ribosome binding sites are indicated in lowercase italics, and predicted translation codons are shown in uppercase italics. Aligned sequences are grouped according to position of the predicted HAIR site relative to the –35/–10 promoter regions.

B. Primer extension experiments in different *D. radiodurans* strain backgrounds. RNA prepared from heat shocked (lanes 2, 4 and 6) and untreated (lanes 1, 3 and 5) wild type (lanes 1 and 2), *sig1* mutant (lanes 3, 4) and Δ*hspR* (lanes 5 and 6) were subjected to primer extension using reverse transcriptase and primers for *dnaK*, *groES* and *hsp20* as indicated in the *Experimental procedures.* To map the promoter and determine the level of transcription from each promoter, primer extension reactions were run on polyacrylamide gels alongside sequencing reactions using the same primer (GATC).

ingly, the *ftsH* promoter region included a HAIR half site with the sequence of CTTGAGT located between the –35/–10 regions. In addition, the spacing between the 7 bp palindromic sequences located upstream of the *ftsH* promoter was only 4 bp rather than the typical 7 (Fig. 5A). Furthermore, the *hsp20* promoter contained a second HAIR sequence overlapping the transcription start site downstream of the –35/–10 regions, similar to the *dnaK* promoter of *S. coelicolor*, which contains three HAIR sites at –75, –49 and +4 (Fig. 5A) (Bucca *et al.*, 1995).

In addition to mapping the promoters in the wild-type strain, we sought to verify the phenomenon of overexpression of *hsp20*, *groES* and *dnaK* observed in the Δ*hspR* backgrounds at 30°C in the microarrray experiments. To do this, we conducted primer extension assays in which the levels of transcription initiation in heat shocked and untreated wild-type and Δ*hspR* backgrounds were compared. Experiments in the *sig1* mutant background served as controls (Schmid and Lidstrom, 2002). As shown in Fig. 5B, and as expected from previous promoter mapping studies (Meima *et al.*, 2001; A. K. Schmid *et al.*, submitted), *groESL* transcription is modestly induced upon heat shock in wild-type cells, and expression of *hsp20* is highly induced at 48°C (Fig. 5B). The *dnaK* transcript also appears to be induced upon heat shock as expected from previous expression studies (Fig. 3; Schmid and Lidstrom, 2002). In contrast, these genes are transcribed at a high constitutive level in the Δ*hspR* mutant background, exhibiting approximately equivalent or, in the case of *groESL*, higher levels of transcription at 30°C compared with the wild-type background at 48°C. In addition, as expected from previous studies (A. K. Schmid *et al.*, submitted), partial induction of *hsp20* and *dnaK* is evident in the *sig1* mutant background, whereas *groESL* transcription under heat shock is nearly abrogated (Fig. 5B).

HspR binds directly to the promoter regions of heat shock genes

To investigate whether HspR regulation of heat shock gene expression is direct or indirect, we conducted electrophoretic mobility shift assays on the promoter regions of *dnaKJgrpE*, *groESL*, *ftsH*, *clpB*, *DR0194*, *hsp20* and *lonB* with partially purified HspR protein. HspR was overproduced from a T7 promoter and accumulated to significantly detectable levels in the heterologous *E. coli* host (data not shown). Much of this protein aggregated in the insoluble fraction (data not shown); nevertheless, we were able to isolate sufficient amounts of HspR protein from the soluble fraction under standard native conditions to allow for efficient gel shift assays. PCR products (300–650 bp) containing the promoter regions of the heat shock genes listed above were incubated with the partially purified HspR protein (Fig. 6). As a control, the DNA fragments were also incubated with *E. coli* extracts containing the empty expression vector (Fig. 6, lane 2). A strong band shift was observed for the promoter regions of *dnaK*, *clpB*, *hsp20*, *lonB* and *ftsH* (Fig. 6, lane 3). Incubation with excess unlabelled specific competitor DNA resulted in complete abrogation of the electrophoretic mobility shifted band in all cases (Fig. 6, lane 4). In contrast, the extent

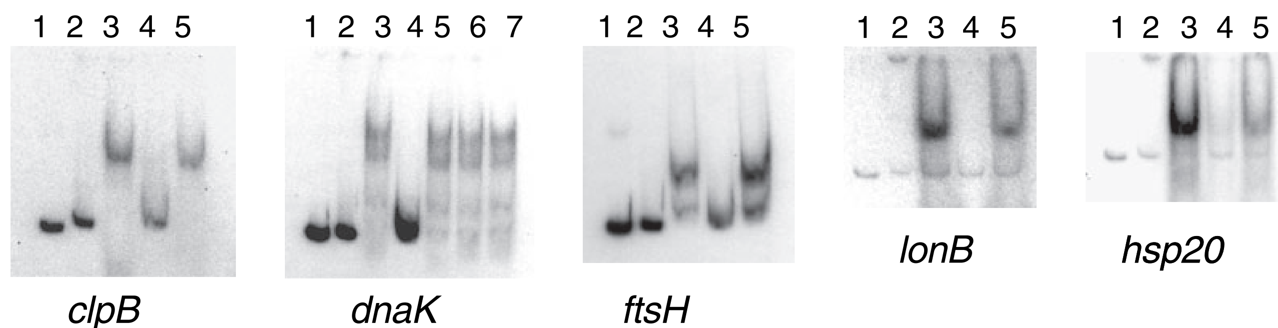

Fig. 6. *D. radiodurans* HspR binds to the promoter regions of heat shock genes. Reactions containing partially purified HspR was incubated with the promoter fragments of *dnaK*, *hsp20*, *clpB*, *ftsH* and *lonB* (lane 3), and subjected to electrophoresis on non-denaturing polyacrylamide gels. Labelled probe alone in binding buffer was run as a reference (lane 1), and reactions in which labelled probe was incubated with *E. coli* extracts containing the empty expression vector served as a control (lane 2). Specificity of interactions were tested by incubating labelled probe with HspR protein and excess unlabelled specific competitor (lane 4), or with an excess of the unlabelled non-specific competitor DR1314 promoter region (lane 5). To test the sequence specificity of HspR for the HAIR site, competition experiments were conducted in which increasing concentrations of the unlabelled *dnaK* promoter region with a mutated HAIR site were incubated with labelled probe and HspR protein (lanes 6 and 7).

of retardation of the shifted band remained unaltered for *dnaK*, *clpB*, *hsp20* and *lonB* upon incubation with up to a fourfold excess of unlabelled non-specific competitor DNA sequence from the promoter region upstream of DR1314, which had been previously identified as a Sig1-controlled gene (A. K. Schmid *et al.*, submitted) (Fig. 6, lane 5). Surprisingly, the DNA fragments corresponding to the *groESL* and *DR0194* promoters resulted in a weak band-shift that was easily competed away with only a fourfold excess of unlabelled DR1314 competitor (data not shown), suggesting that the interaction between the *cis*-acting sequences of these promoter regions and the HspR protein may be weak or non-specific.

To assess whether the HspR-mediated mobility shift observed for the promoter regions of *dnaK*, *clpB*, *lonB*, *ftsH* and *hsp20* resulted from specific binding to the HAIR motif, four residues within the *dnaK* promoter region were mutated, changing the upstream half of the HAIR sequence from CTTGAGT to CAACTGT, thus destroying a potential hairpin structure. The mutant oligonucleotide was then used as a cold competitor against the radiolabelled wild-type *dnaK* probe in gel shift assays with HspR protein. As shown for *dnaK* in Fig. 6, lanes 6 and 7, the mutant HAIR probe is unable to compete with the labelled wild-type oligo for binding of the HspR protein, even when present in fourfold (lane 6) or 40-fold (lane 7) excess. Taken together, these results suggest that HspR binds directly and specifically to the HAIR sequence in the promoter regions upstream of *dnaK*, *ftsH*, *clpB*, *lonB* and *hsp20*.

Discussion

All tested bacteria induce the expression of protective factors in response to the stress of elevated temperature. However, the regulation of the heat shock protective response varies among organisms, including positive and negative regulatory strategies. In *D. radiodurans*, previous studies of heat shock regulation suggested that Sig1, an ECF-type sigma factor, is responsible for the positive heat shock regulation of up to 40 genes (Schmid and Lidstrom, 2002; A. K. Schmid *et al.*, submitted). However, several of these genes, including *hsp20* and members of the *dnaKJgrpE* operon, can still be partially induced in *sig1*-defective cells, suggesting the involvement of other regulators (Schmid *et al.*, 2004). In the current study, we have identified an HspR-like homologue in *D. radiodurans* that governs negative regulation of chaperone and protease gene expression under heat shock. In addition, we have demonstrated that a portion of the regulon is subject to direct regulation by HspR, apparently via specific interaction of the HspR protein with the HAIR element within promoter regions.

Global transcriptional analysis using the whole-genome *D. radiodurans* microarray allowed the identification of 14 genes in the HspR regulon, which included 11 genes predicted to code for heat shock proteins, two genes of unknown function (*DR0194* and *DR1738*), and one predicted membrane ABC transporter. The heat shock genes included *lonB*, *clpB* and the *dnaKJgrpE* and *groESL* operons, which are known to depend on HspR for their heat shock induction in other organisms such as the *Streptomycetes* (Servant and Mazodier, 2001). However, two of these genes with predicted heat shock functions, *ftsH* and *hsp20*, are novel members of the HspR regulon. The *S. albus hsp20* homologue, *hsp18*, predicted to encode a small heat shock protein, is known to be regulated by its own repressor protein RheA (Servant *et al.*, 1999). Furthermore, in *E. coli*, σ^{32} governs *ftsH* heat shock transcription (Gross, 1996); however, in *B. subtilis*, *ftsH* belongs to the class IV heat shock genes, whose regulatory functions remain unknown (Yura *et al.*, 2000). Therefore, this report

represents the first evidence of a *ftsH* regulatory mechanism outside of *E. coli*.

We detected HAIR sequences upstream of *ftsH*, *dnaK*, *clpB*, *lonB*, *groESL*, *DR0194* and *hsp20*. In other organisms, HspR binds to the HAIR sequence, thus conferring repression at heat shock promoters in the absence of stress (Servant and Mazodier, 2001). In *D. radiodurans*, we observed that the promoter regions of all genes but *groESL* and *DR0194* interacted directly and specifically with partially purified HspR via the HAIR sequence. It is possible that the HspR interacted weakly with the HAIR sites within the *groESL* and *DR0194* promoter regions because these sites contained only three available residues for base pairing interactions. Consistent with this hypothesis was the reciprocal observation that two HAIR sites, each identical to the consensus except for one residue, were detected upstream of *hsp20*, which correlated with tight repression in the absence of stress and nearly 100-fold induction under heat shock. Similarly, HspR tightly controls *dnaK* expression in *S. coelicolor*, and the *dnaK* promoter contains three HAIR sites (Bucca *et al*., 1995; 2000). Taken together, these observations are consistent with the hypothesis that, in order for HspR to bind and repress a promoter, a minimum number of four residues must be available for base pairing within HAIR sites.

No HAIR sites were detected upstream of the other three predicted HspR regulon members from the microarray experiments, including the putative signal peptidase (*DR1737*), hypothetical gene *DR1738*, and ABC transporter *DR1655*. These genes are candidates for indirect control by HspR, the mechanism of which is unknown. However, it is possible that elevated levels of protease expression in the Δ*hspR* background could affect the stability of the protein regulators of these three genes, accounting for the observed effects.

Several genes identified in this study as members of the HspR regulon were observed to be under partial positive control by Sig1 in previous studies from our laboratory, including *hsp20*, *DR0194*, *DR1655* and the *dnaKJgrpE* operon, all of which were still induced 1.5- to 4-fold in cells lacking Sig1 (A. K. Schmid *et al*., submitted). The *groESL* operon appeared to be fully dependent on Sig1 in previous studies, but showed weak or perhaps indirect control by HspR in this study, as did *DR0194*. Reciprocally, in the QRT-PCR and microarray experiments of the current study, we observed hyperinduction of *hsp20* (approximately fivefold) and *dnaK* operon members *dnaJ*, *DR0127* and *grpE* (2.5- to 5-fold) in the Δ*hspR* mutant, i.e. an increase in transcription at 48°C above the overexpressed 30°C level compared with wild type. As no residual expression of *hspR* in the deletion strain could be detected in the microarray data (data not shown), these results are consistent with the hypothesis that Sig1 may be responsible for residual heat shock induction of these genes in the absence of HspR. Several examples exist for co-ordinate regulation at the same promoter by an alternative sigma factor and a negative regulator, including HrcA/RpoH-dependent transcription of *groESL* in *C. crescentus* and *A. tumefaciens* (Baldini *et al*., 1998; Nakahigashi *et al*., 1999), and HspR/ClgR/SigH control of *clpP* genes in a complicated transcriptional circuit of *C. glutamicum* (Engels *et al*., 2004). However, it is also possible that a more complex regulatory cascade is at work in *D. radiodurans* and that the overlap in the HspR and Sig1 regulons results from indirect effects.

In summary, from the results of mutant analysis, microarrays, promoter mapping by primer extension and gel mobility shift assays in this study, we conclude that DR0934 encodes an HspR-like negative regulator of heat shock gene transcription in *D. radiodurans* that acts to repress heat shock promoters by binding to the HAIR *cis*-encoded element. Furthermore, combined with results of previous studies (Schmid and Lidstrom, 2002; A.K. Schmid, *et al*., submitted.), it appears that the HspR regulon partially overlaps with that of Sig1.

Experimental procedures

Bacterial strains, growth conditions and media

Deinococcus radiodurans R1 wild-type and Δ*DR0934* strains were routinely grown at 30°C in TGY broth (0.5% tryptone, 0.1% glucose, 0.3% yeast extract) supplemented with 8 μg ml^{-1} kanamycin or 3 μg ml^{-1} chloramphenicol where appropriate. TGY agar supplemented with 10 μg ml^{-1} kanamycin was used for routine growth of Δ*DR0934*, and 15 μg ml^{-1} for the selection of transformants. *E. coli* cultures were grown at 37°C in Luria–Bertani (LB) broth or on LB agar supplemented with 100 μg ml^{-1} ampicillin and/or 50 μg ml^{-1} kanamycin as needed. Routine transformations of *E. coli* were performed using commercially available competent TOP10 (Invitrogen) or JM109 (Promega) as the manufacturer suggested. Transformations of *D. radiodurans* were performed by electroporation as previously described (Schmid and Lidstrom, 2002).

DNA manipulations and mutagenesis.

Plasmid DNA extraction, restriction digests and ligation were performed using standard techniques (Sambrook and Russel, 2001) with enzymes from New England Biolabs (Beverely, MA). Standard PCRs were performed with 1 unit *Taq* DNA polymerase (Invitrogen) and standard concentrations of deoxynucleotides and magnesium chloride, using either genomic DNA from *D. radiodurans*, prepared according to methods described previously (Schmid and Lidstrom, 2002), or plasmid DNA extracted from *E. coli* as templates. Custom oligonucleotide primers (Invitrogen) were each added to the reaction at 0.2 pmol.

The vector pDR0934M was constructed for the purpose of deleting the chromosomal copy of DR0934 as follows. Approximately 1 kb fragments flanking DR0934 were PCR

amplified from *D. radiodurans* genomic DNA using the primers DR0934 5′_fwd (AGATCTCCGACATGGCCTACCAC) and DR0934 5′_rev (GGTACCACCACAAGCAAGGTTGA) to amplify the upstream (5′) flank, and primers DR0934 3′_fwd (TACGTACCGGAGAAGACCAGAAC) and DR0934 3′_rev (GAGCTCGGAACCCAGGTCCAGAA) to amplify the downstream (3′) flank. PCR products were sequenced to confirm the absence of mutations and cloned into pCR2.1-TOPO (Invitrogen). The upstream (5′) sequence was then transferred to pHMR186 (Rothfuss, 2004) as a *Bgl*II–*Bam*HI fragment, and the 3′ flank was cloned as a *Sna*BI–*Sac*I fragment, thus generating pDR0934M, which was used to transform *D. radiodurans* R1 wild type to disrupt the chromosomal copy of DR0934. Resultant clones were screened for kanamycin resistance and chloramphenicol sensitivity, and confirmed by diagnostic PCR on chromosomal DNA to (i) lack a wild-type copy (primers: DR0934RT_fwd, GTCATGCGCCTGCAAC ACCA; DR0934RT_rev, TGGGTCAGCCGCCGAATCTC), (ii) contain the kanamycin resistance gene (primers: kan_fwd, AAGCCAGCTTGTGTCTCA, kan_rev, CCGTCAAGTCAGC GTAAT), (iii) be devoid of the chloramphenicol resistance gene (primers: CmSCO_fwd, AGGACAAATCCGCCGAG CTT, CmSCO_rev, TGCCGCCTCGACGAATTTCT) and (iv) to contain the flanking sequences in the proper chromosomal positions (primers: DR0934Mcheck5′fwd, CGCCGGGC GACCTGATTCAT; Mcheck5′rev, ATCGCGGCCTCGAG CAAGAC; Mcheck3′fwd, CAACTGGTCCACCTACAACA; DR0934Mcheck3′rev, CCCTCAAGCGGGTAGTGGTC).

RNA preparation

Deinococcus radiodurans R1 wild-type and *ΔDR0934* cultures were grown to mid-exponential phase (OD_{600} ≈0.3–0.5), then split into two aliquots, one of which was heat shocked for 5 min at 48°C, the other was maintained at 30°C as a control. One-tenth volume of STOP solution (5% phenol, 95% ethanol) was added before harvesting by centrifugation at 4500 *g* at 4°C for 5 min, followed by bead beating lysis with 0.1 mm zirconium silica beads (BioSpec Products, Bartelsville, OK) for 3 min in a Mini-Beadbeater 8 (BioSpec). RNA was then extracted from lysates using the RNeasy Minikit (Qiagen) according the manufacturer's 'Yeast III' protocol. Incubation of resultant RNA for 1 h with 6–10 U of DNase I (Ambion) removed contaminating genomic DNA.

QRT-PCR

One microgram of *D. radiodurans* wild type and *ΔDR0934* RNA extracted from heat shocked and untreated cultures was used as template in random hexamer-primed first-strand cDNA synthesis reactions with Thermoscript reverse transcriptase (Invitrogen). Parallel reactions without reverse transcriptase were conducted to ensure the absence of contaminating DNA. One microlitre of cDNA was used in quantitative PCRs to amplify 100–200 bp internal gene regions using the following primers: hsp20Qfwd (GGCCT CGAACTGACCTTGGACATTC) and hsp20Qrev (AAAGGT GCCGTAGGCGCGCTCGACA) for measuring *hsp20* expression; dnaKQfwd (TCGGCGGTCAGTTCCTCGAACTTGG) and dnaKQrev (AGCATCTCCCTGCCCTTCATCACCT) for measuring *dnaK* expression; and hpiQfwd (AGACGCGG TAGGTCACGTTGTCCTG) and hpiQrev (TGTCCTCGAAC CCGGCACTGTGAAG) for measuring *hpi* expression. Each 20 μl quantitative PCR reaction also contained SYBR green PCR Core Reagents (Applied Biosystems) in final concentrations of 2 mM $MgCl_2$, 1 mM dNTP, 0.025 U Amplitaq Gold™ polymerase and 0.2 pM of each primer.

Quantitative thermocycling was performed in a RotorGene 3000 (Corbett Research, Sydney, Australia) for 45 cycles with the following parameters: activation of *Taq* polymerase at 95°C for 15 min, melting at 95°C for 20 s, annealing at 55°C for 20 s and extension at 72°C for 20 s. Data were acquired during the extension step and melting curve analysis was performed after each run to ensure the absence of primer dimers and the specificity of products. Data for each of the 30°C and 48°C conditions were quantified separately using the formula $Eff^{-(\Delta C_T)}$ (http://www.wzw.tum.de/gene-quantification), using *hpi* as the reference gene, which has been previously validated as a constitutively expressed housekeeping gene in *D. radiodurans* (A.K. Schmid, *et al.*, submitted). Values of one represent expression equivalent to that of *hpi*, which has high basal expression at 30°C in *D. radiodurans* (data not shown). The averages and standard deviations of three independent experiments are displayed in Fig. 3.

Microarray design and construction

In this study, the same full-genome microarray described previously was used (Tanaka *et al.*, 2004). Briefly, PCR primers were designed to amplify each open reading from the fully sequenced *D. radiodurans* R1 genome (White *et al.*, 1999). PCR products represent internal portions of annotated sequences with a size range of 100–800 bp. Primer pairs were designed for 3180 ORFs. PCR products were generated by combining 20 ng of genomic DNA from strain R1 with oligonucleotide primers (0.2 μmol each, average temperature = 55°C) and 0.1 U *Taq* DNA polymerase (Perkin-Elmer, Wellesley, MA) in a total volume of 100 μl. The other reaction components were as specified by the manufacturer except that 0.3 M betaine was included in the reaction to aid in denaturing *D. radiodurans* genomic DNA. PCR amplification successes were scored (single band, correct size, >50 ng μl^{-1}). Failed PCRs were repeated with an additional 2% success, for an overall efficiency of 93%. PCR products were spotted in triplicate onto UltraGAPS-coated slides (Corning Life Sciences, Corning, NY) using a Lucidea Array Spotter (Amersham Pharmacia, Piscataway, NJ) at a redundancy of 3.0. PCR products were immobilized to the slide surface using a Stratalinker® UV crosslinker (Stratagene, LaJolla, CA). All slides were stored in a desiccator at room temperature before use.

Microarray probe preparation. cDNA probes for microarray hybridization were prepared from three biological replicate total RNA samples of each of wild-type and *ΔDR0934* mutant cultures at 30°C and 48°C as follows. *D. radiodurans* RNA (2 μg) was annealed to 300 pmoles of random hexamer primers (Invitrogen) in a total volume of 18.5 μl by incubating for 10 min at 70°C and subsequent snap freezing in a dry ice-ethanol bath. cDNA was synthesized at 42°C overnight in 30.7 μl of reactions containing 6 μl of 5× first-strand buffer,

1 µM DTT, 0.5 mM dNTP mix containing amino allyl-dUTP (Amersham Biosciences, Piscataway, NJ), and 400 U of SuperScript II reverse transcriptase (Invitrogen). RNA was hydrolysed by adding EDTA to 100 mM and NaOH to 200 mM and incubating at 65°C for 15 min, then neutralized with 25 µl of 1 M Tris (pH 7.0). Unincorporated free amino allyl-dUTPs were removed by washing over Microcon 30 columns (Millipore, Bedford, MA), and resultant cDNA samples were coupled to 1 pmol Cy3 and Cy5 dyes (Amersham) in 0.1 M sodium carbonate buffer for 2 h at room temperature in the dark. Unincorporated dyes were removed by passage over QIAquick MinElute PCR purification columns (Qiagen). Three sets of competitive hybridizations were performed: (i) wild-type untreated (Cy3) versus wild-type heat shocked (Cy5), (ii) *ΔDR0934* untreated (Cy3) versus *ΔDR0934* heat shocked (Cy5) and (iii) wild-type untreated (Cy3) versus *ΔDR0934* untreated (Cy5). Hybridization of probes to prehybridized microarray slides (1 h incubation at 42°C in 5× SSC, 0.1% SDS, 1% BSA) was conducted as previously described (Peterson *et al.*, 2000).

Microarray data acquisition and analysis

Scanning was performed at both 532 nm and 635 nm visible light on hybridized slides using a GenePix 4000B imager (Axon, Union City, CA), and resultant hybridization signals were quantified in the TIGR-SPOTFINDER program (http://www.tigr.org/software.tm4) using the formula spot median > background median + BkgStdDev (roughly twice background intensity). Any signals below this cut-off were omitted from subsequent analysis. Normalization of spot intensities was then performed using the total slide intensity mode of the TIGR_MIDAS program.

Hybridization signal data were analysed as Cy5/Cy3 ratios from nine total signals for each gene resulting from three biological replicate RNA samples, each of which hybridized to triplicate spots on the array. Triplicate spot data were averaged, and a perl script was used to determine which genes were induced under heat shock in the wild type: any genes exhibiting greater than twofold induction in two out of three biological replicate data sets were determined significant. Using *hsp20* and *dnaK* as templates in the Pavlidis Template Matching program of TIGR_MEV, genes meeting the following criteria were considered to be under negative control by DR0934, and therefore members of the DR0934 regulon: (i) induction greater than twofold in the wild type (determined by perl script), (ii) not induced greater than fivefold in *ΔDR0934* under heat shock compared with untreated *ΔDR0934* and (iii) highly induced (i.e. approximately to the wild-type level) in untreated *ΔDR0934* compared with untreated wild-type. Average ratios for each of these three comparisons are presented in Fig. 4.

Primer extension transcription start site mapping

Promoter regions containing approximately 500 bp of sequence upstream and 150 bp downstream of the predicted ATG of *dnaK*, *hsp20*, *DR1314*, *ftsH*, *lonB* and *DR0194* were PCR amplified from *D. radiodurans* chromosomal DNA using Expand DNA polymerase (Roche) with the following primers: dnaK_fwd2, GGAAGATCTCGCACCA ATCCGCACCT; dnaK_rev2, GGAATATCTCGGCGTTGACG ATCACT; Phsp20_up, AGATCTGGAACGCGGTGAAGTG GCTG; Phsp20_PE3, GCTTGACGCCGGGAATGTCC AAGGT; P1314_up, CCAGCTTCACGGCGGCGTTGAA GAC; P1314_PE4, GATCTTGTCGCCGTTCACGCCGTAA; PFtsH_up, GCCGTAGAGCGAGTGCAGGT; PFtsH_PE2, CCACTGCCGGGCAGGTTCGTGAACA; Plon_up, AGATCT GCAGCAGCAGCAGGCTGAGT; Plon_PE2, GTTGATGGA AATGGCGCGGCTGGCA; PDR0194_up, ACCTCGCCGG TGCTGGACTT; PDR0194_PE2, TACCGGCACATTGT GCAGGCCGTTT. The promoter fragments from the genes of interest were then cloned into pCR2.1-TOPO (Invitrogen) and used as templates for sequencing reactions with Sequenase DNA polymerase 7.0 (USB, Cleveland, OH) using the same downstream primer that was used to amplify promoter fragments. For primer extension reactions, primers (the same as were used for sequencing reactions) were first radio-end-labelled by phosphorylation with T4 polynucleotide kinase (Roche). Subsequently, reactions were performed on 5 µg of total RNA extracted from heat shocked and untreated *D. radiodurans* wild type, *sig1* mutant, or *ΔDR0934* cultures using Thermoscript reverse transcriptase (Invitrogen). To map transcription start sites, sequencing ladders were electrophoresed alongside the corresponding primer extension products on gradient polyacrylamide gels (National Diagnostics, Atlanta, GA). Start sites were verified from replicate RNA harvested on different days using two different primers (data not shown). Representative experiments for each gene are shown in Fig. 5.

Overexpression and partial purification of HspR protein

The HspR (DR0934) ORF was PCR amplified from *D. radiodurans* genomic DNA using the following primers: DR0934OVRXN_fwd, CCATGGCTCTTGTGGTATGCTCA ACT; DR0934OVRXN_rev, CTCGAGGTTCTGGTCTTCTCC GGC. Sequencing of the products confirmed the absence of mutation. DR0934 ORF PCR product was cloned into pCR2.1-TOPO, then transferred to pET21d (Novagen) as an *Nco*I–*Xho*I fragment, yielding pET21d::DR0934. Strain BL0934 was subsequently generated by transformation of pET21d::DR0934 into *E. coli* strain BL21(DE3) (Novagen).

To overproduce the DR0934 protein, BL0934 was grown to mid-exponential phase at 37°C ($OD_{600} \approx 0.4$–0.6), and expression of DR0934 was induced by the addition of IPTG to a final concentration of 1 mM and cultivating at 30°C for 6 h. Cells were harvested by centrifugation at 4500 *g* for 15 min at 4°C, pellets were resuspended in 1 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 10 mM β-marcaptoethanol), and lysed by passing twice over a French press at 1000 psi. The soluble fraction was isolated by centrifugation of the lysate at 15 000 *g* for 30 min at 4°C, followed by purification of DR0934 under native conditions by virtue of its polyhistidine tag on a NiNTA mini column (Qiagen) according to the manufacturer's instructions. Protein eluates were concentrated and dialysed against two changes of storage buffer (50 mM Hepes pH 7.5, 300 mM NaCl, 1 mM DTT, 10% glycerol) on a Microcon 30 mini column (Millipore, Bedford, MA) and stored at −20°C. Overpro-

duction and purification was assessed by separating proteins on 12.5% SDS-PAGE gels and visualized by staining with Coomassie brilliant blue.

Electrophoretic mobility shift assays

Promoter fragments upstream of *DR0194*, *groESL*, *ftsH*, *hsp20*, *dnaK* and *lonB* were PCR amplified from *D. radiodurans* chromosomal DNA using Expand polymerase (Roche) with the same primers as those used for transcription start site mapping (see above). Primers PclpB_up (AGATCTA ACTCGCCTTCGTCAACCTG) and PclpB_dn (GGCATGG TTTAACTGCCCTCATTCT) were used to amplify the *clpB* promoter. To generate the HAIR mutant oligonucleotide fragment, the predicted HAIR site of the *dnaK* promoter fragment contained in pRADZ8 (Schmid and Lidstrom, 2002) was changed from CTTGAGT-N7-ACTCAAG to CAACTGT-N7-ACTCAAG with the Quick Change Mutagenesis Kit (Stratagene) using 125 ng of each mutagenesis primer (HAIR_MUTc: GCAAACTTGAGTCTAATACACAGTTGTCT ATTGACTCTGGGGGAT, HAIR_MUTnc: ATCCCCCAGAGT CAATAGACAACTGTGTATTAGACTCAAGTTTGC) according to the protocol supplied by the manufacturer. The resultant mutagenized plasmid was digested with *Bgl*II to release the 564 bp fragment of interest.

End-labelled promoter fragments were incubated for 20 min at room temperature with ≈1.5 µg of partially purified DR0934 protein in 1× gel shift binding buffer (Promega) [5 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 4% glycerol, 0.05 µg ml^{-1} poly-(dI-dC)]. Reactions were then separated at 300 V for 25 min on 6% non-denaturing polyacrylamide gels in chilled 0.5× TBE running buffer and visualized by autoradiography.

Acknowledgements

This work was funded by a grant from the DOE EMSP programme (ER20294), and in part by the University of Washington Molecular and Cellular Biology Training Grant (GM0720), and the DOE MGP programme (DEFG0201ER63151). We also thank Heather Rothfuss for her constant support and helpful advice and Dhileep Sivam for his help with the microarray analysis.

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