Developing a Genetic System in *Deinococcus radiodurans* for Analyzing Mutations

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ABSTRACT

We have applied a genetic system for analyzing mutations in *Escherichia coli* to *Deinococcus radiodurans*, an extremeophile with an astonishingly high resistance to UV- and ionizing-radiation-induced mutagenesis. Taking advantage of the conservation of the β-subunit of RNA polymerase among most prokaryotes, we derived again in *D. radiodurans* the *rpoB/Rif* system that we developed in *E. coli* to monitor base substitutions, defining 33 base change substitutions at 22 different base pairs. We sequenced >250 mutations leading to Rif in *D. radiodurans* derived spontaneously in wild-type and *uvrD* (mismatch-repair-deficient) backgrounds and after treatment with N-methyl-N-nitro-N-nitrosoguanidine (NTG) and 5-azacytidine (5AZ). The specificities of NTG and 5AZ in *D. radiodurans* are the same as those found for *E. coli* and other organisms. There are prominent base substitution hotspots in *rpoB* in both *D. radiodurans* and *E. coli*. In several cases these are at different points in each organism, even though the DNA sequences surrounding the hotspots and their corresponding sites are very similar in both *D. radiodurans* and *E. coli*. In one case the hotspots occur at the same site in both organisms.

As we continue to explore the vast diversity of microorganisms growing in extreme environments, we need to develop genetic systems to study biological processes and to help interpret the information from genome-sequencing projects. But how can we carry out genetic studies in organisms with no characterized genetic systems? One approach is to adapt methods from studying mutations that have been developed in well-studied microorganisms and to derive them again in the new organism of interest. We have recently completed the development of a system for analyzing base substitutions in *Escherichia coli* based on sequencing *rpoB* mutations that generate the rifampicin-resistant (Rif) phenotype (Garibyan et al. 2003). This system extends the previous work of several investigators (Ovchinnikov et al. 1983 and references therein; Jin and Gross 1988; Severinov et al. 1993) and, together with other recent work (Rangarajan et al. 1997; Reynolds 2000; Petersen-Mahrt et al. 2002; Wolff et al. 2004), monitors at least 77 mutations at 37°. It has been used to analyze >1500 mutations derived from a series of mutants and mutagens, as well as from untreated wild-type controls (Garibyan et al. 2003; Kim et al. 2003; Wolff et al. 2004). This work has revealed several prominent hotspots in the spontaneous spectrum and different hotspots in each of the mutagen-induced sets. Because the mutations that result in Rif are clustered within two small regions of *rpoB*, they can be analyzed by using only two primer pairs for amplification and sequencing. The *rpoB*-encoded β-subunit of RNA polymerase is highly conserved among prokaryotes (Musser 1995; Campbell et al. 2001). Rif mutants have been analyzed in a number of microorganisms, including several pathogens (see review by Musser 1995), and the mutations resulting in Rif have been determined. The mutations cluster in the same region as those for *E. coli* and mostly affect the corresponding residues. Thus, the *rpoB/Rif* system offers an opportunity for developing a genetic system to analyze mutations even in organisms that have had little genetic analysis. To evaluate the feasibility of applying this system to other bacteria, we initiated an investigation of mutagenesis in *Deinococcus radiodurans*. R1 (Battista and Rainey 2001), a species within one of the deeply branching phyla of the domain Bacteria. Genetic methods available for the study of *D. radiodurans* are relatively primitive (Battista 1997), but since the regions of *rpoB* containing the sites for the mutations leading to Rif have close to 80% amino acid identity between *E. coli* and *D. radiodurans* (Figure 1), we felt that we could construct a mutagenesis assay system for use in *D. radiodurans* similar to that constructed for *E. coli*. *D. radiodurans* is recognized for its ability to tolerate the lethal and mutagenic effects of DNA damage, exhibiting unusually high resistance to ionizing radiation and ultraviolet (UV) light (Moseley and Mattingsby 1971; Udupa et al. 1994), but the biochemical details of the response of *D. radiodurans* to DNA damage are poorly understood. Being able to analyze the specificity of mutators and mutagens with a system similar
to the E. coli rpoB/Rif system may lead to insights into the nature of mutagenesis and repair in this organism.

**MATERIALS AND METHODS**

**Bacterial strains:** We used *D. radiodurans* strain R1, ATCC 13939, as the wild type (Anderson et al. 1956). We constructed a *uvrD* derivative, NS3113, as detailed below.

**Construction of pNS1165:** A PCR fragment encoding the *uvrD* gene (DR1775) of *D. radiodurans* R1 was amplified directly from purified R1 chromosomal DNA using a pair of primers derived from the published sequence of the R1 genome (http://www.tigr.org/tigr-scripts/CMR2/GenomePage3. spl?database=gdr). Primers uvrDLP (5'-TACAGGATCGCCATCTC-3') and uvrDRP1 (5'-ATAATCCTTAAAAACTCGATTTTCCACCTC-3') were designed for amplification of the first 1165 bp of the *uvrD* coding sequence. This PCR fragment was inserted directly into the vector pGEM-T (Promega, Madison, WI) to generate the construct pNS1165. The insert was sequenced and found to be identical to that of locus DR1775 in The Institute of Genomic Research database.

**Construction of NS3113:** An in vitro transposition protocol (Earl et al. 2002) developed specifically for use in *D. radiodurans* was used to disrupt the *uvrD* coding sequence. Twenty nanograms of purified, circular pGTC101, a derivative of pGPS3 carrying a transposon that is functional in *D. radiodurans*, was combined with commercially available TnsABC* transposase (New England Biolabs, Beverly, MA) and pNS1165 digested with a combination of *Pst* I and *Apa I* and then double picked onto TGY and TGY plates to confirm the Rifr phenotype. Colonies from these TGY plates were used to inoculate cultures for DNA isolation and subsequent sequencing. These plasmids had inserted into the *uvrD* coding sequence. The strain containing the transposon inserted between nucleotides 886 and 887 of the *uvrD* coding sequence. The strain containing the disrupted sequence was designated NS3113. Since the disruption of *uvrD* would result in a mutator phenotype, we used the Rif* assay to test for a mutator character. The frequency of Rif* mutants in NS3113 was determined to be 12 times that of the wild type, R1, indicating that strain NS3113 is a mutator.

**Isolating Rif* mutants:** Spontaneous Rif* mutants were obtained by incubating 5-ml cultures with 100–300 freshly growing cells of R1 and growing for 48 hr at 37°C to saturation. Dilutions of the cultures were plated on TGY plates to determine the cell titers. The cultures were also concentrated 10-fold by centrifugation and 100 μl was plated on TGY containing 50 μg/ml rifampicin (Sigma Chemical, St. Louis) to determine the frequency of Rif* mutations. Mutant frequencies were determined, and the median frequency (f) from a set of cultures (29) was used to calculate the mutation rate (μ) per replication by the method of Drake (1991), using the formula μ = f/N, where N is the number of cells in the culture. Ninety-five percent confidence limits were determined according to Dixon and Massey (1969). Once rifampicin-resistant colonies were obtained, either spontaneously or through the use of a mutagen (see protocols below), they were purified on TGY plates and incubated for 48 hr at 37°C and then double picked onto TGY and TGY + rifampicin plates to confirm the Rif* phenotype. Colonies from these TGY plates were used to inoculate cultures for DNA isolation and subsequent sequencing.

**D. radiodurans genomic DNA:** Genomic DNA was isolated from saturated cultures. Briefly, using the Invitrogen (Carlsbad, CA) DNAzol protocol, cells were pelleted, resuspended in 500 μl of 95% ethanol, and incubated at room temperature for 5 min to remove the outer membrane. The ethanol-stripped cells were collected by centrifugation at 4°C for 5 min at 10,000 × g and resuspended in 200 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The stripped cells were incubated with 3 μl of 50 mg/ml lysozyme (Sigma Chemical) at room temperature for 8 min, followed by another incubation for 1 min with 1 ml of DNAzol reagent (Invitrogen). The cells were centrifuged at 4°C for 10 min at 10,000 × g, and 1 ml of
the supernatant was transferred to a new tube with 500 µl of 100% EtOH. The samples were mixed by inverting and kept at room temperature for 2 min and then centrifuged at 4°C for 10 min at 10,000 x g to precipitate the DNA. The DNA pellet was washed twice with 200 µl of 75% EtOH and allowed to air dry for 30 min. When the ethanol had evaporated, 50 µl of TE buffer was added to the tube and the DNA was allowed to dissolve overnight at room temperature.

**Sequencing the rpoB gene for mutations:** Once the chromosomal DNA was isolated, one of two primer pairs was used to amplify the DNA for direct sequencing. Most of the mutations occurred in the region obtained with 5′-AAACTCTTCCGCGATTCTGACGAGTAAC-3′ (5′ position 1058) and 5′-TAGCTCTAGCAGCTACGACTTCGAC-3′ (5′ position 1945). The rest of the RifR mutations were found using the primer pair 5′-TCTTCCATCGAGCCGCTGTTTTTGGG-3′ (5′ position 173) and 5′-CAGAGATGCAGCGCGGT-3′ (5′ position 325), respectively.

**N′-methyl-N′-nitro-N-nitrosoguanidine mutagenesis:** Six tubes of 5 ml TGY broth were inoculated with R1 and aerated for 48 hr at 37°C. The cultures were pooled together into an Erlenmeyer flask and diluted 1:1 with TGY. A 1-mg/ml N′-methyl-N′-nitro-N-nitrosoguanidine (NTG) solution was made in 1:1 acetonitrile and 0.1 M NaCitrate buffer (pH 5.5). Two microliters of culture was aliquoted into tubes, and NTG was added to a final concentration of 0, 30, or 100 µg/ml. After a 90 min incubation in a 37°C water bath, the mutagenized cultures were washed three times: first with 5 ml of 0.01 M MgSO4, followed by 5 ml of TGY, and finally with 2 ml of TGY (Miller 1992). To determine the percentage of cells surviving exposure to NTG, 50 µl of a 10−5 dilution of each culture was plated onto TGY and incubated at 37°C for 48 hr. Outgrowth cultures were made by adding 500 µl of each mutagenized culture to 5 ml TGY and incubating for 48 hr at 37°C. The outgrowth cultures were concentrated 10-fold and 100 µl was plated on TGY + rifampicin plates to yield RifR colonies for sequencing. Two RifR colonies from each of the 10, 30, and 100 µg/ml NTG TGY + rifampicin plates were sequenced. The experiment was repeated with more cultures at a concentration of 100 µg/ml NTG.

**5-Azacytidine mutagenesis:** An overnight culture was diluted and used to seed overnight cultures with 100–300 cells in TGY with 100 µg/ml of 5-azacytidine (5AZ) and grown for 48 hr on a rotor (Miller 1992). The mutational frequency was determined by plating 100 µl of a 10−5 dilution of each culture on TGY and 50 µl directly on TGY + rifampicin.

**wurD RifR mutants:** D. radiodurans wurD RifR mutants were obtained in the same manner as spontaneous mutants, with the exception that each overnight culture was inoculated with a single wurD colony instead of seeding the cultures with 100–300 cells.

**RESULTS**

**Isolation of RifR mutants and sequence analysis of rpoB mutations:** We isolated RifR mutants at 37°C on TGY plates containing 50 µg/ml rifampicin (see materials and methods) that occurred spontaneously in D. radiodurans wild-type or wurD strains or that were induced by NTG or 5AZ. Table 1 shows the mutation frequencies and rates that we obtained. Although NTG was a potent mutagen for D. radiodurans, ethyl methanesulfonate (EMS) failed to mutagenize this organism at all. Also, although the cytosine analog 5AZ gave positive results, the cytosine analog zebularine, as well as the adenine analog 2-aminopurine, failed to mutagenize D. radiodurans. We isolated one mutant per culture and prepared DNA for sequencing analysis of the relevant regions of the rpoB gene (see materials and methods). Table 2 shows the results for >250 mutations in the D. radiodurans rpoB gene that result in the RifR phenotype, including 185 spontaneous mutations derived in a wild-type background, 33 NTG-induced mutations, and 19 5AZ-induced mutations, as well as 17 spontaneous mutations occurring in a wurD background. From these data we can already define 33 different base substitution mutations at 22 sites (base pairs). Each of the 6 base substitutions can be monitored at a set of 5–7 sites.

**Spontaneous mutations—deletion and base substitution hotspots:** We found that 35 of the 185 (19%) spontaneous mutations detected in the wild-type background are deletions of 9 bp at or adjacent to a 7-bp direct repeat separated by 2 bp and probably are the result of slipped mispairing stimulated at this site (see Figure 2). We detected three different deletions, although one of these (type III in Figure 2) is represented by only 1 occurrence, while type I and type II deletions are represented by 19 and 15 occurrences, respectively (see also Table 2). Deletion type II (Figure 2) is of the exact form found for many deletions in both E. coli and other organisms (see Farabaugh et al. 1978; Albertini et al. 1982 and references therein). Deletion type I is shifted just 1 bp in one direction. In E. coli, one or two small deletions in rpoB that result in RifR mutants have been reported (Jin and Gross 1988), but these are relatively rare compared with the base substitutions that result in RifR in that organism.

In addition to the two deletion hotspots mentioned...
TABLE 2
Distribution of mutations leading to Rif* in *D. radiodurans*

<table>
<thead>
<tr>
<th><em>D. radiodurans</em> site (bp)</th>
<th>Amino acid change</th>
<th>Base-pair change</th>
<th>Wild type</th>
<th>NTG</th>
<th>5AZ</th>
<th>uvrD</th>
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<td><strong>33</strong></td>
<td><strong>19</strong></td>
<td><strong>17</strong></td>
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The DNA sequence change in *rpoB* was determined in each case.
* The corresponding base and base change is different in *E. coli*.

above, the spontaneous spectrum of *rpoB* mutations in *D. radiodurans* shows three base substitution hotspots at positions 1273 and 1303 (G:C → A:T) and 1259 (A:T → C:G). Together, these three hotspots account for 95 of the 150 base substitutions (63%) found among the spontaneous mutations in *rpoB*, even though these are distributed among 33 sites. A comparison of these hotspots with those in *E. coli* *rpoB* reveals that the respective hotspots involve different sites, as shown in Figure 3 (see also Table 2 and Giribyan et al. 2003), with one exception. Mutations of G:C → A:T at 1303 in *D. radiodurans* and the corresponding 1576 in *E. coli* both represent hotspots. Figure 4 shows the DNA sequence alignments surrounding each of the hotspot sites. In three cases, both *E. coli* and *D. radiodurans* have identical nearest neighbors at the respective sites and yet dramatically
different rates in two of these examples. In one case (E. coli 1547), the sequence identity is more extensive. We consider reasons for different hotspots in the discussion.

**Mutations resulting from mutators and mutagens:** We examined the mutational spectrum in a uvrD strain (Table 2). Here, all the mutations are transitions, with G:C → A:T mutations predominating over A:T → G:C mutations, although the spectrum is dominated by a single hotspot at position 1455. We have also employed two mutagens, NTG and 5AZ. It is clear from Table 2 that these agents induce specifically G:C → A:T transitions in the case of NTG and G:C → C:G transversions in the case of 5AZ in D. radiodurans and thus have the same specificity as found in E. coli and other organisms (Guppies and Miller 1989).

**Altered amino acids in RNA polymerase β in Rif₁ mutants:** The amino acid residues affected in Rif₁ mutants are strikingly similar in E. coli and D. radiodurans, as detailed in Figure 5. However, only one site has been detected in D. radiodurans that has not yet been detected in E. coli. In E. coli, in which far more extensive studies have been carried out (Jin and Gross 1988; Severinov et al. 1993; Garibyan et al. 2003; Wolff et al. 2004), alterations at 27 different residues can result in the Rif₁ phenotype, whereas so far 15 such residues have been found in D. radiodurans.

**DISCUSSION**

In this work, we have successfully applied a genetic system developed in E. coli to D. radiodurans, a species that separated from the rest of the bacterial family tree early in evolution (Battista and Rainey 2001). Although assays measuring the frequency of forward mutation to rifampicin (Tempest and Moseley 1982), streptomycin (Kerszman 1975), and trimethoprim (Sweet and Moseley 1974) resistance have been described for D. radiodurans, currently no genetic system allows the analysis of mutations generated in wild-type and repair-
conserved among most prokaryotes, it should be possible to develop a detailed rpoB/Rif\(^{\text{r}}\) system in an organism without sophisticated genetic tools. Investigators from numerous laboratories have analyzed Rif\(^{\text{r}}\) mutants from a variety of pathogens, including *Mycobacterium tuberculosis* (Telenti et al. 1993; Musser 1995), *M. smegmatis* (Karunakaran and Davies 2000), *M. leprae* (Klein et al. 2001), *Bacillus anthracis* (Vogler et al. 2002), *B. cereus* (Vogler et al. 2002), *Rhodococcus equi* (Fines et al. 2001), *Legionella pneumophila* (Neilsen et al. 2000), *Neisseria meningitides* (Stefanelli et al. 2001), *Streptococcus pyogenes* (Aubry-Damon et al. 2002), *Staphylococcus aureus* (Aubry-Damon et al. 1998), and *Helicobacter pylori* (Heep et al. 2000). In all of these cases, the rpoB mutations affect residues corresponding to those altered in *E. coli* Rif\(^{\text{r}}\) mutants, although a complete catalog of possible mutations has not been attempted in these studies.

Table 2 shows the results of sequencing >250 mutations leading to Rif\(^{\text{r}}\) in *D. radiodurans* derived spontaneously in wild-type and *uvrD* (mismatch-repair-deficient) backgrounds, and after treatment with \(N\)-methyl-\(N\)-nitro-\(N\)-nitrosoguanidine and 5-azacytidine. We have used the rpoB-encoded \(\beta\)-subunit of RNA polymerase is highly conserved.

![Figure 4](image)

**Figure 4.**—DNA sequences and homologies surrounding the mutational hotspots in *D. radiodurans* and *E. coli*. The underlined base is the hotspot site (see also Table 2 and Figure 3).

![Figure 5](image)

**Figure 5.**—Amino acid changes in rpoB that lead to the Rif\(^{\text{r}}\) phenotype in *D. radiodurans* and *E. coli*. 

![Table 2](image)
change at 3 sites, the G:C → A:T change at 7 sites, the A:T → T:A change at 5 sites, the A:T → C:G change at 5 sites, the G:C → T:A change at 6 sites, and the G:C → C:G change at 7 sites.

Analysis of the rpoB/Rif<sup>+</sup> system provides the first detailed description of mutagenesis in <i>D. radiodurans</i> and lays the groundwork for studies of the mechanisms that this species uses to avoid mutation. <i>D. radiodurans</i> has an astonishing ability to avoid UV- and ionizing-radiation-induced mutagenesis (Sweet and Moseley 1974; Kerszman 1975; Tempest and Moseley 1982). For example, even when cultures are exposed to 700 J/m<sup>2</sup> UV light, a dose that induces ~5000 thymine-containing pyrimidine dimers per genome in exposed cells and that kills 90% of the irradiated population (Moseley 1983), there is no evidence of UV-induced mutagenesis in this species (Tempest and Moseley 1982). We anticipate that the introduction of the rpoB/Rif<sup>+</sup> system will encourage further investigation of mutagenesis and DNA repair in <i>D. radiodurans</i>.

Several aspects of the results found in this study are worth noting. The spontaneous mutations reveal a deletion hotspot centered near sequence repeats that very probably serve as substrates for the type of slipped-mismatching events that generate deletions in other organisms (Farabaugh et al. 1978; Albertini et al. 1982). Although deletions in rpoB do not usually generate viable Rif<sup>+</sup> strains, the in-frame deletion of 9 bp removes three amino acids that alter the β-subunit of RNA polymerase enough to become resistant to inactivation by rifampicin, but not enough to affect function. The specificities of NTG and 5AZ are the same in <i>D. radiodurans</i> as found for <i>E. coli</i> and other organisms (see, for instance, Cupples and Miller 1989). What is interesting, and not easy to explain, is the failure to detect mutagenesis with EMS, even though NTG is a potent mutagen for <i>D. radiodurans</i>. Also, neither the cytosine analog zebularine (McCormack et al. 1980) nor the adenine analog 2-aminopurine causes mutations in <i>D. radiodurans</i>, whereas they do in <i>E. coli</i> (see Cupples and J. H. Miller 1989; Lee et al. 2004). Finally, the rpoB mutations in the <i>D. radiodurans</i> wrnD strain predominate at a single G:C → A:T site, whereas the major hotspot in <i>E. coli</i> Rif<sup>+</sup> for mismatch-repair-deficient mutations is an A:T → G:C mutation.

There are prominent base substitution hotspots in rpoB in both <i>D. radiodurans</i> and <i>E. coli</i> (Table 2; Gariyan et al. 2003), although with one exception they occur at different sites (see Figure 3). What makes this remarkable is that the DNA sequences surrounding the hotspot sites are very similar, as shown in Figure 4. For instance, the <i>E. coli</i> spontaneous hotspot (31 occurrences among 298 mutations) at position 1714 (A:T → C:G) has a DNA sequence identical to that of the corresponding <i>D. radiodurans</i> site 1441 for 10 bp on one side and the nearest neighbor on the other (Figure 3), yet the <i>D. radiodurans</i> site has only two occurrences of A:T → C:G mutations among 150 base substitutions. Similarly, the G:C → A:T mutation at position 1273 in <i>D. radiodurans</i> is a hotspot, whereas the corresponding change at 1546 is not in <i>E. coli</i>, despite sharing the same 6 bp on one side and the same nearest neighbor on the other. As part of the same sequence segment, the A:T → G:C change at position 1547 is the most prominent hotspot in <i>E. coli</i> (40 occurrences among 298 mutations), but no mutations have been found at the corresponding position (1274) in <i>D. radiodurans</i>. Normally, one would not be able to make conclusions regarding this site in <i>D. radiodurans</i>, since without any occurrences recorded it might be that the A:T → G:C change there does not result in a mutation that yields Rif<sup>+</sup> cells. However, a look at Figure 5 shows that the aspartic acid residue specified by the codon involving 1547 in <i>E. coli</i> and 1274 in <i>D. radiodurans</i> is an amino acid (residue 516 in <i>E. coli</i>; 425 in <i>D. radiodurans</i>) at which many different exchanges yield Rif<sup>+</sup>. In <i>E. coli</i>, where many more mutations have been generated, all five changes at this site have been detected and shown to result in Rif<sup>+</sup>, whereas so far four of these five changes have already been found in <i>D. radiodurans</i>. It is highly probable that the remaining change (to glycine) will also yield a Rif<sup>+</sup> cell and that the zero occurrences of an A:T → G:C change at 1274 in <i>D. radiodurans</i> simply reflect failure to induce the mutation at a detectable rate with the current sample size and not a failure of the resulting change to generate Rif<sup>+</sup> colonies. Position 1259 is a hotspot for the A:T → C:G transversion in <i>D. radiodurans</i>, but not in <i>E. coli</i>. Although this site is part of a 5-bp homology and a 12 of 14-bp homology (Figure 4), the corresponding sites do not have identical nearest neighbors on one side. The lack of a nearest neighbor in an otherwise homologous stretch does not prevent both 1303 in <i>D. radiodurans</i> and 1576 in <i>E. coli</i> (25 occurrences among 298 mutations) from being hotspots for the G:C → A:T transition. That these two sites are hotspots despite being in different organisms is remarkable. It is not clear whether the other sites that are hotspots in one organism but not in the other reflect a requirement for a more extensive sequence environment as a determinant of mutation rate or whether mutation rates at all sites are really organism specific. Additional experiments with engineered sequences are required to answer these questions.

We see no reason why the rpoB/Rif<sup>+</sup> system cannot be applied to other genetically intractable bacterial species. The only requirements for implementing this system are that the investigator knows the rpoB gene sequence and has the ability to isolate Rif<sup>+</sup> mutants from the species of interest. It should now be possible to extend the detailed analysis of spontaneous mutagenesis to a large number of diverse species and to explore how the specifics of this process compare among prokaryotes that evolved to occupy different ecological niches.
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