Survival in Nuclear Waste, Extreme Resistance, and Potential Applications Gleaned from the Genome Sequence of *Kineococcus radiotolerans* SRS30216

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Abstract

*Kineococcus radiotolerans* SRS30216 was isolated from a high-level radioactive environment at the Savannah River Site (SRS) and exhibits γ-radiation resistance approaching that of *Deinococcus radiodurans*. The genome was sequenced by the U.S. Department of Energy’s Joint Genome Institute which suggested the existence of three replicons, a 4.76 Mb linear chromosome, a 0.18 Mb linear plasmid, and a 12.92 Kb circular plasmid. Southern hybridization confirmed that the chromosome is linear. The *K. radiotolerans* genome sequence was examined to learn about the physiology of the organism with regard to ionizing radiation resistance, the potential for bioremediation of nuclear waste, and the dimorphic life cycle. *K. radiotolerans* may have a unique genetic toolbox for radiation protection as it lacks many of the genes known to confer radiation resistance in *D. radiodurans*. Additionally, genes involved in the detoxification of reactive oxygen species and the excision repair pathway are overrepresented. *K. radiotolerans* appears to lack degradation pathways for pervasive soil and groundwater pollutants. However, it can respire on two organic acids found in SRS high-level nuclear waste, formate and oxalate, which promote the survival of cells during prolonged periods of starvation. The dimorphic life cycle involves the production of motile zoospores. The flagellar biosynthesis genes are located on a motility island, though its regulation could not be fully discerned. These results highlight the remarkable ability of *K. radiotolerans* to withstand environmental extremes and suggest that in situ bioremediation of organic complexants from high level radioactive waste may be feasible.
Kineococcus radiotolerans SRS30216 was isolated from HLW within a shielded cell work area at the SRS [2]. K. radiotolerans is an orange-pigmented, aerobic bacterium belonging to the Actinobacteria phylum that is capable of withstanding relatively high concentrations of metals and alkali cations, as well as exposure to extreme doses of ionizing radiation. K. radiotolerans exhibits radiation resistance approaching that of D. radiodurans [2]. D. radiodurans and K. radiotolerans belong to different phyla and it remains unknown whether both organisms attain radiation resistance through a common set of gene products. Because K. radiotolerans survived a HLW environment, it is expected to possess potent cellular defense and repair mechanisms for radiation exposure, osmotic stress and chemical toxicity. The occurrence of all these features in a naturally occurring bacterium may have direct applications for the bioremediation of nuclear waste.

In this work the genome sequence of K. radiotolerans SRS30216 was examined in three different contexts. First, the presence of genes known to confer radiation resistance in D. radiodurans was examined in the K. radiotolerans genome. Second, the capacity for bioremediation was assessed by comparative genomics as well as growth and respiration studies. Finally, the dimorphic life cycle of the organism, in particular the production of mobile zoospores, was examined by identifying genes involved in flagellar motility and chemotaxis.

Results and Discussion

Phylogeny

K. radiotolerans belongs to the suborder Frankineae in the order Actinomycetales and the phylum Actinobacteria. Complete genome sequences are available for only two other members of the Frankineae suborder, Franka alni and Acidothermus cellulolyticus. F. alni is distinguished by its ability to fix nitrogen in symbiosis with alder (Alnus spp.) and myrtle (Myrica spp.), two pioneer plants in temperate regions. The K. radiotolerans genome shows no potential for nitrogen fixation. A. cellulolyticus is a thermotolerant organism isolated from the hot spring in Yellowstone National Park that degrades cellulose. While the K. radiotolerans genome may encode proteins with the potential for degrading complex carbohydrates like cellulose (Krad622, 3823), cellobiose (Krad0408, 2526, 2530, 2531, 2539, 3436, 3480, 3961), glycogen, and starch (Krad1294, 1298), growth on these substrates has not been demonstrated experimentally.

More distant relatives in the same phylum include Streptomyces and Mycobacterium, whose physiology has been extensively examined, and which serve as reference points for comparative genomics.

Genome size and organization

The K. radiotolerans SRS30216 genome was sequenced by the US DOE Joint Genome Institute with the discovery of three replicons. The bulk of the DNA is contained on a 4,761,183 bp linear chromosome (CP000750). In addition there is a 182,572 bp linear plasmid (pKRA01; CP000751) and a 12,917 bp circular plasmid (pKRA02; CP000752). The three contigs were derived from at least 20 reads and average 74.2 mol % G+C. The genome is predicted to contain 4,715 genes.

Linear chromosomes are rare among prokaryotes. Aside from one atypical Agrobacterium isolate, the two principle examples are Streptomyces species [3] and Borrelia burgdorferi [4]. In both Streptomyces and Borrelia bidirectional replication occurs from a single origin of replication (oriC) located near the middle of the replicon. A plot of the GC skew = (C−G)/(C+G) along the chromosome sometimes inverts at the replication origin [5]. The shift in GC skew is thought to be due to accumulated mutational biases during leading vs lagging strand synthesis. There is no obvious GC skew at the center of the chromosome (Figure 1, compare green vs magenta peaks in inner circle). There is however a remarkable GC skew at the telomeres but it seems unlikely that replication proceeds from the telomers toward the center based on the Borrelia and Streptomyces systems. A second method used to locate oriC is the presence of the dnaA gene and DnaA binding sites. dnaA (Krad0001) is near the center of the chromosome, as with Streptomyces and Borrelia. Putative, imperfect blocks of DnaA binding sites, observed using DorIC [6], are widely scattered in the center of the chromosome with the closest being about 94 Kb from the dnaA gene.

The topology of the chromosome was investigated by Southern hybridization using an end-specific probe homologous with Krad2223. Three restriction enzymes were predicted to generate single fragments with this probe. If the chromosome is linear then products with sizes predicted from the DNA sequence should be visible after hybridization. If the chromosome is circular then the hybridization products with the probe should be the sum of the sizes of the predicted restriction fragments from each end plus any missing sequence. Restriction fragments homologous with the Krad2223 probe were similar in size to those predicted by the DNA sequence (Fig. 2). These hybridization results confirm the linear topology predicted by the inability of JGI to close the DNA sequence.

The ends of linear DNA replicons have special features that preserve their integrity. B. burgdorferi linear replicons contain covalently closed hairpin ends [4]. ResT, telomere resolvase, hydrolyzes a phosphodiester bond on each DNA strand then joins the opposite strands to form a covalently closed telomere. The process is reversed during chromosome replication and ResT is essential for B. burgdorferi growth. A ResT homolog is not encoded by the K. radiotolerans genome. Streptomyces coelicolor replicon ends are composed of single-stranded sequences that can anneal to form a noncovalent circular molecule [3]. Streptomyces telomeres bind a family of conserved terminal binding proteins that have no orthologs in the K. radiotolerans genome. Thus, unique mechanisms must protect the K. radiotolerans telomers.

Ionizing radiation resistance

Gamma radiation is one of the most energetic forms of electromagnetic radiation. Gamma rays penetrate tissues and cells, causing direct damage to DNA (namely double strand breaks), proteins, and membranes. Gamma radiation also induces indirect cellular damage through the ionization of water with formation of free radical species, primarily •OH. Oxygen free radicals are extremely reactive, compounded cellular and DNA damage. DNA damage blocks transcription and replication, and if not correctly repaired, could introduce detrimental mutations or cause cell death. Relatively few DNA double strand breaks (DSB) are lethal for most bacteria. Escherichia coli cells succumb to around 10 DSB and Shewanella oneidensis cells die after 1 DSB (based on calculations of 0.0114 DSBS/Gy/Genome; Daly et al., 2004).

Radioreistance has been partially characterized for K. radiotolerans (Figure 3). Acutely irradiated, exponentially grown cultures have a broad shoulder of death, which contrasts with the exponential death of E. coli. This shoulder is due in part to efficient repair systems and in part to the multicellular nature of the organism. In rich medium K. radiotolerans grows in cubical packets that form by alternating cell division planes (Phillips et al. 2002). Thus, the colony forming unit method used to estimate survivorship likely overestimates culture viability. Nevertheless, these results may portray a more ecologically relevant context as cell clustering is common for certain species or developmental
stages of Actinobacteria (e.g., Frankia, Geodermatophilus, Actinoplanes, Micrococcus) or other extremophiles (Kocuria, Deinococcus, Chroococcidiopsis). Remarkably though, Kineococcus can withstand the damaging effects of 20 kGy of $\gamma$-radiation (theoretically generating more than 200 DSB/genome; Daly et al., 2004) and cell division resumes within 4 days (Figure 4).

Several other Actinobacteria species exhibit extraordinary radiation resistance including Rubrobacter radiotolerans, Rubrobacter xylanophilus, and Kocuria rosea [7]. While more ionizing radiation resistant bacterial species are found in the Actinobacteria than any other phylum, radiation resistance is not a widespread trait in this phylum and may have multiple evolutionary origins. The mechanisms that render this trait remain poorly understood. Radiation resistant bacteria suffer severe damage from $\gamma$-radiation [8], which implies that molecular repair processes function with high efficiency.

Analysis of genes common to four ionizing radiation resistant bacteria with fully sequenced genomes indicated that DNA repair
must have played a major role in evolutionary adaptation to ionizing radiation [9]. D. radiodurans has served as the paradigm for radiation resistant organisms and both genetic and biochemical approaches are converging to reveal a complex network of repair and protection processes [7,10]. Many D. radiodurans genes required for ionizing radiation resistance have been identified. In table 1 they are ordered according to the γ-radiation sensitivity of a D. radiodurans strain lacking that gene based on published kill curves. While the roles of only a few of these genes are known with certainty, K. radiotolerans apparently employs a different genetic toolbox from that of D. radiodurans. Absent from K. radiotolerans are homologs of the D. radiodurans ppnA, ddxA, ddxB, ddxC, ddxD genes [11].

Mutations in recA render D. radiodurans as sensitive to ionizing radiation as E. coli illustrating the importance of recombinational repair in repairing DSB (table 1). D. radiodurans cells exposed to 10 kGy γ-radiation accumulate about 100 DSB per genome that are repaired over the course of several hours. D. radiodurans DSB are repaired by homologous recombination in the extended synthesis-dependent strand annealing (ESDSA) process [12]. ESDSA repair is carried out by RecA and PolA, enzymes found in the K. radiotolerans genome (table 2). Based on their genomic analysis, recA and polA exhibit signs of coevolution in ionizing radiation-resistant bacteria [9].

Genes involved in carotenoid biogenesis have been shown to confer a modest level of radiation resistance [13–15] by scavenging electrons from reactive oxygen species (table 1). K. radiotolerans produces carotenoids [2] and the carotenoid biosynthetic pathway is similar to that found in other organisms. In addition to the two genes listed in table 1 encoding phytoene synthetase and phytoene desaturase, K. radiotolerans produces polypropenyl synthase (Krad3227), lycopene cyclase (ectI, Krad0091), and neurosporene dehydrogenase (ectD, Krad3225). A hydroxylase gene (ectZ) was not discovered suggesting that the K. radiotolerans carotenoids are not hydroxylated.

Higher eukaryotes repair DSB using nonhomologous end joining (NHEJ) ([16,17]Riha et al. 2006). NHEJ repair is mediated by the Ku complex and the Ligase IV/XRCC4 complex along with other proteins whose precise biochemical functions remain to be elucidated. While E. coli lacks the NHEJ pathway, some Actinobacteria genera such as Mycobacterium have this repair pathway. Mycobacterial Ku binds DNA ends and recruits a polyfunctional DNA ligase/polymerase (LigD) in vitro (Della et al. 2004). Though repair is mutagenic, it does help maintain cell viability and loss of Ku and LigD increases sensitivity to ionizing radiation (Stephanou et al. 2007). K. radiotolerans appears to lack a Ku-like DNA binding protein. While it does encode an ATP-dependent DNA ligase (Cdc9, or LigB; table 2) NHEJ repair is not likely without Ku.

Ionizing radiation also causes many other types of DNA damage. The K. radiotolerans DNA replication, recombination and repair gene set is overlapping with [9], but generally different from those of D. radiodurans [18] and E. coli [19] which may be expected since these organisms belong to different phyla (table 2). As one illustration of this difference, K. radiotolerans but not D. radiodurans contains RecB and RecC which are involved in recombinational repair in E. coli (reviewed in [20]). DNA replication, repair and recombination systems in K. radiotolerans are more similar to those of Mycobacterium tuberculosis [21], possibly a reflection of the phylogenetic proximity of the two organisms. In addition, M. tuberculosis is at least 10-fold more resistant to ionizing radiation than E. coli [22]. Because M. tuberculosis is an actively studied pathogen its genome is well-annotated. Because it has coevolved the majority of its DNA replication, repair, and recombination mechanisms with K. radiotolerans we chose it as a reference organism, together with E. coli and .

Both K. radiotolerans and M. tuberculosis lack the classical bacterial mismatch repair genes MutS, MutH, MutL, RecJ, ExoVIII, ExoI and Mug (table 2). A compensating factor may be production of DNA polymerases with increased fidelity and proofreading efficiency as K. radiotolerans contains four exonuclease (ε; DnaQ) subunits, three polymerase (α; DnaE) subunits and two β (DnaN) subunits of the replicative DNA polymerase III. Mismatch repair in K. radiotolerans may also be handled by proteins unrelated to
classical bacterial mismatch repair proteins. Some base excision repair genes that may take on such roles are also uniquely overrepresented. There are three Fpg and four Nei base excisionases in *K. radiotolerans*, compared with one Fpg and no Nei homologs in *D. radiodurans*. As an additional example of divergence with *D. radiodurans*, 3-methyladenine DNA glycosylase I (Tag) is present in *K. radiotolerans*, but absent from *D. radiodurans*.

The nucleotide excision repair pathway is also overrepresented in *K. radiotolerans* by three *uvrA* orthologs and by five genes encoding UvrD-like helicases. In addition to these helicases, the *K. radiotolerans* genome, similarly to *M. tuberculosis*, contains an ERCC3 (XPB)-like superfamily II helicase, whose eukaryotic homolog performs essential functions in nucleotide excision repair and transcription [23]. The *K. radiotolerans* and *M. tuberculosis* XPB helicases have been recently demonstrated to be functional *in vitro* (Biswa and Tsodikov, unpublished).

Five homologs of histone-like proteins (IHF or HupB-like) may package DNA in order to protect it from damage, aid recombinational repair, or maintain multiple chromosomes. The direct reversal pathway used to repair pyrimidine dimers includes two copies of the *polB* photolyase gene and an additional, *splB* photolyase gene, which is absent in *E. coli*, *D. radiodurans* and *M. tuberculosis*. Other differences from *M. tuberculosis* include the presence in *K. radiotolerans* of PolA, Topo IV, HolIII, YejH, which are absent in *M. tuberculosis*. The translesion DNA repair system UmuC/UmuD, present in *E. coli* but not *D. radiodurans* or *M. tuberculosis*, is also a part of the *K. radiotolerans* genome.

Similar to mycobacteria, the *K. radiotolerans* replication gene set lacks a well-defined homolog of the helicase loader (DnaC), but contains the other main replication genes (DnaA, DnaB, DnaG). *K. radiotolerans* and *M. tuberculosis* both contain a eukaryotic-like DNA primase gene.

In summary, many of the genes known to confer radiation resistance in *D. radiodurans* are missing from *K. radiotolerans* suggesting novel components to the repair and protection toolbox. Two pathways are involved in DSB repair in other organisms, ESDSA repair mediated by RecA and PolA and NHEJ repair mediated by Ku and LigD. RecA and PolA are present and may be aided by the presence of RecB and RecC. *K. radiotolerans* lacks the Ku protein making the presence of the NHEJ pathway unlikely. Base excision and nucleotide excision repair pathway genes are over represented in *K. radiotolerans* relative to other bacteria.

### Reactive oxygen species detoxification

Most organisms have both RecA and PolA without exhibiting extreme radiation resistance. Remarkably, *D. radiodurans* cells rendered ionizing radiation-sensitive by a polA mutation are fully complemented by expression of the *polA* gene from ionizing radiation-sensitive *E. coli* [24]. Repair proteins, either native or cloned, may function better after irradiation in *D. radiodurans* cells due to protection from protein oxidation [25]. The genetic components and molecular mechanisms of protein repair/protection remain unknown but appear to be correlated with a Mn/Fe ratio in the range of 0.12–0.37 [26]. The Mn/Fe ratio in *K. radiotolerans* is 0.09, slightly lower than *Deinococcus* but much higher than that of radiation sensitive organisms [27].

### Table 1. Genes conferring ionizing radiation resistance in *Deinococcus radiodurans* and their homologs in *Kineococcus radiotolerans*.

| *D. radiodurans* gene | Function | D$_{10}$ (kGy)$^1$ | Reference | Krad locus tag |
|-----------------------|----------|---------------------|------------|---------------|
| recA                  | Homologous recombination | 0.1          | [11]        | 1492          |
| polA                  | DNA polymerase         | 1.0          | [24]        | 2951          |
| pprA                  | Stimulates DNA ligase  | 2.0          | [57]        | none          |
| recQ                  | DNA helicase           | 6.0          | [58]        | 0829          |
| recD                  | Helicase/exonuclease   | 6.0          | [59]        | 0992          |
| ddrB                  | Unknown                | 8.0          | [11]        | none          |
| crtB                  | Phytoene synthase      | 9.0          | [13]        | 3229          |
| crti                  | Phytoene desaturase    | 9.0          | [13]        | 3228          |
| ddrA                  | ssDNA binding protein  | 12.0         | [11]        | none          |
| ddrC                  | Unknown                | >14.0        | [11]        | none          |
| ddrD                  | Unknown                | >14.0        | [11]        | none          |
| sbcC sbCD             | ss endonucleases 3′-5′ ds exonuclease | 15.0 | [60] | 2553 2554 |
| polX                  | ss endonucleases 3′-5′ ds exonuclease | 15.0 | [61] | 4036 |

$^1$Dose of $\gamma$ radiation required for a 90% reduction in cell viability estimated from data supplied in the relevant reference. For comparison, the D$_{10}$ for wild *D. radiodurans* strains ranges from 10–20 kGy depending on the strain and the assay conditions.

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Figure 4. Post-irradiation recovery and growth of *K. radiotolerans*. The irradiated cultures were exposed to 20 kGy $\gamma$-radiation (open circles), and control cultures were incubated under laboratory conditions (closed circles).
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| Protein name | Protein description and comments | Escherichia coli | Deinococcus radiodurans R1 (locus tag) | Mycobacterium tuberculosis H37Rv (locus) | Kineococcus radiotolerans SRS30216 (locus) |
|--------------|---------------------------------|-----------------|----------------------------------------|------------------------------------------|-------------------------------------------|
| Ada          | O-6-methylguanine/O-4-methylthymine DNA methyltransferase | Ada | No homologs | No homologs | Krad_2866 |
| AlkA         | 3-methyladenine DNA glycosylase II; DR_2584 is of eukaryotic type | AlkA | DR_2584, DR_2074 | Rv1317c | Krad_4325, Krad_3854 |
| AlkB         | Alkylation repair protein | AlkB | No homologs | TiGR locus: NT02MT1098 | No homologs |
| Cdc9 (RigB)  | ATP-dependent DNA ligase | No homologs | No homologs | Rv3062 | Krad_4316 |
| ComEA        | DNA uptake protein | ComEA | DR_1855 | Rv2415c | Krad_3435 |
| Dam          | GATC specific N6-adenine methylase | Dam | No homologs | No homologs | No homologs |
| Dcd          | dCTP deaminase | Dcd | No homologs | Rv0321 | Krad_4243 |
| Dcm          | Site-specific C5 cytosine methyltransferase; VSP repair is targeted toward hotspots created by Dcm | Dcm | No homologs | Rv3037c (putative Dcm) | Krad_0734 (putative Dcm) |
| DinB/DinP    | DNA damage inducible protein P (DNA polymerase IV) | DinB | No homologs | Rv3056 Rv1537 (DinX) | Krad_4326 Krad_3213 |
| DinF         | Possible DNA-damage-inducible protein F; integral membrane protein; Na+ -driven multidrug efflux pump | DinF | DR_0792 | Rv2836c | Krad_4334 |
| DinG         | ATP-dependent DNA helicase; SOS inducer | DinG | No homologs | Rv1329c | Krad_1504 |
| DnaA         | Chromosomal replication initiator protein | DnaA | DR_0002 | Rv0001c | Krad_0001 |
| DnaB         | Replicative DNA helicase | DnaB | DR_0549 | Rv0508c | Krad_4333 |
| DnaE         | DNA polymerase III (holoenzyme), α subunit | DnaE | DR_0507 | Rv1547c Rv3370c | Krad_3187 Krad_3215 Krad_0771 Krad_4598 (on pKrad01) |
| DnaG         | DNA Primase | DnaG | DR_0601 | Rv2343c | Krad_3361 |
| DnaN         | DNA polymerase III (holoenzyme), β subunit | DnaN | DR_0001 | Rv0002 | Krad_1769 Krad_0002 |
| DnaQ         | DNA polymerase III (holoenzyme), ε subunit - 3'-5' exonuclease | DnaQ | DR_0856 | Rv3711c | Krad_4419 Krad_3247 Krad_4503 Krad_1768 |
| DnaZ/X       | DNA polymerase III (holoenzyme), γ/τ subunit | DnaZ/X | DR_2410 | Rv3721c | Krad_0466 |
| Dut          | dUTPase | Dut | No homologs | Rv2697c | Krad_1557 |
| ERCC3        | XPB/ERCC3 helicase | No homologs | DR_A0131 | Rv0861c | Krad_3612 |
| Fmu          | rRNA SAM-dependent methyltransferase | Fmu | DR_2168 | Rv1407 | Krad_2983 |
| Fpg/MutM     | Formamidopyrimidine and 8-oxoguanine DNA glycosylase (Homolog of Nei; see below) | Fpg/MutM | DR_0493 | Rv2924c Rv0944 | Krad_1377 Krad_0158 Krad_0151 |
| FtsK         | Chromosome resolution and positioning | FtsK | DR_0400 | Rv2748c | Krad_1482 |
| Gyra         | DNA gyrase, subunit A | Gyra | DR_1913 | Rv0006 | Krad_0007 |
| GyrB         | DNA gyrase, subunit B | GyrB | DR_0906 | Rv0055 | Krad_0006 |
| HAM1/YggV    | Xanthine triphosphate pyrophosphatase, prevents 6-N-hydroxylaminopurine mutagenesis | HAM1/YggV | DR_0179 | Rv1341 | Krad_3762 |
| HeID         | Helicase IV (ATP-dependent 3'-to-5' DNA helicase) involved in the RecF pathway of recombination | HeID | DR_1775 (putative UvrD) | No homologs | Krad_0757 (putative UvrD) |
| HeLY         | Probable helicase, Ski2 subfamily (ATP-dependent RNA helicase) | No homologs | No homologs | Rv2092c (HeLY) | Krad_1885 Krad_0173 |
| HeIZ         | Probable helicase with a Zinc finger domain, Snf2/Rad54 family | No homologs | No homologs | Rv2101 (HeIZ) | Krad_1013 |
| HsdM         | Type I restriction/modification system DNA methylase | HsdM | No homologs | Rv2756c | No homologs |
| Protein name | Protein description and comments\(^a\) | *Escherichia coli* | *Deinococcus radiodurans* R1 (locus tag) | *Mycobacterium tuberculosis* H37Rv (locus) | *Kineococcus radiotolerans* SRS30216 (locus) |
|--------------|-------------------------------------------|------------------|------------------------------------------|---------------------------------------------|---------------------------------------------|
| HsdS         | Type I restriction/modification system specificity determinant | HsdS             | No homologs                             | Rv275c (HsdS')                             | No homologs                                  |
| HNS          | HU-histone protein                        | HNS              | No homologs                             | Rv3852                                     | No homologs                                  |
| HolA         | DNA polymerase III (holoenzyme), \(\delta\) subunit | HolA             | DR\_1244                                | Rv2413c (unrelated to *E. coli* HolA)Krad\_3422 (unrelated to *E. coli* HolA) |                                               |
| HolB         | DNA polymerase III (holoenzyme), \(\delta\) subunit | HolB             | DR\_2332                                | Rv3644c                                    | Krad\_0490                                   |
| HolC         | DNA polymerase III (holoenzyme), chi subunit | HolC             | No homologs                             | No homologs                               | No homologs                                  |
| HolD         | DNA polymerase III (holoenzyme), psi subunit | HolD             | No homologs                             | No homologs                               | No homologs                                  |
| HolE         | DNA polymerase III (holoenzyme), theta subunit | HolE             | No homologs                             | No homologs                               | Krad\_2840                                   |
| HrpA         | ATP-dependent helicase                    | HrpA             | DR\_0420                                | No homologs                               | Krad\_3104 Krad\_1244                         |
| HupB/IHF     | DNA binding protein II, Integration host factor (IHF); histone-like proteins | HupB             | DR\_A0065                               | Rv2986c (HupB) Rv1388 (IHF)                | Krad\_2337 Krad\_1360 Krad\_2805 Krad\_2005 Krad\_3371 |
| LexA         | Transcriptional regulator, repressor of the SOS regulon, autoprotoase | LexA             | DR\_A0344 DR\_A0074                     | Rv2720                                     | Krad\_1506                                   |
| Lhr          | ATP-dependent helicase superfamily II     | Lhr              | No homologs                             | Rv3296                                     | Krad\_1489                                   |
| LigA         | DNA ligase, NAD\(^+\)-dependent           | LigA             | DR\_2069                                | Rv3014c                                    | Krad\_1315                                   |
| LigB         | DNA ligase, NAD\(^+\)-dependent           | LigB             | No homologs                             | Rv938                                      | No homologs                                  |
| LigC         | Probable DNA ligase                      | LigC             | No homologs                             | Rv3731 (LigC)                             | Krad\_0653                                   |
| Mfd          | Transcription repair coupling factor; helicase | Mfd              | DR\_1532                                | Rv1020                                     | Krad\_1067                                   |
| MPG          | 3-Methylguanine DNA glycosylase           | MPG              | DR\_2074 (also see AlkA)                | Rv1688                                     | Krad\_3154                                   |
| Mrr          | Type IV restriction endonuclease          | Mrr              | DR\_1877 DR\_0508 DR\_0587 Rv2528c      | No homologs                               |                                               |
| Mug (ygjF)   | G/T mismatch-specific thymine DNA glycosylase, distantly related to DR\_1751; Present as a domain of many multidomain proteins in many eukaryotes | Mug (ygjF)       | DR\_0715                                | No homologs                               |                                               |
| MutH         | Endonuclease, Component of the MutHLS complex functions in the methyl-directed mismatch repair pathway | MutH             | No homologs                             | No homologs                               | No homologs                                  |
| MutS         | ATPase, Component of the MutHLS complex functions in the methyl-directed mismatch repair pathway | MutS             | DR\_1976 DR\_1039 contains a frameshift | No homologs                               | No homologs                                  |
| MutL         | Predicted ATPase, Component of the MutHLS complex functions in the methyl-directed mismatch repair pathway | MutL             | DR\_1696                                | No homologs                               | No homologs                                  |
| MutT         | 8-oxo-dGTPase, D. r. encodes additional 17 paralogs; only some predicted to function in repair | MutT             | DR\_0261                                | Rv2985c Rv1600c Rv041c                     | Krad\_1131 Krad\_2346 Krad\_3140 Krad\_2697 Krad\_0113 |
| MutY         | 8-oxoguanine DNA glycosylase & AP-lyase, A-G mismatch DNA glycosylase | MutY             | DR\_2285                                | Rv3589c                                    | Krad\_0599                                   |
| Nei          | Endonuclease VIII (also see Fpg above)    | Nei              | No homologs                             | Rv3297 Rv2464c                            | Krad\_1488 Krad\_3521 Krad\_0294 Krad\_3396 |
| NfI          | Endonuclease V                            | NfI              | DR\_2162                                | No homologs                               |                                               |
| NfO          | Endonuclease IV (AP endonuclease)         | NfO              | No homologs                             | Rv0670                                     | No homologs                                  |
| Nth          | Endonuclease III & thymine glycol DNA glycosylase; DR\_0928 and DR\_2438 are of archaeal type and DR\_0289 is close to yeast protein | Nth              | DR\_2438, DR\_0289, DR\_0928 Rv3674c    |                                               | Krad\_0422                                   |
| Ogt          | O-6-methylguanine/O-4-methylthymine DNA methyltransferase | Ogt              | DR\_0428                                | Rv1316c                                    | Krad\_3712                                   |
| Protein name | Protein description and comments | *Escherichia coli* | Deinococcus radiodurans | Mycobacterium tuberculosis | Kineococcus radiotolerans |
|--------------|----------------------------------|-------------------|------------------------|-------------------------|--------------------------|
| ParC         | DNA Topoisomerase IV, subunit A (Type II topoisomerase) | ParC | No homologs | No homologs | Krad_1546 |
| ParE         | DNA Topoisomerase IV, subunit B (Type II topoisomerase) | ParE | No homologs | No homologs | Krad_1534 |
| PepA         | DNA binding (independent of Aminopeptidase activity) protein required for maintenance of plasmid monomers. | PepA | DR_0717 | Rv2213 | Krad_3276 Krad_1149 |
| PinR         | Putative recombinase                | PinR | DR_C0005 | No homologs | Krad_4707 Krad_4374 |
| PolA         | DNA polymerase I                    | PolA | DR_1707 | No homologs | Krad_2951 |
| PolB         | DNA polymerase II                   | PolB | No homologs | No homologs | No homologs |
| PriA         | Putative primosomal protein n’ (replication factor Y) | PriA | DR_2606 | Rv1402 | Krad_2988 |
| PriB         | Core component of the primosome, binds to PriA and single-stranded DNA | PriB | No homologs | No homologs | No homologs |
| PhoB         | Photolyase (direct monomerization cyclobutane-type pyrimidine dimers) | PhoB | No homologs | No homologs | Krad_3554 Krad_4047 |
| RadA         | Predicted ATP-dependent protease    | RadA (Sm) | DR_1105 | Rv3585c | Krad_4702 |
| RadC         | Predicted acyltransferase; predicted DNA-binding protein | RadC | No homologs | No homologs | No homologs |
| RecA         | Recombinase; ssDNA-dependent ATPase, activator of LexA autoproteolysis | RecA | DR_2340 | Rv2737c | Krad_1492 |
| RecB         | Helicase/exonuclease               | RecB | No homologs | No homologs | Rv0630c | Krad_0993 |
| RecB (exo1)  | RecB family exonuclease 1          | No homologs | No homologs | Rv3202c | Krad_1171 |
| RecB (exo2)  | RecB family exonuclease 2          | No homologs | No homologs | No homologs | Krad_4407 |
| RecB (exo3)  | RecB family exonuclease 3          | No homologs | No homologs | Rv2119 | Krad_1855 |
| RecC         | Helicase/exonuclease               | RecC | No homologs | No homologs | Rv0631c | Krad_0992 |
| RecD         | Helicase/exonuclease; Contains three additional N-terminal helix-hairpin-helix DNA-binding modules; closely related to RecD from B. subtilis and Chlamydia | RecD | DR_1902 | Rv0629c | Krad_0994 |
| RecE         | Exonuclease VIII                   | RecE | No homologs | No homologs | No homologs |
| RecF         | Predicted ATPase; required for daughter-strand gap repair | RecF | DR_1089 | Rv0003c | Krad_0004 |
| RecG         | Holliday junction-specific DNA helicase; branch migration inducer | RecG | DR_1916 | Rv2973c | Krad_1368 |
| RecJ         | Single-stranded DNA-specific exonuclease | RecJ | DR_1126 | No homologs | No homologs |
| RecN         | Predicted ATPase                   | RecN | DR_1477 | Rv1696c | Krad_3147 |
| RecO         | Required for daughter-strand gap repair | RecO | DR_0819 | Rv2362c | Krad_3375 |
| RecQ         | Helicase; suppressor of illegitimate recombination | RecQ | DR_1289 DR_2444 | Rv1253 | Krad_0829 RecQ-like: Krad_4305 RecQ-like: Krad_4305 |
| RecR         | Required for daughter-strand gap repair | RecR | DR_0198 | Rv3715c | Krad_0465 |
| RecT         | DNA annealing protein              | RecT | No homologs | No homologs | Krad_1418 |
| RecX         | Regulatory protein for RecA        | RecX | DR_1310 | Rv2736c | Krad_1493 |
| RuvA         | Holliday-junction-binding subunit of the RuvABC resolvosome | RuvA | DR_1274 | Rv2593c | Krad_3054 |

Table 2. Cont.
| Protein name | Protein description and comments | *Escherichia coli* | *Deinococcus radiodurans* R1 (locus tag) | *Mycobacterium tuberculosis* H37Rv (locus) | *Kineococcus radiotolerans* SRS0216 (locus) |
|--------------|---------------------------------|-------------------|------------------------------------------|------------------------------------------|------------------------------------------|
| RuVB         | Helicase subunit of the RuvABC resolvasome | RuvB | DR_0596 | Rv2592c | Krad_3053, Krad_3828 |
| RuVC         | Endonuclease subunit of the RuvABC resolvasome | RuvC | DR_0400 | Rv2594c | No homologs |
| RusA (YbcP)  | Endonuclease/Holliday junction resolvase | RuA (YbcP) | No homologs | No homologs | No homologs |
| SbcB         | Exodeoxyribonuclease I | SbcB | DR_1922 | No homologs | No homologs |
| SbcC         | Exonuclease subunit, Predicted ATPase | SbcC | DR_1921 | Rv1277 (unrelated to *E. coli* SbcD) | Krad_0868, Krad_2554 (Both unrelated to *E. coli* SbcD) |
| SbcD         | Exonuclease | SbcD | DR_1921 | Rv2896c | Krad_4481 |
| SdrA         | DNA or RNA helicase of superfamily II | No homologs | No homologs | Rv2917 | Krad_3772 |
| Smf          | Predicted Rossmann fold nucleotide-binding protein involved in DNA uptake | Smf | DR_0120 | Rv0054, Rv2478c | Krad_0827, Krad_4460 |
| Smb          | Single-strand binding protein; D. radiodurans R1 has three incomplete ORFs corresponding to different fragments of the SSB | Smb | DR_0099 | Rv1210 | Krad_0858 |
| Tag          | 3-methyladenine DNA glycosylase I | Tag | DR_1374 | Rv3646c | Krad_0487 |
| TopA/TopB    | DNA topoisomerase I (Type IA topoisomerase)/DNA topoisomerase III (Type I topoisomerase) | TopA | DR_1374 | Rv3646c | Krad_0487 |
| UDG          | Uracil DNA glycosylase | UDG | DR_1275 | Rv0322 | Krad_3900 |
| UmuC         | Error-prone DNA polymerase; in conjunction with umuD and recA catalyzes translesion DNA synthesis | UmuC | No homologs | No homologs | No homologs |
| UmuD         | In conjunction with UmuC and RecA, facilitates translesion DNA synthesis; autopro tease | UmuD | No homologs | No homologs | No homologs |
| Ung          | Uracil DNA glycosylase; DR_0689 is a likely horizontal transfer from a eukaryote or a eukaryotic virus | Ung | DR_0689, DR_1663 | Rv2976c | Krad_3639 |
| Uve1/BS_YwjD | UV-endonuclease; activity was characterized in Neurospora | Uve1/BS_YwjD | DR_1819 | No homologs | No homologs |
| UvrA         | ATPase, DNA binding | UvrA | DR_1771, DR_10188 | Rv1638c | Krad_2940, Krad_1787, Krad_0057 |
| UvrB         | Helicase | UvrB | DR_2275 | Rv1633c | Krad_2942 |
| UvrC         | Nuclease | UvrC | DR_1354 | Rv1420 | Krad_2935 |
| UvrD         | DNA helicase II (DNA-dependent helicase activity) initiates unwinding from a nick; DR_1572 has a frameshift | UvrD | DR_1775 | Rv0949, Rv3198c (putative) | Krad_0757, Krad_1179, Krad_1172, Krad_4408 |
| UvrD2        | Putative helicase | UvrD2 | DR_1775 (putative UvrD2) | Rv3198c | Krad_1179 |
| Vsr          | Strand-specific, site specific, GT mismatch endonuclease; fixes deamination resulting from Dcm | Vsr | No homologs | No homologs | No homologs |
| XseA/nec7    | Exonuclease VII, large subunit | XseA/nec7 | DR_0186 | Rv1108c | Krad_1122 |
| XseB         | Exonuclease VII, small subunit | XseB | DR_2586 | Rv1107c | Krad_1121 |
| XthA         | Exodeoxyribonuclease III (AP endonuclease) | XthA | DR_0354 | Rv0427c | Krad_4198, Krad_3979, Krad_0544 |
| YbaZ         | Possible methylated-DNA-(protein)-cysteine S-methyltransferase | YbaZ | DR_0428 | Rv3204 | Krad_1169 |
| YhdJ         | Adenine-specific DNA methylase | YhdJ | DR_0020 | No homologs | No homologs |
| YejH         | DNA or RNA helicase of superfamily II | YejH | DR_A0131_1_2 | No homologs | Krad_3612 (see also ERCC3) |

Table 2. Cont.
D. radiodurans is adept at detoxifying reactive oxygen species (ROS) during radiation exposure when many free radicals are generated from hydrolysis of water [13–15]. Like Deinococcus, K. radiotolerans produces carotenoids as one level of protection. K. radiotolerans also possesses an impressive suite of genes involved in ROS detoxification (table 3) comparable to those found in bacterial pathogens of mammals such as Neisseria gonorrhoeae [28]. Pathogenic bacteria are routinely exposed to oxidative stress by the host as part of an innate immune response. These results suggest a thorough ROS detoxification network.

Bioremediation potential

Because K. radiotolerans was isolated from a HLW facility, the genome was examined for gene products that could be used to generate carbon or energy from organic compounds in the tanks. Orthologs belonging to known degradation pathways were not detected for U.S. Departments of Energy (DOE) and Defense (DOD) selected priority pollutants for including benzene, toluene, ethylbenzene, and xylenes (the BTEX compounds), chlorinated hydrocarbons, polynuclear aromatic hydrocarbons, polychlorinated biphenyls, ketones, alkanes, phenols, phthalates, explosives, and pesticides. Several homologues for atrazine degradation were noted \( \text{AtzABZ, Krad}_3031, 4281, 0533 \), respectively, though the pathway seems incomplete.

The SRS HLW also contains low molecular weight organic complexants which interfere with downstream chemical stabilization and processing. Commonly used complexants and decontamination reagents at the SRS include oxalate, glycolate, citrate, and formate. Growth using each organic acid as a sole carbon source was evaluated for K. radiotolerans in comparison with glucose as a positive control and 0.1% yeast extract as a negative control. Respiration was examined using O2 consumption and CO2 evolution. Preliminary studies revealed that citrate and glycolate were unsuitable growth substrates (data not shown). Respiration rates and biomass yields on glucose were higher than those of other carbon sources (table 4). Biomass yields with 5 mM oxalate and 5 mM formate were generally consistent with the 0.1% yeast extract control though higher concentrations were toxic. Similarly, O2 consumption and CO2 production were both better at lower oxalate and formate concentrations. While biomass production was similar between formate, oxalate, and YE, the respiration rates were higher for formate and oxalate.

Table 3. Reactive oxygen species detoxification genes in K. radiotolerans.

| Enzyme                              | Krad Locus Tag |
|-------------------------------------|----------------|
| Alkyl hydroperoxide reductase, ahpC | 3757           |
| Catalase, katE                      | 0865           |
| Mn Catalase, katA                   | 0815           |
| Cytochrome c peroxidase, mouG       | Absent         |
| Glutathionyl spermidine synthase, GSP-Syn | 2757         |
| Glutathione peroxidase, GSHPx       | 1247           |
| Dyp-type peroxidase                 | 3350           |
| Organic hydroperoxidase             | 0813           |
| Peptide methionine sulfoxide reductase, msrA | 1091     |
| Fe/Mn superoxide dismutase, sodA   | 3578           |
| Superoxide reductase, sorA          | Absent         |
While none of the carbon sources stimulated growth, formate and oxalate promote survival and quicken recovery of *K. radiotolerans* following prolonged starvation (Table 5). In these experiments, chloramphenicol was used to accelerate starvation and to uncouple growth from survival. At intervals of 3, 7 or 14 days cells were resuspended in fresh TGY medium and respiration measured. After three days of incubation, substrate specific differences were already apparent. Once starvation was lifted, the highest respiration rates were recorded for cells starved in the presence of oxalate and formate. While all of the cultures recovered, the formate-starved culture was first to enter exponential growth, 4 hours ahead of all other treatments (data not shown).

Remarkably, after 7 days of starvation, the highest rate of respiration was measured from the oxalate treated culture, which recovered and began exponential growth 60 hours after starvation was relieved (Figure 5). The yeast extract treated culture also recovered following more than 100 hours of post-starvation recovery. None of the cultures responded after 14 days of treatment.

*E. coli* contains three formate dehydrogenases. Formate dehydrogenase H (FdhF) is coupled to a hydrogenase and used for hydrogen production by the formate hydrogen lyase reaction. *K. radiotolerans* appears to have this enzyme (Krad1331). Formate dehydrogenase N (FdhGHI) is induced when cells are grown anaerobically with nitrate. Formate dehydrogenase O (FdoGHI) is expressed aerobically and

### Table 4. Metabolism of glucose, oxalate and formate by *K. radiotolerans*.

| Substrate | Consumption/Production Rates (μmol/hr) | Dry Weight (mg) |
|-----------|----------------------------------------|-----------------|
|           | O2 | CO2 | O2:CO2 |                     |
| Glucose (5mM) | 8.08 | 6.71 | 1.2 | 2.77 ± 0.21 |
| Glucose (10mM) | 8.72 | 7.87 | 1.11 | 3.05 ± 0.07 |
| Oxalate (5mM) | 1.83 | 1.36 | 1.35 | 2.17 ± 0.06 |
| Oxalate (10mM) | 1.42 | 1.09 | 1.31 | 1.73 ± 0.21 |
| Formate (5mM) | 6.43 | 6.36 | 1.01 | 2.30 ± 0.10 |
| Formate (10mM) | 5.87 | 5.75 | 1.02 | 1.43 ± 0.25 |
| YE (0.1%) | 4.97 | 4.53 | 1.1 | 2.20 ± 0.00 |
| Kill Control | 0 | 0 | 0 | 0.00 ± 0.00 |

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### Figure 5. Substrate-facilitated survival and recovery of *K. radiotolerans* following starvation.
Following 7 days of incubation, cultures starved in the presence of oxalate (●) and yeast extract (●) recovered following transfer to fresh TGY medium, while no respiratory response was measured from cultures starved in the presence of glucose, formate, citrate, or Tris buffer.

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### Table 5. Starvation recovery of *K. radiotolerans*.

| Substrate | 3 Days | 7 Days | 14 Days |
|-----------|--------|--------|--------|
|           | O2 | CO2 | O2 | CO2 | O2 | CO2 |
| Glucose | 8.63 | 50.42 | 0.25 | 0.15 | 0.3 | 0.09 |
| Formate | 19.57 | 125.9 | 0.49 | 0.22 | 0.3 | 0.05 |
| Oxalate | 11.02 | 58.09 | 15.99 | 14.21 | 0.3 | 0.08 |
| Citrate | 2.65 | 11.32 | 0.37 | 0.13 | 0.3 | 0.05 |
| YE (0.01%) | 1.29 | 1.12 | 9.47 | 8.54 | 0.07 | 0.09 |

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may participate in transfer of electrons from formate to oxygen. Formate dehydrogenases N and O have similar amino acid sequences. *K. radiotolerans* has one of the two (Krad1601, Krad1602, Krad1603), but it is difficult to tell based on the amino acid sequence whether it is an N-type or an O-type.

The *K. radiotolerans* genome encodes glycolysis, pentose phosphate, tricarboxylic acid (TCA) cycle and glyoxylate bypass pathways (table 6). The pentose phosphate cycle [29] and the glyoxylate bypass [30] may both be involved in radiation resistance and post-irradiation recovery as in *D. radiodurans*. Both pathways are important routes for the production of biosynthetic metabolites while minimizing the production of damaging oxygen radicals. *K. radiotolerans* exhibits aerobic respiration on 5- and 6-carbon sugars (eg., fructose, amylose, maltose) and sugar alcohols (eg., glycerol, mannitol, sorbitol) (data not shown). A partial gluconeogenesis pathway was identified (missing pyruvate carboxylase, *pcb*, and glucose-6-phosphatase, *glp*). A partial Entner-Doudoroff pathway was also found, lacking glucose dehydrogenase, *gld*, and phosphogluconate dehydratase, *edd*. Carbohydrate metabolism is supported by many ABC-type sugar transporters.

### Table 6. Central metabolism in *K. radiotolerans*.

| Pathway                  | Krad Locus Tag | Gene Designation | Gene Product Function                  |
|--------------------------|----------------|------------------|----------------------------------------|
| Glycolysis               | 3239           | glcA             | Hexokinase                             |
|                          | 1463           | gpi              | Glucose-6-phosphate isomerase           |
|                          | 4274           | pkA              | Fructose-6-phosphate kinase             |
|                          | 2157           | fba              | Fructose-1,6-bisphosphate aldolase      |
|                          | 2931           | gapA             | Glyceraldehyde-3-phosphate dehydrogenase|
|                          | 2930           | pgk              | Phosphoglycerate kinase                 |
|                          | 0891           | pgm              | Phosphoglyceromutase                    |
|                          | 1073           | eno              | Enolase                                |
|                          | 2959           | pyk              | Pyruvate kinase                         |
| Pentose Phosphate Cycle  | 3372           | ppc              | Phosphoenolpyruvate carboxykinase       |
|                          | 1463           | pgi              | Phosphoglucoisomerase                   |
|                          | Absent         | pcb              | Pyruvate carboxylase                    |
|                          | Absent         | glp              | Glucose-6-phosphatase                   |
| TCA Cycle                | 1140           | gltA             | Citrate synthase                        |
|                          | 1566           | acnA             | Aconitase                              |
|                          | 3988           | icd              | Isocitrate dehydrogenase                |
|                          | 1228           | sucA             | Oxoglutarate dehydrogenase             |
|                          | 3279           | sucB             | Succinyl-transferase                    |
|                          | 3999           | sucC             | Succinyl-CoA synthetase, beta           |
|                          | 3998           | sucD             | Succinyl-CoA synthetase, alpha          |
|                          | 3955           | frdB             | Fumarate reductase                      |
|                          | 3954           | sdhA             | Succinate dehydrogenase                 |
|                          | 1112           | fumC             | Fumarase                               |
|                          | 0742           | mdh              | Malate dehydrogenase                   |
| Pentose Phosphate Cycle  | 1494           | gnl              | Gluconolactonase                        |
|                          | 0597           | gntK             | Gluconate kinase                        |
|                          | 2133           | eda              | 2-keto-3-deoxy-6-phosphogluconate aldolase|
| Entner-Doudoroff         | 1494           | gnl              | Gluconolactonase                        |
|                          | 0597           | gntK             | Gluconate kinase                        |
|                          | 2133           | eda              | 2-keto-3-deoxy-6-phosphogluconate aldolase|
|                          | Absent         | edd              | Phosphogluconate dehydratase            |
|                          | Absent         | gld              | Glucose dehydrogenase                   |
| Glyoxalate Bypass        | 0108           | aceA             | Isocitrate lyase                        |
|                          | 2227           | aceB             | Malate synthase                         |

The *K. radiotolerans* genome was queried by the tblastn algorithm using reference sequences from the genomes of *Frankia alni* ACN14a, *Streptomyces coelicolor* A3(2), *Deinococcus radiodurans* R1, and *E. coli* K12. Cut-off values for confident identification required both amino acid percent identities >45% and an E-score < e-10.

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Electron acceptors other than O₂ increase habitat diversity by enabling growth when oxygen tension is low. Krad1328 has strong identity with Arthrobacter aurescens TC1 nitrate reductase. Nitrate respiration may be a general feature of Arthrobacter species as *Arthrobacter globiformis* and *Arthrobacter nicotianae* have been shown to utilize NO₃⁻ as a terminal electron acceptor [31]. *Arthrobacter* spp. are routinely recovered from subsurface environments contaminated by high level radioactive waste [32,33]. Krad1326 is not related to the *Kuenenia stuttgartiensis* enzyme that participates in anammox, anaerobic ammonium oxidation during which nitrite and ammonium are converted directly into dinitrogen gas.

The *K. radiotolerans* genome possesses a large number of molybdopterin oxidoreductases. Krad4344 has striking identity to the trimethylamine-N-oxide (TMAO) reductase TorA from *E.coli* K12, which is a terminal electron acceptor that is specific for N-oxides. TorA is connected to the quinone pool via a membrane-bound penta-haem cytochrome (TorC) [34]. DorCA in *Rhodobacter* is very similar to TorCA with the difference that DorC catalyses the reduction of both dimethylsulfoxide and TMAO. *K. radiotolerans* lacks a homolog to TorC or DorC and reduction of TMAO has not been experimentally examined. There is no evidence for the type of fumarate reductase that reduces fumarate to succinate during anaerobic growth or for proteins homologous those from *Shewanella* and *Geobacter* involved in metal reduction.

In summary, *K. radiotolerans* exhibits moderate nutritional versatility with genes for growth on a variety of carbon sources and at least two terminal electron acceptors. This organism appears to utilize two of the commonly used complexants, oxalate and formate. These results suggest that *K. radiotolerans* should be investigated for use in scrubbing complexants from HLW tanks. The ability of oxalate and formate to stimulate respiration and prolong viability might provide certain clues into the metabolic ecology of *K. radiotolerans*. *Kineospora* species are commonly found on weathered rock, stone monuments, paintings, desert crust or varnish along with cyanobacteria and fungi which together form biotic crusts, lichens, or endolithic communities (Garrity et al., 2005; West and Coombs, 2006, Schabereiter-Gurtner et al 2001, Torre et al 2003, Tringe et al 2006). Cyanobacteria, fungi, as well as plants, release formate and oxalate for toxic metal chelation and detoxification, and mineral dissolution (Neaman et al., 2005; West and Coombs, 1981). Several studies have demonstrated heterotrophic mineralization of oxalate to CO₂ without assimilation but the mechanism remains unknown.

**Life cycle**

The production of motile zoospores is widespread but patchy among members of the Actinobacteria phylum. All three *Kineospora* species produce zoospores during part of their life cycle, but zoospore formation has not been extensively examined in any species [2,33,36]. Zoospores are produced at the tips of substrate hyphae and in clusters on sporophores in the closely related genus *Kineospora* [37]. However, *K. radiotolerans* cells do not produce hyphae or sporangioles. Cells grown in rich PTYG medium produce large clusters of nonmotile cells that alternate division planes to form cubes of cells connected by a thick extracellular matrix [2]. During stationary phase spherical, dispersed zoospores, approximately 1 μm in diameter, are produced. In PTYG medium, the spores lack flagella. Motile spores were observed only after exposure to 10% sandy loam extract or with certain carbon sources [2]. Thus the production of zoospores and the development of flagella on those spores are subject to environmental cues that remain to be elucidated.

Movement of *Kineospora* SR11 zoospores up chemical gradients has been observed for a variety of inorganic compounds [39]. Remarkable speeds of up to 160 μm s⁻¹ are achieved in *Kineospora* SR11 zoospores [30]. *Kineospora* SR11 is attracted to K⁺, Mg²⁺, and Ca²⁺ salts of phosphate, sulfate and halides, but not other combinations of these ions, suggesting that chemotaxis may be dependent on specific cations and anions pairs. The *K. radiotolerans* genome contains complete pathways for flagellar biogenesis and chemotaxis suggesting that zoospore dispersal is designed to colonize new niches for growth.

The bacterial flagellum is a complex nanomachine that requires dozens of gene products for its assembly and function. Flagellar gene expression is temporally regulated to produce proteins as they are needed for flagellar assembly. Where it has been examined, temporal control of flagellar gene expression is achieved through a transcriptional hierarchy initiated by a master regulator [39,40]. Flagellar genes in *K. radiotolerans* are clustered within a motility island located between Krad1621 and Krad1673 (table 7). Genes within this region encode components of the basal body, hook, filament, flagellar protein export apparatus, flagellar chaperones, flagellar motor and motor switch. None of the genes within the motility island encode proteins that share homology with known master regulators of flagellar biogenesis though some of the genes encode proteins involved in regulating expression of flagellar biogenesis or motility. One such regulatory protein is a FliA (σ₂⁰) homolog encoded by Krad1625. FliA is an alternative σ factor that is required for transcription of flagellar genes in many bacteria whose products are needed late in flagellar assembly [39,40]. Activity of FliA is often regulated by anti-sigma factor FlgM [39–41]. Upon completion of the hook-basal body complex FlgM is secreted from the cytoplasm via the flagellar protein export apparatus, which allows expression of the FliA-dependent flagellar genes. Bacteria that possess a flgA ortholog often also possess an ortholog of flgM [42], suggesting that negative control of FliA activity by FlgM is a general feature of flagellar gene regulation. *K. radiotolerans* does not possess a flgM ortholog, nor do the other flagellated Actinobacteria sequenced to date (*Norcardioides* sp. JS614, *Acidothermus cellulolyticus* and *Lefosnia xyli*). It is possible that *K. radiotolerans* possesses a protein that is functionally equivalent to FlgM but does not share homology with it. Alternatively, regulation of FliA may occur through a novel mechanism in *K. radiotolerans* and related Actinobacteria.

A second regulatory gene within the *K. radiotolerans* motility island is csrA, which encodes the carbon storage regulator. The csrA gene is also associated with flagellum biosynthetic genes in *Norcardioides* sp. JS614, *Acidothermus cellulolyticus* and *L. xyli*. CsrA is a global regulator that binds mRNA to regulate gene expression either positively or negatively [43,44]. CsrA-mediated control of flagellar biogenesis and/or motility has been demonstrated in some Gram-negative bacteria [45–47]. CsrA stimulates expression of *E. coli* fliDC (encodes the master regulator for flagellar biosynthesis) by binding the fliDC operon leader transcript and stabilizing the mRNA [47]. CsrA was recently shown to repress expression of the *Bacillus subtilis* flagellin gene, hag, by preventing ribosome binding to the transcript [48]. RNA footprinting assays identified two CsrA-binding sites in the hag transcript, one of which (BS2) overlaps the Shine-Dalgarno sequence. The corresponding region of the predicted transcript of the *K. radiotolerans* flagellin gene (Krad1626) matches well to the BS2 site of *B. subtilis* hag (9 of 12 bases match). Thus, CsrA may regulate expression of the *K. radiotolerans* flagellin gene as it does *B. subtilis* hag.

Developmental control of flagellar genes has been extensively examined during the dimorphic life cycle of *Caulobacter crescentus*, where a non-flagellated, stalked mother cell undergoes asymmetric binary fission to produce a flagellated swarmer cell [49–51]. The
swarmer cell eventually differentiates into a mother cell, losing its flagellum and elaborating a stalk. Several genes involved in developmental regulation of flagellar biogenesis in C. crescentus have been identified [49–51] including cckA, a histidine kinase in an operon of export genes that activates the master regulator, CtrA [52]. Krad1670, a predicted histidine kinase, appears to be the last gene of an operon that includes fljA, fliR and fliB, genes encoding components of the flagellar protein export apparatus. Given this conservation in synteny K. radiotolerans Krad1670 may play a role in developmental regulation of flagellar biogenesis similar to that of cckA in C. crescentus.

Signal transduction in K. radiotolerans has been examined using the microbial signal transduction database MIST [53]. K. radiotolerans exhibits regulatory complexity comparable to that of Streptomyces spp. with regard to two-component systems. K. radiotolerans is unusual in having 116 di-guanylate cyclase domains. In general these protein domains, which are used to synthesize the second messenger cyclic di-GMP, are over represented in members of the Frankineae suborder of the Actinobacteria but even still K. radiotolerans exhibits resistance to desiccation [2]. Nevertheless, such organisms may have tremendous potential in remediating metabolizable organic constituents of HLW. Removal of organic constituents directly in HLW tanks could greatly improve processing efficiency of HLW. K. radiotolerans appears to utilize two of the commonly used complexants at the SRS, oxalate and formate, and should be investigated for use in scrubbing complexants from HLW tanks.

### Materials and Methods

#### Culture conditions and chemicals

*Kineococcus radiotolerans SRS30216 (BAA-149)* was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cultures were maintained on TGY plating medium (1.0% tryptone, 0.1% glucose, 0.5% yeast extract, 1.4% Difco agar). Except where noted all experiments were conducted in semi-defined growth medium (6.1 g/L Tris (pH 7.0), 0.1% YE) amended with different carbon sources to a final concentration of 5 mM unless otherwise mentioned. All cultures were grown at 28°C and liquid cultures were shaken at 150 rpm.

#### Genome sequencing

The genome of *Kineococcus radiotolerans SRS30216* was sequenced at the Joint Genome Institute (JGI) using a combination of 3 kb, 8 kb and 340 kb DNA libraries. All general aspects of library construction and sequencing performed at the JGI can be found at [http://www.jgi.doe.gov/](http://www.jgi.doe.gov/). Draft assemblies were based on 70,886 total reads. All three libraries provided 9.5× coverage of the genome. The Phred/Phrap/Consed software package (<http://www.phrap.com>) was used for sequence assembly and quality assessment (Ewing and Green 1998; Ewing et al. 1998; Gordon et al. 1998).

After the shotgun stage, 23,746 reads were assembled with parallel phrap (High Performance Software, LLC). Possible misassemblies were corrected with Dupfinisher (Han, 2006,) clone shatter libraries or transposon bombing of bridging clones (Epigenic Biotechnologies, Madison, WI). Gaps between contigs were closed by editing in Consed, custom primer walks, or PCR amplification (Roche Applied Science, Indianapolis, IN.). A total of 4392 primer walk reactions, 5 transposon bombs, 28 clone shatter libraries, and 4 PCR shatter libraries were necessary to close gaps, to resolve repetitive regions, and to raise the quality of the finished sequence. The completed genome sequences of *K. radiotolerans SRS30216* contains 71,381 reads, achieving an average of 14-fold sequence coverage per base with an error rate less than 1 in 100,000. The *K. radiotolerans SRS30216* genome has 3 contigs. The main chromosome and the largest plasmid, pKrad1, both
appear to be linear. PCR and blunt end adapter PCR were used unsuccessfully to attempt to close the gaps.

**Southern blotting and PCR**

A culture of *Kineococcus radiotolerans* was grown to mid log phase in 100 ml of PTYG (0.5% (w/v) yeast extract, 0.5% (w/v) trypclone, 0.5% (w/v) peptone, 0.006% (w/v) MgSO4, 0.0006% (w/v) CaCl2). The cells were collected by centrifugation at 10,000 × g for five minutes, resuspended in 9.5 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and frozen at −20°C for approximately one hour. The cells were quickly thawed at 37°C, mixed with 0.5 ml of 10% SDS and 50 μl of 20 mg/ml proteinase K, and incubated at 37°C for one hour. Then 1.8 ml of 5 M NaCl was added along with 1.8 ml of 1 g CTAB (Hexadecyltrimethylammonium Bromide) and 10 ml of 0.7 M NaCl. The mixture was incubated for 20 minutes at 65°C, extracted with an equal volume of chloroform/isooamyl alcohol (24:1), and centrifuged for 10 minutes at 10,000 × g at room temperature. The aqueous upper phase was transferred to a fresh tube and the DNA precipitated with 0.6 volume of isopropanol then washed with 70% ethanol. The pellet was resuspended in 2 ml TE buffer and incubated at 37°C with 40 μg/ml DNase free RNase for at least 1 hr.

The sample was extracted with an equal volume of Tris buffered phenol (pH 7.9) and the upper aqueous phase was transferred to a fresh tube, where it was extracted with an equal volume of 1:1 phenol and chloroform and then an equal volume of 24:1 chloroform and isooamyl alcohol. The DNA was precipitated with 0.5 M ammonium acetate and 60% ethanol at −20°C for 20 minutes and then pelleted. The pellet was washed with 70% ethanol and allowed to air dry. The pellet was resuspended in 0.5 ml TE buffer and the DNA concentration was about 1 μg/ml.

Probes were created for each theoretical end of the *K. radiotolerans* chromosome using PCR and digoxigenin nucleotides (Roche Biochemicals). Primers Krad2223F 5’CGGCCGACATCCGGGTGTATGGTTC3’ and Krad2223R 5’CGATGTTCGGTGCGGCTGTAGC3’ generated a DNA fragment that was homologous with Krad2223. The PCR reaction contained 0.1 ng of genomic DNA, 0.5 μM each of primer solution. The genomic DNA was initially denatured 2 minutes at 95°C. In subsequent cycles the DNA was denatured for 30 seconds at 95°C, the primers were annealed at 60°C for 30 seconds, and the DNA extended at 72°C for 90 seconds. After 30 cycles the final extension was at 72°C for 3 minutes. The PCR products were separated by agarose gel electrophoresis to determine the success of the PCR reaction.

Genomic *K. radiotolerans* DNA was digested with restriction enzymes, BamHI, BglII or Ncol as specified by the manufacturer. The restriction fragments were separated by electrophoresis in 0.8% agarose. The gel was submerged in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 30 minutes and neutralized (0.5 M Tris-HCl, 1.5 M NaCl, pH 7.5) for 30 minutes. The contents of the gel were transferred to a nylon membrane (Roche Biochemicals) using 20 × SSC (0.3 M sodium citrate, 3 M NaCl, pH 7.0). The membrane was exposed in a UV crosslinker. Hybridizations were performed in roller bottles with 20 ml hybridization solution and 15 μl probe at 60°C overnight as recommended by the manufacturer (Roche Biochemicals). CPDS was added to the membrane, incubated at 25°C for 10 minutes and 37°C for 15 minutes, then exposed to X-ray film for 1–10 minutes.

**Starvation Experiments**

*K. radiotolerans* was grown in TGY medium to mid-exponential phase (~26 hr) at 28°C and 150 rpm. Biomass was harvested by centrifugation, washed in 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4), and split evenly into different tubes containing 50 mM Tris HCl (pH 7.0), 0.01% yeast extract, chloramphenicol (100 μg/ml), and an individual substrate at a final concentration of 5 mM. Cultures were incubated at 28°C. After 3, 7, and 14 days of imposed starvation, the biomass was harvested, suspended in fresh TGY medium (50 ml), and the ability of each culture to recover and resume growth was compared across substrates.

**Respirometry**

Metabolic rates were measured using the Micro-Oxymax multichannel respirometer (Columbus Instruments; Columbus, OH, USA). Respirometry bottles (250 ml capacity) were filled with 50 ml of semi-defined growth medium and headspace gases were analyzed every 2 hr. Bottles were incubated at 28°C and 100 rpm. The Micro-Oxymax respirometer measures the volume of gas consumed (O2) or produced (CO2). Conversions were made to moles of gas using the ideal gas law. End point biomass determinations were made in 125 ml shake flasks using 25 ml of growth medium. Cells were filtered onto 0.22 μm GS filters (Millipore) and dried to a constant weight at 65°C.

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**Author Contributions**

Conceived and designed the experiments: CEB LJS. Performed the experiments: CEB SB GMH BWS ES CSH. Analyzed the data: CEB SB TB TRH OVT LJS. Wrote the paper: CEB OVT LJS.
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