HucR, a novel uric acid-responsive member of the MarR family of transcriptional regulators from Deinococcus radiodurans

Steven P. Wilkinson  
*Louisiana State University*

Anne Grove  
*Louisiana State University*

Follow this and additional works at: [https://digitalcommons.lsu.edu/biosci_pubs](https://digitalcommons.lsu.edu/biosci_pubs)

**Recommended Citation**

Wilkinson, S., & Grove, A. (2004). HucR, a novel uric acid-responsive member of the MarR family of transcriptional regulators from Deinococcus radiodurans. *Journal of Biological Chemistry, 279* (49), 51442-51450. [https://doi.org/10.1074/jbc.M405586200](https://doi.org/10.1074/jbc.M405586200)

This Article is brought to you for free and open access by the Department of Biological Sciences at LSU Digital Commons. It has been accepted for inclusion in Faculty Publications by an authorized administrator of LSU Digital Commons. For more information, please contact [ir@lsu.edu](mailto:ir@lsu.edu).
HucR, a Novel Uric Acid-responsive Member of the MarR Family of Transcriptional Regulators from *Deinococcus radiodurans*†

Steven P. Wilkinson‡ and Anne Grove§

From the Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana 70803

The MarR family of transcriptional regulators comprises a subset of winged helix DNA-binding proteins and includes numerous members that function in environmental surveillance of aromatic compounds. We describe the characterization of HucR, a novel MarR homolog from *Deinococcus radiodurans* that demonstrates phenolic sensing capabilities. HucR binds as a homodimer to a single site within its promoter/operator region with $K_d = 0.29 \pm 0.02 \text{ mM}$. The HucR binding site contains a pseudopalindromic sequence, composed of 8-bp half-sites separated by 2 bp. The location of the HucR binding site in the intergenic region between hucR and a putative uricase suggests a mechanism of simultaneous co-repression of these two genes. The substrate of uricase, uric acid, is an efficient antagonist of DNA binding, reducing HucR-DNA complex formation to 50% at 0.26 mM ligand, compared with 5.2 and 46 mM for the aromatic compounds salicylate and acetylsalicylate, respectively. Enhanced levels in vivo of hucR and uricase transcript and increased uricase activity under conditions of excess uric acid further indicate a novel regulatory mechanism of aromatic catabolism in *D. radiodurans*. Since uric acid is a scavenger of reactive oxygen species, we hypothesize that HucR is a participant in the intrinsic resistance of *D. radiodurans* to high levels of oxidative stress.

Members of the MarR family of winged helix transcriptional regulators control a variety of biological functions in bacteria and Archaea (1). Several transcriptional activators have been identified in this family, but the majority of MarR homologs are transcriptional repressors (2, 3). A number of bacterial MarR proteins regulate environmental stress responses and the expression of pathogenic factors. For example, MexR represses the MexAB-OprM operon of *Pseudomonas aeruginosa*, an operon that encodes a tripartite efflux system responsible for this organism’s intrinsic resistance to multiple antibiotics (4, 5). PecS from *Erwinia chrysanthemi* regulates pectinase and cellulase production, the main virulence determinants of this plant pathogen, and the synthesis of indigoide, an apparent scavenger of reactive oxygen species (6, 7). Other members of the MarR family regulate the catabolism of aromatic compounds, such as HpaR, which mediates the catabolism of 4-hydroxyphenylacetic acid in *Escherichia coli*, and HpcR, which regulates homoprotocatechuate catabolism (8, 9). Autoregulation is a characteristic of a number of MarR proteins, including the operon repressors EmrR and MarR, and several homologs that are not encoded in the context of a contiguous operon, such as MexR and HpaR (8, 10–12).

Phenolic ligands have been shown to regulate gene expression by negatively affecting interactions of MarR homologs with their cognate promoter/operator regions. Repressor activity of EmrR on the multidrug resistance operon, emrRAB in *E. coli*, is antagonized *in vitro* by sodium salicylate and a variety of structurally unrelated phenolic drugs that are putative ligands of the multidrug pump encoded by this operon (10, 13). The affinity of CinR, a repressor of a cinnamoyl ester hydrodrolase from *Butyryrivibrio fibrisolvens* E14, for its binding region is reduced *in vitro* by cinnamic acid derivatives (14). HpcR repression of homoprotocatechuic metabolic genes and HpaR repression of the hpa-meta operon are relieved by the respective aromatic substrates of these catabolic pathways (8, 9). Similarly, the regulators of benzoic acid and 3-chlorobenzoate catabolism, BadR and CbaR, respectively, respond to the aromatic substrates of their pathways (2, 15).

The prototypical member of this protein family, MarR from *E. coli*, negatively regulates the marRAB operon, the expression of which confers an intrinsic phenotypic resistance to structurally diverse antibiotics, organic solvents, oxidative stress agents, and household disinfectants (11, 16, 17). MarR binds as a homodimer to two sites on the operator/promoter (marO) of this operon with an apparent $K_d$ of $\sim 1\text{ mM}$, with site I partially overlapping the $-35$ and $-10$ regions of the promoter and site II overlapping the putative ribosome binding site (18). A range of phenolic compounds induce marRAB transcription *in vivo*, including sodium salicylate, cinnamate, 2,4-dinitrophenol, acetaminophen, sodium benzoate, tetracycline, and chloramphenicol, and a subset of these, as well as plumbagin and menadione, antagonize DNA binding activity of MarR in vitro (16, 19–21). Sodium salicylate has been shown to bind MarR with an apparent $K_d$ value of $0.5–1\text{ mM}$ (18). The 2.3-Å crystal structure of MarR bound to sodium salicylate revealed two binding sites per monomer for this inducer, with each site flanking the putative recognition helix of the DNA binding motif (22).

The heterotrophic, mesophilic bacterium *Deinococcus radiodurans* is best known for its resistance to high levels of ionizing and UV radiation, bulky chemical adducts, and other agents that damage DNA (23–25). Genomic analysis revealed that *D. radiodurans* also encodes orthologs of nearly every known bacterial stress response protein, suggesting mechanisms of resistance to osmotic, temperature, pH, starvation, toxin, phage, dessication, antibiotic, and oxidative stresses (25, 26). The presence of two genes encoding MarR homologs, *dr1159* and *dra0248* (TIGR gene annotation), within the genome sug-
HucR, a Novel Uric Acid-responsive MarR Homolog

HucR, a novel uric acid-responsive MarR homolog, was purified and characterized. The expression and purification of HucR were performed using a bacterial expression system. HucR was overexpressed in E. coli TOP10 (Invitrogen). Purity was verified by SDS-PAGE and Coomassie staining. HucR concentration was determined spectrophotometrically.

**Experimental Procedures**

Cloning, Overexpression, and Purification of HucR—D. radiodurans R1 was kindly provided by J. Battista, and genomic DNA was isolated as described (27). PCR amplification of the hucR ORF (dr1159) from the genome was achieved using primers HucR-fwd (5′-GCT CTT GGT TAT CAT ATG TCA GCC CCC-3′), which introduced an Ndel site (boldface type) overlapping the first codon (underlined), and HucR-rev (5′-CTT TCC CAG AAT G-3′), which introduced an EcoRI site (boldface type) downstream of the hucR stop codon. The resulting 589-bp PCR product was cloned into pGEM5Zf(+) (Promega). The resulting plasmid pSPW1 was transferred into E. coli BL21 (DE3) (pLysS). Cultures were grown in LB containing 100 µg/ml ampicillin at 37 °C to an A600 of 0.5, and HucR overexpression was induced with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside for 1 h. Cells were pelleted at 4 °C and stored at −80 °C. All subsequent steps were carried out at 0–4 °C. Cells were resuspended in lysis buffer (50 mM Tris-HCl (pH 8), 25 mM NaCl, 5% glycerol, 13 mM KCl). Ellipticity measurements were performed in a total volume of 10 µl in Binding Buffer (20 mM Tris-HCl (pH 8), 0.1 mM EDTA, 0.075% BRIJ58, 1.25 mM Na2EDTA, and 4% (v/v) glycerol). Protein-DNA complexes were equilibrated at 22 °C for 1 h. A non-denaturing 8.5% polyacrylamide gel was prerun for 30 min in 0.5× TBE buffer (45 mM Tris borate (pH 8.3), 1.25 mM Na2EDTA), and samples were loaded with the power on. After 1.25 h of electrophoresis, the gels were dried, and protein-DNA interactions were analyzed by phosphorimaging using a STORM 840 PhosphorImager (Molecular Dynamics) and analyzed with TELA software.

**Circular Dichroism Spectroscopy—CD spectroscopy was performed**

Circular Dichroism Spectroscopy—CD spectroscopy was performed on an Aviv 202 CD spectrophotometer. HucR was diluted to 0.2 mg/ml in CD buffer A (20 mM potassium phosphate (pH 7), 34 µM EDTA, 0.8% glycerol, 13 mM KCl). Ellipticity measurements were performed in triplicate at 25 °C using a quartz cuvette with a 0.1-cm path length. Measurements were made at 1-nm steps over the wavelength range from 250 to 190 nm and were corrected for buffer contributions to the signal. HucR secondary structure composition was calculated using the secondary structure algorithm CDSSTR and protein reference set 7 provided by the authors (28–32). The goodness of fit was determined from the NRMSD value of 0.013.

For measurement of thermal stability, HucR was diluted to 0.05 mg/ml in CD buffer B (20 mM potassium phosphate (pH 7), 8 µM EDTA, 0.2% glycerol, 3 mM KCl), and measurements were making using a quartz cuvette with a 1-cm path length. Ellipticity readings from 230 to 200 nm (1-nm steps) were taken over the temperature range 19–70 °C, with steps of 3 °C (19–37 and 61–70 °C) or 1.5 °C (37–61 °C). Each sample also underwent a reverse scan from 67 to 19 °C. 3 min was allowed for thermal equilibration after each step. Wavelength scans from 240 to 200 nm were performed at 19 and 70 °C to verify the native and denatured states of HucR, respectively. CD measurements of HucR melting were performed in triplicate and corrected for buffer contributions to the signal. Calculation of the Tm of HucR was based on ellipticity measurements from 224 to 220 °C. Temperature-dependent ellipticity values were plotted at each wavelength and fit to a two-state model for protein unfolding (33). The Tm of HucR is reported as the average ± S.D.

**HucR-hucO Stoichiometry and Affinity Determination—Primer**

For binding assays under stoichiometric conditions, 0.1 µM 32P-labeled hucO was titrated with HucR up to 0.35 µM, in a total reaction volume of 10 µl in Binding Buffer (20 mM Tris-HCl (pH 8), 0.1 mM EDTA, 0.075% BRIJ58, 1.25 mM Na2EDTA, and 4% (v/v) glycerol). Protein-DNA complexes were equilibrated at 22 °C for 1 h. A non-denaturing 8.5% polyacrylamide gel was prerun for 30 min in 0.5× TBE buffer (45 mM Tris borate (pH 8.3), 1.25 mM Na2EDTA), and samples were loaded with the power on. After 1.25 h of electrophoresis, the gels were dried, and protein-DNA interactions were analyzed by phosphorimaging using a STORM 840 PhosphorImager (Molecular Dynamics) and analyzed with TELA software.

HucR was added last to the binding reactions, at a final concentration of 1 nM. Methidiumpropyl-EDEA (MPE)-Fe(II) and DNase I Footprinting—Complementary 77-mer oligonucleotides were gel-purified, and the top strand was 5’-end-32P-labeled with T4-polynucleotide kinase. Annealing of the oligonucleotides was accomplished by slow cooling from 95 °C to 20 °C in a total volume of 10 µl and included 500 fmol of DNA in modified Binding Buffer with 0.06% BRIJ58, 20 µg/ml BSA, and 1.5% glycerol. Protein-DNA complexes were equilibrated for 1 h at 22 °C. For MPE-Fe(II) footprinting, 1 µl of 10 mM sodium ascorbate was added, followed by 2 µl of MPE mix (25 µM MPE, 25 µM Fe(NH4)2(SO4)2), and digestion was allowed to proceed for 2 min at room temperature. DNase I footprinting samples were incubated with 10–3 to 10–2 units of DNase I (Epicentre) for 20 s at room temperature. Digestion was terminated by phenol-chloroform extraction, and samples were ethanol-precipitated. A/G chemical sequencing ladders were generated according to Sambrook et al. (34). DNA fragments were separated on a 12% polyacrylamide sequencing gel. Density profiles were obtained by phosphorimaging.

**Ligand Binding Assays—A 180-bp sequence spanning bp −158 to +22 relative to the hucO transcriptional start site (bp −76 to +104 relative to the predicted dr1160 transcriptional start site) was amplified, gel-purified, and 32P-labeled with T4-polynucleotide kinase. For each ligand assayed, binding conditions were established to buffer pH effects from the compound. Binding reactions were assembled in Binding Buffer with 0.1 mM DNA, 0.75 mM HucR, and up to 25 mM sodium ascorbate. EMSAs were also performed without BSA in the Binding Buffer with no detectable effect. For acetylsaliclyate assays, acetylsaliclyate was dissolved in ethanol, and binding reactions were assembled in modified Binding Buffer with 400 mM Tris-HCl (pH 8) and 20% ethanol with up to a 25 mM concentration of the compound. Uric acid was dissolved in 0.35 mM NaOH to a concentration of 125 mM. Binding reactions were assembled in modified Binding Buffer with 500 mM Tris-HCl (pH 8), 0.05% BRIJ58, 7.5 µg/ml BSA, and 0.6% glycerol with

The abbreviations used are: EMSA, electrophoretic mobility shift assay; BSA, bovine serum albumin; MPE, methidiumpropyl-EDEA; ORF, open reading frame.
HucR, a Novel Uric Acid-responsive MarR Homolog

Fig. 1. The genetic organization of dr1159 and adjacent ORFs. The relative orientations of the ORFs are represented by open arrows. HucR is encoded by dr1159, dr1160 encodes a hypothetical uricase, and dr1161 encodes a transthyretin-like protein. An intergenic region of 101 bp separating hucR and dr1160/dr1161 contains putative -10 and -35 promoter elements for each gene, designated by bars above (dr1160/dr1161) or below (dr1159) the sequence. Predicted transcription and translation start sites are identified by asterisks and arrows, respectively. The shaded box marks the HucR binding site. Each half-site of the pseudopalindromic sequence within the binding site is identified in boldface type.

up to 20 mM uric acid. Reactions were equilibrated for 1 h at 22 °C, and protein-DNA complexes were analyzed by EMSA as described above. All experiments were carried out in triplicate. Data were fit to a single exponential equation: normalized fractional complex formation = e^{At} + B, where k is the exponential decay constant, and L is the ligand concentration.

RNA Dot Blot Hybridization—10 μl of an overnight culture of D. radiodurans was added to 5 ml of TGY broth (0.5% tryptone, 0.3% yeast extract, 0.1% glucose) or 5 ml of TGY broth supplemented with 10 mM uric acid. Before inoculation, the pH of broths containing uric acid was adjusted to that of TGY broth. Cultures were grown at 30 °C to an A600 of 0.45, and cells were harvested by centrifugation at 4 °C for 10 min and stored at −80 °C. Pellets were resuspended in 3 ml of 95% ethanol and held at 4 °C for 10 min and harvested by centrifugation at 4 °C at 12,000 × g for 10 min. Total RNA was prepared as described (35). DNA contamination was removed by digestion with 1 μg of RNase-free DNase I (Epicentre) at 37 °C for 1 h.

RNA dot blots were performed essentially as described (35). For each RNA sample, 15 μg was adhered to a (+)-charged nylon membrane using a Bio-Dot microfiltration apparatus (Bio-Rad) and cross-linked using a Stratalinker UV cross-linker (Stratagene) at 120,000 μl/cm² for 50 s. Oligonucleotide probes complementary to the dr1160 transcript (+30 to +49 relative to the predicted start site of translation) and to the hucR transcript (+21 to +40 relative to the start site of translation) were used. Since groESL (dr0606–0607) expression in D. radiodurans has been shown to be constitutively expressed under different growth conditions, a probe was designed complementary to residues +310 to +330 of groES to serve as a normalizing factor for RNA samples (36). The probes were 32P-labeled with T4-polynucleotide kinase, separately hybridized to the cross-linked membranes overnight and washed under moderate conditions, as described (35). The densitometric count obtained with the dr1160 or hucR probe for each RNA sample was normalized by multiplying by the ratio of sample 32P-labeled groES counts to control RNA 32P-labeled groES counts. RNA dot blot analysis was performed in duplicate from separate RNA preparations. The levels of hucR and dr1160 transcript from cells grown in 10 mM uric acid are reported as the mean ± S.D. relative to the transcript level from control cells.

Analysis of Uricase Activity—D. radiodurans was grown in TGY or TGY + 10 mM uric acid as described above. Cell pellets were resuspended in chilled HAP buffer, pH 7.5, without 2-mercaptoethanol and lysed by sonication. Insoluble material was removed by centrifugation at 4 °C. Protein concentrations of whole cell lysates were determined from a BSA standard using the modified Lowry protein assay (Pierce). Uricase activity in the whole cell lysate was analyzed using an Amplex® Red Uric Acid/Uricase Assay Kit (Molecular Probes, Inc., Eugene, OR). Each reaction included 45 μl of whole cell lysate. Uricase activity is normalized to the activity recorded for cells grown in the absence of added uric acid and is reported as the mean ± S.D. (n = 5).

RESULTS

The hucR Regulatory Region—Locus dr1159 in chromosome I of D. radiodurans encodes a putative 181-residue, 19.7-kDa MarR homolog. An intergenic region of 101 bp separates dr1159 from the oppositely oriented ORF of a hypothetical uricase (dr1160) (Fig. 1). Pairwise alignment of the predicted protein product of dr1160 with a characterized uricase from Bacillus subtilis indicates 29.5% identity and 54% similarity, suggesting that the D. radiodurans homolog is functional (37). Uricase catalyzes the conversion of uric acid into allantoin during purine catabolism, and its activity is correlated with oxidative stress response in mammals (38, 39). A downstream locus, dr1161, encoding a hypothetical, transthyretin-like protein is oriented in the same direction as dr1160, with the ORFs overlapping by one codon. A study of D. radiodurans promoter elements indicated a strong similarity to the E. coli σ70 consensus –10 and –35 regions among the promoters surveyed (40). We identified σ70-like promoter elements for dr1159 and dr1160 in the sequence between these two genes. These promoter regions are partially overlapping, with the putative transcription initiation site of each gene positioned in the Pribnow box of the other. The spacing and orientation of the hypothetical MarR homolog encoded at locus dr1159, relative to the ORFs of dr1160 and dr1161, suggested a potential regulatory design analogous to systems observed with other MarR proteins, such as MexR from P. aeruginosa and HpaR from E. coli (8, 12). We were therefore prompted to designate dr1159 as hucR (for hypothetical uricase regulator).

Sequence and Structural Analysis of HucR—Pairwise alignment of the amino acid sequence of HucR with the prototype of this family, MarR, reveals 29% identity and 49% similarity between these two proteins. Pairwise alignment with other characterized MarR regulators shows that HucR shares higher homology with EmrR and MexR, demonstrating 34% identity with each and 55 and 49% similarity with these repressors, respectively. Multiple sequence alignment of HucR with eight representative MarR homologs reveals seven identical residues in the C-terminal half of these proteins (Fig. 2). Five of these sites (residues 118, 124, 126, 132, and 133) occur within the β-sheet and turn structural elements of MarR that form the “wing 1” motif, and a sixth identical residue lies in an α-helix immediately adjacent to this region (22, 41). The wing 1 structural element of the winged helix motif flanks the DNA recognition helix and has been shown to make direct contacts with DNA, in either the minor or major groove (41). Of the 14 residues identified from the MarR crystal structure to form the hydrophobic core of the monomeric DNA binding domain, 10 are conserved or identical in the MarR homologs analyzed (22). Direct comparison of HucR and MarR reveals that five of these residues are identical and an additional six are conserved.

The residues in the MarR monomer that were shown to make contacts with the two salicylate ligands are highly conserved in HucR. In ligand binding site “A” of MarR, Thr104 hydrogen-bonds with the salicylate hydroxyl, Arg118 hydrogen-bonds with the salicylate carboxylate, and Pro89 is located within 3.5 Å of the unsubstituted side of the salicylate ring. In HucR, Arg118 and Pro89 are conserved, whereas serine replaces threonine at position 104. Arg109 in MarR, which is conserved in HucR, hydrogen-bonds to the salicylate carboxylate group in...
site “B” of MarR. Val₁²⁸ of MarR is positioned 3.6 Å above the salicylate ring in site “A” of the adjacent half of the dimer. This valine is replaced by alanine in HucR.

The coding region of hucR was cloned into a pET5a expression vector, and the product, HucR, was purified to greater than 95% homogeneity, as revealed by SDS-PAGE (Fig. 3). The far-UV circular dichroism spectrum from 250–190 nm was recorded at 25 °C to determine the secondary structure composition of HucR (Fig. 4). The α-helical and β-sheet content for HucR was calculated to be 47 and 10%, respectively, with 17% turns and 25% random coil (28–32). In comparison, the crystal structure of MarR revealed its composition to be 58% helical and 10% β-sheet (22). The MexR crystal structure also revealed a large α-helical content and a relatively small β-sheet contribution (42). Ellipticity measurements at five wavelengths spanning the negative ellipticity maximum characteristic of α-helices (220–224 nm) were recorded over the temperature range of 19–70 °C and plotted to measure protein denaturation (Fig. 4B). From the CD melting curve, the Tₘ of HucR was calculated to be 51.1 ± 0.0 °C. HucR did not refold during the reverse temperature scan, so ΔHₒ values for folding transitions could not be determined.

Binding of HucR to Its Promoter/Operator Region—Given the prevalence of autoregulatory MarR transcription factors, we biochemically assessed HucR for such a capacity. To test the affinity of HucR for its promoter/operator region (hucO), we amplified a 241-bp region of the D. radiodurans genome, extending from 180 bp upstream of the putative hucR translation start site to 61 bp within the coding region. EMSA revealed HucR to have high affinity for hucO, with an apparent Kₛ of 0.29 ± 0.02 nm (Fig. 5) and a concomitant ΔGₐₚₚ of −12.9
HucR, a Novel Uric Acid-responsive MarR Homolog

Fig. 4. CD spectral analysis of HucR. A, far-UV CD spectrum of HucR at 25 °C. Ellipticity measurements are expressed in machine units (millidegrees). B, thermal unfolding transition of HucR. Ellipticity measurements were collected over the temperature range of 19–70 °C at five wavelengths spanning the negative ellipticity maximum characteristic of α-helices: 220 nm (●), 221 nm (■), 223 nm (□), 225 nm (▲), and 224 nm (●).

kcal/mol. The single complex observed and the goodness of fit to a single-site binding polynomial suggested that HucR binds hucO at a single site. The specificity of this interaction was verified by the inability of pGEM5 to compete for HucR binding (Fig. 5B). Complex formation between 1 nM HucR and 0.1 nM 32P-labeled hucO was reduced to half-saturation at ~1 nM unlabeled hucO, whereas only a minor reduction in complex formation was elicited by 10 nM pGEM5. Since pGEM5 contributes an approximately 12-fold greater molar excess of base pairs than hucO, the competition assay, in conjunction with footprinting analyses (see below), unequivocally demonstrates site-specific binding of HucR and hucO. From EMSA performed under stoichiometric conditions, HucR was shown to bind hucO at a ratio of 1:85:1, suggesting that HucR binds its promoter/operator region as a dimer (Fig. 5D). This finding is consistent with crystallographic and biochemical analyses of other MarR homologs which have also been shown to form homodimers (13, 22, 42).

Methodimpropryl-EDTA-Fe(II) (MPE-Fe(II)) and DNase I footprinting was performed using the top strand of a 77-bp hucO fragment. MPE-Fe(II) footprinting revealed the HucR dimer to protect 21 bp of hucO spanning the region from −19 to +3 relative to the putative uricase (dr1160) transcriptional start site (Fig. 6). The footprint was extended by partial protection of bp −20 and −21. The footprint generated by DNase I (Fig. 6) shows protection from −18 to +6, relative to the uricase transcriptional start site. Analysis of the DNase I footprint also revealed sites of hypersensitive cleavage flanking each end of the HucR dimer binding site (+8, +7, and −19), suggesting that HucR distorts hucO upon binding. Winged helix proteins from the MarR family characteristically bind as dimers to inverted repeat sequences in their cognate recognition sites (12). Footprinting analysis revealed the HucR dimer binding site within hucO to contain an imperfect 8-bp inverted repeat, with 2 bp separating each half of the palindrome (Fig. 1).

Ligand Binding Assays—Members of the MarR family of transcriptional regulators are natural phenolic sensors and hence play critical roles in environmental surveillance. Given the significant conservation in HucR of residues that are involved in binding salicylate in MarR, we tested this anionic lipophilic compound as a potential ligand of HucR. Normalized fractional HucR-hucO complex formation was analyzed as a function of sodium salicylate concentration (Fig. 7). Sodium salicylate antagonized the binding of HucR to hucO, with a 5.2 mM concentration of the compound reducing the normalized fractional saturation to 0.5. At 25 mM sodium salicylate, the fraction of complexed hucO approached zero. Since BSA is known to bind salicylate, it was possible that, due to the BSA in the binding reaction, the antagonistic effect of salicylate on HucR-hucO interaction was greater than we observed. However, removal of BSA from the binding reactions resulted in no observable change in the role of salicylate as a negative effector of HucR (data not shown).

Acetylsalicylate was suggested to induce transcription of the marRAB operon in vivo, yet in vitro analysis suggested that acetylsalicylate does not bind MarR (16, 18). Acetylsalicylate caused a gradual decrease in complexed hucO with increasing drug concentration; however, even at 25 mM acetylsalicylate, the normalized fractional saturation of hucO remained at ~0.6. Approximately 46 mM acetylsalicylate is required to reduce the fractional saturation to 0.5. Therefore, an acetyl group esterified to the 2-hydroxyl of salicylate decreases by 9-fold the negative effector capacity of the ligand.

The apparent phenolic sensing capability of HucR and its high affinity for a site within the promoter/operator region of a putative uricase (Fig. 1) suggested a potential catabolic regulatory system in which HucR regulation of uricase expression is responsive to levels of uric acid, the substrate for this enzyme. Like salicylic acid, uric acid is a planar, aromatic compound but consists of two conjugated ring systems. As seen in Fig. 7, uric acid is a potent regulator of HucR; the normalized fraction of complexed hucO approached zero at ~1 mM uric acid, with 0.26 mM uric acid being sufficient to reduce the normalized fractional complex to 50%. Uric acid is therefore an ~20-fold stronger antagonist of HucR-hucO interaction than salicylate and over 175-fold stronger than acetylsalicylate. This pronounced attenuation of complex formation elicited by uric acid, relative to the effects observed from the other aromatic compounds, suggests that this compound is the natural ligand of HucR.

In Vivo Analysis of Gene Regulation by HucR—The in vitro investigations described above are consistent with a model in which transcription of hucR and dr1160 (putative uricase) is regulated by the high affinity interaction of HucR in the intergenic region. The dramatic reduction in HucR-hucO affinity induced by uric acid suggests that this compound would weaken HucR-mediated repression. Furthermore, the homology between the hypothetical enzyme encoded by dr1160, the only putative uricase in the D. radiodurans genome, and the characterized uricase from B. subtilis suggests that this gene encodes a functional enzyme (37). We investigated this model through a combination of RNA dot blot hybridization and uricase activity experiments.

Transcript levels of hucR and dr1160 were compared from D. radiodurans grown in the presence versus absence of 10 mM uric acid (Fig. 8A). RNA dot blot hybridization of a 32P-labeled transcript probe to total RNA isolated from wild-type D. radiodurans grown under conditions of uric acid induction and repression revealed the expected twofold increase in transcript levels of hucR. Similarly, no measurable difference in the transcript levels of dr1160 was observed.
probe complementary to the sense strand of hucR revealed a higher level of transcript in cells grown in 10 mM uric acid, at a ratio of 1.6 ± 0.1 relative to control cells. Hybridization of a probe complementary to the sense strand of dr1160 revealed up-regulation of the putative uricase in the cells grown in 10 mM uric acid, at a ratio of 1.6 ± 0.0 relative to control cells.

A coupled enzymatic assay was performed to assess uricase activity in protein from whole cell lysate of cells grown in the presence versus absence of 10 mM uric acid (Fig. 8B). The assay links the activity of uricase from the samples to the activity of horseradish peroxidase, producing resorufin, which has an absorption maximum near 560 nm. Uricase activity was detected in cells supplemented with or without uric acid, suggesting that dr1160 does indeed encode a functional uricase. Uricase activity was 1.5 ± 0.0 times higher in cells grown in the presence of 10 mM uric acid compared with its absence. The enhanced levels of hucR and dr1160 transcript and the elevated uricase activity in cells grown in excess uric acid are consistent with the in vitro ligand binding studies with HucR and strongly support a model in which transcription of these two divergent genes is derepressed by the effector uric acid.

**DISCUSSION**

The identification of MarR homologs within the *D. radio-
durans* genome suggested the presence of uncharacterized reg-
ulatory systems responsive to phenolic compounds within this
stress-resistant microorganism. In this study, we demonstrate that HucR binds with very high affinity ($K_d = 0.29 \pm 0.02 \text{nM}$) at a single site in its regulatory region (Figs. 5 and 6). In comparison, *E. coli* MarR binds its cognate site with an apparent $K_d$ of $1 \text{nM}$, and the winged helix protein, Ptr1, binds its cognate sites with apparent $K_d$ values of $1–2 \text{nM}$ (18, 43).

Winged helix proteins from the MarR family characteristically bind to sites containing palindromic or pseudopalindromic sequences. HpaR, for example, binds to sequences possessing two 9-bp half-sites separated by 2 bp, and CinR binds to a 16-bp palindrome composed of two adjacent 8-bp half-sites (8, 14). The binding site of EmrR contains an imperfect 9-bp inverted repeat with each half-site separated by 5 bp (10). The binding site for HucR contains an imperfect 8-bp inverted repeat, with each half-site separated by 2 bp (Figs. 1 and 6). The center of each half-site of the palindrome is therefore separated by 10 bp, thus positioning the binding site for each half of the HucR homodimer on the same face of the double helix. This contrasts with the binding site of MarR, which contains two inverted 5-bp sequences separated by 2 bp, thus positioning the half-sites on different faces of the double helix (18). In the case of MexR, the 5-bp inverted repeat sequences are separated by 5 bp, which would orient the major groove of each half-site on the same face of the DNA helix (12). There is variation within the winged helix family in the mode of DNA binding, but the recognition helix typically makes most of the sequence-specific contacts within the major groove (38, 44). It is therefore likely that HucR shares a similar mode of DNA binding with its two closest characterized homologs, EmrR and MexR, in which the recognition helix of each half of the homodimer binds in the major groove on the same face of the double helix.

A number of autoregulatory members of the MarR family have been described, including repressors that, like HucR, are not encoded in a contiguous operon. The position of the HucR

---

**Fig. 6. Footprinting analysis of HucR-hucO complex.** A, DNase I (lanes 1–3) and MPE-Fe(II) (lanes 4–9) footprinting of the top strand of hucO. Lanes 1 and 4, A/G ladder; lane 2, DNase I treatment of hucO in the absence of HucR; lane 3, DNase I treatment of hucO incubated with 200 nM HucR. Lanes 5–9, MPE-Fe(II) treatment of hucO after incubation with 0, 25, 100, 200, or 400 nM HucR, respectively. The predicted positions of the $dr1160 -10$ promoter element and transcriptional start site are indicated at the right. B, densitometric profile of the HucR-hucO complex, as determined by MPE-Fe(II) footprinting. MPE-Fe(II)-treated hucO in the absence of HucR is represented by the gray trace. The dark line represents DNA incubated with 200 nM HucR (lane 8 in A). C, densitometric profile of the HucR-hucO complex, as determined by DNase I footprinting. The gray line shows the densitometric trace of DNase I-treated hucO in the absence of HucR. DNase I cleavage of hucO incubated with 200 nM HucR is represented by the dark trace. Sequence numbering in A–C is relative to the predicted +1 transcriptional start site of $dr1160$, as in Fig. 1.
binding site in hucO suggests a mechanism of simultaneous transcriptional repression of the divergent hucR and uricase genes, involving steric inhibition of RNA polymerase recruitment to the promoter or elongation. The orientation of hucR and the uricase gene, with an intervening regulatory region, is similar to the genetic organization of other MarR homologs. For example, MexR binds to two sites in the mexR-mexA intergenic region, with each binding site containing promoter elements of each gene, explaining the mechanism of MexR repression (12).

HpaR regulates its own expression and that of an oppositely oriented gene cluster by binding in the central operator/promoter region (8).

Our finding that salicylate is a negative effector of HucR-hucO interaction is consistent with HucR conservation of residues that form the salicylate binding site in MarR (Fig. 7) (22). The decreased affinity of HucR (and MarR) for acetyl salicylate is probably due to the extra acetyl group and the concomitant loss of hydrogen bonding capacity with Ser104 (Thr104 in MarR) (18). An explanation of the apparent higher affinity of HucR for uric acid compared with salicylate awaits structural details of this protein. The efficient antagonism of HucR-hucO interaction by uric acid suggests a regulatory mechanism of uric acid catabolism (Fig. 7). Several repressors of aromatic catabolic pathways from the MarR family have been characterized (45). For instance, HpaR represses the transcription of genes involved in the catabolism of 4-hydroxyphenylacetic acid, and this repression is relieved by the binding of the substrate, 4-hydroxyphenylacetic acid, to HpaR (8). The repression of a cinnamoyl ester hydrolase by CinR is relieved by aromatic substrates of this enzyme (14).

The catabolism of uric acid in D. radiodurans is probably regulated in an analogous manner, with uric acid directly relieving HucR-mediated repression of uricase genes in the cell. This model of co-repression of hucR and dr1160 (predicted uricase) is supported by RNA dot blot analysis, which reveals uric acid-induced up-regulation of both genes to similar transcript levels in D. radiodurans (Fig. 8A) and by the up-regulation of uricase activity in the presence of excess uric acid (Fig. 8B). Since dr1160 is the only uricase homolog in the D. radiodurans genome, we interpret these results as support of a model of HucR-mediated regulation.

The physiological significance of uricase regulation in D. radiodurans is unclear. This enzyme acts as part of the purine degradation pathway in prokaryotes and in eukaryotes. In primates, birds, terrestrial reptiles, and many insects, uric acid is the final product of purine catabolism and is excreted into the environment. Selected soil bacteria are capable of utilising uric acid as its sole carbon, energy, or nitrogen source (46). Bacillus fastidiosus, which requires uric acid (or its degradation products allantoin and allantoic acid) as a substrate for growth (46). B. subtilis can utilize uric acid as its sole nitrogen source (47). Since D. radiodurans is also a soil-dwelling microorganism, it is tempting to speculate on the possibility that it, too, has acquired the capacity to use uric acid as a source of carbon, energy, or nitrogen.
HucR, a Novel Uric Acid-responsive MarR Homolog

Analysis of 16 S ribosomal DNA sequences suggests that Deinococcus forms a separate bacterial phylum with the thermophilic Thermus genus (48). However, the $T_m$ of 51.1 ± 0.0 °C for HucR, is similar to the $T_m$ values (at similar salt concentrations) of other helix-turn-helix transcriptional regulators from mesophilic organisms, such as the LacI DNA-binding domain, which has a $T_m$ of 45.6 ± 0.2 °C, and the DNA-binding domain of MATa2, which has a $T_m$ of 56.5 °C (49, 50). Despite the close relationship of Deinococcus to a thermophilic genus, HucR does not appear to be an unusually thermostable protein. HucR shares the highest sequence similarity to uncharacterized MarR homologs from Sinorhizobium meliloti and Agrobacterium tumefaciens (Fig. 2), both of which are soil-dwelling, mesophilic, plant symbionts from the family Rhizobiaceae. This family is grouped with the Proteobacteria, a eubacterial lineage that is distantly related to Deinococcus. Pairwise alignment of HucR and its homolog from Sinorhizobium shows 38% sequence identity and 55% similarity. Alignment with each of the Agrobacterium homologs reveals 36.5% identity and 55.7% similarity. Since D. radiodurans is a soil-dwelling mesophile, it is plausible that the similarity of HucR to these rhizobial proteins is a result of horizontal gene transfer (25). Multiple genes in D. radiodurans appear to have been acquired via horizontal transfer, including at least seven stress response genes that were probably acquired from species belonging to the family Rhizobiaceae (25). The potential role of HucR in regulating uric acid levels suggests that it too is involved in D. radiodurans stress response. Uric acid is an efficient scavenger of reactive oxygen species, including hydroxyl radicals, superoxide anion, and singlet oxygen, and is thought to be a critical antioxidant in mammals (51, 52). Indeed, D. radiodurans demonstrates extreme resistance to oxidative damage (53–55). HucR-mediated regulation of uric acid levels in D. radiodurans could therefore contribute to this organism’s observed resistance to high levels of oxidative stress. It has been demonstrated that oxygen increases the lethality of ionizing radiation in D. radiodurans presumably by generating reactive oxygen species (56). By regulating levels of uric acid, HucR could therefore also participate in the response of D. radiodurans to ionizing radiation. It is likely that the sensitivity of HucR activity to levels of uric acid serves to maintain an optimum level of this scavenger of peroxynitrite and other reactive oxygen species in the cytoplasm. The low solubility of uric acid is well documented and is manifested in precipitate-related health problems such as gout in humans. Other proteins in this family have been shown to regulate resistance to oxidative stress, including MarR, SlyA, and OhrR (57–59). PecS control of indigoidine, an apparent radical scavenger, regulates E. chrysanthemi resistance to reactive oxygen species (7). It is thus tempting to speculate that HucR shares with these MarR homologs a common functional role of mediating oxidative stress response.

Acknowledgments—We thank members of the Grove laboratory for insightful discussions and John Battista for providing D. radiodurans.

REFERENCES
1. Salavik, M. C., Gambino, L. P., and Miller, P. F. (1995) Mol. Med. 1, 436–446
2. Egland, P. G., and Harwood, C. S. (1999) J. Bacteriol. 181, 2102–2109
3. Oscarsson, J., Mizuno, Y. J., Uhin, B. E., and Haydon, D. J. (1996) Mol. Microbiol. 20, 191–199
4. Poole, K. T., Zhan, Q., Neshat, S., Heinrichs, D. E., and Bianc, N. (1996) Antimicrob. Agents Chemother. 40, 2021–2028
5. Srikumar, R., Paul, C. J., and Poole, K. (2000) J. Bacteriol. 182, 1410–1414
6. Rouzen, C., Nomura, K., Tsuyumu, S., and Nasser, W. (1999) J. Bacteriol. 181, 5948–5957
7. Reverchon, S., Rouanet, C., Expert, D., and Nasser, W. (2002) J. Bacteriol. 184, 654–665