Development of an Improved Cloning Vector and Transformation System in *Amycolatopsis mediterranei* (*Nocardia mediterranei*)

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A versatile plasmid cloning vector pRL60 carrying kanamycin/neomycin resistance (*km/neo*), erythromycin resistance (*ermE*) and α-amylase (*α-amy*) marker genes that replicates in various *Amycolatopsis mediterranei* strains and *Escherichia coli* has been constructed. This cloning vector has been derived from a hybrid plasmid pRL50, which was developed by cloning *ermE* from pIJ4026 into a pRL1 derivative pULAM2. While cloning *ermE* into the *Bam*HI site of pULAM2, only a hybrid plasmid pRL50 with an additional copy of pULAM2 was selected. Thus pRL50 (18.7 kb) contained two copies each of the *km/neo*, *α-amy*, and one copy of *ermE*. When pRL50 was transformed into *A. mediterranei* DSM 40773 through electroporation and selected under erythromycin resistance, the plasmid underwent a spontaneous deletion of 8.5 kb fragment resulting in the formation of plasmid pRL60. pRL60 (10.2 kb) is a shuttle vector between *A. mediterranei* and *E. coli* with three marker genes: *km/neo*, *ermE* and *α-amy*. *ermE* is expressed in *A. mediterranei* thus allowing good selection of transformants. The *α-amy* gene of pRL60 is also expressed in *A. mediterranei* DSM 40773 and its activity can be easily detected on starch containing medium after iodine staining. Most critical parameters evaluated for electrotransformation using pRL60 in *A. mediterranei* were growth phase, electrical field strength, pulse length, pretreatment of mycelia with lysozyme and use of salt free water. At optimized parameters, a transformation efficiency of 4.0×10^4 transformants/μg DNA was reproducibly achieved for *A. mediterranei* DSM 40773. pRL60 could also be transformed into *A. mediterranei* DSM 43304, DSM 46095, MTCC-17 and in mutants F1/24 and T-195, (derived from an industrial strain of *A. mediterranei* N813). The *α-amy* of pRL60 conferred an amylolytic phenotype to all these strains. With the development of pRL60 and a reproducible transformation protocol, the application of recombinant DNA techniques to these industrial microorganisms has now become feasible.

Interest in developing gene cloning methods for species of *Amycolatopsis* stems from the fact that several strains of this genus are known to produce antibiotics. Several species of *Amycolatopsis*, including *A. mediterranei* and *A. orientalis* are of special interest as they produce the commercially and medically important antibiotics rifamycin[1] and vancomycin[2], respectively. A recently introduced glycopeptide antibiotic balhimycin which exhibits antibacterial activity against methicillin resistant *Staphylococcus aureus* strains has been isolated from *Amycolatopsis* sp. Y-86,21022[3].

In most of these organisms, antibiotic yields have been improved through classical strain improvement program primarily through mutations and recombination[4~6]. These methods are extremely useful, but they cannot be used for relieving the rate limiting steps in the biosynthetic pathways and provide little or no information on genes which are most important for high level production. In order to overcome such limitations, transformation methods and cloning vectors have been developed for several species of *Streptomyces*[7,8] and antibiotic biosynthetic genes are now being exploited either for the production of novel antibiotics or to enhance the antibiotic yield in *Streptomyces*[9,10], and *Penicillium*[8]. Unfortunately, species of *Amycolatopsis* and *Nocardia* which form one of the important groups of microorganisms producing antibiotics, remained out of the scope of molecular genetics. This was primarily due to the lack of suitable plasmids (which could be developed into a cloning vector)[11~13], methods of transformation (as

established for *Streptomyces*[13~20] were not applicable to these organisms) and suitable selective marker genes (which could be used for the construction of effective cloning vector).

We had partially overcome the first two difficulties in 1991 by constructing a preliminary cloning vector pRL1 of 10.4 kb with the *km/neo* as selective marker[21]. pRL1 was found to replicate in *A. mediterranei* and *A. orientalis* and a transformation efficiency of 2×10^3 and 1×10^5 transformants/μg DNA in *A. mediterranei* and *A. orientalis*, respectively, was reported. Subsequent studies revealed that the *km/neo* marker gene of pRL1 although expressed effectively in *E. coli*, is not a good selective marker since spontaneous resistant mutants appear with high frequency (80%) among transformants. Thus there was a need to provide pRL1 with suitable markers and also to reduce its size to make it more suitable for transformation and cloning experiments.

Materials and Methods

Bacterial Strains, Plasmids, Culture Conditions and Growth Media

Bacterial strains and plasmids used in the present study are listed in Table 1. *A. mediterranei* and related strains were cultured in liquid TYN medium containing tryptone 10 g, yeast extract 2.5 g and NaCl 5 g per litre (pH 7.2). For growth on agar plates, YM medium containing yeast extract 4 g, malt extract 10 g and glucose 4 g per litre (pH 7.2~7.4) was used. Plasmid pRL1 or its derivative harbouring strains of *A. mediterranei* were grown in liquid or on solid media containing appropriate concentrations of required antibiotics. *E. coli* cultures were maintained in liquid LB medium containing tryptone 10 g, yeast extract 5 g, NaCl 5 g and glucose 1 g per litre (pH 7.5) and kanamycin or neomycin (100 μg/ml) wherever necessary.

Plasmid Isolation and DNA Manipulations

Small scale and large scale DNA preparations of plasmid pRL1 or its derivatives from *A. mediterranei* and related species were performed by a modified procedure as described earlier[21]. Plasmid DNA isolation, purification by cesium chloride-ethidium bromide density gradient centrifugation, analysis by agarose gel electrophoresis and subsequent DNA manipulations were carried out by standard methods as described by SAMBROOK *et al.*[22] and HOPWOOD *et al.*[17].

Table 1. Bacterial strains and plasmids used in this study.

Organism	Strain or plasmid	Characteristic	Source or reference
A. mediterranei	40773	Rifamycin B	DSM[a]
A. mediterranei	43304	Rifamycin SV	DSM
A. mediterranei	46095	Rifamycin SV	DSM
A. mediterranei	46096	Rifamycin derivative	DSM
A. mediterranei	F1/24 ans-13	Protorifamycin	Ciba Geigy, Switzerland
A. mediterranei	T-195 ans-13 thi-8	Protorifamycin	Ciba Geigy, Switzerland
A. mediterranei	14	Rifamycin B	MTCC[b]
A. mediterranei	17	Rifamycin SV	MTCC
Streptomyces lividans	5	—	MTCC
Saccharopolyspora erythreae	1136	Erythromycin	MTCC
E. coli	GM2163	F$^-$ ara-14 Leu B6 thi-1 fhuA31 lacY1 tsx-78 galk^2 gal T22 sup E44 hisG4 str^r cam^r	New England Biolabs
	pULAM2	8.5kb, *km/neo*, *α-amy*	J. F. MARTIN, University of Spain
	pIJ4026	4.2 kb, *ermE*	M. J. BIBB, John Innes Institute Norwich
	pRL1	10.4 kb, *km/neo*	Lab stock (LAL *et al.* 1991)
	pRLM20	12.1 kb, *km/neo*, *ermE*	Lab stock
	pRL50	18.7 kb, *km/neo*, *ermE*, *α-amy*	This study
	pRL60	10.2 kb, *km/neo*, *ermE*, *α-amy*	This study

[a] DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany.
[b] MTCC: Microbial Type Culture Collection.
km/neo and *ermE*: Conferring resistance to kanamycin/neomycin and erythromycin, respectively. *α-amy*: α-amylase.

Improvement of pRL1 and Construction of an Effective Cloning Vector

In order to clone an effective selective marker gene, a derivative of plasmid pRL1, pULAM2 and *ermE* of *Saccharopolyspora erythreae* from pIJ4026 (Fig. 1) were chosen. pULAM2 has been derived from pRL1 and was used for transforming *Nocardia lactamdurans*[23]. The detailed strategy used for the construction of pRL60 is shown in Fig. 1.

Development of a Transformation Protocol and Optimization of Electroporation Parameters

First, a previously described electroporation protocol developed for *A. mediterranei* DSM 40773 was used[21]. However, with this protocol transformation efficiency was very low and needed modifications. For this purpose a 100 ml culture of *A. mediterranei* DSM 40773 was grown to stationary phase (A600, 1.2) in TYN medium at 28°C. Mycelia were collected by centrifugation at 7000 rpm (4°C) for 15 minutes, washed twice in 10 ml ice cold salt free water (from Millipore India Pvt Ltd.) and treated with 100 μl of 4 mg/ml lysozyme for 20 minutes at 20~24°C. The mycelial suspension was washed in 10% glycerol and pelleted again at 3000 rpm for 10 minutes. The mycelia were finally suspended in an appropriate volume of 10% glycerol to get approximately 9×10^9 CFU/ml. Plasmid DNA (ranging from 0.1~5.0 μg/μl) was added to 200 μl of mycelial suspension and the mixture was then transferred to a sterile electroporation cuvette (0.2 cm electrode gap) and pulsed immediately with a Bio-Rad Gene Pulser equipped with a pulse controller (Bio-Rad Laboratories, Regatta Blvd, Richmond, California). Initially, different electric field strengths of 5, 7.5 and 10 kV/cm, (generated by directing 1, 1.5 and 2 kV of voltage, respectively at various resistances) at a capacitance of 25 μF were used. Immediately following the pulse, the cells were spread on YM plates. After an overnight incubation at 28°C, the plates were overlaid with 3 ml soft agar (TYN containing 5 g/litre agar) containing 500 μg/ml erythromycin. Transformants were scored after 3~4 days which were subsequently grown on YM plates containing 200 μg/ml erythromycin or in liquid TYN containing 10~20 μg/ml erythromycin. Simultaneously, the percentage of surviving colonies (CFU) was estimated from serial dilutions of the pulsed mycelial suspension and spread on YM plates without antibiotic pressure. Subsequently, those electrical settings which were found to be suitable were used to study the effect of growth phase, DNA concentration, lysozyme and glycine treatment on transformation efficiency.

Host Range of pRL60 and Its Stability

The transformation protocol through electroporation was only optimized for *A. mediterranei* DSM 40773 and these conditions were used to transform different strains of *A. mediterranei* mentioned in Table 1. The stability of cloning vector pRL60 in *A. mediterranei* DSM 40773 was determined by growing liquid culture inoculated with a single colony of *A. mediterranei* DSM 40773 containing the plasmid pRL60 to stationary phase according to the method described by LAINE *et al.*[24]

Detection of α-Amylase Activity

In order to determine the α-amylase activity and to use it as a morphological marker during selection, transformants containing pRL60 were grown on YM medium containing 1% starch and erythromycin (200 μg/ml). Afterwards, the colonies were exposed to iodine vapours and the activity of α-amylase was detected by the appearance of a halo around colonies harbouring pRL60.

Results and Discussion

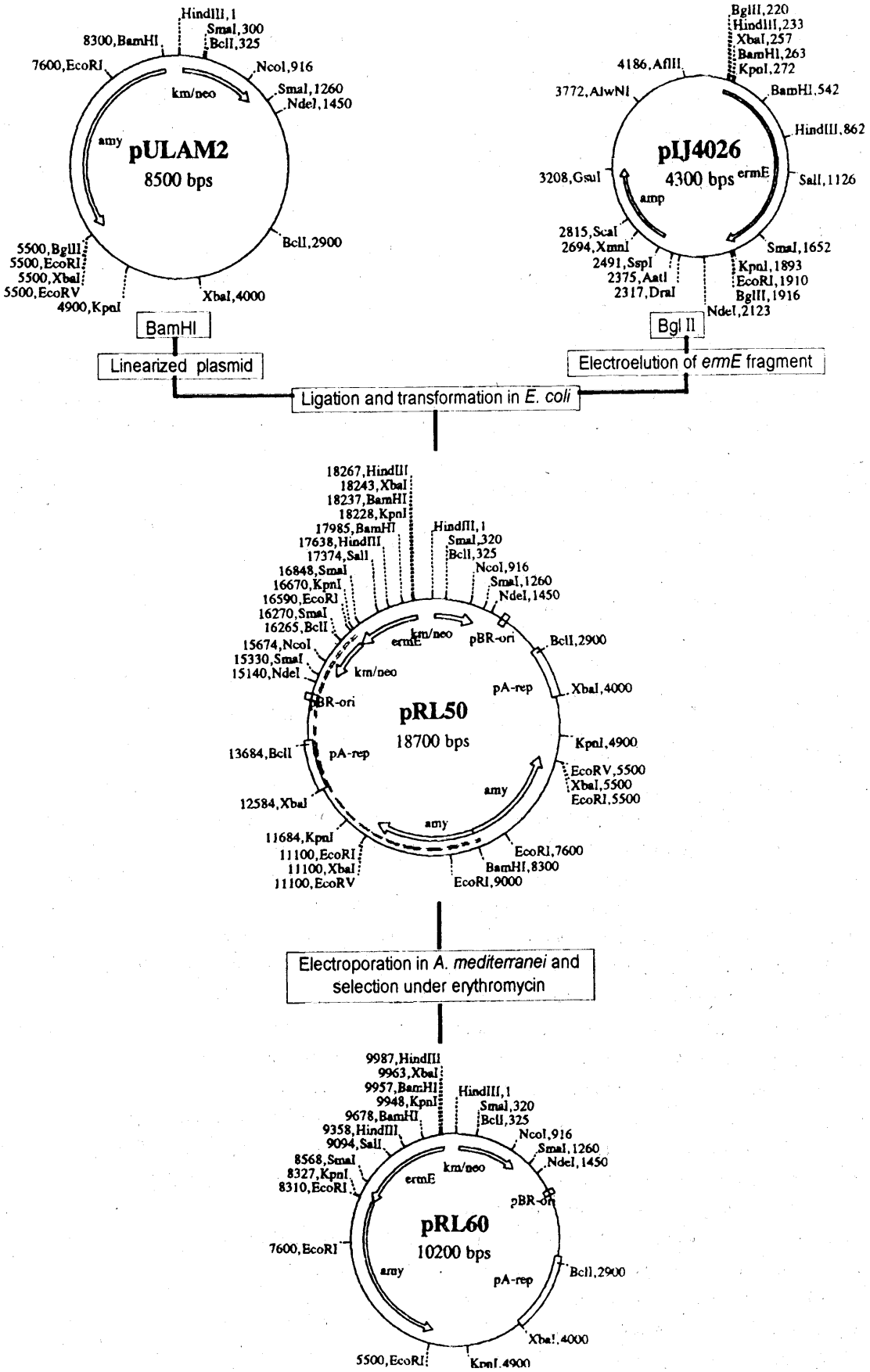
Development of a Hybrid Plasmid pRL50 in *E. coli* GM2163

In order to develop a plasmid cloning vector with two effective marker genes, pULAM2, containing *α-amy* was linearized by digestion with *Bam*HI and ligated with eluted 1.6 kb *Bgl*II fragment carrying the *ermE* gene from pIJ4026 (Fig. 1). After transformation into *E. coli* GM2163, transformants were selected on kanamycin containing plates (Fig. 1). Around 500 transformants were screened through colony hybridization using [α-^{32}P]ATP (sp. act-3000 Ci/mmol) labelled *ermE* as a probe. Out of several clones which gave strong positive signals only one clone was finally selected. Plasmid DNA from this clone was isolated and termed pRL50. The presence of the *ermE* insert and two copies of pULAM2 in pRL50 (18.7 kb) was confirmed by restriction digestion and Southern blot hybridization. Restriction mapping also determined the size of pRL50 to be approximately 18.7 kb (Fig. 1).

Electrotransformation and Expression of *ermE* in *A. mediterranei* DSM 40773

In order to examine the expression of *ermE* in *A. mediterranei*, pRL50 DNA isolated from *E. coli* GM2163 was first transformed into *A. mediterranei*

Fig. 1. Scheme for the construction of cloning vector pRL60.



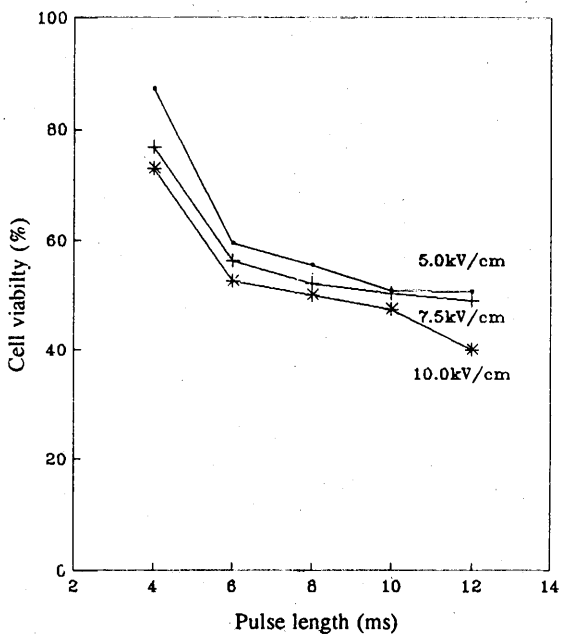
Linearized plasmid pULAM2 was ligated with eluted *ermE* fragment of pIJ4026 and transformed in *E. coli* GM2163. The transformants were selected under kanamycin pressure and subjected to colony hybridization using [α-^{32}P]ATP labelled *ermE* probe. The hybrid plasmid pRL50 when transformed in *A. mediterranei* DSM 40773 through electroporation and selected under erythromycin pressure underwent deletions and rearrangements resulting in the formation of plasmid pRL60 (Dotted lines indicate the region deleted from pRL50 to form pRL60).

through electroporation and transformants were selected on erythromycin containing plates. Interestingly, all the transformants which appeared, showed the presence of a plasmid, termed pRL60 (10.2 kb) which was formed due to a spontaneous deletion of 8.5 kb DNA fragment from pRL50 (18.7 kb). The deletion was very precise as always a specific fragment of DNA which included a region starting from 9 kb to 17.5 kb was deleted from pRL50 resulting in the formation of pRL60. pRL60 thus contained *km/neo*, *α-amy*, *ermE*, pA-*rep*, pBR-*ori* and had a size of 10.2 kb (Fig. 1). The *Bam*HI site of pRL60 can be used for cloning. The absence of any spontaneous mutant in *A. mediterranei* when transformants were selected under erythromycin led to the conclusion that the *ermE* is a suitable marker gene over *km/neo* of pRL1. Unlike *Streptomyces*[25~30], *Amycolatopsis* has been reported to be intrinsically resistant to several other antibiotics like kanamycin, gentamycin, chloramphenicol, bacitracin, spectinomycin, hygromycin and viomycin[19,20,31] thus virtually limiting the choice of marker genes which could be used for the development of cloning vectors in this group of organisms. Although the development of prototype vector pRL1 (10.4 kb) for *A. mediterranei* led to the development of transformation protocol through electroporation, this method was not reproducible[21]. Thus studies on optimization of transformation parameters and host range of pRL1 could not be carried out. In addition CsCl-PEG treatment[19] and protoplasting-PEG[17] methods tried in *A. mediterranei* DSM 40773, were not successful. This difficulty could be solved only through electroporation. In fact electroporation proved successful for species where DNA transfer methods are nonexistent[32], unreliable[33], or less efficient[34]. With the development of pRL60 harbouring three selectable marker genes *km/neo*, *ermE*, *α-amy* and effective expression of *ermE* and *α-amy* in *A. mediterranei*, we have overcome the problems of selection of suitable selection marker genes and transformation methods.

Effect of Electric Field Strength and Pulse Length

In this study the transformation protocol was optimized only for *A. mediterranei* DSM 40773 which was found to be more prone to genetic manipulations as compared to the other strains. The mycelial suspension of *A. mediterranei* DSM 40773 was first pulsed at various field strengths and pulse durations. Increasing electric field strength as well as pulse duration resulted in high rates of cell mortality. For instance, at an electrical field strength of 10 kV/cm and pulse duration of 12 ms, cell viability dropped to 40% (Fig. 2). The transformation efficiency increased with the increase in field strength up to 7.5 kV/cm. However, further increase in field strength, decreased the transformation efficiency (Fig. 3). Effect of pulse length on transformation efficiency was also examined for *A. mediterranei* DSM 40773. Maximum transformation efficiency was obtained at time constant between 14~16 ms (Fig. 4) and further increase in the pulse length resulted in a significant drop in the efficiency due to increased cell mortality. Attempts to enhance the transformation efficiency by repetitively pulsing the cells was found to be ineffective.

Fig. 2. Effect of electric field strength and pulse duration on cell viability in *A. mediterranei* DSM 40773.

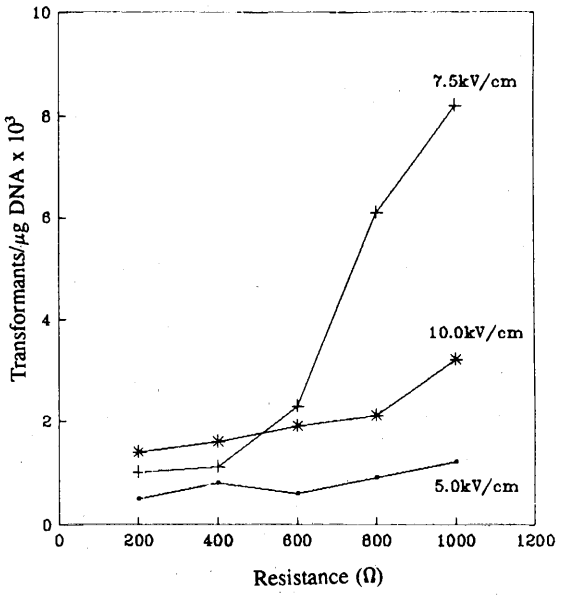


Stationary phase (A_{600}, 1.2) mycelial suspension (200 μl) of *A. mediterranei* was pulsed in a 0.2 cm cuvette. Electric field strength of 5, 7.5 and 10 kV/cm were generated by directing 1, 1.5 and 2.0 kV of electric discharge from a 25 μF capacitor, respectively, with a parallel resistance of 200, 400, 600, 800 and 1000 Ω. The percentage of viable cells was calculated as the ratio of CFU (colony forming unit) after electroporation to the total number of CFU subjected to electroporation.

Effect of Growth Phase, DNA Concentration, Lysozyme and Glycine Treatment on Transformation Efficiency

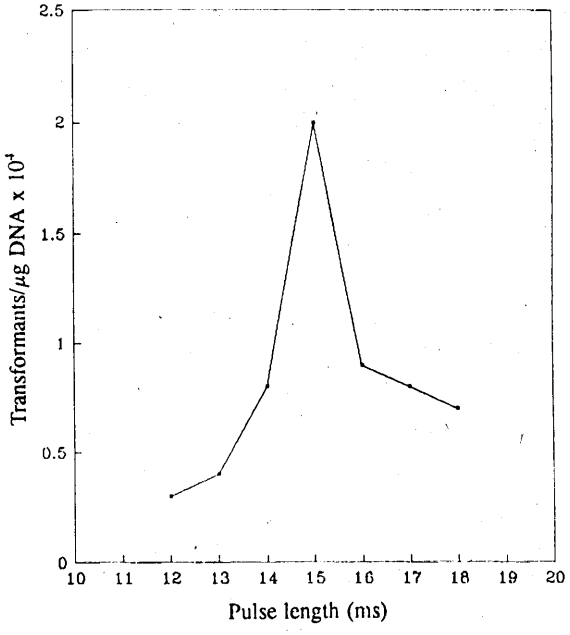
A ten fold increase in transformation efficiency as compared to log phase was observed with mycelia grown to stationary phase. Stationary phase cells gave con-

Fig. 3. Effect of electric field strength and resistance on transformation efficiency of *A. mediterranei* DSM 40773 with plasmid pRL60.



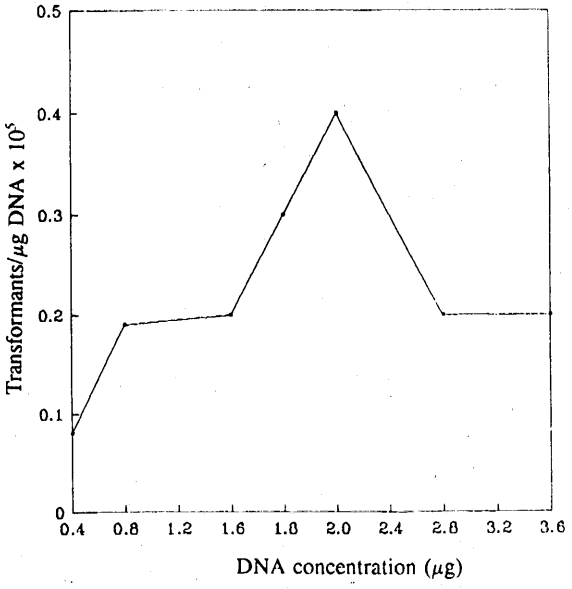
Electric field strength, resistance and capacitance are those described in Fig. 2. Transformation efficiency was calculated as the ratio of total number of transformants obtained to the amount of plasmid DNA used during electroporation.

Fig. 4. Effect of pulse length on transformation efficiency of *A. mediterranei* DSM 40773 transformed with pRL60.



The pulse duration or length was varied by diluting the mycelial suspension with 10% glycerol at an electric field strength, resistance and capacitance of 7.5 kV/cm, 1000 Ω and 25 μF, respectively.

Fig. 5. Effect of varying DNA concentration on transformation efficiency of *A. mediterranei* DSM 40773 transformed with plasmid pRL60.



The electrical parameters used are those mentioned in Fig. 4.

sistently better transformation efficiency in *A. mediterranei* DSM 40773. This observation is in agreement with those reported for *Rhodococcus*[35], *Corynebacterium glutamicum*[36], *Streptomyces rimosus*[37] and *Bacillus brevis*[38]. However there are also variable reports in the literature as far as the effect of the growth phase is concerned and early or mid log phase cells were reported to be more suitable for electroporation[32~34]. A maximum of 4×10^4 transformants/μg DNA was obtained with stationary phase cells pulsed at 7.5 kV/cm, 1000 Ω and 25 μF at a pulse duration of 14~16 ms with 2 μg of DNA. This transformation efficiency is very close to the one reported for *S. rimosus*[37].

Transformation efficiency in *A. mediterranei* DSM 40773 increased linearly with the addition of plasmid DNA between 0.4 μg to 2 μg (Fig. 5) with a maximum of 2 μg of DNA. However, a decrease in transformation efficiency was observed when more than 2 μg DNA was used. Although several workers have previously reported linear DNA dose-response curves over a wide range of DNA concentrations[27,31], a decrease in the transformation efficiency as observed in this study is difficult to explain. With the addition of more DNA, under standard conditions, pulsing time decreases due to changes in the ionic conditions of the mixture which may partly explain the decrease in transformation efficiency.

Mild treatment of *A. mediterranei* mycelium with lysozyme (40 μg/ml) resulted in increase in transformation efficiency by two folds as compared to untreated mycelia. Since, a higher concentration of lysozyme (80 and 120 μg/ml) did not positively affect the transformation efficiency, for all subsequent optimizations, lysozyme treatment of 40 μg/ml for 20 minutes at 20~24°C was used. Supplementation of growth medium with glycine during electroporation was also tested but did not affect the transformation efficiency (data not shown). However, there are reports in literature where both glycine and lysozyme treatment have been found to improve the transformation efficiency.[39,40].

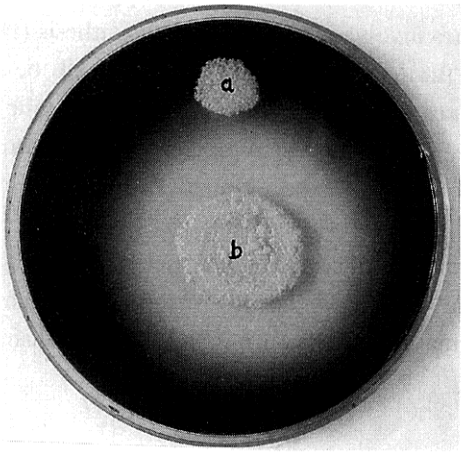
Stability of pRL60

The suitability of a cloning vector is dependent on its stable nature without selection pressure over several generations[41]. In the present investigation, pRL60 was quite stable as more than 60% of the colonies were found to contain the plasmid even in the stationary phase culture grown for 4~5 days without any antibiotic selection pressure. Plasmid DNA from several colonies of *A. mediterranei*, (obtained in several experiments) were analyzed by standard agarose gel electrophoresis. In all cases examined, no differences were observed either in the migration distances or restriction endonuclease digestion patterns when compared with pRL60 DNAs originally used for transformation.

Detection of Transformants by Using *α-amy* as a Marker and Host Range of pRL60

Transformants harbouring pRL60 were grown on YM plates containing 1% starch and once sufficient growth was achieved the plates were exposed to iodine vapours. Transformants containing pRL60 expressed *α-amy* which produced enzyme amylase and secreted to the medium. This resulted in the formation of a colorless halo around the colony due to degradation of starch in the medium (Fig. 6). Thus *α-amy* of pRL60 can be directly used for the selection of transformants in *A. mediterranei* DSM 40773. The use of *α-amy* as a marker has been realized only recently and cloning vectors containing *α-amy* as the marker have been used in *Bacillus subtilis*[42] and *Proteus mirabilis*[43]. In addition, the expression of *α-amy* from *Streptomyces griseus* in *A. mediterranei* and its secretion into the medium provided a direct, rapid and inexpensive assay for the detection of transformants. In the present investigation besides *A. mediterranei* DSM 40773, five different strains of *A. mediterranei* could be successfully transformed with pRL60 and in all the strains

Fig. 6. Use of amylolytic phenotype of *A. mediterranei* harbouring pRL60 for screening.



Transformants containing: (a), pRLM20 (without *α-amy*) and (b), pRL60 (with *α-amy*) were grown on TYN medium containing 1% starch. After sufficient growth the colonies were sprayed with iodine vapours. A large halo appeared around the colony containing pRL60 (b) due to the action of α-amylase on starch.

an amylolytic phenotype could be easily detected by iodine staining. Using standardized conditions of *A. mediterranei* DSM 40773, maximum transformation efficiencies of 7×10^1, 1×10^2, 1.5×10^3, 1×10^3 and 7×10^2 transformants/μg of DNA were obtained for *A. mediterranei* DSM 43304, DSM 46095, MTCC-17, F1/24 and T-195, respectively. There are reports in the literature on the variability of transformation efficiency among different bacterial species and strains[44~48].

A major limitation for the application of plasmid cloning vectors is their host range. The pRL1 derived vector pRL60 appears to have a broad host range as it could replicate in almost all the strains of *A. mediterranei*. pRL60 could be transformed into *A. mediterranei* DSM 43304, an original wild type strain isolated by SENSI and coworkers[49] from French soils. This is the strain from which several modern industrially important rifamycin producers have been derived which are in use for the commercial production of rifamycin in Italy, Korea, China, Russia and India (P.K. Ghosh, personal communication). pRL60 could also be transformed into blocked mutants (F1/24 and T-195) of the rifamycin biosynthetic pathway which are derived from the industrial strain N813 of Ciba Geigy (Switzerland). This opens up possibility of using of pRL60 for cloning and manipulation of antibiotic biosynthetic genes in these

industrial organisms. In fact, there is great interest to identify PKS genes in *Amycolatopsis* and related bacteria and we have already cloned DNA fragments from *A. mediterranei* DSM 46095 which appears to represent PKS genes involved in rifamycin biosynthesis (H. KAUR and R. LAL, Unpublished). pRL60 will be manipulated further in order to provide with unique restriction sites for efficient gene replacement and transposon delivery. We also expect the pRL derived vector and transformation protocol to be successfully applied to the transformation of several other species of *Amycolatopsis* particularly some of the rare species for which genetic transformation systems are difficult to establish.

Acknowledgments

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References

1) SENSI, P. &. J. E. THIEMANN: Production of rifamycin. Prog. Industr. Microbiol. 6: 21～60, 1967
2) BARNA, J. C. J. & D. H. WILLIAMS: The structure and mode of glycopeptide antibiotics of vancomycin group. Microbiol. 38: 339～357, 1984
3) NADKARNI, S. R.; M. V. PATEL, S. CHATTERJEE, E. K. S. VIJAYAKUMAR, K. R. DESIKAN, J. BLUMBACH & B. N. GANGULI: Balhimycin, a new glycopeptide antibiotic produced by *Amycolatopsis* sp. Y-86,21022. Taxonomy, production, isolation and biological activity. J. Antibiotics 47: 334～341, 1994
4) GHIZALBA, O.; J. A. L. AUDEN, T. SCHUPP & J. NÜESCH: The rifamycin: properties, biosynthesis and fermentation. *In* Biotechnology of Industrial Antibiotics. *Ed.*, VANDAMME, E. J., p. 281, Marcel Dekker, Inc., New York, 1984
5) LAL, R.; M. KHANNA, H. KAUR, N. SRIVASTVA, K. K. TRIPATHI & S. LAL: Rifamycin: strain improvement program. Crit. Rev. Microbiol. 21: 19～30, 1995
6) SCHUPP, T.; R. HÜTTER & D. A. HOPWOOD: Genetic recombination in *Nocardia mediterranei*. J. Bacteriol. 121: 128～136, 1975
7) HUTCHINSON, C. R. & I. FUJII: Polyketide synthase gene manipulation: a structure function approach in engineering novel antibiotics. Ann. Rev. Microbiol. 49: 201～211, 1995
8) LAL, R.; R. KHANNA, H. KAUR, M. KHANNA, N. DHINGRA, S. LAL, K. H. GARTEMANN, R. EICHENLAUB & P. K. GHOSH: Engineering antibiotic producers to overcome the limitations of classical strain improvement programs. Crit. Rev. Microbiol. 22: 201～225, 1996
9) HOPWOOD, D. A.: Fine tuning pristinamycin synthesis in *Streptomyces*. Nature Biotechnology 15: 321, 1997
10) SEZONOV, G.; V. BLANC, N. BAMAS-JACQUES, A. FRIEDMAN, J. LUCPERNONODET & M. GUERIN: Complete conversion of antibiotic precursors to pristinamycin II A by overexpression of *Streptomyces pristinaespiralis* biosynthetic gene. Nature Biotechnology. 15: 349～353, 1997
11) MORETTI, P.; G. HINTERMAN & R. HÜTTER: Isolation and characterization of an extrachromosomal element from *Nocardia mediterranei*. Plasmid 14: 126～133, 1985
12) SCHUPP, T. & M. DIVERS: Protoplast fusion and regeneration in *Nocardia mediterranei*. FEMS Microbiol. Lett. 36: 159～162, 1986
13) MATSUSHIMA, P. & R. H. BALTZ: Efficient plasmid transformation of *Streptomyces ambofaciens* and *Streptomyces fradiae* protoplasts. J. Bacteriol. 163: 180～185, 1985
14) LAL, R. & S. LAL: Recent trends in rifamycin research. BioEssays. 16: 211～216, 1994
15) ACEBAL, C.; V. RUBIO & G. MARQUEZ: A method to transform the β-lactam antibiotic producer *Streptomyces wadayamensis*. FEMS Microbiol. Lett. 35: 79～82, 1986
16) BIBB, M. J.; J. M. WARD & D. A. HOPWOOD: Transformation of plasmid DNA into *Streptomyces* at high frequency. Nature (London) 274: 398～400, 1978
17) HOPWOOD, D. A.; M. J. BIBB, K. F. CHATER, T. KIESER, C. J. BRUTON, T. KIESER, H. M. LYDIATE, D. J. SMITH, J. M. WARD & H. SCHREMPF: Genetic manipulation of *Streptomyces*: A laboratory manual. Norwich, John Innes Foundation, 1985
18) LAMPEL, J. S. & W. R. STROHL: Transformation and transfection of anthracycline streptomycetes. Appl. Environ. Microbiol. 51: 126～131, 1986
19) MADON, J. & R. HÜTTER: Transformation system for *Amycolatopsis* (*Nocardia*) *mediterranei*: Direct transformation of mycelium with plasmid DNA. J. Bacteriol. 173: 6325～6331, 1991
20) MATSUSHIMA, P.; M. A. MCHENNEY & R. H. BALTZ: Efficient transformation of *Amycolatopsis orientalis* (*Nocardia orientalis*) protoplasts by *Streptomyces* vectors. J. Bacteriol. 169: 2298～2300, 1987
21) LAL, R.; S. LAL, E. GRUND & R. EICHENLAUB: Construction of a hybrid plasmid capable of replication in *Amycolatopsis mediterranei*. Appl. Environ. Microbiol. 57: 665～671, 1991
22) SAMBROOK, J.; E. F. FRITSCH & T. MANIATIS: Molecular Cloning; a laboratory manual, 2nd ed. Cold Spring Harbor, N.Y., 1989
23) KUMAR, C. V.; J. J. R. COQUE & J. F. MARTIN: Efficient transformation of cephamycin C producer *Nocardia lactamdurans* and development of shuttle and promoter probe cloning vectors. Appl. Environ. Microbiol. 60: 4086～4093, 1994
24) LAINE, M. J.: H. NAKHEI, J. DREIER, K. LEHTILA, D. MELETZUS, R. EICHENLAUB & M. C. METZLER: Stable transformation of the gram positive phytopathogenic bacterium *Clavibacter michiganesis* subspecies *spedonicus* with several cloning vectors. Appl. Environ. Microbiol. 62: 1500～1506, 1995

25) BIBB, M. J.; J. L. SCHTTEL & S. N. COHEN: A DNA cloning system for interspecies gene transfer in antibiotic producing *Streptomyces*. Nature 284: 526~553, 1980
26) BIBB, M. J.; G. R. JANSSEN & J. M. WARD: Cloning and analysis of the promoter region of the erythromycin resistance gene (*ermE*) of *Streptomyces erythraeus*. Gene 42: E357~E362, 1985
27) TAKETO, A.: DNA Transfection of *Escherichia coli* by electroporation. Biochem. Biophys. Acta 949: 318~324, 1988
28) THOMPSON, C. J.; T. KIESER & D. A. HOPWOOD: Physical analysis of antibiotic resistance genes from *Streptomyces* and their use in vector construction. Gene 20: 51~62, 1982a
29) THOMPSON, C. J.; J. M. WARD & D. A. HOPWOOD: Cloning of antibiotic resistance and nutritional genes in streptomycetes. J. Bacteriol. 151: 668~677, 1982b
30) WARD, J. M.; R. JANSSEN, T. KIESER, M. J. BUTTNER & M. J. BIBB: Construction and characterization of a series of multicopy promoter probe plasmid vectors for *Streptomyces* using the aminoglycoside phosphotransferase gene from Tn5 as indicator. Mol. Gen. Genet. 203: 468~478, 1986
31) VRIJBLOED, J. W.; J. MADON & L. DIJKHUIZEN: Transformation of the methylotrophic actinomycete *Amycolatopsis methanolica* with plasmid DNA: stimulatory effect of a pMEA300 encoded gene. Plasmid 34: 96~104, 1995
32) MILLER, J. F.; W. J. DOWER & L. S. TOMPKINS: High voltage electroporation of bacteria: genetic transformation of *Campylobacter jejuni* with plasmid DNA. Proc. Natl. Acad. Sci. U.S.A. 85: 856~860, 1988
33) POWELL, I. B.; M. G. ACHEN, A. J. HILLIER & B. E. DAVIDSON: A simple and rapid method for genetic transformation of lactic streptococci by electroporation. Appl. Environ. Microbiol. 54: 655~660, 1988
34) CHASSY, B. M. & J. L. FLICKINGER: Transformation of *Lactobacillus casei* by electroporation. FEMS Microbiol. Lett. 44: 173~177, 1987
35) YUGTSINGER, M. E. & W. R. FINNERTY: Construction of an *Escherichia coli-Rhodococcus* shuttle vector and plasmid transformation in *Rhodococcus* spp. J. Bacteriol. 170: 63~72, 1988
36) HAYNES, J. A. & M. L. BRITZ: The effect of growth conditions of *Corynebacterium glutamicum* on transformation frequency obtained by electroporation. J. Gen. Microbiol. 136: 225~263, 1990
37) PIGAC, J. & H. SCHREMPF: A simple and rapid method of transformation of *Streptomyces rimosus* R6 and other Streptomycetes by electroporation. Appl. Environ. Microbiol. 6: 352~356, 1995
38) OKAMOTO, A.; A. KOSUGI, Y. KOIZUMI, F. YANAGIDA & S. UDAKA: High efficiency transformation of *Bacillus brevis* by electroporation. Biosci. Biotecnol. Biochem. 61: 202~203, 1997
39) MCDONALD, I. R.; P. W. RILEY, R. J. SHARP & A. J. MCCARTHY: Factors affecting the electroporation of *Bacillus subtilis*. J. Appl. Bacteriol. 79: 213~218, 1995
40) THOMPSON, K. & M. A. COLLINS: Improvement in electroporation efficiency for *Lactobacillus plantarum* by the inclusion of high concentration of glycine in the growth medium. J. Microbiol. Methods 26: 73~79, 1996
41) BLANTY, J. M.; T. BRAUTASET, H. C. WINTHER-LARSEN, K. HAUGAN & S. VALLA: Construction and use of a versatile set of broad host range cloning and transformation expression vectors based on the RK2 replicon. Appl. Environ. Microbiol. 63: 370~379, 1997
42) IKUTA, N.; M. B. N. SOUZA, F. F. VALENCIA, M. E. B. CASTRO, A. C. G. SCHENBERG, A. P. KLEINER & S. A. FILHO: The α-amylase gene as a marker for gene cloning: direct screening of recombinant clones. Biotechnology 8: 241~242, 1990
43) FERNANDEZ, B. L. & S. O. P. DECOSTA: High efficiency transformation of *Proteus mirabilis* with a pUC19 derivative vector directs the expression and secretion of *Bacillus subtilis* α-amylase gene. J. Microbiol. Methods 26: 147~150, 1996
44) MCINTYRE, D. A. & S. K. HARLANDER: Improved electroporation efficiency of intact *Lactococcus lactis* subsp. *lactis* cells grown in defined media. Appl. Environ. Microbiol. 55: 2621~2626, 1989a
45) MCINTYRE, D. A. & S. K. HARLANDER: Genetic transformation of intact *Lactococcus lactis* subsp. *lactis* by high voltage electroporation. Appl. Environ. Microbiol. 55: 604~610, 1989b
46) SOMKUTI, G. A. & D. H. STEINBERG: Genetic transformation of *Streptococcus thermophilus* by electroporation. Biochimie 70: 579~585, 1988
47) WIRTH, R.; A. FREISENEGGER & S. FIEDLER: Transformation of various species of gram negative bacteria belonging to 11 different genera by electroporation. Mol. Gen. Genet. 216: 175~177, 1989
48) DOWER, W. J.; J. F. MILLER & C. W. RAGSDALE: High efficiency transformation of *E. coli* by high voltage electroporation. Nucl. Acids Res. 16: 6127~6145, 1988
49) SENSI, P.; P. MARGALITH & M. T. TIMBAL: Refomycin, a new antibiotic, preliminary report. Farmaco. Ed. Sci. 14: 146~147, 1959