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Transfection in *Micromonospora* spp.

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The introduction of bacteriophage DNA into *Micromonospora* protoplasts, resulting in the production of infective viral progeny, is reported. Transfection was affected by several factors. We observed that it reached a maximum when protoplasts from young mycelium (15 h old) were used. Maximum transfection took place when polyethylene glycol (PEG) was added to the mixtures at a final concentration of 20% (vol/vol) and did not occur at PEG concentrations under 10% or over 35%. The addition of positively charged liposomes to the mixtures was essential, since no transfectants were detected in the absence of liposomes at any PEG concentration. When DNA was present in nonlimiting amounts, a maximum efficiency of around 10^{-3} to 10^{-4} PFU per protoplast was obtained. The efficiency per DNA molecule showed a constant value of around 10^{-4} to 10^{-5} PFU, but the data suggest that transfection could be achieved by a single DNA molecule. The method proved to be equally efficient for the DNAs of at least five *Micromonospora* bacteriophages. On the contrary, we failed to transfect five of seven *Micromonospora* strains. These data suggest that only a minor subpopulation of protoplasts is competent and that the main factors influencing the transfection of *Micromonospora* protoplasts are neither the characteristics nor the origin of the DNA but the properties and status of the protoplasts.

In a previous paper (1) we described the main characteristics of five *Micromonospora* bacteriophages; all of them are temperate and have double-stranded DNA. Since our attempts to isolate spontaneous deletion mutants were relatively unsuccessful (unpublished results), we devised a transfection system which should allow the propagation of deletion mutants generated in vitro, through partial restriction and religation of phage DNAs. This is a necessary step if further attempts to construct phage cloning vectors are to be made for this important group of antibiotic-producing actinomycetes.

MATERIALS AND METHODS

Bacteria and bacteriophages. *Micromonospora* sp. strain IMET 8002, from the collection of the Zentralinstitut für Mikrobiologie und Experimentelle Therapie, Jena, Democratic Republic of Germany, was a kind gift of H. Prauser. This strain was used as the indicator strain and as the transfection host because of its high susceptibility to infection by several phages. Other bacterial strains are mentioned below.

Unless otherwise stated, bacteriophage Mm5 was used as the source of transfecting DNA. DNAs of phages Mm1, ϕM2, ϕM3, and Mm4 were also used. The characteristics of these phages and the methods of culturing and harvesting them were described by Caso et al. (1). Phage DNA was extracted by hot sodium dodecyl sulfate disruption of the particles (11).

Preparation of protoplasts. Spores of *Micromonospora* sp. strain IMET 8002 or other strains, stored at −20°C in 20% glycerol, were inoculated at an initial optical density of 0.1 (at 580 nm) into flasks containing medium 172F supplemented with 0.2% (wt/vol) glycine (Sigma Chemical Co., St. Louis, Mo.). Medium 172F contained (in grams per liter) the following: glucose, 10; soluble starch, 10; yeast extract (Oxoid Ltd., London, England), 5; and Casitone (Difco Laboratories, Detroit, Mich.), 5. After the medium was autoclaved, $MgSO_4$ and $Ca(NO_3)_2$ were added to final concentrations of 10 and 8 mM, respectively. Incubation was carried out at 30°C in an orbital shaker (200 rpm) for various times, usually 15 to 16 h. Mycelium was harvested by centrifugation at 10^4 rpm (12,100 × *g*) for 10 min, washed with 0.3 M sucrose, suspended in a small amount of P buffer (2) containing lysozyme (3 mg/ml), and incubated at 35°C for 2 to 3 h. The protoplast suspensions were centrifuged, washed with P buffer, suspended in the small drop of remaining liquid, and either transfected or stored at 4°C for up to 20 h. Immediately before transfection, another washing with P buffer was done to eliminate any possible nucleases. Protoplast counts were made from diluted aliquots in a Thoma hemacytometer chamber by using a phase-contrast microscope.

Transfection. Small positively charged liposomes were prepared as described by Rodicio and Chater (7) immediately before transfection. Phage DNA in TE buffer (10 mM Tris hydrochloride, 1 mM EDTA [pH 8.0]) was added to preformed liposome suspensions in proportions equal to or less than 1 volume of DNA per 5 volume of liposomes.

Transfection mixtures were made by mixing the ingredients in the following order: (i) a variable amount of protoplasts, usually 10 to 25 μl of a concentrated suspension; (ii) 100 μl of the DNA-liposome mixture, containing from 1 ng to 2.5 μg of DNA; and (iii) 500 μl of a solution of polyethylene glycol (PEG) 1000 or 4000 (Sigma) in P buffer, in variable concentrations up to 60% (vol/vol). The specific concentration of each element will be stated for each individual experiment.

Transfection mixtures were gently homogenized with a 1-ml automatic pipette and allowed to rest at room temperature for 5 to 8 min. Afterwards, 5 ml of P buffer was added to each sample, and protoplasts were collected by centrifugation and suspended in 1 ml of medium 172F supplemented with 0.3 M sucrose. The suspensions were incubated at 30°C and 200 rpm for various times (15 h in the standard proce-

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dure) to allow the production and release of the viral progeny.

Finally, the suspensions were diluted in nonsupplemented medium 172F and assayed by the conventional double-layer method in nonsupplemented medium 172F with *Micromonospora* sp. strain IMET 8002 as the indicator strain. The results were scored after 40 to 48 h of incubation at 35°C.

RESULTS AND DISCUSSION

Preparation and stability of protoplasts. The ability to form protoplasts was very different among several *Micromonospora* strains, depending mainly on the strain, the culture age, and the culture medium (data not shown). As occurs in *Streptomyces* strains (8), protoplast formation seemed to be easier from young cultures than from old ones, and higher yields were obtained when the cultures were grown in the presence of glycine, although glycine concentrations for *Micromonospora* strains had to be lowered with respect to those cited for *Streptomyces* strains (3, 6, 8, 9), since concentrations higher than 0.2% (wt/vol) inhibited the growth of most strains.

After observing protoplast suspensions by phase-contrast microscopy for up to 3 days, we determined that protoplast stability for *Micromonospora* sp. strain IMET 8002 was maximum at sucrose concentrations of around 0.3 to 0.4 M. Unfortunately, plating of spores of *Micromonospora* sp. strain IMET 8002 in medium 172F containing 0.3 or 0.4 M sucrose resulted in very slow growth, with colonies becoming visible only after 5 to 7 days of incubation, instead of the usual 2 days. Furthermore, the efficiency of plating of phage Mm5 in supplemented medium 172F was reduced to less than 20%, as compared with that in nonsupplemented medium 172F. The situation was even worse when other osmotic stabilizers, such as mannitol, sorbitol, and succinate, were used instead of sucrose.

Detection of transfectants. Taking into account the aforementioned facts, we decided not to follow the method commonly used for *Streptomyces* strains, i.e., assaying the transfection mixtures in double-layer plates of osmotically stabilized medium (4, 5, 10, 12), but to incubate the transfection mixtures in an isotonic liquid medium (medium 172F plus 0.3 M sucrose) long enough to allow the release of viral particles, followed by further plating in nonsupplemented medium 172F. Under these conditions, free viral particles were not detected until after 3 h of posttransfection incubation. The release of virions proceeded at practically a constant rate at least until 24 h posttransfection (data not shown). For all further experiments, we arbitrarily chose an incubation time of 15 h in isotonic liquid medium before plating. We are aware that a significant proportion of the newly formed viral particles could be due to second-generation virions originating after infection of mycelium fragments present in the protoplast suspensions by previously released virions. In fact, control samples in which protoplast suspensions were infected with complete virions showed an increase in the phage titer. In any case, individual transfectants could not be counted, since the first-generation virions from a single transfectant would be dispersed in the liquid medium and would give rise to an undetermined number of PFU. Nevertheless, we repeatedly observed transfected samples yielding a total count of 10^1 to 10^2 PFU after 15 h of incubation, and this result indicates that under these conditions, no more than 10^2 PFU could be produced by a single transfection event. Accordingly, the number of individual transfectants should be around 1 to 2 orders of magnitude lower than the observed total PFU in a given sample.

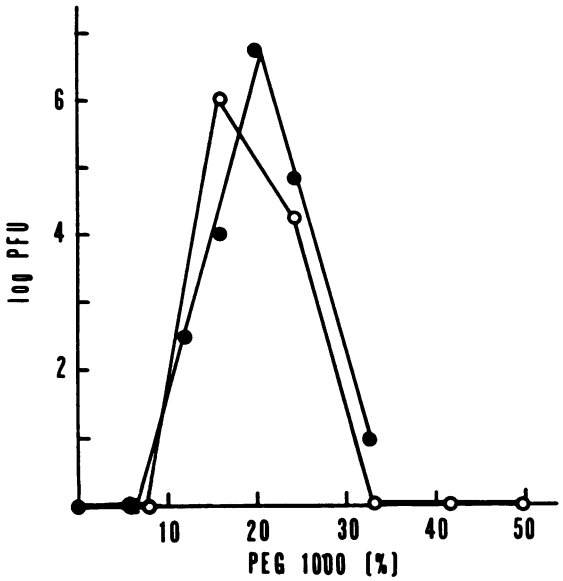

FIG. 1. Effect of PEG 1000 concentration on the transfection of *Micromonospora* sp. strain IMET 8002 protoplasts by phage Mm5 DNA. Transfection mixtures contained 1.4×10^9 protoplasts in 10 μl, 0.12 μg of DNA in 100 μl of liposome suspension, and 500 μl of PEG 1000 at final concentrations of 0 to 49% (vol/vol) (○) or of 0 to 33% (●). All other factors were as in the standard conditions described in the text.

Effect of liposomes. The addition of positively charged liposomes to the transfection mixtures was essential to achieve transfection. No viral particles were produced when liposomes were not present in the mixtures at any of the PEG concentrations assayed (from 12 to 33% [vol/vol]). This fact contrasts with the data for *Streptomyces* strains, for which the addition of liposomes increased over 100-fold the efficiency of transfection (7) but was not strictly necessary (4, 5, 10, 12).

Effect of PEG. The transfection efficiencies obtained with PEG 4000 were usually (but not always) slightly lower than those obtained with PEG 1000, indicating that the molecular weight of PEG was not relevant.

On the contrary, PEG concentration proved to be a most important factor, since transfection took place only in the narrow range between 10 and 35% (vol/vol), with a maximum at around 20% (Fig. 1). These data are again in contrast with those for *Streptomyces* strains, for which the range of PEG concentration is much broader (7, 10) and for which the maximum (when liposomes are used) occurs at 60% PEG (7).

Effect of culture age. Maximum transfection of *Micromonospora* sp. strain IMET 8002 was reached when protoplasts were obtained from 15-h-old cultures (Fig. 2). At this stage, the cultures were mainly composed of young hyphae starting to branch. Young cultures are also optimal for transfection of *Streptomyces parvullus* or *Streptomyces albus* protoplasts (11). It is possible that protoplasts of this age are more readily transfectable than older ones, but the higher efficiencies of transfection in these cultures could also have been due to the fact that the yield and quality of protoplasts were also optimal at this stage.

Effect of DNA concentration. The number of PFU obtained after transfection increased with increasing concentrations of DNA. When log PFU was plotted against log DNA

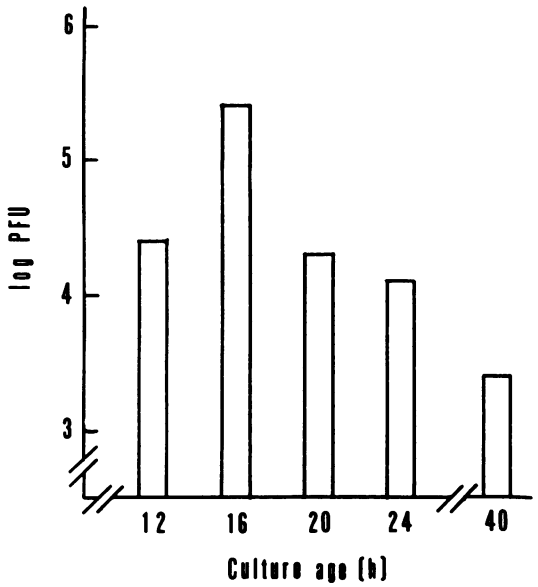

FIG. 2. Effect of culture age on the transfection of *Micromonospora* sp. strain IMET 8002 protoplasts by phage Mm5 DNA. Protoplasts were obtained from mycelium grown for different times under the conditions described in the text. Transfection mixtures contained 3.1×10^9 protoplasts in 25 μl, 2.5 μg of DNA in 100 μl of liposome suspension, and 500 μl of PEG 1000 at a final concentration of 20% (vol/vol). All other factors were as in the standard conditions described in the text.

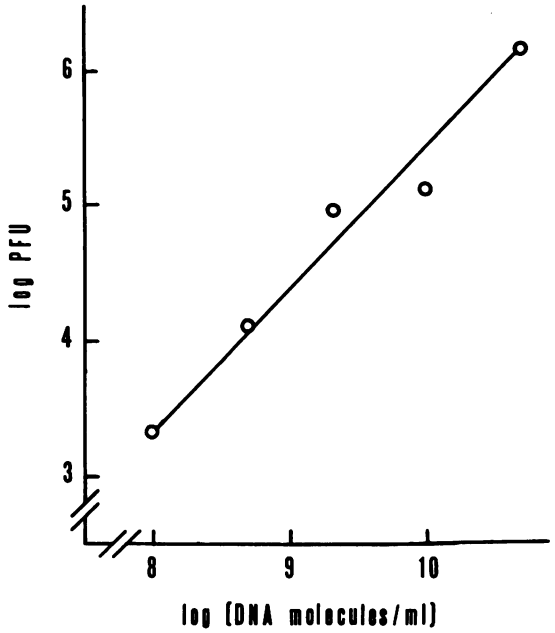

FIG. 3. Effect of DNA concentration on the transfection of *Micromonospora* sp. strain IMET 8002 protoplasts by phage Mm5 DNA. Transfection mixtures contained 6.25×10^9 protoplasts in 25 μl, 500 μl of PEG 1000 at a final concentration of 20% (vol/vol), and variable amounts of phage Mm5 DNA (from 5 ng to 2.5 μg) in 100 μl of liposome suspension. Conversion factor for phage Mm5 DNA: 1 DNA molecule = 4.8×10^{-17} g.

molecules, a linear relationship with a slope nearly equal to 1 could be seen throughout the whole range of DNA concentrations assayed (Fig. 3). This result is very similar to those obtained in other systems, such as transfection of *Bacillus subtilis* with the DNA of phage ϕ29, SPO2, or ϕ105 (13) or of *Streptomyces lividans* protoplasts with ϕC31 or VP5 DNA (10), suggesting that transfection can be achieved by single DNA molecules.

Nevertheless, the efficiency (taken as PFU per DNA molecule) showed constant values of 10^{-4} to 10^{-5} PFU per molecule as the DNA molecules/protoplast ratio increased from 0.015 to 75 (Table 1). These data seem to indicate that transfection was mainly limited by a competent subpopulation of protoplasts rather than by DNA concentration.

The true number of transfectants per DNA molecule, as stated before, should be around 1 to 2 orders of magnitude lower than the number of PFU per DNA molecule, i.e., around 10^{-6}, placing the efficiency of the *Micromonospora* sp. strain IMET 8002-Mm5 DNA system close to those found for several different actinomycete transfection systems (4, 5, 10).

Effect of protoplast concentration. As expected, the total number of PFU increased with increasing amounts of protoplasts in the transfection mixtures. However, when the DNA concentration was not limiting (from 7.5 to 75 DNA molecules per protoplast), the efficiency of transfection almost showed a plateau at around 10^{-3} to 10^{-4} PFU per protoplast (Table 1). This fact, again, seems to imply that only a minor subpopulation of protoplasts (probably around 1 in 10^{-5}) was able to be transfected. This value is in accordance with those found by Suárez and Chater (10) in other actinomycete-actinophage transfection systems.

Transfection with DNAs from different phages. In addition to phage Mm5 DNA, we successfully transfected protoplasts of *Micromonospora* sp. strain IMET 8002 with DNAs from phages Mm1, ϕM2, ϕM3, and Mm4. In all cases (Table 2), the efficiencies were closely similar, again supporting the conclusion that the properties of the protoplasts and not of the DNA are the main factors influencing the transfection of *Micromonospora* protoplasts.

Transfection of different hosts. We tried to transfect protoplasts of six *Micromonospora* strains other than *Micromonospora* sp. strain IMET 8002 with phage Mm5 DNA (obtained after propagation of the phage on strain IMET

TABLE 1. Efficiency of transfection of *Micromonospora* sp. strain IMET 8002 protoplasts with phage Mm5 DNA

Sample[a]	No. of DNA molecules/ protoplast	Total PFU	Efficiency	
			PFU/DNA molecule	PFU/ protoplast
1	0.015	2.2×10^3	2.2×10^{-5}	3.2×10^{-7}
2	0.075	1.6×10^4	3.2×10^{-5}	2.3×10^{-6}
3	0.3	9.7×10^4	4.6×10^{-5}	1.4×10^{-5}
4	1.5	1.0×10^5	1.0×10^{-5}	1.5×10^{-5}
5	7.5	1.4×10^6	2.8×10^{-5}	2.1×10^{-4}
6	15	1.3×10^6	2.5×10^{-5}	3.7×10^{-4}
7	37.5	8.9×10^5	1.7×10^{-5}	6.4×10^{-4}
8	75	5.9×10^5	1.1×10^{-5}	8.4×10^{-4}

[a] Protoplasts were obtained from 15-h-old cultures. All transfection mixtures were as described in the text, had the same final volume, and contained 25 μl of protoplast suspension, 100 μl of the DNA-liposome mixture in a volume ratio of 1:5, and 500 μl of 25% PEG (final PEG concentration in the mixture, 20%). All samples were treated as described in the text and incubated in isotonic medium for 15 h before plating. In samples 1 to 5 (inclusive), the number of protoplasts was kept constant (7.0×10^9 protoplasts per sample), while in samples 5 to 8, the constant factor was DNA concentration (5.2×10^{10} DNA molecules per sample).

TABLE 2. Transfection of *Micromonospora* sp. strain IMET 8002 protoplasts with DNAs from different actinophages

DNA[a]	Total PFU	Efficiency (PFU/DNA molecule)
Mm1	1.0×10^7	2.2×10^{-4}
ϕM2	1.0×10^8	8.0×10^{-4}
ϕM3	1.5×10^7	4.3×10^{-4}
Mm4	1.9×10^7	3.6×10^{-4}
Mm5	1.1×10^7	2.1×10^{-4}

[a] Protoplasts (1.2×10^9) were transfected with 2.5 μg of DNA from phage Mm1, ϕM2, ϕM3, Mm4, or Mm5 under the optimized conditions described in the text. One molecule of phage DNA equals 5.5×10^{-17} g (Mm1), 2.0×10^{-17} g (ϕM2), 7.1×10^{-17} g (ϕM3), 4.7×10^{-17} g (Mm4), or 4.8×10^{-17} g (Mm5).

8002). All strains, except for *Micromonospora halophytica* KCC A-0125, allowed phage propagation in normal infection cycles, but the transfection results were negative for five of them (*M. carbonacea* subsp. *carbonacea* KCC A-0139, *M. chalcea* subsp. *izumensis* ATCC 21561, *M. halophytica* KCC A-0125, *M. melanosporea* KCC A-0063, and *M. narashino* KCC A-0129). The production of phage particles was detected after transfection of *M. chalcea* ATCC 12452, but the efficiency was 10^5-fold lower than that in the control strain (2.7×10^1 PFU versus 2.7×10^6 PFU).

These data could indicate an intrinsic impossibility of transfecting some strains, but we think that they rather reflect differences in the physiological status of the protoplasts, since every attempt was made under conditions optimized for *Micromonospora* sp. strain IMET 8002. Hence, this problem could possibly be solved, provided that the major factors influencing transfection, such as culture age, PEG concentration, etc., are optimized for each particular strain.

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