Journal of Industrial Microbiology, 2 (1987) 71–77
Elsevier
SIM 00065

Genetic recombination by protoplast fusion in *Nocardia asteroides*

M.C. Liechty[a], S.G. Bradley[b] and K.L. Kasweck[a]

[a]*Department of Biological Sciences, Florida Institute of Technology, Melbourne, FL, and* [b]*Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, VA, U.S.A.*

Received 12 August 1986
Revised 19 February 1987
Accepted 23 February 1987

Key words: Conjugation; Genetic recombination; *Nocardia asteroides*; Protoplast regeneration; Protoplast fusion

SUMMARY

Fusion and regeneration of protoplasts of *Nocardia asteroides* strains ATCC 3318, IMRU W3599 and HIK B971 have been used to study genetic recombination in this species. Protoplasts were produced by treatment with lysozyme, following incubation with glycine. Mutants of ATCC 3318 were grown in peptone yeast extract medium at 32°C prior to protoplast production to maximize protoplast frequency, whereas mutants of IMRU W3599 and HIK B971 were grown in trypticase-soy broth. Glycine concentrations favoring protoplast formation varied from 1.5% to 5% depending on strain. For all strains, protoplast formation was complete 1 h after addition of 5 mg/ml lysozyme. Protoplasts were fused by addition of 50% polyethylene glycol-1000. In general, 25% of the protoplasts could be regenerated. The incidence of recombinant recovery was increased up to 750-fold. The distribution of recombinant phenotypes in matings was similar for protoplast fusion and conventional crosses.

INTRODUCTION

Nocardia asteroides, a common soil actinomycete, is an opportunistic pathogen of humans, and is becoming increasingly common in infections in immunosuppressed patients [5]. Treatment of nocardial infections is complicated by the resistance of these organisms to many antibiotics [2,16]. Study of recombination in *N. asteroides* has shown that antibiotic resistance may be stably inherited by recombinants [12]. A number of cryptic plasmids have been discovered in both soil and clinical isolates of *N. asteroides*. These plasmids are also stably inherited by *N. asteroides* recombinants [13], and may serve as vectors for transfer of genes from one strain to another.

Nocardiae exhibit a variety of types of mating behavior, spanning the range from self-fertility in *N. mediterranea* [18] to strict out-breeding among an apparent multiplicity of mating types in *N. asteroides*. Recombination between a number of strains of *N. asteroides* of different origin, even between strains with little homology, has been demonstrated, although strains of common origin are infertile [12]. Analysis of mating behavior and inheritance in *N. asteroides* has been hampered by low

Correspondence: Dr. M.C. Liechty, Department of Biological Sciences, Florida Institute of Technology, Melbourne, FL 32901, U.S.A.

yields of recombinants and by incompatibility between some strains. Protoplasting, fusion, and regeneration techniques were developed for *N. asteroides* to facilitate genetic studies of this organism, and are reported here.

MATERIALS AND METHODS

Strains

Mutants of three strains of *N. asteroides* were used: KK4-64, *his-10 leu-1 str-31,* derived from ATCC 3318; KK5-2, *leu-8,* derived from IMRU W3599; and KK6-119, *met-3 phe-3*, derived from HIK B971. These strains are referred to in the text as KK4, KK5, and KK6. KK5 is in the heterogeneous A group of *N. asteroides,* as defined by numerical taxonomy. KK4 and KK6 are in the homogeneous B group, strains of which share a high degree of DNA homology [17].

Cultivation of strains for protoplast preparation

Several media were tested for their ability to produce cultures that could be readily protoplasted. These media were: (a) trypticase mating medium (TMM) consisting of, per liter, BBL trypticase peptone (Becton, Dickinson and Co., Cockeysville, MD), 17 g; NaCl, 5 g; K_2HPO_4, 2.5 g; glucose, 15 g; (b) supplemented minimal medium (SMM) consisting of, per liter, Na_2HPO_4, 6 g; KH_2PO_4, 3 g; sodium citrate dihydrate, 0.5 g; NH_4Cl, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; glucose, 15 g; nutritional supplements required by auxotrophic strains, 10 μg/ml of each; (c) SMM with 0.4 M mannitol; (d) peptone-yeast extract broth (PY), consisting of, per liter, peptone, 5 g; yeast extract, 3 g; (e) PY with 0.05% Tween-80; (f) PY with 1.5% glucose (PYG); (g) BBL trypticase-soy broth (TSB); (h) TSB with 0.05% Tween-80; (i) TSB with 1.5% glucose (TSG); (j) Difco brain-heart infusion (Difco Laboratories, Detroit, MI); and (k) BBL Actinomyces broth.

Cultures were inoculated from 3-day-old TSG plates to 10 ml of each medium in 125-ml flasks. Cultures were treated by immersion in a sonicating bath to complete fragmentation of mycelia and incubated at 37°C for 18–72 h in a reciprocal shaking incubator. At intervals, 2 ml of each culture was diluted to 10 ml with fresh medium to which glycine was added to a final concentration of 3%. Cells were incubated an additional 18–24 h and protoplasted as described below. Those media described above that gave the greatest yields of protoplasts were selected and growth in these media was measured in the presence of glycine at concentrations of 0.5%–6.0% in 0.5% increments. Cultures were incubated with aeration for 18–24 h at 37°C and growth was compared with that of cultures lacking glycine. A concentration of glycine that was slightly inhibitory to growth was determined for each strain and used in pretreatment of cells for protoplasting. The effect of temperature on protoplasting was tested by growing cells at 30°C, 32°C, and 45°C prior to protoplasting. Following the above tests, standard treatments were adopted for each strain prior to protoplasting.

Standard culture conditions for protoplast preparation

For most efficient protoplasting, KK4 was grown in PY broth with 0.05% Tween-80 for 24 h at 32°C with aeration. The culture was sonicated to homogeneity and diluted 1:5 into 10 ml fresh PY with 0.05% Tween-80 and 1.5% glycine. The culture was incubated another 24 h and harvested. KK5 and KK6 were similarly treated, but were grown in TSB with 0.05% Tween-80 and were treated with 3% and 5% glycine, respectively.

Protoplasting

Protoplasting was done in a hypertonic buffer similar to that of Okanishi et al. [15], incorporating modifications of Baltz and Matsushima [4] and Brownell [8]. Protoplasting buffer (P buffer) consisted of, per liter, mannitol, 73 g (0.4 M); K_2SO_4, 0.25 g; $MgCl_2 \cdot 6H_2O$, 2.03 g (0.01 M); KH_2PO_4, 0.05 g (0.37 mM); $CaCl_2 \cdot 2H_2O$, 3.68 g (0.025 M) in 0.025 M *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), pH 7.2.

Cells were collected from glycine broth by centrifugation and were washed with P buffer. Washed cells were suspended in 2 ml P buffer and divided into two 1-ml portions, one to serve as an unpro-

toplasted control. Lysozyme (Sigma Chemical Co., St. Louis, MO) was added to one sample to a final concentration of 5 mg/ml. Suspensions were incubated at 32°C with gentle agitation for 1 h. Protoplasts were collected by centrifugation at 1000 rpm for 10 min, washed with P buffer and suspended in 1 ml P buffer. Protoplast formation was monitored by phase contrast microscopy.

Fusion

Protoplasts of two strains to be fused were mixed in 1:1 ratio in P buffer. Polyethylene glycol (PEG) 1000 MW (Sigma Chemical Co., St. Louis, MO) in P buffer was added to a concentration of 50% (w/v) to the protoplast suspension and mixed by gentle agitation. Protoplasts were incubated at room temperature for 1 min.

Regeneration

Protoplast suspensions were diluted as appropriate in P buffer and plated on regeneration medium (RM), a modification of that of Okanishi et al. [15] and Brownell [8], consisting of, per liter, mannitol, 73 g (0.4 M); K_2SO_4, 0.25 g; trace elements solution, 2 ml; $MgCl_2 \cdot 6H_20$, 10.12 g (0.05 M); glucose, 10 g; L-asparagine, 2 g; peptone, 1.5 g; yeast extract, 0.75 g; casamino acids, 0.1 g; KH_2PO_4, 0.05 g (0.37 mM); $CaCl_2 \cdot 2H_2O$, 2.95 g (0.02 M); agar, 22 g in 0.025 M TES, pH 7.2. Nutritional supplements required by auxotrophic strains were added to a final concentration of 10 μg/ml. Trace elements solution consisted of, per liter, $ZnCl_2$, 40 mg; $FeCl_3 \cdot 6H_2O$, 200 mg; $CuCl_2 \cdot 2H_2O$, 10 mg; $MnCl_2 \cdot 4H_2O$, 10 mg; $Na_2B_4O_7 \cdot 10H_2O$, 10 mg; $(NH_4)_6Mo_{24} \cdot 4H_2O$, 10 mg. In some experiments protoplasts were mixed with 3 ml molten hypertonic soft agar overlay at 50°C before plating on RM. Overlay agar consisted of, per liter, mannitol, 73 g (0.4 M); $MgCl_2 \cdot 6H_2O$, 10.12 g (0.05 M); $CaCl_2 \cdot 2H_2O$, 3.68 g (0.025 M); agar, 4.1 g in 0.025 M TES, pH 7.2. In all cases, RM plates were inoculated 3 or more days after being poured. RM plates were incubated at 37°C for 7 days. Colonies were picked to TSG plates, incubated at 37°C for 3 days, and replicated to SMM to determine the phenotypes of regenerated cells.

Frequencies of protoplasting, fusion, and regeneration

The percentage of cells protoplasted was determined by diluting protoplast suspensions and unprotoplasted control cultures in distilled water. Lower colony counts from protoplasted cultures were caused by cell lysis and allowed calculation of protoplasting frequency. Fusion frequency was determined by comparing plate counts of PEG-treated and untreated protoplasted cultures. Lower colony counts from PEG-treated cultures resulted from protoplast fusion or aggregation, yielding fewer individual protoplasts. Regeneration frequency was the fraction of all protoplasts surviving on RM, determined by comparing colony counts of protoplasts diluted in P buffer and diluted in distilled water. Plating methods were used in all instances instead of microscopic counts because of the difficulty of accurately counting individual cells in these filamentous organisms.

Mating

Standard matings were done by mixing equal numbers of cells of different strains on TMM agar, as previously described [12]. Mating plates were incubated 3 days at 37°C. The cells were suspended in 0.85% NaCl and plated on SMM selective for recombinants, and on TSG for total population count. Plate counts were used to calculate recombination frequencies.

RESULTS

Culture conditions prior to protoplasting

The composition of the medium in which cells were grown prior to protoplasting appeared to be critical to protoplast formation. Microscopic observation of protoplasted cultures showed that most media resulted in little or no protoplast formation. In addition, the strains differed from each other with respect to the medium most conducive to protoplasting. KK4 protoplasted well when grown in PY and modified PY media, but produced few protoplasts when grown in trypticase-soy (TS)-based media and none when grown on SMM,

brain-heart infusion or Actinomyces broth. In contrast, KK5 and KK6 both protoplasted well when grown in TS-based media, but not when grown in PY media. Addition of 0.05% Tween-80 to the media increased protoplast yield by dispersing clumps of cells, thus aiding in protoplast release. The three strains used in these experiments have similar growth rates overall when grown in the same medium, but they vary considerably in the duration of each stage. In TSB, KK4 tends to persist longer than the other strains as long, well-developed hyphae which are not as readily protoplasted as are younger filaments. KK6 tends to persist longer as cocci and coccobacilli, which are highly refractile to protoplasting.

Culture age was an important factor in protoplast formation. Cultures 24 h old when harvested for glycine treatment were more effectively protoplasted than 18-h cultures or 48-72-h cultures.

The incubation temperature prior to protoplasting had a significant effect on protoplast formation. Initial experiments were done at 37°C, the optimal temperature for growth of *N. asteroides*. At 37°C, the total number of protoplasts formed comprised less than 10% of the cell population, and in the case of KK4 were unstable, lysing readily even when maintained in P buffer. At 30°C or 45°C, growth of the cultures was decreased significantly and no protoplasts were formed. Growth at 32°C was not significantly greater than at 30°C, but resulted in up to 90% protoplasting of the population.

The glycine concentration yielding the slight inhibition of growth necessary for protoplasting varied with strain. Effective glycine concentrations were 1.5% (w/v) for KK4, 3% for KK5, and 5% for KK6.. In some experiments, glycine-treated cultures exhibited morphological features such as football-shaped or club-shaped cells, in contrast to normal rods or filaments, that suggested some cell wall modification induced by glycine prior to lysozyme treatment.

Protoplasting

Microscopic examination of protoplasting suspensions showed that protoplasts began to appear 10–15 min after addition of lysozyme, with most protoplast formation complete within 45 min. Incubation with lysozyme was extended to 1 h to ensure complete protoplasting. The lysozyme treatment temperature was not critical: 32°C, 37°C, or room temperature were equally effective. Most protoplasts formed at the growing tips or branch points of young 10–20 μm long filaments. Few protoplasts were formed from more highly developed filaments, and required several hours incubation with lysozyme before forming. No protoplasts were formed from cocci or coccobacilli.

Each strain was protoplasted in at least four separate experiments, with protoplast frequencies for KK4 of 0.62–0.85 (0.73 avg); for KK5, 0.51–0.79 (0.64 avg); and KK6, 0.55–0.93 (0.74 avg).

Fusion

Fusion was readily accomplished by mixing PEG-1000 with protoplast suspension. Aggregation of protoplasts was immediate and could be assisted by agitation or mixing of the suspension with a pipet. Determination of the decrease in protoplast population resulting from fusion of two or more protoplasts into single colony-forming units allowed calculation of fusion frequencies. Fusion frequencies were variable and not related to strain, ranging from 0.41 to 0.61 for KK4, 0.32–0.73 for KK5, and 0.16–0.83 for KK6. This variability may be related to the amount of mixing to which each fusion mixture was subjected. More mixing resulted in more fusion but suspensions became difficult to pipet, with the formation of clumps of protoplasts. Some variability also may have resulted from different ratios of merely aggregated to truly fused protoplasts.

Regeneration

Regeneration on RM allowed recovery of an average of 25% of protoplasts formed, with the frequency of KK4 recovery being 0.19–0.21, KK5 0.28–0.42, and KK6 0.16–0.35. Regeneration directly on RM without the use of overlay agar, regeneration on freshly prepared RM, or regeneration on RM lacking peptone and yeast extract decreased regeneration frequency up to 50% (data not shown). Without overlays, the regeneration fre-

Table 1

Recombination by protoplast fusion

Standard matings were conducted by co-cultivation on TMM for 3 days at 37°C prior to subculture to SMM to detect recombination. For protoplast fusion, mating strains were grown in glycine broth prior to incubation in P buffer at 32°C for 1 h. Protoplasts were fused in 50% PEG-1000 at 22–25°C for 1 min and regenerated on RM medium at 37°C for 7 days. Colonies were subcultured to TSG at 37°C for 3 days prior to selection on supplemented SMM to detect recombinants.

Parent 1		Parent 2		Recombinant recovery frequency	
strain	reversion freq.	strain	reversion freq.	standard	fusion
KK5-2 *leu-8*	$< 2.9 \times 10^{-10}$	KK6-119 *met-3 phe-3*	$< 1.3 \times 10^{-10}$	0	1.2×10^{-6}
KK4-64 *his-10 leu-1 str-31*	$< 6.3 \times 10^{-7}$	KK6-119 *met-3 phe-3*	$< 1.3 \times 10^{-10}$	1.3×10^{-5}	9.0×10^{-3}

quency for KK4 dropped dramatically from around 0.20 to 0.036. For KK5, this drop in frequency was less dramatic, decreasing to 0.13. KK6 regeneration without overlays was not tested. Use of RM prepared the same day that it was used also lowered regeneration frequencies to 0.084 and to 0.25 for KK4 and KK5, respectively.

Recombination by protoplast fusion

KK4-64 and KK6-119, which yield recombinants in conventional agar matings, were fused, and regenerated colonies were screened for recombinant types. KK5-2 and KK6-119, which do not yield recombinants in conventional matings, were similarly fused and examined (Table 1). The frequency of recombinants recovered from fusion of KK4 and KK6 was 9.0×10^{-3}, or about 750-times greater than recombinant recovery from conventional matings. The frequency of recombinants recovered from fusion of KK5 and KK6 was 1.2×10^{-6}, comparable to the frequency observed in conventional mating of compatible *N. asteroides* strains.

Recombinants of KK4-KK6 fusion were scored for the frequency of individual class types, as shown in Table 2. For recombination both by mating and by fusion, the distribution of recombinant class types among the progeny was similar.

Table 2

Distribution of recombinant phenotypes in crosses of *N. asteroides* KK4-64 *his-10 leu-1 str-31* × KK6-119 *met-3 phe-3* by standard mating and protoplast fusion

The total number of non-parental types was enumerated and their phenotypes were determined by replica plating to appropriate supplemented SMM. The fraction of total recombinants is the number of a given phenotype divided by the total number of non-parental types.

	Fraction of total recombinants	
Phenotype	standard mating[a]	fusion[b]
Met^-	0.38	0.47
Phe^-	0.24	0.19
Leu^-Phe^-	0.12	0.045
Leu^-	0.10	0.10
Leu^-Met^-	0.045	0.045
His^-	0.041	0.059
His^-Phe^-	0.038	0.020
Prototrophy	0.023	0.054
His^-Met^-	0.0034	0.0056

[a] Based on 540 recombinants.
[b] Based on 354 recombinants.

DISCUSSION

Modifications of media developed by Okanishi et al. [15] for protoplasting and regenerating *Streptomyces* were found to be effective for protoplasting and regeneration of *N. asteroides*. The conditions necessary for growth of cells prior to protoplasting proved to be critical to efficient protoplasting and were highly strain-dependent. Culture conditions other than those adopted for these strains resulted in little (less than 10%) or no protoplast formation when such cells were treated with lysozyme. The dependency of efficient protoplasting on culture medium, culture age, cultivation temperature and glycine concentration all suggest that cell wall structure is an important factor in protoplast formation. Nocardiae have a number of complex lipids, peptides, and carbohydrates in their wall in addition to the lysozyme-sensitive peptidoglycan layer. Many of these wall components may contribute to lysozyme resistance in *Nocardia* by masking lysozyme-sensitive bonds in the peptidoglycan layer. The relative ratios of these components and the size of the molecules, as well as the specific identities of the components, are known to vary with medium composition, culture age, and cultivation temperature [7,11,14].

The differences in conditions required to effect protoplasting in the strains of *N. asteroides* tested appear to be related to differences in their life cycle. Nocardiae grow as mycelia in which cross walls are formed, followed by fragmentation of the mycelium into individual cocci and coccobacillary cells, which give rise to new mycelia. The three strains used in these experiments have similar growth rates overall when grown in the same medium, but they vary considerably in the duration of each stage, as noted in 'Results' above. The resistance of coccobacilli to protoplasting may be related to the structure of much of their wall, originally formed as cross walls in mycelia [6]. Since the growth rate of KK4 is different in PY and TSB, 24 h incubation in PY yields the more easily protoplasted shorter filaments. The different percentages of glycine required for each strain, besides allowing incorporation of glycine into the peptidoglycan, also modify growth rates, thus assisting in production of cell populations at the time of harvest that are in the appropriate stage of growth for protoplasting. Failure of younger or older cultures to protoplast may be caused by alterations of cell wall structure that are related to culture age. Such cultures are still largely in the coccobacillary stage, or have already passed into the longer filament stage, or beyond to fragmentation into coccobacilli, and for this reason are unsuitable for protoplasting. Cultures sonicated to fragment, then incubated 24 h, allow new fragments to form the more easily protoplasted short hyphae.

Cultivation temperature prior to protoplasting has been reported to affect the regeneration of protoplasts in species of *Streptomyces* [4]. Cell wall composition may differ enough between cells grown at 30°C and those grown at 32°C to account for the difference in protoplasting ability, or perhaps the distribution of lipids in cells grown at 30°C is unfavorable to stable protoplast formation. Spontaneous lysis of KK4 protoplasts formed from cultures grown at 37°C was observed microscopically, even when protoplasting populations were maintained in the protoplasting buffer. Protoplasts, if formed from cells grown at 30°C, may have been even more transient.

Overlays are commonly used in *Streptomyces* and *Bacillus* regeneration procedures, helping to stabilize protoplasts and assisting regrowth, perhaps by retarding the diffusion of precursors and cell-wall-synthesizing enzymes normally found in the periplasmic space. Inhibitors produced by unprotoplasted cells or by early-regenerating *Streptomyces* and *Bacillus* protoplasts retard regeneration of late-regenerating protoplasts [3,10]. Diffusion of such inhibitors is retarded in drier media, resulting in higher total regeneration frequencies [4,19]. Whether *Nocardia* produces such inhibitors is not known, although a similar effect of overlays and medium age on *Nocardia* regeneration was observed.

Through protoplast fusion of the normally incompatible combination of KK5 with KK6, recombinants were recovered among regenerating colonies at a frequency of 1.2×10^{-6}, still low when compared with a frequency approaching 1% for

KK4 fused with KK6, but adequate for genetic analyses. Some other barriers to recombination, such as a restriction system in one of the strains or simple lack of homology between the chromosomes of the strains involved, may account for the low recombinant recovery frequency. *N. asteroides* is a heterogeneous taxon: KK4 and KK6 are in a well-defined group, group B sharing much homology, while KK5 falls into the more heterogeneous A group [17]. The cell wall can serve as a significant barrier to recombination in *N. asteroides*. It is likely that some factors determining compatibility are normally expressed at the nocardial cell surface as components of the cell wall. This proposal is supported by the lack of fertility between strains of common origin in conventional matings.

In protoplast fusion the entire genome of each cell enters the fusion product, so that all genes have a greater chance to recombine. The frequencies of various class types might be quite different in standard crosses and in fusions if, in standard mating, only a portion of the genome were transferred from donor to recipient, or if transfer were polar. That the distribution of frequencies was similar between the two recombination methods supports the hypothesis that mating in *N. asteroides* in nature occurs by a fusion-like process, as has been observed in growth of *N. asteroides* cultures [1,9].

ACKNOWLEDGEMENTS

This work was supported by grant PCM 7807868 from the National Science Foundation. I would also like to thank Michelle Vahsen and Jane Guiles for excellent assistance in manuscript preparation.

REFERENCES

1 Adams, J.N. and N.M. McClung. 1962. Comparison of the developmental cycles of some members of the genus *Nocardia*. J. Bacteriol. 84: 206–216.

2 Bach, M.C., L.D. Sabath and M. Finland. 1973. Susceptibility of *Nocardia asteroides* to 45 antimicrobial agents in vitro. Antimicrob. Agents Chemother. 3: 1–8.

3 Baltz, R.H. 1978. Genetic recombination in *Streptomyces fradiae* by protoplast fusion and cell regeneration. J. Gen. Microbiol. 107: 93–102.

4 Baltz, R.H. and P. Matsushima. 1981. Protoplast fusion in *Streptomyces*: conditions for efficient genetic recombination and cell regeneration. J. Gen. Microbiol. 127: 137–146.

5 Beaman, B.L., J. Burnside, B. Edwards and W. Causey. 1976. Nocardial infections in the United States, 1972–1974. J. Infect. Dis. 134: 286–289.

6 Beaman, B.L., J.A. Serrano and A.A. Serrano. 1978. Comparative ultrastructure within the nocardiae. In: Nocardia and Streptomyces (Mordarski, M., W. Kurylowicz and J. Jeljaszewicz, eds.), pp. 201–220, Gustav Fischer Verlag, Stuttgart.

7 Beaman, B.L. and D.M. Shankel. 1969. Ultrastructure of *Nocardia* cell growth and development on defined and complex agar media. J. Bacteriol. 99: 876–884.

8 Brownell, G.H. 1981. A protoplasting and transfection procedure for *Nocardia (Rhodococcus)* species. Actinomycetes 16: 54–56.

9 Davis, G.H.G. 1960. The interpretation of certain morphological appearances in a *Streptomyces* sp. J. Gen. Microbiol. 22: 740–743.

10 DeCastro-Costa, M.R. and O.E. Landman. 1977. Inhibitory protein controls the reversion of protoplasts and L forms of *Bacillus subtilis* to the walled state. J. Bacteriol. 129: 678–689.

11 Ioneda, T. and C.L. Silva. 1978. Studies on the lipids from *Nocardia*. In: Nocardia and Streptomyces (Mordarski, M., W. Kurylowicz and J. Jeljaszewicz, eds.), pp. 67–74, Gustav Fischer Verlag, Stuttgart.

12 Kasweck, K.L. and M.L. Little. 1982. Genetic recombination in *Nocardia asteroides*. J. Bacteriol. 149: 403–406.

13 Kasweck, K.L., M.L. Little and S.G. Bradley. 1982. Plasmids in mating strains of *Nocardia asteroides*. Dev. Ind. Microbiol. 23: 279–286.

14 Minniken, D.E. and M. Goodfellow. 1976. Lipid composition in the classification and identification of nocardiae and related taxa. In: The Biology of the Nocardiae (Goodfellow, M., G.H. Brownell and J.A. Serrano, eds.), pp. 160–219, Academic Press, London.

15 Okanishi, M., K. Suzuki and H. Umezawa. 1974. Formation and reversion of streptomycete protoplasts: cultural conditions and morphological study. J. Gen. Microbiol. 80: 389–400.

16 Pulverer, G. and K.P. Schaal. 1978. Pathogenicity and medical importance of aerobic actinomycetes. In: Nocardia and Streptomyces (Modarski, M., W. Kurylowicz and J. Jeljaszewicz, eds.), pp. 417–427, Gustav Fischer Verlag, Stuttgart.

17 Schaal, K.P. and H. Reutersberg. 1978. Numerical taxonomy of *Nocardia asteroides*. In: Nocardia and Streptomyces (Modarski, M., W. Kurylowicz and J. Jeljaszewicz, eds.), pp. 53–62, Gustav Fisher Verlag, Stuttgart.

18 Schupp, T., R. Hutter and D.A. Hopwood. 1975. Genetic recombination in *Nocardia mediterranei*. J. Bacteriol. 121: 128–136.

19 Shirahama, T., T. Furumai and M. Okanishi. 1981. A modified regeneration method for streptomycete protoplasts. Agric. Biol. Chem. 45: 1271–1273.