Protoplast Isolation and Regeneration in *Streptomyces clavuligerus*

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The regeneration of streptomycete protoplasts is a major step following genetic manipulations such as fusion and DNA-mediated transformation. Reports of studies on the regeneration of protoplasts from *Streptomyces clavuligerus* are limited and for this reason the experiments described in this paper were carried out. An investigation of protoplast formation and cytology was made to gain further insight into the loss of protoplast viability in osmotically stabilized support media. Protoplasts with the highest regeneration frequency were isolated from mycelium, grown in a two-stage culture system (without glycine), using lysozyme dissolved in a sucrose osmoticum containing 1% bovine serum albumin. The latter promoted improved protoplast viability. A systematic survey was made of the components of regeneration medium R5, previously used for *S. clavuligerus*, and other potentially advantageous components and conditions, in an attempt to raise the regeneration frequency of the protoplasts. An improved regeneration medium (R6) and protocol which supported higher and more consistent levels of regeneration of *S. clavuligerus* protoplasts resulted from these experiments. These improved procedures for protoplast isolation and regeneration proved to be suitable for other streptomycete species.

INTRODUCTION

The Gram-positive mycelial bacterium *Streptomyces clavuligerus* (ATCC 27064), first isolated from South American soil samples (Higgins & Kastner, 1971), produces at least fifteen antibiotics (Hopwood, 1981*a*). One of these, clavulanic acid, is also a β -lactamase inhibitor and is used clinically in combination with semi-synthetic penicillins such as amoxicillin to treat β -lactam-resistant infections (Rolinson, 1984).

While mutation and selection techniques are widely used to generate strains with improved fermentation characteristics, the more recent methods designed to promote recombination, i.e. protoplast fusion (Hopwood *et al.*, 1977) and transformation (Bibb *et al.*, 1978), can be used to complement this process by enabling the construction of strains with raised antibiotic titre. These procedures require the efficient isolation and regeneration of protoplasts, a technology first pioneered in streptomycetes by Sagara *et al.* (1971) and Okanishi *et al.* (1974). Although there are some common features in the protocols for different streptomycetes, different nutritional and physical conditions are needed for the optimal regeneration of quite closely related species (Hopwood, 1981*b*; Illing, 1987).

Protoplast isolation has previously been described in *S. clavuligerus* (Bailey *et al.*, 1984), but the regeneration frequency of these protoplasts was low and very variable (R. T. Rowlands,

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Abbreviation: DAPI, 4,6-diamidino-2-phenylindole.

personal communication). This paper describes experiments to identify conditions for the production of protoplasts with higher viability. The problem was approached from two aspects: first, a study was made of the procedures used to isolate and maintain the protoplasts; and second, an investigation of protoplast cytology was carried out to test the theory that low regeneration frequencies were a consequence of the presence of protoplasts lacking a chromosome.

METHODS

The methods of protoplast manipulation described by Sagara *et al.* (1971), Okanishi *et al.* (1974), Such (1981) and Bailey *et al.* (1984) provided the basis for the development of the R6 protocol for protoplast isolation and regeneration described in this paper.

Production of mycelium and isolation of protoplasts. Spores from fresh cultures of *S. clavuligerus* (see Table 1 for details of strains) grown on minimal medium [M5D⁻; containing (g l⁻¹): dextrin, 10; (NH₄)₂SO₄, 1; K₂HPO₄, 1; MgSO₄ · 7H₂O, 1; NaCl, 1; CaCO₃, 4; agar, 20; trace elements (Bailey *et al.*, 1984), 1 ml; pH 7·2] were inoculated into 250 ml Erlenmeyer flasks containing 25 ml tryptone soya broth (TSB, 40 g l⁻¹) and maltose (10 g l⁻¹). For auxotrophic strains the medium was supplemented with their specific requirements (amino acids 100 µg ml⁻¹, vitamins 10 µg ml⁻¹, nucleic acids 10 µg ml⁻¹). These seed cultures were incubated on a rotary shaker at 200 r.p.m. and 26 °C for 36 h. Then 1 ml of the seed culture was inoculated into a 250 ml Erlenmeyer flask containing a stainless steel spring (about 2 cm in diameter), and 10 ml TSB, 15 ml yeast extract/malt extract/34% (w/v) sucrose (YEME), 1·25 ml 20% (w/v) maltose and 0·75 ml 0·1 M-MgCl₂ (Chater *et al.*, 1982), and incubated as described above for 10–20 h, depending on the strain used. Growth of these cultures was estimated by optical density readings at 600 nm.

The mycelium was harvested by centrifugation (2000 g, 7–10 min) and washed in 20% sucrose; the supernatant was removed and the mycelium resuspended in the lytic medium, which comprised 1·5 ml medium P (Table 2), containing 2 mg lysozyme (Sigma) ml⁻¹ and 1% (w/v) bovine serum albumin (BSA) to stabilize the protoplasts. The BSA was prepared prior to use by filter-sterilization of a 10% solution of BSA fraction V (Sigma) in sterile distilled water.

The mycelium was digested with lysozyme for 30 min at 26 °C in 3–6 ml portions contained in 12 ml glass tubes. Protoplast formation could be followed microscopically using phase-contrast optics. After digestion, further medium P was added to raise the volume of the suspension to 10 ml. After mixing by vortex agitation the protoplasts were filtered through glass wool (Hopwood *et al.*, 1985) and recovered by centrifugation (2000 g, 7–10 min). The pellet was washed twice in medium P and the final protoplast suspension was adjusted to 1×10^7 ml⁻¹. Dilutions of the protoplasts in the osmotic stabilizer, and a 10⁶ dilution of protoplasts in Tween 80 (0·01%), were prepared and 100 µl samples were inoculated onto regeneration medium R6 (Table 3) and M5D⁺ (Bailey *et al.*, 1984), respectively. Protoplasts diluted in Tween and plated on M5D⁺ provided a control for the presence of non-protoplast-derived colony-forming units. The plates were incubated for 14–20 d at 26 °C to ensure that all the regenerating colonies had developed. Conclusions were based on an analysis of results from three replicate plates, each with 50–200 colonies.

Staining procedures to assess protoplast viability. Protoplasts or mycelium were mixed with 20 µl fluorescein diacetate (stock solution 10 mg ml⁻¹ in acetone) and 1 µl propidium iodide (stock solution 1 mg ml⁻¹ in water). The material was observed immediately under UV illumination using SAL 525 and OG 515 filters and oil-immersion optics. Viable protoplasts had a green fluorescence and non-viable protoplasts were red. A number of fields of view were assessed, and the procedure was repeated to obtain consistent results.

Preparation of protoplasts for transmission electron microscopy. Details of the procedure are given by Illing (1987). Briefly, protoplast pellets were fixed in 20% (w/v) sucrose, 2% (v/v) glutaraldehyde in 0·1 M-phosphate buffer, pH 7, then embedded in 2% (w/v) agar in 0·1 M-phosphate buffer, stained with 1% (w/v) osmium tetroxide and post-stained with 2% (w/v) uranyl acetate. The agar strips were dehydrated in an ethanol series. The ethanol was replaced by propylene oxide and the strips finally impregnated with M-Scope resin, which was polymerized at 60 °C overnight. Sections 70 nm thick were cut.

RESULTS

Factors associated with protoplast isolation which affect regeneration

Lysozyme-sensitive mycelium of wild-type *S. clavuligerus* (strain 12-5) was obtained from spores incubated in TSB, with 1% maltose, on an orbital shaker. For some auxotrophic strains it was necessary to use mycelial seed inocula of different ages (24–72 h). In these cases the lag

Table 1. *Strains of S. clavuligerus*

Strain	Relevant genotype*
12-5 (ATCC 27064)	Wild-type
12-7	<i>cysA208</i>
12-8	<i>proA1</i>
12-9	<i>argA1 cysA320</i>
12-11	<i>hypA1</i>
12-12	<i>lysA1</i>
12-125	<i>proA1 rifA1</i>
12-141	<i>hypA1 cysA44</i>

* *arg*, arginine requirement; *cys*, cysteine/homocysteine requirement; *hyp*, hypoxanthine requirement; *lys*, lysine requirement; *pro*, proline requirement; *rif*, rifampicin resistance.

Table 2. *Composition of osmotic support medium P used in the R5 and R6 protocols*

Constituent	Concn (g l ⁻¹) in medium P used for protocol:	
	R5	R6
Sucrose	103.00	200.00
MgCl ₂	2.03	—
K ₂ SO ₄	0.25	0.10
Trace elements* (ml)	2.00	2.00
CaCl ₂ †	3.70	7.00
KH ₂ PO ₄	0.005	—
MOPS (mM, pH 7.2)†	—	10.00
TES (mM, pH 7.2)†	25.00	—
BSA†‡	—	10.00

* Bailey *et al.* (1984).

† Added after sterilization.

‡ Filter sterilized.

Table 3. *Composition of regeneration media R5, R6 and MR6*

Constituent	Concn (g l ⁻¹) in medium:		
	R5	R6	MR6
Sucrose	100.0	200.0	200.0
Dextrin	10.0	10.0	—
Maltose	—	—	10.0
MgCl ₂	5.1	—	—
Casamino acids	1.0	1.0	—
MgSO ₄ ·7H ₂ O	0.05	0.05	0.05
Arginine·HCl	2.5	—	—
Glutamic acid, sodium salt*	—	11.0	11.0
Trace elements† (ml)	1.0	1.0	1.0
KH ₂ PO ₄	0.05	—	—
K ₂ SO ₄	—	0.1	0.1
CaCl ₂ ·2H ₂ O*	3.7	7.0	7.0
TES (0.25 M) (ml)*‡	100.0	—	—
MOPS (0.1 M) (ml)*‡	—	100.0	100.0
Agar§	20.0	20.0	20.0

* Added after sterilization

† Bailey *et al.* (1984).

‡ pH 7.2.

§ Sigma agar in R5 and R6, Difco agar in MR6.

Fig. 1. Comparison of the loss of protoplast viability in osmotic support medium P with (●) and without (▲) 1% BSA.

phase of growth was not observed. Cells harvested from cultures at the end of the exponential phase gave protoplasts with the highest regenerative potential.

Addition of glycine (0.5%) to the TSB/maltose medium increased the lag phase in the growth cycle and also reduced the efficiency of regeneration of protoplasts prepared from the mycelium obtained from 24 h cultures.

The osmoticum (medium P) used to suspend the protoplasts was not specifically investigated. However, improvements made to the regeneration medium were incorporated where appropriate in medium P (Table 2).

Protoplasts lost viability quite rapidly when held in medium P at room temperature (Fig. 1). Supplementation of medium P with BSA, Casamino acids or polyvinyl pyrrolidone was investigated with respect to the maintenance of protoplast viability. BSA fraction V supported the highest protoplast viability and thus improved regeneration frequencies (Fig. 1). However, incorporation of BSA into medium R6 caused a significant reduction in regeneration frequency.

Viability staining of protoplasts and protoplast cytology

Several compounds, including alkaline methylene blue, crystal violet, tetrazolium chloride, malachite (brilliant) green, Evans blue, neutral red, janus green, eosin, phenolindo-2,6-dichlorophenol, trypan blue, propidium iodide and fluorescein diacetate, were investigated as stains to characterize the viability of protoplasts. The dyes were tested as 1% solutions in 20% sucrose, 10 μ l being added to 500 μ l of protoplast suspension. A combination of propidium iodide and fluorescein diacetate gave the most striking contrast; viable protoplasts fluoresced green and non-viable protoplasts were red. Counting had to be carried out within 10 min as the fluorescein leaked out of the cells. The method was of particular value with very small protoplasts, which appeared dark under phase-contrast microscopy and were difficult to count. Viability counts were tenfold higher than regeneration frequencies, determined on the same protoplast preparation. When mycelium was stained in this way, up to 50% of the mycelium stained red, suggesting the presence of non-viable regions.

Fluorescence staining indicated that 75% of *S. clavuligerus* protoplasts were viable, but this high value was not reflected in regeneration frequencies based on colony counts. Protoplasts, fixed in glutaraldehyde, were stained with either 4,6-diamidino-2-phenylindole (DAPI) or ethidium bromide and examined by UV fluorescence microscopy. The dyes were expected to bind exclusively to the DNA, giving discrete regions of fluorescence; however, whole protoplasts exhibited fluorescence. Nevertheless, thin sections of protoplasts observed by transmission electron microscopy (Fig. 2), confirmed that more than 99% of the protoplasts had a nuclear body.

Fig. 2. Thin section, viewed by transmission electron microscopy, of a mature *S. clavuligerus* protoplast exhibiting complete wall removal leaving a cell with a chromosome and a cytoplasm rich in ribosomes. Bar, 0.1 μm .

Table 4. *Protoplast regeneration (%) at different plating densities*

Volume plated (μl)	Plating density (protoplasts ml^{-1}):		
	5×10^3	10^3	10^2
50	9.7	20.8	35.0
100	14.4	20.1	23.0
200	7.3	13.5	17.8

Optimization of regeneration medium and regeneration conditions

Several media described in the literature were tested for their ability to support the regeneration of *S. clavuligerus* protoplasts. None were as good as medium R5 (Bailey *et al.*, 1984) with respect to both regeneration frequency and aerial mycelium formation. However, the low and variable frequencies obtained on this medium indicated that further improvements might be possible, so a systematic study of the requirement for the components of R5 was made. Each ingredient was assessed alongside alternatives and the optimal concentrations of specific nutrients were formulated as regeneration medium R6 (Table 3). Although this medium generally supported regeneration frequencies about twice those in R5, the results were variable from experiment to experiment. Inoculation of protoplasts on to regeneration plates at different densities highlighted the effects of autoinhibition (Table 4). It was also possible to regenerate protoplasts when shaken in liquid R6; however, fragmentation of mycelial clumps prevented quantification. The flasks were shaken at 200 r.p.m. on an orbital shaker; gentler agitation may have improved the regeneration frequency. The regeneration of protoplasts in liquid medium may ensure that the concentration of autoinhibitors remains at a minimum, preventing foci of high autoinhibitor concentrations.

The optimum temperature for both protoplast isolation and regeneration was 26 °C. An increase in regeneration frequency by dehydration of the basal agar layer (cf. Baltz & Matsushima, 1980, 1981) was only apparent when protoplasts were plated at high density, suggesting that autoinhibition of regeneration was reduced.

Table 5. *Regeneration frequencies of protoplasts from auxotrophic strains of S. clavuligerus*

Regeneration frequencies were assessed on plates inoculated at a density of 10^3 protoplasts per plate and incubated for 20 d at 26 °C. NT, Not tested in this experiment.

Strain	Genotype	Medium	Regeneration frequency (%)	
			Expt 1	Expt 2
12-5	Wild-type	R5	2.50	0.9
		R6	5.47	9.9
12-7	<i>cysA208</i>	R5	NT	0.01
		R6	NT	0.20
12-8	<i>proA1</i>	R5	3.15	NT
		R6	7.18	NT
12-9	<i>argA1, cysA320</i>	R5	NT	0.05
		R6	NT	2.30
12-11	<i>hypA1</i>	R5	4.70	NT
		R6	2.60	NT
12-12	<i>lysA1</i>	R5	5.40	NT
		R6	11.50	NT

Table 6. *Regeneration of protoplasts from S. clavuligerus strains 12-125 (proA1 rifA1) and 12-141 (hypA1 cysA44) on MR6*

Strain	Medium	Supplement(s)*	Regeneration frequency (%)
12-125	R6	Pro	11.3
	MR6	Pro	5.0
12-141	R6	Hyp + Hct	14.2
	MR6	Hyp + Hct	5.1

* Pro, proline ($100 \mu\text{g ml}^{-1}$); Hyp, hypoxanthine ($10 \mu\text{g ml}^{-1}$); Hct, homocysteine thiolactone ($75 \mu\text{g ml}^{-1}$).

Comparison of the R5 and R6 regeneration media

The R5 and R6 media were compared using several mutant strains. Protoplasts were suspended in medium P without BSA. The R6 medium generally gave a twofold improvement in regeneration (Table 5). In one case, i.e. strain 12-11, medium R5 appeared to be better for regeneration; however, this was not true for 12-141, a mutant derivative of 12-11 (not shown). When BSA was added to medium P, further improvement in regeneration frequency was observed.

Despite the inferiority of R5 in terms of regeneration frequency the colonies developed more rapidly on this medium and produced spores. In contrast, the colonies produced on R6 formed only moderate aerial mycelium but no spores, probably due to the inclusion of glutamic acid. The problem of non-sporulation was overcome by subculturing strains on M5D^{-R} (composition as for M5D⁻, but with 2.5 g dextrin, 2 g (NH₄)₂SO₄ and 0.25 g K₂HPO₄).

Colonies were visible on the regeneration plates after 5 d incubation. The majority of viable protoplasts had developed as colonies after 14 d. Strain differences were apparent with respect to the rate of development of regenerants and in the overall regeneration frequency.

Minimal regeneration medium

A minimal medium (MR6) was developed from R6 by making a few modifications (Table 3). This medium, with the necessary specific supplements, was tested using two mutant strains 12-125 (*proA1 rifA1*) and 12-141 (*hypA1 cysA44*). Regeneration of the protoplasts was observed but at a reduced frequency (Table 6).

Table 7. *Regeneration frequencies (%) of three Streptomyces spp. on four media*

Medium	<i>S. clavuligerus</i> 12-5	<i>S. coelicolor</i> M124	<i>S. lividans</i> 1326
R2	0.00	5.20	3.67
R2YE	NT	NT	5.02
RM	4.20	NT	7.15
R6	10.60	13.50	8.70
Control*	0.004	0.017	0.001

NT, Not tested.

* The control was a tenfold dilution in Tween 80 inoculated onto R6 to determine the degree of mycelial contamination.

Regeneration of protoplasts from other streptomycetes on R6 medium

Medium R6 was compared with R2 (Okanishi *et al.*, 1974), R2YE (Thompson *et al.*, 1982) and RM (Robinson *et al.*, 1981) for the regeneration of protoplasts from *S. coelicolor* and *S. lividans*: the newly developed R6 proved to be the most effective in each case (Table 7). In one experiment, protoplasts of *S. jumonjinensis* were also found to regenerate on medium R6.

DISCUSSION

Most of the current techniques for genetic manipulation of streptomycetes are dependent upon the efficient isolation and regeneration of protoplasts. Although earlier workers (Such, 1981; Bailey *et al.*, 1984) had developed protoplast manipulation techniques for *S. clavuligerus* the system was not totally satisfactory; protoplast regeneration, in particular, was inconsistent and generally occurred at a low frequency (R. T. Rowlands, personal communication). All three aspects of protoplast manipulation, i.e. isolation, maintenance and regeneration, were identified as being significant to any improvement of the overall procedure (Bailey *et al.*, 1984).

Physiologically active, but non-growing, mycelium was essential for the isolation of protoplasts with a high regeneration potential. This was obtained using a two-stage culture system (Chater *et al.*, 1982; Ogawa *et al.*, 1983). Although specific periods of incubation could be defined for the seed and mycelium development cultures of the wild-type strain, auxotrophic mutants normally required a longer period of incubation. In contrast to other streptomycetes (Sagara *et al.*, 1971; Keller *et al.*, 1983), growth of *S. clavuligerus* in the presence of glycine was inhibitory to protoplast production and regeneration.

In the development of the R6 protocol a number of major changes were made to the medium P used by Bailey *et al.* (1984). These included increases in sucrose and CaCl₂ concentrations, exclusion of MgCl₂ and KH₂PO₄ and reduction of K₂SO₄, all of which reduced regeneration when included in R6. MOPS buffer improved regeneration and was included in place of TES.

Regeneration frequency was generally reduced if protoplasts were held in medium P for long periods (cf. Ochi *et al.*, 1979; Baltz, 1980). Various compounds, including BSA, Casamino acids and polyvinyl pyrrolidone, have been shown to overcome this problem and improve viability of bacterial protoplasts (Zinder & Arndt, 1956; Gabor & Hotchkiss, 1979; Akamatsu & Sekiguchi, 1981; Liu *et al.*, 1985). Addition of 1% BSA, fraction V, to medium P improved the stabilization of *S. clavuligerus* protoplasts over extended periods and also enhanced regeneration frequencies. It appeared that the BSA acted as a physical support, possibly by reducing the effects of shear forces on the protoplasts during manipulation rather than providing additional nutrients for regeneration. Freshly prepared protoplasts always had the highest regeneration frequency; however, storage at -20 °C proved convenient and caused only a small reduction in viability.

As streptomycete protoplasts are small (1.02–5.4 µm diameter; Illing, 1987) enumeration can be difficult. Optical density measurements, as a means of quantification, have been shown to be unreliable (Hitchcock & Katz, 1978). Other workers have used various staining protocols to aid

observation of protoplasts (Kim *et al.*, 1983; Araujo *et al.*, 1984) or more generally to assess viability of micro-organisms (Calich *et al.*, 1978). Staining the protoplasts made counting easier and led to the development of a procedure, based on membrane integrity, to determine the viability of a protoplast preparation. Comparison of viability assessed by staining and regeneration (colony formation) showed a large discrepancy. Matsushima & Baltz (1985) suggested that the overall viability of protoplasts, rather than efficient protoplast regeneration, has a greater effect on the efficiency of protoplast transformation. Viability staining techniques are therefore potentially useful.

The reasons for the low viability of streptomycete protoplasts, determined as regeneration, is not clear. Hopwood *et al.* (1977) suggested that small protoplasts do not have sufficient volume to contain a chromosome and so lack regeneration potential. Oh *et al.* (1980) observed discrete regions of fluorescence in *Streptosporangium viridogriseum* protoplasts stained with bisbenzimid H33258. Similar experiments with *S. clavuligerus* protoplasts stained with DAPI were not so conclusive; however, transmission electron microscopy of thin sections revealed that more than 99% of the protoplasts had a nuclear region. Clearly lack of this structure could not be the cause of the low regeneration frequencies.

Comparison of regeneration medium R6 with others described in the literature demonstrated its utility for species other than *S. clavuligerus*. It may therefore be useful for the culture of protoplasts from interspecific fusion crosses. Even though the R6 protocol improved protoplast isolation and regeneration frequencies it was not possible to obtain the very high frequencies of regeneration observed with other streptomycete strains. This may be due to a need for additional requirements to support protoplasts of *S. clavuligerus*, but may also be explained by intrinsic variation.

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