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# Efficient Transformation of the Cephamycin C Producer *Nocardia lactamdurans* and Development of Shuttle and Promoter-Probe Cloning Vectors

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**A high transformation efficiency ($1 \times 10^5$ to $7 \times 10^5$ transformants per μg of DNA) of *Nocardia lactamdurans* LC411 was obtained by direct treatment of mycelium with polyethylene glycol 1000 and cesium chloride. A variety of vectors from *Streptomyces lividans*, *Brevibacterium lactofermentum*, *Rhodococcus fascians*, and a *Nocardia* (*Amycolatopsis*) sp. were tested; transformants could be obtained only with vectors derived from an endogenous plasmid of the *Amycolatopsis* sp. strain DSM 43387. Vectors carrying the kanamycin resistance gene (*kan*) as a selective marker were constructed. The transformation procedure has been optimized by using one of these vectors (pULVK1) and studying the influence of the age of the culture, concentrations of cesium chloride and polyethylene glycol, amount of plasmid DNA, and nutrient supplementations of the growth medium. Versatile shuttle cloning vectors (pULVK2 and pULVK3) have been developed by subcloning the pBluescript KS(+) multiple cloning site or a synthetic polylinker containing several unique restriction sites (*Eco*RV, *Dra*I, *Bam*HI, *Sst*I, *Eco*RI, and *Hin*dIII). A second marker, the apramycin resistance gene (*amr*) has been added to the vectors (pULVK2A), allowing insertional inactivation of one of the markers while using the second one for selection. An alternative marker, the *amy* gene of *Streptomyces griseus* (pULAM2), which is easily detected by the release of extracellular amylase in transformants of *N. lactamdurans* carrying this vector, has been added. Two promoter-probe plasmids, pULVK4 and pULVK5, have been constructed, with the promoterless *xylE* gene as a reporter, for utilization in *N. lactamdurans*.**

Several species of *Nocardia* and the related genus *Amycolatopsis* (28) are used industrially for the production of antibiotics. *Nocardia lactamdurans* (46) (previously described as *Streptomyces lactamdurans* [42]) produces the β-lactam antibiotic cephamycin C (4, 11) and the polyether efrotomycin. *Nocardia mediterranei* (recently renamed *Amycolatopsis mediterranei*) (29) produces rifamycin, and *Amycolatopsis orientalis* synthesizes vancomycin. The biosynthetic pathway of cephamycin in *N. lactamdurans* has been extensively studied (5, 11, 26), and the cluster of the cephamycin biosynthetic pathway has been cloned (7–10). However, the difficulty in transforming *N. lactamdurans* has hampered further studies on gene disruption and gene amplification.

Initial attempts in our laboratory indicated that *N. lactamdurans* cannot be transformed with vectors based on *Streptomyces* replicons. Since no endogenous circular plasmids occur in *N. lactamdurans*, we have tried replicons from different bacteria related to the *Nocardia* group, e.g., corynebacteria, *Rhodococcus fascians*, an *Amycolatopsis* sp., and *Streptomyces lividans*. Polyethylene glycol (PEG)-assisted transformation of protoplasts (20) and electroporation have been widely used for transformation of streptomycetes. A new method which uses a combination of PEG and alkaline cations to transform cells of *A. mediterranei* was developed by Madon and Hütter (31). We have developed an efficient procedure for transformation of entire cells of *N. lactamdurans* based on this method, using plasmid vectors developed from the endogenous plasmid pA387 of *Amycolatopsis* sp. strain DSM 43387 (27). This method, which results in efficient transformation of cells of *N. lactamdurans*, has the advantage that it does not require protoplasting and protoplast regeneration.

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## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The bacterial strains and plasmids used are listed in Table 1. *N. lactamdurans* was routinely grown in NYG medium (18) supplemented with 0.85% $MgCl_2$ at 30°C (5). *Escherichia coli* DH5α (19) or JM110 was grown in Luria-Bertani medium (32) at 37°C. *S. lividans* and *R. fascians* were grown in tryptic soy broth at 30°C.

**Transformation procedure.** A seed culture of *N. lactamdurans* LC411 was grown by inoculating 1.5 ml of stock cell suspension (in 20% glycerol) into NYG medium supplemented with 0.85% $MgCl_2$ and incubated for 36 h at 30°C and 250 rpm in an orbital shaker. After growth, 5 ml of this culture was used to inoculate 100 ml of the same medium containing 0.1% Tween 80 in 500-ml baffled Erlenmeyer flasks and the incubation was carried out in the same conditions. Cells (10-ml aliquots) were collected at different times by centrifugation, resuspended in 5 ml of 20% glycerol, and preserved at −20°C until use.

For transformation, cells (5 ml) were centrifuged to form pellets, washed twice with an equal volume of sterile Tris-EDTA buffer and finally with a Tris-HCl 25 mM (pH 8.0) solution, and then resuspended in 1 ml of the same buffer. The transformation mixture consisted of 50 μl of 4 M CsCl, 1 μl of 1 M $MgCl_2$, and 5.0 μl of sonicated calf thymus DNA (5 μg/μl) and plasmid DNA in a 2.5-μl volume. The total volume was brought to 100 μl by adding the *N. lactamdurans* cell suspension. After that, 100 μl of 80% PEG 1000 (Kock Light, Haverhill, United Kingdom) was added to a final concentration of 40%. The components of the transformation mixture were mixed carefully by pipetting up and down and incubated at 30°C for 1 h and then at 42°C for 10 min.

After the incubation, aliquots of the transformation mixture were plated onto S27M medium plates (31) (previously dried

TABLE 1. Bacterial strains and plasmids used in this study

| Microorganism | Strain or plasmid | Main characteristics | Source or reference |
|---|---|---|---|
| *N. lactamdurans* | LC411 | Stable Amy$^+$ derivative of wild-type *N. lactamdurans* NRRL 3802 | 5 |
| | pULVK1 | Derivative from pRL1 obtained by natural deletion | This work |
| | pULVK2 | Derivative of pULVK1 containing synthetic polylinker | This work |
| | pULVK3 | Derivative of pULVK1 containing multiple cloning site of pBluescript KS(+) | This work |
| | pULVK2A | Derivative of pULVK2. Km$^r$ Am$^r$. Contains apramycin resistance gene from pKC505 | This work |
| | pULAM2 | Derivative of pULVK2. Km$^r$ *amy*. Contains *amy* gene from *S. griseus* | This work |
| | pULVKT3 | Derivative of pULVK1. Trifunctional (*E. coli*, *N. lactamdurans*, *Streptomyces* sp.) plasmid | This work |
| *S. lividans* | JI1326 | Wild type | 20 |
| | pIJ699 | Bifunctional *E. coli-Streptomyces* positive selection vector contains *tsr* and *vio* genes | 23 |
| | pIJ702 | *Streptomyces* plasmid containing *tsr* and *mel* (for tyrosinase) genes | 22 |
| | pULBV1 | Contains *tsr* and *amy* gene of *S. griseus* cloned in pGM-7 | 2 |
| | pIJ4083 | *Streptomyces* promoter-probe vector, containing *xylE* gene of *P. putida* as reporter gene | 6 |
| | pKC505 | Shuttle cosmid vector carrying apramycin resistance (Am$^r$) gene of *E. coli* | 39 |
| *B. lactofermentum* | R31 | Derivative of the wild-type *B. lactofermentum* ATCC 21798 with improved transformation efficiency | 40 |
| | pUL340 | Km$^r$. Derivative from pBL1 | 40 |
| | pULRS8 | Km$^r$ Cm$^r$. Derivative from pBL1 | 41 |
| | pULMJ600 | Km$^r$. Derivative from pBL1 | 3 |
| *R. fascians* | D188 | Wild type | 13 |
| | pULRE1 | Bifunctional *E. coli-Rhodococcus* plasmid; Phle$^r$ Ap$^r$ | 35 |
| | pULRE2 | Bifunctional *E. coli-Rhodococcus plasmid; Km$^r$ Hy$^r$ Ap$^r$* | 35 |
| | pULRE3 | Bifunctional *E. coli-Rhodococcus* plasmid; Cm$^r$ Ap$^r$ Km$^r$ | 35 |
| *E. coli* | DH5α | High-efficiency transformation strain. *lacZ* Δm15 *recA1* | 19 |
| | JM110 | *dam dcm* mutant strain | 47 |
| | pRL1 | Bifunctional *Amycolatopsis-E. coli* plasmid. Km$^r$. Contains origin of replication of pA387 endogenous of *Amycolatopsis* sp. DSM 43387 | 27 |
| | pIJ2921 | pUC18 derivative containing modified polylinker. Ap$^r$ | 21 |

in a hood for 2 to 3 h) by mixing the transformed cells with 2 to 3 ml of an overlay of melted R2L agarose (0.7%). PEG elimination from the transformation mixture is not required, since it is not toxic for the microorganisms and does not have any influence on the final result of the transformation. In some experiments (see Results), the transformation mixture was diluted in R2L liquid medium (or in P buffer [20]) and then plated in the same way.

Plates were briefly dried before they were incubated at 30°C. Kanamycin (final concentration of 75 μg/ml in water solution) or apramycin (50 μg/ml) was added after 20 h of incubation. Transformants began to appear 72 to 96 h after addition of the antibiotic. Single colonies were picked up for further analysis.

**Electroporation.** Cells grown in NYG medium as indicated above were harvested at different times and then washed five times with Milli-Q sterile water and finally resuspended in 1/50th of the original volume and preserved in aliquots until use. Cell suspensions (75-μl aliquots) were mixed with 1 to 2 μl of plasmid DNA (5 μg/μl) and then transferred into a chilled electroporation cuvette and exposed to a single pulse of various field strengths (5 to 12.5 kV/cm) and pulse duration from 2.3 to 6.6 ms in a Bio-Rad apparatus. After electroporation, cells were added to 0.5 ml of NYG medium and incubated with agitation at 30°C for 3 to 6 h and then applied to plates containing the antibiotic (kanamycin at 75 μg/ml or apramycin at 50 μg/ml). The efficiencies for transformation reported are the averages of at least two different experiments.

## RESULTS

**Selection of antibiotic resistance markers.** The sensitivity of *N. lactamdurans* to different antibiotics was tested in S27M (31) or modified ELR medium (46). *N. lactamdurans* was resistant to hygromycin and viomycin (MIC higher than 100 μg/ml) or chloramphenicol (MIC higher than 75 μg/ml), but it was sensitive to thiostrepton (5 μg/ml), phleomycin (15 μg/ml), kanamycin (30 μg/ml), or apramycin (25 μg/ml). Thiostrepton-resistant mutants appeared spontaneously with high frequency. Therefore, the thiostrepton resistance (*tsr*) marker was not used. In addition, thiostrepton is known to have multiple regulatory effects on *N. lactamdurans* and other actinomycetes (16, 25). Although *Nocardia* spp. are sensitive to phleomycin, the antibiotic cannot be used for primary selection since it appears that components of S27M medium affect the antibiotic activity. Kanamycin was preferred as the first selective marker because of the high level of resistance that was conferred by the *kan* gene of transposon Tn*5* and the availability of the antibiotic. The apramycin resistance gene was used as a secondary marker. The unique *Xho*I site in the apramycin resistance (*amr*) gene can be used for cloning *Xho*I-compatible fragments, which results in the insertional inactivation of this selective marker.

**Protoplast transformation and electroporation.** Initially, transformation of *N. lactamdurans* protoplasts was tried with vectors pIJ702 or pIJ699 from *S. lividans* (20, 37), pUL340, pULRS8 and pULMJ600 of *Brevibacterium lactofermentum*

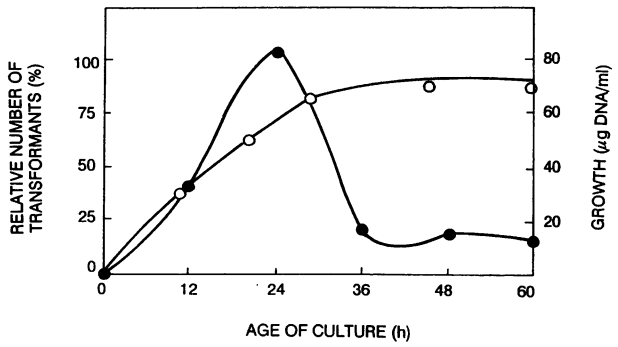


FIG. 1. Effect of the age of *N. lactamdurans* culture in NYG medium on the efficiency of transformation. Growth expressed as micrograms of cellular DNA per ml (○) and the percent transformants relative to the best transformation conditions ($2 \times 10^5$ transformants per μg of DNA was considered 100%) (●) are indicated.

(33, 40, 41), pRL1 of *Amycolatopsis* sp., and pULRE1, pULRE2, and pULRE3 of *R. fascians* (35). No transformants were obtained with any of these plasmids because of their inability to replicate in *N. lactamdurans* or the low efficiency of protoplast DNA uptake, although *N. lactamdurans* can be easily protoplasted and regenerated in the modified ELR or S27M medium described for this purpose.

A variety of electroporation conditions (with the same plasmids) were tested by varying the field strength from 5 to 12.5 kV/cm and pulse duration from 2.3 to 6.6 ms. The results of the electroporation were very poor (0.1 to 1 transformant per μg of DNA) with plasmid pRL1 and did not improve for cells grown in the presence of glycine (0.5 to 1%) and/or Tween 80 or with pretreatment of the mycelium with lysozyme (100 μg/ml) for 5 min. No transformants were obtained after electroporation with plasmids from corynebacteria, *R. fascians*, or *S. lividans*.

**Initial PEG- and cesium chloride-assisted transformation of intact cells.** A successful transformation of *N. lactamdurans* with plasmid pRL1 obtained from *E. coli* DH5α was attained when intact cells were incubated with plasmid DNA in the presence of PEG and CsCl. The low initial efficiency (1 to 10 transformants per μg of DNA) was significantly increased (40 to 80 transformants per μg of DNA) when pRL1 was obtained from *E. coli* J110 (a *dam dcm* mutant strain) instead of *E. coli* DH5α.

The age of the *N. lactamdurans* culture was very important to achieve high transformation efficiencies. Two- to five-fold higher efficiencies (up to 400 transformants per μg of DNA) were obtained by using 24-h-old mycelium than by using 36-h-old mycelium. Cells from cultures grown for 48 to 72 h were much more difficult to transform (Fig. 1). The best efficiency of transformation was obtained when the culture was in the exponential phase of growth, 8 to 10 h before the organism reached the stationary phase.

**Isolation of a stable deleted plasmid from *N. lactamdurans*.** pRL1 was notoriously unstable in *N. lactamdurans*. About 70 to 75% of the 70 transformants tested that were obtained with pRL1 isolated from *E. coli* JM110 showed a deleted plasmid of 6.0 kb. A similar proportion (about 80%) of the 60 transformants obtained with plasmid pRL1 isolated from *E. coli* DH5α yielded the same deleted plasmid, in several different experiments. The deleted plasmids from 50 separate clones showed the same size in gel electrophoresis. Linearization and mapping with restriction endonucleases indicated that all the deleted plasmids were identical.

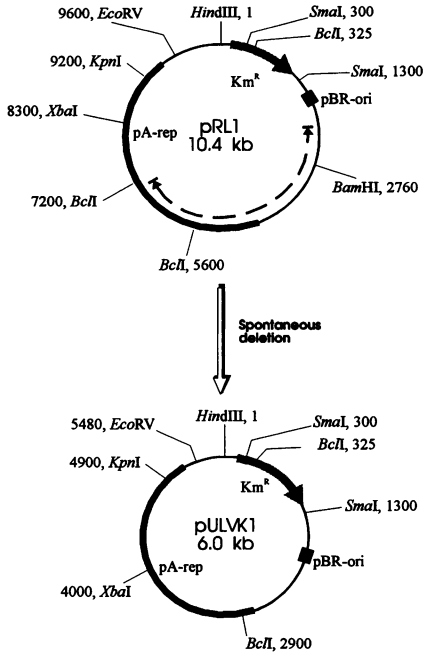


FIG. 2. Formation of the stable plasmid pULVK1 by spontaneous deletion in *N. lactamdurans* LC411 of plasmid pRL1. Thin line, DNA originating from *E. coli* plasmids carrying the pBR322 origin of replication (pBR-ori) and the kanamycin resistance gene from Tn*5* ($Km^r$). Thick line, DNA from *Amycolatopsis* sp. containing the pA387 origin of replication (pA-rep). The dashed arc indicates the fragment of DNA that was deleted in pULVK1. The numbers indicate nucleotide positions.

One of the deleted plasmids, named pULVK1 (6.0 kb), was mapped in detail by restriction analysis (Fig. 2). A region of 4.4 kb was deleted from the parental pRL1, which had a size of 10.4 kb. The deleted plasmid pULVK1 retained the origins of replication of *E. coli* and *Amycolatopsis* sp. and the kanamycin resistance marker. Plasmid pULVK1 was stable and could be propagated in either *E. coli* or *N. lactamdurans* and has been routinely used in cloning experiments. In *N. lactamdurans*, it has an approximate copy number of 20 to 30 copies per cell, whereas in *E. coli* it showed about 15 to 20 copies per cell (without spectinomycin amplification).

**Optimization of PEG, cesium chloride, Tween 80, and DNA concentrations.** Initial studies indicated that growth of *N. lactamdurans* in NYG medium supplemented with $MgCl_2$ (8.5 g/liter) and Tween 80 (0.1%) supported a higher transformation efficiency than tryptic soy broth with the same added components. Other compounds such as glycine (a known inhibitor of cell wall biosynthesis) at a concentration of 0.5 to 1%, alone or in combination with Tween 80, and/or $MgCl_2$ always yielded a smaller number of transformants.

The addition of PEG 1000 to the transformation mixture was strictly required. No transformants were obtained in the absence of PEG. A high transformation efficiency was obtained in a narrow range of PEG concentrations (from 30 to 48%); the optimal efficiency was consistently observed at 40% PEG (Fig. 3A).

Another important factor in obtaining efficient transformation was the CsCl concentration. Relatively high concentrations of CsCl (above 0.4 M) were required. The optimal efficiency of transformation was observed at 1 M concentrations of this salt, although a large number of transformants was

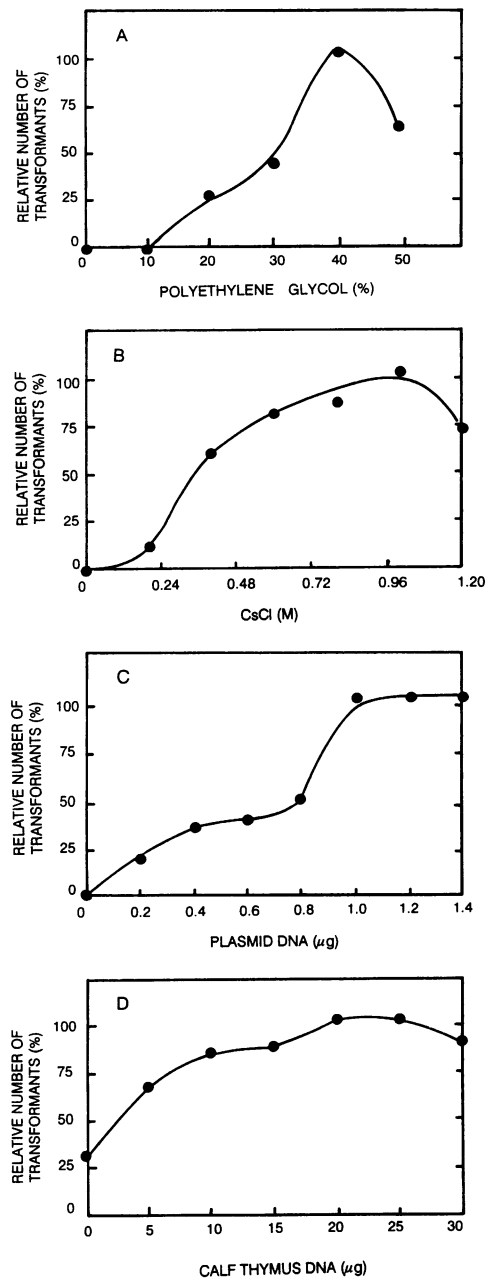


FIG. 3. Optimization of different parameters affecting the efficiency of transformation. (A) Concentration of PEG 1000. (B) Concentration of CsCl. (C) Concentration of plasmid (pULVK1) DNA per transformation experiment. (D) Concentration of carrier (calf thymus) DNA. The transformation conditions were as follows: PEG 1000, 40% (except in the experiments for panel A); cesium chloride, 1.0 M (except in the reactions for panel B); plasmid DNA, 1 μg per transformation reaction (except in the experiments for panel C); and calf thymus DNA, 20 μg per transformation reaction (except in the reactions for panel D).

also obtained at lower or higher CsCl concentrations (Fig. 3B). Replacement of CsCl by either RbCl, LiCl, or KCl at 1 M concentration, yielded only 36% (for LiCl) or 0% (for KCl or RbCl) of the transformants obtained with CsCl (Table 2). Likewise, substitution of $Ca^{2+}$ for $Mg^{2+}$ at the same concentration in the presence of CsCl (1 M) yielded 30 to 40% as many transformants as control transformations with $Mg^{2+}$.

TABLE 2. Effect of different alkaline salts on the transformation of cells of *N. lactamdurans*

| Alkaline salt(s) (concn)[a] | Efficiency of transformation | |
|---|---|---|
| | No. of transformants/ μg of DNA | % |
| CsCl (1 M) | $6.7 \times 10^5$ | 100 |
| RbCl (1 M) | 0 | 0 |
| LiCl (1 M) | $2.4 \times 10^5$ | 36 |
| KCl (1 M) | 0 | 0 |
| CsCl (1 M), $CaCl_2$ (5 mM) (replacing $MgCl_2$ [5 mM]) | $2.6 \times 10^5$ | 38 |

[a] $MgCl_2$ (5 mM) was used in all experiments except in the last transformation, in which it was replaced by $CaCl_2$ (5 mM).

Concentrations of plasmid DNA higher than 1 μg per transformation reaction were required to obtain high transformation efficiencies, and saturation of the transformation reaction was obtained by using 2 μg of plasmid DNA (Fig. 3C). The concentration of calf thymus DNA (in the range of 10 to 25 μg) used as a carrier was not very important, although the transformation efficiency decreased when the amount used was less than 10 or more than 25 μg per reaction (Fig. 3D). Under the optimized transformation conditions, efficiencies of $1 \times 10^5$ to $7 \times 10^5$ transformants per μg of DNA were routinely obtained.

**Development of improved cloning vectors.** New improved cloning vectors were developed by replacing the *Eco*RV-*Hin*dIII fragment of pULVK1 (nucleotide positions 5480 to 1, Fig. 2) by a synthetic linker with several unique cloning sites (*Eco*RV*, *Dra*I*, *Bam*HI*, *Eco*RI*, *Hin*dIII*, and *Sst*I*), resulting in plasmid pULVK2 (5.7 kb) (Fig. 4). In a different strategy, the multiple cloning site of pBluescript KS(+) (44) was subcloned as a 0.40-kb *Pvu*II fragment. The resulting plasmid, pULVK3, has a size of 6.4 kb (Fig. 4). These plasmids can be easily isolated from *N. lactamdurans* by the alkaline sodium dodecyl sulfate-lysis method reported for *S. lividans* (24).

DNA fragments ranging from 2 to 10 kb were successfully cloned in the polylinker sites without affecting the stability of the plasmid.

**Construction of vectors with two markers.** The apramycin resistance gene was isolated as a 1.2-kb *Eco*RI-*Pst*I fragment from cosmid pKC505, cloned in the *Eco*RI-*Pst*I sites of pBluescript SK(+) and subcloned as an *Eco*RI-*Bam*HI fragment into pULVK2, yielding plasmid pULVK2A (Fig. 4).

A different marker was introduced at the *Bam*HI site of pULVK2 by inserting the *amy* gene of *Streptomyces griseus* (45) from pULBV1 as a 2.7-kb *Bgl*II fragment which is efficiently expressed in *N. lactamdurans* from its own promoter (Fig. 5) (25). The new vector with the *kan* and *amy* markers was named pULAM2 (Fig. 4). Clones of *N. lactamdurans* transformed with pULAM2 were easily detected in starch-based minimal medium by the surrounding halo of degradation of starch when exposed to iodine vapors.

By replacing the *Hin*dIII-*Eco*RV fragment of pULVK1 with the 4.4-kb *Hin*dIII-*Eco*RV region of pIJ699 containing the origin of replication of *S. lividans* pIJ101, we obtained a new multifunctional vector, pULVKT3 (not shown), able to replicate in *E. coli*, *N. lactamdurans*, and several *Streptomyces* species, using the kanamycin resistance gene as a selective marker in the three microorganisms.

pULVKT3 was used successfully to transform *Streptomyces*

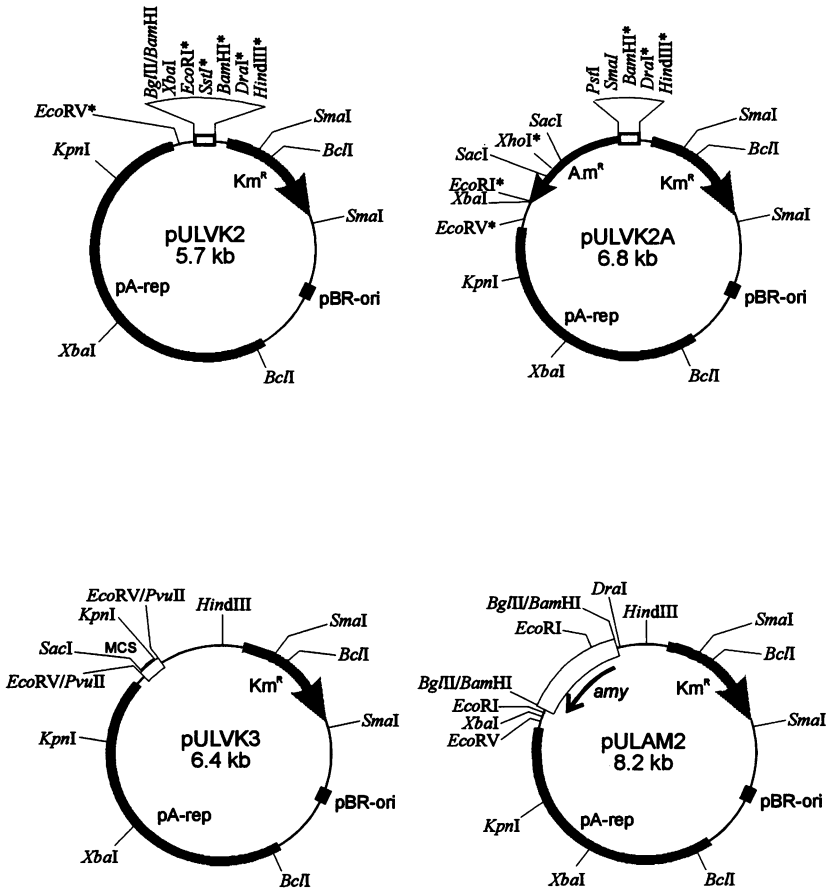


FIG. 4. Improved plasmids for transformation of *N. lactamdurans*. pULVK2 carries a synthetic polylinker with several unique restriction sites (asterisks). pULVK3 carries the multiple cloning site from pBluescript KS(+). pULVK2A contains the apramycin resistance gene ($Am^r$) in addition to the kanamycin resistance gene ($Km^r$), and pULAM2 carries the α-amylase gene (*amy*) of *S. griseus*. All these plasmids have the origin of replication of pA387 of *Amycolatopsis* sp. (pA-rep) and the origin of replication of pBR322 (pBR-ori). The *S. griseus* DNA fragment in pULAM2 containing the *amy* gene is indicated by a double line.

*clavuligerus* and still retain the *E. coli* DNA fragment, unlike other *Streptomyces* plasmids such as pIJ699 in which the *E. coli* part is deleted when introduced in *S. clavuligerus*. The multifunctional vector pULVKT3 may be useful to transfer cephamycin biosynthetic genes directly from *N. lactamdurans* to *S. clavuligerus* and vice versa.

**Promoter-probe vectors.** The promoterless *xylE* of *Pseudomonas putida* (6) was subcloned as a 1.6-kb *Bgl*II fragment from the *Streptomyces* promoter-probe vector pIJ4083 in both orientations in the *Bam*HI site of pULVK2A, yielding the 8.4-kb promoter-probe vectors pULVK4 and pULVK5 (Fig. 6).

The usefulness of these vectors was confirmed by subcloning the *lat* gene promoter of the cephamycin cluster (7) as a *Hin*dIII-*Bam*HI fragment in pULVK5 and observing the change to yellow in the presence of catechol (0.5%). In random cloning of fragments containing promoters, it is advisable to replicate the transformants into either MEY (20) or a minimal medium before testing for the change to yellow to avoid the interference of the pink pigment produced by *N. lactamdurans* in complex media. A minimal medium containing isoleucine as the sole carbon source was found to be the best, since in this medium *N. lactamdurans* grows as white colonies and the

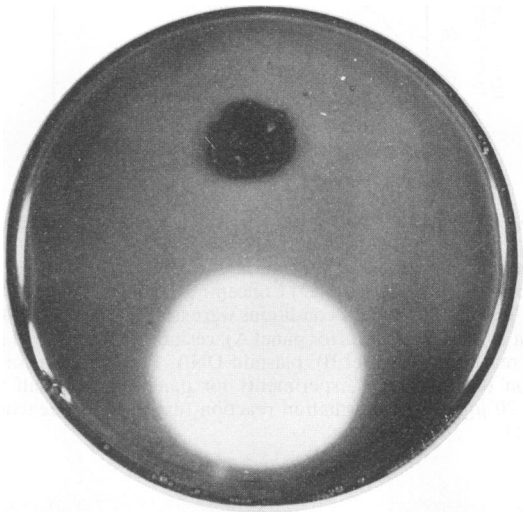

FIG. 5. Use of the *amy* gene of *S. griseus* as a marker in *N. lactamdurans*. A patch of control *N. lactamdurans* transformed with pULVK2A shows no amylase activity (upper area), whereas a patch of *N. lactamdurans* transformed with pULAM2 shows a large white halo on starch plates after iodine staining (lower area).

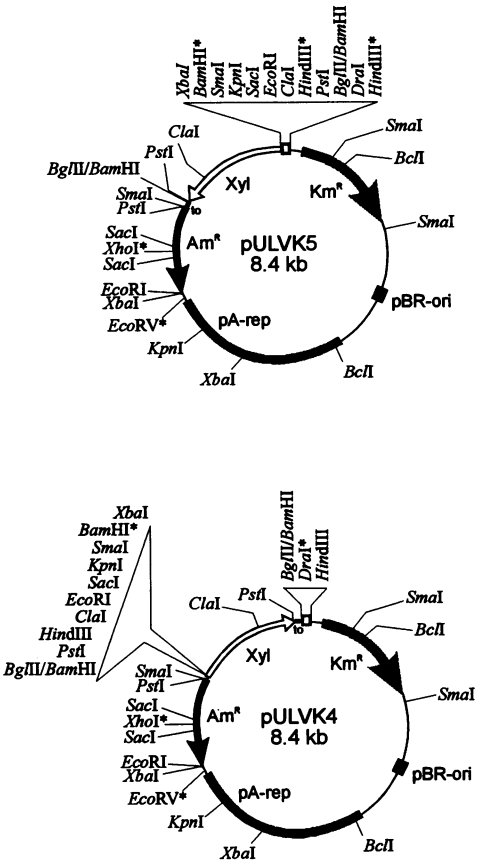


FIG. 6. Promoter-probe plasmids pULVK4 and pULVK5 containing the promoterless *xylE* from *P. putida* in addition to the apramycin resistance ($Am^r$) and kanamycin resistance ($Km^r$) genes. The origins of replication pA-rep and pBR-ori are as defined in the legend for Fig. 2.

change to yellow after spraying with catechol can easily be observed.

## DISCUSSION

Methods for efficient transformation of *S. lividans* and several other species of *Streptomyces* have been developed previously (36, 43). However, transformation of many species of *Streptomyces* and other rare actinomycetes of industrial relevance is difficult to achieve. We developed an efficient plasmid transformation of the β-lactam producer *S. clavuligerus* (17), but repeated attempts to transform the cephamycin C producer *N. lactamdurans* by using *Streptomyces* vectors were unsuccessful since, as shown in this work, plasmids containing origins of replication from *S. lividans*, *R. fascians*, and *Brevibacterium lactofermentum* do not transform *N. lactamdurans*. The successful transformation of *N. lactamdurans* with vectors derived from the endogenous plasmid pA387 of *Amycolatopsis* sp. strain DSM 43387 (27) is of great interest since the genera *Nocardia* and *Amycolatopsis* appear to be very closely related (28). In fact, *N. mediterranei* has been reclassified as *A. mediterranei* (29).

PEG-assisted transformation of protoplasts is well known. The lack of transformation of *N. lactamdurans* protoplasts even with pULVK1 appears to be related to the poor DNA uptake of *N. lactamdurans* protoplasts. Regeneration of the complex cell wall of *N. lactamdurans* protoplasts might be incomplete, as described also for regeneration of corynebacterium protoplasts, apparently because of the need to synthesize mycolic acids (34).

Electroporation has proved useful for transformation of bacteria for which alternative DNA transfer methods did not exist or were inefficient. Electroporation has been applied to transformation of corynebacteria (14) and lactic acid bacteria (38). The inefficient transformation of *N. lactamdurans* by electroporation prompted us to try direct transformation of entire cells with PEG and alkaline cations, a method reported for transformation of *A. mediterranei* (31). For this method, the mycelium does not need any special preparation and the critical steps of protoplast formation and regeneration are avoided.

Several factors were optimized to achieve high efficiencies of transformation. The age of the culture was critical for high transformation efficiency. The best rate of transformation was obtained at 24 h when the cells were in the logarithmic phase of growth, and efficiency decreased sharply after 36 h probably because of the modification of the cell wall or the formation of nucleases. In *S. clavuligerus*, a similar effect of the cell growth phase on protoplast transformation was observed (17).

The source of plasmid DNA used was important for successful transformation of *N. lactamdurans*. A 40- to 80-fold increase in the efficiency of transformation was obtained when plasmid DNA was isolated from a *dam dcm* mutant strain of *E. coli* instead of *E. coli* DH5α, which indicates that the methylation of the plasmid DNA in *E. coli* differently from the normal modification in *Nocardia* spp. is responsible for the low efficiency of initial transformation. This problem was avoided by the use of an *E. coli dam dcm* mutant strain or by isolating plasmid DNA directly from previously transformed *N. lactamdurans*. The presence of DNA restriction systems in *N. lactamdurans* may explain the low transformation efficiency obtained by using modified DNA from heterologous hosts. In fact, a methyl-specific restriction system has been found in the actinomycete *Streptomyces avermitilis* (30) and similar restriction systems are likely to occur in many other actinomycetes.

An interesting finding was the observation of the deletion of a constant 4.4-kb fragment from plasmid pRL1, which gave rise to the stable plasmid pULVK1 of 6.0 kb. A similar deletion from unstable corynebacterial plasmid pULRS61 which formed the stable plasmids pUL330 and pUL340 was found in *B. lactofermentum* (40, 41). Such deletions occur frequently with plasmids that contain short repeats of a certain sequence (1, 12) or that replicate via the rolling circle mechanism (15). The deleted plasmid pULVK1 gave very high efficiencies of transformation (up to $2 \times 10^5$ transformants per μg of DNA) compared with those for the undeleted pRL1 form, which indicates that many cells transformed with the undeleted form were probably unable to replicate the plasmid in a stable form and it was lost from the cells.

The copy number of pULVK1 that we have observed in *N. lactamdurans* (20 to 30 copies per cell) is smaller than the copy number reported for pRL1 in *A. mediterranei* (about 90 copies per cell) (27). The difference might be due to a less efficient recognition of the origin of replication of the *Amycolatopsis* sp. plasmid by the *N. lactamdurans* plasmid replication machinery. The stable plasmid pULVK1 has served as the starting point to construct a variety of plasmids with two selective markers (Fig. 4) and promoter-probe vectors (Fig. 6) for use in *N. lactamdurans*. These vectors are very useful instruments for advanced molecular genetics studies of *N. lactamdurans*.

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## REFERENCES

1. **Albertini, A. M., M. Hofer, M. P. Calos, and J. H. Miller.** 1982. On the formation of spontaneous deletions: the importance of short sequences homologies in the generation of large deletions. Cell **29:**319–328.
2. **Blanco, J., and J. F. Martín.** Unpublished data.
3. **Cadenas, R. F., and J. F. Martín.** Unpublished data.
4. **Castro, J. M., P. Liras, J. Cortés, and J. F. Martín.** 1985. Regulation of α-aminoadipyl-cysteinyl-valine, isopenicillin N synthetase, isopenicillin N isomerase and deacetoxycephalosporin C synthetase by nitrogen sources in *Streptomyces lactamdurans*. Appl. Microbiol. Biotechnol. **22:**32–40.
5. **Castro, J. M., P. Liras, L. Láiz, J. Cortés, and J. F. Martín.** 1988. Purification and characterization of isopenicillin N synthase of *Streptomyces lactamdurans*. J. Gen. Microbiol. **134:**133–141.
6. **Clayton, T. M., and M. J. Bibb.** 1990. *Streptomyces* promoter-probe plasmids that utilize the *xyl*E gene of *Pseudomonas putida*. Nucleic Acids Res. **18:**1077.
7. **Coque, J.-J. R., P. Liras, L. Láiz, and J. F. Martín.** 1991. A gene encoding lysine 6-aminotransferase, which forms the β-lactam precursor α-aminoadipic acid, is located in the cluster of cephamycin biosynthetic genes in *Nocardia lactamdurans*. J. Bacteriol. **173:**6258–6264.
8. **Coque, J. J. R., P. Liras, and J. F. Martín.** 1993. Genes for a β-lactamase, a penicillin-binding protein and a transmembrane protein are clustered with the cephamycin biosynthetic genes in *Nocardia lactamdurans*. EMBO J. **12:**631–639.
9. **Coque, J. J. R., J. F. Martín, J. G. Calzada, and P. Liras.** 1991. The cephamycin biosynthetic genes *pcb*AB, encoding a large multidomain peptide synthetase, and *pcb*C of *Nocardia lactamdurans* are clustered together in an organization different from the same genes in *Acremonium chrysogenum* and *Penicillium chrysogenum*. Mol. Microbiol. **5:**1125–1133.
10. **Coque, J. J. R., J. F. Martín, and P. Liras.** 1993. Characterization and expression in *Streptomyces lividans* of *cef*D and *cef*E genes from *Nocardia lactamdurans*: the organization of the cephamycin gene cluster differs from that in *Streptomyces clavuligerus*. Mol. Gen. Genet. **236:**453–458.
11. **Cortés, J., J. F. Martín, J. M. Castro, L. Láiz, and P. Liras.** 1987. Purification and characterization of a 2-oxoglutarate-linked ATP-independent deacetoxycephalosporin C synthase of *Streptomyces lactamdurans*. J. Gen. Microbiol. **133:**3165–3174.
12. **Criado, L. M., J. F. Martín, and J. A. Gil.** 1993. The *pab* gene of *Streptomyces griseus*, encoding p-aminobenzoic acid synthase, is located between genes possibly involved in candicidin biosynthesis. Gene **126:**135–139.
13. **Desomer, J., P. Dhaese, and M. Van Montagú.** 1988. Conjugative transfer of cadmium resistance plasmids in *Rhodococcus fascians* strains. J. Bacteriol. **170:**2401–2405.
14. **Dunican, L. K., and E. Shivnan.** 1989. High frequency transformation of whole cells of amino acid producing coryneform bacteria using high voltage electroporation. Bio/Technology **7:**1067–1070.
15. **Fernandez-Gonzalez, C., R. F. Cadenas, M. F. Noirot-Gros, J. F. Martín, and J. A. Gil.** 1994. Characterization of a region of plasmid pBL1 of *Brevibacterium lactofermentum* involved in replication via the rolling circle model. J. Bacteriol. **176:**3154–3161.
16. **García-Domínguez, M., P. Liras, and J. F. Martín.** 1991. Cloning and characterization of the isopenicillin N synthase gene of *Streptomyces griseus* NRRL 3851 and studies of expression and complementation of the cephamycin pathway in *Streptomyces clavuligerus*. Antimicrob. Agents Chemother. **35:**44–52.
17. **García-Domínguez, M., J. F. Martín, B. Mahro, A. L. Demain, and P. Liras.** 1987. Efficient plasmid transformation of the β-lactam producer *Streptomyces clavuligerus*. Appl. Environ. Microbiol. **53:**1376–1381.
18. **Ginther, C. L.** 1979. Sporulation and serine protease production by *Streptomyces lactamdurans*. Antimicrob. Agents Chemother. **15:**522–526.
19. **Hanahan, D.** 1985. Techniques for transformation of *E. coli*, p. 109–136. *In* D. M. Glover (ed.), DNA cloning, vol. 1. IRL Press, Oxford.
20. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf.** 1985. Genetic manipulation of *Streptomyces*: a laboratory manual. The John Innes Foundation, Norwich, United Kingdom.
21. **Janssen, G. R., and M. J. Bibb.** 1993. Derivatives of pUC18 that have *Bgl*II sites flanking a modified multiple cloning site and that retain the ability to identify recombinant clones by visual screening of *Escherichia coli* colonies. Gene **124:**133–134.
22. **Katz, E., C. J. Thompson, and D. A. Hopwood.** 1983. Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. J. Gen. Microbiol. **129:**2703–2714.
23. **Kieser, D., and R. D. Melton.** 1988. Plasmid pIJ699, a multicopy positive selection vector for *Streptomyces*. Gene **65:**83–91.
24. **Kieser, T.** 1984. Factors affecting the isolation of ccc DNA from *Streptomyces lividans and Escherichia coli*. Plasmid **12:**19–36.
25. **Kumar, C. V., and J. F. Martín.** 1994. Thiostrepton induced proteins in *Streptomyces*, *Amycolatopsis* and *Nocardia* species. FEMS Microbiol. Lett. **118:**107–112.
26. **Láiz, L., P. Liras, J. M. Castro, and J. F. Martín.** 1990. Purification and characterization of the isopenicillin N epimerase from *Nocardia lactamdurans*. J. Gen. Microbiol. **136:**663–671.
27. **Lal, R., S. Lal, E. Grund, and R. Eichenlaub.** 1991. Construction of a hybrid plasmid capable of replication in *Amycolatopsis mediterranei*. Appl. Environ. Microbiol. **57:**665–671.
28. **Lechevalier, H. A.** 1989. Nocardioform actinomycetes, p. 2348–2361. *In* S. T. Williams, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 4. The Williams & Wilkins Co., Baltimore.
29. **Lechevalier, M. P., H. Prauser, D. P. Labeda, and J.-S. Ruan.** 1986. Two new genera of nocardioform actinomycetes: *Amycolata* gen. nov. and *Amycolatopsis* gen. nov. Int. J. Syst. Bacteriol. **36:**29–37.
30. **MacNeil, D. J.** 1988. Characterization of a unique methyl-specific restriction system in *Streptomyces avermitilis*. J. Bacteriol. **170:**5607–5612.
31. **Madón, J., and R. Hütter.** 1991. Transformation system for *Amycolatopsis* (*Nocardia*) *mediterranei*: direct transformation of mycelium with plasmid DNA. J. Bacteriol. **173:**6325–6331.
32. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Martín, J. F., R. F. Cadenas, M. Malumbres, L. M. Mateos, C. Guerrero, and J. A. Gil.** 1990. Construction and utilization of promoter-probe and expression vectors in corynebacteria. Characterization of corynebacterial promoters, p. 283–292. *In* H. Heslot, J. Davies, J. Florent, L. Bobichon, G. Durand, and L. Penasse (ed.), Proceedings of the 6th International Symposium on Genetics of Industrial Microorganisms. Societé Française de Microbiologie, Paris.
34. **Martín, J. F., R. Santamaría, H. Sandoval, G. del Real, L. M. Mateos, J. A. Gil, and A. Aguilar.** 1987. Cloning systems in amino acid-producing corynebacteria. Bio/Technology **5:**137–146.
35. **Mateos, L. M., and J. F. Martín.** Unpublished data.
36. **Matsushima, P., and R. H. Baltz.** 1985. Efficient plasmid transformation of *Streptomyces ambofaciens* and *Streptomyces fradiae* protoplasts. J. Bacteriol. **163:**180–185.
37. **Matsushima, P., M. A. McHenney, and R. H. Baltz.** 1987. Efficient transformation of *Amycolatopsis orientalis* (*Nocardia orientalis*) protoplasts by *Streptomyces* plasmids. J. Bacteriol. **169:**2298–2300.
38. **Powell, I. B., M. G. Achen, A. J. Hiller, and B. E. Davidson.** 1988. A simple and rapid method for genetic transformation of lactic streptococci by electroporation. Appl. Environ. Microbiol. **54:**655–660.
39. **Rao, R. N., M. A. Richardson, and S. Kuhstoss.** 1987. Cosmid

shuttle vectors for cloning and analysis of *Streptomyces* DNA. Methods Enzymol. **153:**166–198.

40. **Santamaría, R. I., J. A. Gil, and J. F. Martín.** 1985. High-frequency transformation of *Brevibacterium lactofermentum* by plasmid DNA. J. Bacteriol. **162:**463–467.
41. **Santamaría, R. I., J. F. Martín, and J. A. Gil.** 1987. Identification of a promoter sequence in the plasmid pUL340 of *Brevibacterium lactofermentum* and construction of new cloning vectors for corynebacteria containing two selectable markers. Gene **56:**199–208.
42. **Stapley, E. O., M. Jackson, S. Hernández, S. B. Zimmerman, S. A. Currie, S. Mochales, J. M. Mata, H. B. Woodruff, and D. Hendlin.** 1972. Cephamycins, a new family of β-lactam antibiotics. I. Production by actinomycetes, including *Streptomyces lactamdurans* sp. n. Antimicrob. Agents Chemother. **2:**122–131.
43. **Thompson, C. J., J. M. Ward, and D. A. Hopwood.** 1982. Cloning of antibiotic resistance and nutritional genes in streptomycetes. J. Bacteriol. **151:**668–677.
44. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13 mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
45. **Vigal, T., J. A. Gil, A. Daza, M. D. García-González, and J. F. Martín.** 1991. Cloning, characterization and expression of an α-amylase gene from *Streptomyces griseus* IMRU3570. Mol. Gen. Genet. **225:**278–288.
46. **Wesseling, A. C., and B. D. Lago.** 1981. Strain improvement by genetic recombination of cephamycin producers, *Nocardia lactamdurans* and *Streptomyces griseus*. Dev. Ind. Microbiol. **22:**641–651.
47. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.