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REVIEW PAPER

**Gauri Dhingra · Rekha Kumari · Shashi Bala
Swati Majumdar · Shweta Malhotra
Poonam Sharma · Sukanya Lal · John Cullum
Rup Lal**

Development of cloning vectors and transformation methods for *Amycolatopsis*

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Abstract The genus *Amycolatopsis* is of industrial importance, as its species are known to produce commercial antibiotics. It belongs to the family *Pseudonocardiaceae* and has an eventful taxonomic history. Initially strains were identified as *Streptomyces*, then later as *Nocardia*. However, based on biochemical, morphological and molecular features, the genus *Amycolatopsis*, containing seventeen species, was created. The development of molecular genetic techniques for this group has been slow. The scarcity of molecular genetic tools including stable plasmids, antibiotic resistance markers, transposons, reporter genes, cloning vectors, and high efficiency transformation protocols has made progress slow, but efforts in the past decade have led to the development of cloning vectors and transformation methods for these organisms. Some of the cloning vectors have broad host range (pRL series) whereas others have limited host range (pMEA300 and pMEA100). The cloning vector pMEA300 has been completely sequenced, while only the minimal replicon (pA-*rep*) has been sequenced from pRL plasmids. Direct transformation of mycelia and electroporation are the most widely applicable methods for transforming species of *Amycolatopsis*. Conjugational transfer from *Escherichia coli* has been reported only in the species *A. japonicum*, and gene disruption and replacements using homologous recombination are now possible in some strains.

G. Dhingra · R. Kumari · S. Bala · S. Majumdar
S. Malhotra · P. Sharma · S. Lal · R. Lal (✉)
Molecular Biology Laboratory, University of Delhi,
Department of Zoology, Delhi 110007, India
E-mail: duzdel@del2.vsnl.net.in
Tel.: +91-11-27666254
Fax: 91-11-27666541

J. Cullum
LB Genetik, University of Kaiserslautern, Fachbereich Biologie,
Postfach 3049, 67663 Kaiserslautern, Germany

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Introduction

The actinomycetes are a diverse group of gram-positive bacteria that produce more than two-thirds of the known biologically active microbial natural products, including many commercially important antibiotics, anticancer agents, other pharmacologically useful agents, animal health products and agrochemicals. They include the genera *Streptomyces, Corynebacterium* and *Amycolatopsis* to mention a few. Although the importance of molecular genetic methods for *Streptomyces* spp. and *Corynebacterium* spp. has been described in several reviews, there is no corresponding overview available for species of *Amycolatopsis*.

The genus *Amycolatopsis* was first proposed by Lechavalier et al. [25] to accommodate four species that had been misclassified in the genus *Nocardia*. Currently, the genus *Amycolatopsis* contains 17 validly described species, and belongs to the family *Pseudonocardiaceae*. Species of *Amycolatopsis* are important for antibiotic production: rifampicin, which is the major drug for treating tuberculosis, is a semi-synthetic derivative of rifamycin from *A. mediterranei* and vancomycin, from *A. orientalis* is a drug of last resort for treating multiple-resistant *Staphylococcus aureus* infections. In addition to producing these antibiotics, *Amycolatopsis* species have other potential applications (Table 1) [4, 8, 9, 10, 11, 25, 29, 34, 38, 39, 42, 43, 44, 52x, 53]. The genetic manipulation of *Amycolatopsis* species has proven slow and frustrating and there are far fewer genetic tools available than for *Streptomyces* [17]. Despite all these technical difficulties, there has been steady progress and the time now seems ripe for a major expansion in our knowledge of *Amycolatopsis*. The present work summarizes this progress on the development of

Table 1 Some species of *Amycolatopsis* and their importance

Organism	Antibiotic/compound	Application/importance	Reference
Amycolatopsis sp.	Epoxyquinomicins	Antibacterial	[29]
A. orientalis	Chloroeremomycin	Antibacterial	[52x]
Amycolatopsis sp.	Epoxyquinomicins A and B	Antibacterial	[44]
Amycolatopsis sp.	Azicemicins A and B	Antibacterial	[43]
Amycolatopsis sp.	Azicemicin A	Antibacterial	[42]
A. orientalis	Vancomycin	Antibiotic activity	[4, 52x]
A. mediterranei	Rifamycin	Anti-leprosy and anti-tuberculosis	[38]
Amycolatopsis mediterranei DSM 5908	Balhimycin	Against methicillin resistant *S. aureus*	[34]
A. mediterranei var. Kanglensis	Homorifamycin	Antibacterial	[53]
*Amycolatopsis Sp.*MI481–42F4	Amythiamycins	Against gram-positive *MDR* strains	[39]
Amycolatopsis spp.	–	Degradation of aromatic hydrocarbons	[11]
A. methanolica	–	Methanol-utilizing bacterium	[8, 9]
A. japonicum	Ethylenediamine disuccinic acid	Chelating agent	[10]

molecular genetic tools and provides a platform for further work.

Replicative plasmids for development of vectors

As *Amycolatopsis* is related to *Streptomyces*, there have been several attempts to use *Streptomyces* vectors. However, in *A. orientalis* it was not possible to obtain satisfactory levels of transformants [31] by using protocols developed for transformation of *Streptomyces* [3, 5, 30, 41] and *Saccharopolyspora* [54]. Nonetheless, when the growth media and the protoplast preparation and regeneration protocols were optimized, plasmids based on pIJ101, pFJ103 and SCP2 replicons could be used in *A. orientalis* [31]. In contrast, *Streptomyces* vectors do not seem to work in *A. mediterranei*. Plasmids pIJ61, pIJ702 and pIJ922 [17], which are based on SLP2, pIJ101 and SCP2 replicons respectively, did not give transformants in *A. mediterranei* [37]. However, all these vectors carry the thiostrepton resistance gene, which might not be selectable in this species. When pMEA52 (a pIJ101 derivative carrying an erythromycin resistance gene that can be selected in *A. mediterranei*) was used, there initially appeared to be a high transformation frequency [27], but the colonies grew extremely slowly and the clones did not grow in a liquid medium.

Another reason why initial attempts were focused on *Streptomyces* vectors is that the occurrence of plasmids in species of *Amycolatopsis* is rare and only a few plasmids have been reported from different species of the bacteria (Table 2) [24, 33, 49]. Plasmid pMEA100 (23.7 kb) was isolated from *A. mediterranei* strain LBG A3136 (Fig. 1a) [33]. The copy number of the plasmid was very low (0.5 copies per haploid genome) and an integrated copy was found in the chromosome. Another strain, *A. mediterranei* ATCC 13685, also carried an integrated copy of pMEA100, but no free plasmid was detected. pMEA100 was not very stable and underwent internal rearrangements including deletions and amplifications [26, 33, 56]. However, it elicited a lethal zygosis phenotype that allowed its transfer to be detected on agar plates. The integrated form could be excised site-specifically from the chromosome and, after transfer to a plasmid-free strain, underwent site-specific integration into the chromosome [26]. Excision and integration of pMEA100 occurred by site-specific recombination in a 47-bp sequence identical in both the plasmid and the chromosome [26]. Starting from a spontaneous deletion derivative that had lost the integration region, it was possible to construct the 11.3-kb vector pMEA123 carrying *erm*E (erythromycin resistance gene from *Saccharopolyspora erythraea*) as a selection marker [27] (Fig. 1b). While the plasmid has a single *Sac*I restriction site available for cloning experiments, there are no further studies described in the literature on pMEA100-based vectors.

Table 2 Naturally occurring plasmids for *Amycolatopsis spp.*

Organism	Plasmid [reference]	Features
A. mediterranei LBG3136	pMEA100 [33]	23.0 kb
A. orientalis DSM43387	pA387 [24]	29.6-kb cryptic plasmid
A. methanolica	pMEA300 [49]	13.3kb conjugative plasmid; integrates into chromosome

The second well-known plasmid of the genus *Amycolatopsis* is pMEA300, from *A. methanolica* NCIB 11946 [49] (Fig. 2). This plasmid is smaller (13.3 kb) than pMEA100 but it integrates into the chromosome at a unique site and shows a lethal zygosis phenotype. Unlike pMEA100 [56], pMEA300 did not show frequent deletions and DNA amplifications. Plasmid regions were identified that encode site-specific integration [49], replication [50], and conjugational transfer [48] functions. A *stf* (stimulation of transfer) gene was also identified whose deletion resulted in reduced transformation frequencies [50]. On the basis of these analyses it was possible to construct a series of vectors that had deleted integration functions and were present only as free plasmids in *A. methanolica*. In particular, *E. coli–A. methanolica* shuttle vectors have proved useful. These vectors have been used for cloning genes involved in

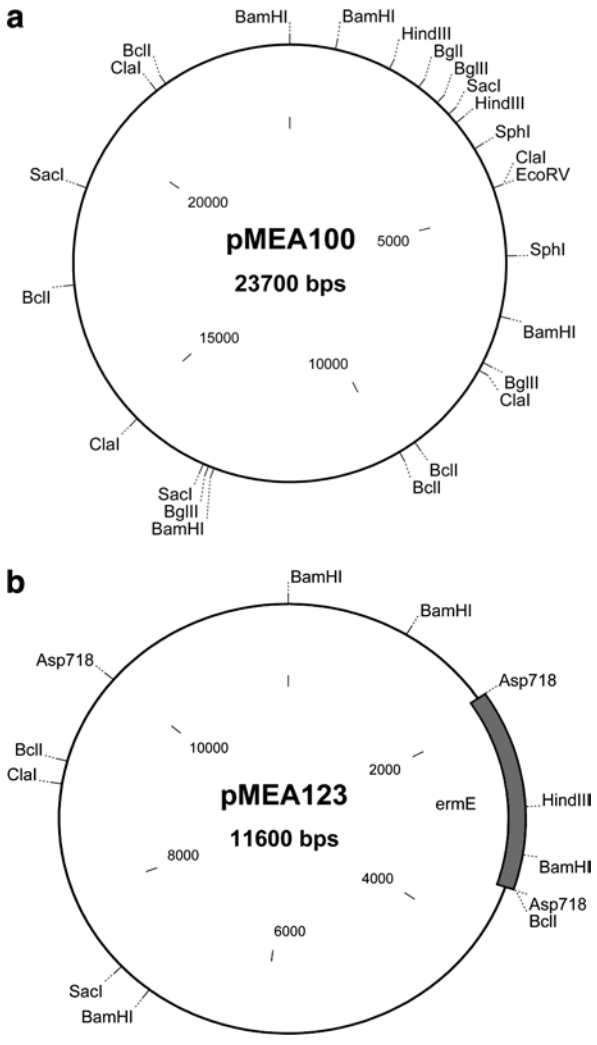

Fig. 1 Restriction map of **a** plasmid pMEA100 from *Amycolatopsis mediterranei* LBG A3136, **b** plasmid pMEA123

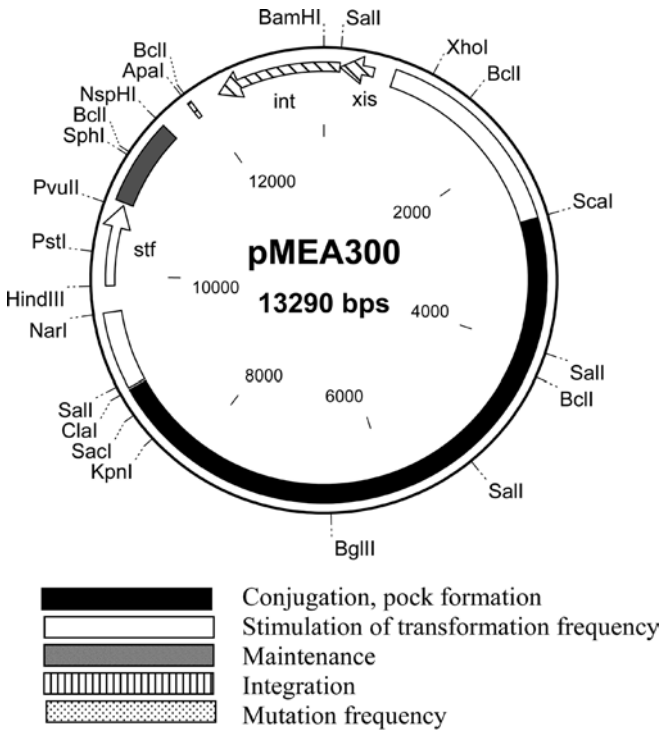

Fig. 2 Restriction map of plasmid pMEA300 from *Amycolatopsis methanolica*

glucose [1] and methanol utilization [12, 50] and in aromatic amino acid biosynthesis [47].

A comparatively large plasmid, pA387 (29.7 kb), was reported from *A. orientalis* DSM 43387 [24] (Fig. 3a). Unlike pMEA100 and pMEA300, pA387 occurred only in a free state and did not integrate into the chromosome. pA387 has a relatively low copy number (10 copies per cell) and is fairly stable as it could be cured only at low frequency (1.5%) by protoplasting or ethidium bromide treatment. pA387 is a cryptic plasmid whose loss was not associated with the disappearance of any phenotypic character from the organism. Lal et al. [24] constructed the *E. coli–Amycolatopsis* shuttle vector pRL1 by cloning a 5.1-kb fragment of pA387 in the *E. coli* cloning vector pDM 10. pRL1 is 10.4 kb in size and carries the *km*/*neo* resistance gene as a selective marker (Fig. 3b). pRL1 replicates in both *A. mediterranei* and *A. orientalis* and transformation efficiencies of 2×10^3 and 1×10^5 transformants/μg DNA in *A. mediterranei* and *A. orientalis,* respectively, were reported [24].

A major problem in developing cloning vectors is that most species of *Amycolatopsis* are resistant to some of the antibiotics commonly used for vector construction in actinomycetes. Many *A. mediterranei* strains [16, 22, 27, 35] show a natural high resistance against thiostrepton and kanamycin. *A. orientalis* is resistant to chloramphenicol, bacitracin, spectinomycin, neomycin, hygromycin B and viomycin [31]. However, many *Amycolatopsis* strains are sensitive to erythromycin and apramycin [22, 27, 40]. *Nocardia lactamdurans* is resistant to hygromycin, viomycin and chloramphenicol but sensitive to thiostrepton, phleomycin, kanamycin and apramycin [19]. Even when the *km*/*neo* gene of pRL1 was used as a selectable marker for transformation of *A. mediterranei* strains, there was a high background of mutant colonies lacking plasmid DNA when selection was done on either kanamycin or neomycin [16]. To overcome these problems, an erythromycin resistance gene *ermE* was cloned into the *Bam*HI site of pRL1, which resulted in the construction of pRLM20 and pRLM30 [16]. Despite their large size of 12.1 kb, these cloning vectors could be successfully transferred by electroporation and *ermE* was effectively expressed in *A. mediterranei*. Subsequently, *ermE* was cloned [22] into the pRL1 derivatives pULAM2 and pULVK2A [19]. In order to develop better cloning vectors based on the pA387 replicon, it was important to reduce the sizes of the plasmids. When pRL1 was introduced into *N. lactamdurans* [19], spontaneous deletions resulted in plasmid pULVK1, which has proved useful in developing further vectors. An interesting method for obtaining smaller vectors was discovered

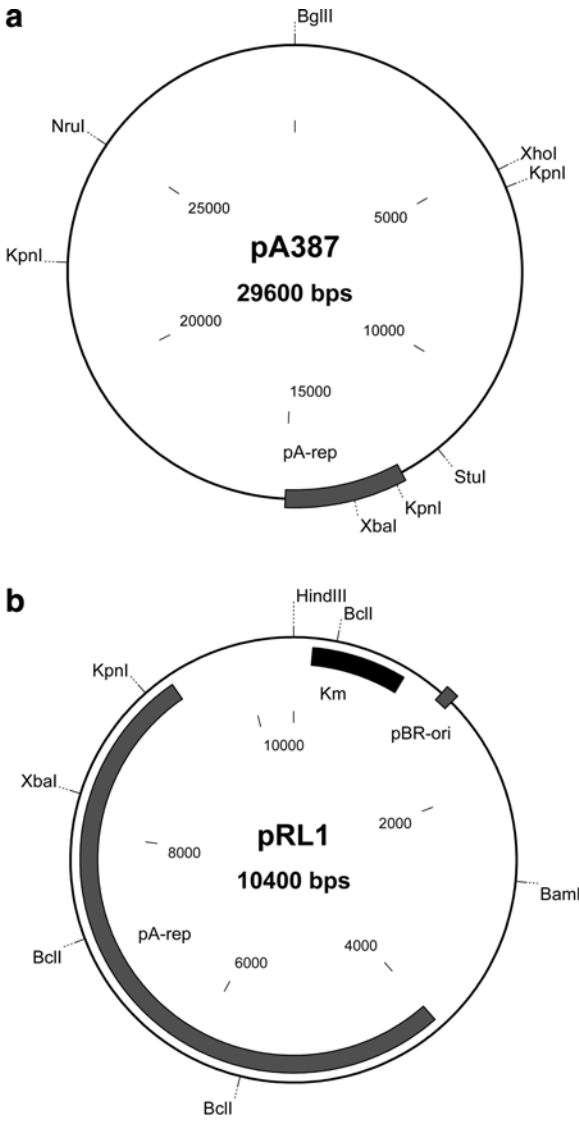

Fig. 3 Restriction map of **a** plasmid pA387 (29.6 kb) from *Amycolatopsis orientalis* DSM 43387, **b** plasmid pRL1

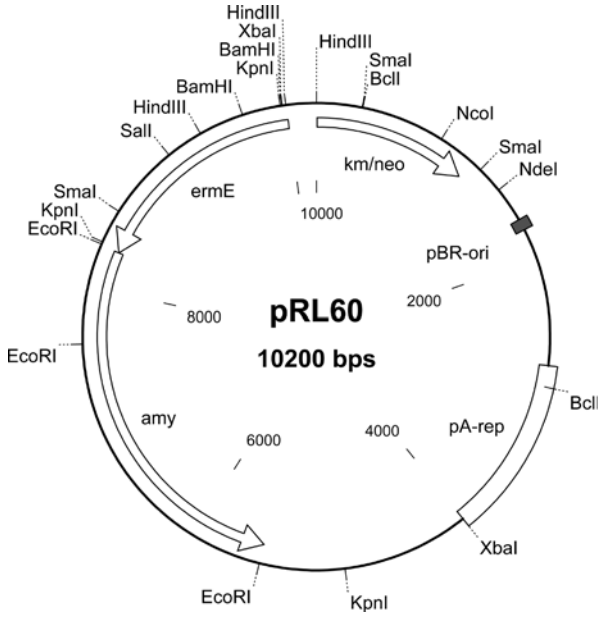

Fig. 4 Restriction map of pRL60

while cloning *ermE* into a vector [22]. A large hybrid plasmid (pRL50) containing two copies of the vector was first selected in *E. coli* and, upon transformation in *A. mediterranei* DSM 40773 and selection with erythromycin, it underwent deletions and rearrangements leading to the formation of plasmid pRL60. The rearrangement was specific, which reflected that large repeats in the hybrid plasmid and the antibiotic pressure played a crucial role in selecting the plasmid retained by the organism. This strategy was then utilized for the construction of a series of smaller, stable plasmids [45]. Two large concatemers, pRL50 and pRL80, were first constructed in *E. coli* and then transferred into *A. mediterranei* DSM 40773. The transformants were selected by different antibiotic selections. This approach not only resulted in the formation of several smaller plasmids (pRL51, pRL52, pRL53, pRL60, pRL81 and pRL82) but also helped in redefining the size of the minimal replicon (*pA-rep*). The *pA-rep* was found to be limited to ~1.0 kb [45]. Sequence analysis of *pA-rep* indicates a G + C content of 67.4%, and the presence of several direct and inverted repeat structures as well as an 18-bp palindrome sequence. There does not seem to be any protein coded by the *pA-rep* sequence and it is speculated that the replication mode may be *polA*-dependent like ColE1 [45]. However, further work is needed to define the minimal replicon size and the mode of replication.

The pRL series of vectors includes stable cloning vectors of different sizes containing one or more marker gene(s) as well as *E. coli–Amycolatopsis* shuttle vectors. They can be easily transferred in *A. mediterranei* strains by electroporation, and a transformation efficiency of 1×10^6 transformants/μg DNA was achieved with pRL60 [45] (Fig. 4). Apart from the *km*/*neo* resistance gene, these cloning vectors contain *ermE* or *am* (apramycin resistance gene) as the second selectable marker. *ermE* and *am* were expressed well in *A. mediterranei* strains and no spontaneous mutants could be observed among transformants [16, 22, 45].

In addition to *km*/*neo* and *ermE*, pRL60 also contains α-amy (encoding α-amylase from *Streptomyces griseus*), which is secreted into the medium by *A. mediterranei* thereby providing a direct, rapid, and inexpensive assay for the detection of transformants.

Transformation methods

Transformation methods are often strain dependent and may differ for members of the same species. Development of a transformation system also depends on having suitable DNA to transform. Plasmid DNAs

have been used, but such plasmids must be able to replicate and express a marker gene in *Amycolatopsis* spp., and problems could arise due to restriction systems. Thus, the development of transformation methods for *Amycolatopsis* has not been easy and no single method appears to be applicable to all strains. Basically, five methods have been utilized in order to transfer plasmid DNA into *Amycolatopsis*. These methods are: (a) induced protoplast transformation [31], (b) direct transformation with PEG and CsCl [27], (c) electroporation [24], (d) electroduction [45], and (e) conjugation [40].

Induced protoplast transformation

The first attempt to develop a transformation system for *A. mediterranei* based on protoplasting was made by Schupp and Divers [37]. Although protoplast formation was apparently efficient in *A. mediterranei* using the *Streptomyces* procedure [7], these protoplasts could not be regenerated after plating on regeneration agar R2YE. The procedure that finally proved successful made use of mannitol as an osmostablizer in a treatment medium called R2L, otherwise similar to R2YE. However, Schupp and Divers [37] could not demonstrate the transformation of these protoplasts with *Streptomyces* cloning vectors. Subsequently, Matsushima et al. [31] observed that protocols developed for transformation of *Streptomyces* [3, 5, 30, 41] and *Saccharopolyspora* [54], did not give satisfactory levels of transformants when *Streptomyces* plasmids were used in *A. orientalis*. However, they successfully identified several factors, including type of medium, number of protoplasts, and concentration of PEG, that played a crucial role in transformation. After optimization, three *Streptomyces* plasmid vectors were transformed. There seemed to be some restriction and a transformation efficiency of 10^6 transformants/μg DNA was obtained with plasmid pIJ702 isolated from *A. orientalis* compared to 2×10^4 transformants/μg DNA when isolated from *S. lividans*. The second *Amycolatopsis* strain from which the PEG-induced protoplast transformation has been successfully established is *A. japonicum* MG417-CF17 [40]. The source of PEG was important and only a very low transformation frequency of 0.56 transformants/μg DNA was achieved. For many other *Amycolatopsis* or *Nocardia* strains, PEG-induced transformation of protoplasts has been unsuccessful [19, 24, 26, 35, 50, 55].

Direct transformation of mycelia with plasmid DNA

A method of direct transformation of mycelial suspensions was developed by Madon and Hütter [27] for *A. mediterranei* LBG A3136. The method utilizes, in addition to PEG, cations such as potassium, cesium and rubidium. With pMEA100, the transformation efficiency was 1.2×10^6 transformants/μg DNA. When transformants of the pMEA100 derivative pMEA123 were selected using erythromycin, 0.4×10^5 transformants/μg DNA were obtained. Modified direct transformation was the only method that was successful in introducing DNA into *A. mediterranei* DSM 5908 [35]; as plasmids that integrate by homologous recombination were used, a direct comparison of transformation frequencies with other systems is not possible. The method could also be used to transform *A. methanolica* [50] using pMEA300. Optimizing the method for *A. methanolica* by reducing the concentration of CsCl resulted in a transformation of 9×10^5 transformants/μg DNA. In *Amycolatopsis* sp. strain HR167 a transformation efficiency of 7.1×10^5 transformants/μg DNA was reported [36] and the method also worked for *N. lactamdurans* (6.7×10^5 transformants/μg DNA) [19]. Successful transformation at a very low frequency (< 1 transformant/μg DNA) was also observed in *A. japonicum* [40].

Electroporation

Electroporation was first carried out in *A. mediterranei* DSM 40773 and *A. orientalis* DSM 40040 and the transformation protocol was optimized using pRL1 [24]. Mycelial suspensions in distilled water were pulsed at high field strengths and a transformation efficiency of 1×10^3 transformants/μg DNA was achieved in *A. mediterranei* DSM 40773. As the *km*/*neo* marker in pRL1 is not useful in many strains, the electrotransformation of *A. mediterranei* was optimized using plasmid pRL60, which also contains α-amylase and *ermE* marker genes [16, 22]. The most critical parameters evaluated for electrotransformation using plasmid pRL60 were growth phase (late exponential phase), electrical field strength (7.5 kV/cm), pretreatment of mycelia with lysozyme, and use of salt-free water [22, 45]. With optimized parameters a transformation efficiency of 1×10^4 transformants/μg DNA could be obtained in *A. mediterranei* DSM 40773. pRL60 could also be transferred into *A. mediterranei* DSM 46095, DSM 46096, MTCC-14, MTCC-17, MTCC-18, F1/24, T-195, DSM 43387 and transformation efficiencies of 1×10^4, 1×10^4, 1×10^4 1×10^5, 1×10^5, 1×10^4, 1×10^4, 1×10^3 transformants/μg DNA respectively were obtained under conditions described above [22]. In *Amycolatopsis* strain HR167, which produces vanillin [36], electroporation gave 2×10^2 transformants/μg DNA using plasmid DNA pRLE6. The results of electroporation were very poor in *N. lactamdurans* [19] and the method did not yield transformants in *A. mediterranei* DSM 5908 [35].

Electroduction

Serious problems have been encountered while isolating plasmid DNA from *A. mediterranei* strains. With the routine methods, which we follow [30], plasmid DNA

yields are very low and DNA preparations are usually heavily contaminated with chromosomal DNA. One alternative, which has been employed in *Streptomyces* spp. to overcome this problem, is the direct transfer of plasmid DNA from *Streptomyces* to *E. coli* through electroporation, a method known as electroduction [51]. A prerequisite for this method is the availability of a shuttle vector between the donor and the recipient bacteria. Having constructed a series of shuttle plasmids between *A. mediterranei* and *E. coli* as well as a method of transformation through electroporation [14, 16, 22, 24], we have been able to apply the method of electroduction developed for *Streptomyces* [51] to *A. mediterranei* [45]. With this approach a series of shuttle plasmids of the pRL series were transferred directly from *A. mediterranei* to *E. coli* [45]. The numbers of transformants obtained in each experiment were independent of the size of plasmid used. Furthermore, the plasmid DNAs did not undergo any deletions or rearrangements during their direct transfer from *A. mediterranei* to *E. coli*. In addition to *A. mediterranei* strains, this method is also expected to work in several other bacterial strains where plasmid isolation is extremely difficult, especially in *A. orientalis*, *A. methanolica*, and *Nocardia spp.* including *N. lactamdurans*, *N. asteroides*, *N. autotrophica*, *N. brasiliensis*, *N. bravicatena*, *N. carnea,* and *N. cellulans*, in which *pA-rep* has been reported to work [19].

Conjugation

pMEA100 in *A. mediterranei* LBG A3136 [33] and pMEA300 in *A. methanolica* [48] can transfer between strains by conjugation. However, this property has not yet been used for genetic manipulation in *Amycolatopsis*.

Several versatile vectors that can be transferred from *E. coli* to *Streptomyces* spp. by conjugation have been developed [6]. The vectors contain the *ori*T sequence from IncP-group plasmid RK2 (also designated RP1/RP4) but require the transfer function to be supplied *in trans* by the *E. coli* donor strain [32]. This system can transfer DNA to almost any bacterial species. Most of these vectors are non-replicative in *Streptomycetes* but integrate into the chromosome to yield stable recombinant strains. The integration can be achieved by homologous recombination via cloned chromosomal fragments or by site-specific integration using plasmid pSAM2 [32] or phage-derived functions (ϕC31) [18]. Recently Stegmann et al. [40] have used such a system with plasmid pSET152, which carries an apramycin resistance marker and the ϕC31-attachment site to allow integration into the chromosome. A frequency of 2.4×10^{-4} transconjugants/recipient was obtained, which, though low, is still useful for many purposes. It seems likely that such conjugation will also work for other *Amycolatopsis* strains.

Host range of *Amycolatopsis* cloning vectors and the potential of these cloning vectors and transformation methods for gene cloning and gene replacements

Although pMEA100 [27] and pMEA300 [46, 49] derived cloning vectors could be used for transformation of *A. mediterranei* strain LBG A3136 and *A. methanolica* respectively, these plasmids have a limited host range. In contrast, plasmid cloning vectors derived from the pA387 replicon have a broader host range. Apart from *A. mediterranei* DSM 40773 and *A. orientalis* DSM40040 [31], other *A. mediterranei* strains that can be transformed by using plasmids containing *pA-rep* are DSM 43304, DSM 46095, DSM 46096, MTCC14, MTCC 17, MTCC 18, F1/24, and T-195 [21, 22]. The latter two strains were derived from an industrial strain of Ciba-Geigy (*A. mediterranei* N/813). Martin et al. [28] even succeeded in transforming *N. lactamdurans*, *N. autotrophica* and *N. brasiliensis* using pRL1 or cloning vectors derived from pRL1 [28]. Pelzer et al. [35] also transformed *A. mediterranei* DSM 5908, which produces balhimycin, using pRL1 albeit with a very low transformation efficiency that may have been due to the presence of a strong restriction system. *A. japonicum* [40] and *Amycolatopsis* sp. strain HR167 [36] have also been transformed by plasmids based on the pA387 replicon.

pMEA300-based shuttle vectors were used to clone several genes of *A. methanolica* including phenylalanine biosynthesis genes [46, 47]. Cloning vectors of the pRL series have not yet been used extensively for cloning purposes, because the manipulation of antibiotic biosynthesis clusters usually relies on integrative vectors rather than freely replicating vectors. However, they offer a useful set of vectors applicable to many and perhaps all *Amycolatopsis* species. Nonetheless, there is a need to further improve these cloning vectors and develop better strategies to carry out genetic engineering experiments especially in species of *Amycolatopsis* that produce antibiotics.

The rifamycin biosynthetic gene clusters have been cloned and characterized from *A. mediterranei* S699 [2], *A. mediterranei* LBG A3136 [13] and *A. mediterranei* DSM 46095 [15], but there is still no report of their manipulation for the generation of rifamycin analogues. This is primarily due to the recalcitrant nature of *A. mediterranei* strains to genetic manipulation. In contrast, the erythromycin gene cluster in *Saccharopolyspora erythraea* has been manipulated extensively and a number of analogues have been produced [23]. In our laboratory, we have used homologous recombination for rifamycin domain replacement in *A. mediterranei* [20]. In this approach, a cassette was constructed to replace the acyltransferase domain of module 8 (AT8) of the rifamycin biosynthetic gene cluster, which recruits propionate into the growing chain, by an acyl transferase domain (AT2) of the rapamycin biosynthetic gene cluster, which recruits acetate into the growing chain.

The cassette was constructed by flanking AT2 with sequences adjacent to AT8 (Fig. 5). It was then cloned into *E. coli* plasmid pIJ4026, which contained *ermE* as the marker gene and which does not replicate in *A. mediterranei*. The construct was transferred by electroporation into *A. mediterranei* MTCC18 and single-crossovers were selected with erythromycin. The integration of the construct by a single-crossover was demonstrated by Southern blot hybridization. These transformants were then grown without erythromycin selection pressure and a second crossover event in which plasmid DNA looped out and the AT8 domain was replaced by AT2 occurred (Fig. 5). This was again confirmed by Southern blot hybridization. The same strategy was also used to replace AT6 with AT2.

Encouraged by these developments, we developed a new vector for homologous recombination in *A. mediterranei* [20]. A derivative of pUC18, pKVE was constructed that contains the *ori*T, *erm*E and *lac*Zα genes (Fig. 6a). pKVE does not replicate in *A. mediterranei* but, when chromosomal sequences are cloned in it, there is high efficiency of integration into the chromosome. The cassette to replace the AT6 domain of the rifamycin biosynthetic gene cluster (Fig. 6b) was cloned in pKVE and the replacement carried out as described above. A facile host-vector strategy for genetic engineering of rifamycin PKS in *A. mediterranei* S699 has also been reported [2]. In this system, the genes of six or ten modules of rifamycin polyketide synthase (PKS) were replaced with a hygromycin resistance marker gene. The deletion host retained the ability to synthesize the starter unit 3-amino-5-hydroxy benzoic acid. A suicide plasmid carrying a short fragment from the 5′ flanking end of the engineered deletion, an apramycin resistance marker gene and suitably engineered PKS genes could be introduced via electroporation into the deletion host, resulting in the integration of PKS genes and the biosynthesis of reporter polyketides. This strategy requires

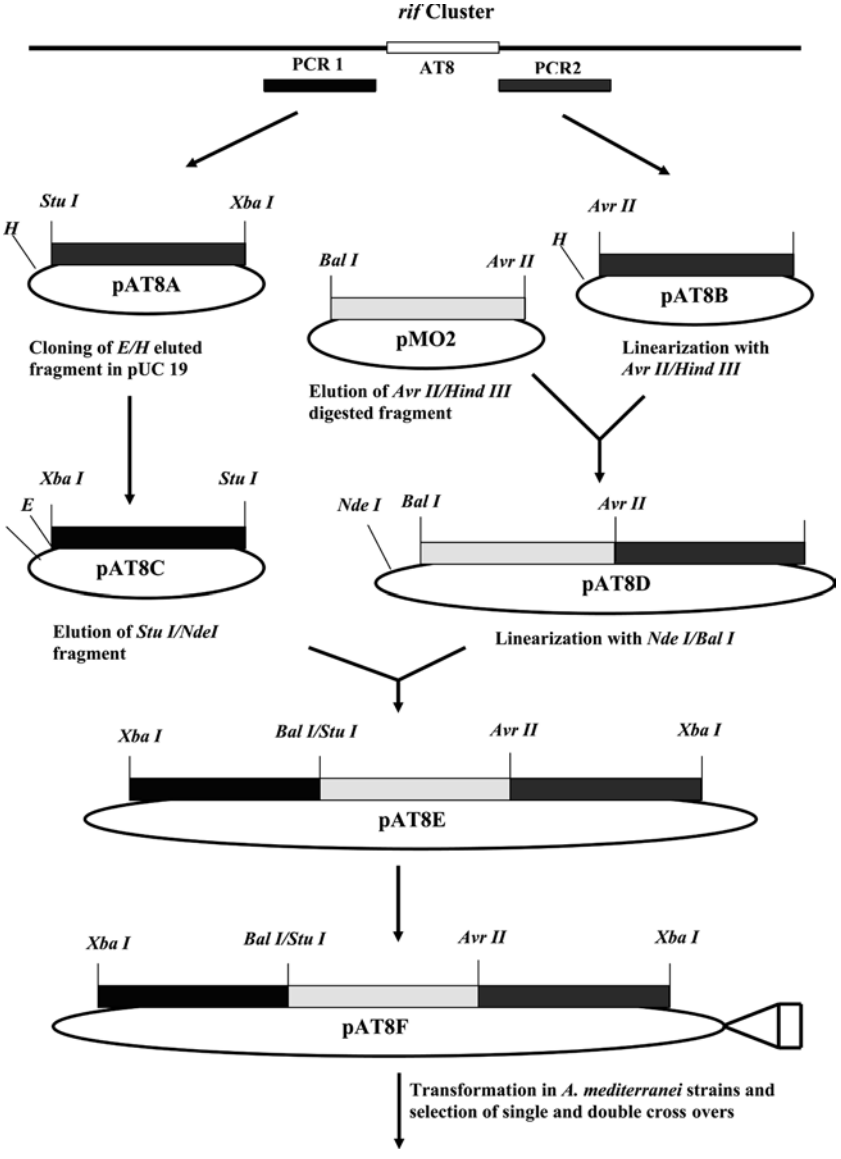

Fig. 5 Strategy for domain replacement for rifamycin biosynthetic gene cluster. The flanking regions of AT8 (acyl-transferase domains of rifamycin biosynthetic gene cluster), PCR1 and PCR2, were amplified and cloned in pUC18/19 (pAT8A and pAT8B respectively). Also, the AT2 (acyl-transferase domains of rapamycin biosynthetic gene cluster) was cloned in pUC18. The final cassette was prepared by ligation of cloned fragments, yielding pAT8E. The final fragment, containing flanks of AT8 and AT2, was cloned in the *Xba*I site of pIJ4026 containing the erythromycin gene (pAT8F). The construct was transformed by electroporation and single- and double-crossovers were scored

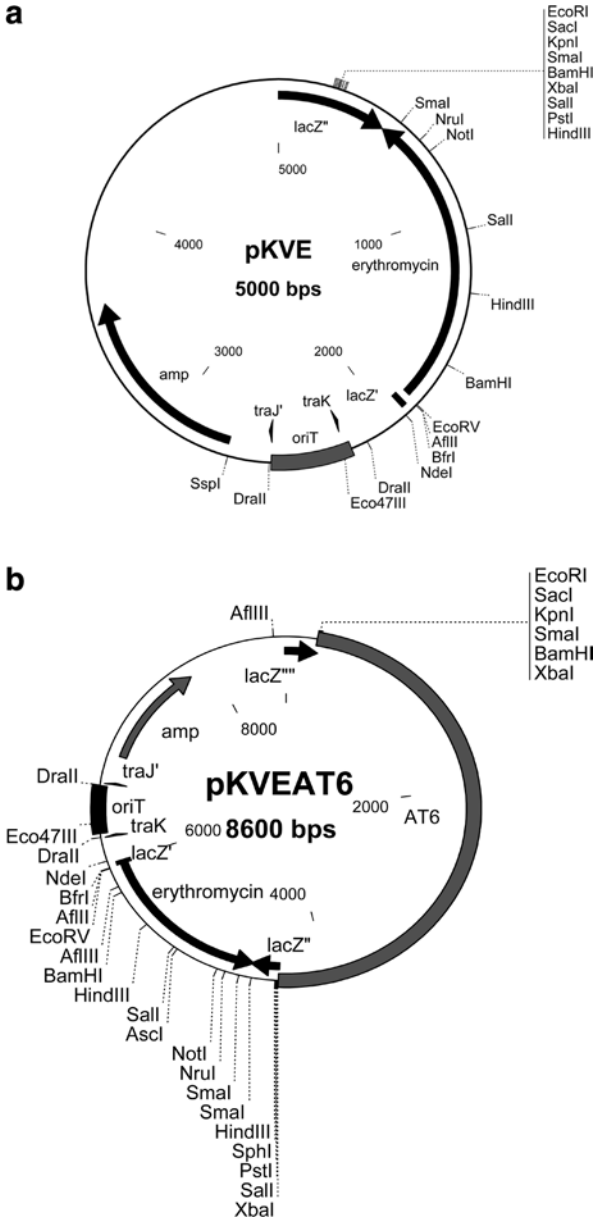

Fig. 6 Restriction map of **a** pKVE, **b** pKVEAT6

only a single-crossover, thus eliminating the need for tedious, time-consuming, non-selectable double-crossovers.

Conclusions and future prospects

Reasonably good progress has been made in the development of molecular genetic techniques for *Amycolatopsis* species during the past 5 years. The lack of cloning vectors and transformation methods had placed *Amycolatopsis* far behind *Streptomyces* as a subject of molecular genetic research, but the development of plasmid cloning vectors, transformation methods and gene replacement strategies for several species of *Amycolatopsis* has begun to open up the possibilities for studying the molecular genetics of these bacteria. One of the plasmids, pMEA300, from *A. methanolica* has been completely sequenced and its encoded functions are already deduced. The wider host range of the pA387 replicon makes it more suitable for general cloning vectors and the resulting pRL series of cloning vectors has led to the development of transformation methods and the identification of suitable marker genes for several species of *Amycolatopsis*, including *A. mediterranei*. These cloning vectors, however, require further improvements but the transformation methods developed have been demonstrated to be good enough for carrying out gene or domain replacement in *A. mediterranei* through homologous recombination.

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