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Optimal Cultural and Physiological Conditions for Handling *Streptomyces rimosus* Protoplasts

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A general procedure for manipulating protoplasts of three *Streptomyces rimosus* strains was developed. More than 50% regeneration efficiency was obtained by optimizing the osmotic stabilizer concentrations and modifying the plating procedure. Preparation and regeneration of protoplasts were studied by both phase-contrast and electron microscopy. After cell wall degradation with lysozyme, protoplasts about 1,000 to 1,500 nm in diameter appeared. The reversion process exhibited normal and aberrant regeneration of protoplasts to hyphae and to spherical cells, respectively. Spherical cells contained no α,ε-LL-diaminopimelic acid and were colorless or red after Gram staining. They showed consistent stability during at least five subsequent subcultivations. However, the omission of glycine from the precultivation medium reduced the unusual process of regeneration almost completely. After normal protoplast regeneration, the production of oxytetracycline by single isolates was not affected.

Streptomycetes produce most industrially important antibiotics. To improve existing methods of production and to introduce new ones, novel genetic techniques have been recently developed. The most significant advances in *Streptomyces* genetics involve protoplast fusion, facilitating the high frequency of in vivo genetic recombination, and the transformation or transfection of protoplasts by plasmid or actinophage DNA, resulting in the development of in vitro recombinant DNA technology (10). Therefore, procedures for manipulating protoplasts of different species and even of different strains are indispensable. The basic conditions for handling *Streptomyces* protoplasts were largely developed by Okanishi et al. (13) and adapted with minor modifications by many investigators (9).

In this paper, we describe the best procedure for the preparation and regeneration of protoplasts of three different *Streptomyces rimosus* strains and report morphological details of the regeneration process itself. The strains differed in growth kinetics, morphology, and oxytetracycline (OTC) yield and were chosen to develop a general procedure for manipulating their protoplasts. Therefore, typical examples are shown in tables and figures.

MATERIALS AND METHODS

Bacterial strains. Mutants of three different strains of *S. rimosus* were used: ATCC 10970 (R7) 252 (*his-4 pro-1 tyr-1*) and soil isolates R6 750 (*ade-37*) and R8 1322 (*his-11 arg-69*) from the culture collection of the Faculty of Food and Biotechnology, University of Zagreb. For simplicity these mutants are referred to throughout the text as R6, R7, and R8.

S. rimosus strains R7 and R8 grow well with abundant sporulation and form pale yellowish brown (R7) and reddish brown (R8) pigments. Strain R6 forms compact dark brown colonies and sporulates with much less intensity.

When cultivated in the same media, the strains show different growth kinetics. Although the difference in growth kinetics is not significant in the lag and stationary phases, it is pronounced in the exponential growth phase. Strains R7 and R8 grow faster and attain stationary phase earlier than does strain R6.

Strains R7 and R8 have very low antibiotic activity, with relative productions of 0.12 and 0.01, respectively, where 1.00 is the activity of strain R6.

Growth of mycelia and protoplast preparation. Growth of mycelia for protoplast preparation was determined by cultivating *S. rimosus* strains in liquid medium (1% inoculum) on a rotary shaker (220 rpm) at 28°C. The number of colony-forming units (CFU) was estimated at 6-h intervals. The strains were cultivated in medium S (13) with the addition of glycine at concentrations of 1% (wt/vol) for strain R6, 2.5% (wt/vol) for R7, and 3% (wt/vol) for R8, or in complete precultivation medium consisting of (grams per liter of de-ionized water): tryptic soy broth (Difco Laboratories), 30.0; yeast extract (Difco), 10.0; sucrose, 103.0; $MgCl_2 \cdot 6H_2O$, 10.12. After autoclaving, 10 ml of $CaCl_2 \cdot 2H_2O$ (3.68%) was added. The mycelia were harvested by centrifugation and were washed with a solution of 0.3 M sucrose.

For the preparation of *S. rimosus* protoplasts the following procedure was used. Washed mycelia were incubated in medium P (13) with the addition of 2 mg

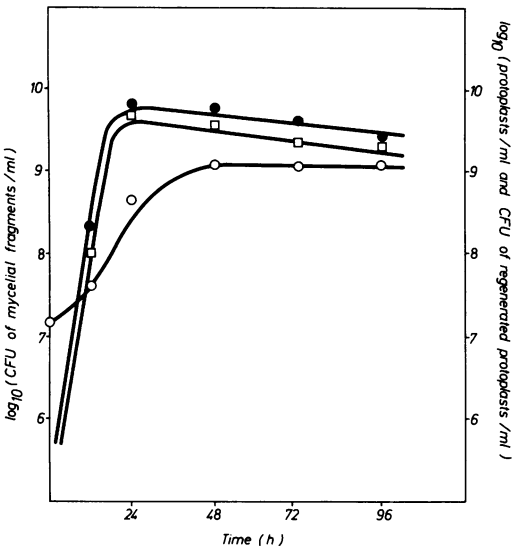

FIG. 1. Effect of *S. rimosus* R7 growth phase (○) on the formation (●) and regeneration (□) of protoplasts.

of lysozyme (Serva) per ml at 28°C for 1 to 2 h. After the incubation, medium P was added, and the suspension was pipetted up and down several times. Thoroughly homogenized protoplasts were washed three times with medium P by centrifugation at 1,000 × *g* for 5 min. The number of protoplasts was determined with a hemacytometer.

Leakage from protoplasts during preparation. Leakage from protoplasts was determined by the method of Okanishi et al. (13).

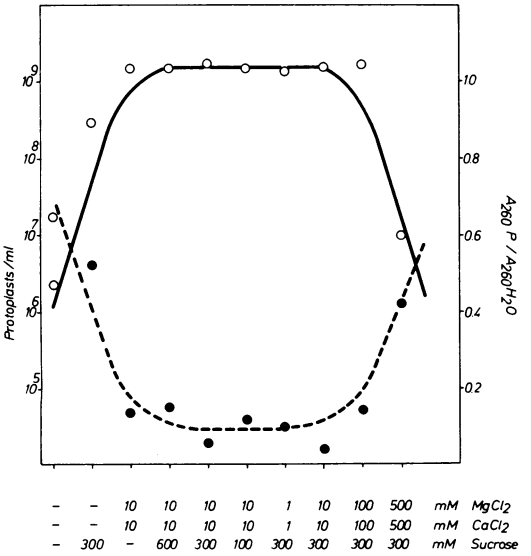

FIG. 2. Effect of osmotic stabilizers on the formation of *S. rimosus* R7 protoplasts expressed as number of protoplasts counted (○) or equivalent of bursting measured as a decrease in light absorbance at 260 nm (A_{260}; ●).

TABLE 1. Effect of Casamino Acids and gelatin on regeneration of *S. rimosus* R7 protoplasts

Added to regeneration medium		Protoplasts per ml	CFU/ml on regeneration medium	Regeneration (%)
Substance	Amt (%)			
None		6.0×10^8	1.2×10^7	2.0
Casamino Acids	0.1	2.0×10^9	3.4×10^7	1.7
	0.5	1.0×10^9	2.0×10^7	2.0
	1.0	1.6×10^9	3.0×10^7	1.9
Gelatin	0.1	4.0×10^9	6.3×10^7	1.6
	0.5	2.5×10^9	1.5×10^8	6.0
	1.0	2.5×10^9	2.5×10^8	10.0

Reversion of protoplasts. Purified protoplasts were diluted in medium P and plated on regeneration media for viable counts to determine cell reversion. They were plated either by being spread with a glass rod or by being embedded in a soft agar overlayer. The plates were incubated at 28°C for 72 h. Growing colonies were counted, and the reversion rate was expressed as a percentage of the total number of inoculated protoplasts. Regeneration of protoplasts was monitored on complete regeneration medium, i.e., complete precultivation medium solidified by the addition of 2% (wt/vol) agar (Difco). Minimal regeneration medium was the synthetic medium described by Alačević et al. (1) supplemented with (grams per liter): gelatin, 1; sucrose, 103; $MgCl_2 \cdot 6H_2O$, 10.12. After autoclaving, 10 ml of $CaCl_2 \cdot 2H_2O$ (3.68%) was added. Soft agar of complete and minimal media contained 0.6% agar.

Colony formation from nonprotoplasted units was determined by diluting protoplasts in water and plating on nonhypertonic minimal medium (1).

Microphotography. Morphology of the reverting protoplasts was observed by taking photographs in a Leitz Wetzlar microscope with phase contrast illumination. The samples were studied by in situ microphotography at high magnification (×1,280) as described by Hadlaczky et al. (8).

Electron microscopy. For examination by electron microscope, the biological material was fixed in 1% glutaraldehyde and postfixed in 1% OsO_4. After dehydration, it was embedded in Araldite. Sections were cut on a Reichert Om U2 ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a

TABLE 2. Effect of plating procedure on regeneration of *S. rimosus* R7 protoplasts[a]

Plating procedure	CFU/ml on regeneration medium	Regeneration (%)
Spreading with glass rod	2.4×10^8	9.5
Overlaying with agar:		
0.1%	2.2×10^8	8.6
0.3%	8.0×10^8	32.0
0.6%	1.4×10^9	56.0

[a] Protoplast suspension contained 2.5×10^9 protoplasts per ml.

TABLE 3. Formation and regeneration of *S. rimosus* protoplasts

Strain	Protoplasts per ml in:		Absorbance ratio[a]	Mycelial fragments per ml in:		Regeneration of protoplasts		
						CFU/ml in:		Frequency (%)
	Medium P	Water		Medium P	Water	Medium P	Water	
R6	1.3×10^9	1.0×10^7	0.08	5.0×10^6	5.0×10^6	8.0×10^8	1.6×10^5	62
R7	4.0×10^9	2.6×10^7	0.05	7.2×10^6	8.2×10^6	3.0×10^9	5.6×10^6	75
R8	3.6×10^9	2.8×10^7	0.07	5.0×10^6	5.0×10^6	2.0×10^9	2.1×10^4	56

[a] Ratio of light absorbance at 260 nm in the supernatant fluid of protoplasts suspended in protoplasting medium P to absorbance in the supernatant fluid of protoplasts suspended in water.

Siemens Elmiskop I microscope.

α,ε-LL-Diaminopimelic acid assay. The assay of α,ε-LL-diaminopimelic acid for mycelia, protoplasts, and spherical cells was performed as described by Bošnjak et al. (5).

Determination of OTC concentration. The production of OTC was determined in cultures of nonprotoplasted and protoplasted *S. rimosus* R6 colonies by the method of Vešligaj et al. (18). The relative OTC yield was calculated in relation to production in the nonprotoplasted culture.

RESULTS AND DISCUSSION

Optimal conditions for protoplast formation and regeneration. Previous experiments have shown that effective formation of protoplasts can be accomplished by lysing diffused filamentous mycelia. This was achieved by growing mycelia in the presence of subinhibitory concentrations of glycine (11, 13, 15). For *S. rimosus* strains R6, R7, and R8, the optimal formation of protoplasts was obtained by growing mycelia in medium S (13) in the presence of 1 to 3% glycine, giving 40 to 80% survivors. However, considerable clumping and pelleting occurred and could not be completely eliminated. In addition, colonies obtained after the regeneration of protoplasts prepared from glycine-containing liquid medium exhibited aberrant morphology, which will be discussed below. For these reasons, mycelia were prepared by precultivation of hyphal fragments in complete precultivation medium rather than in glycine-containing medium S, which appeared to give uniform filamentous growth irrespective of the strain used. It was observed that for most species the presence of glycine was not crucial for good protoplast formation when mycelia were grown in various complex media different from medium S. This was presumably due to differences in the amounts of glycine they contained and probably to concentrations of other materials that might affect the susceptibility of the peptidoglycan to lysozyme (9).

There have been several reports correlating the mycelial growth phase with the efficiency of protoplast preparation and regeneration. Rodicio et al. (14) found that 3-h-old germ tubes for some species yield protoplasts even without glycine. For the transfection of *Streptomyces parvulus* and *Streptomyces albus*, protoplasts have to be made from very young mycelia (17), whereas for optimum frequencies of plasmid transformation in *Streptomyces lividans*, protoplasts prepared from mycelia taken from an intermediate period in the growth cycle are required (9). Baltz (2) reported a critical transition stage between exponential and stationary growth for the preparation and regeneration of *Streptomyces fradiae* and *Streptomyces griseofuscus* protoplasts.

The effect of the growth phase on protoplast formation and regeneration of *S. rimosus* is presented in Fig. 1. During the first 6 h of growth, very few protoplasts were formed, even with prolonged lysozyme treatment. After that time protoplasts could be readily obtained and cell walls could be regenerated during both the exponential and stationary phases. Therefore, it was possible to prepare protoplasts that were capable of good regeneration for use in fusion (D. Hranueli et al., manuscript in preparation) from all but the lag phase.

Okanishi et al. (13) described suitable osmotically stabilizing solutions for handling mycelia during protoplast formation and developed osmotically stabilizing agar media for the regeneration of *Streptomyces* protoplasts. To examine the effect of osmotic stabilizers on preparation and storage, the stability of protoplasts from strains R6, R7, and R8 was tested in a basal solution of medium P (13) containing different concentrations of Ca^{2+}, Mg^{2+}, and sucrose. The results are expressed as numbers of protoplasts counted, or an equivalent of bursting measured as a decrease in light absorbance at 260 nm (Fig. 2). It appears that the concentration range of osmotic stabilizers sufficient for maintaining protoplast stability is rather broad. More than 50% of the protoplasts were stable in the solutions which contained 1 to 100 mM Ca^{2+} and Mg^{2+}. With no salt or with an excess of salts (500 mM Ca^{2+} and Mg^{2+}), rapid lysis of protoplasts occurred. The effect of sucrose in the concentrations of 100 to 300 mM did not have

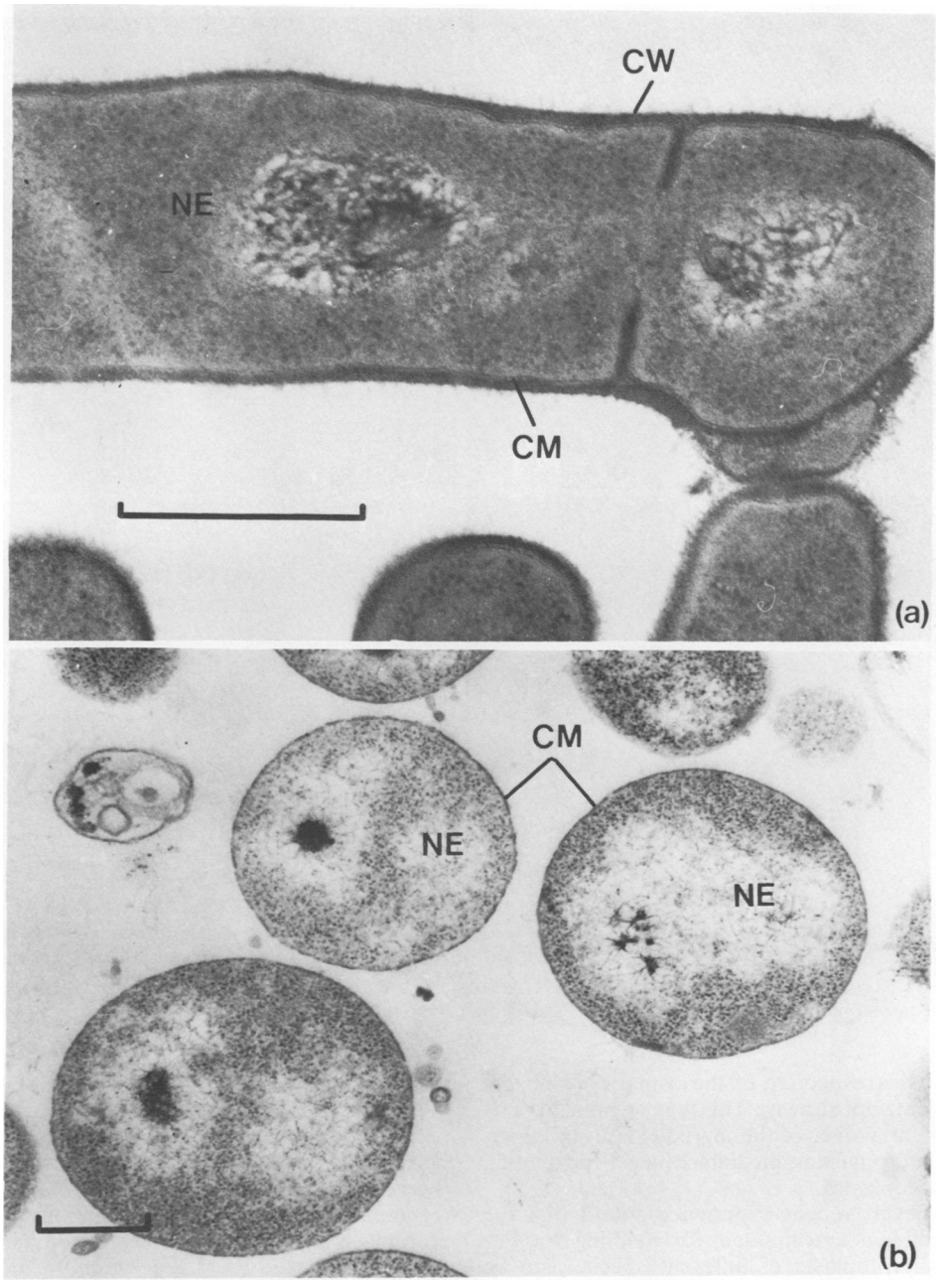

FIG. 3. Electron microphotographs of ultrathin sections of *S. rimosus* R7 hyphae (a) and protoplasts (b). CM, Cytoplasmic membrane; CW, cell wall; NE, nuclear equivalent; bars, 500 nm.

such dramatic consequences. However, sucrose enhances protoplast stability in media lacking Ca^{2+} and Mg^{2+}, so it was not omitted from solutions containing the salts. Therefore, protoplasting medium P (13) was adopted and used throughout experiments.

Since the successful regeneration of protoplasts depends on the composition of the regeneration media, complete regeneration medium and minimal regeneration medium were developed. They were based on media routinely used in genetic work with *S. rimosus* strains, supplemented with osmotic stabilizers. For that purpose, various concentrations of Ca^{2+}, Mg^{2+}, and sucrose were tested. From the results it was obvious that 1 to 3% regeneration could be

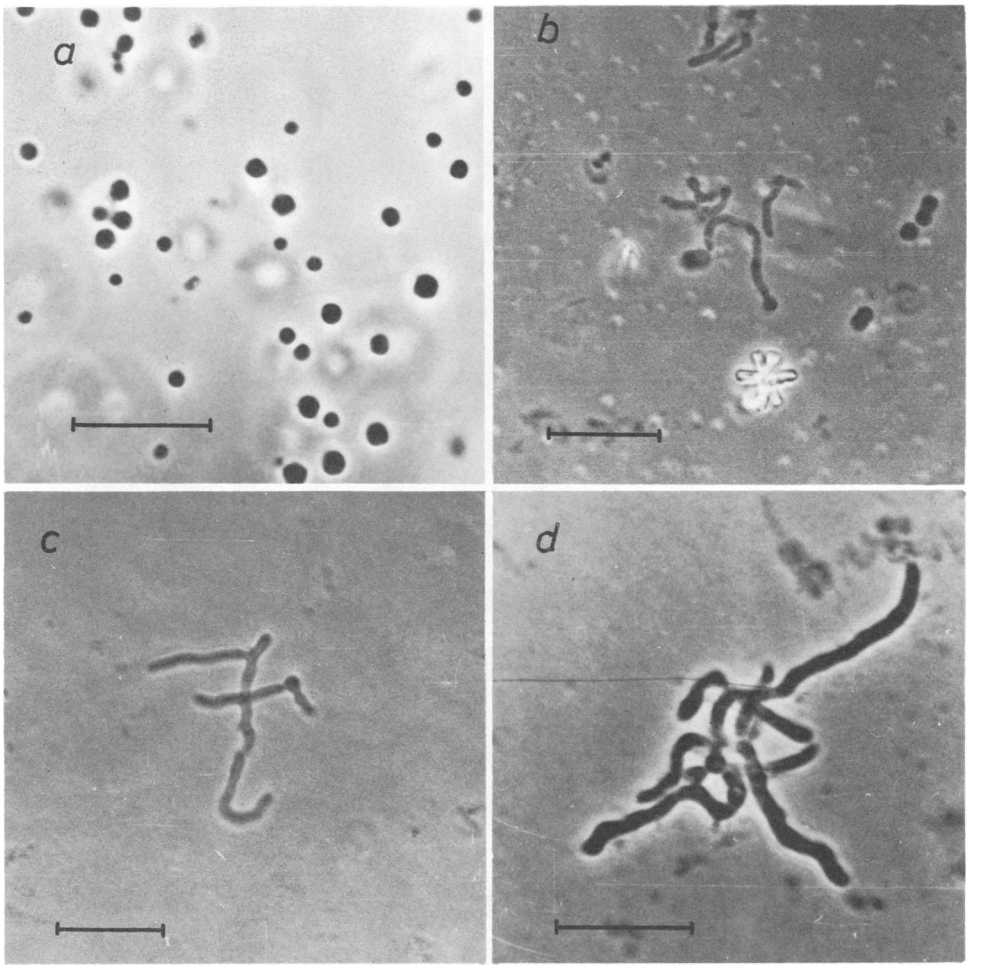

FIG. 4. Regeneration process of *S. rimosus* R7 protoplasts on thin agar blocks of regeneration medium under phase-contrast microscopy after incubation for 18 h (a), 26 h (b, c), and 48 h (d). Bars, 10 μm.

obtained irrespective of the osmotic stabilizers used (data not shown). This was presumably due to the carry-over of the ingredients from hypertonic protoplasting medium P, used for diluting the protoplasts.

However, recent experience shows that the basic biological techniques for handling *Streptomyces* protoplasts of different species can be improved considerably. It has been shown that the addition of amino acids or proteins to a regeneration environment can lead to substantial improvement and reproducibility (6). Therefore, the influence of Casamino Acids and gelatin in regeneration media were tested. An example of this series of experiments is shown in Table 1. The presence of gelatin improved the regeneration yield by three- to fivefold. The presence of Casamino Acids exhibited no effect. Besides the optimization of medium components, the choice of physiological conditions can also be important for regenerating protoplasts. For example, dehydration of the regeneration plates, the temperature at which mycelia are grown and protoplasts are regenerated (3), and modification of the plating procedure (12, 16) can improve the regeneration efficiency. Embedding *S. rimosus* protoplasts in soft agar overlayer on hypertonic medium improved the regeneration yield an additional fivefold. This was achieved by overlaying the protoplasts with 0.6% soft agar instead of spreading them with a glass rod (Table 2). By using this procedure, it is possible to obtain regeneration frequencies of *S. rimosus* protoplasts from 40 to 80%, depending on the strain used.

A typical experiment showing the results of protoplast formation and regeneration is presented in Table 3. It is evident that during protoplast formation, a certain fraction of mycelial fragments always remains after lysozyme

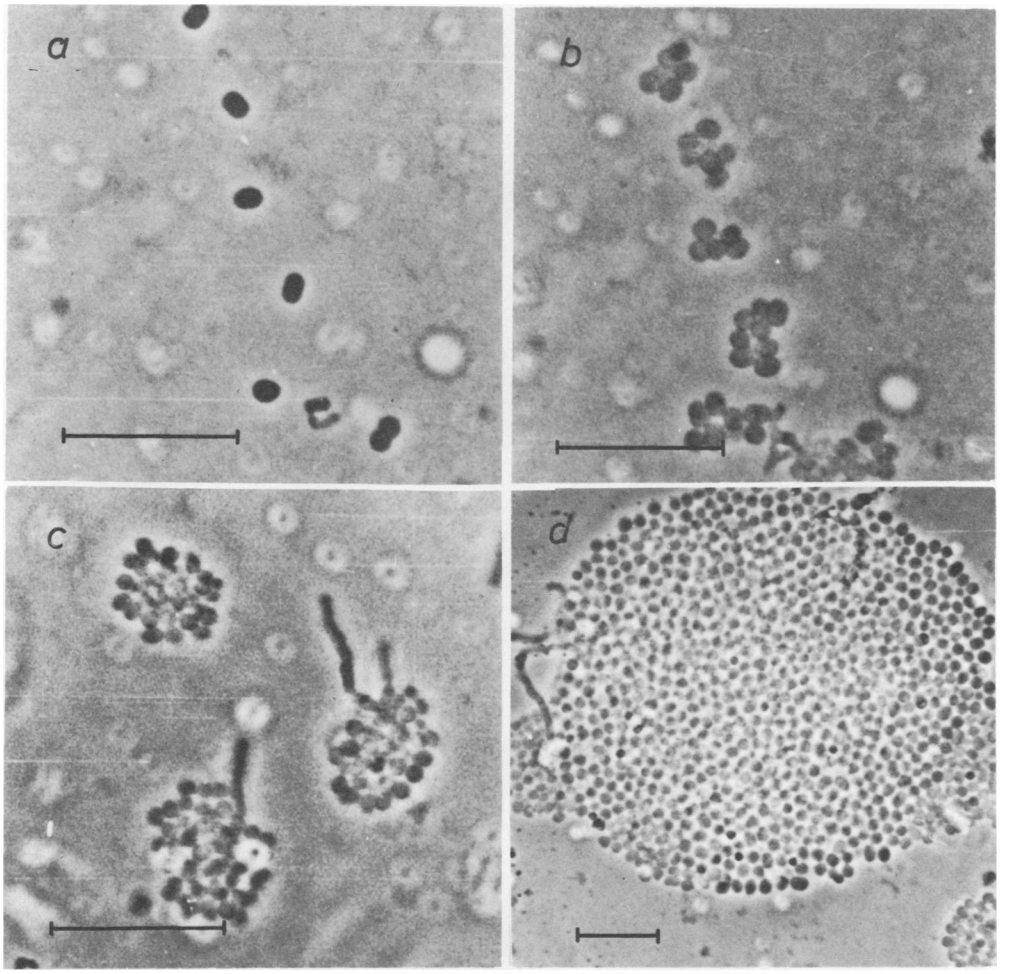

FIG. 5. Unusual sequence of regeneration of *S. rimosus* R7 protoplasts. (a) Dividing protoplasts at 26 h; (b) morula-like clusters at 36 h; (c) regeneration of individual apparent protoplasts in morula-like clusters at 48 h; (d) microcolony consisting of spherical cells at 96 h. Bars, 10 μm.

treatment. The size of this fraction varies from experiment to experiment and can influence the estimation of regeneration frequencies. It was observed that colonies from nonprotoplasted units appeared before those originating from regenerated protoplasts (data not shown). After successful protoplast regeneration, the fraction of nonprotoplasted units represented 0.01 to 0.1% of the entire population. However, the autoinhibition of protoplast regeneration by nonprotoplasted units (2, 11) was not observed.

Morphological studies of protoplast formation and regeneration. Cell wall lytic enzymes such as lysozyme are able to hydrolyze the cell walls of *Streptomyces* mycelia. The effect of lysozyme on *S. rimosus* R7 cell walls was studied in thin sections of intact mycelia (Fig. 3a). After the extrusion of mesosomal content, the progressive degradation of cell walls took place. The cell wall layer, 23 ± 2.0 nm thick, became thinner and more relaxed until it disappeared completely, leaving spherical units surrounded by cytoplasmic membrane. These units are considered to be protoplasts (Fig. 3b). *S. rimosus* R7 protoplasts did not contain α,ε-LL-diaminopimelic acid and were nearly the same size, about 1,000 to 1,500 nm in diameter. Similar protoplast sizes have already been reported for *Streptomyces griseus* (13) and *Streptomyces hygroscopicus* (7) (diameters, 1,000 to 2,000 nm).

The regeneration of protoplasts was studied by phase-contrast microscopy on thin agar blocks of regeneration medium. During the first 24 h, the picture was dominated by protoplasts which were not changed in shape or size (Fig. 4a). The swelling of protoplasts over 10-fold reported by Okanishi et al. (13) was never observed. After 20 to 24 h of incubation, most of

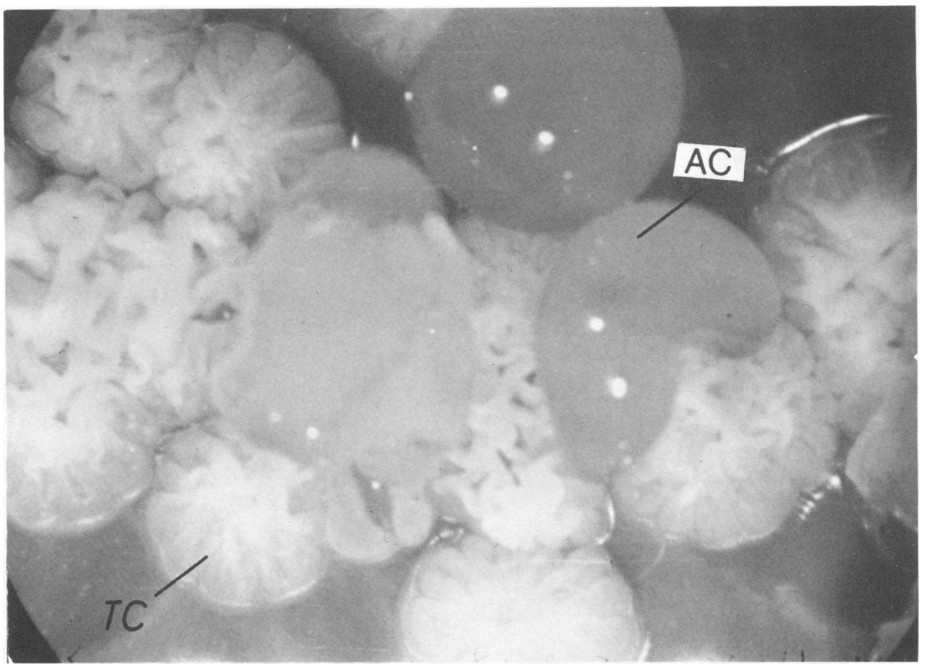

FIG. 6. Morphology of typical and atypical colonies obtained after regeneration of *S. rimosus* R7 protoplasts. TC, Typical colony consisting of mycelial growth; AC, atypical eubacterium-like colony. Magnification, ×5.

the protoplasts regenerated filamentous hyphae, sometimes with more than one protrusion from each (Fig. 4b and c). Extensive branching of hyphae surrounding the initial protoplasts was observed during the next 24 h (Fig. 4d).

Occasionally, an alternative and unusual se-

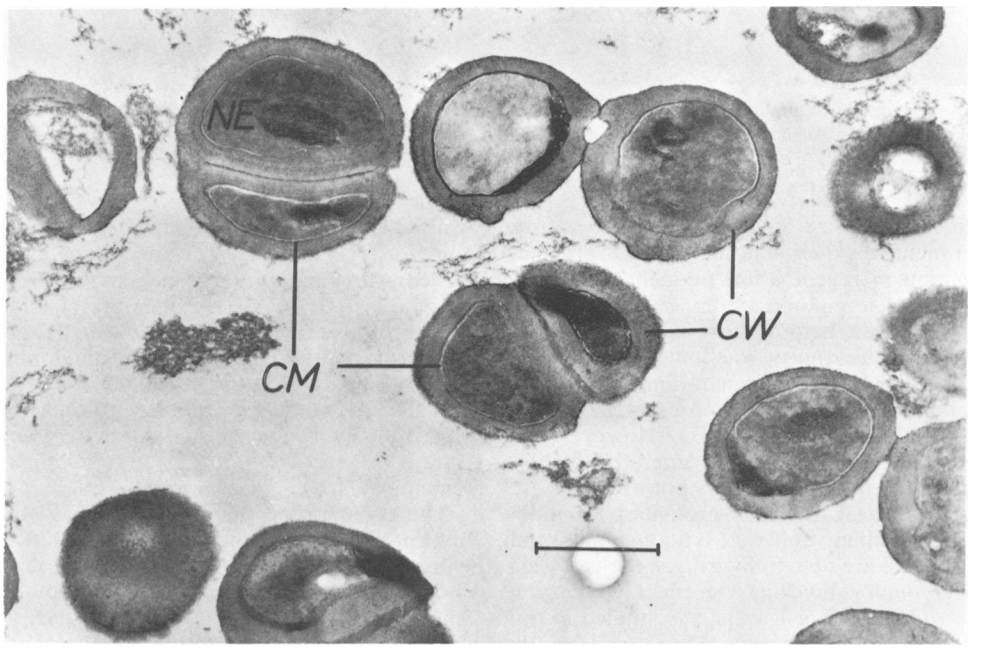

FIG. 7. Electron microphotograph of cross section of spherical cells obtained after unusual regeneration of *S. rimosus* R7 protoplasts. CM, Cytoplasmic membrane; CW, cell wall; NE, nuclear equivalent. Bar, 500 nm.

TABLE 4. Relative OTC yields of nonprotoplasted colonies (R6) and colonies isolated after protoplast regeneration (R6p) on submerged cultivation

Colony	Relative OTC yield
R6-1	1.00
R6-2	0.98
R6-3	1.02
R6p-1	0.93
R6p-2	1.00
R6p-3	0.93
R6p-4	1.02
R6p-5	1.07
R6p-6	0.92
R6p-7	1.04
R6p-8	0.95
R6p-9	0.98
R6p-10	1.05

quence of events took place. After 24 h of incubation, division of apparent protoplasts had occurred (Fig. 5a). The division proceeded, forming morula-like clusters (Fig. 5b). During division, individual protoplasts in some clusters successfully regenerated hyphae (Fig. 5c), giving rise to colonies consisting of a mixture of hyphae and spherical cells. However, the apparent protoplasts in most clusters continued to divide, producing colonies consisting of spherical cells only (Fig. 5d). A similar sequence of events has been observed in studies of the reversion processes of protoplasts of *Bacillus megaterium* (8) and *Brevibacterium flavum* (12). The morphological diversity of the reversion sequence may depend upon many physiological and environmental factors, and we could not direct the sequence entirely at will. However, the colonies consisting of spherical cells retained this property during at least five subsequent subcultivations. It was observed that the omission of glycine from precultivation media reduced the unusual process of regeneration almost completely. Glycine is known as an inhibitor of murein biosynthesis and of regeneration of the protoplast-type L-forms of streptomycetes (4). Therefore, it could prevent regeneration with normal filamentously growing hyphae.

Colonies consisting of spherical cells exhibited atypical eubacterium-like morphology. They were soft, smooth, and translucent, in contrast to the hard, rough, and turbid colonies of mycelial growth (Fig. 6). Baudler and Gumpert (4) have shown that on agar media, L-form cells of *S. hygroscopicus* grow as characteristic fried egg colonies with dark centers and brighter peripheries. Electron microscopic investigations revealed that L-form cells have no cell wall structure and are surrounded only by the cytoplasmic membrane. However, the electron microscopy of samples taken from atypical *S. rimosus* R7 colonies showed spherical cells with thick cell walls ranging from 67.5 to 95.5 nm (Fig. 7). Spherical cells contained no α,ε-LL-diaminopimelic acid and were colorless or red after Gram staining. The origin of spherical cells isolated after unusual regeneration of protoplasts was confirmed by genetic analysis. Histidine, proline, and tyrosine requirements were assayed by auxonography. Colonies of spherical cells retained the parental requirements present in the original strain.

The consistency of OTC productivity after preparation and regeneration of *S. rimosus* R6 protoplasts was tested. The production of 10 typical regenerated colonies was assayed on submerged cultivation (Table 4). The relative production varies from 0.92 to 1.07, which is within the range of natural variation of OTC production by random isolates. Since the procedure for the formation and regeneration of *S. rimosus* protoplasts had not changed the ability of the organism to produce OTC, it could be successfully applied during the breeding of these industrially important strains.

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