REVIEW

The genus *Amycolatopsis*: Indigenous plasmids, cloning vectors and gene transfer systems

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Abstract The genus *Amycolatopsis* is a member of the phylogenetic group nocardioform actinomycetes. Most of the members of the genus *Amycolatopsis* are known to produce antibiotics. Additionally, members of this genus have been reported to metabolize aromatic compounds as the sole sources of carbon and energy. Development of genetic manipulation in *Amycolatopsis* has progressed slowly due to paucity of genetic tools and methods. The occurrence of indigenous plasmids in different species of *Amycolatopsis* is not very common. Till date, only three indigenous plasmids viz., pMEA100, pMEA300 and pA387 have been reported in *Amycolatopsis* species. Various vectors based on the indigenous plasmids, pMEA100, pMEA300 and pA387, have been constructed. These vectors have proved useful for molecular genetics studies of actinomycetes. Molecular genetic work with *Amycolatopsis* strains is not easy, since transformation methods have to be developed, or at least optimized, for each particular strain. Nonetheless, methods for efficient transformation (polyethyleneglycol (PEG) induced protoplast transformation, transformation by electroporation and direct transformation) have been developed and used successfully for the introduction of DNA into several *Amycolatopsis* species. The construction of plasmid cloning vectors and the development of gene transfer systems has opened up possibilities for studying the molecular genetics of these bacteria.

Key words: *Amycolatopsis* · plasmid · cloning vector · gene transfer system

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Actinomycetes

Actinomycetes, one of the major communities of the microbial population present in soil, comprise a diverse group of Gram-positive, aerobic, mycelial bacteria. They were initially considered to be an intermediate group between bacteria and fungi but now are recognized as prokaryotic organisms. Actinomycetes belong to the order *Actinomycetales*¹ and are characterized by the formation of substrate and aerial mycelium on solid media, presence of spores and a high GC content of DNA (60–70%). They include the genera *Streptomyces*, *Corynebacterium*, *Micromonospora*, and *Amycolatopsis* to mention a few.

Actinomycetes are metabolically diverse microbes and exist in various habitats in nature. They produce many extracellular enzymes in soil. By decomposing complex mixtures of polymers in dead plant, animal and fungal material^{2–4}, they play an important role in the mineralization processes in nature. They are well known for their economic importance as producers of many bioactive compounds, such as antibiotics and enzymes^{5–7}. Infact, actinomycetes make some two-thirds of the known antibiotics that are produced by microorganisms. *Streptomyces* and *Amycolatopsis* species are widely used in industrial production processes.

The genus *Amycolatopsis*

The genus *Amycolatopsis* was proposed by Lechevalier *et al.*⁸ and was assigned to the family *Pseudonocardiaceae*^{9, 10}. It can be distinguished from all of the other genera classified in the family *Pseudonocardiaceae* by a combination of chemical and morphological markers¹¹. The organisms in the genus *Amycolatopsis* produce branched, fragmenting aerial and substrate mycelia and are characterized by the

following cell chemistry: a type IV cell wall (*meso*-diaminopimelic acid, arabinose and galactose in the cell wall)⁸; the absence of mycolic acids⁸; complex mixtures of saturated and branched-chain fatty acids^{12, 13}; muramic acid in an *N*-acetyl form¹²; a major menaquinone of MK-9(H₄) with a minor amount of MK-9(H₂)^{8,14}; and phosphatidylethanolamine as a diagnostic phospholipid (a phospholipid type PII pattern according to the classification of Lechevalier *et al.*¹⁵). Members of all of these species can be separated from one another by using various biochemical, morphological and physiological properties^{16–18}.

The genus *Amycolatopsis* encompasses bacteria that are well known for the production of commercially and medically important antibiotics¹⁹, two of which, rifamycin and vancomycin, are currently commercially important (Table 1). Additionally, members of this genus have been reported to metabolize aromatic compounds as the sole sources of carbon and energy²⁰ (Table 1).

Plasmids of *Amycolatopsis*

Development of genetic manipulation in *Amycolatopsis* has progressed slowly due to scarcity of sophisticated genetic tools and methods. Till date, only three indigenous plasmids viz., pMEA100³⁵, pMEA300³⁶ and pA387³⁷ have been re-

ported in *Amycolatopsis* species. The development of gene transfer methods for *Amycolatopsis* has proved difficult since gene cloning systems have to be developed, or at least optimized, for each particular strain. However, methods for efficient transformation have been developed and used successfully for the introduction of DNA into various *Amycolatopsis* species^{37–40}. There has been a steady progress in the development of molecular genetic tools for the genetic engineering of *Amycolatopsis*. The focus of this review is on the indigenous plasmids of *Amycolatopsis* as well as on the construction of cloning vectors and the development of gene transfer systems for the genetic manipulation of *Amycolatopsis*.

Plasmid pMEA100

Amycolatopsis mediterranei LBG A3136 is a rifamycin-producing actinomycete⁴¹. It contains a low-copy-number covalently closed circular extrachromosomal element, pMEA100, which could only be isolated from mycelium grown on agar plates³⁵. A restriction map of pMEA100 is shown in Fig. 1a.

Plasmid pMEA100 (23.7 kb) exists in a chromosomally integrated form as well as in a free form in *A. mediterranei* LBG A3136, whereas in a closely related strain ATCC 13685, only an integrated form was detected³⁵. It is capable

Table 1 Some species of *Amycolatopsis* and their importance.

Organism	Antibiotic/compound	References
<i>Amycolatopsis</i> sp. strain MK299-95F4	Epoxyquinomicins A, B, C and D	21
<i>Amycolatopsis</i> sp. strain MJ126-NF4	Azicemicins A and B	22
<i>A. orientalis</i> subsp. <i>orientalis</i> DSM 40040	Vancomycin	23
<i>A. coloradensis</i> NRRL 3218	Avoparcin	24
<i>A. orientalis</i> ATCC 53884	Quartromycin	25
<i>A. vancoresmycina</i> DSM 44592	Vancoresmycin	26
<i>Amycolatopsis</i> sp. Y-86,21022	Balhimycin	27
<i>A. tolypomycina</i> IFO 14664	Tolypomycin	26
<i>A. rifamycinica</i> DSM 46095	Rifamycin	28
<i>A. alba</i> NRRL 18532	Glycopeptide antibiotic related to vancomycin	13
<i>A. decaplanina</i> DSM 44594	Decaplanin	29
<i>Amycolatopsis</i> sp. strain HT-6	Polyester-degrading bacteria	30
<i>A. mediterranei</i> MI710-51F6	Dethymicin	31
<i>A. lurida</i> DSM 43134	Ristocetin A, ristocetin B	32
<i>A. japonicum</i> MG417-CF17	(S,S)-N,N'-ethylenediaminedisuccinic acid	33
<i>A. methanolica</i> NCIB 11946	Methanol-utilizing bacteria	34
<i>A. mediterranei</i> DSM 40501	Degrades aromatic compounds	20
<i>A. rugosa</i> DSM 43194	Degrades aromatic compounds	20
<i>A. benzoatilytica</i> DSM 43387	Degrades aromatic compounds	20
<i>A. orientalis</i> subsp. <i>orientalis</i> DSM 43388	Degrades aromatic compounds	20

Fig. 1 (a) Map of plasmid pMEA100^{35,40}. (b) Map of plasmid pMEA123⁴⁰. Black line represents plasmid pMEA100 sequences. Box represents the erythromycin resistance gene.

of site-specific excision and integration and is subject to internal rearrangements^{42,43}. Plasmid pMEA100 was found to be self-transmissible, eliciting the lethal zygosis phenotype (pock formation)³⁵. A common phenotype associated with transfer of actinomycete plasmids is that of pock formation, zones of growth inhibition that become apparent when plasmid-carrying cells develop in a confluent lawn of plasmid-lacking recipient cells. This feature makes *Amycolatopsis* and *Streptomyces* the only microorganisms where genetic exchange is visible with the naked eye.

The indigenous plasmid of *A. mediterranei* LBG A3136, pMEA100, was used to construct a cloning vector, pMEA123⁴⁰. The restriction map of vector pMEA123 is presented in Fig. 1b. It contains the erythromycin resistance gene, and a single *SacI* restriction site that can be used for cloning experiments. However, there are no further studies described in the literature on pMEA100-based vectors.

Plasmid pMEA300

Amycolatopsis methanolica NCIB 11946³⁴ is a versatile methanol-utilizing bacterium, closely related to *Amycolatopsis mediterranei* and *Saccharopolyspora erythraea*^{8,44}. It contains the 13.3 kb element pMEA300, that is present mostly in an integrated state, but it can also replicate autonomously³⁶. The restriction map of the integrated copy was found to be identical to that of the free copy³⁶ (Fig. 2).

Fig. 2 Map of plasmid pMEA300^{36, 45–47}. Arrows indicate the positioning of the putative open reading frames (ORFs) (GenBank accession no. L36679).

Depending on the growth conditions, plasmid pMEA300 is maintained either integrated at a unique genomic location or occurs as an autonomously replicating plasmid, coexisting with the integrated form⁴⁵. Generally, covalently closed circular pMEA300 was found in less than 1 copy per 5–10 chromosomes. However, the copy number of the free plasmid DNA increased to approximately 50 per chromosomes when cells of *A. methanolica* were cultivated in media with autoclaved sucrose (or fructose).

Plasmid pMEA300 is the first integrative actinomycetes plasmid that has been completely sequenced (GenBank accession no. L36679). Regions on pMEA300 encoding functions for site-specific chromosomal integration (2.1 kb)³⁶, autonomous replication (maintenance; 2.4 kb and 0.8 kb)⁴⁵, conjugational transfer (6.2 kb)⁴⁶, stimulation of transformation frequency (1.5 kb)⁴⁷, and enhanced mutation frequency have been identified and localized on pMEA300 (Fig. 2). The latter two characteristics, as well as the observed effect of medium composition on the copy number of autonomously replicating pMEA300, are novel and unique features for actinomycetes plasmids.

Various vectors based on the *A. methanolica* indigenous plasmid pMEA300 have been constructed⁴⁵ and successfully employed as *A. methanolica*-*E. coli* shuttle vectors. In particular, shuttle vector pWV138 has proved useful⁴⁸ (Fig. 3). Plasmid pWV138 contains a unique BamHI site that can be used for cloning, resulting in insertional inactivation of the tetracycline resistance marker gene in *E. coli*⁴⁸. A gene bank, covering the entire genome of

Fig. 3 Map of plasmid pWV138⁴⁸. Arrows on pWV138 indicate open reading frames encoding functions required for maintenance and autonomous replication (*korA*, *orfA*, *orfB*), conjugational transfer (*traA*), stimulation of transformation frequency (*stf*) and high mutation frequency (*mut*). Km, kanamycin resistance gene; Tc, tetracycline resistance gene.

Fig. 4 Map of plasmid pA387. pA-rep designates the region carrying the information for autonomous replication in *A. benzoatilytica* DSM 43387 (GenBank accession no. EF375609).

A. methanolica, was constructed in *E. coli* using a pMEA300-derived shuttle vector, pWV138⁴⁸. In general, pMEA300-derived shuttle vectors have been used for cloning genes involved in glucose utilization⁴⁹ and in aromatic amino acid biosynthesis⁴⁸.

Plasmid pA387

The recently reclassified actinomycete *Amycolatopsis benzoatilytica* DSM 43387 (formerly *Amycolatopsis orientalis* DSM 43387)⁵⁰, a clinical isolate from submandibular mycetoma tissue, has been reported to metabolize aromatic compounds²⁰. It harbours a 30,157 bp low copy number (approximately 10 copies per cell) autonomously replicating element, pA387³⁷ (GenBank accession no. EF375609). It is a stable plasmid that is present only in the free form in *A. benzoatilytica* DSM 43387³⁷. Plasmid pA387 has been characterized completely at the sequence level (GenBank accession no. EF375609). The physical map of plasmid pA387 is presented in Fig. 4. There are four sites for KpnI, single sites for StuI, XbaI, XhoI, NruI, and BglII and no recognition sites for EcoRI, BamHI, and HindIII in this plasmid.

Various vectors based on the replicon of plasmid pA387 have been constructed. A hybrid plasmid, termed pRL1, was constructed by cloning a 5.1-kb fragment of pA387 into the *Escherichia coli* vector pDM10³⁷. Plasmid pRL1 (Fig. 5) is the first hybrid plasmid which successfully transformed *Amycolatopsis mediterranei* DSM 40773 and *Amycolatopsis orientalis* DSM 40040. It contains two unique restriction sites (HindIII and BamHI) that can be used for cloning purposes. The stable plasmid pRL1 served as the starting point for the construction of a variety of *E. coli*-*Amycolatopsis* shuttle vectors.

Fig. 5 Map of plasmid pRL1³⁷. Km, kanamycin resistance gene; pBR-ori, origin of replication of vector pBR322; pA-rep, replicon region of plasmid pA387.

Fig. 6 Map of plasmid pULVK1⁵¹. Thin line indicates DNA originating from *E. coli* plasmids carrying the pBR322 origin of replication (pBR-ori) and the kanamycin resistance gene from Tn5 (km). pA-rep, replicon region of plasmid pA387.

Introduction of DNA into *Amycolatopsis lactamdurans* LC411

Plasmid pRL1 could be successfully transformed in *Amycolatopsis lactamdurans* LC411⁵¹ (formerly *Nocardia lactamdurans* LC411). However, pRL1 was found to be unstable in *A. lactamdurans* LC411 and underwent deletions to yield plasmid, named pULVK1 (6.0 kb) (Fig. 6). The deleted plasmid pULVK1 retained the origins of replication of *Amycolatopsis* sp. and *E. coli* and the kanamycin resistance marker. Plasmid pULVK1 was stable and has been routinely used in cloning experiments. It served as the starting point for the construction of a variety of plasmids with two selective markers and promoter-probe vectors for use in *A. lactamdurans*.

Versatile shuttle cloning vectors, pULVK2 (5.7 kb) and pULVK3 (6.4 kb), were developed by cloning the synthetic polylinker containing several unique restriction sites (EcoRV, DraI, BamHI, SstI, EcoRI and HindIII) and subcloning the pBluescript KS(+) multiple cloning site, respectively, into pULVK1 (Fig. 7). These plasmids could be transformed in *A. lactamdurans* LC411 and have been used successfully for the cloning of DNA fragments ranging from 2 to 10 kb in the polylinker sites without affecting the stability of the plasmid⁵¹.

A second marker, the apramycin resistance gene (*amr*) was inserted into pULVK2 resulting in pULVK2A (Fig. 8), allowing insertional inactivation of one of the markers while using the second one for selection⁵¹. A different marker, the amylase gene (*amy*), which is easily detected by the release of extracellular amylase in transformants of *A. lactamdurans* carrying this vector, was introduced into pULVK2, resulting in pULAM2⁵¹ (Fig. 8). Additionally, two 8.4 kb promoter-probe plasmids, pULVK4 and pULVK5, with the promoterless *xylE* gene as a reporter, were also constructed for utilization in *A. lactamdurans*. These vectors are useful instruments for molecular genetics studies of *A. lactamdurans*.

Introduction of DNA into Several *Amycolatopsis* Species

Plasmid pRL1 (10.4 kb) could be successfully transformed in *A. mediterranei* DSM 40773 and *A. orientalis* DSM 40040³⁷. However, a subsequent study revealed that the kanamycin/neomycin gene of pRL1 was not a very effective selectable marker for *A. mediterranei* DSM 40773 and related strains, as resistant mutants appeared spontaneously with high frequency⁵². Thus, there was a need to introduce suitable genetic markers in pRL1 as well as to reduce its size to make it more suitable for transformation and cloning experiments. The in vitro and in vivo gene manipulations of pRL1 or its derivatives led to the development of the series of plasmids illustrated in Fig. 9.

Initially, the size of plasmid pRL1 was reduced from 10.4 to 7.0 kb by using an exonuclease deletion kit. The resulting plasmid was designated as pRLM10. However, pRLM10 could not be used for cloning additional marker genes since it lacked suitable restriction sites and even the BamHI site of pRL1 was eliminated⁵².

To introduce more selectable marker genes, thiostrepton (*tsr*) and erythromycin (*ermE*) resistant genes were used. Although *tsr* could not be suitably expressed in *A. mediterranei* DSM 40773, the cloning of erythromycin resistant gene in pRL1 resulted in the construction of cloning vectors pRLM20 and pRLM30 (erythromycin resistant gene

Fig. 7 Map of plasmids pULVK2 and pULVK3⁵¹. Km, kanamycin resistance gene. Both these plasmids have the origin of replication of pA387 (pA-rep) and the origin of replication of pBR322 (pBR-ori). Asterisk indicates unique restriction sites.

Fig. 8 Map of plasmids pULVK2A and pULAM2⁵¹. Am, apramycin resistance gene; km, kanamycin resistance gene; amy, -amylase gene. pBR-ori, origin of replication of vector pBR322; pA-rep, replicon region of plasmid pA387. Asterisk indicates unique restriction sites.

(*ermE*) was cloned in anticlockwise and clockwise directions, respectively) (Fig. 9).

Despite their relatively large sizes (12.1 kb), cloning vectors pRLM20 and pRLM30, were successfully transformed in *A. mediterranei*. The erythromycin resistant gene (*ermE*) was also cloned in pULVK2A, resulting in plasmid pRL90 (8.5 kb), which could be transformed in *A. mediterranei* DSM 40773 (data not shown). Apart from *A. mediterranei* DSM 40773, other strains that could be transformed with pRLM20, pRLM30 and pRL90 are *A. mediterranei* strains,

DSM 43304, MTCC 17, F1/24, T-195 and *Amycolatopsis rifamycinica* DSM 46095⁵².

Further reduction in plasmid size was not accomplished in vitro, but rather, a new strategy based on in vivo recombination between large homologous repeats was used⁵³ (Fig. 9). This strategy was based on the observation that large repeats in the hybrid plasmid as well as the antibiotic pressure plays a crucial role in selecting the plasmid retained by the organism⁵⁵. The approach involved the cloning of a selectable marker, erythromycin resistance gene (*ermE*), into

Fig. 9 Scheme for construction of shuttle plasmids by intraplasmid recombination in *A. mediterranei*. Construction of plasmid pRLM10, pRLM20 and pRLM30 is described in the text. All other plasmids were generated via in vivo intraplasmid recombination. Solid lines indicate common events, whereas broken lines indicate rare events. Abbreviations: Km, kanamycin resistance gene; ermE, erythromycin resistance gene; Amy, -amylase gene; Am, apramycin resistance gene; pBR-ori, origin of replication of vector pBR322; pA-rep, replicon region of plasmid pA387 (Data adapted from Tuteja *et al.*⁵³).

plasmids pULAM2 and pULVK2A (derivatives of pRL1), followed by selection of the hybrid or concatemeric plasmids pRL50 and pRL80 (with large homologous repeats) in *E. coli* GM2163. These plasmids were then introduced into *A. mediterranei* DSM 40773, with selection in the presence

of different antibiotics. During the process of transformation and selection in *A. mediterranei*, pRL50 and pRL80 underwent intraplasmid recombinations, yielding derivatives that retained a common region essential for replication and maintenance, as well as the selected resistance genes.

This strategy resulted in the formation of several smaller plasmids designated as pRL51, pRL52, pRL53, pRL60, pRL81, and pRL82. These plasmids, isolated from *A. mediterranei* DSM 40773, could be transferred to other *Amycolatopsis mediterranei* strains, DSM 43304, MTCC 17, F1/24, T-195 and *Amycolatopsis rifamycinica* DSM 46095. The pRL series of vectors⁵⁴ includes stable cloning vectors containing one or more marker gene(s) for utilization in *Amycolatopsis* species.

Introduction of DNA into *Amycolatopsis* sp. strain HR167

Plasmid pRL60 is a 10.2-kb shuttle vector comprising an origin of replication (pA-rep) (needed for the replication in *Amycolatopsis*) and the pBR-ori for *E. coli*. It contains a kanamycin resistance gene, an erythromycin resistance gene (*ermE*) and an -amylase (-amy) marker gene⁵⁵ (Fig. 10). Plasmid pRL60 was found to be suitable for transformation of *Amycolatopsis* sp. strain HR167; however, erythromycin resistance gene (*ermE*) could not be used as a selectable marker due to an inherent erythromycin resistance of this strain⁵⁶. When plasmid pRL60 was transformed in *Amycolatopsis* sp. strain HR167, some regions of the plasmid were spontaneously deleted, resulting in plasmid named as pRLE6 (Fig. 10).

Plasmid pRLE6 has a size of 5,844 bp (GenBank accession no. AY062236) and lacks the marker genes -amy and *ermE* of pRL60. Plasmid pRLE6 was transformed into

Amycolatopsis sp. strain HR167, where it was maintained without further deletion under selection on kanamycin. It is a suitable shuttle vector, with single restriction sites for EcoRI and HindIII suitable for cloning genes for introduction into *Amycolatopsis* sp. strain HR167.

Introduction of DNA into *Amycolatopsis mediterranei* U-32

Amycolatopsis mediterranei U-32 could be successfully transformed with plasmid pULVK2A⁵¹ and a derivative of plasmid pRL1³⁷ called pDXM32⁵⁷. A map of pDXM32 is shown in Fig. 11. Plasmid pDXM32 was constructed by inserting the apramycin resistance determinant, *aac3(IV)*, from pULVK2A into pRL1⁵⁷. It has a size of 11.7 kb and possesses a kanamycin resistance gene and an apramycin resistance determinant. Despite its large size, plasmid pDXM32 is likely to be of general use in *Amycolatopsis* research.

In general, pA387 based shuttle vectors are broad host range vectors that have been found to be maintained in a wide variety of *Amycolatopsis* species, including *A. orientalis* DSM 40040³⁷, *Amycolatopsis* sp. strain HR167⁵⁶, *A. lactamdurans* LC411⁵¹, *A. rifamycinica* DSM 46095^{52,53,55}, *A. japonicum* MG417-CF17⁵⁸, *A. mediterranei* U-32⁵⁷, and other *A. mediterranei* strains DSM 40773, DSM 43304, MTCC 17, F1/24 and T-195^{37,52,53,55}. The construction of pA387 based shuttle vectors has proved

Fig. 10 Map of plasmids pRL60⁵⁵ and pRLE6⁵⁶ (GenBank accession no. AY062236). Amy, -amylase gene; km/neo, kanamycin/neomycin resistance gene; ermE, erythromycin resistance gene. pBR-ori, origin of replication of vector pBR322; pA-rep, replicon region of plasmid pA387.

Fig. 11 Map of plasmid pDXM32⁵⁷. The open reading frames predicted by sequence analysis derived from pA387 are shown as black arrows (GenBank accession no. AY256857). pBR-ori, origin of replication of vector pBR322. Neo, neomycin resistance gene; acc(3)VI, apramycin resistance gene; orf7 is a chimera containing only 79 aa from pA387.

valuable for molecular genetics studies of actinomycetes^{59–62}. Recent studies have also demonstrated the potential utility of these vectors for mutagenesis studies, which were carried out in *Nocardia uniformis* subsp. *tsuyamanensis* ATCC 21806^{63, 64}.

Gene Transfer Systems for *Amycolatopsis*

Several methods for efficient transformation have been developed and used successfully for the introduction of DNA into several *Amycolatopsis* species. However, the development of gene transfer methods for *Amycolatopsis* has proved difficult since gene cloning systems have to be developed, or at least optimized, for each particular strain. The following methods have been utilized in order to transfer plasmid DNA into *Amycolatopsis* species: (a) protoplast transformation, (b) direct transformation of mycelia, (c) electroporation, (d) electroduction, and (e) conjugation.

The first attempt for the introduction of DNA by transformation of protoplasts was made by Schupp and Divers³⁸. A combination of important variables for successful transformation of actinomycetes protoplasts were then identified by Matsushima *et al.*³⁹. Transformation of protoplasts has been successfully used to transform *A. mediterranei*, *A. orientalis* ATCC 19795, and *A. japonicum* MG417-CF17^{38, 39, 58}.

A method for direct transformation of mycelia with plasmid DNA was developed by Mado and Hütter for *A. mediterranei* LBG A3136⁴⁰. It is a simple, rapid transformation procedure that gives high transformation efficiency. The transformation procedure includes treatment of mycelium with PEG as well as with potassium, cesium, or rubidium cations in high concentrations. The addition of magnesium ions and calf thymus DNA was found to increase the transformation efficiency. This method could also be applied for transformation of *A. lactamdurans* LC411⁵¹, *A. methanolica* NCIB 11946⁴⁷, *A. japonicum* MG417-CF17⁵⁸, *Amycolatopsis mediterranei* DSM 5908⁶⁵ and *Amycolatopsis* sp. strain HR167⁵⁶.

Electroporation was first carried out in *A. mediterranei* 40773 and *A. orientalis* DSM 40040³⁷. A variety of electroporation conditions (such as growth phase of the culture, pretreatment of mycelia with lysozyme, electrical field strength, resistance, and pulse duration) were optimized to achieve high efficiencies of transformation. This method could also be applied for transformation of *A. mediterranei* U-32⁵⁷, *Amycolatopsis* sp. strain HR167⁵⁶, *A. rifamycinica* DSM 46095^{52,53,55}, and other *A. mediterranei* strains DSM 40773, DSM 43304, MTCC 17, F1/24 and T-195^{37,52,53,55}.

Electrodeposition is a method which allows direct transfer of plasmid DNA between donor and recipient under conditions of electroporation. It is a simple technique that allows a significantly faster analysis of plasmid DNA. The method is based on two basic features, i.e. the fact that DNA molecules may be introduced or released from a bacterial cell submitted to electroporation, and the inherent ability of shuttle plasmids to replicate in at least two distant bacterial species. Difficulties have been encountered while isolating plasmid DNA from *Amycolatopsis* strains. Using this method, a series of shuttle plasmids of the pRL series could be transferred directly from *A. mediterranei* strains into *E. coli* for the analysis of plasmid DNA⁵³.

Bacterial conjugation is a powerful tool for gene transfer into various bacteria. Conjugative transfer in actinomycetes takes place only on solid media under prolonged physical contact of the parental strains⁶⁶. A conjugal transfer system for mediating gene transfer into the genus *Amycolatopsis* is virtually non-existent. Till date, there is only one report demonstrating conjugal transfer of the plasmid DNA from *E. coli* to *A. japonicum* MG417-CF17⁵⁸. It seems likely that this method of DNA transfer will also be applicable to other *Amycolatopsis* species.

Conclusion and future prospects

Substantial progress has been made in the development of molecular genetic techniques for *Amycolatopsis* species over

the past two decades. The construction of plasmid cloning vectors and the development of gene transfer systems for several species of *Amycolatopsis* has opened up possibilities for studying the molecular genetics of these bacteria. Although different methods for efficient transformation (polyethyleneglycol (PEG) induced protoplast transformation, transformation by electroporation and direct transformation) have been developed and used successfully for the introduction of DNA into various *Amycolatopsis* species, there is a need to develop a conjugal transfer system for mediating gene transfer into the genus *Amycolatopsis*. The development of a conjugal transfer system would undoubtedly broaden the applicability of gene transfer systems and would significantly facilitate the genetic manipulation of *Amycolatopsis* for which existing DNA transfer methods are inefficient.

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