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Gene manipulation in *Streptomyces*

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The gene cloning and recombination in the genus *Streptomyces* offers a good possibility to improve secondary metabolite production and creating new antibiotics. This type of genetic manipulation facilitated by the construction of different novel cloning vectors like plasmids, phages and transposable elements and also robust gene cloning methods. This review article describes some of these vectors and regulation of genes in *Streptomyces*.

Keywords: *Streptomyces*, gene cloning, plasmid, phage, transposon

Introduction

The versatile genus, *Streptomyces* includes quite a large number of species, which have been classified according to their physiological, morphological and genetical characters[1-5]. The spores of the genus germinate to produce multicellular substrate mycelium followed by aerial mycelium, which grows on the substrate mycelium. The aerial hyphae after maturation give rise to spores by fragmentation[6,7]. These changes support the formation of various metabolites extracellularly, particularly the enzymes like proteases[8-11], lipases[12-14] and nucleases[15-17]. Moreover, these bacteria are used now-a-days in bioconversion also[18-20]. The genus *Streptomyces* is the source of nearly 75% of the total naturally occurring antibiotics[21]. Out of its many strains *S. coelicolor* A3(2) is genetically the best known[22]. Bentley *et al*[23] have sequenced its complete genome. They have reported 8,667,507 base pair linear chromosome of this organism, containing the largest number of genes so far discovered in a bacterium. The 7,825 predicted genes include more than 20 clusters coding for known or predicted secondary metabolites. But the wild strains are not generally very much useful in the industrial applications because of their low production potential. So, it is obligatory to recognise the rate limiting steps of the antibiotic biosynthetic pathway. In recent years, much progress has been made in understanding the regulatory and biosynthetic pathways of these complex macro molecules[24-28]. Molecular genetic approach provides the potential to alleviate the rate limiting steps and to provide information on gene regulation, precursor flux and secondary metabolite biosynthesis[29-31]. The available information can be used to guide strain improvement strategies that can also be blended to random mutagenesis, selection and recombination for molecular cloning to optimise production[32-35]. In this review article some of these aspects have been discussed.

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Construction of Cloning Vectors

Several types of cloning vectors are now available for gene cloning in the genus *Streptomyces*.

Plasmids

Plasmids are the most frequently used vectors for gene cloning in microorganisms. But the plasmids, which are constructed for gene cloning in other bacterial organisms, cannot be applicable in *Streptomyces*. Because they do not replicate within the *Streptomyces* cells[36]. Schrempf *et al*[37] first reported the high copy number and endogenous plasmids in *Streptomyces,* which were also characterised[38]. The vectors may be natural or constructed, which carry a particular antibiotic resistance gene as a marker. Table 1 gives some examples of plasmids used in *Streptomyces* gene cloning.

Plasmid SLP1 from *S. coelicolor* A3(2) and pSAM2 from *S. ambofacies* can integrate into the host chromosome[42, 43]. The integration occurs between the attP-site of the plasmid and attB-site on the chromosome via recombination in both cases[44]. Nie *et*

Table 1—Plasmids used for gene cloning in *Streptomyces*

Plasmid	Size (Kb)	Characteristics*	References
pKC436	11.1	Am^R, MCS, CoS, OriT, $int^{\varphi C31}$	Bierman *et al* [39]
pOJ260	3.5	Am^R, MCS, OriT	Bierman *et al* [39]
pCZA213	11.2	pGM 160 with OriT, Tn5099 delivery vector	Solenberg & Baltz [40]
pRHB126	17.2	Rep^{ts}, pac, Tn5096	McHenney & Baltz [41]
pCZA186	10.1	pGM160, with OriT, Tn5096	Solenberg & Baltz [40]
pCZA185	7.8	Am^R, Ts^R, Rep^{ts}, pGM160 with OriT	Solenberg & Baltz [40]

*Am^R–apramycine resistance gene; MCS–multiple cloning site; CoS-λ cohesive ends; OriT–origin for conjugal transfer from plasmid RP4; $int^{\varphi C31}$–integration factor from bacteriophage φC31; Rep^{ts}–temperature sensative replicon; pac–packaging origin from bacteriophage FP43 to facilitate transduction, Ts^R–thiostrepton resistance

al[45] constructed a recombinant plasmid pNL2200. The construction was done with a 7.5 kb fragment of *S. ansochromogenes,* which was involved in nikkomycin biosynthesis and the fragment was inserted into pBluescript M13.

Zhou *et al*[46] reported a new replicon pH2808. This vector was constructed after insertion of streptomycin (str) resistance gene into the pIJ101 replication region of pHZ806 and hygromycin (hyg) resistance gene between 3.5 and 3.8 kb DNA fragments. Any DNA fragment cloned between 3.5 and 3.8 kb fragments of this vector, can be suitably integrated between the two corresponding regions following introduction into the wild type *S. lividans* strain. Xue *et al*[47] developed a multiplasmid approach. They showed a three plasmid system for heterologous expression of 6-deoxyerythronolideb synthase (DEBS) to facilitate combinatorial biosynthesis of polyketides made by type I modular polyketide synthase (PKS). These plasmids have mutually selectable antibiotic resistance markers. A strain of *S. lividans* was transformed with all three plasmids and the resulting strains produced wide range of 6-deoxyerythronolide B analogs. Magarvey *et al*[48] transformed *S. lividans* KAAA1 by plasmid pIJ702 carrying *S. venezuelae* genomic library. It was found that the transformed cells have stable recombinant vector containing a 2.5 kb *S. venezuelae* DNA fragment. Two recombinant plasmids, pUWL-219 and pLNSP have been developed with hsCTLA-4 gene, which were used to transform *S. lividans* resulting into two engineered *S. lividans* strains, pUWL219-vc and pLNSP/CTLA-4[49].

One of the short comings in the gene cloning via plasmid vector in *Streptomyces* is the presence of various restriction in the cells. To get rid of such problems, restriction deficient mutants are developed; *S. fradiae* is a good example of this[50]. With the desired DNA from such organisms, successful transformation was carried out in *S. avermitilis*. The other way is to use single stranded DNA of phage origin, which is reported to be 100 times efficient[51].

Matsushima and Baltz[52] also showed that restriction barriers would be bypassed by introducing plasmid vectors from *E. coli* into actinomycetes by conjugation. This has two advantages. Firstly, it is not constrained by the host range of FP43 bacteriophage, which is primarily limited to *Streptomyces*[53]. Secondly, versatile conjugal vectors are now available that integrate into the host chromosome[32]. Guangdong *et al*[54] developed a *Streptomyces*/*E. coli* shuttle plasmid pKc 1139 (AmR), which has tsr gene as selection marker for homologous recombination. Using this vector, bioengineered strain, *S. spiramyceticus* wsj-1 was constructed by integrating the 4′′-O-acyltransferase gene ist from *S. mycarofaciens* 1748. This was possible by homologous recombination into the chromosome of the spiramycin producing strain *S. spiramyceticus* F21.

Phage

Host restriction is a major problem for cloning of genes in *Streptomyces*. It can be caused by bacteriophage mediated transduction (Table 2). The linear DNA may be introduced into the host cells during physiological phases, which minimises the expression of host restriction[32].

The phage is one of the most widely used cloning vector in case of gene cloning in *Streptomyces*[61]. Glucose kinase gene (glk) is used as a counter selectable marker in φC31 phage vector lacking aHP function to select for combination events in construction of gene replacement mutants[32]. The recombinant strain, *Saccharopolyspora spinosa* was developed by φC31 mediated transduction. Prior to transduction the phage vector was developed by

integrating plasmid pOJ436 (11.1 kb) into the att BI site of the phage. The recombinant strain produced 96% of the yield of the spinosys as compared to the control strain. This result suggests that insertion into φC31 att B site can be nearly neutral in some cases, which is sufficient for applications but may not be adequate for the construction of over producer[24]. Similarly, several vectors that have the bacteriophage φC31 attachment and integration function (att/int) have also been developed[39,62]. Matsushima and Baltz[63] analysed several exconjugants, which indicated that plasmid pOJ434 and pRHB304 could be inserted into a unique φC31 attB site of *S. toyocaensis* chromosome. They also found that some of the insertions had minimal deleterious effect on recombinant strains of *S. toyocaensis* producing antibiotic A 47934. Thorpe *et al*[64] showed that the genome of the *Streptomyces* temperate phage φC31 integrates into the host chromosome via a recombinase belonging to a novel group of phage integrase related to resolvase/invertase enzymes.

FP43 phage mediated transduction of *S. toyocaensis* by plasmid pRHB126 has been reported[63]. The plasmid was introduced into the host cell at a frequency of $1X10^{-6}$ $(PFU)^{-1}$ when selections were made for Am^R or Ts^R. The plasmid pRHB126 was also introduced into *S. roseosporus* by bacteriophage FP43 mediated transduction. Transductions were obtained at a frequency of about 10^{-5} per PFU[65].

The generalised transducing phages for *Streptomyces* species are able to transduce chromosomal markers or plasmids between derivatives of *S. coelicolor*[66]. They have reported four phages (DAH2, DAH4, DAH5, DAH6) for transducing chromosomal markers of frequency range from 10^{-5} to 10^{-6} per PFU. Two commercially important species, *S. avermitilis* and *S. verticillus* have been found competent host to such transducing phages.

Table 2—Bacteriophages used in genetic manipulation in *Streptomyces*

Bacteriophages	Characteristics	References
FP4, FP46, FP55, FP56, FP60, FP61	Broad host range	Cox & Baltz [55]
FP43	Broad host range, transduces pRH126	Cox & Baltz [55] Hahn *et al* [56] Mchenney & Baltz [41]
FP22	Broad host range	Cox & Baltz [55] Hahn *et al* [57]
R4	Broad host range	Chater & Chater [58] Cox & Baltz [55]
SH10	Broad host range	Klaus *et al* [59]
VP11	Broad host range	Dowding [60] Cox & Baltz [55]

Table 3—Transposons for gene transfer in *Streptomyces*

Transposon	Size (kb)	Characteristics*	References
Tn5096	3	Am^R	Solenberg & Baltz [67]
Tn5099	4.4	Hm^R, contains DraI, AseI & SspI sites	Hahn *et al* [68]

*Am^R – apramycin resistance; Hm^R–hygromycin resistance gene

Transposons

The ultimate goal for the insertion of genes in the neutral site of the host chromosome which do not have any negative effects on the biosynthesis of secondary metabolites. The location of neutral site can be identified readily by transposon mutagenesis (Table 3). But the transposons have no resistance marker genes. So the recombinant transposons are constructed for gene cloning in *Streptomyces*. Solenberg and Baltz[67] reported transposon Tn5096 containing apramycin resistance gene, which is the derivative of IS493 of *S. lividans*[69]. Transposon Tn4556 of *S. fradiae* has recombinant transposon Tn4560, which contains a viomycin resistance gene[70-72]. Herron *et al*[73] constructed a mini transposon Tn1792 based on IS6100 for improving transposon mutagenesis of antibiotic producing *Streptomyces*. They also developed easily manageable transposition assays to demonstrate inducible transposition of Tn1792 into the *Streptomyces* genome from a temperature delivery plasmid. The temperature sensative plasmid delivery system also observed in case of intermolecular transposition of insertion sequence IS6100 to the genome of avermetin producing *S. avermitilis*[74]. The terminal inverted repeats of the linear plasmid SCP1 of *S. coelicolor* A3(2) possess a truncated copy of the transposon Tn4811 of *S. lividans* 66[75].

The genes of transposons generally recombine with the host chromosome and thus produce mutants. The transposons Tn5096, Tn5098 and Tn5099 a derivative of Tn5096 containing tyl F and tyl J genes, recombined into the chromosome of the host cell[76]. Recombinants obtained by transposon exchange produced higher yields of Ty. Volff and Altenbuchner[77] observed Tn5493 as a promising tool for genetic and molecular analysis and manipulation of antibiotic producing *Streptomyces*. It transposes at

a frequency 3% in *S. lividans* and insertion occurs into the target cell genome randomly. Engel *et al*[78] developed a nikkomycin non-producing mutant from wild type *S. tendae* ATCC 31160 by cloning a 2.6 kb Bam HI fragment from the genome of the wild type strain containing transposon Tn 4560.

Regulation of Gene in Antibiotic Biosynthesis

Streptomyces has complex gene expression system with a cascade of regulatory mechanism. Thus, antibiotic is the result of function of several gene products of biosynthetic cluster and regulatory cluster (Table 4). About 100 genes are sequenced in *Streptomyces*. A tRNA $_{Lev}$ (bld A) gene is responsible for one level of control in *Streptomyces* more particularly in *S. coelicolor*[82]. This tRNA can recognise the UUA codon, which is present only in 13 of them[83]. These 13 genes are mainly responsible for regulation of antibiotic biosynthesis, antibiotic resistance and antibiotic export[44].

Regulation of antibiotic biosynthesis is controlled by afs B and afs C genes in *S. coelicoler*[84]. The over-production of actinorhodin is achieved after cloning of afs B gene to *S. coelicolor*[85, 86]. Regulatory genes of antibiotic biosynthetic pathways are reported to control bialaphos, actinorhodin, methylenomycin, undecyl prodigiosin, streptomycin, and daunorubicin biosynthesis[79,80, 87-90].

The disruption of regulatory region of the methylenomycin cluster leads to an increment in production, thus suggesting a negative regulation. A pathway-specific regulation has been observed. The presence of additional cloned copies of the regulatory genes brpA, actII-orf4, redD-orf1, strR and $dnrR_2$ leads to an over production of the corresponding antibiotic[44]. AbsA1 and AbsA2 are the two genes that regulate the expression of multiple antibiotic gene clusters in *S. coelicolor*. The response regulator encoded by the AbsA2 gene is a negative regulator of antibiotic gene clusters. Genetic analysis reveals that the phosphorylated form of the AbsA2 response regulator (phospho-AbsA2), generated by the cognate AbsA1 sensor histidine kinase, is required for normal growth phase regulation of antibiotic biosynthesis[91].

Manipulation of Biosynthetic Genes

The manipulation of biosynthetic genes is also considered in *Streptomyces* for strain improvement programme. Improvement encompasses enhanced production efficiency or change in the chemical nature of metabolite from the wild strain. Malpartida *et al*[92] identified the genes involved in the polyketide antibiotic biosynthesis. Each of the antibiotics of this group has a common biosynthetic pathway[93,94]. Hybridization experiments also suggest the presence of homologous DNA not only in polyketide antibiotic producers but also in *S. venezuelae* (chloramphenicol) and *S. parvulus* (actinomycin)[92]. It may also be in other chemical class of antibiotics as well. The chemically modified antibiotics (hybrid antibiotic) are generally prepared from the β-lactam group of antibiotics. The novel structured antibiotics are generated through genetic engineering. Dihydro-granatirhodin and mederrhodin A & B are prepared by the transfer of actinorhodin biosynthetic genes into the producer organism[95]. A bacterial artificial chromosome (BAC) that can be shuttled among *Escherichia coli*, where it replicates autonomously within a suitable *Streptomyces* host and integrates into the chromosome site specifically, has been developed[96]. The existence of gene clusters of genetically amenable host strains, such as *S. coelicolor* or *S. lividans* makes it a sensible approach. Due *et al*[97] characterized, sequenced and cloned a set of thirty biosynthetic gene clusters from the blemycin (BLM) producer *S. verticillus* ATCC15003. Carreras

Table 4—Example of some positive regulatory genes in *Streptomyces*

Strain	Antibiotic	Gene	Effect	References
S. hygroscopicus	Bialaphos	brpA	Transcriptional activation of bialaphos resistance gene (bar) and six other regulatory genes (bap)	Anzai *et al* [79]
S. coelicolor	Actinorhodin	actII	30-40 fold increase in actinorhodin production	Hopwood *et al* [80]
S. coelicolor	Methylenomycin	unnamed	Insertional inactivation of the gene causes overproduction of methylenomycin; deletion of the region also results in overproduction	Chater & Bruton [81]

et al[98] developed a method for large-scale bioconversion of novel 6-deoxyerythronolide B (6dEB) analog into erythromycin analogs. This genetic manipulation is based on efficient generation of novel 6-dEB analogs using a plasmid based system in *S. coelicolor*. 6-dEB and 13-substituted 6-dEB analogs produced in this manner were fed to *S. erythraea* mutants, which could not produce 6-dEB, yet retained this 6-dEB modification system and resulted in generation of erythromycin A and 13-substituted erythromycin A analogs. Miyamoto *et al*[99] identified a glycosyltransferase gene rhoG, which is responsible for the glycosylation of epsilon-rhodomycinone in β-rhodomycin biosynthesis.

Conclusions

Biosynthetic genes for secondary metabolite production are organised in clusters. Characterization of these clusters provides us the basis for more knowledge based molecular genetic manipulation in *Streptomyces*. The *in vitro* mutagenesis of active sites of the biosynthetic enzymes results in the modification of substrate specificity and thus produces new products. The increase in the industrial production of many antibiotics can be achieved by introducing the multiple copies of positive regulatory genes. Gene aplification can also remove the bottle-neck in the antibiotic biosynthetic pathways.

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