Gene disruption and gene replacement in *Streptomyces* via single stranded DNA transformation of integration vectors

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ABSTRACT

For the isolation of single stranded plasmid DNA, various *E. coli* and *E. coli – Streptomyces* shuttle plasmids were equipped with the phage f1 replication origin. The transformation of some representative *Streptomyces* species with plasmid vectors occurred irrespective of whether single or double stranded DNA was used. In contrast, the transformation of *Streptomyces* was 10 to 100 times more efficient when an integration vector was in the single stranded form as opposed to the double stranded form. *Streptomyces viridochromogenes* was transformed by single stranded DNA integration vectors in order to replace the *pat* by the *tsr* gene and generate mutants unable to synthesize phosphinothricin – tripeptide (PTT).

INTRODUCTION

Two striking features distinguish *Streptomyces* from other bacteria: a complex cycle of morphological development and the capacity to synthesize a variety of different metabolites. In recent years, the interest on *Streptomyces* host-vector systems has increased dramatically. Many *Streptomyces* plasmid vectors were constructed (1) and successfully applied for gene isolation and gene characterization (2). Mutants with defective antibiotic biosynthetic pathways have proved useful in the elucidation of antibiotic synthesis. For this reason, gene disruption and gene replacement methods have been developed to produce such mutants.

The *Streptomyces* temperate phage ΦC31 was modified by a deletion in the attachment site (3). The stable maintenance of the integrated phage is possible via recombination of the cloned fragments. By using ΦC31 derivatives, the mutation and isolation of genes participating in antibiotic production such as the methylenomycin biosynthetic gene cluster was possible (4). The application of the ΦC31 system, however, is restricted to the host range of the phage which comprises about 50% of the *Streptomyces* strains tested so far (5). Another system described by Anzai *et al.* (6) is based on the efficient elimination of plasmids from *S. hygroscopicus* by protoplasting and regeneration. In this case, an *in vitro* altered DNA fragment was cloned into the *Streptomyces* vector pIJ680 (7), and *S. hygroscopicus* was transformed with the resulting plasmid. After curing the cells, colonies were detected which carried the mutation on the chromosome. The mutation had apparently resulted from a gene replacement. However, to date, the application of this method has not been reported for other *Streptomyces* strains. In a recent paper (8), we reported the application of a vector system based on the inherently temperature-sensitive *S. ghanaensis* replicon pSG5, for gene disruption. When the vectors contained DNA fragments homologous to the host chromosome, mutants carrying integrated plasmids could be selected as a result of a temperature shift to 39°C. The application of temperature-sensitive plasmids is complicated by the occasional disintegration of the plasmids at permissive temperatures.

Therefore, a system which results in stable mutants and which is generally applicable to all *Streptomyces* strains, is desirable. Such systems are known for gram-negative organisms like rhizobia and make use of non-replicative vectors (9). Recently the application of non-replicative *E. coli* plasmids for generating mutants in the avermectin (10) and spiramycin (11) biosynthesis has been reported. We now have developed a method for *Streptomyces* using transformation with single stranded DNA of *E. coli* plasmids unable to replicate in *Streptomyces*. Thus, the advantages of the *E. coli* system such as efficient DNA isolation and rapid cloning procedures, can be applied to *Streptomyces*.

For the first time, a method is described in which non-replicative *E. coli* vectors in single stranded DNA form are used to generate *Streptomyces* mutants unable to synthesize antibiotics. We have demonstrated that the frequency of integration into the *S. viridochromogenes* chromosome was significantly higher when the donor DNA was single stranded, rather than double stranded.

MATERIALS AND METHODS

Bacterial strains, phages and plasmids

S. viridochromogenes Tü494 (12) was obtained from Prof. H.Zähner, University of Tübingen and *S. lividans* TK23 (13) from Prof. D.A.Hopwood, John Innes Institute, Norwich (U.K.).

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Table 1. Plasmids used in the present study

Plasmid	Structure[a]	Ref.
pFl = pT7/T3α-19	*E. coli* multifunctional vector for DNA-cloning, *in vitro* transcription and dideoxysequencing: derived from pUC19 (17), carrying *bla*, MCS[b], *lacZ'*, T7 and T3 promotors and phage f1 intergenic region	15
pSLE41	*E. coli* vector based on pACYC184, carrying *cat*, *tet*, *tsr*	18
pGM160	*E. coli*–*Streptomyces* shuttle vector: pSG5[c] replication region fused with pUC19 (17) carrying *aacC1 bla* and *tsr*	8
pGM4	*Streptomyces* vector: pSG5 replicon carrying *tsr* and *aph*I genes, cloning into the *Bam*HI site inactivates *aph*I	19
pES4	pGM4, carrying the 4 kb *Bam*HI fragment of *S. viridochromogenes* with PTT biosynthetic genes and the internal 0.8 kb *Bgl*II fragment (phosphinothricin *N*-acetyltransferase gene, *pat*)	20

[a]Genes: *aacC1*, gentamicin acetyltransferase gene from *Pseudomonas aeruginosa* (Selection in *E. coli* and *Streptomyces*); *aph*I, neomycin phosphotransferase gene from *S. fradiae* (Selection in *Streptomyces*); *bla*, β-lactamase gene from *E. coli* (Selection in *E. coli*); *cat*, chloramphenicol acetyltransferase gene from *E. coli* (Selection in *E. coli*); *lacZ'*, truncated β-galactosidase (*lacZ*) gene; *tet*, tetracycline resistance gene from *E. coli* (Selection in *E. coli*); *tsr*, thiostrepton resistance gene from *S. azureus* (Selection in *Streptomyces*) (21)
[b]MCS: multiple cloning site
[c]pSG5, endogenous *S. ghanaensis* plasmid (8), temperature–sensitive replication (22)

For transformation experiments, *S. ghanaensis* ATCC 14672 and *S. venezuelae* DSM40755, served as recipients. All cultures were grown under *Streptomyces* culture conditions (7).

E. coli JM83 (14) was used in cloning experiments. For the isolation of single stranded DNA after infection with helper phage M13K07 (15), *E. coli* NM522 (16) was applied.

The plasmid vectors are described in Table 1.

Construction of vectors

The E. coli Streptomyces shuttle plasmid pDH303, test vector for the efficiency of single/double stranded DNA transformation. To construct the shuttle plasmid pDH303 (Fig. 1), plasmid pFl (Table 1), carrying the gene conferring resistance to ampicillin for selection in *E. coli* (15), was linearized with *Sst*I and fused with the 3.4 kb *Sst*I fragment of pGM160 (8). This 3.4 kb *Sst*I fragment of pGM160 contains the replication region of the *Streptomyces* plasmid pSG5 (22) and the thiostrepton resistance marker enabling selection in *Streptomyces*. pDH303 could be isolated as double stranded or single stranded DNA (Materials and Methods).

The integration vector pDH5. Plasmid pDH5 (Fig. 3) is an integration vector for *Streptomyces*, based on vector pF1 (Table 1) with the intergenic region of phage f1 for single stranded DNA isolation. It contains the thiostrepton resistance gene which enables the primary selection of *Streptomyces* clones which carry integrated plasmids.

A 1.05 kb *Bcl*I fragment of pSLE41 (18) containing the gene conferring resistance to thiostrepton, was inserted into an *Xho*II site which is located in a nonessential region of the plasmid pF1. Since seven *Xho*II sites were present on this plasmid (two within the ampicillin resistance gene, one in the MCS and four between the resistance marker and the origin of replication of the plasmid) the 1.05 kb *Bcl*I fragment was ligated with *Xho*II partially digested pF1. Plasmid pDH5 carries the thiostrepton marker in an *Xho*II site which is located between the origin of replication and the ampicillin resistance gene. Hence, the intact MCS and the possibility of cloning into, and thus, inactivating the *lacZ* gene, were retained.

DNA isolation

Isolation of double stranded DNA. S. viridochromogenes chromosomal DNA was isolated according to the method of Altenbuchner and Cullum (23), *Streptomyces* plasmid DNA according to Kieser (24). *E. coli* double stranded plasmid DNA was isolated as described by Holmes and Quigley (25).

Isolation of single stranded phage DNA. E. coli single stranded phage DNA was obtained after the plasmid-carrying strain was infected with helper phage, M13K07 (15). This phage was developed in order to maximize interference of phage replication, thereby increasing the ratio of plasmid DNA to phage DNA. Preparation of the single stranded DNA was carried out as described by Messing et al. for M13 vectors (26). After PEG-mediated pelleting (4% polyethylene glycol 6000, 0.5 M NaCl), the phages were resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). The phage suspension was treated twice with 1 volume phenol/chloroform (pH 8.0) and single stranded phage DNA was precipitated overnight at −20°C with 1/10 volume 3 M Na acetate and 2.5 volume ethanol. By this method, up to 5 μg of single stranded DNA could be extracted from a 10 ml culture. The yield depended on the insert size of the plasmid and on the multiplicity of infection (27).

Assay of PTT-production

PTT production was tested on agar plates seeded with *Bacillus subtilis* ATCC6633 spores, as described elsewhere (28). PTT-producing and non-producing mutants could be distinguished by the appearance of an inhibition zone which indicated the synthesis of the antibiotic PTT.

Southern hybridization

Hybridization experiments were performed using a non-radioactive DNA labelling and detection kit, as recommended by the commercial supplier (Boehringer, Mannheim).

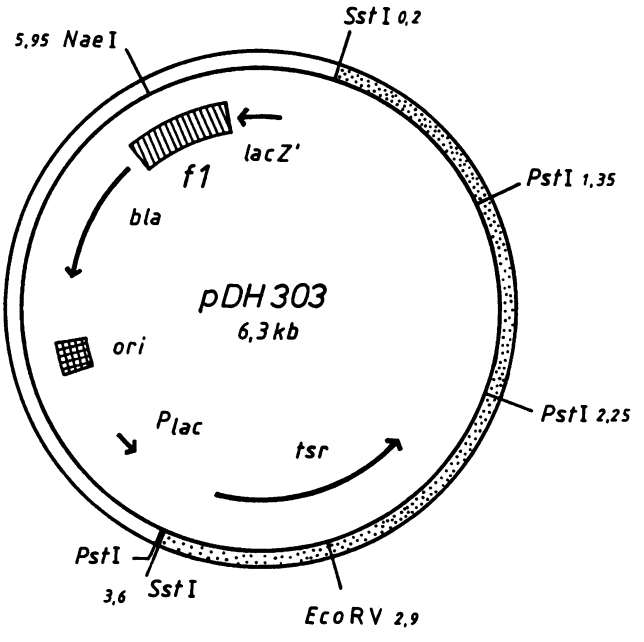

Fig. 1. The *E. coli Streptomyces* shuttle plasmid pDH303. The shuttle plasmid pDH303 consists of the *E. coli* replicon pF1 (open bars) (15) fused with the 3.4 kb *Sst*I fragment of the plasmid pGM160 (8) (dotted). Abbreviations: *bla*: β-lactamase gene, f1 (hatched): phage f1 intergenic region, kb: kilobase pairs, *lacZ'*: truncated β-galactosidase gene, ori (double hatched): origin of pUC plasmid replication, P_{lac}: β-galactosidase gene promotor, *tsr*: thiostrepton resistance gene.

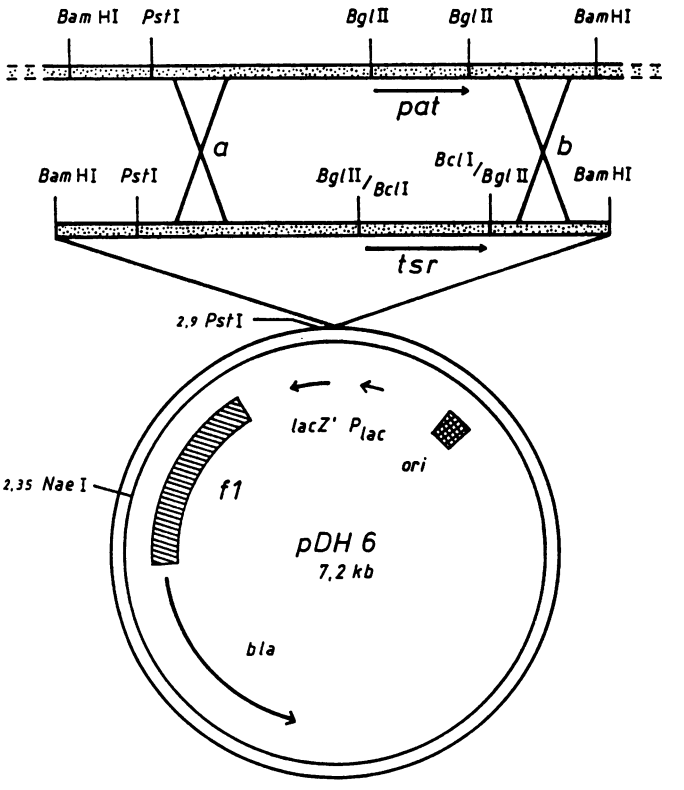

Fig. 2. The integration vector pDH6 and its integration into the *S. viridochromogenes* chromosome. The plasmid pDH6 consists of the *E. coli* vector pF1 (15), which contains a 4 kb *S. viridochromogenes* DNA fragment (dotted) (20). The internal *Bgl*II fragment with the *pat* gene (31) was replaced by the *Bcl*I fragment carrying the *tsr* gene (21). Homologous recombination of plasmid pDH6 into the *S. viridochromogenes* chromosome was possible due to the DNA fragments adjacent to the antibiotic resistance gene. Single crossover (a or b) led to integration of pDH6 into the chromosome, double crossover (a and b) delivered gene replacement mutants. Abbreviations are as in the legend to Fig. 1, *pat*: phosphinothricin *N*-acetyltransferase gene.

RESULTS

Transformation of *Streptomyces* species with plasmid vectors using single and double stranded DNA

The very effective restriction system of various *Streptomyces* strains (29), prevents efficient transformation with plasmid DNA derived from foreign strains. The efficiency of the transformation would be further reduced when integration has to occur. The use of plasmids in the single stranded DNA form, could possibly overcome these difficulties. In order to test this, the transformation efficiencies obtained with single and double stranded DNA forms isolated from *E. coli*, had to be compared. For this purpose, the shuttle plasmid pDH303 was constructed. This plasmid consisted of two *E. coli* origin of replication regions (pUC, f1), one *Streptomyces* replicon (pSG5) and two marker genes; one selectable in *Streptomyces* and one in *E. coli* (Fig. 1). Plasmid DNA was obtained from *E. coli* in the single stranded form by the phage DNA isolation method (Materials and Methods), and in the double stranded form by the plasmid DNA isolation procedure (Materials and Methods). The transformation efficiencies with pDH303 single and double stranded DNA, were compared in *E. coli* and *Streptomyces* strains. Using *E. coli* NM522 as the recipient, the transformation efficiency with the double stranded pDH303 shuttle plasmid was 10 fold higher than that with single stranded DNA (Table 2). When *Streptomyces* were transformed, there was no significant difference between the double or single stranded DNA, even if one takes into consideration that 1 μg of single stranded DNA contains twice as many plasmid molecules as 1 μg of double stranded DNA. The variation in transformation frequencies corresponds to the different transformation efficiencies reported for these strains (18, 30).

Table 2. Transformation efficiencies with the shuttle plasmid pDH303

Species/strain	Transformants/μg single stranded DNA[a]	Transformants/μg double stranded DNA[b]
E.coli NM522	3.4×10^5	3.8×10^6
S. lividans TK23	1.9×10^4	2.5×10^4
S. ghanaensis	4×10^0	8×10^0
S. venezuelae	1.2×10^1	1.6×10^1
S. viridochromogenes	1×10^2	7×10^1

[a]prepared by the phage DNA isolation method
[b]prepared by the plasmid DNA isolation method

Transformation of *Streptomyces viridochromogenes* with integration vectors using single and double stranded DNA

It has been observed in various bacteria, that integration via homologous recombination is greatly facilitated by the presence of single stranded DNA (31). By analogy to this, single stranded rather than double stranded integration vectors could lead to higher rates of insertion into the host chromosome. Plasmid pDH6 (Fig. 2) was used to investigate this possibility. This plasmid is a derivative of pF1 (Table 1), which contains a *Streptomyces* chromosomal fragment, and which could be isolated in the single

or double stranded DNA forms. The chromosomal fragment contained in pDH6 was a 4 kb *Bam*HI fragment (20) of the PTT-producer *S. viridochromogenes* (pES4, Table 1). This 4 kb fragment contained at least three genes required for the biosynthesis of PTT (28), including the *pat* gene, located on an internal 0.8 kb *Bgl*II fragment (32). This BglII fragment was replaced by a 1.05 kb *Bcl*I fragment containing the thiostrepton resistance gene from *S. azureus* (21), to enable the primary selection of transformants in *S. viridochromogenes* to be made (Fig. 2).

In order to determine whether the integration frequency was influenced by the DNA configuration, *S. viridochromogenes* was transformed with the vector pDH6 in single and double stranded DNA forms. The use of single stranded DNA resulted in 10 to 100-fold more thiostrepton resistant colonies which carried plasmid pDH6 integrated into the chromosome, than when double stranded DNA was used (Table 3). Since the transformation frequency itself was the same for double and single stranded DNA (Table 2) it can be concluded that the recombination rate was indeed enhanced by single stranded DNA.

The integration of plasmid pDH6 into the chromosome was verified by three methods:

(1) *E. coli* was transformed with DNA isolated from the thiostrepton resistant colonies. No *E. coli* transformants were obtained, demonstrating that autonomous plasmid DNA was not present in the thiostrepton resistant cells.

(2) Further proof was provided by hybridizing pDH6 plasmid DNA with total DNA from the transformants demonstrating that recombination occurred between the cloned *Bam*HI fragment and the corresponding chromosomal region (data not shown).

(3) Furthermore, total DNA isolated from *S. viridochromogenes*, cleaved with *Bam*HI or *Pst*I, and religated, resulted in transformants of *E. coli*. A restriction analysis revealed the original pF1 plasmid in the case of the *Bam*HI digestion, and in the case of the *Pst*I cleavage, the pF1 plasmid with adjacent DNA fragments (Fig. 2). Thus, the entire plasmid had integrated via homologous recombination into the *S. viridochromogenes* genome. This procedure can be used to isolate a DNA fragment of interest together with its flanking chromosomal region.

Gene replacement in *Streptomyces viridochromogenes*: A chromosomally located *pat* gene is replaced by *tsr*

The integration of plasmid pDH6 into *S. viridochromogenes* did not disrupt any genes, since the *Streptomyces* DNA fragments of pDH6 contained no fragments which were incomplete central fragments of genes. However, gene replacement could occur after double crossover in the homologous DNA situated adjacent to the thiostrepton resistance gene (Fig. 2). The exchanged 0.8 kb *Bgl*II homologous fragment would result in the PTT-non-producing, PTT-sensitive phenotype.

Table 3. Transformation efficiencies with the integration vector pDH6

Protoplast isolation[a]	Transformants/μg single stranded DNA[b]	Transformants/μg double stranded DNA[c]
1	202	3
2	623	20
3	78	7

[a]three different protoplast isolations of *S. viridochromogenes* Tü494
[b]prepared by the phage DNA isolation method
[c]prepared by the plasmid DNA isolation method

Therefore, 100 colonies containing the vector pDH6 integrated into the chromosome were examined further after single spore passage. 96 colonies were phenotypically the same as the wild type with respect to PTT production and resistance, but their resistance to thiostrepton indicated that plasmid pDH6 was chromosomally integrated. 4 colonies were PTT sensitive and unable to produce PTT. Hybridization experiments indicated that the chromosomal *Bgl*II fragment was deleted in those mutants unable to synthesize PTT, confirming that gene replacement by double crossover, had occurred.

Hence, by using *E. coli* single stranded DNA integration plasmids, it was possible to generate mutants by plasmid integration followed by gene replacement.

Gene disruption in *Streptomyces viridochromogenes*: Generation of mutants unable to synthesize phosphinothricin-tripeptide

Exploiting the highly efficient integration of transformed *Streptomyces* with single stranded DNA, an integration vector was constructed, which should have widespread use in gene disruption and gene replacement experiments in *Streptomyces* strains. Vector pDH5 (Fig. 3), consisted of the plasmid pF1 (Table 1) containing the thiostrepton resistance gene to enable selection in *Streptomyces*. The cloning can be performed in *E. coli* applying standard techniques. The MCS and the *lacZ'* system facilitate the cloning of DNA fragments into the vector. Following transformation of *Streptomyces*, plasmid integration into the chromosome, by homologous recombination of the cloned fragments, can occur. If the vector contains internal fragments of genes or operons the integration will result in gene disruption and thus mutation. The vector pDH5 can also be used for gene replacement experiments. The mutated DNA of the gene of interest cloned into pDH5, can insert itself into the host genome and replace the wild type DNA by double recombination.

In order to prove the applicability of pDH5, it was employed to construct mutants unable to produce PTT. *Xho*II digested fragments (approximately 200–600 bp in size) of the cosmid

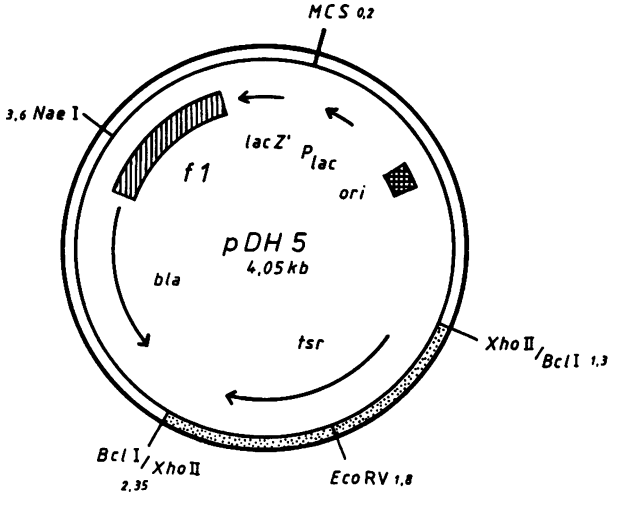

Fig. 3. The physical map of the general integration vector pDH5. The general integration vector pDH5 is based on the *E. coli* plasmid pF1 (15). It contains the thiostrepton resistance gene (dotted) and a multiple cloning site (MCS). The MCS comprises the following single restriction sites: *Eco*RI, *Sst*I, *Kpn*I, *Xma*I, *Ava*I, *Sma*I, *Bam*HI, *Xba*I, *Acc*I, *Hin*cI, *Pst*I, *Sph*I, *Hin*dIII. Abbreviations are as in the legend to Fig. 1.

pPTcos1 which contains most of the PTT biosynthetic gene cluster (28), were inserted into the *Bam*HI site of pDH5. After primary selection for *lacZ* inactivation in *E. coli*, 16 different clones were chosen for further study.

The PTT-producing strain *S. viridochromogenes* was transformed with double stranded plasmid DNA as well as single stranded phage DNA, isolated from the 16 *E. coli* clones. *Streptomyces* colonies containing the plasmid integrated into the chromosome, were selected on medium supplemented with thiostrepton and tested for their ability to synthesize PTT (Materials and Methods). Three of sixteen clones tested were unable to produce PTT.

As previously observed, the transformation of *S. viridochromogenes* and subsequent integration of these constructs was 10 to 100 times more efficient when single stranded DNA was used rather than double stranded DNA. The smallest fragment which was inserted was about 200 bp in length. Since the integration of all vectors was confirmed, an homologous DNA fragment of 200 bp, is apparently sufficient in length for recombination in *S. viridochromogenes* to occur.

DISCUSSION

In this paper we describe an efficient and generally applicable integration system for *Streptomyces* based on *E. coli* replicons, which are unable to replicate in *Streptomyces*. The plasmids can be integrated into the *Streptomyces* chromosome by a single reciprocal recombination of fragments located on the plasmid. The selection of the integrated plasmid in *Streptomyces* is made possible by the thiostrepton resistance gene of *S. azureus* inserted into a non-essential region of the plasmid. Because of the inherently low transformation and integration rates with the *E. coli* plasmid DNA, the phage f1 replication origin was cloned into the vectors to enable the isolation and transformation with single stranded DNA. The use of single stranded as compared to double stranded DNA, enhanced the efficiency of the system by a factor of 10 to 100. This was sufficient to generate numerous colonies, which carried an integrated plasmid. Since no difference in the transformation frequency with shuttle vectors could be detected when double or single stranded DNA was used, we assume that the integration into the chromosome is stimulated by the single stranded DNA form. It is known that in *E. coli* single stranded DNA rather than double stranded DNA is the substrate of the RecA protein, which is necessary for integration via homologous recombination (31).

Working with this system is convenient, because all cloning procedures can be carried out in *E. coli*. Additionally, the isolation of single and double stranded DNA is easy and can be efficiently performed. If internal fragments of genes or operons were cloned into these vectors, the integration via homologous recombination into the corresponding region of the chromosome would lead to gene disruption. The applicability of the system has been proven by generating PTT-non-producing mutants. It is highly likely that the system will work in almost every *Streptomyces* strain, but it cannot be excluded that there may be species in which the procedure is not possible. Furthermore the advantages described for integration systems in other organisms (9) also apply to the *Streptomyces* system; integrated plasmids can be rescued by circularization of the appropriate restriction fragments and by retransformation of *E. coli*.

The inability of plasmid pDH5 to replicate in *Streptomyces* confers additional uses for the plasmid. Cloning of certain genes into plasmid pDH5 and integration into the chromosome via an additional chromosomal fragment may be a promising alternative to cloning into low copy number plasmids.

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