CLONING OF ANTIBIOTIC-RESISTANCE GENES IN STREPTOMYCES

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Antibiotic-resistance genes were shotgun cloned from antibiotic-producing *Streptomyces* sp. using pock-forming plasmids (pSF689 and pSF765), as cloning vectors. *Streptomyces chartreusis* SF1623 and *S. lividans* 66 were used as host strains.

The ribostamycin (RSM) resistance gene was cloned from *S. ribosidificus* SF733 DNA (on a 2.3 Md *Pst*I fragment) into both *S. chartreusis* SF1623 and *S. lividans* 66, using pSF689 as vector.

Kanamycin (KM), novobiocin (NB), destomycin (DM) and racemomycin (RM) resistance genes were cloned from *S. kanamyceticus* M1164, *S. spheroides* M1469, *S. rimofaciens* M1470 and *S. lavendulae* A249 genomic DNA into *S. lividans* 66, using pSF765 as vector.

Furthermore two types of KM resistance determinants derived from *S. kanamyceticus* M1164 were cloned using *S. lividans* 66, the pSF689 vector.

The RSM resistance gene showed no homology to plasmid pSF733 of *S. ribosidificus* SF733, but hybridized to *Pst*I or *Bcl*I digested total DNA of *S. ribosidificus* SF733.

In Streptomyces, recombinant DNA technology is an important technique for the development of genetic knowlege, increasing the yield of antibiotics, and for potential of producing hybrid antibiotics.

This technology has developed rapidly since the discovery of the *Streptomyces coelicolor* A3(2) pock-forming plasmid, SCP2*, by HOPWOOD and his group[1]. Plasmid transformation in *Streptomyces* was established by BIBB *et al.*[2] and using plasmids, SCP2* and SLP1.2, the methylenomycin resistance gene was cloned[3]. Subsequently, THOMPSON *et al.*[4,5,6] cloned thiostrepton, neomycin (NM), viomycin and erythromycin resistance genes in *S. lividans* 66.

We have examined pock-forming plasmids from various antibiotic-producing Streptomyces. Four plasmids, pSF689, pSF765, pSF601 and pSF674, had this ability and have been transferred to other species of Streptomyces[7]. This has enabled us to develop other Streptomyces host-vector systems.

The cloning of resistance genes from antibiotic-producing Streptomyces is important in the study of antibiotic biosynthetic genes[8], as well as to provide selective markers for plasmid cloning vectors.

In this study, various antibiotic resistance genes have been cloned from various antibiotic-producing Streptomyces to facilitate the application of recombinant DNA technology to antibiotic-producing Streptomyces having diverse antibiotic-sensitivity.

Abbreviations: KM; kanamycin, KMA; kanamycin A, KMB; kanamycin B, DKB; dibekacin (dideoxykanamycin B), GM; gentamicin, LVDM; lividomycin, NB; novobiocin, DM; destomycin, RM; racemomycin, RSM; ribostamycin, AAC; aminoglycoside acetyltransferase, APH; aminoglycoside phosphotransferase, Md; megadalton.

Materials and Methods

Bacterial Strains

The recipient strains used as hosts for recombinant plasmids were *S. chartreusis* SF1623[9,10] and *S. lividans* 66. Antibiotic-producing Streptomycetes used as donor strains were *S. ribosidificus* SF733, *S. kanamyceticus* M1164, *S. spheroides* M1469, *S. rimofaciens* M1470 and *S. lavendulae* A249. *S. lividans* 66 was kindly supplied by Dr. N. D. LOMOVSKAYA and Dr. K. F. CHATER. Other strains were obtained from the Meiji Seika Kaisha Collection.

Growth Conditions for Chromosomal DNA Preparation

The donor strains were grown in MYG medium[7] at 28°C for 48 hours. Chromosomal DNA was isolated as described by SMITH[12].

Isolation of Plasmid DNA

Plasmid DNA was prepared from *S. chartreusis* SF1623 as previously described by NOJIRI *et al.*[13] or OKANISHI *et al.*[14]. The method for *S. lividans* 66 was as described by HANSEN *et al.*[15].

Shotgun Cloning

1) Digestion and Ligation Conditions: Foreign DNA was inserted into the vector as follows: *Pst*I-digested total DNA of the donor strains was inserted into *Pst*I site of pSF689 or *Bcl*I digested chromosome DNA of the donor strains was inserted into *Bcl*I site of pSF765. Vector (1.5 μg) and donor DNA (6.0 μg) were mixed and digested at 37°C for 2 hours in 100 μl of digestion buffer which contained 10 mM Tris-HCl (pH 7.4), 6 mM dithiothreitol, 10 mM $MgCl_2$ and 5 units of endonuclease *Pst*I. In the case of *Bcl*I, incubation was at 50°C in the same digestion buffer containing 50 mM NaCl. The digestion mixture was shaken with an equal amount of phenol saturated with TESH buffer (0.2 M Tris-HCl, pH 8.0, 20 mM EDTA and 50 mM NaCl). The upper layer was removed and the aqueous phase extracted twice with ethyl ether to remove remaining phenol. 300 μl of ethanol was added to the aqueous phase and the mixture stored at -80°C for 2 hours. After centrifugation (10,000$\times g$, 5 minutes), the pellets were washed once with 500 μl of cold ethanol, the pellets were dried under vacuum and dissolved in 40 μl of sterilized water. The DNA solution was heated at 70°C for 10 minutes, and 5 μl of ligation buffer which contained 66 mM Tris-HCl, pH 7.6, 6.6 mM $MgCl_2$, 10 mM dithiothreitol and 1.0 mM ATP (each denoting the final concentration) was added and ligation accomplished by incubation with 5 μl (1 unit) of T4 DNA ligase at 22°C for 2 hours.

2) Preparation and Transformation of Protoplasts: Protoplasts were prepared as described by OKANISHI *et al.*[16]; transformation of *S. lividans* 66 was as described by THOMPSON *et al.*[5] or CHATER *et al.*[17]. *S. chartreusis* SF1623 was transformed as described by TOYAMA *et al.*[10].

3) Selection of Resistance Clones: Transformed protoplasts were regenerated and after spore formation (at 32°C for 4 days) the lawns were replicated to plates of BENNETT's medium (*S. lividans* 66) or YS medium[11] (*S. chartreusis* SF1623), containing antibiotics at 20 μg/ml. After incubation for 2~3 days at 32°C resistance clones appeared. Resistance clones were picked up, gently homogenized and spread on R2YE medium[1,17]. Spores appearing after incubation at 32°C for 4 days were inoculated to YEME medium containing 34% sucrose and 5 mM $MgCl_2$[1,5,17], and the plasmid was isolated from each culture.

Nick Translation and Southern Hybridization

Nick translation and Southern hybridization with *Streptomyces* sp. was as described by BIBB *et al.*[3]. A labelled probe of the 2.3 Md RSM resistance fragment from pMS1 was made by nick translation[18] using [α-^{32}P]dCTP. This probe was then hybridized to *Pst*I or *Bcl*I-cut total DNA of *S. ribosidificus* SF733 using the modified method of SOUTHERN[19].

Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out on a horizontal slab gel of 1% agarose in E buffer (Tris-CH_3COOH 40 mM, EDTA 1 mM, CH_3COONa 5 mM, pH 8.0). After electrophoresis for 2 hours at a constant voltage of 100 volts, the bands were visualized by fluorescence under 302 nm UV light.

Result

Shotgun Cloning Experiments

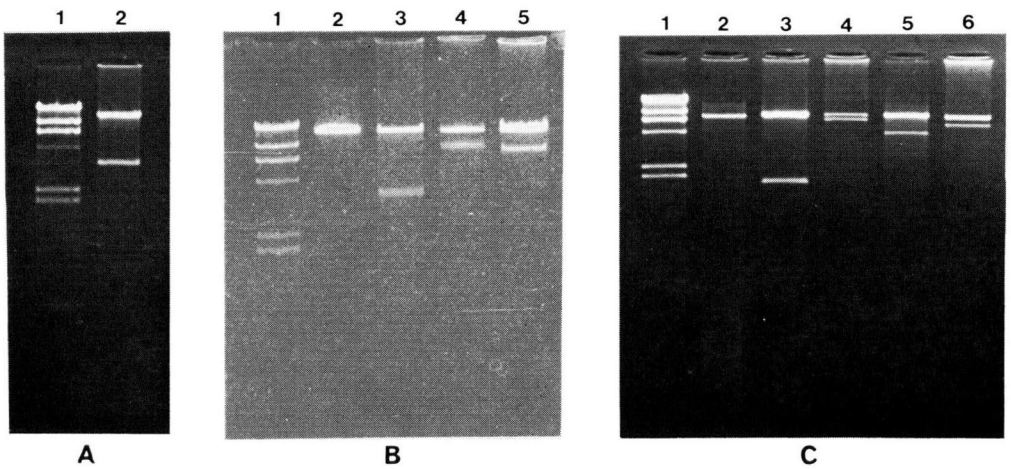
In the first approach (expt. A), *Pst*I fragments of *S. ribosidificus* SF733 DNA were ligated into the *Pst*I site of pSF689 vector, and *S. chartreusis* SF1623 protoplasts were transformed with the ligated

Table 1. Antibiotic resistance clones obtained by shotgun cloning.

Experiment	Cloning vector	Cloning site	Host strain	Donor strain				
				S. ribosidificus SF733 (RSM)*	*S. kanamyceticus* M1164 (KM)*	*S. spheroides* M1469 (NB)*	*S. rimofaciens* M1470 (DM)*	*S. lavendulae* A249 (RM)*
Expt. A	pSF689	*Pst*I	*S. chartreusis* SF1623	+	NT**	NT**	NT**	NT**
Expt. B	pSF689	*Pst*I	*S. lividans* 66	+	+	−	−	−
Expt. C	pSF765	*Bcl*I	*S. lividans* 66	−	+	+	+	+

* Product.
** Not tested.

Fig. 1. Agarose gel electrophoresis of recombinant plasmids cleaved by restriction enzymes.
A: *Pst*I digests of plasmid from *S. chartreusis* SF1623.
1: *Hin*d III digested phage λcI DNA.
2: pMS1 (contains pSF689 and the 2.3 Md RSM resistance fragment).
B: *Pst*I digests of plasmids from *S. lividans* 66.
1: *Hin*d III digested phage λcI DNA.
2: pSF689
3: pMS1 (contains pSF689 and the 2.3 Md RSM resistance fragment).
4: pMS19 (contains pSF689 and the 6.0$_4$Md KM resistance fragment I).
5: pMS21 (contains pSF689 and the 5.2 Md KM resistance fragment II).
C: *Bcl*I digests of plasmids from *S. lividans* 66.
1: *Hin*d III digested phage λcI DNA.
2: pSF765
3: pMS18 (contains pSF765 and the 1.3 Md KM resistance fragment I).
4: pMS32 (contains pSF765 and the 4.1 Md NB resistance fragment).
5: pMS14 (contains pSF765 and the 2.8 Md DM resistance fragment).
6: pMS33 (contains pSF765 and the 3.5 Md RM resistance fragment).



mixture.

In the second (expt. B), *Pst*I fragments of *S. ribosidificus* SF733, *S. kanamyceticus* M1164, *S. spheroides* M1469, *S. rimofaciens* M1470 and *S. lavendulae* A249 DNAs were separately ligated into the single *Pst*I site of the pSF689 vector. *S. lividans* 66 protoplasts were transformed with each ligated mixture respectively.

In the third (expt. C), *Bcl*I fragments of the above donor strains were ligated into the single *Bcl*I site of pSF765 and the mixtures used to transform *S. lividans* 66 protoplasts.

In expt. A, *S. chartreusis* SF1623 failed to form spores on regeneration medium. In expt. B and expt. C, transformation was detected by the appearance of "pocks" on a lawn of *S. lividans* 66 growth arising from the regenerated protoplast population on R2YE medium.

When the lawns were replicated to plates, RSM resistance clones appeared in expt. A and expt. B, KM resistance clones in expt. B and expt. C, NB, DM and RM resistance clones in expt. C respectively (Table 1).

Recombinant plasmids prepared from transformants could re-transform *S. lividans* 66 protoplasts and resistance clones appeared coincidently with pock-forming clones.

Electrophoretic Analysis of Recombinant Plasmids

A 2.3 Md *Pst*I fragment was obtained from the plasmid (pMS1) of RSM resistant *S. chartreusis* SF1623 (Fig. 1A).

*Pst*I digests of plasmids (pMS1, pMS19 and pMS21) recovered from RSM and KM resistant transformants of *S. lividans* 66 were analyzed by agarose gel electrophoresis. The RSM resistance gene in this case was also encoded by the 2.3 Md fragment. The KM resistance genes were coded on either the 6.0 Md or 5.2 Md fragments (Fig. 1B).

*Bcl*I digests of plasmids (pMS18, pMS32, pMS14 and pMS33) obtained from KM, NB, DM and RM resistant transformants of *S. lividans* 66 were also analyzed. The KM resistance gene was encoded by a 1.3 Md fragment, the NB resistance gene by a 4.1 Md fragment, the DM resistance gene by a 2.8 Md fragment and the RM resistance gene by a 3.5 Md fragment (Fig. 1C).

The 6.0 Md and 5.2 Md KM resistance fragments were extracted, digested with *Bcl*I and re-electrophoresed. The digestion patterns of the two fragments were distinct. The 6.0 Md fragment contained the 1.3 Md *Bcl*I fragment (Fig. 2). The 1.3 Md fragments from pMS18 and pMS19 (contains pSF689 and the 6.0 Md KM resistance fragment I) were identical and conferred similar resistance spectra in cloning vectors.

Fig. 2. Agarose gel electrophoresis of two different KM resistance fragments and pMS18, digested with *Bcl*I.

1: *Hin*d III digested phage λcI DNA. 2: KM resistance fragment (6.0 Md) from pMS19, produced by *Pst*I. 3: KM resistance fragment (5.2 Md) from pMS21, produced by *Pst*I. 4: pMS18 (contains pSF765 and the 1.3 Md KM resistance fragment I). The arrow indicates the 0.46 Md fragment.

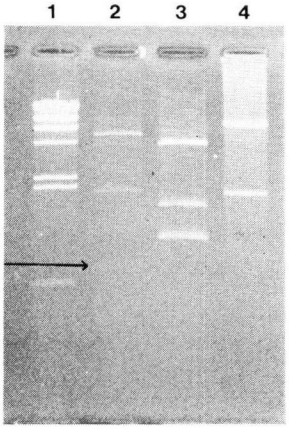


Table 2. MCG* of *S. chartreusis* SF 1623 and *S. lividans* 66 harboring antibiotic-resistance gene.

Plasmid name	Resistance gene	Cloning vector	Cloning site	Fragment size (Md)	MCG* of *S. chartreusis* Plasmid (+)	MCG* of *S. chartreusis* Plasmid (−)	MCG* of *S. lividans* Plasmid (+)	MCG* of *S. lividans* Plasmid (−)
pMS1	RSM	pSF689	*Pst*I	2.3	1,000	5	1,500	10
pMS18	KM-I	pSF765	*Bcl*I	1.3			100	2.5
pMS21	KM-II	pSF689	*Pst*I	5.2			250	2.5
pMS32	NB	pSF765	*Bcl*I	4.1			50	10
pMS14	DM	pSF765	*Bcl*I	2.8			500	5
pMS33	RM	pSF765	*Bcl*I	3.5			1,000	5

* Maximum concentration permitting growth, μg/ml.

Table 3. MIC* of *S. lividans* 66 transformant by pMS plasmids.

Plasmid	KMA	KMB	DKB	GM	RSM	NM	LVDM
pMS1	2.5	2.5	5	5	>80	40	20
pMS18	>80	40	>80	5	>80	20	20
pMS21	>80	>80	>80	>80	10	2.5	20
none	2.5	2.5	5	5	10	2.5	20

* μg/ml.

Antibiotic-resistance of Transformants

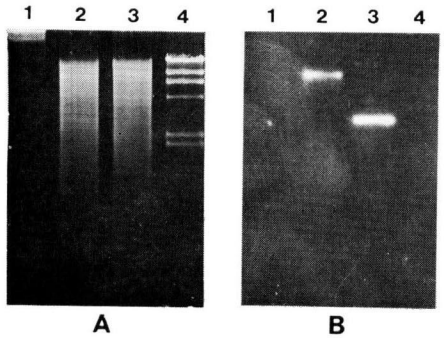
The cloned antibiotic-resistance genes (fragment sizes summarized in Table 2) were transformed into *S. chartreusis* SF1623 and *S. lividans* 66.

The resistance level of each transformant was indicated by the maximum concentration permitting growth (MCG). As shown in Table 2, the values of the MCG increased in all transformants, for example, with RSM and RM, 100 fold and for NB, 5 fold. The KM resistance gene I and KM resistance gene II differed not only in size of the fragment, but also in their MCG to KM, 100 μg/ml and 250 μg/ml, respectively.

Fig. 3. Southern hybridization of ^{32}P-labelled RSM resistance gene obtained from pMSI to total DNA preparation from *S. ribosidificus* SF733.

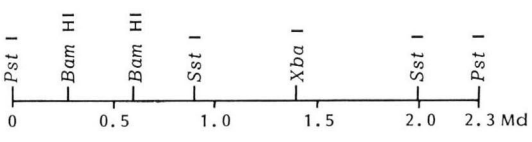
Agarose gel electrophoresis patterns (A) and corresponding autoradiograms (B).

1: pSF733 DNA. 2: *Bcl*I digested total DNA. 3: *Pst*I digested total DNA. 4: *Hin*d III digested λCI DNA.



S. lividans 66 was transformed by pMS1, pMS18 or pMS21; resistance to the aminoglycoside antibiotics are shown in Table 3. *S. lividans* 66 harboring pMS1 was resistant to RSM and NM; when harboring pMS18 was resistant to many aminoglycoside antibiotics, except GM and LVDM; pMS21 encoded resistance to the hexose-type aminoglycosides (KM, DKB and GM), but not to the pentose-type aminoglycosides (RSM, NM and LVDM).

Fig. 4. Restriction map of RSM resistance fragment of *S. ribosidificus* SF733.



Origin of the RSM Resistance Gene

To determine whether the RSM resistance gene was coded on the chromosome or the pSF733 plasmid[13] of *S. ribosidificus* SF733, a ^{32}P-labelled probe of the RSM resistance fragment obtained from pMS1 was made by nick translation and hybridized to *Pst*I or *Bcl*I-cut *S. ribosidificus* SF733 or pSF733 plasmid DNA. The RSM resistance fragment showed homology to fragments of *S. ribosidificus* SF733, but not to pSF733 plasmid DNA. In the case of *Bcl*I-digested chromosomal DNA, the labelled probe hybridized to a fragment of about 10 Md, in case of *Pst*I digested chromosomal DNA, to the fragment of 2.3 Md.

Cleavage Map of RSM Resistance Gene

A restriction endonuclease cleavage map of the RSM resistance gene of *S. ribosidificus* SF733 is shown in Fig. 4.

Discussion

Interspecific shotgun cloning of antibiotic resistance genes in Streptomyces has been described by BIBB *et al.*[3] and THOMPSON *et al.*[4,5] using SCP2* or SLP1.2 as vectors.

We described the use of the pock-forming plasmids pSF689 and pSF765 as vectors for interspecific shotgun cloning in Streptomyces; both *S. lividans* 66 and *S. chartreusis* SF1623 could be used as hosts possessing good protoplast formation and regeneration properties and weak restriction systems[10,11].

We have suggested previously that the RSM resistance gene was not coded on the plasmid pSF733[13]. With the cloning of the gene described here, DNA hybridization experiments indicate that the RSM resistance gene is coded by the chromosome of *S. ribosidificus* SF733.

The RSM resistance gene encodes an RSM phosphotransferase (APH)[20] (in preparation), different from the NM resistance gene (APH) cloned from *S. fradiae* ATCC 10745 by THOMPSON *et al.*[4], since radioactively labelled 2.3 Md RSM fragment probe failed to hybridize to *Bcl*I or *Pst*I digests of total DNA of *S. fradiae* ATCC 10745 (data not shown). As confirmation of this, the restriction cleavage map of the RSM resistance gene (Fig. 4) was different from that of the NM resistance gene reported by THOMPSON *et al.*[6].

S. ribosidificus SF733 accumulated 3-*N*-acetyl RSM in culture[21], and the presence of an RSM acetyltransferase activity has been reported[20,22]. We have confirmed the presence of RSM acetyltransferase in *S. ribosidificus* SF733 by the [^{14}C]acetyl CoA transfer studies, however we have so far been not successful in our attempts to clone the RSM acetyltransferase gene.

DNA hybridization studies revealed that the *Bcl*I fragment hybridized with the RSM resistance gene was large (about 10 Md); this may be the reason why this fragment was not cloned in expt. C; in spite of the fact that the 2.3 Md RSM resistance gene had no *Bcl*I site.

It has been shown that AAC(6′) is coded on the 1.3 Md fragment of pMS18 containing the KM resistance gene I cloned from *S. kanamyceticus* M1164 (in preparation), however the resistance mechanism encoded by the 5.2 Md KM resistance fragment II of pMS21 has not been elucidated.

S. lividans 66 harboring pMS18 is sensitive to GM but resistant to RSM. On the other hand *S. lividans* 66 harboring pMS21 is resistant to GM but sensitive to RSM. This confirms that two independent KM resistance genes are present in *S. kanamyceticus* M1164. It has been reported that AAC(6′) is involved in the biosynthesis of KM[22].

We have expanded our survey of pock-forming plasmids to various antibiotic-producing Streptomyces using *S. lividans* 66 as indicator strain[7]. However since most high-yielding antibiotic-producing strains are unable to form spores, the pock-forming phenotype is not universally applicable as a genetic and phenotypic marker.

Using cloned antibiotic resistance genes as genetic markers we have examined the host range of several Streptomycetes plasmids and found that the small recombinant plasmid pMS18 had a broad host range. It has been transferred to *S. chartreusis* SF1623 (cephamycin producer), *S. griseus* (strep-

tomycin), *S. ribosidificus* (ribostamycin), *S. hygroscopicus* SF619 (paromomycin), *S. wedmorensis* (fosfomycin) by protoplast transformation, and is maintained by the expression of KM resistance.

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