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The Evolution of RecD Outside of the RecBCD Complex

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Abstract The common understanding of the function of RecD, as derived predominantly from studies in *Escherichia coli*, is that RecD is one of three enzymes in the RecBCD double-stranded break repair DNA recombination complex. However, comparative genomics has revealed that many organisms possess a *recD* gene even though the other members of the complex, *recB* and *recC*, are not present. Further, bioinformatic analyses have shown that there is substantial sequence dissimilarity between *recD* genes associated with *recB* and *recC* (*recD1*), and those that are not associated with *recBC* (*recD2*). *Deinococcus radiodurans*, known for its extraordinary DNA repair capability, is one such organism that does not possess either *recB* or *recC*, and yet does possess a *recD* gene. The *recD* of *D. radiodurans* was deleted and this mutant was shown to have a capacity to repair double-stranded DNA breaks equivalent to wild-type. The phylogenetic history of *recD* was studied using a dataset of 120 *recD* genes from 91 fully sequenced species. The analysis focused upon the role of gene duplication and functional genomic context in the evolution of *recD2*, which appears to have undergone numerous independent events resulting in duplicate *recD2* genes. The role of RecD as part of the RecBCD complex appears to have a divergence from an earlier ancestral RecD function still preserved in many species including *D. radiodurans*.

Keywords RecD · RecB · RecC · *Deinococcus radiodurans* · *Bacillus halodurans* · Gene duplication · DNA damage

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Introduction

There are two known types of RecD: *recD1* and *recD2* (Rocha et al. 2005). The only biological context for our understanding of RecD comes primarily from the study of *recD1*, most notably in *Escherichia coli*, in which it functions as part of the RecBCD heterotrimer consisting of three polypeptides, RecB, RecC, and RecD. The heterotrimer complex is involved in homologous recombination, and possesses a number of catalytic activities that are used to initiate the repair of both double-stranded DNA breaks (DSBs) and single-stranded DNA gaps (SSGs), which are generated during the course of DNA replication or exposure to DNA-damaging agents (Chedin and Kowalczykowski 2002; Spies and Kowalczykowski 2005). *E. coli*, isolated in 1885, has been used as a model organism as a result of the fact that it grows quickly in easy to make media, and because of its presence as an enteric symbiote of humans. As a model system, it has been used to study gene structure (Benzer 1961), recombination (Emmerson 1968), and historical contingent evolution (Blount et al. 2008).

In *E. coli*, the RecBCD complex as a whole binds tightly to dsDNA ends, and using a combination of helicase and

$3' \rightarrow 5'$ nuclease activities, digests away the DNA until a Chi, crossover hotspot instigator [5′-GCTGGTGG-3′] is encountered. At this point, RecBCD pauses, switches the polarity of its nuclease activity and creates a 3′ ssDNA tail. RecA is then loaded onto the ssDNA by RecBCD, and carries out strand invasion of homologous dsDNA to initiate homologous recombination (Singleton et al. 2004).

The *E. coli* RecD protein has been shown to be a DNA-dependent ATPase, as well as a $5' \rightarrow 3'$ helicase (Dillingham et al. 2003; Taylor and Smith 2003). Genetic studies in *E. coli* have revealed that the RecB and RecC gene products are necessary for the repair of DSBs in *E. coli*. Deletion of either *recB* or *recC* increases sensitivity to DNA-damaging agents, such as UV radiation and mitomycin C (Emmerson 1968; Howard-Flanders and Theriot 1966; Spies and Kowalczykowski 2005; Willetts and Mount 1969). In contrast, *E. coli* cells deleted for *recD* display no obvious phenotypes when exposed to DNA-damaging agents. They do, however, exhibit higher levels of plasmid instability than wild-type cells (Biek and Cohen 1986). Although *recD* null *E. coli* cells lack ATP-dependent nuclease activity, they are proficient for DSB repair and catalyze homologous recombination at high rates in a Chi independent manner (Amundsen et al. 1986; Biek and Cohen 1986; Lovett et al. 1988). It should be noted that homologous recombination in these *recD* mutants is dependent on the functions of RecBC and the RecJ nuclease (Lloyd et al. 1988; Lovett et al. 1988).

Results from many genome sequencing projects as well as other studies have revealed that the *recB*, *recC*, and *recD* genes are conserved in many bacteria (Rocha et al. 2005). However, some bacteria, such as *Bacillus subtilis*, do not contain *recB* and *recC* genes. Instead, these bacteria contain a two subunit helicase/nuclease enzyme, AddAB, which functions in a similar manner to the RecBCD complex in that it recognizes an organism-specific Chi site, and uses a cognate RecA protein. The AddA subunit displays regions of homology to the RecB subunit, including an ATP-binding motif, several potential helicase motifs as well as the nuclease domain, whereas the AddB subunit does not appear to share substantial homology to either the RecC or RecD subunit (Chedin and Kowalczykowski 2002; Yu et al. 1998). The RecDs of several other organisms (almost exclusively RecBC organisms closely related to *E. coli*) have been deleted or mutated with a variety of phenotypes as is examined in "Discussion" section (Bidle and Bartlett 1999; Chaussee et al. 1999; Khairnar et al. 2008; Kickstein et al. 2007; Mehr and Seifert 1998; Miesel and Roth 1994; Regha et al. 2005; Servinsky and Julin 2007; Zhou et al. 2007).

An analysis of the *recD* sequence from many species, taking care to include representative members of *recBC* species, *addAB* species, and as many cases as possible of species that contained *recD* in the absence of *recBC* or *addAB* was made. Evidence was found to suggest that *recD* had been the subject of numerous independent events that result in duplicate *recD* within the same genome throughout various bacterial lineages including at least one very ancient event. Gene duplication has been widely observed in many lineages and hypothesized as a major driver of the evolution of novel function (Zhang 2003). Alternative hypotheses have suggested that purifying selection would prevent duplicate genes that were not already bi-functional from developing novel functions (Hughes 1994). To explore the role of *recD* outside the *recBCD* pathway as understood in *E. coli* and to understand the role of gene duplication in *recD* evolution, we studied the *recD* of *Deinococcus radiodurans* because of its remarkable DNA repair activity coupled with the dissimilarity of its RecD sequence from homologs in other species.

Deinococcus radiodurans is among a small number of known organisms that do not contain *recB* and *recC* or *addA* and *addB* genes, but still contain RecD (Rocha et al. 2005; White et al. 1999). This Gram-positive bacterium is highly resistant to large doses of ionizing and ultraviolet radiation as well as other DNA-damaging agents. While its DNA suffers numerous DSBs upon exposure to these agents, the organism has the remarkable ability to restore its genome without any apparent mistakes (Battista 1997). The molecular basis of this DNA repair is at present poorly understood, although a novel mechanism has been proposed (Slade et al. 2009). Phylogenetic analysis of the *D. radiodurans recD* gene indicates that it is divergent from all other known *recD* sequences, therefore we created a *D. radiodurans* strain that is null for the *recD* gene. We tested the ability of this strain to withstand several kinds of DNA-damaging agents. We found that, compared to wild-type, the mutant strain was able to repair DSBs from ionizing radiation, and hydrogen peroxide with unchanged kinetics. Its growth was also insensitive to the presence of hydroxyurea relative to wild-type.

The results, presented below, suggest that the RecD of *D. radiodurans* is not essential to DSB repair. Phylogenetic analysis suggests that our understanding of *recD* biology related to DSB repair may only be applicable to the shorter (*recD1*) *recBC*-associated version of the gene. The longer (*recD2*) version of the gene appears to represent a more ancestral form.

Materials and Methods

Bioinformatic Dataset Construction

Bioinformatic investigations of various *recD* sequences were performed to further elucidate the differences between *recD's* role in the presence of *recBC*, in the presence, of

addAB, and alone. To do so, a dataset of various *recD* protein sequences was constructed. Initially, *recDs* were selected from the species analyzed for the presence or absence of various DNA repair enzymes in Rocha et al. 2005. This initial list of *recDs* was then augmented by searching the functional annotation database on the CMR (Peterson et al. 2001) with the intent of including a representative *recD* from each of the frequently used bacterial phylogenetic subdivisions as described in Ciccarelli et al. (2006). Additional sequences were selectively added from COG0507 (Tatusov et al. 2003), PF04257 (Finn et al. 2008), and from NCBI annotation searches. Genes that were annotated as "RecD," "Exonuclease V, alpha subunit," "RecD/TraA," or "TraA" were initially included. In genomes where a *recD* was not annotated as present, or where the annotation of a potential *recD* was ambiguous, a BLAST search was performed versus the genome in question using the *E. coli recD* as a query, and the expect value set to 1,000, or by alignment, see below. Likewise, the presence or absence of *recB*, *recC*, *addA*, and *addB* were confirmed by BLAST searches in addition to using the genome annotations. Due to their high similarity with *addA* & *addB*, *rexA* & *rexB* were treated as *addAB* for the purpose of this analysis.

Several RecD sequences were initially included in the dataset but then removed upon closer examination. Sequences that upon alignment clustered by neighbor joining closely with the F plasmid *traA* or *traI* genes were removed from the data set. This was because they did not align convincingly with other sequences annotated as *recD*. The Archaea *Methanocaldococcus jannaschii* has a *recD* gene, encoded at locus MJ1519, while it clustered in the general vicinity of the *Mycoplasma recDs* on the tree, it had no close neighbors, and the decision was made to limit the analysis to bacterial lineages. The final version of the dataset contained 120 *recD* genes from 91 species; see Table S1 in the Supplementary Data.

In the Genbank annotation of the *Bacillus halodurans* C-125 genome (BA000004.3 GI:47118318), a *Bacillus Firmicute* (Takami et al. 2000), the *recD* gene is truncated. In the CMR annotation, there are instead two side by side *recD* genes. For this reason, the sequence of the *B. halodurans recD* was checked. Genomic DNA was amplified and sequenced. The forward and reverse primer sequences for both amplification and sequencing were 5′-ctcattcctaagatt-attcagc-3′ and 5′-tgaatgaacctgattttatggc-3′, respectively. Two separate amplification reactions were sequenced from both forward reverse primers. All sequences agreed that the single nucleotide frame shift mutation in the completed genome sequence was not present. For this reason, the composite amino acid sequence of the two *B. halodurans recD* genes was used in the phylogenetic analysis.

Many organisms contributed two or three separate *recD* genes to the dataset. Each *recD* gene is referred to in this article after the name of the species it came from. Multiple genes from the same species were assigned a number, with the shortest sequence from a given species receiving the number "1," the next shortest sequence receiving the number "2," and so forth. For a complete description of all sequences added to the dataset, see Table S1 in the Supplementary Data.

Phylogenetic Analysis

The *recD* sequences in the above described dataset are from a very diverse collection of bacterial species, and their sequences are highly divergent. Therefore, in order to reduce noise in the phylogenetic signal, only strongly aligning positions were used for phylogenetic analysis. An alignment was constructed using ClustalX v1.83 (Thompson et al. 1997) using default settings, and then manually edited. Aligned positions where the alignment quality was manually determined to be poor, where sequence conservation was extremely low, or which contained a preponderance of gap characters were deleted. This resulted in a collection of aligned *recD* amino acid positions, containing 267 informative positions, corresponding to the following amino acid positions of the *E. coli recD*: 156–188, 198–219, 237–245, 256–289, 292–314, 350–380, 437–465, 476–489, 493–503, 529–554, 560–595, 596–600, plus a small number of additional positions that did not align with the *E. coli* sequence mostly at the N-terminal side of the alignment. The regions that remain in the edited alignment are predominantly in the helicase domain, and are structurally conserved between the *E. coli* and *D. radiodurans* proteins (Saikrishnan et al. 2008).

A phylogenetic tree was constructed using the maximum likelihood method as implemented in the Phylip phylogenetic package (Felsenstein 1989) using default parameters except as noted here. Hundred bootstrap replicates were used to evaluate these trees. Automation of bootstrapping was carried out using the phylipfasta script written by Jonathan Badger (personal communication). Branch lengths were inferred by using Tree Puzzle (Schmidt et al. 2002) with the default options set. A neighbor joining tree constructed from the same alignment had a similar topology to the maximum likelihood tree (data not shown). The tree was rendered with the use of the software package Dendroscope (Huson et al. 2007).

Bacterial Strains

The bacterial strains used in this study are *D. radiodurans* R1 (obtained from ATCC, Manassas, VA), SV1 (R1Δ*recD*), described below and *rec30* (Moseley and Copland 1975) (kindly provided by Dr. John Battista).

Creation of the *recD* Knockout Vector

The knockout vector was created by first ligating double-stranded (ds) oligonucleotides 5′-cggaattcatttaaarcgc gaggcctcgagcggccgcggctagcagatctgttaacgagctcggtacccc-3′ into *Eco*RI/*Kpn*I cleaved pGEM3Zf (Promega Corp) to add a multiple cloning region. A second multiple cloning region was then added to the above product by ligating ds-oligonucleotides 5′-cccaagcttgatcatcgatatcgggcccgttaa cggcgccgcatgcctgcagaa-3′ at the *Hin*dIII and *Pst*I sites to yield pGEMSV. The chloremphenicol marker was amplified using pRAD1 (Meima and Lidstrom 2000) (a kind gift from Dr. Mary Lidstrom) as template and oligonucleotides 5′-cgggatcccccgggtcgttctgacgctcc-3′ and 5′-ggggtaccttacgc cccgccctgccactc-3′ as primers and then ligated into *Bam*HI/*Kpn*I cleaved pGEMSV to yield pGEMSVCm. Five hundred base pairs (bp) of upstream and downstream sequences of *recD* were amplified using R1 wild-type genomic DNA as template and oligonucleotides 5′-ccatcgatgcaactt-caaagtgcgggg-3′ and 5′-cgggatccgccaggcaggatagcgcgg-3′, and oligonucleotides 5′-gaaggcctgcgaccgcgctcacgttg-3′ and 5′-ggggtaccgacgccttaagagttctg-3′, respectively, as primers. The *recD* upstream and downstream sequences were sequentially ligated into pGEMSVCm cleaved by *Bam*HI/*Cla*I and *Kpn*I/*Stu*I, respectively. The chloramphenicol marker was then replaced from this vector by cleaving with *Kpn*I and *Bam*HI, making the cleaved vector blunt-ended and ligating it to a blunt-ended fragment containing a kanamycin marker (from pET28a, Novagen) under the control of the *D. radiodurans groEL* promoter to yield pGEMSVKanRecDDownUp.

Creation of a *recD* Deletion Strain

To begin to characterize RecD in *D. radiodurans*, we created a knockout strain in which the entire *recD* open reading frame (ORF) was replaced by a kanamycin resistance gene (*kan*) by homologous recombination. PCR as well as Southern blot analysis were performed to confirm the deletion of *recD* and to ensure that the *kan* marker had integrated solely into the *recD* locus (Supplementary Data, Figure S1).

The *recD* null strain was created as described by Earl et al. with some modifications (Earl et al. 2002). pGEMSVKanRecDDownUP was first passaged through *E. coli* INV110 (Invitrogen, Carlsbad CA) to obtain unmethylated DNA. The plasmid was then cleaved with *Bcl*I and *Stu*I and the resulting linear 2.5 kb fragment containing the *kan* marker, flanked by *D. radiodurans* sequences to the left and right of the *recD* gene, was used to transform competent R1 wild-type cells as described by Masters et al. (1991). Transformants were selected on TGY agar plates containing 5 μg/ml kanamycin. Several individual transformants were grown to saturation in TGY broth containing 5 μg/ml kanamycin and used to inoculate TGY broth containing 25 μg/ml kanamycin. The resulting saturated cultures were then plated on TGY agar media containing 25 μg/ml kanamycin and resulting individual colonies were screened by PCR for *recD* null mutants.

After obtaining the appropriate results, the *recD* null strain was observed under the microscope and evaluated for growth. The mutant cells appeared to be slightly larger than the isogenic $recD^+$ cells and they grew a little slower than wild-type with a doubling time of app. 110 min versus 90 min, respectively.

Ionizing Radiation Experiments

Cultures each of exponential ($\sim$0.2 OD_{600}) wild-type and *recD* null cells were exposed to a ^{60}Co source (Gammacell model 220 s/n 232, MDS Nordion at NIST, Gaithersburg, MD) at 4°C at a rate of 2.97 kGy/h.

Recovery experiments were performed by harvesting, washing once with PBS and exposing exponential ($\sim$0.2 OD_{600}) wild-type and *recD* null cultures in PBS to 2,000 Gy of γ radiation as above. The cells were then diluted into TGY broth and incubated at 30°C. At the indicated times, aliquots were harvested and genomic DNA from an equivalent number of cells was isolated in agarose plugs as described by Mattimore and Battista (1996). The genomic DNA was analyzed by pulsed-field gel electrophoresis (PFGE) with 0.5× TBE (Tris–borate–EDTA) at 4 V/cm^2 and 14°C with a 10- to 60-s ramp for 22 h. DNA was visualized by staining with ethidium bromide.

Hydrogen Peroxide Experiments

The indicated amounts of hydrogen peroxide were added directly to exponential ($\sim$0.2 OD_{600}) wild-type and *recD* null cultures and incubated at 30°C for 30 min. The cells were then harvested, washed once with TGY broth, and allowed to recover in TGY broth at 30°C. At the indicated times, aliquots were removed, processed, and analyzed by PFGE as above.

Hydroxyurea Experiment

Ten-fold serial dilutions of exponential ($\sim$0.2 OD_{600}) wild-type, *recA* mutant (*rec30*), and *recD* null cultures were spot-plated onto TGY agar media containing the indicated amounts of hydroxyurea and then grown at 30°C for several days.

Results

Gene-Length, and Phylogenetic Analysis of *recD*

As Rocha et al. pointed out, *recD* genes seem to exist in two different types, which they referred to as *recD1* (short sequence, present in *recBC* organisms) and *recD2* (longer sequence containing an additional N-terminal domain, and present in *recBC* minus organisms). In this article, we have used amino acid length to assign most RecDs as either 'short' (less than 655 amino acids and more than 450) or 'long' (more than 710 amino acids). These criteria classify 113 of 120 *recD* genes from the 91 species included in our dataset in a manner that approximately mirrors the Rocha et al. *recD1* and *recD2* classifications. A phylogenetic analysis of *recD* protein sequences from selected organisms demonstrates that, as a whole, short *E. coli*-like *recBC*-associated *recDs*, and long *recDs* are phylogenetically distinct from one another. One of the most striking qualities of our phylogenetic analysis is that many organisms have multiple copies of the *recD* gene, and that different copies from the same genome often do not phylogenetically segregate together (Fig. 1).

Ciccarelli et al. identified *Thermoanaerobacter tengconge* as the modern organism most proximal to the last universal common bacterial ancestor (Ciccarelli et al.

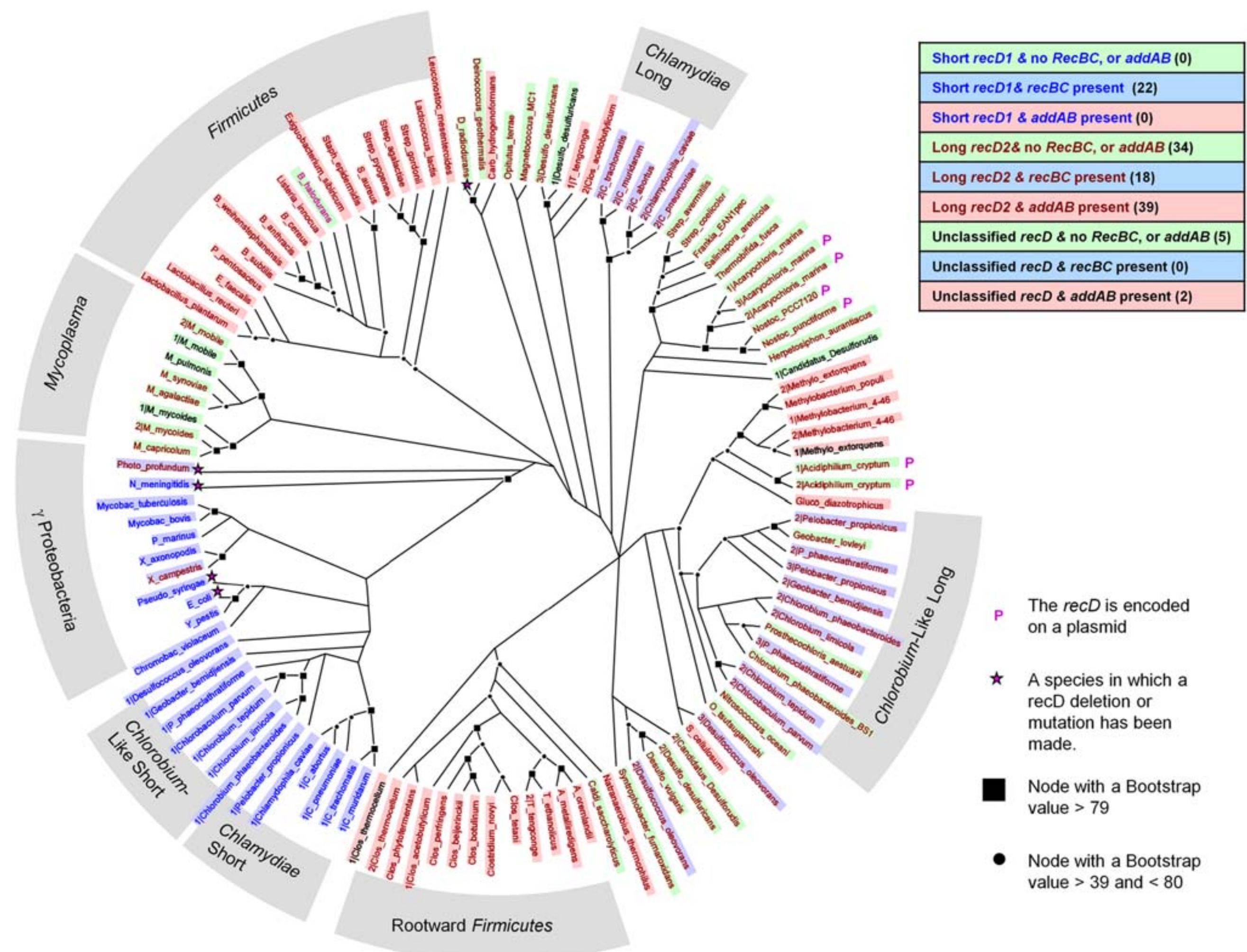

Fig. 1 Maximum likelihood phylogenetic tree derived from a partial alignment of 120 *recD* genes from 91 bacterial species. Each *recD* gene is labeled by its species of origin. Multiple *recD* genes from the same organism were prefixed with a number followed by a | character for readability. Numbers were assigned to *recD* genes from the same species in order of shortest amino acid sequence to longest. Each label is also *color coded*, with *background color* representing the presence or absence of the *recBC* or *addAB* genes, and the *foreground color* representing whether a gene belongs to the short *recD1* (450–655 amino acids) or long *recD2* (more than 710 amino acids) classifications of *recD*. Organisms, which have had their *recD* genes knocked out or mutated in the literature, are marked with a star. One such organism, *Neisseria gonorrhoeae*, is not represented in this dataset because its genome is not completely sequenced and thus a determination of the presence of *recBC* or *addAB* could not be made with certainty. However, the related species *Neisseria meningitidis* is marked with a *star* in its place. For details concerning the sequences represented in this tree, see supplementary data

2006). This was based upon a tree constructed from an alignment of concatenated highly conserved proteins. This is in keeping with the belief that the last bacterial common ancestor was a thermophile even if that may not have been the case for the last common ancestor of Archaea and Bacteria (Boussau et al. 2008). *T. tengconge* along with the *Clostridium* species make up what we refer to as "root ward *Firmicutes*." Three root ward *Firmicutes* (*Clostridium thermocellum*, *Clostridium acetobutylicum*, and *T. tengconge*) have two *recD* genes each. In most of these three species, both of their genes are, by length, of the long (*recD2*) type; one copy of their *recD* genes segregates with the other root ward *Firmicutes* in the tree, and the other copy is relatively divergent and not clearly a member of any group of *recDs*. The fact that *C. acetobutylicum* and *T. tengconge* both have a diverged and undiverged copy of *recD* suggests that an ancient gene duplication occurred in the last bacterial ancestor. The *C. thermocellum* divergent copy clusters relatively close to the other root ward firmicutes, but with low bootstrap support. It may represent a more recent duplication of the *recD* gene, or a less diverged product from the same the ancient gene duplication that gave rise to the diverged copies of *C. acetobutylicum* and *T. tengconge*. The root ward *Firmicutes* do not have *recBC*, but instead have *addAB*.

Conversely, the *Chlamydia* species are *recBC* organisms; they each possess a long and a short *recD*. In the phylogenetic tree (Fig. 1), their long *recDs* are phylogenetically distinct with high bootstrap support from other long *recD* sequences The short *Chlamydia recDs* are also a phylogenetically distinct group, but cluster with the (also short) *recDs* from *Gamma Proteobacter* such as *E. coli*. The internal branching pattern of the long *Chlamydia recDs* is identical to the internal branching pattern of the short *Chlamydia recDs*. Clearly, there was a single gene duplication event in a common ancestor of the *Chlamydia.*

Similar to the *Chlamydia* species, there is another collection of *recBC* organisms, composed mostly of *Chlorobium* but also some *Delta proteobacteri.* Most of this *Chlorobium*-like group of species (*Pelobacter propionicus*, *Pelodictyon phaeoclathratiforme*, *Geobacter bemidjiensis*, *Chlorobium phaeobacteroides*, *Chlorobium limicola*, *Chlorobium tepidum*, and *Chlorobaculum parvum*) have both a short *recD* gene that clusters with *Proteobacter recD's* such as *E. coli*, and a long *recD*. Two of them, *P. propionicus* and *P. phaeoclathratiforme*, also have a third copy of *recD*, which in both cases is a long that clusters close to the other long copy from those genomes. Interestingly, several related species to the *Chlorobium*-like group have *recDs* that cluster with the long *Chlorobium recDs* (*Geobacter lovleyi*, *Prosthecochloris aestuarii*, and *C. phaeobacteroides BS1*). However, these three species do not have short *recD* genes, and also do not have copies of *recB* or *recC*. This correlation is especially striking in the case of *C. phaeobacteroides BS1* (different strain of the other *C. phaeobacteroides*), which does have *recBC* and a short *recD*. Whereas the short *recD* genes from the *Chlorobium*-like group and those from the *Chlamydia* species both group with the *Proteobacter recD's*, the longs of these two species do not show a strong phylogenetic relationship. The internal branching patterns of the long *Chlorobium*-like *recDs* are very similar to those of the short *Chlorobium*-like *recDs*. This again suggests a single event in the common ancestor of the *Chlorobium*-like species leading to duplicate genes. However, the third copy in *P. propionicus* and *P. phaeoclathratiforme* argues that at least one additional duplication event must have occurred in a common ancestor to those two species.

The gene duplication event(s) that happened in a common ancestor of the *Chlamydia* species, the common ancestor of the *Chlorobium*-like species, and the common ancestor of *C. thermocellum*, *C. acetobutylicum*, and *T. tengconge*, may be a separate, or they may all be the same event preserved in different lineages.

Nine other species have duplicate *recD* genes. In six of those (*Mycoplasma mobile*, *Mycoplasma mycoides*, *Acaryochloris marina*, *Methylobacterium extorquens*, *Acidiphilium cryptum*, and *Methylobacterium 4-46*), the duplicate genes cluster with the other gene of the same organism. *A. marina* has three such *recD* genes that all cluster together phylogenetically; two of them are plasmid copies. *Candidatus Desulforudis audaxviator*, *Desulfovibrio desulfuricans*, and *Desulfococcus oleovorans*, each have one long *recD* gene that are weakly related to one another, and at least one other relatively divergent long *recD*, and in the case of *D. oleovorans*, a short copy of the gene that clusters with the *Proteobacter*. The simplest way to account for these nine organisms with two or three *recD* genes is to posit multiple independent gene duplication events in various lineages and points in history. In the cases of *M. mobile*, *M. mycoides*, and *M. extorquens*, these gene duplications seem to have been accompanied by truncations leading to *recD* genes that are dramatically shorter (204, 318, and 192 amino acids, respectively) than those of the long (greater than 710 amino acids) or short (between 450 and 655 amino acids) classifications.

The *recD* of *B. halodurans*, a *B. Firmicute*, as annotated in the reference genome (Takami et al. 2000), initially appears to be an additional example of a truncated *recD* gene. Upon further investigation, it became apparent that a single nucleotide mutation had caused a frame shift that split the *recD* gene into two slightly overlapping reading frames of 391 amino acids each. Both ORFs retained high conservation on the nucleotide and amino acid levels to closely related *recD* genes from *Bacillus anthracis* and *B. subtilis*. Due to this conservation and the single nucleotide nature of the frame shift mutation, *B. halodurans strain* JCM 9153 was acquired from ATCC, and the sequence of the region of the

B. halodurans recD containing the frame shift mutation was amplified and sequenced. The frame shifting single nucleotide insertion was not detected and thus determined to be a sequencing error. Therefore, the full length 744 amino acid sequence of the *B. halodurans recD* was used in the analysis.

Many organisms have a *recD* gene without having either *addAB* or *recBC*. All organisms with *recD* and with out *recBC* or *addAB* have *recDs* of the long type dissimilar from that of *E. coli*. One such organism is *D. radiodurans*. The *recD* of *D. radiodurans* (715 amino acids long) is very divergent from most other *recD* sequences in the tree with its only close neighbor being *Deinococcus geothermalis*. The absence of the *addAB* or *recBC* genes, combined with the extraordinary DNA repair capacity of *D. radiodurans*, suggest that a knock out of the RecD in *D. radiodurans* would be informative of the role of RecD in organisms significantly different from *E. coli.*

Repair of Double-Stranded Breaks from Ionizing Radiation

Given the importance of the *E. coli* RecBCD enzyme in DNA repair (Spies and Kowalczykowski 2005), we asked whether the RecD protein of *D. radiodurans* played a similar role. To directly evaluate whether the *recD* mutant strain was able to repair DSB damage of its genomic DNA in response to γ radiation, wild-type and *recD* deletion cells were exposed to 2,000 Gy of ionizing radiation and then allowed to recover in TGY liquid media. At various time points, aliquots were removed and chromosomal DNA was isolated for analysis by PFGE. Figure 2 clearly shows that the DNA of both wild-type and *recD* null cells were similarly fragmented at this dose of radiation. However, by 90 min post-irradiation, both wild-type and mutant cells had begun to repair their genomes as indicated by the appearance of some reformed chromosomes (shown by *, Fig. 2). After 2½ h of recovery, the chromosomes were repaired effectively in both strains, strongly suggesting that the RecD of *D. radiodurans* is not necessary for the repair of DSBs resulting from ionizing radiation.

Repair of Double-Stranded Breaks from Hydrogen Peroxide

As DSBs are the major form of DNA damage by ionizing radiation, the results from the experiments above raised the possibility that in *D. radiodurans*, RecD, in general, may not be required for their repair. A simple prediction of this idea is that *recD* null cells should also be somewhat tolerant to other agents that primarily cause DSBs. To test this possibility, we first queried the degree to which wild-type and *recD* null cells were able to withstand exposure to hydrogen peroxide, which cleaves DNA via a free radical

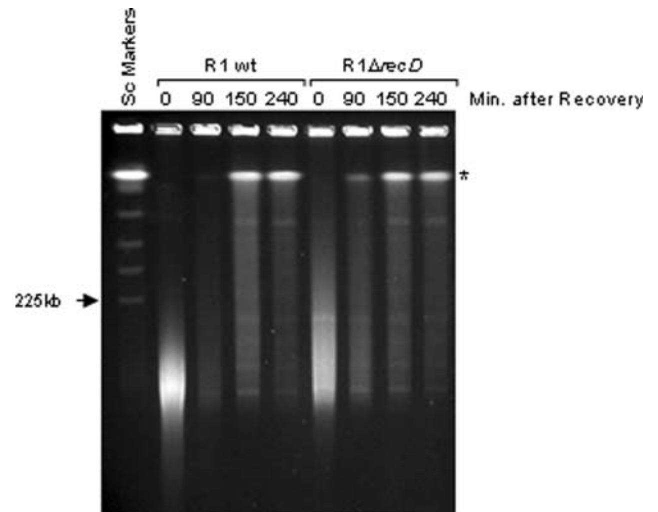

Fig. 2 RecD is not required for DSB repair from ionizing radiation in *D. radiodurans*. Exponentially growing cells were exposed to 2,000 Gy of IR at 4°C and then recovered in TGY liquid media at 30°C. At the indicated times, samples were removed, genomic DNA was isolated in agarose plugs, and analyzed by pulsed-field gel electrophoresis (PFGE). The markers are those derived from *Saccharomyces cerevisiae* genomic DNA. Reformed chromosomes are denoted by an *

mechanism (Imlay and Linn 1988). In these assays, we incubated either wild-type or *recD* deletion cells with various concentrations of hydrogen peroxide for 30 min. The hydrogen peroxide was then removed and the cells were allowed to recover in rich liquid media. Similar to the previous recovery experiment, chromosomal DNA was isolated at appropriate time points and analyzed by PFGE. At all concentrations of hydrogen peroxide tested, chromosomal DNA from both wild-type and *recD* null cells was fragmented (Fig. 3). The degree of fragmentation corresponded to the concentration of hydrogen peroxide; progressively more fragmentation of DNA was observed with higher concentrations of hydrogen peroxide. The mutant cells showed no reduced capacity to repair these hydrogen peroxide induced DSBs. These hydrogen peroxide induced DSBs may be a primary lesion, or they may be a secondary consequence of base excision repair acting on damaged bases, however, the exact mechanism of the creation of the DSBs is irrelevant to the observation that the *recD* null cells repair the damage with kinetics equivalent to the wild-type. This DNA repair capacity exists regardless of the over-all sensitivity of the organism to hydrogen peroxide, which we do not test here.

Lack of Sensitivity of the *recD* Deletion Strain to Hydroxyurea

As another test, we compared the relative sensitivities of either wild-type, *recA* mutant (*rec30*) or *recD* null cells to

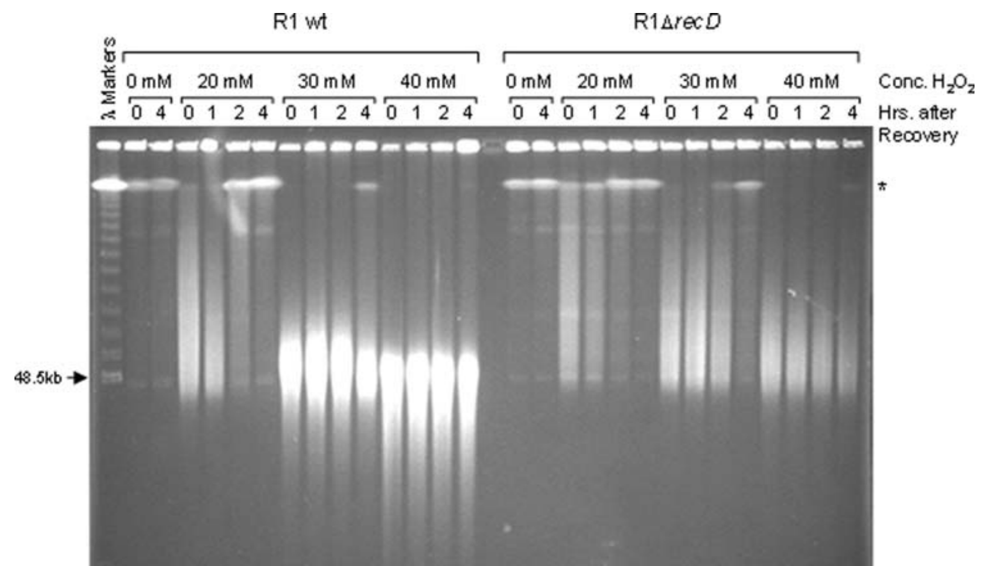

Fig. 3 RecD is not required for DSB repair from hydrogen peroxide in *D. radiodurans*. The *recD* deletion strain is better able to withstand exposure to hydrogen peroxide. Exponential wild-type and *recD* deletion cells were exposed to various concentrations of hydrogen peroxide for 30 min at 30°C and then allowed to recover in TGY liquid media lacking peroxide. At the indicated times, samples were analyzed as described in Fig. 2. The markers are a 50 kb ladder derived from bacteriophage λ DNA. Reformed chromosomes are denoted by an *

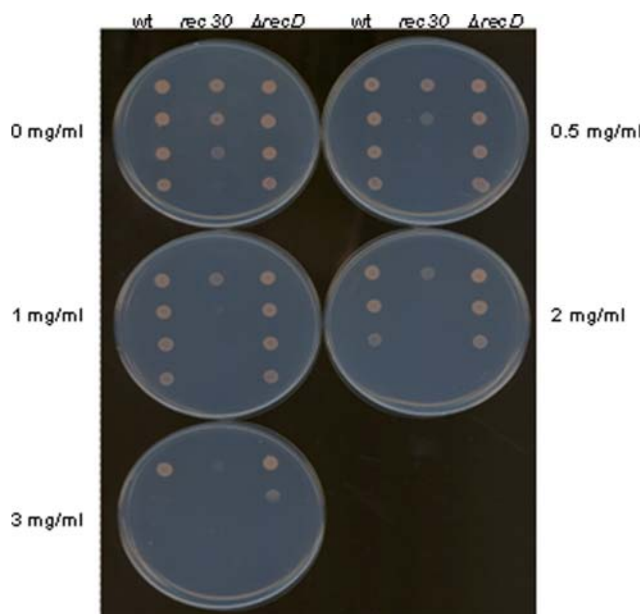

Fig. 4 The *recD* deletion strain is no more susceptible to hydroxyurea than wild-type. Ten-fold serial dilutions of exponential wild-type, *rec30*, and *recD* deletion cells were spot-plated onto TGY agar media containing the indicated amounts of hydroxyurea and then allowed to grow at 30°C for 2–4 days

hydroxyurea, which can produce DSBs by stalling DNA replication complexes. This was done by spot-plating 10-fold serial dilutions of each strain onto TGY agar media containing different amounts of the drug and allowing the cells to grow for several days. Qualitatively, the wild-type strain began to show appreciable sensitivity to 2 mg/ml hydroxyurea and was severely sensitive to 3 mg/ml hydroxyurea. On the other hand, the *rec30* strain demonstrated sensitivity at the lowest concentration of HU tested (0.5 mg/ml) and showed almost no growth on plates containing 3 mg/ml hydroxyurea, reflecting the central role of RecA in DNA repair (Fig. 4). The *recD* null cells did not show any increased sensitivity to hydroxyurea above that of wild-type cells. Together, the results from the ionizing radiation experiment and this experiment suggest that RecD may not be necessary for the repair of DSBs in *D. radiodurans*.

Discussion

The bacterial RecBCD enzyme, a complex of three subunits encoded by the *recB*, *recC*, and *recD* genes, is required for nearly all of the recombination processes that involve a DSB. The enzyme utilizes its multiple activities, that include DNA-dependent ATPase, DNA helicase, ssDNA endo- and exonuclease, and dsDNA exonuclease, to generate ssDNA which is necessary for RecA to catalyze strand exchange for homologous recombination (Spies and Kowalczykowski 2005). Several genetic studies have described phenotypes associated with mutations in the *recD* gene. As far as we can ascertain, with the exception of two organisms [*Neisseria gonorrhoeae* (Chaussee et al. 1999; Mehr and Seifert 1998) and *D. radiodurans* (this article, (Khairnar et al. 2008; Servinsky and Julin 2007; Zhou et al. 2007)], these studies have focused on organisms that belong to the class of gamma proteobacteria, which includes the bacterial model organism *E. coli* (Amundsen et al. 1986; Bidle and Bartlett 1999; Biek and Cohen 1986; Kickstein et al. 2007; Lovett et al. 1988; Miesel and Roth 1994; Regha et al. 2005). Our analysis of these studies, as well as our own, suggests that the *recD* mutant phenotypes may be classified into several groups. Furthermore, our analysis also suggests that RecD may have evolved different functions in various organisms.

The first phenotypic group, represented by the *E. coli* and *Salmonella typhimurium*, is one in which *recD* mutant strains behave similarly to wild-type. Although the ATP-dependent exonuclease activity is abolished in *recD* mutants of both these organisms, they are viable and respond to DNA-damaging agents, such as ultraviolet radiation, as well as wild-type (Amundsen et al. 1986; Biek and Cohen 1986; Lovett et al. 1988; Miesel and Roth 1994). These strains are proficient in recombination; however, the exchanges occur near double-stranded ends independently of Chi sites (Chaudhury and Smith 1984; Lovett et al. 1988; Miesel and Roth 1994; Thaler et al. 1989). As stated earlier, recombination in these strains is dependent on the activities of RecBC and the RecJ nuclease (Lloyd et al. 1988; Lovett et al. 1988). Another organism that belongs to this group is *Acinetobacter baylyi*, in which a *recD* deletion does not compromise viability, efficiency of DNA transformation or response to UV radiation when compared to wild-type (Kickstein et al. 2007). It should be pointed out that this insensitivity to the loss of the RecD activity is in the presence of the RecBC activities. Simultaneous loss of all these activities is associated with a dramatic reduction in the ability to repair DSBs. We do not place *D. radiodurans* in this phenotypic class because it does not have the *recBC* genes.

The second phenotypic group is characterized by a requirement for a function of RecD for viability when an extremophilic organism is exposed to its challenging environment. This property is observed in the deep-sea bacterium *Photobacterium profundum* SS9 and the Antarctic psychrotrophic bacterium *Pseudomonas syringae* Lz4W. In the case of *P. profundum* SS9, the *recD* gene was first identified by its ability to complement the pressure-sensitive phenotype of mutant EC1002 (Bidle and Bartlett 1999). Subsequent experiments demonstrated that mutant EC1002 contains a G-to-A transition at nucleotide position 258 in the *recD* coding sequence resulting in the creation of a stop codon at amino acid position 86. The same study also showed that two other independent *recD* mutations in SS9 severely affect growth at high pressure. The *E. coli recD* is unable to complement the high-pressure growth defect of EC1002. On the other hand, the SS9 *recD* gene allows growth at high pressure when placed into a *recD* mutant strain of *E. coli*, suggesting that SS9 RecD has an activity at high pressure that is not provided by *E. coli recD*. In the Antarctic bacterium *P. syringae* Lz4W, Regha et al. have identified several gene disruption *recD* mutants that do not grow at 4°C, indicating that a functional RecD is essential for growth at that temperature (Regha et al. 2005). These mutants are surprisingly more sensitive to mitomycin C and UV radiation than wild-type. In addition, they do not display a corresponding morphological change in cell size, like wild-type Lz4W, when cells are shifted to growth at 4°C. *P. syringae recD* complements the exonuclease deficiency of *recD* mutants of *E. coli* in plaque size assays; however, *E. coli recD* fails to complement either the cold-sensitive phenotype of a *P. syringae recD* mutant or the increased sensitivity of UV radiation, suggesting that even *P. syringae* Lz4W RecD may possess a different activity than *E. coli* RecD.

Our results as well as others with *recD* mutants in *D. radiodurans* suggest a third phenotype (this article, (Khairnar et al. 2008; Servinsky and Julin 2007; Zhou et al. 2007) and also raise the possibility of a new function for RecD. In contrast to the *E. coli recD* null mutant, which respond similarly to wild-type when exposed to a variety of DNA-damaging agents (Biek and Cohen 1986; Lovett et al. 1988), *D. radiodurans recD* mutant strains show a variety of responses to different DNA-damaging agents (Khairnar et al. 2008; Servinsky and Julin 2007; Zhou et al. 2007). Servinsky and Julin (2007) and Zhou et al. (2007) have shown that *D. radiodurans recD* mutants are more sensitive to UV radiation and hydrogen peroxide than wild-type. Servinsky and Julin have also shown that their *recD* mutant is more sensitive than wild-type to ionizing radiation (Servinsky and Julin 2007). However, two other groups have reported no difference in sensitivity to ionizing radiation between a *recD* mutant strain and wild-type (Khairnar et al. 2008; Zhou et al. 2007). Although, we do not understand the difference in results, we believe the defect in *recD* mutant strains is not related to its ability to repair the resulting DSBs. CHEF Gel analysis revealed that chromosomes broken by exposure to either 2 kGy of ionizing radiation or various concentrations of hydrogen peroxide are repaired with similar kinetics in a *recD* deletion strain we created and a wild-type strain (Figs. 2, 3). However, *D. radiodurans recD* mutants do not respond more sensitively than wild-type to all types of DNA-damaging agents tested. For example, Servinsky and Julin have shown that their *recD* mutant responds similarly to wild-type to exposure to mitomycin C or methyl methanesulfonate (Servinsky and Julin 2007). In addition, our own *recD* deletion responds like wild-type when grown on media that contains hydroxyurea (Fig. 4). In summary, the third *recD* mutant phenotype is characterized by a lack of essential function at an organism's natural environment (different from *P. profundum*, *P. syringae*, and *E. coli*, etc.) and increased sensitivity to selective groups of DNA-damaging agents (different from *E. coli*), leading to the possibility of an unrelated function to that of *E. coli* for the protein.

The *recD* mutants of *N. gonorrhoeae* presented a dilemma as to which phenotypic group they belong. Two groups have reported apparently conflicting results. One, Chaussee et al. (1999), found that an insertional *recD* mutant in strain MS11 is viable, does not affect survival

following UV exposure and reduces pilin phenotypic variation. In contrast, Mehr and Seifert 1998 found that although viable, a disrupted *recD* in human isolate FA 1090 1-81-S2 is more sensitive than wild-type to both UV and ionizing radiation and does not affect pilin antigenic variation. Thus, one study, the former, places *Neisseria gonorrheae* in the first phenotypic group which includes *E. coli* while the latter study places *Neisseria gonorrheae* in the same group as *D. radiodurans*. Regardless, RecD appears to have a spectrum of biological roles in different organisms.

To understand these diverse functions of *recD*, it is necessary to place it within its evolutionary context. Based upon the phylogenetic tree of *recDs*, and the multiple copies of the *recD* gene in certain lineages, correlated against the presence of *recBC* and *addAB*, it is clear that *recD* has undergone several duplications and truncations in various bacterial lineages. These duplications could have occurred by two mechanisms: recombination within a single ancestral cell followed by divergence of the two copies, or acquisition of a divergent copy by lateral transfer from another organism. Due to their high degree of sequence similarity, the three copies of *A. marina recD* seem a likely candidate for the first mechanism of duplication, whereas the second copy of the *D. desulfuricans recD*, which is very divergent from the other two copies from *D. desulfuricans*, appears to be an example of the lateral transfer mechanism of gene duplication.

It is also clear that the short version of *recD* is an adaptation to its participation in the RecBCD complex. Although the absence of *addAB* is a prerequisite for the presence of *recBC*, the presence of *addAB* in an organism does not entirely correlate to the copy number or length of the *recD* in that same organism. It appears that the divergence of the duplicated *recD* into the version associated with the RecBCD complex was gradual with numerous stable intermediates remaining to this day in which the short RecBC adapted version of the gene coexists with one or more long copies.

The simplest explanation for most of multiple-copy *recD* organisms is that a single extremely ancient duplication event occurred in a Firmicute-like ancestor common to all current bacterial species. Different parts of the aftermath of that gene duplication are preserved in the phylogenetic record: *C. acetobutylicum*, *C. thermocellum*, and *T. tengconge* preserve a state similar to what must have been present just after this duplication event, with a long *recD* gene that is similar to other *Clostridium* species, and the other copy diverging. Next, many *Chlorobium*-like species and the *Chlamydia* species have acquired *recBC* and simultaneously specialized one copy of *recD* into a short form, while still retaining a relatively un-diverged long *recD*. Finally, proteobacter, such as *E. coli*, have further specialized by losing their long *recD*, retaining only their short *recBC* adapted copy. This sequence of events, inferred from the phylogenetic tree, (Fig. 1) is in keeping with the theory that following gene duplication, one of the gene copies is free to diverge and acquire new roles (Hughes 1994; Zhang 2003).

The *recD* of *D. radiodurans* is phylogenetically unrelated to most other *recD*s. It is possible that although divergent, the *recD* of *D. radiodurans* has retained the ancestral Firmicute-like activity of long *recD*s such as *Clostridium tetani*. Alternatively, it may have acquired a new function altogether. Regardless, the phylogenetic, and in vivo evidence suggest that the familiar *recD* function, as derived from *E. coli*, is not applicable to *D. radiodurans*. The ancestral *recD* function is associated with the long form of the *recD* gene as seen in species like *C. tetani* and remains largely uninvestigated.

It can be inferred that *M. mobile* and *M. mycoides* have also independently duplicated, and then truncated, their pre-existing copies of *recD* with the loss of *addAB*, most likely a result of the gene loss common in intracellular parasites (Hutchison and Montague 2002). The Mycoplasma, however, did not simultaneously acquire *recBC*, so shortened *recD*s may not be entirely a result of specialization to *recBC*. This is further confirmed by the example of *M. extorquens*, which appears to be another independent example of duplication and truncation, this time in the presence of *addAB*.

It is striking that so many distantly related species contain multiple copies of *recD*. Distantly related species could share the trait of containing multiple *recD*s for two reasons: they have all inherited duplicate copied from a common ancestor, or because second and third copies were acquired separately in different lineages. While the central nodes of the tree are not well supported by bootstrap, except the node differentiating the short *recDs* (*recD1*) from other *recDs* (*recD2* and unclassified), many of the local nodes are well supported. This is significant because it suggests that at least some of these duplications must have been independent events. Even if we assume that only one ancient duplication took place giving rise to the duplicate copies in *C. acetobutylicum*, *C. thermocellum*, and *T. tengconge*, the *Chlorobium*-like species, the *Chlamydia* species, and the short *recD* in *D. oleovorans*, that still leaves 12 duplicate copies of *recD* in various species un-accounted for as well as a third copy in *Chlorobium*-like species *P. propionicus*. In two of those cases, *A. marina* and *Acidiphilium crytum*, the additional gene can be accounted for by the fact that the duplicates are encoded on plasmids. Some of these species with multiple copies of *recD* can be accounted for by positing a relatively recent common ancestor in which the duplication occurred because the duplicate copies are of very similar sequence. Thus, the duplicate genes in the

M. mobile and *M. mycoides* may be from a single event. Likewise, the duplicate copies in *M. extorquens* and *Methylobacterium 4-46*, may be separate descendents of a single gene duplication or lateral transfer from a closely related species. The third copies in *P. phaeoclathratiforme* and *P. propionicus*, may be descendents of the same event, but the third copy in *D. desulfuricans* appears to be quite recent due to the high degree of conservation between the first and third copies. The three copies of *recD* in *D. oleovorans*, are harder to account for due to their relative divergence from on another, but may account for yet another independent event, probably a lateral transfer.

Regardless of what its exact function may be, the long *recD* retains a helicase domain suggesting that even in the absence of *recBC* it is a recombination or repair protein. Although pure *traA* sequences were screened from the data set many of the retained *recD* sequences are annotated as "recD/traA." TraA is a strand and site specific knickase, sometimes referred to as a "relaxase," associated with the replication and transmission of conjugative plasmids. A conjugative mechanism may account for some of the duplications, particularly those on or related to plasmid encoded copies of *recD*. However, it does not account for, the *Mycoplasma* duplicate genes, and the third copies in *D. desulfuricans*, *P. propionicus*, and *D. oleovorans.* Nor can it account for the ancient duplication, which we theorize gave rise to the short *recD1* copes in the *Chlorobium*-like species, the *Chlamydia* species and the divergent copies in *C. acetobutylicum*, *C. thermocellum*, and *T. tengconge*. That makes at least four and perhaps as many as seven separate events leading to duplicate genes in the phylogenetic history of *recD2*. Others have characterized a similar tendency of *recD1* to be subject to duplication or lateral transfer as part of the acquisition or duplication of the *recBCD* gene complex. It has been hypothesized that the *recBCD* complex may influence the lateral transfer of the genes encoding it (Cromie 2009). That *recD2* may also display this characteristic in the absence of *recBC* is interesting.

Due to the intermediates that appear to have been preserved from the ancient gene duplication and adaptation to *recBC*, and because of the many more recent independent duplication events that it has undergone, *recD* is an ideal model for the exploration of gene duplication as a precursor to acquisition of new function. To that end, it would be interesting to compare and contrast various aspects of the biology of both *recD* copies in such organisms as *C. acetobutylicum* and *Clostridium tepidum* with one another as they represent different stages of *recD*'s evolution away from the presumed ancestral state similar to the *recD* as represented in the undiverged copies of *recD* in root ward *Firmicutes* organisms like *C. tetani*. As each of these organisms retain an undiverged copy, the new function and the ancestral function can be compared directly. Just as intermediate states in *recD*'s evolution appear to have been preserved in the biological diversity of bacterial genomes, one would expect the corresponding changes in the folded structure of the protein, its expression and regulation, and is binding partners to also be preserved. The study of *recD*, outside of the proteobacter clade represents an opportunity to understand the acquisition of new function with regards to the larger genomic context as well as gene duplication.

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