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Translesion-synthesis DNA polymerases participate in replication of the telomeres in *Streptomyces*

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ABSTRACT

Linear chromosomes and linear plasmids of *Streptomyces* are capped by terminal proteins that are covalently bound to the 5′-ends of DNA. Replication is initiated from an internal origin, which leaves single-stranded gaps at the 3′-ends. These gaps are patched by terminal protein-primed DNA synthesis. *Streptomyces* contain five DNA polymerases: one DNA polymerase I (Pol I), two DNA polymerases III (Pol III) and two DNA polymerases IV (Pol IV). Of these, one Pol III, DnaE1, is essential for replication, and Pol I is not required for end patching. In this study, we found the two Pol IVs (DinB1 and DinB2) to be involved in end patching. *dinB1* and *dinB2* could not be co-deleted from wild-type strains containing a linear chromosome, but could be co-deleted from mutant strains containing a circular chromosome. The resulting Δ*dinB1* Δ*dinB2* mutants supported replication of circular but not linear plasmids, and exhibited increased ultraviolet sensitivity and ultraviolet-induced mutagenesis. In contrast, the second Pol III, DnaE2, was not required for replication, end patching, or ultraviolet resistance and mutagenesis. All five polymerase genes are relatively syntenous in the *Streptomyces* chromosomes, including a 4-bp overlap between *dnaE2* and *dinB2*. Phylogenetic analysis showed that the *dinB1*-*dinB2* duplication occurred in a common actinobacterial ancestor.

INTRODUCTION

The linear chromosomes and linear plasmids of *Streptomyces* are capped by terminal proteins (TPs) that are covalently bound to the 5′-ends of the DNA. Most TPs identified in *Streptomyces* belong to an archetypal Tpg family with highly conserved amino acid sequences and sizes (~185 aa) (1,2). The Tpg proteins cap a family of highly conserved telomere DNA sequences found in most chromosomes and some linear plasmids of *Streptomyces*. There are atypical telomeres with heterologous sequences, such as those of linear plasmid SCP1 of *Streptomyces coelicolor* (3). So far, only one atypical TP has been identified, i.e. Tpc that caps SCP1 (4). Tpc is distinct from Tpgs in sequence and size (259 aa).

Replication of these linear replicons is initiated from an internal origin and proceeds bidirectionally to the telomeres, which results in a 3′-single-stranded gap at each end. The gaps are presumably filled by DNA synthesis (‘end patching’) using the TPs as protein primers [reviewed in (5,6)]. That the *Streptomyces* TP acts as a primer for DNA synthesis has been supported by *in vitro* deoxynucleotidylation, in which dCTP (the first nucleotide of the conserved telomere sequences) was specifically linked to a Thr residue of Tpg (7). In this system, a crude extract of *Streptomyces* was used as the source of the participating enzymes, and therefore it was not clear which DNA polymerase catalyzed the reaction.

TP-primed DNA synthesis was initially discovered in replication of other TP-capped linear viral replicons, of which adenoviruses [reviewed in (8)] and *Bacillus* phage ϕ29 [reviewed in (9)] are best studied. These systems differ from that of *Streptomyces* in that the TP-primed synthesis initiates replication at a telomere and proceeds to the other end without invoking discontinuous lagging strand synthesis. The DNA polymerases catalyzing the TP-primed DNA synthesis in these systems are of Family B.

No Family-B DNA polymerase is encoded by the *Streptomyces* genome. Instead, five DNA polymerases belonging to three other families are found in the sequenced *Streptomyces* genomes—one Pol I enzyme (encoded by *polA*) of Family A, two Pol III enzymes (encoded by *dnaE*) of Family C, and two Pol IV enzymes (encoded by *dinB*) of Family Y. One or more of these is presumed to catalyze the end patching synthesis. Of these, deletion of *polA* was achieved in *S. coelicolor* strains with linear

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chromosomes (10), indicating either that it is not involved in TP-primed end patching, or that it is, but its function may be substituted by another DNA polymerase(s).

In *S. coelicolor*, *dnaE1* has been previously shown to be essential for chromosome replication (11). The role of *dnaE2* in *Streptomyces* has not been investigated. In Firmicutes, a second Pol III is encoded by *polC*, which catalyzes leading strand synthesis of the chromosome, while DnaE catalyzes lagging strand synthesis (12). It is possible that the two DnaE homologs also divide their responsibility similarly in *Streptomyces*. Alternatively, DnaE2 may be involved in translesion synthesis during DNA repair as in *Mycobacterium tuberculosis* (13). Lastly, DnaE2 may catalyze TP-primed end patching synthesis.

Multiple copies of *dinB* homologs are more common than those of *dnaE* homologs in bacterial genomes. Interestingly, in all available *Streptomyces* sequences, one of the duplicate *dinB* homologs (*dinB2*) is tightly coupled with *dnaE2*, in that the initiation codon (ATG) of *dinB2* (SCO1738 in *S. coelicolor*) overlaps the stop codon (TGA) of *dnaE2* (SCO1739) to from an ATGA overlapping sequence. The other homolog, *dinB1* (SCO1380), stands alone. *dinB*-encoded Pol IV catalyzes error-prone translesion synthesis (TLS) in *Escherichia coli* and several bacteria. However, in *M. tuberculosis*, deletion of two *dinB* homologs individually or in combination had no effect on the susceptibility to compounds that form N^2-dG adducts and alkylating agents, and the rate and the spectrum of spontaneous mutations (14). It was suggested that the DinB homologs in *Mycobacterium* differ in biological functions from their counterparts in other bacteria.

Which one(s) of these DNA polymerases is involved in end patching synthesis? Thus far, only *polA* and *dnaE1* had been studied. In this study, we therefore investigated *dnaE2*, *dinB1* and *dinB2* for possible roles in end patching. We found that *dnaE2* may be deleted without affecting replication of linear chromosomes. *dinB1* and *dinB2* could also be deleted singly, but deletion of both genes was possible only on a circular chromosome but not on a linear chromosome. These results indicate that these Pol IV homologs participate in end patching DNA synthesis. Moreover, *dinB1* and *dinB2*, but not *dnaE2*, were found to be involved in ultraviolet radiation resistance and mutagenesis. Phylogenetic studies indicated that the *dinB1*-*dinB2* duplication and evolution occurred only in actinobacteria, while independent duplications occurred sporadically in various other bacterial clades. In contrast, the duplication of *dnaE* appeared to have occurred in an earlier bacterial ancestor, leading to widespread *dnaE* homologs.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1.

Microbiological and genetic manipulations

Genetic manipulations of *E. coli* and *Streptomyces* were performed according to the methods of Kieser *et al.* (18).

Gene disruption

The PCR-targeting system of Gust *et al.* (16) was used for gene disruption in *Streptomyces*. The gene disruption cassette was generated by PCR using a pair of primers containing sequences flanking the target gene on a template containing *oriT* and a resistance marker. For disruption of *dnaE2* and *dinB2*, the template was pIJ773 [containing apramycin resistance gene *aac(3)IV*]. For disruption of *dinB1*, the template was pIJ778 (containing spectinomycin resistance gene *aadA*). The PCR product was used to transform *E. coli* BW25113/pIJ790 harboring a plasmid or cosmid of *S. coelicolor* containing a kanamycin resistance (*aph*) gene and the target gene to replace the latter by the gene cassette. The resulting vectors were used for targeted gene replacement in *S. coelicolor via* conjugation from *E. coli* ET12567/pUZ8002. Transformants resistant to kanamycin, spectinomycin, or apramycin were selected initially. From spores of these transformants, kanamycin-sensitive segregants were scored for possible candidates, in which the wild type alleles had been removed by a second crossover.

UV sensitivity and mutagenesis

For UV sensitivity tests, diluted spore suspensions were spread on R2YE medium, irradiated at various dosages with a UV Stratalinker 1800 (Stratagene), and incubated at 30°C for 4 days in the dark to minimize photoreactivation repair. For the mutagenesis test, the UV irradiated plates were incubated at 30°C for 24 h to allow mutation fixation, and overlaid with 11 µg/ml rifampicin to score rifampicin-resistant mutants (13,19).

Reverse transcription polymerase chain reaction (RT–PCR) assay for gene expression

RT-PCR assay was performed on *Streptomyces* cultures treated with ultraviolet (UV) irradiation or methyl methanesulfonate (MMS). For UV irradiation, *S. coelicolor* M145 was grown on a cellophane membrane laid on R2YE agar for 2 to 3 days. The plates were irradiated with a UV Stratalinker 1800 (Stratagene, 200 J/m^2), and the mycelial mass was collected, and dispersed in STE buffer (0.1 M NaCl, 10 mM Tris–HCl, pH8.0 and 1 mM EDTA). For MMS treatment, *S. coelicolor* M145 was cultivated in liquid YEME medium containing 0.5% glycine to log phase, and MMS was added to a final concentration of 25 µg/ml. After different lengths of time, the mycelium was harvested by centrifugation, and resuspended in STE. The collected mycelium was treated with lysozyme (1 mg/ml) at 37°C for 10 min, and RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The reverse transcription reaction was carried out by using QuantiTect Rev. Transcription Kit (Qiagen) according to the manufacturer's instructions. A 5-µl aliquot of the RT reaction product was used as a

Table 1. Bacterial strains and plasmids used in this study

Strains/plasmids	Description	Source/reference
S. coelicolor M145	SCP1⁻ SCP2⁻	(15)
E2ko	Δ*dnaE2*::*aac(3)IV* mutant of M145	This study
HH9	Δ*dinB2*::*aac(3)IV* mutant of M145	This study
HH10	Δ*dinB1*:: *aadA* mutant of M145	This study
HH11	Sp^r Km^r exoconjugant isolated from HH9 that has received pLUS898	This study
HH12	Chloramphenicol-sensitive derivative of HH11 containing a circular chromosome	This study
HH13	Δ*dinB2*::*aac(3)IV* and Δ*dinB1*:: *aadA* segregant of HH12	This study
S. lividans TK64hyg	Spontaneous hygromycin resistant mutant of TK64, *pro-2 str-6*	This study
E. coli BW25113/pIJ790	K12 derivative; Δ*araBAD* Δ*rhaBAD*/ΔRED (*gam bet exo*) *cat araC rep101*(Ts)	(16)
E. coli ET12567/pUZ8002	*dam-13*::Tn*9 dcm cat tet hsdM hsdR zjj-201*::Tn*10*/*tra neo* RP4	(16)
pIJ773	*aac(3)IV* oriT	(16)
pIJ778	*aadA* oriT	(16)
StI11	*S. coelicolor* cosmid containing dnaE2 and *dinB2*	(17)
pLUS897	pCRII-TOPO plasmid containing SCO1379-SCO1382	This study
pLUS898	pLUS897 derivative in which *dinB1* is replaced by the *aadA* gene cassette	Figure 2
pLUS899	Plasmid containing the ARS of pSLA2, *tsr*, *tap-tpg* and a pair of *S. lividans* telomeres	Figure 3
pLUS899L	Linear version of pLUS899	Figure 3
pLUS899dinB1	pLUS899 derivative containing *dinB1*	Figure 3
pLUS899dinB1L	Linear version of pLUS899dinB1	Figure 3
pLUS899dinB2	pLUS899 derivative containing *dinB2*	Figure 3
pLUS899dinB2L	Linear version of pLUS899dinB2	Figure 3

template and amplified with FastStart Taq DNA polymerase (Roche). The program used for the PCR consisted of 2 min of initial denaturation at 95°C, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1 min/kb. The final extension step was done at 72°C for 7 min. The oligonucleotide primers are listed in Supplementary Table S1.

Phylogenetic analysis

Sixty-eight bacterial strains were used to assess the phylogeny of DinB and DnaE homologs in them. These sequences were retrieved from KEGG Orthology (KO) database (http://www.genome.jp/kegg/ko.html). The homologous sequences were aligned using MAFFT (PMID: 18372315, PMID: 16362903, PMID: 15661851), and the aligned sequences were used to reconstruct the phylogenetic trees in a maximum-likelihood manner with PhyML (PMID: 14530136). To acquire accurate and reliable phylogeny, equilibrium frequencies and proportion of invariable sites were optimized and estimated in the substitution model. In addition, the tree topology was searched using SPR moves (PMID: 16234323). For evaluation of the branching significance, the aLRT statistical test was applied to compute the branch supports (PMID: 16785212).

K_a/K_s analysis

The coding sequences of *dinB* and *dnaE* were retrieved from KEGG Orthology (KO) database (http://www.genome.jp/kegg/ko.html), and aligned by the codon-based alignment using MAFFT and RevTrans (PMID: 18372315, PMID: 16362903, PMID: 15661851, PMID: 12824361). The Li93 method (PMID: 8433381) was then exploited to calculate the K_a/K_s values. For sliding K_a/K_s calculations, the windows size was 90 bases and the step was 15 bases.

RESULTS AND DISCUSSION

DnaE2 is not essential and is not involved in end patching

To examine the role of DnaE2 in *Streptomyces,* the REDIRECT procedure of Gust *et al.* (16) was used to delete *dnaE2* (SCO1739) and replace it with the *aac(3)IV* (apramycin resistance, Am^r) gene cassette on the chromosome of *S. coelicolor* M145. Δ*dnaE2* mutants were readily isolated, in which the deletion was confirmed by Southern hybridization (Figure 1a). The mutants exhibited no detectable difference in growth or morphology. The result indicated that DnaE2 was not essential for chromosome replication in *S. coelicolor*. A representative designated E2ko was chosen for further studies.

To check the possibility that DnaE2 might be involved in end patching, and that the chromosomes of the Δ*dnaE2* mutants might be circularized, genomic DNA of E2ko was subjected to restriction and Southern hybridization using the telomere DNA as the probe ('end probe'). The results showed the presence of intact telomere sequences (Figure 1c), indicating that DnaE2 was not necessary for end patching.

dinB1 and *dinB2* can be individually knocked out in *S. coelicolor*

To test the roles of DinB1 and DinB2 in *Streptomyces*, attempt was made to delete *dinB1* (SCO1380) and *dinB2* (SCO1738) individually from *S. coelicolor* M145. For deletion of *dinB2, dinB2* on cosmid StI11 (containing *aph* gene conferring kanamycin resistance, Km^r; http://streptomyces.org.uk/) was replaced by the *aac(3)IV* gene cassette in *E. coli*, and the cosmid was conjugally transferred to *S. coelicolor* M145. From the resulting Am^r transconjugants, kanamycin-sensitive (Km^s) segregants (putative double-crossover products) were readily isolated after one sporulation cycle at a frequency of

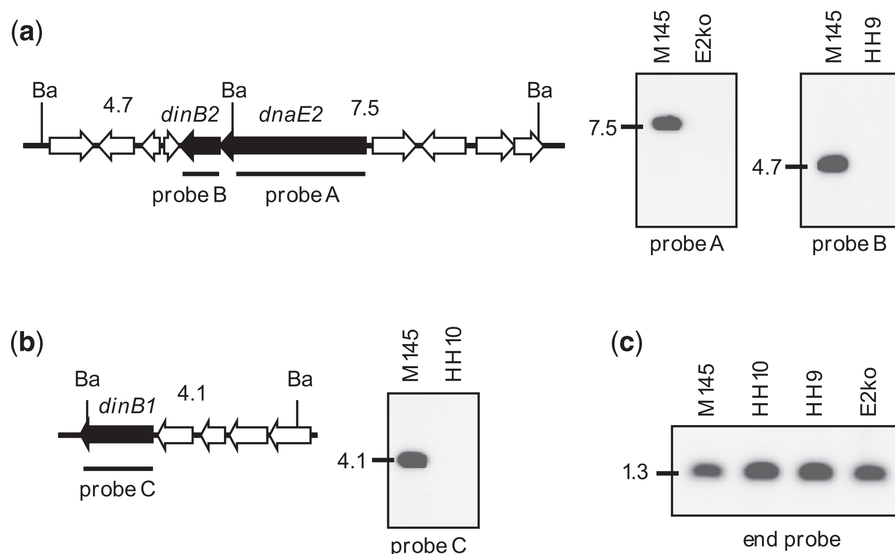

Figure 1. Creation of Δ*dnaE2*, Δ*dinB1* and Δ*dinB2* mutations in *S. coelicolor*. (**a**) *Left*. Physical map of *dnaE2-dinB2* (filled arrows) showing the surrounding ORFs (open arrows), the *Bam*HI sites (with the distance in between in kb) on the *S. coelicolor* chromosome, and the two probes used in hybridization. *Right*. Hybridization analysis of the Δ*dnaE2* (E2ko) and Δ*dinB2* (HH9) mutants. Genomic DNA was digested with *Bam*HI and subjected to Southern hybridization after agarose gel electrophoresis using probes A and B. (**b**) *Left*. Physical map of *dinB1* (filled arrows) showing the surrounding ORFs (open arrows), the *Bam*HI sites (with the distance in between in kb), and probe C used in hybridization. *Right panel*. Hybridization analysis of the Δ*dinB1* (HH10) mutant. Genomic DNA was digested with *Bam*HI and subjected to Southern hybridization using probe C. (**c**) Genomic DNA of E2ko, HH9 and H10 was digested with *Bam*HI and subjected to Southern hybridization using the 1.3-kb 'end probe' (1.3-kb *Bam*HI terminal fragment).

10%. The deletion of *dinB2* in the Am^r Km^s segregants was confirmed by Southern hybridization (Figure 1a). A representative of the Δ*dinB2* mutants, HH9, was chosen for further studies.

For deletion of *dinB1*, *E. coli* plasmid pLUS898 containing *aph* and SCO1379-SCO1382, in which *dinB1* (SCO1380) had been replaced by an *aadA* gene cassette (conferring spectinomycin resistance, Sp^r; Figure 2a) was conjugally transferred to M145 by conjugation. Sp^r exoconjugants were isolated, from which Km^s segregants were readily isolated at a 5% frequency. The deletion of *dinB1* in these segregants was confirmed by Southern hybridization (Figure 1b). A representative of the Δ*dinB1* mutants, HH10, was chosen for further studies.

The chromosomes in HH9 and HH10 remained linear, as evident by the presence of intact telomeres in these strains (Figure 1c). These results indicated that *dinB1* and *dinB2* were not essential for end patching. However, there was a possibility that the two genes might complement each other in end patching function. To test this, attempts were made to delete both genes together.

dinB1 and *dinB2* can be deleted together only on circular chromosomes

To create such a double mutant, we attempted to delete *dinB1* in HH9 (Δ*dinB2*) using pLUS898 (Figure 2a). Sp^r Km^r exoconjugants (putative single-crossover products) were readily isolated. However, no Sp^r Km^s segregants (putative double-crossover products) could be isolated after screening about 10^4 colony-forming units from spores of the Sp^r Km^r exoconjugants. The failure to delete *dinB1* in HH9, compared to the relative ease of deleting it in M145, suggested that *dinB1* and *dinB2* could not be deleted together on the linear *S. coelicolor* chromosome.

The substrate mycelium of *Streptomyces* contains multiple nucleoids, and spores are haploid. It is possible that double crossovers had occurred in some chromosomes in the mycelium, but spores harboring these chromosomes with *dinB1 dinB2* double deletions are non-viable. To investigate this possibility, DNA was isolated from the mycelium of the Sp^r Km^r HH9 exoconjugants growing in the absence of Km, and examined by *Bam*HI digestion and Southern hybridization using the DNA spanning SCO1379-SCO1382 (probe D) as the probe (Figure 2b). The results revealed not only the 6.1-, 4.1- and 2.0-kb hybridizing *Bam*HI fragments that were expected from single crossovers, but also the 5.0- and 2.8-kb hybridizing fragments that were expected from double crossovers (exemplified by a representative culture, HH11, in Figure 2c, top). In addition, the chromosomes remained linear, as evident from the presence of intact telomere DNA (Figure 2c, bottom). The results indicated that although Δ*dinB1* Δ*dinB2* mutants could not be isolated, double crossing over did occur on some of the chromosomes in the substrate mycelium.

We interpreted these results to indicate that DinB1 and DinB2 are required for end patching, and that these two polymerases overlap in the end patching function and may complement each other. Under these premises, both the Δ*dinB1* Δ*dinB2* (double-crossover) and Δ*dinB2* (single-crossover) chromosomes could employ DinB1 produced by the latter in the same hypha for end patching, but, in haploid spores, end patching was possible only for the

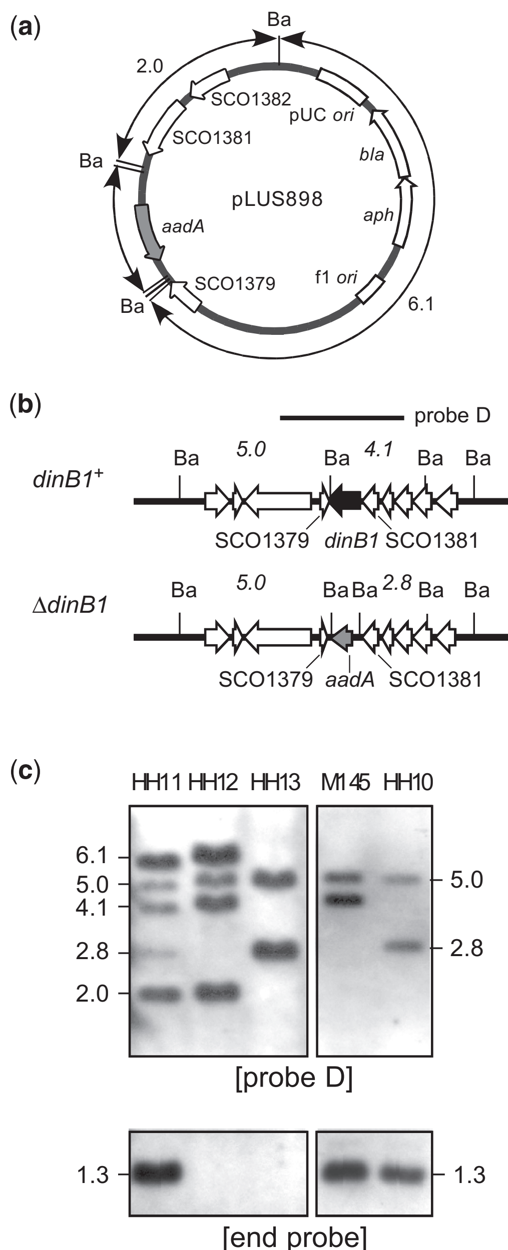

Figure 2. Creation of Δ*dinB1*Δ*dinB2* double mutation in *S. coelicolor*. (**a**) Physical map of pLUS898, containing the Δ*dinB1*::*aadA* alleles. The open arrows with their gene designations depict ORFs neighboring *dinB1* from the *S. coelicolor* chromosome, and *bla* (beta-lactamase), *aph*, and two replication origins (*ori*) on the pCRII TOPO vector. (**b**) Physical maps of the *dinB1*$^+$ and Δ*dinB1*::*aadA* alleles, the *Bam*HI (Ba) sites (and the distances in between), and the hybridization probe D. (**c**) Hybridization analysis of Sp^r exconjugants. Genomic DNA was digested with *Bam*HI and subjected to Southern hybridization (top: probe D; bottom, end probe) after agarose gel electrophoresis. HH11 is a representative Sp^r Km^r exconjugant containing pLUS898 DNA inserted into the chromosome through a single crossover. Genomic DNA of HH11 used here was isolated from a liquid culture growing in the absence of kanamycin to allow loss of pLUS898 through a second crossover. HH12 is a chloramphenicol-sensitive mutant isolated from HH11, containing a circular chromosome (no hybridization to end probe). HH13 is a representative Sp^r Km^s segregant isolated from HH12. HH10 is a Δ*dinB1* mutant. (See the text for analysis and interpretation of the results.).

Δ*dinB2* chromosomes, but not for the Δ*dinB1* Δ*dinB2* chromosomes.

This model predicted that deletion of double deletions of *dinB1*and *dinB2* were possible on a circularized chromosome. To test this, a chloramphenicol-sensitive mutant, HH12, containing a circularized chromosome (lacking the chromosomal telomeres; Figure 2c, bottom) was isolated from HH11. As expected from the model, Sp^r Km^s mutants were isolated readily (at frequencies of ~5%) from the spores of HH12. Restriction and hybridization analysis of the genomic DNA of the Sp^r Km^s mutants (exemplified by HH13) revealed only the 5.0- and 2.8-kb *Bam*HI fragments as expected from double crossovers (Figure 2c, top). Thus, double deletion of *dinB1* and *dinB2* was readily achieved on a circular chromosome.

Replication of linear plasmids requires *dinB1* or *dinB2*

Our model also predicted that (at least some) linear plasmids containing archetypal telomeres (such as those of the *S. coelicolor* and *S. lividans* chromosomes) could not replicate in a Δ*dinB1* Δ*dinB2* mutant. To test this, the procedure of Qin and Cohen (20) was employed to construct linear plasmids in *Streptomyces*. Plasmid pLUS899 (Figure 3a left) containing the *tap*-*tpg* operon, an autonomously replicating sequence (ARS) of pSLA2, and *tsr* flanked by a pair of *S. lividans* chromosomal telomeres was constructed in *E. coli*. *Ase*I-linearized pLUS899 DNA was used to transform HH12. A linear plasmid (designated pLUS899L) was generated in all the 12 thiostrepton-resistant (Thio^r) transformants examined. The linearity of pLUS899 was confirmed by the presence of the expected 5.1- and 7.6-kb *Sac*I fragments (Figure 3a right).

Transformation of HH13 by *Ase*I-cleaved pLUS899 DNA also produced Thio^r transformants, but the transformation frequency was about two orders of magnitudes lower than that of HH12. In all the (11) transformants examined, the plasmid DNA was digested by *Sac*I into a single DNA molecule, indicating that these plasmids were circularized products. No linear plasmid was detected (Figure 3a, right).

To complement for the deficiency, *dinB1* and *dinB2* were individually inserted into pLUS899 to generate pLUS899dinB1 and pLUS899dinB2, respectively (Figure 3b and c, left). These plasmids were linearized by *Ase*I and introduced into HH12 and HH13. In both cases, linear plasmids (pLUS899dinB1L and pLUS899dinB2L, respectively) were detected (Figure 3b and c, right). These results supported the notion that replication of linear plasmids, like that of linear chromosomes, requires either DinB1 or DinB2.

dnaE2, dinB1 and *dinB2* are not involved in conjugal transfer

During conjugation, the circular plasmids in *Streptomyces* are presumably transferred in double-stranded form (21) through the TraB ring (22,23), unlike the classical rolling circle replication model of transfer of single-stranded DNA in most other bacteria. To test whether *dnaE2*, *dinB1*, or *dinB2* may be involved in replication of

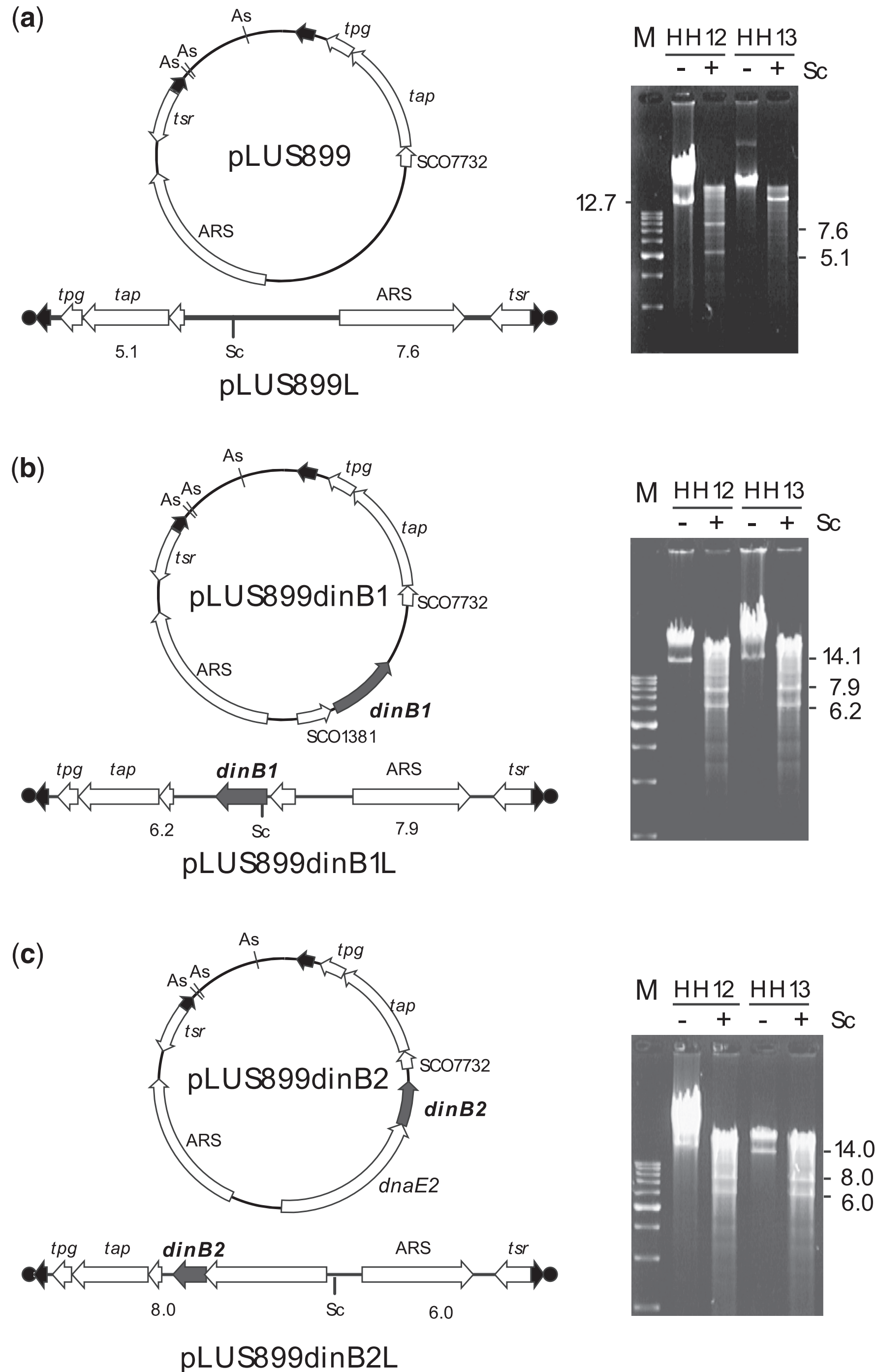

Figure 3. *dinB1* or *dinB2* is required for linear plasmid replication. (**a**) pLUS899. *Left panels*. pLUS899 contains an ARS of the pSLA2 linear plasmid, *tsr* (thiostrepton resistant gene), *tap-tpg* and a pair of the 365-bp telomere sequences of the *S. lividans* chromosome (filled arrows). The *Ase*I (As)-containing sequence between the telomere sequences is the *E. coli* vector pMTL23. The expected linear derivative of pLUS899, designated pLUS899L, is shown below. The unique *Sac*I site and the sizes of the *Sac*I fragments are shown. *Right panel*. *Ase*I-linearized pLUS899 DNA was used to transform HH12 and HH13. HH13 was transformed at an efficiency about one hundred fold lower than HH12. Thiostrepton-resistant transformants were isolated and their genomic DNA was fractionated by agarose gel electrophoresis with (−) or without (+) *Sac*I (Sc) digestion. In all HH12 transformants, linear pLUS899L DNA was evident by the digestion of the 12.7-kb DNA into 7.6- and 5.1-kb fragments. In all HH13 transformants, the plasmid DNA appeared to be circular as evident by the production of a single *Sac*I fragment of ~13 kb. (**b**) pLUS899dinB1. (Left panel) pLUS899dinB1 is a derivative of pLUS899 containing a copy of *dinB1* and its upstream ORF (SCO1381). The expected linear derivative, designated pLUS899dinB1L, is shown with the unique *Sac*I site. (Right panel) *Ase*I-linearized pLUS899dinB1 DNA transformed HH12 and HH13 at about the same efficiency. The transformants of both strains harbored linear DNA (pLUS899dinB1L), as evident by the cleavage of the uncut plasmid DNA into the expected 7.9- and 6.2-kb *Sac*I fragments. pLUS899dinB1. (**C**) (Left panel) pLUS899dinB2 is a derivative of pLUS899 containing a copy of *dinB2* and the upstream overlapping *dnaE2*. The expected linear derivative, designated pLUS899dinB2L, is shown with the unique *Sac*I site. (Right panel) *Ase*I-linearized pLUS899dinB2 DNA transformed HH12 and HH13 at about the same efficiency. The transformants of both strains harbored linear DNA (pLUS899dinB2L), as evident by the cleavage of the uncut plasmid DNA into the expected 8.0- and 6.0-kb *Sac*I fragments.

circular plasmids during conjugal transfer, circular plasmid pIJ303 was introduced into M145, E2ko HH9, HH10, HH11, HH12 and HH13 by conjugal transfer, and tested for conjugal transfer of the plasmid to TK64hyg (a spontaneous hygromycin resistant mutant isolated in this study). No significant difference in the plasmid transfer frequencies were observed among these matings (data not shown), indicating that transfer of these the circular plasmid did not depend on *dnaE2*, *dinB1* or *dinB2*.

Similarly, SLP2tsr, a SLP2 derivative containing an insert of *tsr* (24), was introduced to M145, E2ko HH9, HH10, HH11 and HH12, and tested for transfer to TK64hyg. Again, the mutations in the DNA polymerase genes did not cause defects in plasmid transfers (data not shown). In *E. coli*, replication of circular plasmids during conjugal transfer is catalyzed by Pol III (25). In *Streptomyces*, this role is probably also mainly played by the Pol III composed of DnaE1.

dinB1 and *dinB2* are involved in translesion repair

None of the Δ*dinB1* (HH10), Δ*dinB2* (HH9) and Δ*dinB1* Δ*dinB2* (HH13) mutants exhibit any detectable anomaly in morphology or growth characteristics. In other bacteria, *dinB*-encoded DNA polymerase IV is involved in translesion repair of DNA damage, and *dinB* mutants exhibit higher sensitivity to UV and increased UV-induced mutagenesis [reviewed in (26,27)]. Are *dinB1* and *dinB2* also involved in these processes in *Streptomyces*? Compared with the wild-type parent M145, the Δ*dinB1* and Δ*dinB2* single mutants did not exhibit increased sensitivity to UV (Figure 4a). However, the Δ*dinB1* Δ*dinB2* double mutations caused a slight increase in UV sensitivity. These results indicated that *dinB1* and *dinB2* also assume complementary roles in repair of UV damage. They apparently play only a relatively minor role in the repair due to the presence of multiple other repair systems, such as excision repair, photoreactivation and recombinational repair, in *Streptomyces*.

Mutation to rifampicin resistance was used to test UV-induced mutagenesis in these mutants (Figure 4b), and the results showed that the mutagenesis rates in HH10 (Δ*dinB1*) and HH9 (Δ*dinB2*) were reduced to 50%, compared to M145. The mutation rate in HH13 (Δd*inB1* Δ*dinB2*) was further reduced to about 10% of that in M145. It is noteworthy that HH13 differs from M145 not only in the *dinB1* and *dinB2* mutations but also in possessing a circular chromosome with large deletions. These results indicated that both DinB1 and DinB2 polymerases produced about the same extent of errors during TLS repair of UV damage.

E2ko exhibited no differences in UV damage repair and UV-induced mutagenesis as M145 (data not shown), indicating that DnaE2 was not involved in these processes.

It was possible that DNA repair in spores and mycelia involved different DNA polymerases and different mechanisms in *Streptomyces*. These repair and mutation studies were performed using UV-irradiated spores, and any UV damages on the chromosomes that required translesion repair by DinB1 and/or DinB2 polymerases must be repaired at the germination stage for the cultures to survive. Therefore, the observed effects of Δ*dinB1* and Δ*dinB2* mutations on UV damage repair (Figure 4a) reflected the involvement of these polymerases at the germination stage. This was also true for UV-induced mutagenesis, which accompanied the translesion repair. The involvement of these polymerases in DNA repair during mycelial growth remained to be investigated.

The *M. tuberculosis* genome also carries two *dinB* homologs, *dinB1* (*dinX*) and *dinB2* (*dinP*). Recently, Kana *et al.* (14) discovered that deletion of them singly or in combination did not appear to cause increases in sensitivity to DNA damaging agents or mutation frequencies. The authors suggest that the DinB homologs

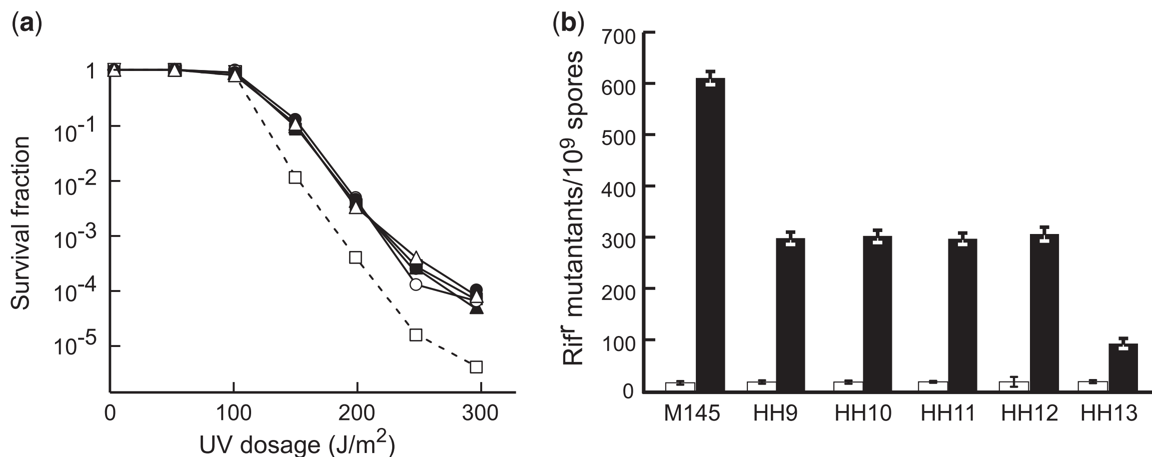

Figure 4. UV sensitivity and UV-induced mutagenesis of the DNA polymerase mutants. (**a**) Sensitivity of the polymerase mutants to UV. Spores of the mutant cultures were irradiated with ultraviolet radiation to various dosages, and the survivals were scored by plating and incubating on R2YE agar for 4 days. Filled circles, M145; open circles, HH9; filled triangles, HH10; open triangles, HH11; filled squares, HH12; open squares, HH13. The results shown are the means of five independent experiments. The standard deviations were all <2.6% of the means, and therefore not shown. (**b**) Frequency of UV-induced rifampicin-resistant mutations in the mutants. Spores of the mutant cultures were irradiated with 200 J/m^2 of UV, and the numbers of rifampicin resistant mutants were scored (filled bars) and compared with unirradiated spores (open bars). The results shown are the means of five independent experiments, and the error bars indicate the standard deviations.

in *Mycobacterium* differ significantly in biological functions from their homologs in other bacteria. It is likely that one or both of these DinB homologs also take part in end patching for replication of TP-capped linear plasmids found in some *Mycobacterium* species, such as *M. xenopi, M. branderi* and *M. celatum* (28,29).

dnaE2 and *dinB2* are induced by UV irradiation and DNA alkylation

The involvement of *dinB1* and *dinB2* in resistance to UV and UV-induced mutagenesis suggested that they are inducible by the SOS response. In *E. coli*, the single *dinB* gene is induced by the SOS response (30). A LexA-binding SOS box is present in the promoter of *dinB* in *E. coli*. A Gram-positive bacterial SOS box, GAACN$_4$RTTY, (31–33) is present in the promoter region of *dnaE2* in *S. coelicolor, S. avermitilis, S. scabiei and S. griseus* (with one mismatch). This further suggests that the putative *dnaE2-dinB2* operon is under SOS response.

To test this notion, M145 was irradiated with UV and the expression of *dinB1*, *dinB2* and *dnaE2* was determined by RT–PCR. Interestingly, the results (Figure 5) show that the expression of *dinB2* and *dnaE2* occurred at a relatively low level, and was increased by ~6-fold in 60 min after UV irradiation. In contrast, *dinB1* was constitutively expressed and insensitive to UV irradiation. *recA*, which is known to be under SOS regulation, was induced to about the same extent as *dinB2* and *dnaE2* in M145. Treatment of M145 cultures with MMS gave similar results with about the same levels of induction for *dinB2*, *dnaE2* and

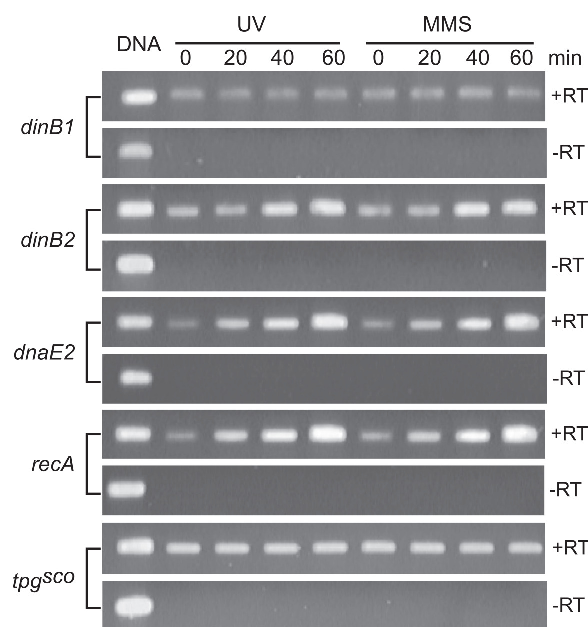

Figure 5. Expression of *dnaE2*, *dinB1* and *dinB2*. M145 cultures were irradiated with UV or treated with MMS as described in 'Materials and Methods' section. RNA was isolated from the cultures and 0.5 µg of RNA each was used for RT-PCR ('+RT') using primers specific for *dnaE2*, *dinB1* and *dinB2* (Supplementary Table S1). The time (0, 20, 40 and 60 min) after UV or MMS treatment is indicated. Controls without the reverse transcription step ('-RT') are included. 'DNA', control PCR with genomic DNA as template.

recA, and none for *dinB1*. In comparison, the expression of the TP gene *tpg* appeared to be constitutive and not induced by UV or MMS treatment.

Constitutive expression of the two Pol IV enzymes during active growth of *S. coelicolor* is expected for their roles in replication of the chromosomes. However, the lack of SOS response by *dinB1* is surprising considering its involvement in repair of UV damage and UV-induced mutagenesis. The identical constitutive expression and SOS response patterns of *dnaE2* and *dinB2* are consistent with the notion that these two genes form an operon under the same transcriptional regulation.

TLS DNA polymerases are biochemically suitable for end patching

TP-primed replication of the linear genomes of *Bacillus* phage ϕ29 and adenoviruses is catalyzed by a viral-encoded DNA polymerase that belongs to the same Family B as Pol II. In bacteria, both Pol II and Pol IV are TLS DNA polymerases involved in DNA repair and induced by the SOS response (34,35). Perhaps it makes biochemical sense that TLS DNA polymerases were adopted to catalyze TP-primed DNA synthesis during evolution. The use of TP as primer to carry out patching synthesis at the terminal 3′-overhangs is not unlike the switch of Pol III to a TLS polymerase in bypassing a bulky adduct in that proper Watson-Crick base pairing between the template and the incoming nucleotides is not present, and TLS polymerases have been shown to possess a more spacious active site that could accommodate bulky adducts or non-Watson-Crick base pairing (36,37).

Then why do ϕ29 and adenoviruses adopt Pol II, while *Streptomyces* adopts Pol IV for TP-primed synthesis? There are two possible considerations. First, Pol II possesses a 3′–5′ proofreading function that Pol IV lacks, and is therefore less error-prone than Pol IV. Second, Pol II has a higher processivity than Pol IV. These two features of Pol II would be advantageous for achieving high fidelity in replication of whole viral genomes. In contrast, patching of the relatively short (~300-nt) of *Streptomyces* telomeres probably does not demand high fidelity. Interestingly, there is no Pol II homolog in *Streptomyces*.

Since a single deletion of either *dinB1* or *dinB2* did not prevent replication of the linear replicons, it is apparent that one of them is sufficient for end patching. It is not clear whether both of them participate in end patching *in vivo*, or one plays the main role, while the other represents a backup. In the latter case, which one is the major player?

The DNA polymerase genes are relatively conserved in synteny in *Streptomyces*

A total of five DNA polymerase homologs (*dnaE1, dnaE2*, *polA*, *dinB1* and *dinB2*) are identified in nine sequenced *Streptomyces* chromosomes (*S. coelicolor*, *S. lividans*, *S. avermitilis*, *S. scabiei*, *S. griseus*, *S. bingchenggensis*, *S. cattleya, S. flavogriseus* and *S. venezuelae*). Interestingly, they are all located on the 'left arm' of the

chromosome (based on the reference species *S. coelicolor*) (Figure 6), and oriented in the same direction as replication. All of them exhibit a conserved synteny on these chromosomes except for the *dnaE2*-*dinB2* pair in *S. griseus*, which is translocated to the leftmost position. The relatively terminal locations of *dinB1* and *dinB2* in all nine species are consistent with hypothesis that genes involved in end patching would be near the termini as remnants of a linear plasmid that recombined with an ancestral circular chromosome in an event that linearized the chromosome during evolution (38). The chromosome of the milbemycin-producing *S. bingchenggensis* possesses a third *dnaE* homolog (39), designated *dinE1b*, which, unlike the other DNA polymerase genes, lies close to the right end of the chromosome (Figure 6).

dinB2 emerged by duplication in actinobacteria and fused with *dnaE2* in *Streptomyces*

Duplication of *dinB* is seen in all the sequenced chromosomes of *Streptomyces* species as well as many other clades of actinobacteria. Phylogenetic analysis of the DinB homologs from five *Streptomyces* and 63 other bacterial species (Figure 7) clearly separated the DinB1 and DinB2 homologs of *Streptomyces* into two distinct branches with similar topology, which consists exclusively of actinobacteria. The DinB homologs in other bacterial classes are distributed outside of these two branches. This indicates that the duplication that produced *dinB1* and *dinB2* occurred in an ancestral actinobacterium.

Of the other actinobacteria, some (*Kytococcus sedentarius*, *Propionibacterium freudenreichii*, *Nocardia farcinica* and *Rhodococcus* rha1) possess both DinB1 and DinB2 homologs. Two of these species (*Nocardia farcinica* and *Rhodococcus* rha1) and *M. tuberculosis* possess duplicate DinB1 homologs (designated DinB1a and DinB1b). None possesses duplicate DinB2 homologs. Many actinobacteria possess only one DinB homolog, either of the DinB1 family or the DinB2 family. *Mesorhizobium* bnc1 is unique in possessing DinB2 plus a DinB homolog that is clustered intriguingly in a sub-branch with gram-positive *Bacillus melitensis* and gram-negative *Caulobacter crescentus*. These results indicate that while all the *Streptomyces* species have maintained both *dinB1* and *dinB2*, some actinobacteria appear to have lost one of them, duplicated one of them (*dinB1*), or exchanged one of them with other bacteria.

The 4-bp fusion of *dnaE2*-*dinB2* is found in all *Streptomyces*, another actinobacterium *Kytococcus sedentarius* and Gram-negative *Mesorhizobium* bnc1 (Figures 6 and 7). The latter most likely has acquired the fusion by horizontal transfer. The gene fusion strongly suggests that these two genes form an operon under the same control. This is supported by operon prediction on MicrobesOnline (40,41) and their similar SOS responses (Figure 5). In non-actinobacterial species, *Geobacter* m21 (δ-proteobacterium), *Geobacter bemidjiensis* (δ-proteobacterium), *Nitrospira defluvii* (Nitrospirae), *Syntrophobacter fumaroxidans* (δ-proteobacterium), *Desulfococcus oleovorans* (δ-proteobacterium), *Dehalogenimonas lykanthroporepellens* (Chloroflxi), *Bacillus tusciae* (Firmicute), *Thermincolar* Jr (Firmicute) and *Flavobacterium johnsoniae* (which possesses two DinB homologs), *dnaE2* also lies next to a *dinB* homolog (mostly without fusion). However, in these cases, the order of these two genes is reversed.

Interestingly, the coupling of *dinB* and *dnaE* homologs occurred mainly in duplicate genes (except in *D. lykanthroporepellens*). The physical coupling presumably puts the two genes under the same regulatory controls as in *Streptomyces*. Many bacteria (particularly α-proteobacteria) that possess duplicate *dnaE* homologs often possess *imuB* (*i*nducible *mu*tagenesis), a more distant homolog of *dinB*, and often also an accompanying gene, *imuA* (42). In α-proteobacteria, the three genes form an operon-like organization in the order *imuA*-*imuB*-*dnaE2* with an SOS box in the promoter

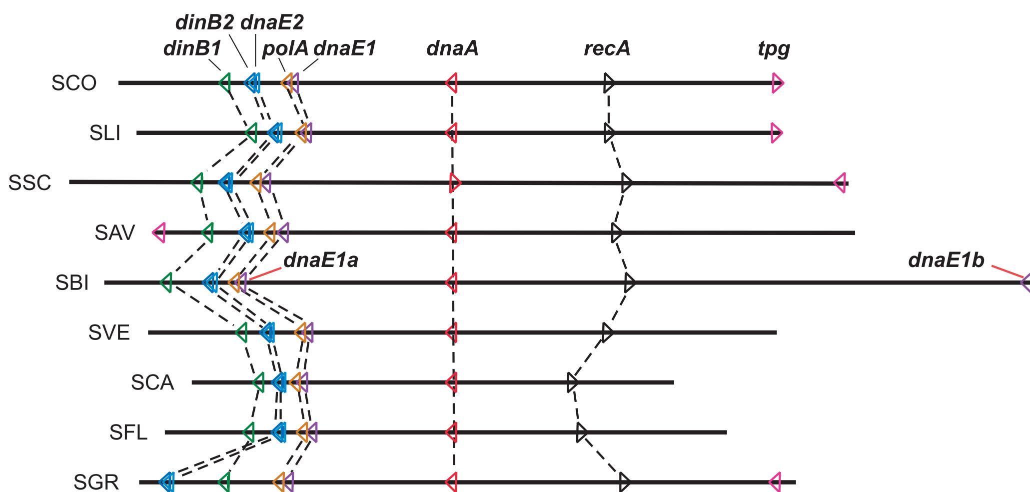

Figure 6. Locations of DNA polymerase genes in *Streptomyces* genomes. Locations and direction of transcription (colored arrowheads) of *dnaA*, *recA*, *tpg* and the DNA polymerase genes are indicated on nine sequenced *Streptomyces* chromosomes (oriented according to the *S. coelicolor* chromosome). The chromosomes are aligned at *dnaA*, which is typically adjacent to *oriC*. Chromosome abbreviations: SCO, *S. coelicolor*; SLI, *S. lividans*; SSC, *S. scabiei*; SAV, *S. avermitilis*; SBI, *S. bingchenggensis*; SVE, *S. venezuelae*; SCA, *S. cattleya*; SFL, *S. flavogriseus*; SGR, *S. griseus*.

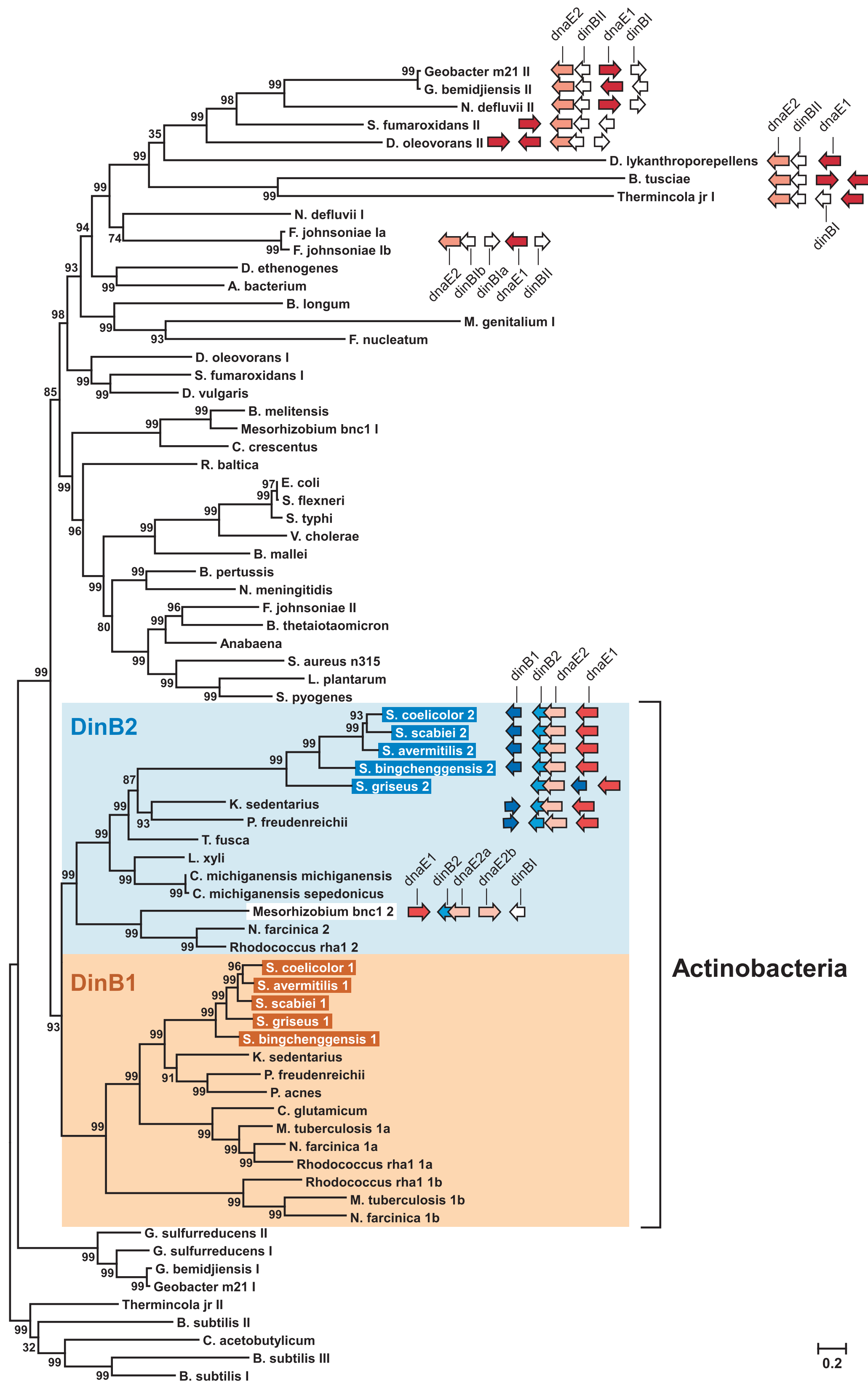

Figure 7. Phylogenetic analysis of DinB homologs. DinB homologs in 68 bacteria are subjected to multiple sequence alignment followed by phylogenetic tree construction as described in 'Materials and Methods' section. The bootstrap numbers are indicated at the branch junctions. '1' and '2' following the species names depict DinB1 (shaded in brown) and DinB2 (shaded in blue) in *Streptomyces*, respectively. 'I', 'II' and 'III' following the species names depict DinB homologs in other species. For those species in which *dnaE* and *dinB2* appear to form an operon, the synteny of the *dnaE* and *dinB* homologs relative to other DNA polymerase genes is displayed by the arrays of colored arrows: Dark red, *dnaE1*; light red, *dnaE2*; Dark blue, *dinB1*; light blue, *dinB2*; white, other *dinB* homologs (outside of actinobacteria except in *Mesorhizobium* bnc1).

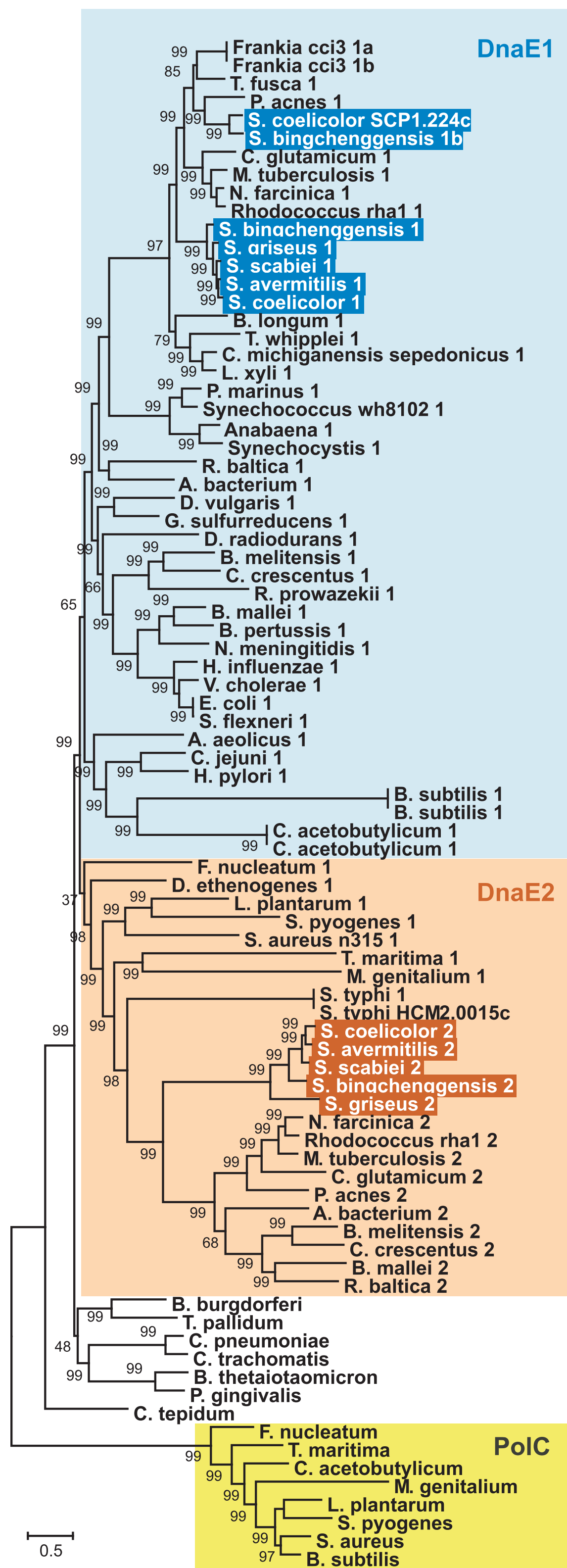

Figure 8. Phylogenetic analysis of DnaE homologs. DnaE homologs in 68 bacteria are subjected to multiple sequence alignment followed by phylogenetic tree construction as described in 'Materials and Methods' section. The bootstrap numbers are indicated at the branch junctions. '1' and '2' following the species names depict DnaE1 (shaded in blue) and DnaE2 (shaded in brown) in *Streptomyces*, respectively. PolC family is shaded in green. The *Streptomyces* homologs are emphasized by dark reverse colors.

region. In contrast to *dnaE2* of *Streptomyces*, *dnaE2* (as well as *imuA* and *imuB*) of *C. crescentus* are involved in error-prone DNA repair (42).

Some *dnaE* homologs are acquired by horizontal transfer

The biological role of DnaE2 in *Streptomyces* is a mystery. This study shows that it is not essential for chromosome replication or end patching, and deletion of *dnaE2* did not cause any changed sensitivity to UV-induced killing and mutagenesis despite the fact that it is induced by UV irradiation and exposure to MMS. In contrast, the downstream gene *dinB2*, which most likely is co-transcribed and co-regulated, is involved in end patching, repair of UV-induced damage, as well as UV-induced mutagenesis.

Despite its cryptic biological function in *Streptomyces*, *dnaE2* homologs are present in diverse clades of bacteria. Of 23 actinobacterial chromosome sequences examined, all except *Tropheryma whipplei* and *Thermobifida fusca* contain a *danE* homolog. Multiple (two or more) copies of *dnaE* homologs are also found in α-, β- and γ-proteobacteria, acidobacteria, planctomycete and chloroflexus. Phylogenetic analysis of DnaE homologs in 68 bacteria including 19 actinobacteria (Figure 8) separated them into two large families (herein designated DnaE1 and DnaE2 families) represented by DnaE1 and DnaE2 of *Streptomyces*, respectively, and a small family. The DnaE1 family includes those homologs present singly in bacterial genomes. This suggests that *dnaE1* represents the primordial archetype, and that *dnaE2* emerged by duplication.

In the DnaE1 branch, while all the DnaE1 homologs encoded by various *Streptomyces* chromosomes are clustered together in a single sub-branch, the DnaE1 homolog encoded by the SCP1 plasmid is clustered with the second DnaE1 homolog of *S. bingchenggensis* (designated DnaE1b) in a separate sub-branch. Unlike the other DNA polymerase genes, *dnaE1b* is located near the right-hand telomere (104 kb from the end; Figure 6). The phylogenetic distances and the unique genome locations (on a linear plasmid and near the end of the chromosome) suggest that *dnaE1* of SCP1 and *dnaE1b* of *S. bingchenggensis* were acquired by horizontal transfer. It is not known whether *dnaE1b* plays a biological role in *S. bingchenggensis*, but *dnaE1* (SCP1.224) on SCP1 is accompanied by *dnaN* (SCP1.119; encoding β sliding clamp), and both of these genes appear to be important for replication of SCP1 (H.-H. Tsai, unpublished results).

dnaE2 and *dinB2* evolved rapidly in *Streptomyces*

The longer branch lengths in the phylogenetic trees showed that *dinB2* has evolved more rapidly than *dinB1* in *Streptomyces* (Figure 7). Analysis of synonymous *vs.* non-synonymous substitutions shows relatively low (<0.5) values of K_a/K_s ratios for *dinB1* (Supplementary Figure S1a). These results indicate that it has evolved relatively rapidly toward a higher degree of purifying (stabilizing) selection. In contrast, *dinB2* exhibits significantly larger and more variable K_a/K_s. Sliding K_a/K_s analysis between each *dinB1* and *dinB2* pair in five *Streptomyces* species further revealed K_a/K_s ratios

significantly larger than 1 in specific regions, suggesting these regions had undergone positive selection in *dinB2* (Supplementary Figure S1b).

dnaE1, which encodes the chromosomal replicase, exhibits very low K_a/K_s ratios as expected for a conserved replicase (Supplementary Figure S1c). In comparison, *dnaE2* has larger and more variable K_a/K_s ratios, even more so than *dinB2* (Supplementary Figure S1b). This and the longer branch length in the DnaE phylogenetic tree (Figure 8) indicate that *dnaE2* has also evolved more rapidly toward a new but unclear function. Sliding K_a/K_s analysis between each *dnaE1* and *dnaE2* pair in five *Streptomyces* species revealed positive selection in specific regions, notably the 'thumb' domain and the region between the PHP and 'palm' domains, of *dnaE2* (Supplementary Figure S1c).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table S1, Supplementary Figure S1.

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