Topoisomerase IV is required for partitioning of circular chromosomes but not linear chromosomes in Streptomyces

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ABSTRACT

Filamentous bacteria of the genus Streptomyces possess linear chromosomes and linear plasmids. Theoretically, linear replicons may not need a decatenase for post-replicational separation of daughter molecules. Yet, Streptomyces contain parC and parE that encode the subunits for the decatenase topoisomerase IV. The linear replicons of Streptomyces adopt a circular configuration in vivo through telomere–telomere interaction, which would require decatenation, if the circular configuration persists through replication. We investigated whether topoisomerase IV is required for separation of the linear replicons in Streptomyces. Deletion of parE from the Streptomyces coelicolor chromosome was achieved, when parE was provided on a plasmid. Subsequently, the plasmid was eliminated at high temperature, and ΔparE mutants were obtained. These results indicated that topoisomerase IV was not essential for Streptomyces. Presumably, the telomere–telomere association may be resolved during or after replication to separate the daughter chromosomes. Nevertheless, the mutants exhibited retarded growth, defective sporulation and temperature sensitivity. In the mutants, circular plasmids could not replicate, and spontaneous circularization of the chromosome was not observed, indicating that topoisomerase IV was required for decatenation of circular replicons. Moreover, site-specific integration of a plasmid is impaired in the mutants, suggesting the formation of DNA knots during integration, which must be resolved by topoisomerase IV.

INTRODUCTION

Most bacterial chromosomes consist of covalently closed circular DNA with negative superhelicity. Two counter-acting topoisomerases, gyrase and topoisomerase I (Topo I), are responsible for the maintenance of balanced negative superhelicity of these circular DNA molecules. Gyrase, a GyrA2GyrB2 heterotetramer, cuts and reseals two strands of DNA simultaneously using energy supplied by ATP to create negative supercoiling (Type II topoisomerase). In contrast, Topo I relaxes the negatively supercoiling by cutting and resealing one strand of DNA at a time (Type I topoisomerase).

The gyrase–Topo I pair also acts in concert to relieve the superhelicity generated during replication and transcription, i.e. the local positive supercoiling ahead of the replication forks and transcription bubbles is relaxed by gyrase, and the local negative supercoiling behind the transcription bubbles is compensated by Topo I. Because of these important physiological roles, gyrase and Topo I are basically essential for viability of bacterial cells, although some defects in one of these proteins may be tolerable or suppressed by mutation in the other.

Another topological issue arises at the termination of replication of circular chromosomes and plasmids, i.e. the resolution of the interlocking catenane daughter molecules. Playing this role is another Type II topoisomerase, topoisomerase IV (Topo IV), which is a homolog of gyrase (1,2). Mutations in parC or parE result in defective decatenation of the circular chromosomes and plasmids.
and are generally lethal (3–5), although they may be partially suppressed by simultaneous overexpression of both gyrase subunits in *Escherichia coli* (6).

Most bacteria possess both gyrase and Topo IV. A few exceptions are Corynebacteria, *Campylobacter jejuni*, *Deinococcus radiodurans*, *Treponema pallidum* and some Mycobacteria (such as *Mycobacterium leprae*, *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*) (7,8), which lack *parC* and *parE*. Presumably, decatenation of the circular chromosomes in these bacteria is carried out by gyrase. This notion was supported by the demonstration that the gyrase of *M. smegmatis* indeed possesses a strong decatenation activity as well as supercoiling activity in vitro (9).

An interesting question arose when linear chromosomes were discovered in some bacteria such as *Borrelia burgdorferi* and *Streptomyces* spp., i.e. do these linear chromosomes require a Type II topoisomerase for decatenation? In theory, replication of linear DNA does not result in catenated molecules, and therefore may not require a decatenase for resolution. However, *parC* and *parE* homologs are present in the chromosomal sequences of these bacteria. In Gram-positive bacteria, *gyrA* and *gyrB* usually form an operon near *oriC*, and *parC* and *parE* lie separately in opposite orientations distally from *oriC*. This is also true for *Streptomyces*. For example, in *S. coelicolor*, the *gyrAB* operon (SCO3873–3874) is near *oriC*, whereas the *parC* and *parE* homologs (SCO5836 and SCO5822) lie in opposite orientations separated by 13 kb on the right arm of the chromosome (Figure 1). Phylogenetic analysis shows that the *parC* and *parE* homologs are grouped in the ParC and ParE branches with those of other bacteria, distinct from the GyrA and GyrB branches, respectively (Supplementary Figure S1). That SCO5822 and SCO5836 of *S. coelicolor* encode the Topo IV subunits was confirmed in vitro by Schmutz et al. (8) using purified and assembled heterotetrameric topoisomerases. They showed that (SCO5822)₂(SCO5836)₆, like other Topo IV (6), possessed both decatenation and relaxation activity, but not supercoiling activity.

Whereas gyrase is essential in *Streptomyces* as well as other bacteria, the role of Topo IV is not clear in *Streptomyces*. It is likely that Topo IV is required for post-replication decatenation of circular plasmids in *Streptomyces*. In addition, spontaneous circularization of the chromosomes through fusion of the two arms occurs at relatively high frequencies (about 5 × 10⁻⁷ per sporulation cycle) in *Streptomyces* [reviewed in (10)]. One would expect that these circular chromosomes would require a decatenase for post-replication segregation.

A more interesting question is whether the linear chromosomes and linear plasmids require Topo IV for decatenation in *Streptomyces*. These linear replicons are capped by terminal proteins (TPs) covalently bound at the 5'-ends of the DNA (11). It was shown recently that these TP-capped telomeres interact *in vivo*, resulting in the formation of a circular configuration with negative superhelicity, despite the linearity of these replicons (12). If the telomere–telomere interactions persist throughout and after the completion of replication, the requirement of decatenation would seem imperative.

Moreover, in eukaryotes, despite the linearity of their chromosomes, a type II topoisomerase, Topo II, appears to be required for untangling of the intertwined daughter chromosomes after replication. Mutations that inactivate the decatenation activity of Topo II in yeast result in interlocked chromosomes in S phase (13,14). Topo IV may also perform a similar role for the linear chromosomes in *Streptomyces*.

In this study, we addressed the question whether Topo IV was essential for the linear or circular DNA in *Streptomyces*. We investigated this issue by attempts to delete a Topo IV gene. Our results showed that deletion of *parE* could be achieved on a linear chromosome but not on a circular chromosome, indicating that Topo IV was essential for circular DNA but not for linear DNA in *Streptomyces*. This was confirmed by the ability of the *parE* deletion mutants to support replication of linear plasmids but not circular plasmids. In the *parE* deletion mutants, the linear replicons presumably bypass the requirement of Topo IV through dissociation and reestablishment of the telomere–telomere complex to achieve segregation. Nevertheless, Topo IV was likely important for efficient untangling of the linear daughter chromosomes, because the *parE* deletion mutants exhibited retarded growth and sporulation and temperature sensitivity. Moreover, it was also discovered that Topo IV was involved in resolution of DNA knots formed during site-specific integration of circular plasmids.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids**

Strains and plasmids used in this study are listed in Table 1. Microbiological and genetic manipulations in *E. coli* and *Streptomyces* were according to Sambrook *et al.* (27) and Kieser *et al.* (26). *Streptomyces* strains were cultured on six solid media, LB (Difco), low-salt LB (LB Lennox, Difco), protoplast regeneration medium R5 (26), SFM (0.2% mannitol, 0.2% soya flour and 0.2% agar) (26), PYM (0.5% peptone, 0.3% yeast extract, 0.3% malt extract, 1% glucose and 2% agar) (28) and DNA (Difco Nutrient Agar), and two liquid media, TSB (0.3% tryptone soya broth powder) and YEME (0.3% yeast extract, 0.5% peptone, 0.3% malt extract, 1% glucose, 34% sucrose and 5 mM MgCl₂) (26). pLUS355 was constructed from pLUS970 (21) by removing a 3.3-kb *BclI–SphI* fragment containing the rlf locus required for replication in linear form and replacing a 0.6-kb *BclI–SphI* fragment downstream of *tsr* with multiple cloning site sequence.

**Construction of parE mutants in Streptomyces**

The gene replacement method based on Gust *et al.* (17) was used to generate the deletion mutants in this study. Basically, the *parE*-disrupting cassette was generated by polymerase chain reaction (PCR) (primers H4-5' and H4-3'; Supplementary Table S2), which contained a
unique priming site annealed to the apramycin-resistant (Apr') cassette from pIJ773 and 36-bp sequences flanking each side of parE on the chromosome. This amplified fragment was introduced by transformation into E. coli BW25113/pIJ790 harboring a cosmid clone—St5B8 of S. coelicolor (19) containing parE, and Apr' transformants were selected, which harbored the cosmid with a ∆parE::aac(3)IV allele (designated

Table 1. Bacterial strains and plasmids used in this study

| Culture/plasmid | Genotype/description | Source/reference |
|-----------------|----------------------|-----------------|
| S. coelicolor   |                      |                 |
| M145            | Wild type, SCP1 SCP2 | (15)            |
| M145/pLUS379    | pLUS379 integrated into the chromosome via single crossover | This study |
| M145/parE       | M145 containing ∆parE::aac(3)IV mutation | This study |
| M145/parE/pLUS385 | M145/parE harboring pLUS385 | This study |
| 3456            | pSL(SCP1)^{NF} SCP2 | (16)            |
| 3456/pLUS379    | pLUS379 integrated into the chromosome via single crossover | This study |
| 3456/parE       | 3456 containing ∆parE::aac(3)IV mutation | Figure 2, this study |
| 3456/parE/pLUS385 | 3456/parE harboring pLUS385 | This study |
| E. coli        |                      |                 |
| BW25113/pIJ790 | K12 derivative; araBAD rhaBAD)/x-RED (gam bet exo) cat araC rep101^r | (17) |
| ET12567/pUZ8002 | dan-13:Tn9 dem cat tet hsdM hsdR zj-201::Tn10/tra neo RP4 | (18) |
| pIJ773         | E. coli plasmid, aac(3)IV oriT | (17) |
| St5B8          | S. coelicolor cosmid containing spanning recA and parE | (19) |
| pLUS379        | St5B8 derivative in which parE is replaced by the Apr' cassette | This study |
| pHZ132         | E. coli–Streptomyces temperature-sensitive shuttle plasmid containing pSG5 ARS, cos, oriT, tsr and bla | (20) |
| pLUS355        | Derivative of pLUS970 (21), in which the rfr locus is removed and a multiple cloning site is inserted. | This study |
| pLUS356        | Derivative of pLUS970 (21), in which the HindIII–SfiI fragment is replaced by a multiple cloning site | Figure 5A, this study |
| pLUS383        | Derivative of pHZ132 (20) containing tsr and bla and a deletion of the 1.6-kb HpaI–HindIII fragment containing cos | This study |
| pLUS385        | pLUS383 derivative containing parE | Figure 2A, this study |
| pIJ702-117     | Derivative of pIJ702, tsr, melC (with a up-regulating promoter) | (22) |
| pLUS891        | Plasmid containing pSLA2 ARS (23) and tsr flanked by a pair of 320-bp telomere sequences of SCP1 | (24) |
| pIJ82          | pSET152 (25) derivative containing hyg replacing aac(3)IV | (26) |
| pIJ82–parE     | pIJ82 containing parE | Figure 4A, this study |

Figure 1. Synteny of parC and parE genes in Streptomyces genomes. Locations and direction of transcription (colored arrowheads) of parC, parE, gyrAB, dnaA, recA, and the five DNA polymerase genes are indicated on eight sequenced Streptomyces chromosomes (oriented according to the S. coelicolor chromosome). The chromosomes are centered and aligned at dnaA. Chromosome abbreviations: SCO, S. coelicolor; SLI, S. lividans; SSC, S. scabiei; SAV, S. avermitilis; SVE, S. venezuelae; SCA, S. castellii; SFL, S. flavogriseus; SGR, S. griseus. The accession numbers and other details of the sequences used are in Supplementary Table S1.
pLUS379, pLUS379 was transferred by conjugation from E. coli ET12567/pUZ8002 (18) to S. coelicolor M145 and 3456 for gene replacement. Apr<sup>t</sup> exoconjugants were selected. These exoconjugants were kanamycin-resistant (Kam<sup>t</sup>), indicating that they contained integrated pLUS379. Further attempts to isolate kanamycin-sensitive (Kam<sup>t</sup>) Apr<sup>t</sup> segregants among the exoconjugants to obtain Apr<sup>t</sup>:aad(3)/IV mutants failed. For complementation, a 2.6-kb sequence spanning parE and 200 bp upstream of it from S. coelicolor was inserted into pLUS383, a derivative of the temperature-sensitive plasmid pHZ132 (20), which conferred thiostrepton and viomycin resistance. The resulting plasmid, designated pLUS385, was introduced into S. coelicolor by transformation. From thiostrepton-resistant (Thio<sup>t</sup>) exoconjugants, Kam<sup>t</sup> segregants were obtained that contained the ΔparE deletion. Subsequently, loss of pLUS385 was achieved by screening at 40°C.

Complementation of parE mutants

To complement the parE mutation in 3456ΔparE and M145ΔparE, the parE coding sequence with upstream promoter region (761 bp) was generated by PCR (primers C4-XbaI-5′ and C4-EcoRV-3′; Supplementary Table S2). The amplified fragment was cloned into an integrative plasmid, pIJ82, giving rise to pIJ82–parE. The resulting plasmid was introduced into E. coli ET12567/ pUZ8002 and further integrated into S. coelicolor 3456 and M145 φC31 attB site via E. coli–Streptomyces conjugal transfer. Hygromycin-resistant transconjugants were selected and verified by Southern blotting.

Microscopy

Aerial mycelium and spore chains were collected on sterile coverslips, inserted in minimal medium containing mannitol for 13 days, according to the methods of Kim et al. (8). The coverslips were stained with 5 μg/ml DAPI (4′,6-diamidino-2-phenylindole) in phosphate-buffered saline containing 50% glycerol, and then examined with a fluorescence microscope (Leica DMLB) with 360-nm excitation light and a 425-nm emission filter.

Phylogenetic analysis

Sixteen bacteria were selected to represent Streptomyces, actinobacteria and other bacteria (see Supplementary Table S1 for genomic source information). The orthologs of gyrase and Topo IV from each bacterium were extracted from the Kyoto Encyclopedia of Genes and Genomes database (29), and used for the construction of a phylogenetic tree using the Neighbor-Joining method in Molecular Evolutionary Genetics Analysis (MEGA) software version 5 (30).

RESULTS

The Topo IV component, parE, was deleted in two steps

We first attempted to delete parE (SCO5822) in wild-type S. coelicolor M145 using the REDIRECT procedure of Gust et al. (17). Cosmid pLUS379 contained a kanamycin resistance gene (aph) and a segment of S. coelicolor DNA, in which parE was replaced by an apramycin-resistance gene [aad(3)/IV] cassette. Conjugal transfer of the cosmid from E. coli to M145 produced Apr<sup>t</sup> exoconjugants. The insertion of the cosmid in the parE region by homologous recombination in these exoconjugants was confirmed by PCR analysis (data not shown). Subsequent attempts to isolate kanamycin-sensitive (Kam<sup>t</sup>) segregants from the M145/pLUS379 exoconjugants, which would have undergone a second crossover and deleted parE, failed among more than 600 colonies screened.

We have previously experienced similar difficulties in attempts to delete polA (DNA polymerase I) and recA from M145, but succeeded with relative ease in 3456 (a strain containing an integrated plasmid SCP1<sup>NF</sup>) (31,32). Thus, we attempted to delete parE in 3456 using the same procedure. Apr<sup>t</sup> 3456/pLUS379 exoconjugants were similarly isolated using pLUS379. However, attempts to isolate Kan<sup>t</sup> segregants also failed among 450 exoconjugants screened.

To check the possibility that parE was essential for viability, we introduced a temperature-sensitive plasmid, pLUS385, which contained a viomycin-resistance gene (vph), a thiostrepton-resistance gene (trs) and parE (Figure 2A), into M145/pLUS379 and 3456/pLUS379. From the Thio<sup>t</sup> transformants, Kan<sup>t</sup> segregants were readily isolated at frequencies of approximately 10<sup>−1</sup> in M145/pLUS379 and 10<sup>−2</sup> in 3456/pLUS379. That these Thio<sup>t</sup> Kan<sup>t</sup> segregants had suffered deletion of parE through double crossovers was confirmed by restriction and hybridization (Figure 2B and 2C). These ΔparE mutants still possessed pLUS385 (being Thio<sup>t</sup>), and were designated M145ΔparE/pLUS385 and 3456ΔparE/ pLUS385, respectively.

If parE was essential for viability of Streptomyces, it was expected that M145ΔparE/pLUS385 and 3456ΔparE/ pLUS385 would exhibit temperature sensitivity, because replication of the vector (pHZ132) that carried parE was defective in replication at elevated temperature (20). Indeed, compared with the control cultures (M145/ pLUS385 and 3456/pLUS385), the plating efficiencies of M145ΔparE/pLUS385 and 3456ΔparE/pLUS385 at 40°C were reduced by 60–70% on LB and DNA agar, and by more than two orders of magnitude on PYM agar (Figure 2D).

The colonies that survived the elevated temperature from plating of the M145ΔparE/pLUS385 and 3456ΔparE/ pLUS385 spores were analyzed for the presence of pLUS385. If parE was essential, it was expected that these surviving cultures would have retained pLUS385. Instead, all these surviving cultures were plasmid-less and Thio<sup>t</sup> (data not shown). Moreover, these cultures were all Apr<sup>t</sup>, and restriction and Southern hybridization confirmed deletion of the parE sequence (data not shown). Therefore, these results indicated that parE was not essential for either of these strains, which were designated M145ΔparE and 3456ΔparE, respectively. The initial failure to isolate these deletion mutants directly was most likely due to their retarded growth and sporulation (see following text).
parE exhibited defective growth, and temperature sensitivity

Compared with the wild type, M145ΔparE and 3456ΔparE also grew more slowly at the normal temperature (30°C) on several solid media, particularly R5 and LB agars (Table 2). They grew also very slowly in YEME broth, but normally in TSB broth. We suspected that the poor growth might be correlated to higher osmolality of these media (Table 2 and Supplementary Table S3). This notion was supported by the comparison of LB (containing 10 g/l NaCl) and low-salt (Lennox) LB (containing 5 g/l NaCl), in which the lower salt appeared to benefit the growth of the mutants.

On the medium (SFM) used for conjugation during the construction of the mutants, the vegetative growth of M145ΔparE and 3456ΔparE was comparable with that of the wild-type strains. However, sporulation of the mutants was significantly retarded on this medium. Whereas white aerial hyphae formed in the mutant colonies at about the same time (about 4 days after plating of the spores) as in the wild type, gray spores appeared only sparsely even 10 days after plating (Figure 3A).

The poor sporulation of the mutants was probably due to deficiency in decatenation of the chromosomes during sporulation, where proper partitioning is critical (33). When the 3456ΔparE colonies were examined by DAPI staining under the microscope, the aerial hyphae contained relatively few spores of normal shapes. In the rare spore chains, anucleate spaces (lacking spore-like structure) were often observed (Figure 3B upper panel). The frequency of these anucleate spaces was about 18%. In comparison, the frequencies of anucleate spores in M145 and M145ΔparE/pIJ82–parE were below 1%. In addition, the aerial hyphae of 3456ΔparE often contained very large bulges and extrusions that were packed with excess of DNA (lower panel). This was in contrast to the regularly distributed nucleoids in the aerial mycelial segments and the spore chains in 3456 and 3456ΔparE/pIJ82–parE.

ΔparE exhibited defective growth, and temperature sensitivity

Compared with the wild type, M145ΔparE and 3456ΔparE also grew more slowly at the normal temperature (30°C) on several solid media, particularly R5 and LB agars (Table 2). They grew also very slowly in YEME broth, but normally in TSB broth. We suspected that the poor growth might be correlated to higher osmolality of these media (Table 2 and Supplementary Table S3). This notion was supported by the comparison of LB (containing 10 g/l NaCl) and low-salt (Lennox) LB (containing 5 g/l NaCl), in which the lower salt appeared to benefit the growth of the mutants.

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ΔparE mutants were complemented by integrated parE

To complement the ΔparE mutation, the parE coding sequence with its upstream promoter region (761 bp; from the termination codon of the upstream gene to the
initiation codon of \( \text{parE} \) was inserted into an integrative plasmid, pIJ82 (26), and the resulting plasmid, pIJ82–parE (Figure 4A), was introduced into \( M145/\text{parE} \) and \( 3456/\text{parE} \). Hygromycin-resistant transformants were isolated, and integration of the plasmid into the \( \phi C31 \) attB site was confirmed by restriction and hybridization (Figure 4B). These complementation strains, designated \( M145/\text{parE}/\text{pIJ82–parE} \) and \( 3456/\text{parE}/\text{pIJ82–parE} \), grew as well as wild type on solid media and in liquid media tested (Table 2). The retarded sporulation on SFM and abnormal nucleoid distribution displayed by \( M145/\text{parE} \) and \( 3456/\text{parE} \) were also eliminated (Figure 3B). These results confirmed that the observed growth defects of \( M145/\text{parE} \) and \( 3456/\text{parE} \) were due to deletion of \( \text{parE} \).

**AparE mutants cannot maintain circular plasmids**

The ability to tolerate the \( \Delta \text{parE} \) mutation in \( M145 \) and \( 3456 \) indicated that these linear chromosomes in \( \text{Streptomyces} \) do not require Topo IV for resolution. This is intriguing in view of the fact that the linear chromosomes and plasmids form a circular configuration (herein termed ‘pseudo-circle’) \textit{in vivo} through telomere–telomere association in \( \text{Streptomyces} \) (12). There are two possible bypass mechanisms for the post-replicational untangling of the daughter replicons of these pseudo-circles: (i) gyrase may substitute for Topo IV for the untangling function, and (ii) the interacting telomeres of these linear replicons may transiently dissociate from each other for the untangling. These hypotheses may be tested with a circular plasmid. The first bypass mechanism would allow a circular plasmid to propagate in the deletion mutants, whereas the second mechanism would not.

First, we tested pIJ702-117, which contained \( \text{tsr} \) and the \( \text{melC} \) operon (22). This plasmid transformed \( M145 \) and \( 3456 \) at ‘normal’ frequencies, but failed to produce any transformants in \( M145/\text{parE} \) and \( 3456/\text{parE} \). Next, we tested two circular plasmids, pPLUS356 and pPLUS891, both of which included a linear plasmid sequence (Figure 5A) (24). These plasmids may appear and replicate as free linear molecules (with TP-capped telomeres) in the transformants under certain conditions. \( M145 \) and \( 3456 \) were transformed by these two plasmids at ‘normal’ frequencies, but \( M145/\text{parE} \) and \( 3456/\text{parE} \) were

Table 2. Growth defect of \( \Delta \text{parE} \) mutants

| Medium       | R5 | LB | Low-salt LB | SFM | PYM | DNA | YME | TSB |
|--------------|----|----|-------------|-----|-----|-----|-----|-----|
| Growth at 30°C | parE<sup>a</sup> | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
|              | \( \Delta \text{parE} \) | +  | +  | +  | ++  | ND  | ND  | ND  |
| Growth at 42°C | parE<sup>b</sup> | +  | +++ | +++ | +++ | ND  | ND  | ND  |
|              | \( \Delta \text{parE} \) | +  | −  | +  | ++  | ND  | ND  | ND  |

<sup>a</sup>M145, 3456, M145/\text{parE}/\text{pIJ82–parE} and 3456/\text{parE}/\text{pIJ82–parE}.

<sup>b</sup>M145/\text{parE} and 3456/\text{parE}.

+++ , normal growth; ++ , slightly reduced growth; + , poor growth; − , no growth; *, retarded sporulation; ND, not determined.

**Figure 3.** Growth characteristics of the \( \text{parE} \) mutant and the complementation strain. (A) 3456, 3456/\text{parE}, and 3456/\text{parE}/\text{pIJ82–parE} were grown on SFM agar for 4 days at 30°C. 3456/\text{parE} remained white with aerial hyphae, while the other two strains had produced gray spores. (B) 3456, 3456/\text{parE}, and 3456/\text{parE}/\text{pIJ82–parE} were grown over coverslips on MM containing mannitol for 13 days, and the spores were collected from the coverslips, stained with DAPI and imaged under a fluorescence microscope. Image contrast has been increased for better clarity. More sample photos are in Supplementary Figure S2.
Figure 4. Complementation of the parE mutants of 3456 and M145. (A) The integrative plasmid pIJ82-parE containing the parE coding sequence and 761-bp upstream promoter region. hyg, hygromycin resistance gene. int, integrase gene of φC31 phage. attP, φC31 attachment site. Nc, NcoI site. (B) The restriction maps of the hrdB-parE region on the chromosome of 3456, 3456ΔparE, and 3456ΔparE/pIJ82-parE (harboring the integrated pIJ82-parE). Probe II used in Southern blotting (below) is indicated by the horizontal bar. The NcoI cutting sites are indicated by the vertical lines, and the sizes of the restriction fragments are indicated in kb. (C) Confirmation of integration of parE complementation. Genomic DNA was digested with NcoI, and subjected to Southern hybridization using the Probe II. The sizes (kb) of the hybridizing fragments are indicated on the right.

Figure 5. Inability of circular plasmids to replicate in the ΔparE mutants, M145ΔparE and 3456ΔparE. (A) Circular plasmids pLUS356 and pLUS891 (left) used for transformation and the linear versions, pLUS356L and pLUS891L, generated in the transformants (right). bla, beta-lactamase gene; tsr, thiostrepton resistance gene; ARS, autonomously replicating sequence of pSLA2; filled arrows, telomeres of the S. lividans chromosome (on pLUS356) or SCP1 plasmid (on pLUS891); filled circles, TPs. As, AseI site; Ba, BamHI site. The sizes (kb) of the BamHI fragments of the linear DNA are indicated. (B) M145ΔparE and 3456ΔparE were transformed by pLUS356 or pLUS891. Genomic DNA was isolated from the Thio⁺ transformants, digested with BamHI, and subjected to Southern blotting using the transforming DNA as the respective probes. Representative transformants are shown. The sizes (kb) of the hybridizing fragments are indicated.
transformed at frequencies of about two orders of magnitude lower. The transformants were examined for the presence of plasmids. Interestingly, all of the 21 pLUS356 transformants (9 in 3456ΔparE and 12 in M145ΔparE) and 26 pLUS891 transformants of 3456ΔparE examined contained only the linear versions of these plasmids (i.e. pLUS356L and pLUS891L; Figure 5B), and no circular plasmids. In contrast, the M145 and 3456 transformants contained only circular but no linear plasmids. For comparison, we tested pLUS355, a variant of pLUS970 that lacked the rlr locus required for replication in linear form and thus can replicate only in circular form in Streptomyces. No Thio<sup>+</sup> transformants could be produced in M145ΔparE or 3456ΔparE.

Finally, another transformation was performed using a mixture of 3456 and 3456ΔparE protoplasts at comparable concentrations of colony forming units (Table 3). AseI-linearized pLUS356 DNA produced 221 transformants of 3456 and 120 transformants of 3456ΔparE, representing transformation frequencies of 9.6 x 10<sup>-5</sup> and 2.3 x 10<sup>-5</sup>, respectively. The plasmids present in these transformants were all linear (pLUS356L; not shown). In contrast, pLUS355 DNA produced 594 Thio<sup>+</sup> transformants of 3456, but none of 3456ΔparE.

These results showed that Topo IV was required for the maintenance of circular plasmids, but not linear plasmids, in Streptomyces, and that gyrase could not functionally substitute for Topo IV in the decatenation of the circular plasmids.

### DISCUSSION

Topo IV is essential for segregation of circular daughter chromosomes and plasmids in bacteria. Mutations in the coding genes have been found to cause either lethality or thermosensitivity in bacteria with circular chromosomes (4,35). Initially, we also failed to delete parE directly in M145 and 3456, and the preliminary results were reported previously (36). However, in this study, we showed that in Streptomyces cultures with a linear chromosome, the chromosomal parE could be deleted in the presence of a complementing copy on a plasmid, and the removal of the plasmid produced ΔparE mutants that exhibited growth and sporulation deficiencies. The deficiencies explain the earlier difficulties in isolating the mutants directly by screening for segregants through double crossovers.
The successful isolation of the ΔparE mutants demonstrated that Topo IV is not essential for wild-type *Streptomyces* with a linear chromosome. This is interesting because the linear chromosomes and linear plasmids have been demonstrated to exit in circular configuration through telomere–telomere interactions (12). If the telomere–telomere interactions persist through replication, ‘pseudo-catenanes’ would be produced, which would have to be resolved for proper segregation. Thus, in the ΔparE mutants, it is likely that dissociation of the telomere–telomere complex occurs during or after replication to allow the decatenation of the pseudo-catenane.

Such dissociation of the telomere–telomere complex has been proposed as a possible mechanism for solving another post-replication segregation problem for linear replicons posed by intramolecular telomere–telomere associations. It was pointed out that if the ‘old’ TPs on the parental strains of the linear plasmid or chromosome remain associated with each other through replication, and the new TPs capping the daughter DNA become associated with each other, a pseudo-dimeric (Möbius strip-like) structure would result (12,36). These pseudodimers may be resolved by exchanging the interacting TPs, i.e. the ‘old’ TPs switching to associate with the ‘new’ ones at the opposing telomere. It is likely that this partner changing mechanism is also used to resolve the pseudo-catenanes at least in the ΔparE mutants.

In some actinobacteria (such as Corynebacteria and *M. smegmatis*), which lack *parC* and *parE* in their genomes, the role of decatenation of their circular replicons must fall on gyrase. However, in *Streptomyces*, gyrase is unlikely to perform decatenation, because circular plasmids and circular chromosomes cannot be maintained in the ΔparE mutants.

Why did the ΔparE mutants exhibit poor growth and sporulation? There are two plausible non-mutually exclusive possible reasons. First, it may be due to low efficiency of decatenation of the pseudo-catenanes by dissociation of the telomere–telomere complexes. The dissociated telomeres must reconnect in correct topology to achieve separation. Second, it could be that Topo IV is also important in untangling daughter linear chromosomes, as shown in yeasts (37–40). The *Streptomyces* chromosomes are typically 6–10 Mb, significantly larger than the chromosomes of *Saccharomyces cerevisiae* (2.2–0.2 Mb) and *Schizosaccharomyces pombe* (5.7–3.5 Mb). The anomaly of nucleoid size and distribution in the hyphae of the parE mutants (Figure 3B) supports these notions.

The poor growth of the ΔparE mutants was more severe on rich media and at high temperature. Presumably, the faster chromosomal replication in the cultures under these conditions demand timely post-replication resolution and partitioning of the chromosomes before the next round of replication is completed. Alternatively, the higher osmotic pressure in the richer media (Supplementary Data and Supplementary Table S3) may be an important factor. Such elevated sensitivities to rich media, osmotic pressure and/or thermosensitivity have been often observed in topoisomerase mutants of bacteria (41–43).

It is intriguing that some actinobacteria with circular chromosomes lack Topo IV, whereas *Streptomyces* spp. with linear chromosomes possess Topo IV, which is not absolutely required. The linear chromosomes of *B. burgdorferi* also possess Topo IV. In this case, the telomeres are hairpinned instead, and there is no evidence for telomere–telomere interactions in this bacterium.

Why do *Streptomyces* spp. possess a decatenase that is not absolutely required? There are at least three possible advantages: (i) it decatenates better than the proposed transient dissociation of the telomere–telomere complex, (ii) it allows harboring of circular plasmids and (iii) it allows the survival of mutants in which the chromosomes have circularized. The last point is intimately related to the extremely high occurrence of chromosome circularization in *Streptomyces*, a puzzle that is poorly understood in...
terms of molecular mechanism and evolutionary significances. As we have shown here, without Topo IV, no circular chromosomes can survive.

The ΔparE mutants also exhibited low transformation efficiencies of pIJ82, which integrates into the Streptomyces chromosomes at a specific attB site. Site-specific recombination involving a circular DNA molecule may produce knots, which are harmful for cells if not efficiently removed. Zechiedrich et al. (44) have shown that the products of site-specific recombination by λ Int or Tm3 resolvase were decatened by Topo IV, not gyrase. López et al. (45) also showed Topo IV to be the topoisomerase that unknots DNA during replication. Presumably, Topo IV is also important for unknotting in Streptomyces.

The pIJ82 transformants of 3456ΔparE appeared in very few numbers very slowly and sporadically. In these transformants, the knots had presumably been removed by fortuitous events at various times before cell death, which allowed the hyphae to resume growth. One possibility was the occurrence of a double-strand break in the knot region followed by repair, which removed the knots by chance. In any case, on replating, these transformants grew like 3456ΔparE without wide variations in colony size. This supports the notion that the Topo IV was important only during the site-specific recombination stage when knots were formed.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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