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STRUCTURAL STABILITY OF HETEROLOGOUS GENES CLONED IN *STREPTOMYCES* PLASMID pIJ702

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A recombinant plasmid pWCL1 containing *Streptomyces* plasmid pIJ702, *E. coli* plasmid pUC12, and hepatitis B viral surface antigen (HBsAg) gene was stably maintained in *E. coli*, but exhibited structural instability in *S. lividans* 1326. The deletions were found ranging from 2.75 to 5.65 kilobases (kb) and most of them occurred within the melanin (mel) gene of pIJ702, resulting in the loss of part of the *mel* gene sequence plus the insert. The removal of the pUC12 sequence from pWCL1 eliminated the instability. However, pUC12 alone inserted in either orientation on pIJ702 also caused the deletion in *S. lividans* 1326. The results indicated that the structural instability of hybrid plasmid of pIJ702 depended on the interaction between the *mel* sequence and the inserted sequence.

Streptomyces has attracted considerable attention as a highly differentiated prokaryote, and as primary sources of economically important antibiotics. Moreover, their versatile activity of transcription and translation systems may prove of practical use for the expression of heterologous genes (1, 2), because *E. coli* and *B. subtilis* are relatively fastidious in this respect. However, there is no guarantee that heterologous genes would be satisfactorily replicated in *Streptomyces*. Notable is also the fact that *Streptomyces* species have a genomic instability problem (3, 4). Thus, the stability of heterologous genes cloned in *Streptomyces* is one of the important factors pertinent to the expression of these genes.

In this communication the stability of *E. coli* plasmid pUC12 (5) and the hepatitis B viral surface antigen (HBsAg) gene (6) in a *Streptomyces* plasmid pIJ702 (7) was reported. The results indicated that while HBsAg

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gene alone was stable in pIJ702, but insertion of pUC12 destabilized the plasmid.

MATERIALS AND METHODS

Enzymes and Reagents. Restriction endonucleases, T4 DNA ligase, bacterial alkaline phosphatase and S1 nuclease were from Bethesda Research Laboratory (BRL, Gaithersburg, MD, USA) or Boehringer Mannheim (Mannheim, West Germany) and were used as suggested by the supplier. Thiostrepton was kindly supplied by S. J. Lucania (E. J. Squibb and Sons, New Brunswick, NJ, USA).

Plasmids. *E. coli* plasmid pUC12 has been described previously (5). *Streptomyces* plasmid pIJ702 (7) carrying two markers, thiostrepton-resistance determinant (tsr) and *mel* gene, was kindly provided by Professor E. Katz (Georgetown University, Washington D.C., USA). Plasmid pTC13 was constructed by inserting the 1.4 kb *Bam*HI fragment of the HBsAg gene from pTWS1 (6) at the downstream *Bam*HI site of the *lac* promoter of pUC12.

Bacterial Strains, Culture Conditions, and Transformation. *S. lividans* 1326 and *E. coli* JM103 were used as recipient hosts for recombination plasmids. *E. coli* strain JM103 was grown in L broth or L agar and transformed by the method of Cohen *et al.* (8). Preparation and transformation of *S. lividans* 1326 protoplasts were as described by Thompson *et al.* (9). Protoplasts were allowed to regenerate on R2YE plates (10) for 16 h and then overlayed with soft nutrient agar (Difco) containing 200 μg/ml thiostrepton.

Plasmid DNA Preparation. Plasmids were isolated from *E. coli* by the procedure of Birnboim and Doly (11). Plasmid pIJ702 or its derivatives were isolated from *S. lividans* 1326 according to the procedure of Kieser (12).

Miscellaneous Methods. Colony hybridization for screening the presence of specific DNA sequence within the transformants was performed as described previously (13). Nick translation of plasmid DNA (14) and Southern hybridization (15) were carried out according to the procedures previously described.

RESULTS

Stability of pIJ702 derivative carrying pUC12 and HBsAg genes

An *E. coli-Streptomyces* shuttle vector pWCL1 (9.75 kb) was constructed in *E. coli* by ligating pIJ702 cleaved at the unique *Sst*I site and the *Sst*I-cut pTC13 which is a pUC12 derivative containing the HBsAg gene inserted downstream from the *lac* promoter (Fig. 1A). The restriction map of pWCL1 was confirmed by the restriction enzyme digestion analysis and Southern hybridization experiments using the 1.4 kb *Bam*HI fragment of HBsAg gene as a probe (data not shown).

The plasmid pWCL1 was transferred to *S. lividans* 1326 and screened for the thiostrepton-resistant transformants. Restriction endonuclease digestion and dot hybridization with three different probes (pUC12, HBsAg, and tsr genes) were utilized for the analysis of the plasmids isolated from the

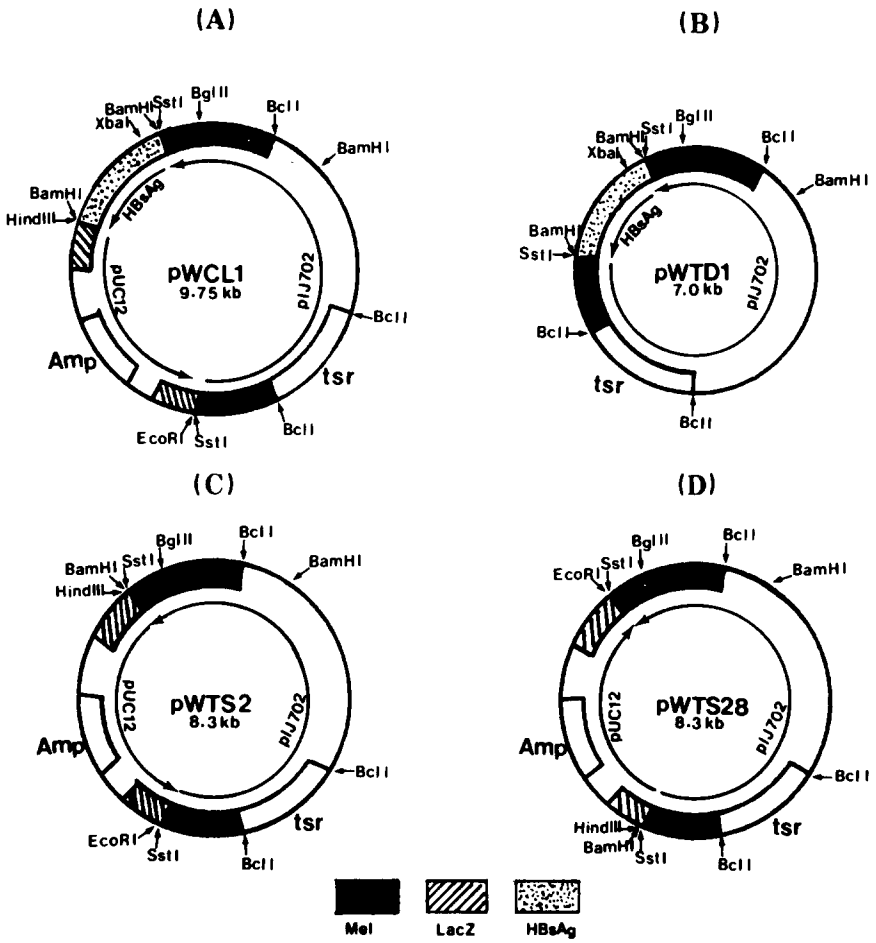

Fig. 1. Restriction maps of plasmids pWCL1, pWTD1, pWTS2, and pWTS28.

pWCL1 transformants. As indicated in Table 1 and Fig. 2, several plasmids isolated from pWCL1 transformants (e.g. 1-14, 1-17) were found to lack part of pUC12 or HBsAg genes. Plasmids with the complete deletion of pUC12 and HBsAg sequences were also found in the transformants 1-11 and 1-15. The final sizes of these deleted plasmids ranged from 4.1 to 7.0 kb.

Some transformants (e.g. 1-10, 1-14, 1-17, 1-215) contained mixed groups of plasmids, indicating that the deletion events were probably occurring during the growth of mycelium. Although a few initial transformants (e.g. 1-13) appeared to harbor an entire intact pWCL1 plasmid, successive culturing of the mycelium on the solid medium led to a loss of 5.65 kb from the vector. Southern hybridization results indicated that both HBsAg gene and pUC12 vector

Table 1. Properties of the plasmid DNA from the variants of pWCL1 transformants

transformants	size (kb) of plasmids	hybridization to		
		pUC12	HBsAg	tsr
1-13	9.75	+++	+++	+
1-10, 215, 216	9.75, 4.1	++	++	+
1-14	7.0*, 5.1	++	+++	+
1-3	7.0, 5.1*	±	+	+
1-17	7.0, 4.1*	±	±	+
1-11, 15	5.1	-	-	+

Plasmid DNA was treated with the unique cut *Cla*I endonuclease which lies within the tsr gene. The symbol of star indicates the predominant form. Hybridization results were from Fig. 2. The relative intensity of the hybridization are scored as +++, ++, +, ±, and -, in decreasing order.

were completely lost. The resulting plasmid of 4.1 kb is even smaller than pIJ702. Thus, none of the pWCL1 transformants in *S. lividans* 1326 stably maintained the whole plasmid.

Stability of pIJ702 derivative carrying HBsAg gene

To investigate whether the pUC12 sequence was involved in the instability of pWCL1, the 2.7 kb pUC12 sequence was excised from pWCL1 by *Eco*RI and *Hind*III digestion and the resulting sticky ends were repaired with the klenow fragment of DNA polymerase I and then ligated. The ligated DNA was used to transform into the protoplasts of *S. lividans* 1326. Six out of 41 transformants had been selected to have the HBsAg gene by colony hybridization. These 6 transformants contained plasmids of identical size (7.0 kb) and restriction map (Fig. 1B), as expected for pWCL1 deleted of the

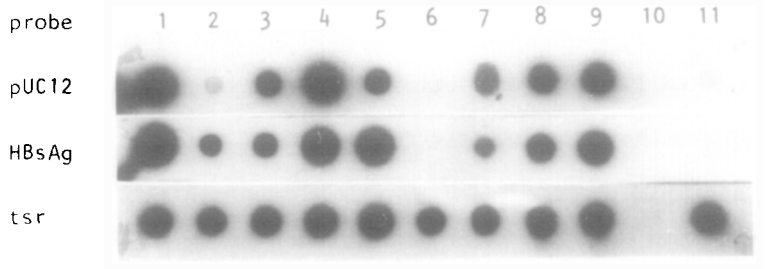

Fig. 2. Dot hybridization of 32P-labelled DNA probes to plasmid DNA isolated from pWCL1 transformants.
Aliquots (0.5 μg) of host chromosomal DNA, pWCL1 plasmid DNA, and DNA isolated from each of the pWCL1 transformants, were spotted on nitrocellulose paper and hybridized with DNA probes as indicated.
1: pWCL1; 2: 1-3; 3: 1-10; 4: 1-13; 5: 1-14; 6: 1-11; 7: 1-17; 8: 1-215; 9: 1-216; 10: chromosomal DNA of *S. lividans* 1326; 11: 1-15.

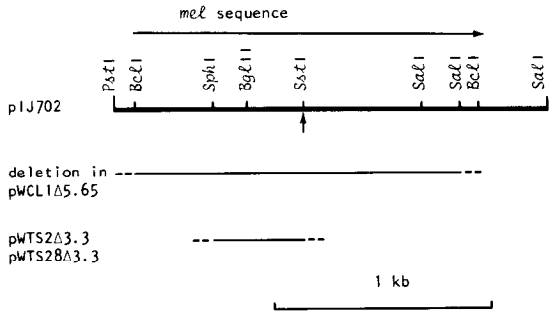

Fig. 3. Deletion on pWCL1Δ5.65, pWTS2Δ3.3, and pWTS28Δ3.3 plasmids. The thick line represents the *mel* gene and its flanking sequence (*PstI-SalI*). The horizontal arrow indicates the *mel* gene. The vertical solid arrow indicates the *SstI* site at which pUC12 and HBsAg sequences were inserted. The sizes of the deletion are shown as solid lines. The dashed lines represent the uncertain end points.

pUC12 sequence. These plasmids were designated pWTD1 to pWTD6. They were all stably maintained in the initial transformants as well as many subsequent subculturings without any deletion observed. This is in contrast with the pronounced deletion observed for pWCL1 which contained both HBsAg and pUC12 sequences.

Stability of pIJ702 derivative harbouring *E. coli* pUC12 sequence

The stability of pUC12 cloned in pIJ702 was further investigated. For this purpose plasmids, pWTS2 and pWTS28 with 2.7 kb pUC12 insert in either orientation in pIJ702 *SstI* site (Fig. 1C, D), were constructed in *E. coli*. Plasmids isolated from *S. lividans* 1326 transformed with either pWTS2 or pWTS28 initially exhibited no detectable deletion. However, after subculturing 4 to 5 times, the sizes of the plasmids isolated from both groups of transformants all were decreased to 5.1 kb. Thus, the pUC12 insert in pIJ702 eventually caused a deletion of 3.3 kb, larger than pUC12 itself, again indicating that deletion occurred inside the pIJ702.

Mapping the deletion plasmids

To learn more about the molecular basis of the instability of pWCL1, pWTS2, and pWTS28 in *S. lividans* 1326, the extent of deletion was analyzed with a number of restriction endonucleases. As shown in Fig. 3, the deletion end points fell within the segment of *mel* sequence flanking the insert. In the deleted plasmid of pWCL1 (pWCL1Δ5.65), the whole 1.55 kb *mel* gene

moiety was completely deleted; whereas in the deleted plasmid of pWTS2 (pWTS2Δ3.3) or pWTS28 (pWTS28Δ3.3), 0.43 kb segment containing the *Sph*I and *Sst*I sites of the *mel* gene was missing. There was no DNA rearrangement of the plasmids other than these deletion.

DISCUSSION

The *Streptomyces* plasmid pIJ702 has been used widely in cloning experiments (16, 17). The *mel* gene on it provides a very convenient marker for insertional inactivation. However, structural instability of the resulting hybrid plasmid was detected (2). In this study we showed that the HBsAg gene was stably maintained on pIJ702, while insertion of pUC12 alone or in conjunction with the HBsAg gene induced deletion.

The cause of this structural instability is not clear, but a few clues may be deduced. The instability induced by the pUC12 insertion cannot be due to its larger size alone, because stable inserts larger than pUC12 have been reported (17, 18). Nor can the instability be only a function of a particular orientation, because both orientations of pUC12 caused deletions. The deletions appeared to occur in discrete sizes during subculturing. The eventual stable plasmids tend to lose all the inserted heterologous sequences. The deletion would not be result of homologous recombination, because there is no large stretch of repeated sequences in the regions of deletions (19). Nevertheless, whatever the mechanism of the deletion, it appears to result from the characteristic of insert introduced.

The occurrence of plasmid deletion is not peculiar to *Streptomyces*. In *B. subtilis*, instability was also frequently observed when foreign DNA was inserted into plasmid vectors (20). Such instability may be due to intramolecular rearrangement and is often independent of the general recombination systems of the host strains (21, 22). At present little is known about the molecular basis of *Streptomyces* plasmid instability. It will be of further interest to understand whether these deletional mechanism of *Streptomyces* is mediated by the host recombination system.

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