CURRENT MICROBIOLOGY Vol. 23 (1991), pp. 265–269

Current Microbiology

Formation, Regeneration, and Fusion of Protoplasts in a *Cellulomonas* Strain

Hilda Rodriguez,[1] Boris Garcia,[1] Odelsa Ancheta,[2] and M. Sipiczki[3]

[1]Department of Microbiology, Cuban Research Institute of Sugarcane By-products, Havana, Cuba; [2]National Center of Scientific Research, Havana, Cuba; and [3]Department of Genetics, L.K. University, Debrecen, Hungary

Abstract. The effect of different conditions on protoplast formation was studied in the streptomycin-resistant strain *Cellulomonas* sp. *M32Bo*. The greatest efficiency (75% protoplasts) was achieved by use of 0.5 *M* sodium succinate as osmotic stabilizer, supplemented with 20 m*M* $MgCl_2$, 200 μg/ml of lysozyme, and 0.01 *M* EDTA at pH 7.4. Cells harvested at the mid-exponential growth phase were more suitable for protoplast formation than those of the stationary phase. Electron microscopy observations showed the presence of both protoplasts and spheroplasts in the treated samples, some of them still showing a rod shape. Two regeneration media were developed that showed similar regeneration frequencies (52%). Strain *M32Bo* was fused with a tetracycline-resistant strain (*Cellulomonas* sp. Sz). Segregation analysis of fusant colonies suggested the existence of a temporary diploid stage in which both parental genotypes were expressed.

Protoplast fusion provides a means of genetic recombination and is a promising technique in the improvement of industrially important microorganisms. Improvements in dehydrodivanillin degradation [4], lignin degradation [13], and cellulase production [18] have already been achieved by these methods in different microorganisms. For cellulolytic microorganisms it seems particularly attractive because of the supposed multigenic nature of their polysaccharide degradation systems (cellulases and xylanases).

The possibility of protoplast formation, regeneration, and fusion has been demonstrated in *Cellulomonas* [7, 9, 10]. However, the methods of formation and regeneration of protoplasts used in the various laboratories varied markedly, and no genetic analysis of the fusants has been reported. In this paper studies on the formation, regeneration, and fusion of protoplasts of *Cellulomonas* strains developed for single cell protein production are presented.

Materials and Methods

Strains. *Cellulomonas* sp *M32Bo* is a streptomycin-resistant mutant obtained from a hypercellulolytic derepressed mutant [1]. *Cellulomonas* sp. Sz is a tetracyclin-resistant strain obtained from the Pasteur Institute (Paris, France).

Media and culture conditions. The strains were maintained on nutrient agar (NA) [1]. Cultures were grown in a rotatory shaker at 100 rpm and 32°C with saline MO medium [14] and 1% cellobiose as the carbon source. Hypertonic medium for protoplast formation contained 0.5 *M* sodium succinate or 0.5 *M* sucrose as osmotic stabilizer, 20 m*M* $MgCl_2$, 0.05 *M* Tris-hydroxymethyl-aminomethane-HCl (Tris-HCl), and lysozyme. For the regeneration of protoplasts two different media were used: RM1, a lower layer containing MO saline plus 1.5% agar and an upper layer composed of 0.5 *M* sucrose, 20 m*M* $MgCl_2$, 0.2% walseth cellulose, and 0.8% agar; RM2, a lower layer of nutrient agar (NA) and an upper layer containing 0.5 *M* sucrose, 20 m*M* $MgCl_2$, and 0.8% agar. The effect of supplementation with caseine and that of reduced sucrose concentrations on regeneration was tested in RM1. RM2 supplemented with 500 μg/ml streptomycin and 5 μg/ml tetracycline in the upper layer was used as the selective medium for fusant strains.

Protoplast formation and regeneration. Samples taken from cellobiose cultures at middle and late exponential growth phase were centrifuged at 10,000 *g* for 15 min and washed with 0.9% NaCl. The pellet was resuspended in the hypertonic medium with lysozyme and incubated at 32°C without shaking. The EDTA was added according to the method of Weiss [19]. The protoplast formation was monitored by microscopic observations and by osmotic shock. The sample was diluted tenfold in distilled water and in hypertonic medium simultaneously, and the optical density of the suspension was measured after different time intervals. The efficiency of protoplast formation was calculated from the difference between the optical density (OD) in the hypertonic medium and the OD in distilled water.

After treatment with lysozyme the protoplasts were harvested by centrifugation at 10,000 *g* for 15 min. The pellet was

Address reprint requests to: Dr. M. Sipiczki, Department of Genetics, L.K. University, P.O. Box 56, H-4010, Debrecen, Hungary.

resuspended in an equal volume of hypertonic medium without lysozyme, diluted with the same medium, and spread on or imbedded in the regeneration medium RM1 and the nutrient agar. Colonies were counted after 6 days. The regeneration frequency was calculated from the difference between the total colony counts on the regeneration medium and those on NA (osmotically resistant cells).

Fusion of protoplasts. Equal amounts (1.5 ml) of each protoplast suspension were gently mixed and centrifuged at 10,000 *g* for 15 min. The pellet was resuspended in 0.3 ml of hypertonic medium without lysozyme. Then 2.7 ml of 55% PEG solution was added with gentle mixing to the suspension. The mixture was incubated for 10 min at 30°C and then plated on the selective medium RM2. Samples of the suspension without PEG treatment were also plated on the selective medium as controls. Fusion frequency was taken as the ratio of the number of colonies grown on the selective medium (with both antibiotics) to the number of protoplasts mixed from each strain.

Single colony analysis. One of the fusant colonies obtained was suspended in 0.9% NaCl, diluted, and plated on NA without antibiotic. After 72 h of incubation individual colonies were picked and streaked on NA plates supplemented as required to test their phenotypes (antibiotic concentrations were: streptomycin, 500 μg/ml; and tetracycline, 5 μg/ml). One colony of the progeny showing the same phenotype as the original fusant colony was again picked and submitted to the same procedure.

Transmission electron microscopy. Samples were fixed in Riter-Kellenberger fixer [15] processed by the classic procedure of electron microscopy and imbedded in Spurr resin [17].

Results and Discussion

Sucrose, the osmotic stabilizer in the Gokhale medium [7], was found to induce cell agglutination in our strain, thus precluding the monitoring of protoplast formation by turbidimetry. Further, this made it difficult to collect the protoplasts by centrifugation. Its substitution by 0.5 *M* sodium succinate eliminated both difficulties, so we used sodium succinate instead of sucrose as osmotic stabilizer in all the following experiments.

For further improvement of protoplast formation we supplemented the medium with 0.01 *M* EDTA. This agent is known to act by the removal of the LPS layer of the Gram-negative bacteria, thus making the peptidoglycan sensitive to the lysozyme attack [6]. Although *Cellulomonas* is considered to be Gram positive [2], a stimulating effect of EDTA in its protoplasting has been observed [10]. In our strain a similar effect was detected: 0.01 *M* EDTA increased the efficiency of protoplast formation by a factor of two (Table 1). The probable effect of pH was also assayed. With 0.05 *M* Tris-HCl, pH 7.4 allowed the highest efficiency. All the subsequent experiments were done at this pH and in the presence of 0.01 *M* EDTA.

Table 1. Effect of EDTA and time of incubation on protoplast formation[a]

Type of treatment	Time of incubation (min)	Efficiency of protoplasting (%)
Without EDTA	180	25
With EDTA	180	51
With EDTA	120	63
With EDTA	90	70
With EDTA	60	74
With EDTA	30	75

[a] Lysozyme concentration, 200 μg/ml.

The optimal duration of treatment was also investigated. As seen in Table 1, the highest efficiency was obtained at 30 min. Longer incubation times did not increase the efficiency. Moreover, after 90 min a gradual reduction of the proportion of protoplasts was observed. This was probably owing to a gradual lysis of protoplasts, as a similar decrease was also detected in samples transferred to the hypertonic medium without lysozyme (Fig. 1). Thus, the longer treatment seems to threaten the stability of protoplasts.

Although protoplast formation was evident from the osmotic shock procedure, the phase contrast observations were doubtful, since no marked differences could be observed between the treated and the untreated samples. This was probably due to the pleomorphism of *Cellulomonas*, which can show bacillary and round cell shapes in the same culture [2].

Observations of the treated samples by electron microscopy (Fig. 2) showed that the lysozyme was indeed acting on the cells, but its action was not complete in many cases. Furthermore, besides round protoplasts completely depleted of their cell wall, rod-shaped cells with partially degraded or even completely degraded cell walls were also observed.

Cells harvested at the middle exponential phase were more susceptible to the action of lysozyme than those from the late exponential growth phase. This is in accordance with the results of Kim and Lee [9] obtained in another *Cellulomonas* strain. The efficiency of protoplast formation increased with the increase of the lysozyme concentration (Table 2).

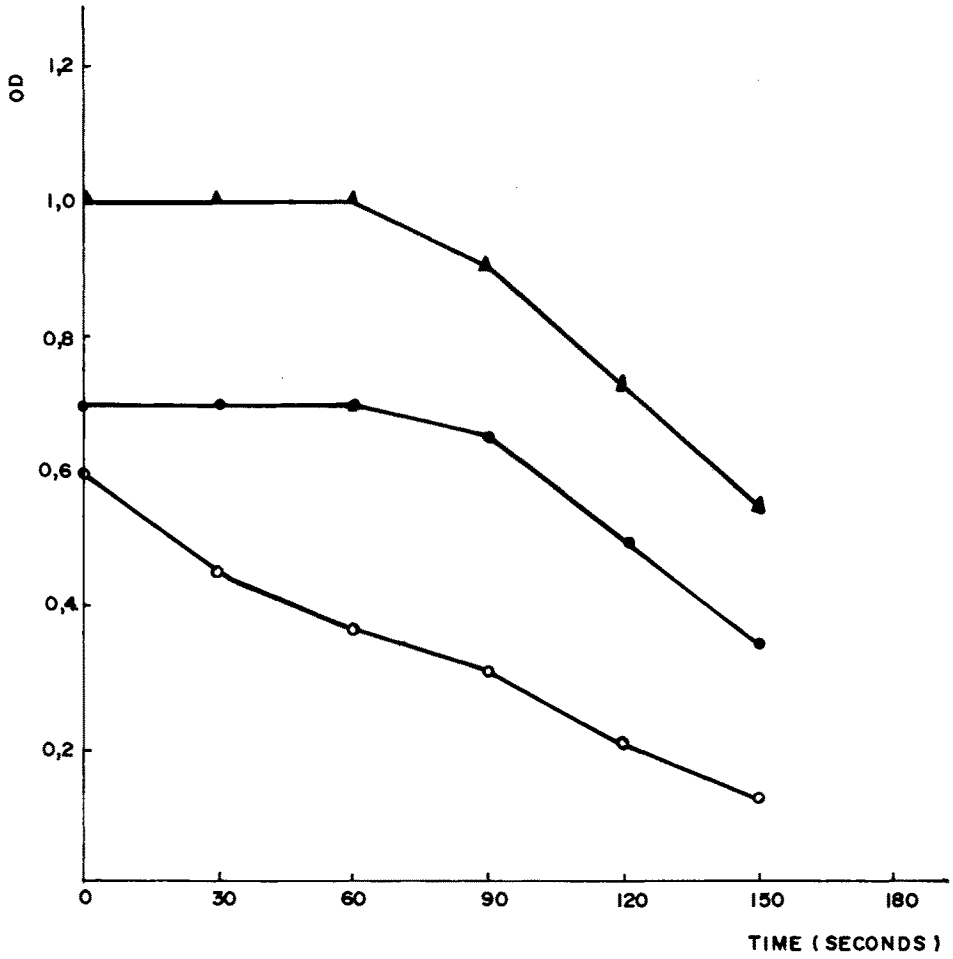

Fig. 1. Stability of protoplasts of *Cellulomonas* sp. *M32Bo* after dilution in hypertonic medium and distilled water. ▲, Samples treated with 200 μg/ml lysozyme for 1 h and diluted in hypertonic medium; ●, samples treated with 200 μg/ml lysozyme for 30 min and diluted in hypertonic medium; and ○, samples treated with 200 μg/ml lysozyme for 30 min and diluted in distilled water.

Numerous authors have reported a positive effect of $CaCl_2$ on the stability of protoplasts in other bacterial species [3, 11]. In our case, however, the presence of 20 m*M* $CaCl_2$ caused an aggregation of cells without detectable protoplast formation. Otts and Day [12] also reported an inhibitory effect of Ca^{2+} on protoplast formation in *Leuconostoc mesenteroides*.

Two ways of plating the protoplasts on the regeneration medium were tried: spreading on the medium surface and imbedding in the soft agar upper layer. The frequencies attained were 32% and 5%, respectively. Supplementation of the medium with casein increased the regeneration frequency by 11%. However, it decreased when the sucrose concentration was reduced to 0.3 *M*, and colonies of two different sizes appeared. The reason for this phenomenon is unknown. RM2 gave similar regeneration rates (51%), and so this medium can be used when no cellulosic substrate is required in the fusion experiments.

Fusion between the streptomycin-resistant strain and the tetracycline-resistant strain gave rise to the formation of hybrid colonies on the streptomycin plus tetracycline selective medium (RM2) at a frequency of 2.0×10^{-7}. No growth was detected on this medium when the partner strains were plated separately. Gokhale et al. [7] obtained lower frequencies when four auxotrophic markers were used for selection, but higher frequencies when two markers were used.

The products of protoplast fusion in *Bacillus subtilis* and *Bacillus megaterium* were found to be diploids or haploid recombinants. The presence of a diploid stage could be demonstrated by their capacity to generate different types of segregants [5, 8]. In order to investigate the genetic nature of our fusants, segregation analysis of colonies grown on the selective medium (primary colonies) was made as described in Materials and Methods. The segregation pattern is demonstrated in Table 3 with results obtained from the analysis of one fusant colony. Most cells of the fusant formed colonies of a phenotype

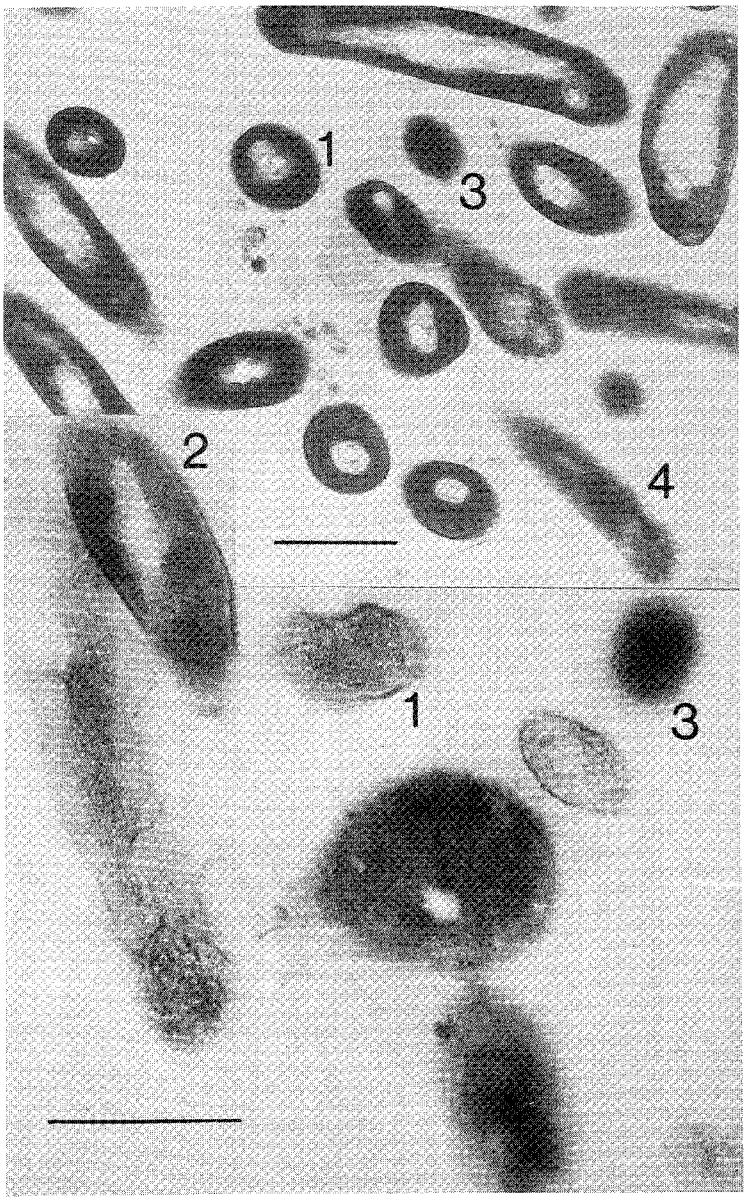

Fig. 2. Electron micrographs of *Cellulomonas* sp. *M32Bo* samples submitted to lysozyme. (1) round-shaped spheroplasts, (2) rod-shaped spheroplast, (3) round-shaped protoplasts, and (4) rod-shaped protoplast. Samples were treated with 200 μg/ml lysozyme for 30 min in the presence of 0.01 *M* EDTA. Bar represents 0.5 μm.

(S^r T^r) identical to that shown by the original colony. However, other segregants also appeared: one parental genotype (S^r T^s) and a recombinant type (S^s T^s). This indicates that the fusant colony was diploid (or partially diploid), but genetically unstable. Diploid hybrids were also described in *Bacillus subtilis*, but in their cells only one of the parental chromosomes was expressed [8, 16]. In our case both parents expressed their genotypes. This could be simply owing to the fact that we used antibiotic resistance markers (probably located on bacterial plasmids), whereas in *Bacillus subtilis* auxotrophic markers were used, located in the main bacterial chromosomes.

In contrast to the primary colony, the progeny colony did not segregate, its cells giving rise only to colonies with the double resistant recombinant phenotype, identical to the phenotype of the primary colony. This could indicate that the progeny colony was already a genetically stable haploid recombinant. Thus the primary fusants were probably diploid cells, but the diploid constitution was then gradually broken down by segregation of haploids showing various parental and recombinant phenotypes.

The results summarized in this paper show that *Cellulomonas* sp *M32Bo* is amenable to protoplast fusion and thus demonstrate that this technique can

Table 2. Effect of lysozyme concentration and growth phase on protoplast formation

Lysozyme concentration (μg/ml)	Growth phase	Efficiency of protoplasting (%)
200	Mid-exponential	75
40	Late-exponential	6
100	Late-exponential	19
200	Late-exponential	25
400	Late-exponential	32
4000	Late-exponential	50

Table 3. Segregation patterns of a primary and a secondary fusion colony

Colony	Total colony number	Phenotypes frequency (%)			
		S^s T^r	S^r T^s	S^s T^s	S^r T^r
1 (primary colony)	173	0	4.6	1.2	94.2
2 (secondary colony)	160	0	0	0	100

be applied to the construction of strains for industrial lignocellulosic conversion.

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