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Escherichia coli Genes and Pathways Involved in Surviving Extreme Exposure to Ionizing Radiation

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To further an improved understanding of the mechanisms used by bacterial cells to survive extreme exposure to ionizing radiation (IR), we broadly screened nonessential *Escherichia coli* genes for those involved in IR resistance by using transposon-directed insertion sequencing (TraDIS). Forty-six genes were identified, most of which become essential upon heavy IR exposure. Most of these were subjected to direct validation. The results reinforced the notion that survival after high doses of ionizing radiation does not depend on a single mechanism or process, but instead is multifaceted. Many identified genes affect either DNA repair or the cellular response to oxidative damage. However, contributions by genes involved in cell wall structure/function, cell division, and intermediary metabolism were also evident. About half of the identified genes have not previously been associated with IR resistance or recovery from IR exposure, including eight genes of unknown function.

Organisms have evolved mechanisms to maintain genomic integrity in the face of extreme environmental stresses. One class of extremophiles, typified by the bacterium *Deinococcus radiodurans* (1, 2), exhibits extraordinary resistance to the effects of high doses of ionizing radiation (IR). The repair of damaged DNA, stalled replication forks, and other damaged cellular components is critical for cells to survive exposure to IR. The DNA sugar-phosphate backbone is particularly susceptible to both direct and indirect damage caused by IR (3, 4). Direct damage is caused by absorption of IR by the DNA molecule, which can lead to strand breakage and chemical alterations of bases. In contrast, indirect damage occurs when reactive oxygen species (ROS), such as hydroxyl radicals, which are formed when IR is absorbed by water, interact with DNA. Hydroxyl radicals produce single-strand DNA breaks. Double-strand DNA breaks (DSBs) can occur when two IR-induced single-strand DNA breaks are in close proximity (5). DSBs are the most lethal form of DNA damage, because they halt DNA replication, cause the collapse of the replication fork, and are difficult to repair (1, 6, 7). Cells repair DSBs and other DNA damage caused by IR by utilizing recombinational DNA repair and nonhomologous end joining (6–10). Because ROS are also by-products of aerobic respiration and general metabolism, it is likely that genes involved in IR survival are also involved in preserving DNA integrity under normal conditions, suggesting an essential role in bacteria.

The capacity of cells to repair DNA, particularly double-strand breaks, has long been linked to cell survival after IR exposure (11–19). DNA repair similarly plays a major role in the extremophile IR resistance phenotype of *Deinococcus* (15, 17–19). More recently, the Daly group, and later the Radman group, focused attention on the importance of amelioration of oxidative damage to proteins (20–24). In this mechanism, specialized DNA repair pathways are not necessary. Passive protection of proteins from oxidative processes (including a generic complement of DNA repair functions) facilitate survival at high levels of IR. Nevertheless, clear evidence has indicated that adaptations to the cellular DNA repair systems can make substantial contributions to extreme levels of IR resistance (25). Given the complexity of bacterial metabolism, it seems unlikely that the list of processes contributing to IR resistance is limited to DNA repair and amelioration of oxidative damage to proteins. Thus, a broader assessment is needed.

We have carried out an exercise in directed evolution in which the *Escherichia coli* K-12 strain MG1655 acquired the phenotype of extreme resistance to IR (25, 26). Four evolved populations of *E. coli* were obtained, and they exhibited levels of IR resistance approaching that of *D. radiodurans*. Analysis of numerous sequenced isolates from these populations allowed us to identify the genetic alterations accounting for most of the acquired IR resistance phenotype (25). In one highly evolved isolate, the phenotype was largely explained by mutations in three DNA metabolism genes, *recA*, *dnaB*, and *yfjK*. The modified genes provide the beginning of a more complete molecular accounting of adaptations needed to survive extreme radiation resistance.

Efforts to understand this phenotype have focused to a large extent on *Deincoccus radiodurans* and related bacteria (1, 2, 19, 21, 22, 24). Analysis of transcriptome (27, 28) and proteome (29, 30) changes upon IR exposure, as well as careful analysis of how the genome is reconstructed over time (1, 31–34), have provided some important insights into this bacterium's response to IR. However, broad genetic screens to identify all contributing processes are very difficult to perform with *Deinococcus*, reflecting its multigenomic status (1, 2, 19, 24). In contrast, *E. coli* strains with an extreme IR resistance phenotype provide an opportunity to utilize a highly tractable and insight-fertile genetic system to more broadly explore the molecular basis of this phenotype. One step toward a complete description of the genetic requirements for IR resistance would be the identification of all contributing genes

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TABLE 1 Strains used in this study

Name	Description or genotype	Reference
WT	MG1655	
Founder	*E. coli* MG1655 single colony isolate	Harris et al. (26)
CB2000	Population IR-2-20 isolate	Harris et al. (26)
EAW7704	Founder Δe14	Byrne et al. (25)
EAW247	Founder Δe14 Δ*ftsP*::*kan*	This study
EAW327	Founder Δe14 Δ*rdgC*::*kan*	This study
EAW262	Founder Δe14 Δ*topB*::*kan*	This study
EAW243	Founder Δe14 Δ*rsxB*::*kan*	This study
EAW230	Founder Δe14 Δ*recX*::*kan*	This study
EAW252	Founder Δe14 Δ*radA*::*kan*	This study
EAW242	Founder Δe14 Δ*uup*::*kan*	This study
EAW326	Founder Δe14 Δ*sbcB*::*kan*	This study
EAW232	Founder Δe14 Δ*yejH*::*kan*	This study
EAW250	Founder Δe14 Δ*uvrA*::*kan*	This study
EAW251	Founder Δe14 Δ*uvrB*::*kan*	This study
EAW231	Founder Δe14 Δ*recF*::*kan*	This study
EAW229	Founder Δe14 Δ*recN*::*kan*	This study
RTB003	Founder Δe14 Δ*recG*::*kan*	This study
EAW386	Founder Δe14 Δ*pgi*::*kan*	This study
EAW392	Founder Δe14 Δ*speA*::*kan*	This study
EAW390	Founder Δe14 Δ*yhgF*::*kan*	This study
EAW389	Founder Δe14 Δ*yabI*::*kan*	This study
EAW379	Founder Δe14 Δ*ybjN*::*kan*	This study
EAW397	Founder Δe14 Δ*yafC*::*kan*	This study
EAW398	Founder Δe14 Δ*ybgI*::*kan*	This study
EAW463	Founder Δe14 Δ*yebC*::*kan*	This study
EAW434	Founder Δe14 Δ*ompA*::*kan*	This study
EAW435	Founder Δe14 Δ*tolA*::*kan*	This study
EAW438	Founder Δe14 Δ*prc*::*kan*	This study
EAW439	Founder Δe14 Δ*pstS*::*kan*	This study
SLHC1	Founder Δe14 Δ*crr*::*kan*	This study
EAW467	Founder Δe14 Δ*rsxA*::*kan*	This study
EAW471	Founder Δe14 Δ*dnaJ*::*kan*	This study

TABLE 2 Oligonucleotides used in this study

Name	Sequence
ORB1	5′ CTG TCT CTT ATA CAC ATC TC
ORB5	5′ CAA GCA GAA GAC GGC ATA CGA GAT TCC TCA GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC T
ORB6	5′ CAA GCA GAA GAC GGC ATA CGA GAT ATT GGC GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC T
ORB7	5′ CAA GCA GAA GAC GGC ATA CGA GAT ATT GGC GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC T
ORB8	5′ ATT GAT ACG GCG ACC ACC GAG ATC TAC ACT AAT ACG ACT CAC TAT AGG GAG ACC GGC CTC AG
ORB9	5′ TAG GGA GAC CGG CCT CAG GGT TGA GAT GTG TA

that are not modified in directed evolution trials. That identification requires a genetic screening approach.

Many screens have been carried out to identify genes involved in DNA repair in *E. coli* (35–42). These have resulted in the discovery of many of the key DNA repair enzymes we continue to study today. Screens to identify genes involved in radiation resistance were part of these efforts. The *recN* and *recG* genes were characterized to an extent as genes involved in radiation resistance and given a rad nomenclature (*radB* and *radC*, respectively) until their functions were further understood (12, 43). However, modern screening methods are much more robust and are sensitive methods for discovering new genes with particular functions. We sought to identify the genes involved in survival after extreme IR exposure for three additional reasons. (i) We do not understand the physiological function of nearly one-third of the genes of *E. coli*, despite its role as the most extensively studied organism. (ii) Radiation resistance is a complex phenotype whose molecular basis remains the subject of some controversy (1, 2, 22, 44). (iii) Current research tends to focus on either DNA damage or protein oxidation, and contributions from other processes are possible. We thus set out to provide a more global assessment of the cellular processes that contribute to IR resistance.

A range of modern screening methods have been described (45–49) that utilize transposon mutagenesis in combination with Illumina sequencing. These techniques measure each gene's contribution to fitness on a genomic scale through massive sequencing of transposon-genome junctions in highly mutagenized populations. We employed a relatively new procedure called transposon-directed insertion sequencing (TraDIS) (45). In this method, saturating transposon mutagenesis is performed and the resulting insertion mutants are pooled to make an insertion mutant library. This library is then subjected to repeated exposures to IR. Genomic DNA from the nontreated population as well as the irradiated populations is isolated, and the location of each transposon insertion as well as the frequency of each insertion mutant within the population are determined. The change in frequencies of insertion mutants within the population are calculated for each gene, reflecting the effect the insertion has on a strain's ability to survive radiation exposure.

Using TraDIS, we have identified 46 candidate genes that appear to have a significant role in survival after IR exposure. These are the focus of this report.

MATERIALS AND METHODS

Bacterial strains and primers used in this study. All strains used in this study are *E. coli* K-12 derivatives and are listed in Table 1. Genetic manipulations were performed as previously described (50). Oligonucleotide primers are listed in Table 2.

Transposome preparation. Transposon mutagenesis was performed using the Epicentre EZ-Tn*5* transposition system, which consists of a transposase dimer conjugated to transposon DNA (51). The transposon is EZ-Tn*5* <KAN-2>Tnp and was amplified by using the oligonucleotides ORB1 and phusion polymerase (Stratagene). One hundred nanograms of this DNA was incubated with Tnp EK54/MA56/LP372, a hyperactive transposase with reduced target specificity (52), at room temperature for 3 h. Transposome complexes were dialyzed against Tris-EDTA (TE) to remove all salt from the reaction mixture before electroporation.

Preparation of electrocompetent cells for mutagenesis. Cells were cultured in Luria-Bertani (LB) broth at 37°C with aeration to an optical density at 600 nm (OD_{600}) of 0.4 to 0.6, chilled at 4°C for 30 min with stirring, harvested by centrifugation, and washed three times with ice-cold 10% glycerol. In the final wash, cells were resuspended in 1/500 (vol/vol) ice-cold glycerol-yeast extract medium and stored at −80°C. One hundred microliters of cell suspension was mixed with 10 μl of transposomes and electroporated in a 2-mm electrode gap cuvette with a GenePulser II (Bio-Rad). Cells were recovered in 1 ml of SOC medium (53) and incubated at 37°C for 1 h and then spread on plates containing 40 mg/ml kanamycin and incubated overnight. The total number of colonies was estimated by counting colonies on several plates. The colonies on each plate were pooled sterilely in LB plus 20% glycerol and stored at −80°C. Approximately 5 or more electroporations were performed per strain to generate an insertion mutant pool. The number of mutants per electroporation ranged from 20,000 to 175,000. By estimating the total number of mutants per batch, volumes containing similar numbers of mutants

from each batch were pooled to create mutant libraries to contain 5×10^5 mutants.

IR treatment. An IR dose of 1,000 Gy was applied iteratively to the mutant libraries by using a Mark I ^{137}Cs irradiator (from J. L. Shepherd and Associates). Mutant pools were inoculated into 100 ml of LB at an initial OD_{600} of 0.02 and were grown to an OD_{600} of 0.2. Cells were spun down and resuspended in 0.5 ml LB, IR treated, and then allowed to grow for approximately 7 generations to stationary phase before being used to inoculate the next cycle. This was repeated five times. Nonirradiated mock cultures were taken through all five passages in parallel but sat outside the irradiator during treatment.

Fragment library sample preparation, sequencing, and data analysis. Genomic DNA was isolated after each cycle of irradiation and from the nonirradiated cultures. DNA from the first and fifth cycles was sheared to an average size of 300 bp by using hydroshear sonication. Preparation of the fragment library for sequencing was performed as described by Illumina, except the PCR amplification step was modified so that only sequences flanking transposons were amplified, using primers ORB5, -6, or -7 and ORB8. ORB5 to -7 are custom reverse primers with different indexes for multiplexing. ORB8 was the forward primer, complementary to the adapter used (Table 2). Amplified fragment libraries were separated on an E-gel size select 2% agarose gel (Promega), and 270-bp fragments were purified. The amplified DNA fragment libraries were sequenced on single-end Illumina flow cells for 75 cycles in an Illumina genome analyzer IIx. The sequencing primer, ORB9, was modified for only sequence transposon-containing DNA fragments. Sequence reads from the Illumina FASTQ files were separated into reads with tag and reads without. Reads containing the transposon tag sequence were retained for analysis. The 10-bp tag was removed, and then reads were trimmed to 50 bp and mapped to the *E. coli* genome by using BOWTIE (54), omitting insertion locations with less than 10 reads and allowing for 1 mismatch. All insertion locations in the first 1% and last 10% of gene regions were removed from further analysis. Further, genes with three or fewer insertion locations that met our criteria were considered essential. Reads per gene were normalized by the total millions of reads collected for the sample to normalize for variations of total reads in different sequencing runs. Contribution values were calculated as the log ratio of reads in the irradiated sample, $n_{g,B}$, to reads in the nonirradiated sample, $n_{g,A}$, for each gene, g [$\log(n_{g,B}/n_{g,A})$]. Genes were analyzed for the decrease in insertion locations per gene in a parallel analysis. The genes shown below in Table 4 had a contribution value of -0.5 to -2, indicating a loss of 0.5 to 2 logs of reads under the irradiated versus nonirradiated condition. When genes were found to have a low contribution value yet a large decrease in insertion locations, the genes were checked for single insertion locations with over 1,000 reads, which can be artifacts of library amplification. The genes listed below in Table 4 that were discovered by loss-of-insertion locations rather than decreased read counts were in the top 99.999% of genes for insertion location losses.

IR survival assays for gene validation. Of the 46 genes discovered, 19 genes were verified by deleting the gene from the Founder strain via the Wanner method (50). Deletions strains were tested for their ability to survive increasing doses of ionizing radiation in comparison to an isogeneic wild-type strain. All strains were tested in biological triplicates. Cells from a fresh single colony of each strain were cultured in LB broth (55) at 37°C with aeration. After growth overnight, cultures were diluted 1:1,000 into 25 ml fresh LB broth in 125-ml flasks and grown at 37°C with shaking until an OD_{600} of ~0.4 was reached. For each sample, 15 ml of culture was spun down and resuspended in 0.8 ml of fresh LB. One-hundred-microliter aliquots were set on ice as the nonirradiated controls, and the other 700 μl was irradiated in a Mark I ^{137}Cs irradiator (from J. L. Shepherd and Associates) for the times corresponding to 1 and 2 Gy (~7 Gy/min). Irradiated samples as well as the nonirradiated control samples for each culture were diluted appropriately and plated on LB–15% agar medium to determine the total number of CFU. Percent survival was calculated by dividing the titer of the surviving population by the titer of the nonirradiated control sample. For each strain, 3 to 5 biological replicates were performed.

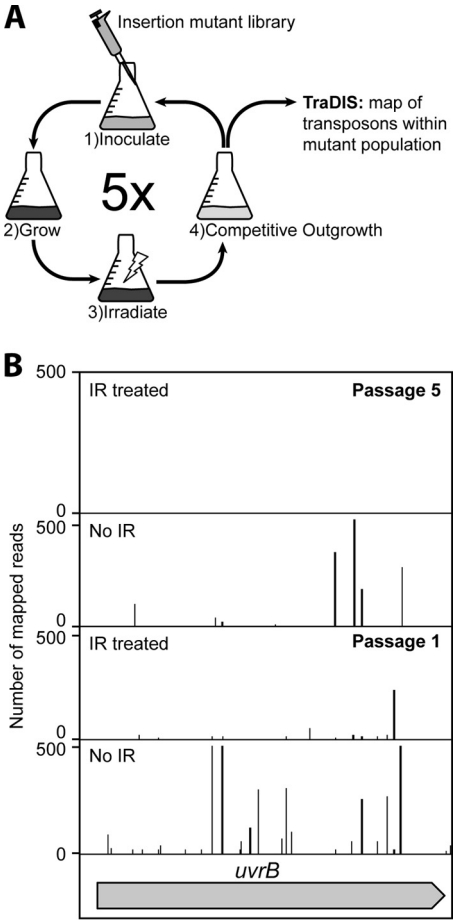


FIG 1 (A) IR treatment and passaging of the mutant pool. The mutant library, consisting of 500,000 insertion mutants, was grown to mid-log phase. A nontreated sample was taken, and the rest of the library was irradiated with a dose of 1,000 Gy before both samples were diluted into fresh medium for competitive outgrowth. After outgrowth, genomic DNA was extracted from both the irradiated and nonirradiated samples (passage 1 results). Outgrown cultures were then used as the inoculum for the next passage of IR treatment. This was repeated five times. After each treatment passage, genomic DNA was extracted for TraDIS analysis. (B) TraDIS profile for *uvrB*. Detailed plots generated used MochiView (107) show the frequency and distribution of transposon-directed insertion site sequences across a chromosomal region containing *uvrB* for a pool of 500,00 transposon mutants. "No IR" indicates that the mutant pool was subjected to the permissive nonirradiated condition; +IR indicates that the pool was subjected to 1,000 Gy. Data are presented after 1 and 5 passages of irradiation and outgrowth. The *y* axis shows the number of mapped sequence reads, and the position of annotated genes relative to the plotted sequence reads is indicated below the distribution plot.

RESULTS

TraDIS was performed to identify genes involved in IR survival. The original directed evolution trials were carried out with an aliquot of *E. coli* strain MG1655 obtained from F. R. Blattner (56). Deep sequencing revealed 6 mutations in this strain (designated Founder) relative to the type strain database (26). For TraDIS, a mutant library consisting of 500,000 insertion mutants was generated in Founder and also in one highly evolved strain, CB2000. Each library was subjected to 5 rounds of irradiation followed by competitive outgrowth, as diagrammed in Fig. 1. A nontreated

TABLE 3 General sequencing results

Wild type (Founder)	IR treatment	Total no. of reads	No. of unique insert sites	Avg distance between inserts (bp)	Gene length cutoff
Passage 1	−	22,778,780	90,920	51.0	122.9
Passage 5	−	33,338,903	25,590	181.3	436.6
Passage 1	+	30,935,721	125,644	36.9	88.9
Passage 5	+	19,557,186	39,185	118.4	285.1

control was taken through the entire experiment, treated identically except that it sat outside the irradiator during treatment. To identify genes that contribute to IR survival, genomic DNA from the mutagenized population was isolated after the first and fifth IR treatment passages. TraDIS was carried out using Illumina high-throughput sequencing to identify genes that, when disrupted, caused cells to drop out of the population after IR treatment, as illustrated for *uvrB* in Fig. 1B. The method can be verified in part by examining the effects of irradiation on the insertion patterns of genes known to have major roles in the repair of DNA damage inflicted by IR. As expected, *uvrB*, which encodes a key component of the complex that promotes nucleotide excision repair, exhibited a diagnostic two-part insertion pattern reflecting an essential role in IR resistance (step 1). Numerous insertions were present in the *uvrB* gene in the nonirradiated populations, indicating that the gene is not essential for normal growth (step 2). Transposon-directed sequence reads were reduced in this gene after IR treatment passage 1, and there were no sequence reads in this gene after IR treatment passage 5 (Fig. 1). These results suggest that any cells that had insertions in *uvrB* rapidly dropped out of the population upon treatment.

General sequencing results. The protocol described above generated 15 to 30 million reads per sample. The reads were mapped to the *E. coli* genome, and the number of unique transposon insertion sites and the average base pair distance between inserts for each sample were calculated (Table 3). These results suggested that the mutagenesis was saturating with 1 insertion per 40 to 50 bases for the mutant pool after 1 passage. For the mutant pool after 5 passages, 1 transposon insertion per 100 to 200 bases was detected. The decline was expected, as it was previously reported that passaging reduces the number of unique mutants in the pool, even in the absence of stress (45). This is due to genetic bottlenecks that occur during passaging and competition between strains with different mutations. Insertion densities were calculated for the mutant pools from each passage, as previously described (Table 3) (57). The gene length boundaries were calculated to determine the minimum length of a gene (in bp) required to ensure that the absence of sequenced transposon insertions signaled an essential gene function rather than a random chance occurrence ($P < 0.05$). This value differed by sample due to the varied insertion densities obtained for each sample.

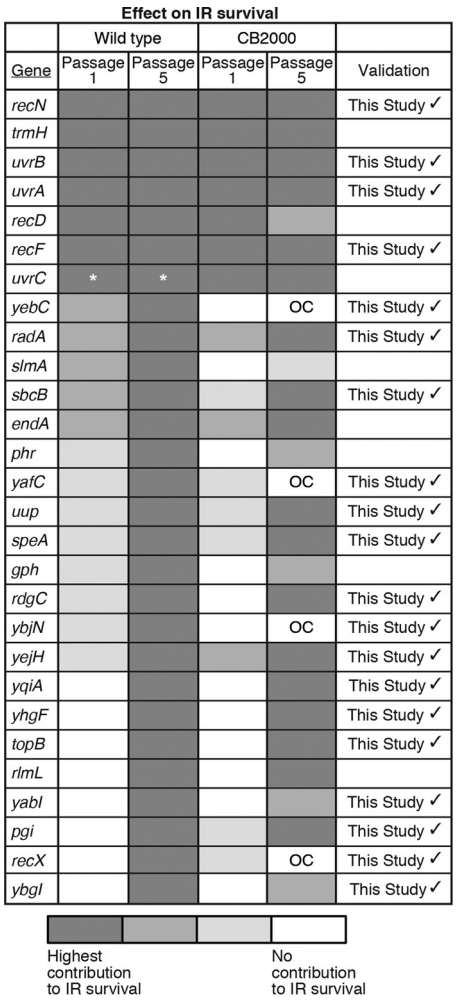
We note that approximately 670 genes in the *E. coli* genome are required for normal growth in an unstressed environment under our growth conditions as indicated by the absence of insertions in these genes in our nonirradiated control that met our threshold criteria (see Materials and Methods). These genes are summarized in Table S1 in the supplemental material. We are thus effectively screening the approximately 3,555 genes denoted nonessential in our nonirradiated sample (approximately 84% of the genome). Our goal was to identify those genes that are not necessary during normal growth but which become important when cells are heavily dosed with ionizing radiation.

Essential genes have previously been surveyed in *E. coli*. Of 620 genes denoted essential in one survey that covered 87% of the genomic open reading frames in *E. coli* (58, 59), approximately 55% overlap the essential genes found in our study. A second survey carried out under different conditions produced a list of 300 essential genes (60), of which 94% appeared essential in our study. The differences observed between these three studies are likely due to different growth conditions, the presence or absence of competitive outgrowth, the approach for distinguishing essential versus nonessential genes, and the depth in which the mutant libraries were assayed. Because of the requirement for outgrowth in our protocol, any gene inactivation that produces a sufficient decline in growth rate under our conditions will lead to that gene's inclusion on the list of essential genes.

Identification of genes involved in IR survival. After removal of the transposon tag sequence, each read was mapped to the *E. coli* genome. The first genome-derived base pair of each read defined the genomic location of each transposon insertion within the mutant pool. The number of transposon insertion locations for each gene was used to calculate the relative contributions of nonessential genes to IR survival. Contribution values were only calculated for genes with at least three independent insertion sites to reduce variability that can result in misleading fitness calculations (61). We identified genes that, when disrupted, resulted in reduced IR survival fitness after passage 1 and after passage 5. A total of 46 genes were thus identified in the Founder strain (Tables 4 and 5). We also noted that well over 90% of the nonessential genes in the *E. coli* genome exhibited little or no difference in the observed insertion patterns with or without irradiation. The genes of interest in this study are those exhibiting transposon insertion patterns similar to *uvrB* in Fig. 1, and they are listed in Tables 4 and 5.

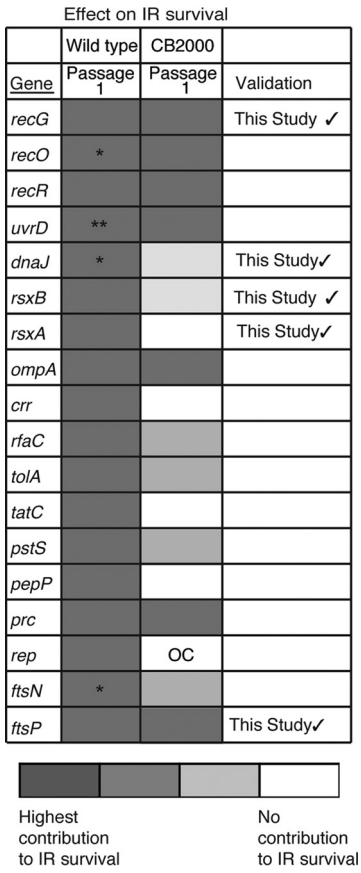
Deletion or alteration of some genes involved in DNA repair are known to result in slow growth phenotypes in rich media (62–70). The otherwise-nonessential *recA* protein, clearly important for IR resistance, is not present in our list because strains with alterations resulting in *recA* gene inactivation grow somewhat slower and are unable to compete with the broader population during outgrowth. A total of 18 of the 46 genes listed in Tables 4 and 5 exhibited patterns that reflected somewhat slow growth, although the decline in growth rate was insufficient to remove them from our screen at least in passage 1 (Tables 4 and 5). Cells disrupted for these genes had reduced fitness upon irradiation in passage 1. By passage 5, insertions in these genes disappeared from both the irradiated and the nonirradiated samples, eliminated competitively during outgrowth. Interestingly, two genes, *recR* and *rep*, appeared to be essential for IR survival as early as the first IR exposure. By passage 5, there were no insertions in these genes

TABLE 4 Genes with the largest contribution to radiation resistance after five passages[a]



[a] Contributions were calculated as described in Materials and Methods. Genes listed here had the largest decrease in reads upon irradiation compared to the nonirradiated control, with contribution factors between −0.5 and −2 logs. *, the gene was discovered by analyzing loss of unique insertion locations. Validation in this study indicates that the gene was assayed for its contribution to IR resistance as shown in Fig. 2.

TABLE 5 Genes that when disrupted resulted in reduced fitness upon irradiation in passage 1[a]



[a] These genes were identified to be important for passage 1, but cells lacking these genes were likely outcompeted due to growth defects associated with the gene deletion, and there were no data for passage 5 (in either the nonirradiated control or the irradiated sample). Contributions were calculated as described in Materials and Methods. *, the gene was discovered by analyzing loss of unique insertion locations. **, *uvrD* exhibited the greatest observed loss of unique insertion locations. Validation in this study indicates that the gene was assayed for its contribution to IR resistance as shown in Fig. 2.

in the nonirradiated control samples. We hypothesize that these genes are essential for surviving IR but also make a modest contribution to growth in rich media. These genes have been reported to be important for normal growth (59, 60).

To further investigate the importance to general radiation resistance of the 46 genes identified here in Founder, TraDIS was performed on CB2000, a strain of *E. coli* previously reported to be highly radiation resistant (26). The 9 genes with the largest contributions to IR resistance (Table 4) were also identified as top contributors in CB2000 after passage 1, in spite of the presence of all CB2000 mutations that confer an IR resistance phenotype. This result validates their importance for timely recovery from damage inflicted by IR. A total of 37 of the reported genes for MG1655 (80%) were identified as important in CB2000 as well. Data were ambiguous for 5 of the 9 genes that were required for MG1655 but not CB2000. This was likely due to the genetic bottlenecks that occur during passaging or slow growth of these mutants. Four genes, *pepP*, *rsxA*, *crr*, and *tatC*, appear to be important for survival in wild-type *E. coli* but not the directly evolved CB2000. This suggests that one or more of the 69 mutations arising in CB2000 (25) render these four genes dispensable for IR survival.

IR resistance gene validations. To directly verify a subset of genes identified as putative IR resistance genes by TraDIS, we separately deleted 31 of the 46 genes identified from *E. coli* MG1655 (Δe14) and assayed each deletion mutant for survival following exposure to 1,000 and 2,000 Gy (Fig. 2). Two of these (*tatC* and *waaC*) had viability issues that made it impossible to generate survival curves. The *tatC* gene is nonessential, as it encodes a component of a system that transports folded proteins from the cell to the outer membrane. Deletions of the gene compromise the integrity of the outer membrane and render it sensitive to many stresses (71–74). The *waaC* gene (formerly *rfaC*) encodes heptosyltransferase I, which catalyzes a step in the synthesis of outer membrane lipopolysaccharide. As in the case of *tatC*, deletions of *waaC* may render the cell particularly sensitive to stress (75), even though the

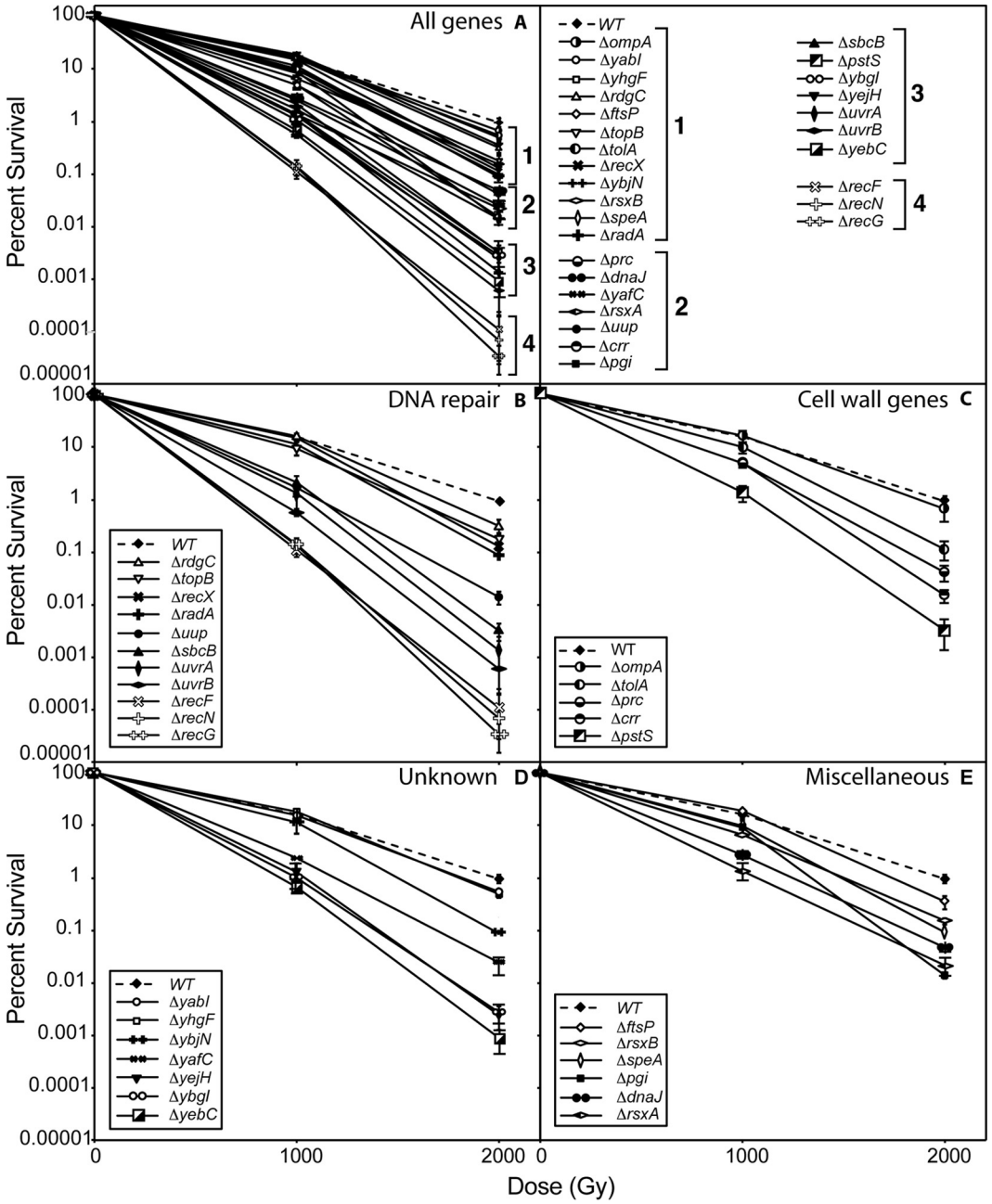


FIG 2 Effects of gene deletions on survival of *E. coli* to increasing doses of radiation. A total of 26 genes identified using TraDIS were individually deleted in MG1655 and assayed for survival to 1,000 and 2,000 Gy IR. Gene deletion strains appeared to cluster into four sensitivity groups, numbered 1 through 4, with increasing severity of IR sensitivity. The successive panels highlight particular groupings of deletion strains, as indicated by the titles in each panel. WT, wild type.

gene may be scored nonessential. The other 29 exhibited IR recovery deficiencies that were readily documented with survival curves, helping to validate that the overall screen was identifying genes of interest. From our tests, these 29 deletion mutants were clustered into four different groups based on the overall decline in cell survival upon their deletion. They were numbered 1 through 4 in order of increasing severity of the observed sensitivity to ionizing radiation. The genes in group 1 (*rdgC*, *ftsP*, *radA*, *rsxB*, *topB*, *recX*, *speA*, *yabI*, *yhgF*, *ybjN*, *tolA*, and *ompA*) exhibit relatively modest effects when deleted, with a decline in survival of just over an order of magnitude or less at 2,000 Gy. Those in group 2 (*pgi*, *rsxA*, *dnaJ*, *yafC*, *prc*, *uup*, and *crr*) exhibit a decline in survival of 1 to 2 orders of magnitude at 2,000 Gy. The group 3 genes (*uvrA*, *uvrB*, *yejH*, *sbcB*, *yebC*, *ybgI*, and *pstS*) had declines in survival of approximately 2 to 3 orders of magnitude. Those gene products in group 4 produce the most dramatic effects, a 3- to 5-log decline in survival at 2,000 Gy when deleted (Fig. 2). This final group features three genes (*recF*, *recG*, and *recN*) that have long been associated with IR survival. We note that one other gene initially identified in the screen and subjected to validation by this method (the *mrcB* gene, not included in Table 5) turned out to be a false positive. Although the screen exhibited a very low error level, it is thus

possible that one or two additional listings in Table 4 are errant. We thus estimate the false-positive rate to be <5%.

Contributions to IR resistance. The list of gene functions required for survival after extreme IR exposure (Table 4) generally continues themes that were evident in the directed evolution study that examined the genetic adaptations required for extreme resistance to IR (25). In the overall list, 20 of the 46 genes (or 43%) can be clearly defined as DNA repair or DNA metabolism functions. DNA repair functions have already been amply implicated in recovery from IR exposure (11–19), and the heavy representation of DNA repair functions in this list helps to verify the screen. In the directed evolution study (25), a small number of mutations in genes involved in DNA repair provide the major contributions to extreme IR resistance in one isolate (CB2000) derived from directed evolution. Whereas the idea that DNA is the major target of IR that results in lethality has been challenged in recent years (20–24), a range of key DNA repair systems must be intact in order for the cell to survive extreme IR exposure.

Additional contributions are evident. An additional 8 of the genes identified (17%) have not been functionally characterized and represent a new class of genes to be studied for a role in surviving IR exposure (Fig. 2D). We have directly validated 7 of these (for reasons that are unclear, it was not possible to make deletion mutants of *yqiA*), demonstrating that they are indeed important to survival when cells are exposed to IR. In three cases (*yabI*, *yhgF*, and *ybjN*), elimination of gene function has just a modest effect on IR survival and the genes fall into group 1. One gene, *yafC*, falls into group 2. Three genes (*yejH*, *ybgI*, and *yebC*) have quite substantial effects on IR survival and fall into group 3.

This result represents the first observed phenotype for many of these genes. A few things are known about the genes that fall into category 3. Although the function remains enigmatic, the structure of the YbgI protein is known. It is a toroidal structure consisting of a trimer of dimers, wherein each subunit exhibits two metal binding sites on the inside of the toroid (76). The *ybgI* gene shares an operon with the gene that encodes endonuclease VIII, eliciting some speculation about a possible DNA repair function (76). The product of the *yebC* gene appears to have a function in the transcriptional regulation of the RuvABC proteins, which are all involved in recombinational DNA repair (77). The *yejH* gene is worthy of special mention. It encodes a putative DNA helicase with significant homology to the human XPB gene, which encodes a nucleotide excision repair helicase conserved in eukaryotes and archeans. By sequence analysis, the protein possesses the 7 helicase motifs central to superfamily 2 helicases in the N-terminal 350 amino acids. It plays a substantial role in survival after IR exposure.

The remaining 19 genes cluster into 5 major categories, which are defined in Table 6. Four of the genes fall into a category of oxidative stress signaling (*pgi*, *speA*, and *rsxAB*), in line with the idea that amelioration of protein oxidation is a major mechanism of IR resistance (20–24). Four of these were directly validated. The *pgi* and *rsxA* genes fall into category 2, while *rsxB* and *speA* are category 1 genes. The *pgi* gene has a complex involvement, as described below. Also as described below, genes in other categories may affect oxidative stress. The remaining genes have roles in cell wall structure and biosynthesis (7), protein stability and turnover (5), cell division (4), and central metabolism (2). This listing does not include a few gene products, such as *pgi*, that fall into more than one functional category (Table 6). Of interest, 5 of the genes listed in Table 6, *pgi*, *prc*, *tatC*, *recF*, and *pepP*, are part of a broader network of 93 genes believed to play a role in promoting the stress-induced mutagenesis (SIM) response of *E. coli* K-12 (78).

TABLE 6 Clustering of identified genes by cellular function

Cellular function	No. of genes clustered	% of identified genes	Genes[a]
DNA metabolism	21	46	*recN*, *uvrABCD*, *recD*, *recF*,* *recO*, *sbcB*, *endA*, *phr*, *uup*, *gph*, *rdgC*, *yejH*, *topB*, *recX*, *recR*, *rep*, *radA*, *recG*
Cell wall structure and biosynthesis	7	15	*rfaC*, *prc*,* *ompA*, *tolA*, *pstS*, *tatC*,* *crr*
Unknown	7	15	*yafC*, *ybjN*, *yqiA*, *yhgF*, *yabI*, *ybgI*, *yebC*
Cell division	4	9	*tolA*,* *slmA*, *ftsN*, *ftsP*
Oxidative stress signaling	4	9	*pgi*,* *rsxAB*, *speA**
Protein stability and turnover	3	6	*pepP*,* *prc*,* *dnaJ*
Central metabolism	2	4	*pgi**, *speA**
SIM response	5	11	*pgi**, *prc**, *tatC**, *recF**, *pepP**

[a] *, the indicated gene is listed more than once.

Two genes identified in the screen are not included in Table 6: *trmH* and *rlmL*. These may contribute to IR resistance. However, they may have been identified as IR resistance genes due to possible polar effects on genes immediately downstream that are known to be involved in radiation survival: *recG* and *uup*, respectively. We have not directly tested these insertions to confirm the presence of polar effects. However, each of these genes is in the same operon as and coexpressed with the indicated downstream genes.

The requirements for genes involved in protein stability and turnover, as well as those involved in oxidative stress signaling, can likely be understood in the context of current research from the Daly and Radman groups that indicates that protein oxidation is a major deleterious effect of IR (21, 24, 44, 79, 80). As is the case for DNA, proteins are a target of IR-mediated damage. Among the mutations underlying the acquired extreme resistance to IR documented in the directed evolution study (25), mutations in *rsxB*, which encodes part of a system that controls the cellular response to reactive oxygen species, and in *gsiB*, which encodes a glutathione transporter, were apparently fixed in population IR-2-20. Each makes a small but measurable contribution to the acquired IR resistance of evolved strain CB2000. The current study (Table 6) indicates that multiple cellular systems involved in ameliorating the effects of oxidative damage play a significant role in IR resistance.

The *pgi* gene deserves special mention. The product of the *pgi* gene, phosphoglucose isomerase, catalyzes the second step in glycolysis. However, it is not an essential gene due to the metabolic bypass provided by the pentose phosphate pathway. The pentose phosphate pathway generates NADPH, which is interconverted with NADH by the NADH/NADPH transhydrogenases encoded by the genes *udhA* and *pntAB*. One result is a substantial increase in the electrons fed into oxidative phosphorylation and a resultant

increase in the production of damaging reactive oxygen species. Cells deleted for *pgi* generate suppressor mutations in *rpoS*, *udhA*, and *pntAB* under some growth conditions (81). They also lose the e14 prophage to deletion (81). We note that deletion of the e14 prophage is a stress indicator, and this was the first genomic alteration detectable in all trials of our experiment in directed evolution of radiation resistance (26). The role of *pgi* in oxidative stress may be much more complex. The Pgi protein was identified as an interaction partner with the YejH protein in a global search for *E. coli* protein interactions (82), a result we have confirmed (R. Byrne, unpublished results). YejH is one of the proteins of unknown function identified in the current study as essential for recovery from heavy IR exposure (Table 4). A *pgi* deletion mutant is hypersensitive to oxidative stress induced by paraquat (83), UV sensitive (78), defective for the *rpoS* reponse (78), and defective for spontaneous SOS induction (78). The *pgi* gene is part of the *soxR* regulon (84, 85). The *pgi* gene product may have functions outside its role in glycolysis, perhaps working upstream of *rpoS* in a pathway that leads to stress-induced mutagenesis (78).

A few of the genes listed under other categories may actually affect the cellular oxidative response. For example, cells with a deletion of the *speA* gene, which is involved in polyamine biosynthesis, are sensitive to H_2O_2 (86), and we have thus listed *speA* under both categories. At least one of the genes of unknown function may also be linked to the oxidative damage response. The *ybjN* gene encodes a protein with structural homology to the DR1245 protein of *Deinococcus radiodurans* and type III secretion system chaperones (87). Overexpression of *ybjN* leads to induction of the SOS response (88). In general, the product of the *ybjN* gene appears to play a broad role in cellular survival under conditions of stress (88).

Selected aspects of intermediary metabolism are also doubtlessly linked to stress responses. The *crr* gene identified in the current screen encodes the phosphotransfer protein EIIA(glc), which is a component of three different sugar transport systems (glucose, trehalose, and maltose) (89). EIIA(glc) is also a negative regulator of other carbohydrate utilization pathways (glycerol, lactose, melibiose, and maltose) and negatively regulates *rpoS*. It may positively regulate adenylate cyclase, which controls transcription of genes involved in the stress response. Survival after IR exposure may require some flexibility in carbon source utilization. PstS is a periplasmic protein that binds phosphate as part of the phosphate transport system (71, 90–93). Phosphate limitation itself can trigger a stress response in bacteria. The combination of limiting phosphate and radiation damage may be synergistic in the effects on lethality.

The requirements for several genes involved in outer membrane structure and biosynthesis continue an additional theme seen in our directed evolution study (25). In the evolved strain CB2000, mutations in the genes *wcaK* and *nanE* again made small but measurable contributions to the acquired IR resistance phenotype (25). These genes encode enzymes involved in the synthesis and/or recycling of peptidoglycan or surface polysaccharides. In the present study, eight additional genes that contribute to cell wall structure and biosynthesis were identified that make significant contributions to IR survival. After DNA repair, this is the largest number of genes concentrated in any particular function. The importance of the bacterial cell wall as a target of IR-mediated damage has not yet been adequately assessed. The results of these two studies suggest that, in addition to DNA damage and protein inactivation via oxidation, the integrity of the bacterial cell wall, or particular substructures within it, may represent a significant factor in the overall lethality of IR. In each case, the gene functions identified in the new study can be dispensed with under normal growth conditions, but they become important upon IR exposure. We can suggest at least three mechanisms that might be at work. First, the cell wall, particularly the outer membrane, could have substructures that are effectively weak points that are particularly sensitive to damage inflicted by IR. Alternatively, there may be key enzymatic or transport steps that have broad significance for cell wall or membrane integrity. The relevant enzymes or transporters could become essential under stress, as we suggested above for *tatC* and *waaC*. The TolA protein is the inner membrane protein that links to Pal, an outer membrane protein, to maintain cell envelope integrity. This linkage is likely important under stressed conditions, such as irradiation (94–98). Second, there may be alterations to the peptidoglycan that are part of a general cellular response to stress that are critical to IR survival. Peptidoglycan plays an important role in osmotic regulation and cell shape (99). Changes to its structure accompany a number of cell stresses, including the nutritional stress that leads to the onset of stationary phase (99, 100). Third, a somewhat different cell shape may be more optimal for IR survival in ways that are hard to predict. The *ompA* gene, which was mutated in a few isolates studied in the original directed evolution study (25) and found to contribute to survival in this study, has a proposed role in mediating cell shape. Cells deleted for *ompA* have unstable outer membrane structures and the cells tend to be spherical (101, 102). Additionally, alterations in one of the 12 penicillin binding proteins that catalyze synthesis of peptidoglycan were documented in a long-term evolution experiment that showed increased cellular fitness in a particular medium, and this in turn produced alterations in cell shape (103). Additional mechanisms may be considered, and this list is not intended to be exhaustive.

The present study was carried out to identify genes that are critical for survival when cells are exposed to ionizing radiation but which were not necessarily targets of mutation in our recent directed evolution study (25). However, of the 46 genes identified in this study, 9 (19%) acquired mutations in one or more of the sequenced isolates characterized as part of that earlier study (25). As the present study indicates that loss of many of these gene functions results in significant radiation sensitivity, it is tempting to speculate that the mutations identified in the earlier study may be either neutral or gain-of-function mutations. This conclusion must be tempered by the fact that each of the IR-resistant *E. coli* strains in which the mutations appear has dozens of additional mutant loci that could potentially act as functional suppressors (25). Of the nine, the nonsynonymous mutation in *rsxB* has already been discussed. A mutation upstream of *tolA* was fixed in another of the four separately evolved populations (IR-3-20) (25), indicating that a change in the expression of this operon could be beneficial to survival. A nonsynonymous mutation was also fixed in *prc* in IR-3-20. While no mutations were found among the evolved isolates in *ftsN* or *ftsP*, mutations were common in *ftsW* and *ftsZ* among isolates of two subpopulations in IR-1-20, suggesting that alterations of the cell division process might contribute to IR resistance. Other mutations in our previous study were identified in *recG*, *dnaJ*, *ompA*, *tatC*, and *yejH*. The *yejH* and *dnaJ* mutations appeared to be fixed in the isolates taken from the further evolution of strain CB1000, and it is possible that the ob-

served mutations in these two genes provide a useful gain of function in the context of extreme exposure to ionizing radiation. However, the mutations in *tatC*, *recG*, and *ompA* appeared in only one or a few IR-resistant isolates, providing little in the way of a pattern to indicate that the mutations in these genes contribute significantly to IR resistance.

DISCUSSION

Combined with the directed evolution study (25), this work reveals a multifaceted and nuanced cellular approach to surviving IR. The published directed evolution study documents that enhancements to DNA repair processes can make major contributions to an extreme IR resistance phenotype acquired by directed evolution (25), even in a genetic background that is otherwise unaltered. At the same time, contributing mutations appear that provide potential enhancements to cellular systems for protein oxidation amelioration, protein folding and stability, cell division, and maintenance of cell wall structure and function. Roles for the same cellular functions are evident in the screen carried out here. A more complete description of the molecular basis of extreme IR resistance might thus consist of (i) enhanced DNA repair processes, (ii) an enhanced capacity to prevent or ameliorate the effects of protein oxidation, including protein stabilization/refolding, (iii) an appropriate control of cell division to ensure that DNA repair can be completed, and (iv) an enhancement of key processes affecting the structure and function of the cell wall and maintenance of cell wall integrity.

The current study provides a general screen of gene functions that are not required for normal growth but which become necessary when cells are exposed to high levels of ionizing radiation. There are at least 46 genes required for cells to recover from IR exposure. As befits the need to repair IR damage to DNA, DNA repair functions predominate, with 20 identified genes falling into this category. Several of these genes, particularly *recF*, *recN*, and *recG*, make very substantial contributions to survival and represent genes long known to be required for survival after IR exposure (12, 104–106). The results, not surprisingly, highlight the importance of general DNA repair when cells are exposed to ionizing radiation. At the same time, the requirements for gene functions involved in protein structure stabilization and turnover, the response to oxidative damage, and the maintenance of bacterial cell wall structure and function continue themes that were evident in the directed evolution study (25).

Our assessment of genes that contribute to IR survival implicated eight genes of previously unknown function in the recovery of cells, with five of them validated. By utilizing high-throughput screening techniques with a simple organism such as *E. coli* under various growth conditions, we can begin to identify the functions of these enigmatic genes by identifying growth conditions or stress conditions where these genes become essential. A follow-up study on the cellular role of these eight genes will begin to unravel the basis of their contributions and potentially define their cellular functions. This in turn may help define the role of their homologs in archaeans and eukaryotes. Of the eight genes, three have homologs identified in all three domains of life, and all eight have homologs in eukaryotes. In no instance has the function of one of these homologs been described in detail, although some hints about YbjN, YebC, YejH, and YbgI functions have appeared (76, 77, 87, 88). In several cases, we provide here the first phenotype described for cells lacking the functions of these genes.

Among the genes with no previously described phenotype, we were particularly interested in *yejH* because of the dramatic TraDIS profile and the IR sensitivity of cells lacking a functional copy. We have found that this gene exhibits significant homology to the human gene encoding XPB. This gene clusters into category 3, along with two other genes of unknown function (*yebC* and *ybgI*) and the *uvrA*, *uvrB*, and *sbcB* genes of known function that have been studied for their roles in DNA repair for decades. Based on the gene sequence, an initial characterization (R. Byrne and S. Chen, unpublished data), and the the new phenotype of the *yejH* gene described here, we hypothesize that YejH is involved in cellular repair of DNA after IR exposure. Characterization of this gene is the subject of ongoing work.

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