Intestinal Microbiota as Novel Biomarkers of Prior Radiation Exposure

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There is an urgent need for rapid, accurate, and sensitive diagnostic platforms to confirm exposure to radiation and estimate the dose absorbed by individuals subjected to acts of radiological terrorism, nuclear power plant accidents, or nuclear warfare. Clinical symptoms and physical dosimeters, even when available, do not provide adequate diagnostic information to triage and treat life-threatening radiation injuries. We hypothesized that intestinal microbiota act as novel biomarkers of prior radiation exposure. Adult male Wistar rats (n ¼ 5/group) received single or multiple fraction total-body irradiation of 10.0 Gy and 18.0 Gy, respectively. Fresh fecal pellets were obtained from each rat prior to (day 0) and at days 4, 11, and 21 post-irradiation. Fecal microbiota composition was determined using microarray and quantitative PCR (polymerase chain reaction) analyses. The radiation exposure biomarkers consisted of increased 16S rRNA levels of 12 members of the Bacteroidales, Lactobacillaceae, and Streptococcaceae after radiation exposure, unchanged levels of 98 Clostridiaceae and Peptostreptococcaceae, and decreased levels of 47 separate Clostridiaceae members; these biomarkers are present in human and rat feces. As a result of the ubiquity of these biomarkers, this biomarker technique is non-invasive; microbiota provide a sustained level of reporting signals that are increased several-fold following exposure to radiation, and intestinal microbiota that are unaffected by radiation serve as internal controls. We conclude that intestinal microbiota serve as novel biomarkers of prior radiation exposure, and may be able to complement conventional chromosome aberrational analysis to significantly enhance biological dose assessments.

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INTRODUCTION

In the event of a nuclear terrorist event or accident, fast and reliable bioassays are needed to identify radiation-exposed individuals, and assess an individual's radiation dose, especially during the first few days after exposure, when dose information would identify those victims who could benefit most from medical intervention. Effective biodosimeters are needed in triage environments to exclude individuals who have not been exposed so that medical management can be a top priority for first responders after radiological and nuclear events (1). Humans and other mammals are remarkably sensitive to acute doses of ionizing radiation. Without appropriate medical care, the median lethal dose of radiation, the $LD_{50/60}$ (the dose that kills 50% of the exposed population within 60 days after exposure), is estimated to be 4.5 Gy (2). Therefore, evaluating doses between 1 and 10 Gy, which have high relevance after radiological and nuclear events, is especially important because exposure in this dose range poses risks of serious acute health effects. Yet, survival is possible (3).

Humans and other animals are hosts to highly-complex ecosystems of colonizing microbes. The vast majority of these microbes (10–100 trillion), represent all three domains of life, live in the intestines, and are excreted with the feces (4); microbiota exhibit a large variation in sensitivity to total-body radiation (5–8). We rationalized that variations in response of the intestinal microbiota to radiation would serve as unique biomarkers of prior radiation exposure. Radiation would be expected to increase, decrease or have no effect on the abundance of specific intestinal microbiota expelled with the feces.

Several studies carried out in the 1950s and 1960s identified specific bacteria present in feces that are sensitive to irradiation of the host. Total-body irradiation (13.6 Gy) or local intestinal radiation alone (19.4 Gy) in the rat resulted in bacterial overgrowth of fecal-type organisms in the small intestine (9). Maximal changes were observed 3 days after irradiation. There was a 100–10,000-fold increase in Proteobacteria, especially Enterobacteriaceae, with similar increases in Enterococci. Normally-predominant Lactobacilli decreased slightly in number or remained unchanged. Feces were not examined.

Irradiation of the small intestine with 6.3 Gy in the rat resulted in a 4-fold decrease in *Lactobacilli* and a 1000-increase in *Pseudomonas*. These changes were maximal 6–7 days after irradiation (*10*). In this study, feces were not examined. Fungi were present in the feces of control, nonirradiated rats, but were absent 12 days after irradiation. Total-body irradiation with single dose 10.2 Gy resulted in increased *Proteobacteria* in the feces 5 days after irradiation. Results of these studies suggest a dose- and time-dependent effect of single-dose total-body irradiation on fecal bacteria and fungi in rats. In pigs surviving detonation of a nuclear weapon, *Streptococci*, *Clostridium*, *Salmonella*, and *Bacillus* levels in the feces were elevated on days 3–6 post detonation (*11*). Total-body irradiation with a single dose of 4.4 Gy in the dog resulted in increased coliforms and *Staphylococci* in the feces (*12*). The bacterial microbiota of the feces of one man was studied after he was accidentally exposed to total-body irradiation (5.4 Gy). Between 12 and 23 days after exposure, increased populations of *Streptococcus salivarius* and a pleomorphic *Clostridium* sp. were present in fecal specimens (*13*). Other bacteria were unaffected by total-body irradiation.

These previous animal and human studies indicate that radiation alters the abundance of specific intestinal microbiota present in the feces. However, changes observed post-radiation exposure in specific intestinal microbiota were transient and unsustained. For a biomarker to have utility as a radiation biodosimeter, a biologically-significant and sustained signal from the intestinal microbiota needs to be present. We hypothesized that intestinal microbiota excreted into the feces can act as biomarkers of radiation exposure, and that the abundance of specific intestinal microbiota are diagnostic of exposure to radiation. Previous studies have relied on the cultivation of fecal bacteria followed by manual inspection and classification. However, the majority of bacteria colonizing the intestines are not readily cultivatable by current techniques. For this reason, we applied high throughput molecular approaches based on 16S rRNA gene sequences to provide a more comprehensive view of the response of this complex ecosystem to radiation. Each bacterial species has a fixed number of genomic copy numbers for the 16S rRNA gene; abundance of 16S copies provides an index of the relative amount of the microbe. The objectives of this study were to (1) measure shifts in intestinal microbiota populations in rat feces observed pre- and post-irradiation, (2) identify candidate intestinal microbial biomarkers of prior radiation exposure, and (3) determine whether microbiota designated as biomarkers in rats are observed in human feces.

MATERIALS AND METHODS

Animals

Male WAG/RijCmcr (Wistar) rats at 5 weeks of age used in this study received humane care in compliance with the ''Guide for the Care and Use of Laboratory Animals'' published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). Rat handling and use protocols were approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin. Rats were fed laboratory rodent diet 5010 (LabDiet, St. Louis, MO) and given water *ad libitum* (*14*).

Irradiation

Littermate rats ($n = 5$/group) were assigned at random to receive single- or multiple-fraction total-body X-irradiation (TBI) of 10.0 Gy and 18.0 Gy, respectively. TBI was done with a posterior-anterior field at a dose rate of 1.95 Gy/min (*14*). Following irradiation, rats in each group were housed with a maximum of three per cage for subsequent monitoring.

Feces Collection and DNA Extraction

Fresh fecal pellets were obtained from each rat prior to (day 0) and at days 4, 11, and 21 post-irradiation. Pellets were homogenized in 1 ml PBS, and 200 ul of the homogenate were used for microbial DNA isolation using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA).

Microarray Analysis

The bacterial diversity and comparative community structure of rat fecal DNA samples was characterized by Second Genome Inc. (San Francisco, CA) using the high-density G3 PhyloChip™ 16S rRNA microarray-based assay (PN49-0002A) and bioinformatic methods. The microbiota analysis focused on calculating inter-sample distances and assessing the significance of microbiome dissimilarity (*15*). Data analysis incorporated several separate stages: preprocessing and data reduction, summarization, normalization where needed, sample-to-sample distance metrics, ordination/clustering, sample classification, and significance testing. Details of the microarray analysis and data analysis are included in Supplementary files (see Supplementary Material, data supplement 1; http://dx.doi.org/10.1667/RR2691.1.S1).

Quantitative PCR

Isolated DNA samples were subjected to quantitative PCR using an iCycler (Bio-Rad, Hercules, CA) for microbial population enumeration. The PCR reaction mixture consisted of 50% iQ SYBR Green Supermix (Bio-Rad), 0.4 μ*M* forward and reverse primers, and 3.8% template solution in RNase/DNase free water. The forward (F) and reverse (R) 16S rRNA primer pairs used include: BactF285 (GGTTCTGAGAGGAGGTCCC) and UniR338 (GCTGCCTCCCGTAGGAGT) for the *Bacteroidetes* taxon; UniF338 (ACTCCTACGGGAGGCAGC) and CcocR491 (GCTTCTTAGTCAGGTACCGTCAT) for the *Clostridia* taxon; and Uni515F (GTGCCAGCMGCCGCGGTAA) and Ent826R (GCCTCAAGGGCACAACCTCCAAG) for the *Proteobacteria* taxon (*16*). Genomic DNA from *Bacteroides fragilis*, *Ruminococcus products*, and *Escherichia coli* were obtained from the American Type Culture Collection and used as standards. The forward (F) and reverse (R) primer pairs used for confirming radiation exposure biomarkers include: StableF (TTCGCTTCTCTTCGTATGCGGC); StableR (TCTTCACACACGCGGCATGGC); DownF (CGCGTGGGTAACCTGCCCTG); DownR (CGCGGGTCCATCCTATACCGCA); AcuteF (TCGGGCCTCTTGCCATCGGA); AcuteR (CCGGTTAACGCTTGCACCCCT); ChronicF (CTGGGATGGACCTGCGGTGT); and ChronicR (TTACGAGCCGAAACCCTTCTTCAC). The paired Student's t test was used to find significant differences among variables in the qPCR data. PCR data variance is shown in representative scatter plots see Supple-

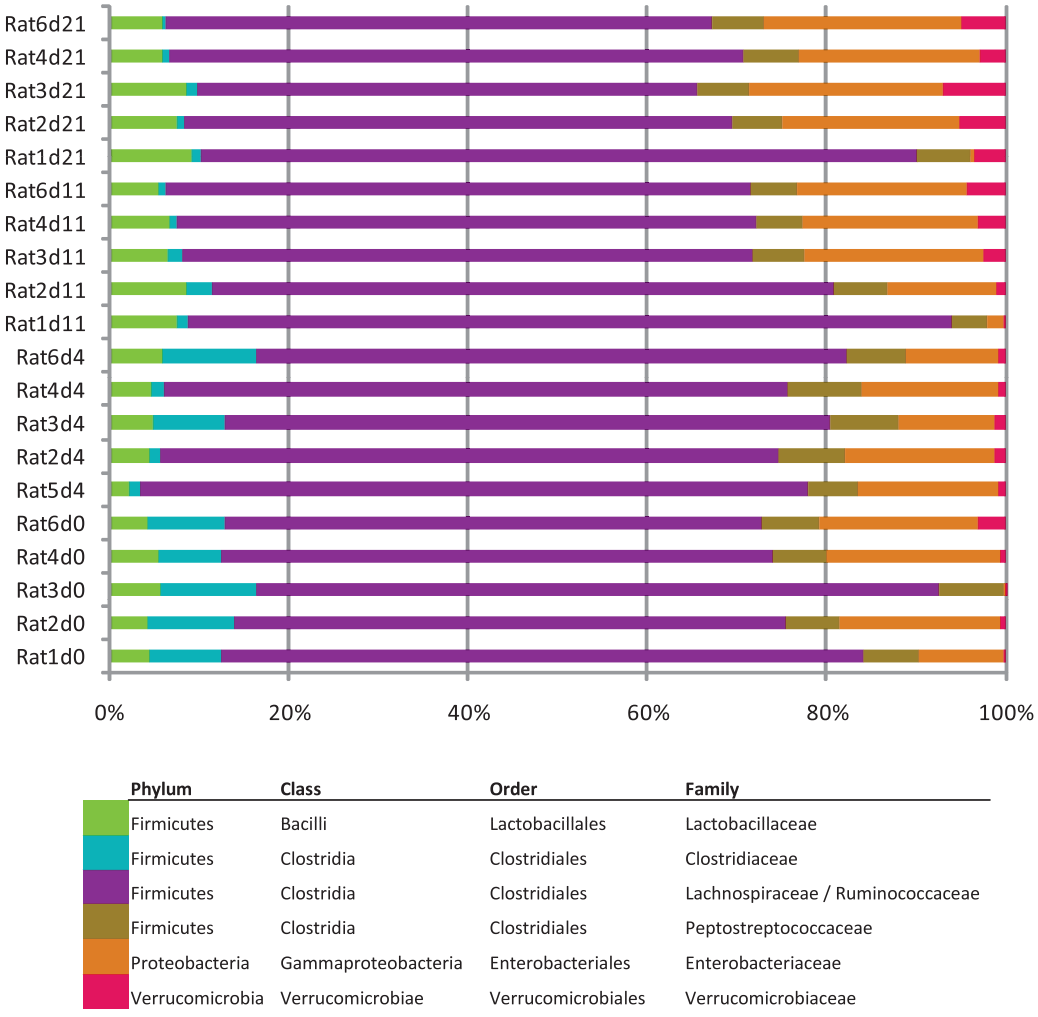

FIG. 1. Proportions of OTUs present in rat feces classified at the family level. The bar chart shows the top 6 most-rich members of the family rank. Each color block represents the percentage of OTUs detected within a family compared to the total number of OTUs detected within the top 6 most-rich families.

mentary Material, data supplement 2; http://dx.doi.org/10.1667/RR2691.1.S1.

Human and Rat Similarities

We performed an analysis to determine if the taxa designated as microbial biomarkers of radiation exposure in our rat studies are also observed in normal human fecal material. This is an important comparison to make as microbiota taxa are known to be dissimilar across species. We used a set of radiation biomarkers consisting of three lists of operational taxonomic units (OTUs) that were consistently decreased, stable, or increased over the 21-day period following irradiation (Table 1). Each OTU was queried by multiple 25 nucleotide oligomers that specifically hybridize to one or more 16S rRNA gene sequences. Wherever an OTU contained a 16S gene originating from a bacterium in human feces, that gene was selected as the representative sequence. Next, the OTU identifications were compared to those detected by the G3 PhyloChip™ assay in composite human fecal samples obtained from six subjects. The three lists were also compared to taxa found in 373 healthy human stool samples surveyed by the NIH Human Microbiome Project and accessed from http://www.hmpdacc.org on March 24, 2011.

RESULTS

Microarray Studies of Microbial Abundance after Radiation Exposure

Bacterial diversity and comparative community structure of rat fecal DNA samples were characterized using the G3 PhyloChip 16S microarray-based assay and bioinformatic methods. The sequence of the 16S ribosomal RNA (rRNA) gene is unique to each eubacteria taxon, and changes in the quantity of 16S rRNA genes across total DNA extraction products are indicative of changes in the population of the species. Feces were obtained from five independent rats at all time points (0, 4, 11, and 21 days) after exposure to 10-Gy single-fraction total-body irradiation for analysis. Members of the *Firmicutes* and *Proteobacteria* phyla were the most abundant microbiota present in the feces (Fig. 1). The microbiota analysis focused on calculating inter-sample distances and assessing the significance of microbiome

TABLE 1
List of representative OTUs that are indicative of irradiation

OTU	Full Taxonomy	Day Averaged Log_2 Signal Values 0	4	11	21	Log_2 Fold Change Pre vs. Post Radiation Log_2	p-value
OTUs that increase following irradiation:							
31902	Bacteria:Cyanobacteria:YS2_SP:Rs-H34_CL:Unclassified:Unclassified:sfA	10.94	15.29	15.04	14.28	-3.93	2.10E-04
31674	Bacteria:Cyanobacteria:YS2_SP:Rs-H34_CL:Unclassified:Unclassified:sfA	13.28	16.12	16.06	15.44	-2.60	7.29E-04
10703	Bacteria:Firmicutes:Bacilli_SP:Lactobacillales_CL:Lactobacillales:Streptococcaceae:sfA	13.72	14.28	15.37	15.16	-1.21	5.13E-03
10461	Bacteria:Firmicutes:Bacilli_SP:Lactobacillales_CL:Lactobacillales:Lactobacillaceae:sfA	12.88	12.96	15.75	16.16	-2.07	3.70E-03
46156	Bacteria:Bacteroidetes:p-184-o5_SP:C9_B12_CL:Bacteroidales:Unclassified:sfA	9.53	10.27	11.54	15.92	-3.04	2.90E-03
46174	Bacteria:Bacteroidetes:p-184-o5_SP:Bacteroidales_CL:Bacteroidales:Unclassified:sfA	10.87	11.73	12.58	15.82	-2.50	3.23E-03
Unchanged controls:							
42924	Bacteria:Firmicutes:Clostridia_SP:RF30_CL:Clostridiales:RF6_FM:sfA	15.56	15.14	16.23	15.49	-0.06	
41704	Bacteria:Firmicutes:Mollicutes_SP:RF39_CL:Unclassified:Unclassified:sfA	15.49	14.63	16.30	15.73	-0.07	
8943	Bacteria:Firmicutes:Bacilli_SP:Lactobacillales_CL:Lactobacillales:Leuconostoc_FM:sfA	14.59	14.17	15.22	14.46	-0.03	
39693	Bacteria:Firmicutes:Clostridia_SP:Clostridiales_CL:Clostridiales:Clostridiaceae:sfA	15.18	15.09	15.61	14.84	0.00	
OTUs that decrease following irradiation:							
39153	Bacteria:Firmicutes:Clostridia_SP:Clostridiales_CL:Clostridiales:Clostridiaceae:sfA	14.89	13.95	13.45	12.86	1.47	8.04E-03
39143	Bacteria:Firmicutes:Clostridia_SP:Clostridiales_CL:Clostridiales:Clostridiaceae:sfA	15.33	14.40	13.04	12.71	1.94	1.88E-03
39286	Bacteria:Firmicutes:Clostridia_SP:Clostridiales_CL:Clostridiales:Clostridiaceae:sfA	15.43	14.56	13.15	12.80	1.92	2.37E-03
39546	Bacteria:Firmicutes:Clostridia_SP:Clostridiales_CL:Clostridiales:Clostridiaceae:sfA	15.20	14.39	12.94	12.59	1.90	2.49E-03
40020	Bacteria:Firmicutes:Clostridia_SP:Clostridiales_CL:Clostridiales:Eubacterium_FM:sfA	14.96	14.13	13.80	13.11	1.27	1.39E-02

Note. Heatmaps highlight the trends of OYUs that increase (blue) and decrease (red) after irradiation. Log_2 fold changes: day 0 – averaged (days 4, 11 and 21) are shown along with *t* test, *P* values.

dissimilarity without the use of pre-exposure controls. This aspect of the analysis is very important for translation, ultimately, to a radiation triage situation in which pre-exposure controls for each individual will not be available. A total of 7,484 bacterial OTUs were detected in at least one of the samples. The Adonis test demonstrated that bacterial communities were more dissimilar across days than they were within the same day ($P < 0.001$). Samples separated more distinctly by day than by rat when all taxa present in at least one of the samples was considered using the Bray-Curtis dissimilarity measurement (Fig. 2A). Hierarchical clustering in the form of dendrograms using the complete linkage method revealed a close relationship at days 0, 4, 11, and 21 (Fig. 2B).

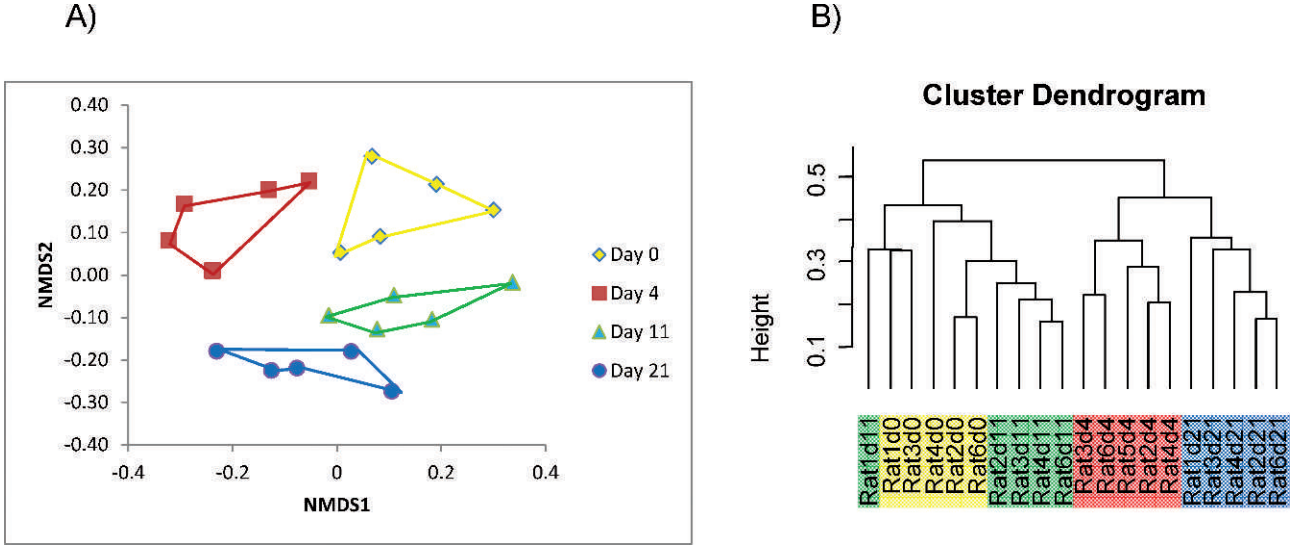

FIG. 2. Intestinal microbial community analysis in feces pre- and post-irradiation. Panel A: Differences in composition of 16S rRNA sequences measured by PhyloChip were used to calculate the Bray-Curtis distance between rat feces samples. The presence-absence scoring for each hybridizing signal in all 7484 OTUs was incorporated in the analysis. Non-metric multidimensional scaling ordination of the samples showed microbial communities were significantly different by day ($P < 0.001$) but not by rat ($P < 0.09$), as determined by the Adonis test, and delineated with lines for clarity. Panel B: Hierarchical clustering showing the phylogenetic relationships of the microbiota in rat feces. Samples were clustered using the farthest neighbor distance (complete linkage) algorithm to show the strong dependence of microbiota on day post irradiation.

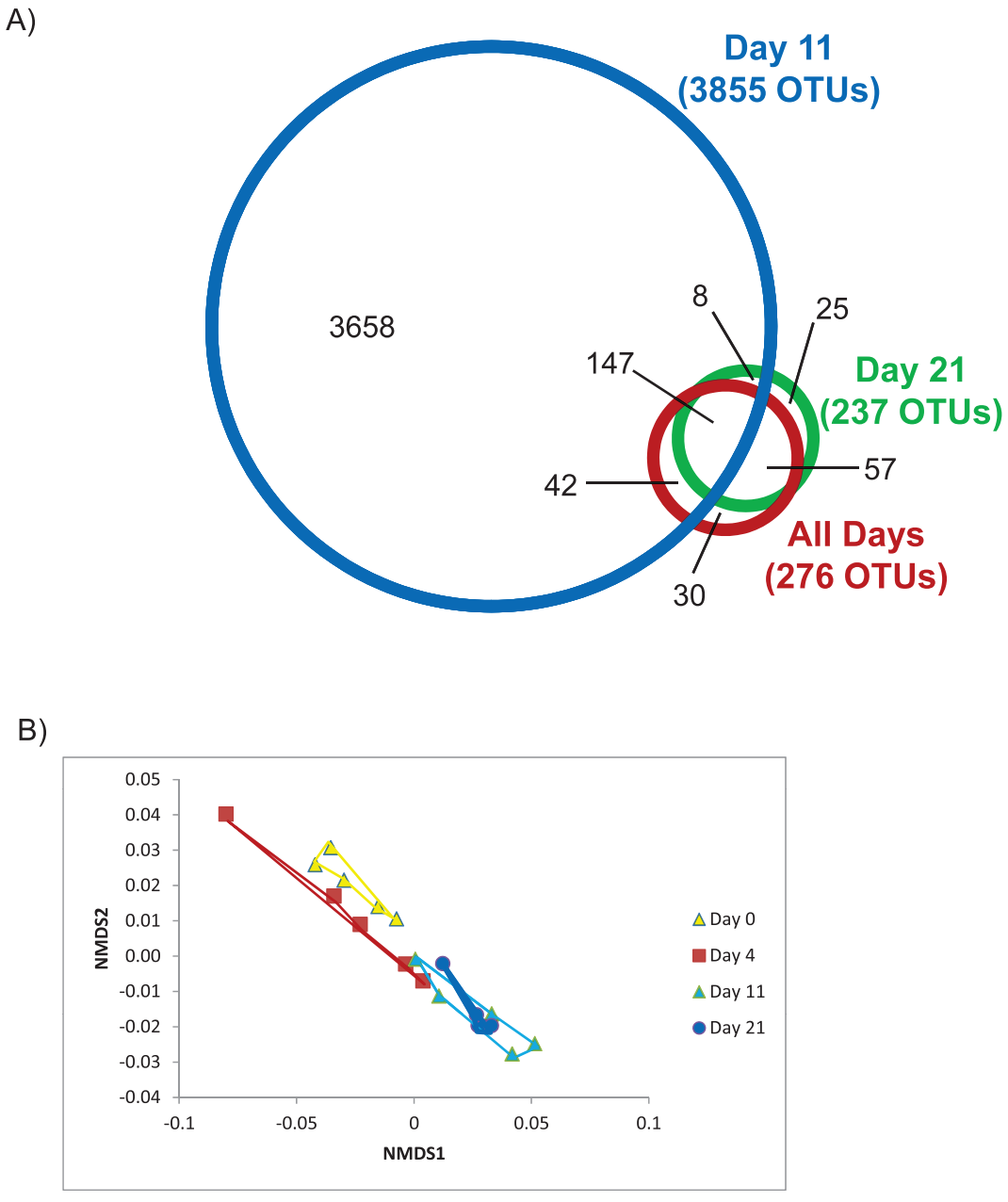

FIG. 3. Candidate biomarkers for radiation exposure. Panel A: Venn diagram illustrating abundance of OTUs exhibiting-statistically significant changes between background, day 0, and day 11 (Day 11); background and day 21 (Day 21); and background and combined days 4–21 (All Days). Numbers in black indicate the number of OTUs that are shared between each analysis. B) Non-metric multidimensional scaling ordination of the samples based on the 147 common OTUs found in A) showed distance separation by day ($P < 0.001$) but not by rat ($P < 0.09$) and delineated with lines for clarity.

Candidate Biomarkers of Prior Radiation Exposure

To find candidate biomarkers of prior radiation exposure, we searched for OTUs that exhibited changes in abundance that were persistent from days 4–21 post-irradiation. We found abundance levels of 276 OTUs that were changed at days 4–21 when we limited the number of false discoveries to 5 (total), as estimated by the q-value (all days, Fig. 3) (*17*). We then compared these 276 OTUs with the 3855 and 237 OTUs that were significantly altered on days 11 and 21 as compared to background (days 11 and 21, Fig. 3). A common set of 147 OTUs were found between the three comparisons and were used as an initial list of biomarker candidates.

Ordination using these 147 OTUs separated the data points by days post-irradiation using non-metric multidimensional scaling (Fig. 3B; $P < 0.001$). (Representative OTUs from this list are shown in Table 1.) From the list of 276 OTUs that showed significant changes between background, day 0, and days 4 through 21, we further

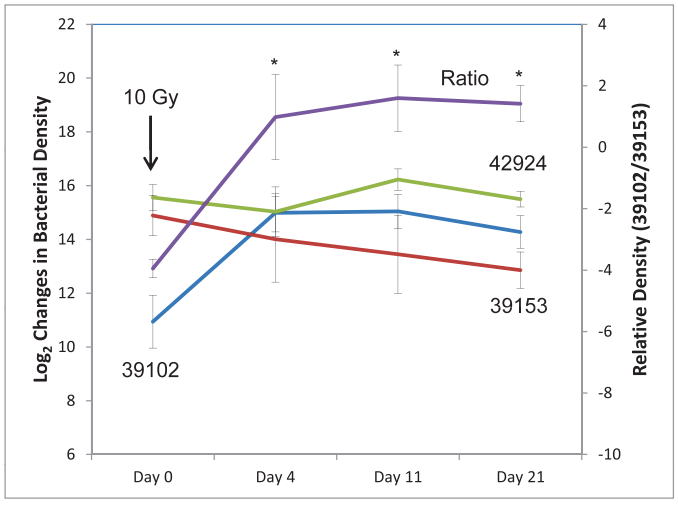

FIG. 4. Persistent changes in specific OTU abundance following radiation exposure. Abundance of three representative OTUs 31902, 42924, and 39153 showing increased, stable, and decreased 16S rRNA expression. The error bars represent within group variation for 5 rats at each time point. The ratio of 39102 abundance relative to 39153, i.e. $\log_2$ 39102 – $\log_2$ 39153 served as a potential composite biomarker for irradiation. The values of this biomarker are −3.95, 0.98, 1.60, and 1.42 at day 0, 4, 11, and 21, respectively. Values were calculated independently for each day and are statistically significant as compared to day 0 ($*P < 0.001$).

selected OTUs that exhibited a persistent change in abundance following irradiation (Table 1). Even though the changes in abundance of these OTUs were not as large as the top 147 candidates discussed above, they represented a distinctly different phenotype. We found 165 members of the *Bacteroidales*, *Lactobacillaceae* and *Streptococcaceae* OTUs with increased expression following radiation exposure, 142 *Clostridiaceae* and *Peptostreptococcaceae* OTUs with unchanged abundance that may serve as internal controls, and 47 separate *Clostridiaceae* OTUs with decreased expression. The complete listing of OTUs that increased, decreased or were unchanged following irradiation is provided in Supplementary Material, data supplement 3, 4 and 5; http://dx.doi.org/10.1667/RR2691.1.S1.

Results from the microarray studies are also specific to individual bacteria. For example, abundance of OTU 31902 (*Cyanobacteria*) increased, OTU 39153 (*Clostridia*) decreased, and OTU 42924 (*Clostridia*) was unchanged in the 4–21-day period post radiation exposure (Fig. 4). The increased/decreased ratio of 31902/39153 increased from −4 to +2 $\log_2$ difference indicating a 64 fold-change at days 4, 11, and 21 post-irradiation (Fig. 4), and may also be used as a possible biomarker of prior radiation exposure. The use of a ratio in developing intestinal microbiota as biomarkers for radiation biodosimetry may be advantageous because pre-exposure samples will not be available in the event of a radiological device being detonated.

Quantitative PCR Confirmed Changes in Intestinal Microbiota Abundance after Irradiation

To determine whether specific groups of bacteria identified by microarray analysis can be detected by an independent method, feces were also analyzed for expression of 16S rRNA in selected groups of intestinal microbiota using qPCR. Abundance of *Proteobacteria* increased almost 1000-fold 4 days after 10 Gy total-body irradiation and then returned to control values (Fig. 5A). Abundance of *Clostridia* and *Bacteroidetes* was less affected over this period suggesting particular microbial taxa (e.g., order or family), whose abundance are unaffected by radiation may serve as internal controls (Fig. 5A, Table 1, see Supplementary Material, data supplement 4; http://dx.doi.org/10.1667/RR2691.1.S1). In these studies, the primers

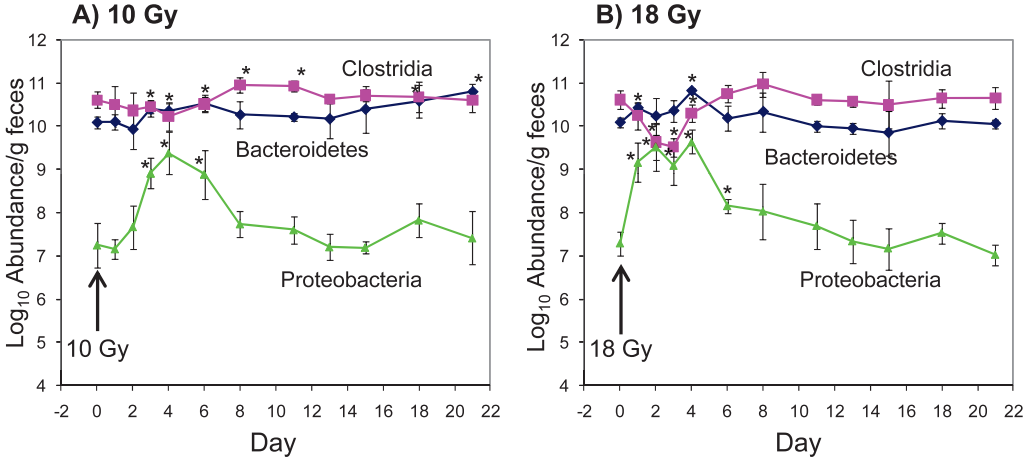

FIG. 5. Transient or no changes in multiple individual human bacteria present in feces of rats exposed to 10 Gy and 18 Gy irradiation. Abundance of Proteobacteria was increased by almost 1000-fold following irradiation while the *Clostridia* and *Bacteroidetes* abundances were relatively stable. A 10-fold drop in *Clostridia* was observed only in feces of 18 Gy irradiated rats. Data are mean ± SD, $n = 5$/group. $*P < 0.05$ vs. Day 0.

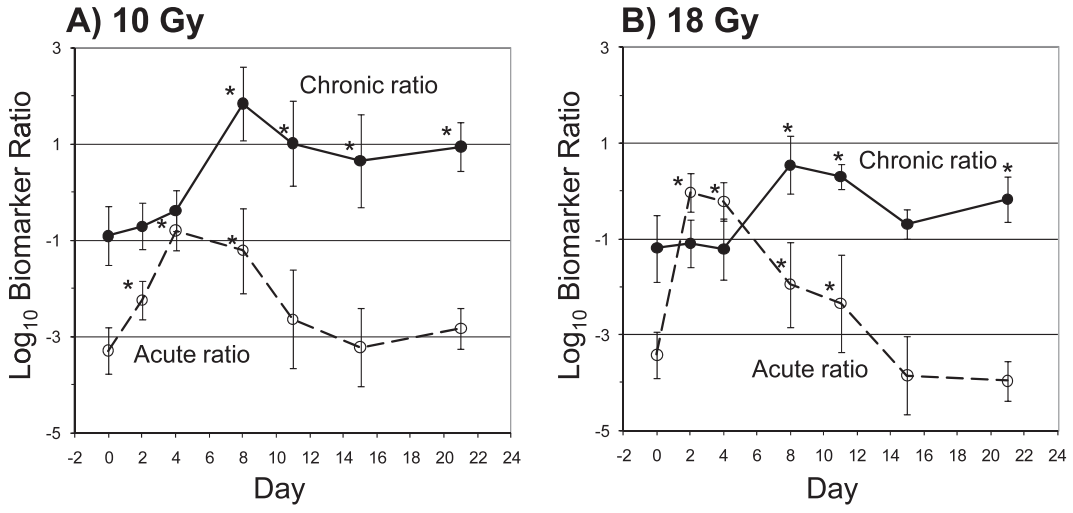

FIG. 6. PCR confirmation of biomarker dynamics in feces of rats exposed to 10 and 18 Gy irradiation. The dashed lines show the ratio of acutely increased/decreased biomarkers and the solid lines show the ratio of chronically increased/decreased biomarkers. Data are mean ± standard deviation, n = 5/group. *$P < 0.05$ vs. Day 0.

for 16S rRNA were detected over 100 separate members in each bacterial group.

Dose-Response Studies

To determine the impact of radiation dose, studies were conducted using six fractionated exposures totaling 18 Gy over a 3-day period to model therapeutic radiation as used clinically. The results show 18 Gy irradiation induced a prolonged increase in *Proteobacteria* over 5 days (Fig. 5B), as compared to over 3 days observed after 10 Gy (Fig. 5A). Further, 18 Gy irradiation induced a 10-fold reduction in *Clostridia* at days 1–3 that was not observed with 10 Gy irradiation. The increases in *Proteobacteria* at 2 days after 18 Gy irradiation correspond with equivalent responses observed at 4 days after 10 Gy irradiation since the 6 fractions of the 18 Gy regimen were administered over 3 days instead of 1 day.

Quantitative PCR Confirmed Biomarkers of Prior Radiation Exposure

Guided by the biomarker OTUs discovered using PhyloChip analysis, we designed PCR primers for quantitative PCR studies to confirm irradiation-induced changes in biomarker abundance. We were able to find biomarkers that were stable, decreased, acutely increased (within the first week), and chronically increased (for more than 21 days) following irradiation (see Supplementary Material, data supplement 6; http://dx.doi.org/10.1667/RR2691.1.S1). The biomarker ratio, acute increase/decrease, was increased from 2 to 8 days following 10 and 18 Gy irradiation, while the ratio ''chronic increase/decrease'' was increased from 8 to 21 days post-irradiation (Fig. 6A and B). These results confirm the existence of individuals, or groups of bacteria, that can serve as biomarkers of prior radiation exposure.

Impact of Age on Bacterial Populations

To determine the impact of age on the abundance of intestinal bacterial populations, feces were collected from non-irradiated 5-week-old rats over the next 21 days. The abundance of *Bacteroidetes*, *Proteobacteria*, and *Clostridia* did not change over the 21-day study period; the abundance of three major (>90% of microbiota) groups of bacteria affected by radiation were unchanged over time in rats not exposed to radiation (see Supplementary Material, data supplement 7A; http://dx.doi.org/10.1667/RR2691.1.S1).

Impact of Genetic Background and Diet on Bacterial Populations

To determine the impact of genetic background and diet on the abundance of bacterial populations present in rats, feces were collected from inbred WAG/RijCmcr rats fed Teklad 8604 chow, outbred Sprague Dawley rats fed LabDiet 5010 chow, and inbred Dahl S rats fed LabDiet 5010 chow; the three rat strains studied are permanently maintained on these diets. The abundance of *Bacteroidetes*, *Proteobacteria*, and *Clostridia* in these three rat strains was comparable and stable over a 6-day study period. Thus, strain and diet did not exert an effect on these three bacterial populations present in rat feces (see Supplementary Material, data supplement 7B; http://dx.doi.org/10.1667/RR2691.1.S1).

Human and Rat Similarities

Investigation of six human fecal samples analyzed using G3 PhyloChip revealed that all 47 OTUs that decreased in rats are present in humans, 98 of the stable 142 OTUs in rats are present in humans, and 12 of the 165 OTUs that increased in rats are found in humans (see Supplementary Material, data supplement 8; http://dx.doi.

org/10.1667/RR2691.1.S1). These 157 OTUs form the basis of the proposed microbiota signature that correlates with, and appears to be diagnostic of, prior exposure to radiation.

We further broadened our rat-to-human analysis by comparing rat OTUs to bacterial taxa detected in 373 stool samples collected during the human microbiome project (http://www.hmpdacc.org/data_browser.php). Rat fecal OTUs were binned at the genus-level to match pyrosequencing results from human samples (see Supplementary Material, data supplement 9; http://dx.doi.org/10.1667/RR2691.1.S1). The 47 OTUs that decreased in the rat were mapped to two genera: *Clostridium* and *Sarcina*, and both are present in humans. Eighty-nine of the stable 142 OTUs were mapped to 14 genera in the *Firmicutes* phylum (see Supplementary Material, data supplement 9; http://dx.doi.org/10.1667/RR2691.1.S1), of which 13 are present in humans. One hundred forty-one of the 165 OTUs that increased in rat were mapped to three genera: *Barnesiella*, *Lactobacillus*, and *Streptococcus*; all three are present in humans. Results show that more than 96% of the classified rat biomarkers are matched to bacterial genera present in humans.

DISCUSSION

This study demonstrates the potential for intestinal microbiota to provide a non-invasive measurement to rapidly identify prior exposure to ionizing radiation. Our OTU-level analysis of feces of irradiated rats showed changes in the abundance levels of 212 genomically-distinct bacteria, of which 59 (12 increased and 47 decreased following irradiation) were found in normal human feces. 16S rRNA levels in 98 intestinal microbiota unaffected by radiation served as internal controls. Intestinal microbiota affected by irradiation provided a sustained level of reporting signals that persisted at least 21 days following exposure to radiation. The increased/decreased ratio of two individual bacteria increased 64-fold at day 21 compared with day 0, and it may have utility as a biomarker of prior exposure to ionizing radiation, as demonstrated by our PCR results. These biomarkers may also be indicative of early gastrointestinal system injury following fractionated therapeutic radiation. Future studies focusing on partial radiation exposure, in conjunction with dose and dose-rate experiments will allow us to better assess the utility of these biomarkers. Evidence that abundance of copies of the 16S rRNA gene in response to ionizing radiation is robust suggests that gene-abundance signatures are likely to be translatable from discovery platforms to developing ''fieldable'' assay platforms more suitable for practical biodosimetry. Genetic background and age did not appear to exert changes in abundance for multiple bacterial taxa including *Bacteroidetes*, *Proteobacteria*, and *Clostridia* in control rats not exposed to radiation. The abundance of multiple intestinal bacterial taxa were also unaffected by diet in control rats. However, diets used in the present study have well-defined protein, carbohydrate, and fat contents and may not represent the divergent composition of human diets and their impact on the intestinal microbiota (*18*). Additional studies are needed to define the impact of divergent human diets over different time periods on the response of intestinal microbiota to radiation. In the event that areas of the body may be partially shielded, an absence of change in intestinal microbiota may also be useful to diagnose that the intestines have not been exposed to prior irradiation.

In addition to the OTU-level comparison of rat and human feces, we also analyzed taxa detected in 373 stool samples from the Human Microbiome Project and compared them with our findings. Fourteen of the 15 bacterial genera that were up, down, and stable following irradiation in rats were found in the human samples (see Supplementary Material, data supplement 9; http://dx.doi.org/10.1667/RR2691.1.S1). This similarity accounted for more 96% of the classified biomarker OTUs. Biomarkers we aim to extract from these studies are commensal bacteria ubiquitous to the rat and human populations. As such, their abundance will be relatively stable and independent of a normal and healthy individual's immune activity. Even though there are variances in microbiota composition between individuals, the human microbiota can generally be categorized into three main enterotypes that are independent of body mass index, age, and gender (*19*).

Estimates of prior radiation exposure must be made at the individual level, and provide timely and accurate information to help with medical triage decisions following acts of nuclear terrorism, warfare, or accidents (*20*). While chromosomal aberrational analysis is the reigning gold standard for radiation biodosimetry, scoring this method requires stimulation of cell division, thus needing several days before results are available. In addition, very few centers exist to conduct cytogenetic dosimetry, and their capacity is quite limited. Since gene expression does not require cell division, microarray-based approaches may provide results within several hours of drawing blood. However, the number of genes present in blood that are affected by radiation [14 genes in reference (*21*) and 74 genes in reference (*22*)] represents a relatively small number, changes in expression are modest, and this diagnostic test requires invasive sample collection. The present study has identified multiple intestinal microbiota present in feces that act as novel biomarkers of prior radiation exposure, where changes in expression are increased 100-1000-fold and where levels of reporting signals are detectable by 2 days post-exposure and persist for a minimum of 3 weeks after exposure. Complementary methodologies are needed that yield information that is both forward-deployable and capable of driving immediate triage decisions. Biologically-based dosimetry measures biological (cellular) processes affected by ionizing radiation. An

advantage of biological dosimetry is the measurement of cellular damage rather than dose received. Disadvantages are that the method may not be completely specific to damage from radiation, it may be affected by damage from pre-existing pathology, and it requires collection of invasive samples. For example, collection and processing of blood from tens of thousands of individuals would not be practical within a one-day period. Physically-based dosimetry estimates radiation dose via physical changes to body structures, such as teeth and nails. An advantage of physical dosimetry is that there is no complex variation by time- or delayed-response. Disadvantages include low sensitivity, absence of an internal control, heterogeneity of the reporting signal, and limited resolution. We believe radiation-induced changes in the intestinal microbiota will be most useful when they are integrated with independent information about prior radiation exposure.

The complete sequencing of the human genome and commercially-available platforms has enabled global transcriptional profiling studies. Microarray experiments have characterized the response of multiple human genes to ionizing radiation. The current study has applied microarray analysis specific for bacterial 16S rRNA gene sequences for the first time to develop the intestinal microbial community present in feces pre- and post-irradiation. Our objective was to describe a biological signature of prior radiation exposure using microarray analysis. Our approach was to analyze a large microarray data set in a coordinated and integrated manner, identify 16S rRNA sequences that are increased, unchanged or decreased in response to irradiation, and to define a biological signature that may be used to diagnose prior exposure to irradiation.

To accomplish this we integrated large-scale-expression profiling studies of the intestinal microbiome with quantitative PCR to understand the relationship between intestinal microbiota and radiation and we identified multiple individual members of the intestinal microbiota that are sensitive to ionizing radiation. The innovative application of the PhyloChip technology in our study permit the simultaneous identification of a comprehensive list of bacterial OTUs including all, but not limited to, 16S rRNA gene sequences present in the March 2008 NCBI database. Because the PhyloChip is able to use the entire bacterial 16S rRNA gene sample pool rather than just a few thousand to tens-of-thousands of sequences from the microbial community, it has a greater capacity for reproducibly quantifying changes in populations even, among the low abundance members. Considering that a typical fecal sample contains $>10^{10}$ cells per gram, the sampling effort by sequencing is usually too small to give reliable estimates of diversity. However, each sample analysis by the PhyloChip provides detailed information on microbial composition at these low levels, and the highly-parallel and reproducible nature of this array allows tracking community dynamics over time and with exposure to radiation.

In the setting of radiological terrorism or nuclear accident, stool could be collected in addition to blood and urine. The technology needed to process stool is currently available (ex. QIAamp DNA Stool Mini Kit) and can be automated (ex. QIAcube). Use of intestinal microbiota as biomarkers of prior irradiation will require considerable research and development before it may be used in an emergency situation. The hardware needed to extract DNA, prepare and read the PhyloChip, or perform qPCR and then analyze large amount of data already exists. Other groups have already established this technology and are developing it for blood-based radiation biodosimetry. We envision that DNA extraction, amplification, and microarray analysis will be automated with the analytical system delivered by air to a medical field's facility in proximity to, but not inside, the radioactive contamination zone.

The microarray results showed a limited number of OTUs that exhibit statistically-significant differences between day 0 and day 4. In particular, the OTUs in the *Proteobacteria* phylum measured by microarray did not exhibit the 100-1000-fold increase that was observed using PCR (Fig. 5). The cause of this difference is evident from the hybridizing score of the PhyloChip data. Except for three outlier samples (Rat 3 day 0, Rat 1 day 11, and Rat 1 day 21), $>75\%$ of the OTUs exhibited hybridizing signal that were within the saturation limit of the Phylochip (data not shown). Microchip saturation may prevent the quantitation of the dramatically-increased *proteobacteria* abundance. Furthermore, examination of all OTUs showed a similar trend of saturation to suggest that additional non-*proteobacteria* OTUs may have been under-quantified. This result suggests that even though we were successful at obtaining multiple OTUs for use as biomarkers, many more may become evident once the challenge of signal saturation in the PhyloChip analysis is addressed.

Microarray analysis of peripheral blood has been used to predict radiation doses received *in vivo* in a heterogeneous population of total-body irradiation patients, and to compare the predictive ability of signatures derived from *in vivo* versus *ex vivo* exposures (*23*). A total of 413 genes were significantly expressed following irradiation. Recently, NMR has been used to identify small molecule metabolites as biomarkers in mouse urine after exposure to 8 Gy whole-body X rays. Changes in these biomarkers were detected on the first day after irradiation, and typically reached their maximum on the third and fourth days after irradiation (*24*). In this study, the biomarker was temporally dependent, suggesting this biomarker is useful only at certain times following irradiation.

Limitations of our current study are that the lowest dose of radiation detectable and earliest response (within the first 48 h), by intestinal microbiota to irradiation have yet to be determined. In addition, the impact of health status on intestinal microbiota after irradiation is unknown. As an example, antibiotic use throughout the U.S. population is common (*25*); we need to determine the impact of pre-

existing use of antibiotics on abundance of intestinal microbiota following exposure to radiation. In the period immediately following nuclear detonation, supportive care including antibiotics can improve the prognosis for some irradiated casualties (26); we need to determine whether bacteria affected by antibiotics are the same as those affected by radiation. In addition, the skin functions as a critical barrier that can be severely compromised by exposure to ionizing radiation; when radiation injury is combined with traumatic wound injury, the normal wound healing process is disrupted, resulting in greater morbidity and mortality (27–31). In the event of a radionuclear attack, skin injury will likely be associated with additional injuries such as blunt trauma and wounds (32–35). Therefore, the impact of combined radiation injury and wound injury on intestinal microbiota needs to be determined.

We conclude that using intestinal microbiota as biomarkers of prior radiation exposure represents a novel approach that can complement conventional chromosome aberrational analysis and may significantly enhance biological dose assessments. Further studies are warranted to improve our fundamental understanding of the interaction between radiation and the microbiota present in the intestines and expelled into the feces.

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REFERENCES

1. Swartz HM, Flood AB, Gougelet RM, Rea ME, Nicolalde RJ, Williams BB. A critical assessment of biodosimetry methods for large-scale incidents. Health Phys 2010; 98(2):95–108.
2. Mole RH. The LD50 for uniform low LET irradiation of man. Br J Radiol 1984; 57(677):355–69.
3. Coleman CN, Blakely WF, Fike JR, MacVittie TJ, Metting NF, Mitchell JB, et al. Molecular and cellular biology of moderate-dose (1–10 Gy) radiation and potential mechanisms of radiation protection: report of a workshop at Bethesda, Maryland, December 17–18, 2001. Radiat Res 2003; 159(6):812–34.
4. Crawford PA, Gordon JI. Microbial regulation of intestinal radiosensitivity. Proc Natl Acad Sci USA 2005; 102(37):13254–9.
5. Battista JR, Earl AM, Park MJ. Why is Deinococcus radiodurans so resistant to ionizing radiation? Trends Microbiol 1999; 7(9): 362–5.
6. Gunter SE, Kohn HI. Effect of x-rays on the survival of bacteria and yeast. II. Relation of cell concentration and endogenous respiration to sensitivity. J Bacteriol 1956; 72(3):422–8.
7. Kelner A, Bellamy WD, Stapleton GE, Zelle MR. Symposium on radiation effects on cells and bacteria. Bacteriol Rev 1955; 19(1): 22–44.
8. Witkin EM. Inherited Differences in Sensitivity to Radiation in Escherichia Coli. Proc Natl Acad Sci USA 1946; 32(3):59–68.
9. Kent TH, Osborne JW, Wende CM. Intestinal flora in whole-body and intestinal x-irradiated rats. Radiat Res 1968; 35(3):635–51.
10. Vincent JG, Veomett RC, Riley RF. Relation of the indigenous flora of the small intestine of the rat to post-irradiation bacteremia. J Bacteriol 1955; 69(1):38–44.
11. Noyes HE, Evans JR, Baker HJ. Effects of a Nuclear Detonation on Swine–Bacteriologic Studies. Ann N Y Acad Sci 1963; 105:653–65.
12. Furth FW, Coulter MP, Howland JW. Bacteriologic studies of the X-radiated dog. Am J Pathol 1952; 28(2):171–83.
13. Balish E, Pearson TA, Chaskes S. Irradiated humans: microbial flora, immunoglobulins, complement (C'3), transferrin, agglutinins, and bacteriocidins. Radiat Res 1970; 43(3):729–56.
14. Baker JE, Fish B, Su J, Haworth S, Strande J, Komorowski R, et al. 10 GY Total body irradiation increases risk of coronary sclerosis, degeneration of heart structure and function in a rat model. Int J Radiat Biol 2009; 85(12):1089–1100.
15. Hazen TC, Dubinsky EA, DeSantis TZ, Andersen GL, Piceno YM, Singh N, et al. Deep-sea oil plume enriches indigenous oil-degrading bacteria. Science 2010;330(6001):204–8.
16. Croswell A, Amir E, Teggatz P, Barman M, Salzman NH. Prolonged impact of antibiotics on intestinal microbial ecology and susceptibility to enteric Salmonella infection. Infect Immun 2009; 77(7):2741–53.
17. Storey JD, Tibshirani R. Statistical significance for genomewide studies. Proc Natl Acad Sci USA 2003; 100(16):9440–5.
18. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. Sci Transl Med 2009; 1(6):6ra14.
19. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, et al. Enterotypes of the human gut microbiome. Nature 2011; 473(7346):174–80.
20. Grace MB, Moyer BR, Prasher J, Cliffer KD, Ramakrishnan N, Kaminski J, et al. Rapid radiation dose assessment for radiological public health emergencies: roles of NIAID and BARDA. Health Phys 2010; 98(2):172–8.
21. Brengues M, Paap B, Bittner M, Amundson S, Seligmann B, Korn R, et al. Biodosimetry on small blood volume using gene expression assay. Health Phys 2010; 98(2):179–85.
22. Paul S, Amundson SA. Development of gene expression signatures for practical radiation biodosimetry. Int J Radiat Oncol Biol Phys 2008; 71(4):1236–44.
23. Paul S, Barker CA, Turner HC, McLane A, Wolden SL, Amundson SA. Prediction of in vivo radiation dose status in radiotherapy patients using ex vivo and in vivo gene expression signatures. Radiat Res 2011; 175(3):257–65.
24. Chen C, Brenner DJ, Brown TR. Identification of urinary biomarkers from x-irradiated mice using NMR spectroscopy. Radiat Res 2011; 175(5):622–30.
25. Grijalva CG, Nuorti JP, Griffin MR. Antibiotic prescription rates for acute respiratory tract infections in US ambulatory settings. JAMA 2009; 302(7):758–66.
26. DiCarlo A, Maher C, Hick J, Hanfling D, Dainiak N, Chao N. Radiation injury after a nuclear detonation: medical consequences and the need for scarce resources allocation. Disaster Med Public Health Preparedness 2011; 5:S32–S44.
27. Augustine AD, Gondre-Lewis T, McBride W, Miller L, Pellmar TC, Rockwell S. Animal models for radiation injury, protection and therapy. Radiat Res 2005; 164(1):100–9.
28. DiCarlo AL, Hatchett RJ, Kaminski JM, Ledney GD, Pellmar TC, Okunieff P, et al. Medical countermeasures for radiation combined

injury: radiation with burn, blast, trauma and/or sepsis. Report of an NIAID Workshop, March 26–27, 2007. Radiat Res 2008; 169(6):712–21.

29. Kiang JG, Jiao W, Cary LH, Mog SR, Elliott TB, Pellmar TC, et al. Wound trauma increases radiation-induced mortality by activation of iNOS pathway and elevation of cytokine concentrations and bacterial infection. Radiat Res 2010; 173(3):319–32.
30. Messerschmidt O. Whole-body irradiation plus skin wound: animal experiments on combined injuries. Br J Radiol Suppl 1986; 19:64–7.
31. Ran X, Cheng T, Shi C, Xu H, Qu J, Yan G, et al. The effects of total-body irradiation on the survival and skin wound healing of rats with combined radiation-wound injury. J Trauma 2004; 57(5): 1087–93.
32. Coleman CN, Stone HB, Moulder JE, Pellmar TC. Medicine. Modulation of radiation injury. Science 2004; 304(5671):693–4.
33. Gottlober P, Steinert M, Weiss M, Bebeshko V, Belyi D, Nadejina N, et al. The outcome of local radiation injuries: 14 years of follow-up after the Chernobyl accident. Radiat Res 2001; 155(3): 40–16.
34. Mettler FA, Jr., Gus'kova AK, Gusev I. Health effects in those with acute radiation sickness from the Chernobyl accident. Health Phys 2007; 93(5):462–9.
35. Moulder JE, Cohen EP. Future strategies for mitigation and treatment of chronic radiation-induced normal tissue injury. Semin Radiat Oncol 2007; 17(2):1418.
36. Tibshirani RJ, Efron B. Pre-validation and inference in microarrays. Stat Appl Genet Mol Biol 2002;1:Article1.

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