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Involvement of Two Putative Alternative Sigma Factors in Stress Response of the Radioresistant Bacterium *Deinococcus radiodurans*

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Two genes bearing similarity to alternative sigma factors were identified in the *Deinococcus radiodurans* genome sequence and designated *sig1* and *sig2*. These genes were cloned and inactivated, and both were found to be important for survival during heat and ethanol stress, although the *sig1* mutants displayed a more severe phenotype than the *sig2* mutants. Reporter gene fusions to the *groESL* and *dnaKJ* operons transformed into these mutant backgrounds indicated that *sig1* is required for the heat shock induction of *groESL* and *dnaKJ*, whereas *sig2* mutants show a more moderate defect in *dnaKJ* induction and are not impaired for *groESL* induction. Essentiality tests suggested that neither *sig1* nor *sig2* is essential under all conditions. Sequence comparisons demonstrated that the *sig1* gene product is classed distinctly with extracytoplasmic function (ECF) sigma factors, whereas Sig2 appears to be a more divergent sigma factor ortholog. These results suggest that *sig1* encodes the major ECF-derived heat shock sigma factor in *D. radiodurans* and that it plays a central role in the positive regulation of heat shock genes. *sig2*, in contrast, appears to play a more minor role in heat shock protection and may serve to modulate the expression of some heat protective genes.

Deinococcus radiodurans is an extremely radioresistant, nonpathogenic bacterium that forms one of the major known phylogenetic branches of the bacterial domain, with *Thermus* as the only closely related genus (3). The genome of *D. radiodurans* has been sequenced and annotated and has been shown to contain four high-G+C genetic elements, including two chromosomes, a megaplasmid, and a plasmid (40). *D. radiodurans* maintains 4 to 10 copies of the genome, depending on the growth phase (2).

The radioresistance mechanisms of *D. radiodurans* have been studied intensively because of the organism's ability to survive an acute dose of up to 5 megarads of γ-irradiation and up to 1,000 J of UV irradiation/m^2 without mutation (2, 35). Despite this interest, little is known about how the organism responds to other environmental stressors. The few studies of *D. radiodurans* stress response systems indicate that the organism mounts a regulated protective response against DNA damage induced by γ-irradiation (2, 35, 36), UV light (8), and oxidative stress (23, 39); however, the mechanisms of this regulation have not yet been investigated.

In other bacteria, transcriptional regulation of genes involved in stress response is mediated by alternative sigma factors, which direct RNA polymerase to specific stress promoter sequences. Sigma factors have been divided into two major groups based on sequence and function, the σ^{70} and σ^{54} classes. Of the σ^{70} class, groups 1 and 2 are responsible for transcription of housekeeping promoters, whereas group 3 sigma factors (21), such as σ^{S} and σ^{H} in *Escherichia coli* and σ^{B} in *Bacillus subtilis*, transcribe stationary-phase and osmotic stress (14), heat shock (11, 42), and general stress-protective (12) genes, respectively. A subfamily of group 3 sigma factors, extracytoplasmic function (ECF) sigma factors (22), controls the transcription of genes involved mainly in extracytoplasmic cell protective functions, including production of aerial mycelia in *Streptomyces coelicolor* (4), virulence in *Salmonella enterica* serovar Typhimurium (18) and osmotolerance and heat shock survival in *B. subtilis* (15, 16). Most bacterial genomes contain one vegetative sigma and several alternative sigma factor and ECF orthologs.

Surprisingly, searches of the complete and annotated genome sequence of *D. radiodurans* (40) have revealed only three predicted sigma factors, including one that is classed as a vegetative σ^{70} (*rpoD*/*sigA*, DR0916), one that is classed as an ECF subfamily member (*sig1*, DR0180), and one putative sigma factor (*sig2*, DR0804). Conspicuously missing are orthologs for the nitrogen regulation (*rpoN*), general starvation and osmotic stress (*rpoS*), general stress (*sigB*), and heat shock (*rpoH*) sigma factor genes. Other known stress-regulatory gene orthologs are also not recognized in the genome sequence, including genes that code for heat shock negative regulators, such as *hrcA*, *ctsR*, and *hspR* (30), the oxidative-stress regulators *oxyS* (1) and *soxRS* (34), and osmotic-stress regulators such as *ompR*. Such regulators either are absent from the genome or are so divergent that they are not recognizable. This suggests that stress response regulation in *D. radiodurans* may differ significantly from that of established paradigms. We therefore sought to identify the function of the two alternative sigma factors of *D. radiodurans* in stress response regulation. Here we report the cloning, mutation, and phenotypic characterization of these two putative sigma factors.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source or reference
E. coli		
JM109	(*mcrA*) *recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1* Δ(*lac-proAB*) [*F' traD36 proAB*] *lacI*^{q}ZΔM15]	Promega
TOP10	*F*$^-$ *mcrA*Δ (*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 deoR araD139*Δ(*ara-leu*)*7679 galU galK rpsL*(Str^r) *endA1 nupG*	Invitrogen
D. radiodurans R1		
R1	Wild-type strain	J. R. Battista
AKS10	R1 *sig1*::*aph* (Km^r) [Sig1$^-$]; result of recombination of pAKS10 into chromosome of R1	This study
AKS11	R1 *sig2*::*aph* (Km^r) [Sig2$^-$]; result of recombination of pAKS11 into chromosome of R1	This study
plasmids		
pCR2.1-TOPO	Cloning vector for PCR-generated products, Ap^r Km^r, 3.9 kb; uses covalently linked topoisomerase instead of DNA ligase	Invitrogen
pMTL23	General cloning vector for *E. coli;* Ap^r, 2.5 kb	5
pUC19	General cloning vector for *E. coli*; Ap^r, 2.7 kb	41
pAKS10	pMTL23 carrying the *D. radiodurans sig1* gene disrupted by the pCR2.1 Km^R gene driven by the *D. radiodurans groESL* minimal promoter; Km^R Ap^R, 4.7kb	This study
pAKS11	Like pAKS10; carrying the disrupted *D. radiodurans sig2* gene	This study
pAY/K2	pUC-based insertion vector for carrying the *D. radiodurans amyE* gene disrupted by the pUC4K Km^r gene; Ap^r Km^r	24
pGROES4Z	*D. radiodurans* suicide vector for the disruption of *amyE* containing the *groES* promoter insert; Ap^r Km^r, 9 kb	25
pRADZ1	Promoter probe vector that uses *lacZ* as a reporter, derived from pRAD1, 6.8 kb	24
pRADZ3	pRADZ1 carrying the R1 *groESL* promoter	25
pRADZ8	pRADZ1 carrying the R1 *dnaK* promoter	This study

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. *E. coli* cultures were grown in Luria-Bertani broth or on Luria-Bertani agar at 37°C; *D. radiodurans* cultures were grown in TGY broth or on TGY agar at 30°C (29). Ampicillin (100 μg/ml), kanamycin (50 μg/ml for *E. coli*, 20 μg/ml for the screening of *sig1* and *sig2* mutants on plates in *D. radiodurans*, 10 μg/ml for routine growth on plates, and 8 μg/ml for growth in liquid), or chloramphenicol (3 μg/ml for *D. radiodurans*) was added to culture media as appropriate. Transformations of *E. coli* were performed with commercially available cells (TOP10 and JM109 from Invitrogen, Carlsbad, Calif., and Promega, Madison, Wis., respectively). Transformations of *D. radiodurans* were performed by electroporation (H. Rothfuss, unpublished data). Electrocompetent cells were prepared by washing in 1 mM HEPES as previously described (9). TGY broth (900 μl) was added immediately after electroporation, and cells were allowed to recover for 16 h before plating on selective medium.

DNA manipulations. DNA was isolated from *E. coli* by the alkaline lysis method according to Sambrook and Russel (32). *D. radiodurans* chromosomal DNA for Southern analysis and PCR amplification was prepared by using a detergent lysis method as follows. Cells were collected from overnight cultures by centrifugation (16,000 × *g* for 2 min) and resuspended in 500 μl of $T_{50}E_{50}$ buffer (50 mM Tris-HCl, 50 mM EDTA [pH 8.0]) supplemented with lysozyme (2 mg/ml) and NaCl (0.15 M). The suspension was incubated at 37°C for 1 h. Proteinase K (0.6 mg/ml; Roche Diagnostics Corp., Mannheim, Germany) and *Streptomyces griseus* protease (0.6 mg/ml; Sigma Chemical Corp., St. Louis, Mo.) were then added, and the suspension was incubated at 37°C for another hour. After lysis with 100 μl of 10% sodium dodecyl sulfate, the aqueous phase was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (24:24:1) (Roche Corp.), and chromosomal DNA was precipitated with 0.5 volume of isopropanol. Finally, the pelleted DNA was resuspended in 100 μl of $T_{10}E_1$ (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and stored at 4°C.

Restriction enzymes were obtained from New England Biolabs (Beverly, Mass.), Roche Corp. (Indianapolis, Ind.), or Promega and were used as the supplier described. T4 polymerase and T4 DNA ligase were purchased from New England Biolabs. *Taq* polymerase and custom primers were obtained from Gibco BRL (Grand Island, N.Y.).

Disruption of *sig1* and *sig2* genes. pAKS10 and pAKS11 insertion vectors for the purpose of disrupting *sig1* and *sig2* genes were constructed as follows. First, *sig1* (DR0180) and *sig2* (DR0804) open reading frames (ORF) and flanking regions were PCR amplified from *D. radiodurans* genomic DNA with the following primers: 0180 fwd (TGGCTACGACCCCCTGT) and 0180 rev (GGTCAGCCAGAAGCGGT) to amplify *sig1* and 0804 fwd (CTGCTGGTCTTCGCTGCT) and 0804 rev (GGGGCACTCTTCCCATTT) to amplify *sig2*. *sig1* and *sig2* PCR products were cloned into pCR2.1-TOPO (Invitrogen) and then transferred as a *Kpn*I-*Xho*I fragment to pMTL23 (5), yielding pSIG123 and pSIG223, respectively. To construct the kanamycin resistance (Km^r) cassette, the *Bam*HI-*Bgl*II *groESL* promoter fragment from pGROES4Z (25) was cloned into the unique *Bgl*II site of pCR2.1 upstream of the promoterless Km^r marker. The *groESL*-Km^r fusion was then PCR amplified with blunt-ended, restriction-tagged primers and cloned into pCR2.1-TOPO to generate pPgroK. The *groESL*-Km^r fusion was then removed from pPgroK as an *Eco*RV-*Eco*47III fragment and inserted into the T4 DNA polymerase-filled, unique *Eco*NI site of pSIG123 or the unique *Eco*47III site of pSIG223, yielding pAKS10 and pAKS11, respectively. pAKS10 and pAKS11 were then transformed into *D. radiodurans* R1 to disrupt the chromosomal copies of *sig1* and *sig2*, respectively. Transformants were screened for double-crossover mutation by diagnostic colony PCR analysis using the primers 0180 304 bp ups (TGCTGGTCCTCGCCTAT) and 0180 171 bp dns (AACGCGCTGATGTCAGG) to screen for disruption of *sig1*, and 0804 fwd and 0804 rev (see above) were used to screen for disruption of *sig2*. Positive clones were retested by Southern blot analysis on *Drd*I-digested chromosomal DNA using the enhanced chemiluminescence (ECL) kit from Amersham Pharmacia Biotech (Piscataway, N.J.) according to the manufacturer's protocol.

Stress survival assays. To characterize stress conditions for the wild type, cultures of *D. radiodurans* R1 were grown to mid-exponential phase (optical density at 600 nm, ~0.3 to 0.4) and shifted to a range of stress conditions. After 4 h of exposure to stress, survival was assessed by plating serial dilutions in 50 mM phosphate buffer in duplicate on TGY agar. After 3 days of growth at 30°C, surviving colonies were counted. Conditions that resulted in a 10- to 100-fold loss of survival after 4 h were chosen as the stress conditions for *D. radiodurans* R1 and used for comparison with sigma mutants in subsequent experiments (see below).

To characterize sigma mutant phenotypes, *D. radiodurans* R1, AKS10 (*sig1*

mutant), and AKS11 (*sig2* mutant) were grown to mid-exponential phase at 30°C in TGY without selection and then shifted to stress by dividing each culture into two aliquots. One aliquot was induced to stress as follows: heat shock was induced by transfer to 48°C, salt stress was induced by addition of NaCl to a final concentration of 1.2 M, acid shock was induced by addition of 0.3 N HCl to shift the pH of the culture from 6.8 to 3.7, or oxidative stress was induced by the addition of 120 mM H_2O_2. The second aliquot was continued at 30°C for a nonstress control. After induction to stress, cultures were plated every 2 h up to 8 h to assess survival. Survival is expressed as CFU per milliliter.

Cloning of the *dnaK* promoter. The putative *dnaKJ* promoter was PCR amplified from the *D. radiodurans* genome as a 564-bp fragment by using the following primers: dnaK_fwd2 (CGCACCAATCCGCACCT), which anneals 468 bp upstream of the predicted *dnaK* (DR0129) start codon, and dnaK_rev2 (CGGCGTTGACGATCACT), which anneals 89 bp downstream of the predicted ATG. The PCR fragment was cloned into pCR2.1-TOPO (Invitrogen) and subcloned as a *Bgl*II fragment into pRADZ1, yielding pRADZ8 (Table 1).

β-Galactosidase assays. *D. radiodurans* cultures expressing *lacZ* were permeabilized with toluene as previously described (25), and quantitative analyses of *lacZ* expression were conducted according to Miller (26). Results are expressed in Miller units, and the averages ± standard deviations of at least three replicate experiments are reported.

Sequence comparison and predictions. Computational analyses of DNA and predicted amino acid sequences were performed by using internet-based programs as follows. *D. radiodurans* sequence data were obtained from the completed and annotated genome at the Institute for Genomic Research (http://www.tigr.org) (40). Sequences from other bacteria were obtained from GenBank (http://www.ncbi.nlm.nih.gov) or the Pasteur Institute (http://genolist.pasteur.fr). Multiple alignments comparing these sequences to those of *D. radiodurans* were performed using ClustalW 1.7 (http://searchlauncher.bcm.tmc.edu) and analyzed with BoxShade software (http://www.ch.embnet.org/software/BOX_form.html).

RESULTS

Identification, cloning, and disruption of *D. radiodurans* alternative sigma factors. Two ORFs predicted to encode alternative sigma factors were identified in the complete and annotated genome sequence of *D. radiodurans* R1 (40). The first, DR0180, which we designated *sig1*, is a 687-bp predicted ORF whose product bears identity to the ECF family of sigma factors (22), with the highest identity (38%) to *Mycobacterium tuberculosis sigE*. The second, DR0804, which we designated *sig2*, is a 846-bp predicted ORF whose deduced amino acid sequence bears similarity to other sigma factors, with 31% identity to a putative *Magnetococcus* sp. ECF-like sigma factor. Genome searches using the conserved sigma factor regions 2 and 4 (10, 13) did not identify any other potential sigma factor genes.

Both *sig1* and *sig2* genes were cloned as PCR products amplified from *D. radiodurans* genomic DNA and disrupted by allelic exchange insertion of a kanamycin resistance marker as described in Materials and Methods. One of the difficulties for gene disruption in this strain has been the high natural resistance of *D. radiodurans* R1 to kanamycin, up to 4 μg of kanamycin per ml (R. Meima, unpublished data) and the low-level kanamycin resistance obtained with existing cassettes, 8 μg of kanamycin per ml (25). The result is a high percentage of colonies with no insert due to spontaneous resistance. Therefore, a new kanamycin cassette was constructed for *D. radiodurans*, with a kanamycin resistance marker driven by the previously characterized *D. radiodurans groESL* minimal promoter region, which contains only the σ^{70}-controlled major start site (25). This new cassette allowed screening at concentrations of kanamycin of up to 25 μg/ml, which greatly reduced the number of colonies arising from spontaneous resistance. To disrupt *sig1*, this new cassette was inserted at the unique *Eco*NI site by double-crossover allelic exchange insertion; 217 bp of DNA in the *sig1* gene are left upstream of the insertion and 563 bp are left downstream. *sig2* was disrupted by insertion of the same kanamycin resistance marker at the unique *Eco*47III site; 546 bp of DNA in the *sig2* gene are left upstream of the insertion and 299 are left downstream.

Cultures transformed to kanamycin resistance with pAKS10 and pAKS11 were screened at 20 μg of kanamycin per ml for double-crossover insertion into *sig1* and *sig2* by a diagnostic colony PCR strategy using primers flanking the gene of interest. Because *D. radiodurans* contains multiple copies of its genome (2), it has the potential to retain both wild-type and mutated copies. This PCR strategy detects allelic differences at the locus of interest, allowing us to identify clones that are homozygous for the insertion. Approximately half of the clones tested for each of the *sig1* and *sig2* insertions were heterozygous, maintaining the insertion at high copy numbers and also the wild-type gene at low copy numbers. Sequencing of the PCR products confirmed their identity. One-sixth of the clones did not contain an insertion and were presumably spontaneous kanamycin-resistant clones. However, about one-third of the clones tested were homozygously null, and their genotype was verified by Southern analysis. One representative homozygous clone for each mutation was then chosen for subsequent experiments. Single crossover recombinants were not obtained at either the *sig1*or *sig2* mutant loci, which is in contrast to our previous experience with insertion into the DR0405 locus (H. Rothfuss, unpublished).

Assessment of *sig1* and *sig2* gene essentiality. Because *E. coli rpoE* is known to be an essential ECF sigma factor gene (7) and the *D. radiodurans sig1* and *sig2* genes seem to class with ECF sigma factors, it is possible that one or both of the *sig1* and *sig2* genes may also be essential under all growth conditions. Since *E. coli rpoE* mutants require acquisition of a suppressor mutation at a second site in order to grow, they form colonies three times slower following transformation than mutants carrying the complementing gene in *trans* (7). Therefore, if *D. radiodurans sig1* and *sig2* were essential genes, we expected heterozygous clones to form larger colonies faster after transformation than homozygous null clones. However, no differences in colony size or time to colony formation were observed between homozygous null and heterozygous mutants (data not shown).

To further test the possibility of gene essentiality, we compared the efficiency of disruption of *sig1* or *sig2* to that of a nonessential amylase gene (*amyE*) (25). *D. radiodurans* cells were transformed with pAKS10, pAKS11, or pAY/K2, which disrupt *sig1*, *sig2*, and *amyE*, respectively; and efficiency of transformation was assessed. Since acquisition of a compensatory mutation depends upon the background mutation rate ($\sim 1/10^6$), we expected to observe orders of magnitude fewer transformants for pAKS10 and pAKS11 than for pAY/K2 if *sig1* or *sig2* null mutants had acquired a second site suppressor mutation to survive. However, insertion into *amyE* was only about 10-fold more efficient than insertion into *sig1* and about 5-fold more efficient than into *sig2* (Table 2). This trend reflects the length of the DNA flanking regions available for recombination in allelic exchange, a factor that has been observed to affect recombination frequencies in *D. radiodurans*. Since pAKS10 carries 300-bp flanking sequences for recombi-

TABLE 2. Comparison of insertion efficiencies into *sig1* and *sig2* versus a nonessential amylase gene (*amyE*)

Gene	Plasmid	Transformants/μg of DNA
amyE	pAY/K2	5,501 ± 370.0
sig1	pAKS10	476 ± 230.0
sig2	pAKS11	1,703 ± 967

nation into *sig1*, pAKS11 has 500-bp flanking sequences for insertion into *sig2*, and pAY/K2 carries 700-bp flanking sequences for disruption of *amyE*, the variability in transformation efficiency observed between the three insertion plasmids is consistent with the differences in flanking-region length. Taken together, these data suggest that *sig1* and *sig2* may not be essential genes, and the mutants do not appear to have acquired suppressor mutations at a second site in order to survive.

Determination of stress conditions. Since previously published studies tested only survival of UV (8), gamma irradiation (35), and oxidative stress (23, 39) in *D. radiodurans*, stress survival was first assessed under osmotic, ethanol, acid, and heat stress conditions as described in Materials in Methods. The condition that caused a 10- to 100-fold loss of survival and significantly impaired growth in wild-type *D. radiodurans* during 4 h of cultivation following a shift to stress was chosen as the stress condition in subsequent experiments with the mutant strains. These conditions were 1.2 M NaCl, 20% ethanol, 120 mM H_2O_2 (39), pH 3.7, and 48°C.

The role of *sig1* and *sig2* in stress protection. To determine the involvement of *sig1* and *sig2* in a protective stress response, the mutants were tested for growth and survival compared to the wild type under the stress conditions noted above. No growth differences between *D. radiodurans* R1 wild type and the *sig1* and *sig2* mutants were observed at 30°C (data not shown). In addition, no differences in survival between the mutants and wild type were observed under salt, oxidative, or acid stress conditions (data not shown). However, the cells defective in *sig1* exhibited impairment of survival of up to 3 orders of magnitude compared to wild type following heat shock (Fig. 1A) and up to 2 orders of magnitude after a shift to ethanol stress (Fig. 1B). Cells mutant for *sig2* were also more susceptible to heat and ethanol stress than wild-type cells but less sensitive to heat and ethanol stress than *sig1* mutants (Fig. 1). Taken together, these results suggest that both the *sig1* and *sig2* gene products are involved in survival following heat and ethanol stress. However, the *sig1* gene product seems to be more important in heat shock and ethanol stress survival than the *sig2* gene product.

Expression of heat shock genes in the *sig1* and *sig2* backgrounds. In *E. coli* and many other bacteria, a heat shock-specific sigma factor is responsible for transcription of a number of heat shock genes, including chaperone operons such as *groESL* and *dnaKJ* under heat stress conditions (42). To determine if either *sig1* or *sig2* functions similarly in the heat stress response in *D. radiodurans*, expression of the *groESL* (25) and *dnaKJ* (see Materials and Methods) promoters was assessed in the *sig1* and *sig2* backgrounds. Mutant and wild-type *D. radiodurans* strains were transformed with pRADZ3 (24) or pRADZ8 (Table 1), promoter-probe vectors carrying

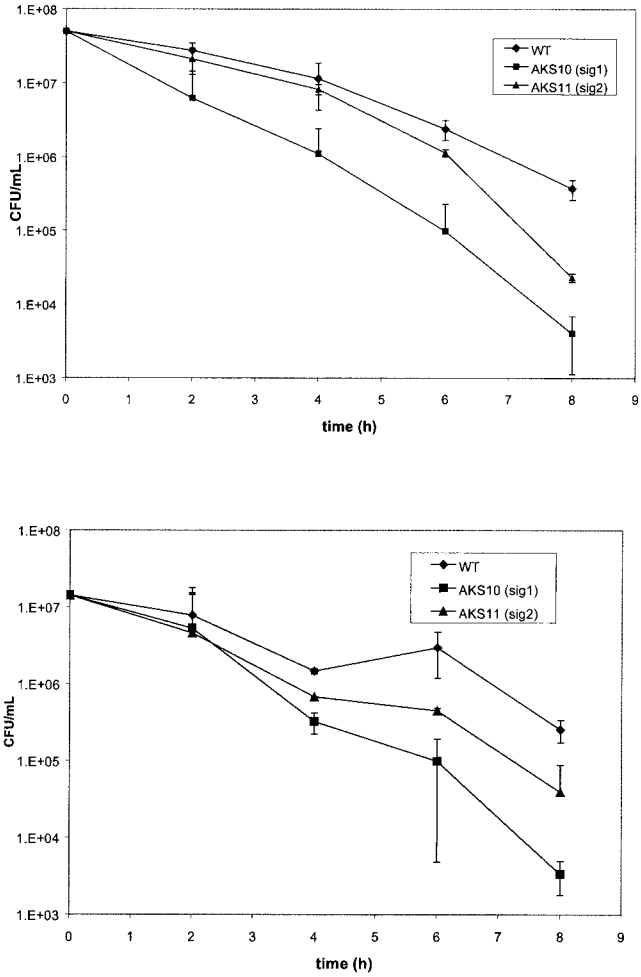

FIG. 1. Survival of wild-type *D. radiodurans* R1 and *sig1* (AKS10) and *sig2* (AKS11) mutant strains under stress conditions. (A) Survival of R1 and mutants during heat shock. (B) Survival of R1 and mutants during ethanol stress. R1, AKS10, and AKS11 were grown in TGY broth at 30°C until mid-exponential phase (optical density at 600 nm = 0.3 to 0.4) and shifted to heat shock at 48°C (time zero) or ethanol stress by the addition of 20% ethanol. Samples were removed from each culture every 2 h, and serial dilutions were plated to assess survival. Error bars represent standard deviations from the averages of at least three experiments.

promoterless *lacZ* transcriptional fusions to the *groESL* and *dnaKJ* promoters, respectively. The pRADZ1 empty vector, which encodes low β-galactosidase activity that is not induced during heat shock, was transformed into all three strains as a control. The pRADZ1 construct is present at about the same copy number as the chromosomes in *D. radiodurans* (24). Transformants were grown to mid-exponential phase at 30°C and subsequently shifted to heat shock at 40°C during mid-exponential phase to induce promoter-reporter activity. In this experiment, 40°C was used as the heat shock temperature instead of 48°C to avoid heat instability of β-galactosidase.

At 30°C, the basal level of β-galactosidase activity from the *groESL* promoter remained relatively constant (500 to 700 Miller units) in each of *D. radiodurans* R1, *sig1* and *sig2* strains throughout the course of the experiment (Fig. 2A). Within 60

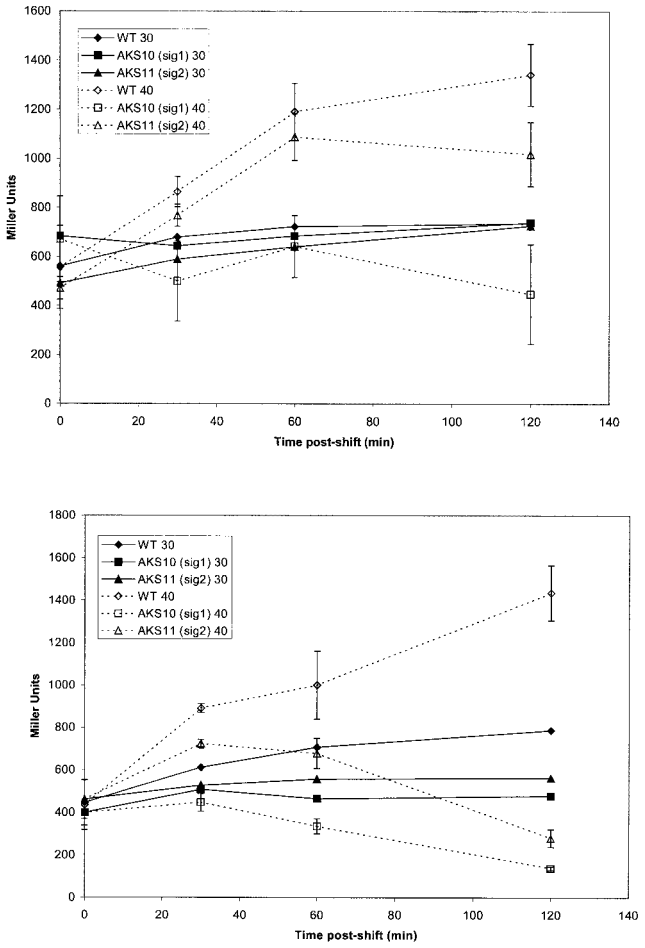

FIG. 2. Induction of β-galactosidase activity in *sig1* (AKS10) and *sig2* (AKS11) mutant and wild-type (R1) strains carrying a plasmid-borne *lacZ* transcriptional fusion to *groESL* (A) or *dnaKJ* (B). Heat shock protocol was as for Fig. 2, except the temperature was shifted to 40°C. Duplicate samples were removed at 0, 30, 60, and 120 min postinduction to assess β-galactosidase activity. Error bars represent standard deviations from the averages of three experiments. Error bars for 30°C background data points overlap (not shown).

min of shift to heat shock at 40°C, however, expression of *lacZ* from the *groESL* promoter was induced approximately twofold in R1 (wild type), after which the increase in activity slowed to reach its maximum at 2 h postinduction (Fig. 2A) (25). In contrast, *sig1* mutants grown at 40°C failed to induce *groESL* expression above the 30°C background level during the course of the experiment (Fig. 2A). Similarly, although *dnaKJ* expression in the wild type increased twofold at 40°C, *sig1* mutants were impaired for *dnaKJ* induction during heat shock (Fig. 2B).

In contrast, *sig2* mutants transformed with pRADZ3 were not impaired for induction of *groESL* compared to the wild type, exhibiting a twofold induction of *groESL* within 60 min of shift to heat stress (Fig. 2A). However, *sig2* mutants showed a slight impairment for the induction of *dnaKJ* expression during heat shock compared to the wild type (Fig. 2B).

One difficulty in interpreting these results is that the lack of induction of chaperones in the mutants might affect stability of β-galactosidase, invalidating its use as a reporter. This possibility was tested by using a control, the vector pRADZ1, which exhibits low-level constitutive expression of β-galactosidase. Activity for pRADZ1 in the *sig1*, *sig2*, and R1 wild-type strains remained constant at approximately 60 Miller units under both nonstress and heat shock conditions during the first 60 min of the experiment (data not shown). However, 2 h after a shift to heat shock, β-galactosidase activity in the mutants decreased 1.6-fold, whereas the wild type maintained activity (not shown). Similarly, although β-galactosidase activity from the *groESL* and *dnaKJ* promoters during heat shock remained at or above the 30°C background level for the first hour of the experiment in both *sig1* and *sig2* mutants, prolonged exposure ($\geq$2 h) of the mutant strains to the elevated temperature resulted in a significant reduction in enzyme activity (Fig. 2). This suggests that the results for the first hour of exposure reflect promoter activity, while the later time points reflect a combination of promoter activity and β-galactosidase instability.

Taken together, the results of the promoter-probe fusion experiments suggest that (i) Sig1 is required for the heat shock induction of the *groESL* and *dnaKJ* promoters, (ii) Sig2 is involved in *dnaKJ* but not *groESL* induction, and (iii) stability of β-galactosidase, and presumably other proteins, is compromised in both mutant strains after prolonged exposure to elevated temperatures.

ClustalW analysis of *sig1* and *sig2*. Although many ECF sigma factors are known to transcribe some cytoplasmic genes (27), the only example of an ECF family member known to transcribe cytoplasmic heat shock chaperones is SigH of *M. tuberculosis*, which controls *clpB* and *dnaK* transcription (31). It was therefore surprising that Sig1 may function as a cytoplasmic heat shock sigma factor despite its original annotation as an ECF-like sigma factor. We therefore compared the Sig1 and Sig2 amino acid sequences to those of group 3 σ^{70} family members (21) and to ECF subfamily members using the ClustalW 1.7 program (http://searchlauncher.bcm.tmc.edu) (Fig. 3). Since *D. radiodurans* is a divergent organism based upon 16S rRNA sequencing, representative sigma factor sequences were selected from a wide range of bacteria for the multiple sequence alignment. As shown in Fig. 3, the Sig1 amino acid sequence appears to cluster more closely with ECF sigma factors than sigma factors of the group 3 σ^{70} class. In addition, conservation between Sig1 and other ECF sigma factors seems highest in region 4.2, which is thought to be responsible for binding to the −35 region of promoters (19, 20, 33). These results correlate with our initial BLAST analysis, which indicated that Sig1 is 38% identical at the amino acid level to *M. tuberculosis* SigE, but found no hits to any group 3 σ^{70} factors (data not shown).

Sig1 also exhibits features typical of an ECF sigma factor subfamily member (22). First, the Sig1 sequence contains a large gap in the alignment of region 3, indicating that this region is shorter than that of group 3 σ^{70} family members (Fig. 3). Second, like other ECF factors, Sig1 lacks hydrophobic residues in region 2.3. Taken together with the BLAST results, this sequence comparison suggests that Sig1 is a true sigma factor that may be evolutionarily derived from an ECF family ancestor.

The Sig2 amino acid sequence was more divergent from the other sigma factors (Fig. 3) (28). The Sig2 sequence showed the highest number of conserved residues in region 2, which is

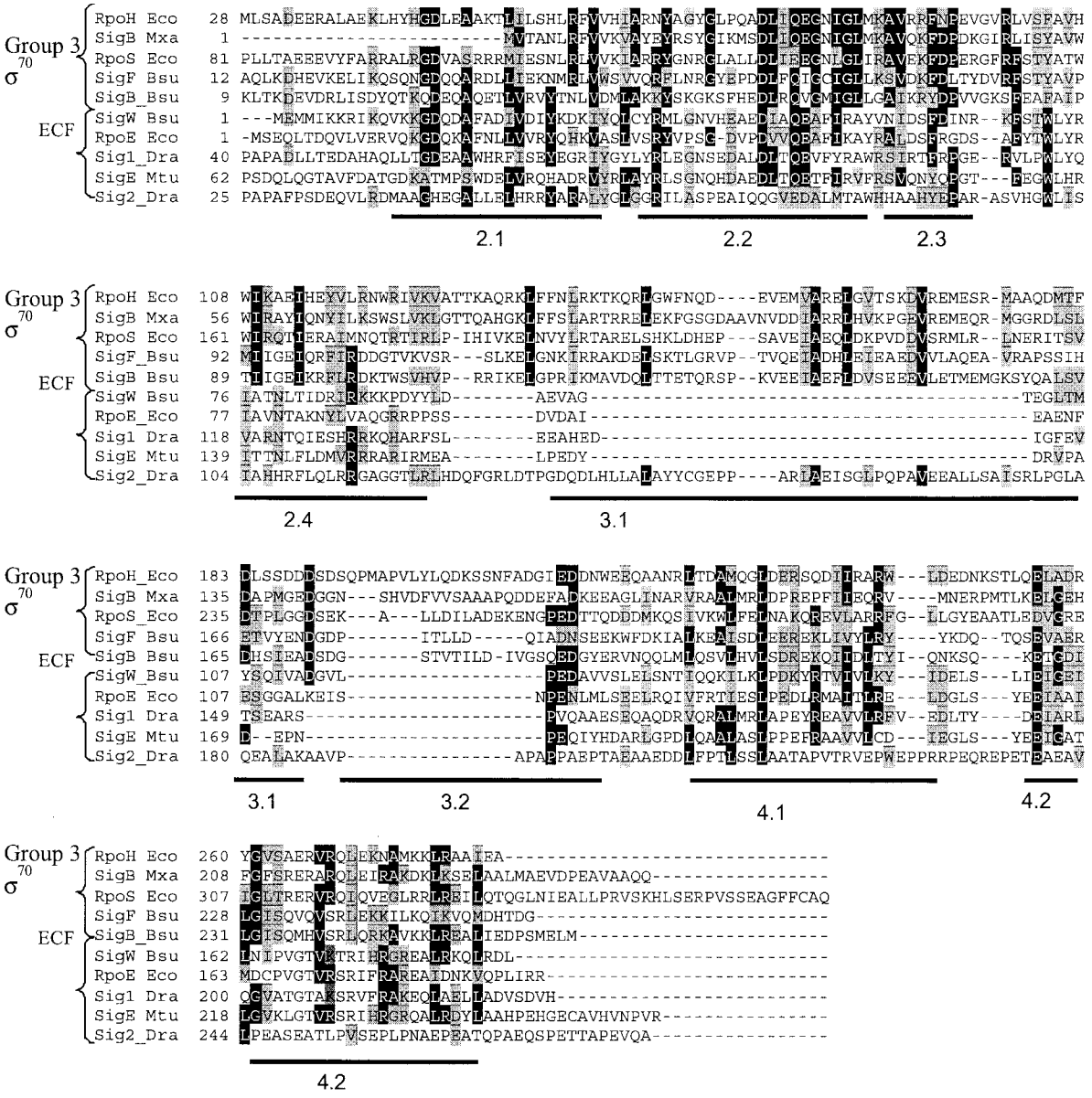

FIG. 3. ClustalW alignment of *D. radiodurans* Sig1 and Sig2 amino acid sequences with those of other characterized ECF sigma factors and group 3 σ^{70} family alternative sigma factors. Identical residues are shaded in black, similar residues are shaded in gray, and gaps are indicated by dashes. Lines and numbers below the alignment indicate conserved regions 2 through 4. Region 1 is not included because several ECF sequences lack this region. Numbers to the left of each sequence represent the amino acid residue number relative to the sequence start. Sequences for *E. coli* (Eco), *B. subtilis* (Bsu), and *M. tuberculosis* (Mtu) were obtained from the Pasteur Institute Internet genome sites, located at http://genolist.pasteur.fr. *D. radiodurans* (Dra) Sig1 (DR0180) and Sig2 (DR0804) sequences were obtained from the Institute for Genomic Research (www.tigr.org) (40). *Myxococcus xanthus* (Mxa) SigB was obtained from GenBank, accession number X55500.

responsible for sigma subunit interaction with the RNA polymerase core (38). Region 2 is also the most highly conserved among all sigma factor sequences included in the alignment. This evidence suggests that Sig2 may encode a sigma factor, but further characterization would be required to confirm this hypothesis.

DISCUSSION

Searches of the *D. radiodurans* genome have indicated that although stress response genes appear to be present, stress response regulatory systems in this bacterium are either absent or highly divergent. Therefore, a study of the role of sigma factors in stress response regulation was undertaken in the organism. Our results suggest that both of the recognizable alternative sigma factor orthologs in the genome (*sig1* and *sig2*) are involved in survival following a shift to ethanol and heat stress. In other bacteria these two stresses both involve accumulation of misfolded proteins, and so it is likely that the regulon senses and/or responds to this signal. The difference in survival between the mutants and wild type also demonstrates that *D. radiodurans* mounts a protective response towards heat

and ethanol stress, which is a novel finding, since previous reports have only focused on the protective response against DNA damage (2, 8, 23, 36, 39).

Phenotypic characterization of these mutants suggests that Sig1 is required for the positive regulation of *groESL*, *dnaKJ*, and presumably other genes whose products protect protein stability at elevated temperature. This function is similar to that of *E. coli* σ^H, which is the 32-kDa heat shock factor responsible for the transcription of heat shock chaperone and protease genes such as *groESL*, *dnaKJ*, *clpXP*, and others (11, 42). However, our ClustalW results indicate that the *D. radiodurans* Sig1 sequence is classed more distinctly with ECF sigma factors, whereas *E. coli* σ^H is known to be classed with group 3 σ^{70} factors (21) (Fig. 3). These results suggest that *sig1* may encode the major heat shock sigma factor of *D. radiodurans* responsible for the positive regulation of heat shock genes such as *groESL* and *dnaKJ* and that the Sig1 alternative sigma factor appears to have been derived from an ECF family ancestor. The only other example of such a system is in *M. tuberculosis*, a high-G+C gram-positive organism in which an alternative sigma factor transcribes classical chaperones such as *dnaK* and *clpB* although it belongs to the ECF family (31). However, here we present the first example of an ECF-like sigma factor involved in the transcription of *groESL*. Together, these ECF-derived alternative sigma factors may represent a novel class of heat shock regulators among high-G+C organisms. Although no obvious ECF consensus was found in the *groESL* or putative *dnaKJ* promoter sequences, ECF promoters tend to be more conserved within an organism than between organisms (6, 17, 27, 31). In addition, *D. radiodurans* promoters in general appear to be divergent, and a consensus among any promoter type remains unclear (25).

In contrast to *sig1* mutants, *sig2* mutants exhibit heat and ethanol survival that are only moderately impaired relative to those of the wild type and the level of *dnaKJ* heat shock induction is intermediate between that of wild type and the *sig1* mutants. Sig2 was also shown not to be involved in *groESL* regulation. Our ClustalW results indicate that the Sig2 sequence is more divergent and is not closely related to either ECF factors or group 3 σ^{70} factors (28). Our results so far suggest that the *sig2* gene product may be a divergent putative sigma factor that plays a more minor role in protection against heat and ethanol stress in *D. radiodurans.*

It is surprising that only two alternative sigma factor orthologs were found in the genome, since most other bacteria with a similar genome size contain considerably more sigma factor genes. It is also surprising that the two alternative sigma factor orthologs in *D. radiodurans* appear to protect against the same stress. It is possible that other, more divergent sigma factor orthologs that are involved in protection against stresses other than heat and ethanol stress are present in the genome, but this is unlikely because so far sigma factors are well conserved throughout the bacterial domain (21). The *D. radiodurans* genome was searched with conserved regions 2 and 4 of *sig1* and *sig2*, but no other genes with significant identity to these regions were found. Because genes encoding other known regulators of stress response, such as *soxRS*, *rpoS*, and *ompR*, are not recognizable in the *D. radiodurans* genome, it remains to be determined how other stresses are regulated in the organism.

We performed essentiality tests on *sig1* and *sig2* by comparing transformation efficiencies to those of a nonessential gene. Our results are suggestive that *sig1* and *sig2* are not essential under all conditions, but definitive proof would require transformation of the mutating vector in the presence and absence of a wild-type gene in *trans*. Attempts to perform this experiment were not successful due to interference by the highly efficient recombination system of *D. radiodurans* (data not shown). In addition, no tightly regulated inducible promoter is available with which to titrate gene activity in *D. radiodurans*. Therefore, definitive proof of gene essentiality in this system awaits more amenable genetic tools.

During the course of the phenotypic characterization of the sigma mutants, it was observed that *D. radiodurans* R1 survived better than many other organisms during stress. For example, *D. radiodurans* R1 loses only 10-fold survival in 20% ethanol and 1.2 M NaCl during 8 h of cultivation. In contrast, *B. subtilis* 168 survival has been reported to decrease up to 100-fold in 9% ethanol over 4 h and 150-fold in 200 mM salt over 3 h (37). In addition, *B. subtilis* shows a 100-fold loss of survival within 2 h of a shift from pH 7 to pH 4.3. *D. radiodurans*, on the other hand, can withstand pH 3.7 for 4 h with only a 10-fold loss of survival (data not shown). Out of the stress conditions we have tested, heat shock is the only stress during which *D. radiodurans* exhibits susceptibility typical for soil bacteria (37). From these observations, we conclude that *D. radiodurans* is extremely resistant to most environmental stress conditions compared to other soil organisms, such as *B. subtilis*. The lack of recognizable stress response regulators in the *D. radiodurans* genome sequence suggests that *D. radiodurans* may employ novel mechanisms for stress survival and represents a new model organism for understanding the diversity of stress response regulation in bacteria.

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