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# Analysis of functions in plasmid pHZ1358 influencing its genetic and structural stability in *Streptomyces lividans* 1326

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**Abstract** The complete DNA sequence of plasmid pHZ1358, a widely used vector for targeted gene disruption and replacement experiments in many *Streptomyces* hosts, has been determined. This has allowed a detailed analysis of the basis of its structural and segregational instability, compared to the high copy number plasmid pIJ101 of *Streptomyces lividans* 1326 from which it was derived. A 574-bp DNA region containing *sti* (strong incompatibility locus) was found to be a determinant for segregational instability in its original *S. lividans* 1326 host, while the structural instability was found to be related to the facile deletion of the entire *Escherichia coli*-derived part of pHZ1358, mediated by recombination between 36-bp direct repeats. A point mutation removing the *Bam*HI site inside the *rep* gene encoding a replication protein (*rep**) and/or a spontaneous deletion of the 694-bp region located between *rep* and *sti* including the uncharacterized ORF85 (*orf85*$^-$) produced little or no effect on stability. A pHZ1358 derivative (pJTU412, *sti*$^-$, *rep**, *orf85*$^-$) was then constructed which additionally lacked one of the 36-bp direct repeats. pJTU412 was demonstrated to be structurally stable but segregationally unstable and, in contrast to *sti*$^+$ pHZ1358, allowed efficient targeted gene replacement in *S. lividans* 1326.

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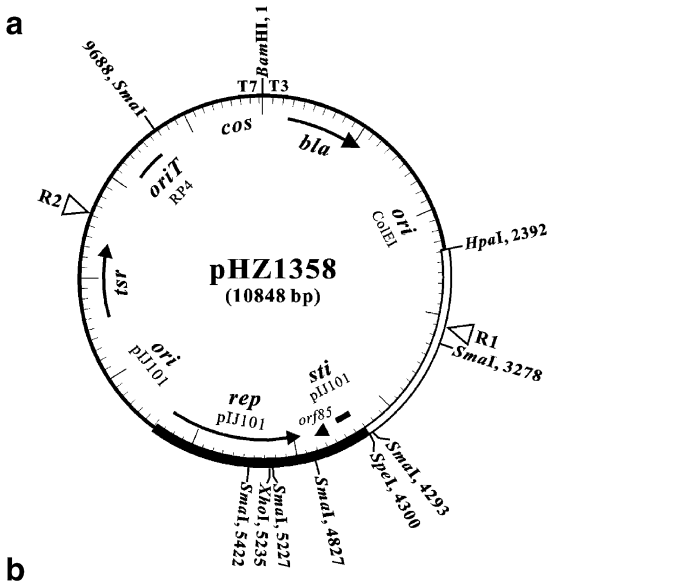
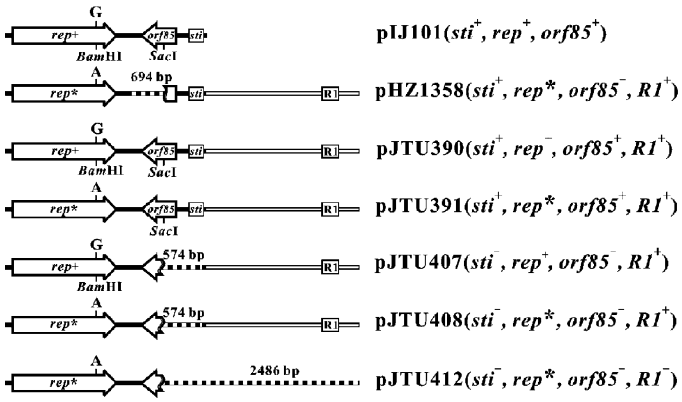
## Introduction

pIJ101 is a 8,830-bp covalently closed circular (CCC) *Streptomyces* plasmid (Kendall and Cohen, 1988) (accession number NC_001387). pIJ101 has a copy number of ~300 and it replicates by the rolling circle mechanism (Deng et al. 1988; Kieser et al. 1982). Popular pIJ101-derived cloning vectors like pIJ702 (Katz et al. 1983) contain the double-strand origin (*pIJ101-ori*) and the *rep* gene, but lack the single-strand origin which is located in the probably untranslated *sti* region (*sti* stands for strong incompatibility) (Deng et al. 1988). Lack of *sti* results in the accumulation of single-stranded plasmid replication intermediate and in a reduction of the double-stranded plasmid copy number (Deng et al. 1988).

*sti*$^+$ pIJ101-derived plasmids have only rarely been used in gene cloning and functional analysis in *Streptomyces*, but one such plasmid, pHZ1358 (Fig. 1a), has found wide use in the efficient generation of mutations by targeted gene disruption or replacement in many *Streptomyces* hosts. For reasons that have remained obscure, it has proved unsuccessful for *S. lividans* and the closely related *S. coelicolor*, an important limitation given the continuing importance of these strains. pHZ1358 differs from most pIJ101-derived cloning vectors in that it carries *sti*, which contains the pIJ101 origin of second-strand synthesis, and therefore, does not accumulate the single-stranded replication intermediate. Additionally, it has the origin of transfer of the conjugative plasmid RP4 for efficient conjugative transfer from *Escherichia coli* to *Streptomyces* and a phage λ *cos* site flanked by T3 and T7 promoters for the efficient

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| | 1326 | | FR-008 | | 5008 | |
|---|---|---|---|---|---|---|
| | GS(%) | SS | GS(%) | SS | GS(%) | SS |
| pIJ101(sti+, rep+, orf85+) | >99 | − | <5 | − | <5 | − |
| pHZ1358(sti+, rep*, orf85−, R1+) | >99 | − | <5 | − | <5 | − |
| pJTU390(sti+, rep−, orf85+, R1+) | >99 | − | <5 | +/− | <5 | +/− |
| pJTU391(sti+, rep*, orf85+, R1+) | <5 | − | <5 | +/− | <5 | +/− |
| pJTU407(sti−, rep+, orf85−, R1+) | <5 | − | <5 | +/− | <5 | +/− |
| pJTU408(sti−, rep*, orf85−, R1+) | <1 | + | <1 | + | <1 | + |
| pJTU412(sti−, rep*, orf85−, R1−) | | | | | | |

**Fig. 1** **a** Map of the bifunctional cosmid vector pHZ1358 deduced from the complete nucleotide sequence. The positions of the 36-bp direct repeats (R1 and R2), from nt 3,110 to nt 3,145, and from nt 8,526 to nt 8,561, are marked with *triangles*. The *thick black arc* highlights a DNA segment derived from pIJ101 which is the focus of the present study. **b** Schematic representation of the mutations of the region highlighted in **a** for pHZ1358 and its derivatives pJTU390, pJTU391, pJTU407, pJTU408, and pJTU412. The corresponding region from wild-type pIJ101 is also shown for reference. *bla* β-lactamase gene, *tsr* thiostrepton resistance (Thio$^R$) gene, *cos* phage λ *cos* site, *oriT* origin of transfer of plasmid RP4, *T3* and *T7* promoters specifically recognized by the phage T3 and T7 RNA polymerases, *ori ColE1 E. coli*-derived replication origin, *sti* strong incompatibility region containing a single-strand origin of replication, *ori pIJ101 Streptomyces*-derived replication origin, *rep*$^+$ G at nt 5,194 of pHZ1358 within a *Bam*HI site in the *rep* region of pIJ101, *rep** A at nt 5,194 of pHZ1358 removing the *Bam*HI site in the *rep* region of pIJ101, *orf85* a putative open reading frame of unknown function, *R1* and *R2* two 36-bp direct repeats. **c** Percentage genetic stability (*GS%*) and structural stability (*SS*) of pHZ1358 and plasmids derived from it, in *S. lividans* 1326, *Streptomyces* sp. FR-008, and *S. hygroscopicus* 5008. *Plus sign* full-sized plasmid detected, *minus sign* truncated plasmid detected, *plus/minus sign* mixture of full and truncated plasmids detected

generation of ordered cosmid libraries. In contrast to suicide plasmids or temperature-sensitive *Streptomyces* plasmid vectors frequently used for gene disruption and replacement, pHZ1358 is replicative but genetically very unstable in many *Streptomyces* hosts. Typically, 99.99% colonies lose thiostrepton resistance after one round of nonselective growth on mannitol soya flour medium (SFM) plates. pIJ702 lacks the *sti* function and is known to be structurally stable in *S. lividans*, and a case of segregational instability of pIJ702 and its use for gene disruption experiments has been reported in *Saccharopolyspora erythraea* (Weber et al. 1990), but it remains unclear whether *sti* or a spontaneous deletion of the 694-bp fragment which was characterized between *rep* and *sti* (a region including a partial *orf85*, an *orf* of unknown function previously suggested to be required for plasmid maintenance; Kieser et al. 1982) has anything to do with the structural or segregational stabilities of the plasmids. Also, a spontaneous mutation removing the unique *Bam*HI site in the *rep* region of pIJ101 was detected by Kieser et al. (1982). This change from ccgGatcctc to ccgAatcctc would lead to a single amino acid change (P373S) inside the Rep

protein (456 aa), but a possible effect of this alteration on plasmid stability has not been examined so far.

In this study, we report the complete nucleotide sequence of this widely used plasmid and the discovery that pIJ101-derived *sti*$^+$ plasmids are segregationally more stable than *sti*$^-$ plasmids in its native host *S. lividans* 1326, although not so in some other *Streptomyces* strains tested. We also report that facile deletion of the *E. coli* part of pHZ1358 derivatives is mediated by a 36-bp direct repeat sequences in *Streptomyces* species. The results in this study suggest that both segregational and structural instability have contributed to the efficiency and usefulness of pHZ1358 and its derivatives as vectors for making gene replacements in most *Streptomyces* because the *Streptomyces* replicon is rapidly lost in the absence of antibiotic selection and inserts mediating gene replacements (cloned in the *E. coli* part) are rapidly separated from the *Streptomyces* replicon by double crossovers between two direct repeats. The information obtained has led to the logical construction of *sti*$^-$ pHZ1358 derivatives (pJTU407/pJTU408, pJTU412) as improved plasmid vectors for making gene replacements in the widely used strain *S. lividans* 1326.

## Materials and methods

### Strains, plasmids, and culture conditions

*Streptomyces* and *E. coli* strains and plasmids that were used and constructed in this study are listed in Table 1.

**Table 1** Strains and plasmids used in this study

| Strains and plasmids | Description/derivation | Reference |
|---|---|---|
| *E. coli* DH5$^\alpha$ | General cloning host | Hanahan (1983) |
| *E. coli* ET12567/pUZ8002 | Strain deficient in methylation, for intergeneric conjugation | MacNeil et al. (1992) |
| *S. lividans* 1326 | Used as *Streptomyces* host, JIC culture collection number 1326 | Kieser et al. (1982) |
| *S. lividans* ZX1 | Used as control strain of *dnd*$^-$, SJTU collection number ZX1 | Zhou et al. (2004, 2005) |
| *S. lividans* HXY6 | *S. lividans* 1326-derivated mutant strain with *dnd*$^-$, SJTU collection number HXY6 | Liang et al. (2007) |
| *S. lividans* HXY7 | *S. lividans* 1326-derivated mutant strain with *dnd*$^-$, SJTU collection number HXY7 | This work |
| *S. lividans* SYH40 | *S. lividans* 1326-derivated mutant strain with *dnd*$^-$, SJTU collection number SYH40 | This work |
| *S. hygroscopicus* 5008 | Used as *Streptomyces* host, SJTU collection number 5008 | Bai et al. (2006) |
| *Streptomyces* sp. FR-008 | Used as *Streptomyces* host, SJTU collection number FR-008 | Chen et al. (2003); Hu et al. (1994) |
| pIJ101 | *sti*$^+$, *rep*$^+$, *orf85*$^+$ | Kieser et al. (1982) |
| pIJ649 | *sti*$^+$, *rep**, *orf85*$^+$ | Deng et al. (1988) |
| pHZ1358 | *sti*$^+$, *rep**, *orf85*$^-$, R1$^+$ | Sun et al. (2002); Fig. 1a |
| pJTU390 | 1,625-bp *Xho*I–*Spe*I fragment from pIJ101 was cloned into pHZ1358 digested with the same enzymes; *sti*$^+$, *rep*$^+$, *orf85*$^+$, R1$^+$ | This work; Fig. 1b |
| pJTU391 | 1,625-bp *Xho*I–*Spe*I fragment from pIJ649 was cloned into pHZ1358 digested with the same enzymes; *sti*$^+$, *rep**, *orf85*$^+$, R1$^+$ | This work; Fig. 1b |
| pJTU407 | Self-ligation of a 10,960-bp *Sac*I–*Spe*I blunt-ended fragment after removal of a 574-bp *Sac*I–*Spe*I fragment from pJTU390; *sti*$^-$, *rep*$^+$, *orf85*$^-$, R1$^+$ | This work; Fig. 1b |
| pJTU408 | Self-ligation of a 10,960-bp *Sac*I–*Spe*I blunt-ended fragment after removal of a 574-bp *Sac*I–*Spe*I fragment from pJTU391; *sti*$^-$, *rep**, *orf85*$^-$, R1$^+$ | This work; Fig. 1b |
| pJTU412 | Self-ligation of a 9,052-bp *Hpa*I–*Sac*I blunt-ended fragment after removal of a 2,486-bp *Hpa*I–*Sac*I fragment from pJTU391; *sti*$^-$, *rep**, *orf85*$^-$, R1$^-$ | This work; Fig. 1b |
| pJTU413 | Two amplified fragments (3,830 and 4,497 bp, respectively) from the *S. lividans* 1326 chromosome were cloned into the blunted *Hpa*I–*Sac*I site of pJTU412 | This work; Fig. 3 |
| pJTU191 | Two amplified fragments (3,830 and 4,497 bp, respectively) from the *S. lividans* 1326 chromosome were cloned into the blunted *Hin*dIII–*Eco*RV site of pOJ260 | This work; Fig. 3 |
| pJTU194 | Two amplified fragments (3,830 and 4,497 bp, respectively) from *S. lividans* 1326 chromosome were cloned into the unique *Hpa*I site of pJTU408 | This work; Fig. 3 |
| pOJ260 | Vector for pJTU191 construction | Bierman et al. (1992) |
| pUC18 | Vector for DNA sequencing | Kieser et al. (2000) |

*sti* strong incompatibility region containing an origin of single-strand replication, *rep*$^+$ G at nt 5,194 of pHZ1358 within a *Bam*HI site, *rep**, A at nt 5,194 of pHZ1358 removing the *Bam*HI site, *orf85* a putative open reading frame with unknown function, *R1* one of two 36-bp direct repeat sequences

*Streptomyces* and *E. coli* techniques were made as described by Kieser et al. (2000) and Sambrook et al. (1989). The total DNA treated with restriction enzymes was fractionated on 0.8% agarose gel and blotted to Hybond N nylon membrane (Amersham Biosciences) for Southern hybridization. A FLA3000 phosphoimager (Fujifilm) was used to detect radioactivity of the Southern blots.

All *Streptomyces* strains used in this study were grown on SFM agar medium (20 g of soy flour, 20 g of mannitol, 20 g of agar per liter) at 28°C for 7 days for sporulation or in tryptone soya broth with yeast extract liquid medium (30 g tryptone soya broth powder, 10 g yeast extract, 103 g sucrose per liter) at 28°C for 2 days for growth of mycelium and isolation of total DNA. Thiostrepton was used at 10 μg $mL^{-1}$ in agar media and at 5 μg $mL^{-1}$ in liquid media.

To determine the genetic stability (GS) of plasmids in a *Streptomyces* host, the exconjugants carrying the plasmids were inoculated onto SFM plates without thiostrepton to allow nonselective growth. Spores of inoculated exconjugants were then replicated onto plates, either with or without thiostrepton. The GS was calculated as the number of colonies on thiostrepton plates, as a percentage of the number on plates without antibiotics. To investigate the structural stability (SS) of plasmids, DNA was extracted from the exconjugants, digested with *Sma*I and subjected to Southern blot analysis using the full-sized plasmid as a probe.

### Sequence analysis and assembly

For shotgun DNA sequencing, plasmid DNA was sonicated with a 550 Sonic Dismembrator (Fisher Scientific) and fragments of 1.6–2.0 kb were recovered from 0.7% low melting agarose gel using a Geneclean II reagent kit (Bio 101), then subcloned into pUC18. For automated sequencing, plasmid DNA templates were prepared by alkaline lysis using the Prep 96 Plasmid Kit (Qiagen). Sequencing reactions were carried out with BigDye Terminator Cycle Sequencing kits (Applied Biosystem Division, Perkin Elmer). The sequences of custom-designed sequencing primers were 5′-GTA AAA CGA CGG CCA GT-3′ (forward) and 5′-GCG GAT AAC AAT TTC ACA CAG G-3′ (reverse). Primers for the detection of possible deletion of *E. coli* DNA mediated by the 36-bp direct repeats were 5′-CGG CTG CAT ACG CTT GAT-3′ (forward) and 5′-GTC TTC CGC CTC GGT TTC-3′ (reverse). Sequence reads were obtained from 377 DNA sequencers (PE/ABD).

Sequence contig assembly and base editing utilized the Phred/Phrap/Consed package (Ewing et al. 1998; Gordon et al. 1998). The sequence data were analyzed by comparison with the sequences of pIJ101 and Supercos1, deposited under accession numbers NC_001387 and M99566, respectively, and by using FramePlot 2.3 (Ishikawa and Hotta 1999). DNA and deduced protein sequence homology searches were performed using PSI-BLAST (Altschul et al. 1997; Altschul and Koonin, 1998; Schaffer et al. 2001). The nucleotide sequence of pHZ1358 has been deposited in the National Center for Biotechnology Information database under the accession number AY667410.

## Results

### Complete nucleotide sequence of the widely used *Streptomyces–E. coli* plasmid vector pHZ1358

Determination of the nucleotide sequence of pHZ1358, constructed in a multistep process from several *E. coli* and *Streptomyces* vectors (Sun et al. 2002), allowed all the changes compared to the parent plasmid pIJ101 to be pinpointed. A schematic map of pHZ1358, containing relevant restriction sites, is presented in Fig. 1a, highlighting the *E. coli*-derived replication origin (*ColE1-ori*), selective marker (*bla*), *oriT* from plasmid RP4, phage λ *cos* site, and T3 and T7 promoters for use to prepare single strands, and the *Streptomyces*-derived thiostrepton resistance gene (*tsr*), strong incompatibility locus (*sti*), replication origin (*pIJ101-ori*), and replication protein (*rep*). A spontaneous mutation removing the unique *Bam*HI site in the *rep* region of pIJ101, as previously detected in pHZ1358 (Kieser et al. 1982), was confirmed as a change from ccgGatcctc to ccgAatcctc, so in the present study, the altered *rep* is designated *rep**. A spontaneous deletion which removed a 694-bp fragment of pIJ101 (nucleotides from 7,739 to 8,432) and created a *Nar*I site at the junction (CGAC*GG–CGCC*TG) was localized in a region thought to be important for plasmid maintenance (Kieser et al. 1982).

An additional five apparent nucleotide differences were detected between pIJ101 and pHZ1358. C-1215, G-1219, and A-1243 of pIJ101 were detected as G-6407, C-6411, and G-6435 in the pHZ1358 sequence, all in noncoding regions, and T-6913 and G-6914 in pHZ1358 were detected as G-1721 and T-1722 in pIJ101, apparently lying in a region for a hypothetical stem–loop structure in pIJ101.

### Genetic instability in *S. lividans* 1326 associated with loss of *sti* function of pHZ1358 derivatives derived from pIJ101

Deletions or point mutations in *rep*, *sti*, and/or *orf85* of pIJ101 (Fig. 1b) were each examined for their possible association with the segregational stability of pHZ1358 in *S. lividans* 1326. First, neither the silent mutation removing the *Bam*HI site in the *rep* gene (*rep**, a transversion from G to A in the indicated *Bam*HI site of pHZ1358 and pJTU391) nor a 694-bp deletion involving part of *orf85* in pHZ1358 (Fig. 1b) affected the stable maintenance of

pHZ1358, pJTU390, and pJTU391 in *S. lividans* 1326. In contrast, deletion of a 574-bp DNA fragment carrying the *sti* site (a second-strand site for plasmid replication in its functional orientation) obviously destabilized plasmid maintenance in *S. lividans* 1326 and immediately resulted in the frequent loss of pJTU390/pJTU391 derivatives (pJTU407/pJTU408, $sti^-$) in subsequent generations. Therefore, the function of *sti* is a key determinant for maintaining genetic stability of pIJ101-derived plasmids in its native host, *S. lividans* 1326.

The effect of *sti* on segregational maintenance in two other representative *Streptomyces* strains, *Streptomyces* sp. FR-008 and *S. hygroscopicus* 5008, was also examined. None of the plasmids tested, whether they possessed (pHZ1358 and pJTU390/pJTU391) or lacked (pJTU407/pJTU408) *sti* function, had an altered stability: more than 95% of the exconjugants of the plasmids lost their thiostrepton resistance in all cases after one round of nonselective growth.

## Structural instability of pHZ1358 derivatives in *S. lividans* 1326

All of the plasmid vectors studied, whether $sti^+$ (pJTU390 and pJTU391) or $sti^-$ (pJTU407 and pJTU408), $orf85^+$ (pJTU391) or $orf85^-$ (pHZ1358), $rep^+$ (pJTU390) or *rep** (pJTU391), displayed structural instability in *S. lividans* 1326, and the same region was deleted in each case, as shown by restriction analysis and Southern hybridization (Fig. 2). However, these plasmids displayed a different pattern of behavior in *Streptomyces* sp. FR-008 and *S. hygroscopicus* 5008. The same deletion as found in *S. lividans* 1326 could be detected with $sti^+rep^+$ (pJTU390) and $orf85^-$ (pHZ1358) derivatives both in *Streptomyces* sp. FR-008 and *S. hygroscopicus* 5008. However, both deleted and full-sized plasmids were detected after introduction of vectors with $sti^+$ (pJTU391), $rep^+$ or *rep** (pJTU407/pJTU408), and $orf85^+$ (pJTU391) (Fig. 2), although the deleted plasmids of pJTU408 is much less.

## Common DNA deletion is mediated by 36-bp direct repeat sequences at the boundaries between *E. coli*- and *Streptomyces*-derived DNA

PCR analysis of pHZ1358 deletions from *S. lividans* 1326, *Streptomyces* sp. FR-008, and *S. hygroscopicus* 5008 revealed that the deletion fusion generates a 36-bp sequence (5′-GAC CAA AGC GGC CAT CGT GCC TCC CCA CTC CTG CAG-3′), which is present as two separate copies (from nt 3,110 to nt 3,145 and from nt 8,526 to nt 8,561, respectively) in the same orientation in pHZ1358 (indicated as R1 and R2 in Fig. 1a,b). To prove that the deletion event results from an intramolecular homologous recombination event mediated by these 36-bp direct repeats, rather than a host mechanism to distinguish and

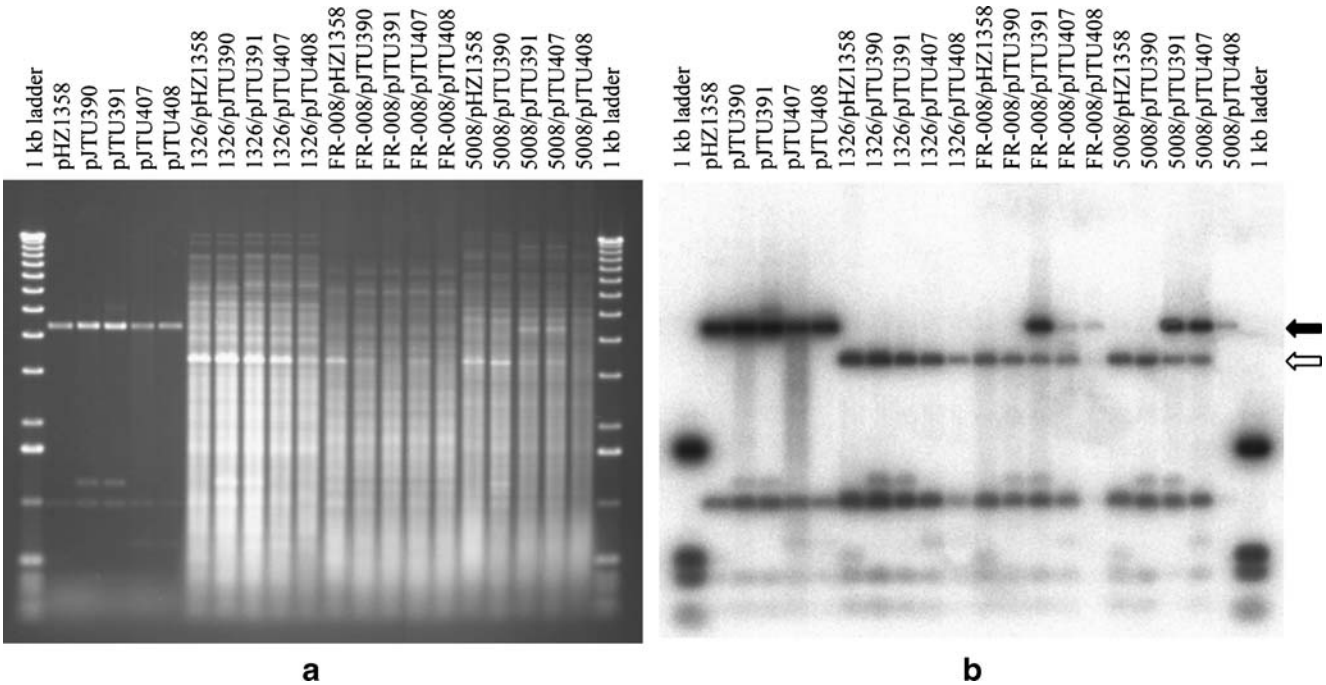


**Fig. 2** Detection of pHZ1358 and its derivatives in *S. lividans* 1326, *Streptomyces* sp. FR-008, and *S. hygroscopicus* 5008. **b** is the autoradiograph of **a** in which all of the indicated samples were digested with *Sma*I, blotted onto Hybond N membrane, and hybridized with $^{32}$P-dCTP-labeled pHZ1358. The *open arrow* points to the band generated after in vivo deletion of one of the two larger bands (*solid arrow*) mediated by the 36-bp direct repeats (shown as *triangles* in Fig. 1a)

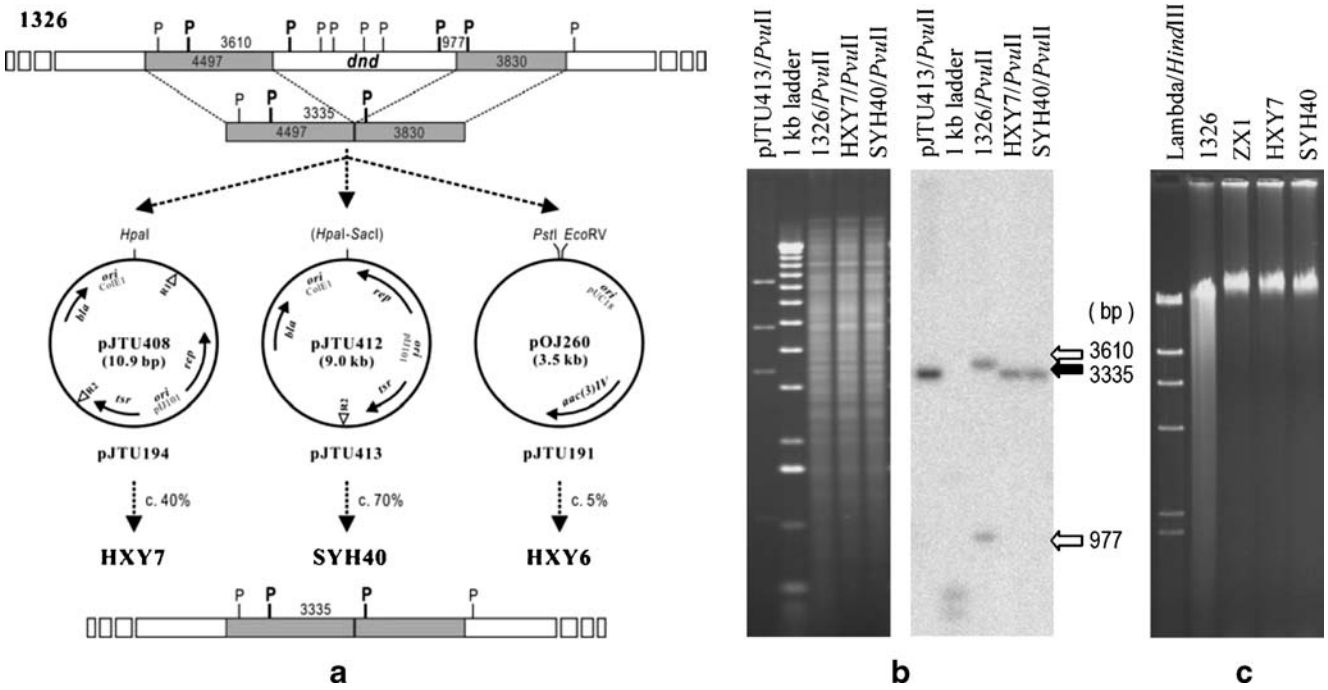


**Fig. 3** Use of pHZ1358 derivatives pJTU408 and pJTU412 for targeted gene replacement of the *dnd* gene cluster in *S. lividans*. Efficiency was ~70% when pJTU412 was used and ~40% when pJTU408 was used, compared to only ~5% with the suicide plasmid vector pOJ260. HXY7, SYH40, and HXY6 (**a**) are expected to be identical. This was confirmed for HXY7 and SYH40 by a Southern hybridization experiment (**b**) in which *Pvu*II-digested total DNA of 1326, HXY7 and SYH40 was probed with the isolated 3,335-bp *Pvu*II-digested DNA fragments from pJTU413. The expected positive fragments in negative control (1326) and positive control plasmid (pJTU413) or mutants (HXY7 and SYH40) were marked with *numbers* between two *bold Pvu*II sites (abbreviated in *P*) in **a** and indicated by *open* and *solid arrows* in **b**, respectively. The loss of the Dnd phenotype from all of the generated mutants in **a** was confirmed in **c** where wild-type 1326 DNA was seen to be degraded in vitro during electrophoresis, while both HXY7 and SYH40 are as stable as the control strain ZX1 (*dnd*$^-$). Phage DNA digested with *Hin*dIII provided as size standards

remove the *E. coli* DNA, a 2,486-bp *Sac*I–*Spe*I fragment (from nt 1,813 to nt 4,299 in pHZ1358, and the corresponding sequences in pJTU391; Fig. 1a,b), including one of the two 36-bp direct repeats, was deleted. When the resultant plasmids (pJTU412; Fig. 1b) was introduced into *S. lividans* 1326, *Streptomyces* sp. FR-008, and *S. hygroscopicus* 5008, their structures was found to be stable in every case.

## Use of structurally stable *sti*$^-$ pHZ1358 derivatives for efficient targeted gene replacement in *S. lividans* 1326

Two fragments (3,830 and 4,497 bp, respectively, shaded regions in Fig. 3a) flanking both sides of the sequenced *dnd* gene cluster from *S. lividans* 1326 (Zhou et al. 2004; Zhou et al. 2005), which encodes a sequence-selective, stereospecific phosphorothioate DNA modification that renders DNA susceptible to degradation during agarose gel electrophoresis (Zhou et al. 2004; Zhou et al. 2005; He et al. 2007; Wang et al. 2007), were cloned from cosmids as the two arms for the targeted deletion of the complete *dnd* gene cluster by gene replacement. They were cloned into the unique *Hpa*I site of pJTU408 and into the blunt-ended *Hpa*I–*Sac*I site of pJTU412, giving rise to pJTU194 and pJTU413, respectively (Fig. 3a). After individual conjugal transfer from *E. coli* ET12567 carrying RP4 into *S. lividans* 1326, approximately $10^{-3}$ exconjugants per donor could be obtained after selection for thiostrepton. Exconjugants were subsequently inoculated onto SFM plate for two rounds of nonselective growth for 7–10 days before selection by replica plating for Thio$^S$ colonies. Almost all of the tested colonies lost thiostrepton resistance, and about 40% of pJTU194 exconjugants and about 70% of pJTU413 exconjugants were found to have an obvious deletion of the 6,462-bp DNA containing the entire *dnd* gene cluster from the chromosome of *S. lividans* 1326, as confirmed by Southern hybridization (Fig. 3b). Total DNA of *S. lividans* 1326 and of the suspected *dnd* deletion mutants (HXY7 and SYH40) was subjected to electrophoresis under conditions that would degrade the DNA of *S. lividans* 1326. The DNA of both HXY7 and SYH40 remained undegraded (Fig. 3c), confirming the expected deletion of the *dnd* gene cluster from *S. lividans* 1326.

The deletion construct with the same arms as pJTU194 and pJTU413 was also inserted into a blunt-ended site of a

suicide vector pOJ260 (Bierman et al. 1992) after digestion with *Pst*I and *Eco*RV. When the resulting plasmid pJTU191 was used to obtain gene replacement in *S. lividans* 1326 using the procedure as described above, only about 5% of pJTU191 exconjugants were found to have the expected *dnd* deletion. When representative clones of mutant HXY6 (Liang et al. 2007) were compared with HXY7 and SYH40, either by Southern hybridization or assay of Dnd phenotype, they were found to be indistinguishable (not shown).

## Discussion

The successful use of pHZ1358 for targeted gene replacements in many *Streptomyces* species, but not in its native host, *S. lividans*, depends on its segregational instability in the heterologous strains. This prompted us to characterize the genetic determinant(s) originating from pIJ101 that confer the segregational stability in *S. lividans*. Our results show that *sti* is the responsible element, while additional factors examined (including a silent mutation removing the *Bam*HI site in the *rep* gene (*rep**) and a 694-bp deletion involving part of *orf85*) have no effect. The function of *orf85* remains unknown.

The finding of *sti*$^-$-mediated genetic instability for pIJ101-derived plasmids in *S. lividans* 1326 is consistent with a phenomenon observed for many commonly used *sti*$^-$ pIJ101 derivatives (e.g., in *S. lividans* 1326 transformed with pIJ702 [carrying the $MelC_1C_2$ operon encoding melanogenesis leading to black colony formation], the white colonies arose at a rate of about 12% after one round of nonselective growth on plate).

The *sti* element is known to contain a major site for the second-strand synthesis of pIJ101 by the rolling circle mechanism, and its absence severely reduces the conversion of single-stranded plasmid DNA into double-stranded forms. The exact mechanism of the genetic instability constantly observed with *sti*$^-$ pIJ101 derivatives remains obscure, but since the absence of *sti* does not seem to cause segregational instability in other two *Streptomyces* strains tested, the mechanism may require an unidentified *trans*-acting element specific for *S. lividans*.

The importance of a 36-bp direct repeat sequence at the boundaries between *E. coli*- and *Streptomyces*-derived DNA in mediating deletions in *S. lividans* 1326, *Streptomyces* sp. FR-008, and *S. hygroscopicus* 5008 accords with previous findings for bifunctional *Streptomyces–E. coli* vectors (Bai et al. 2006; Chen et al. 2003) and supports the idea that such deletions are generally caused by homologous recombination, rather than a specific host mechanism to distinguish and remove the *E. coli*-derived DNA.

The present study has demonstrated that the structurally stable *sti*$^-$ plasmids, pJTU408 and pJTU412, allow efficient targeted gene replacement in the widely used strain *S. lividans*, in contrast to pHZ1358 or the suicide plasmid pOJ260 (Fig. 3a). The higher frequency of targeted gene replacement obtained using pJTU412 than pJTU408 is likely due to the removal of one of the two 36-bp direct repeat sequences in pJTU412 because this prevents the rapid loss of the plasmid insert(s) which is the substrate for the replacement event. Plasmid pYH7 containing an additional apramycin resistance gene in pJTU412 has recently been used for the successful disruption of biosynthetic gene clusters encoding the macrocyclic polyketides meridamycin and polyether nigericin in *Streptomyces* sp. DSM4137 (Sun et al. 2006; Harvey et al. 2007) and seems likely to prove a useful tool for targeted gene replacements in many other *Streptomyces*, as well as in *S. lividans*.

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