Genetic Recombination by Protoplast Fusion of *Cellulomonas* sp CS 1-1

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原形質体 融合法에 의한 *Cellulomonas* sp. CS 1-1의 遺伝子 再組合

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Auxotrophic mutants of a cellulolytic baterium *Cellulomonas* sp. CS 1-1 were grown at 30°C for 6 hr using a complete medium containing 0.5M sucrose and for another 90 min after addition of 0.3 U/ml penicillin G, and were protoplasted by 0.2 mg/ml lysozyme for 2 hr. Prototrophic recombinants were obtained at the rates of 10^{-3} to 10^{-5} by fusing the protoplasts in the presence of 40% polyethyleneglycol 3350. Nystatin could be used to eliminate fungal contamination during the regeneration of the plotaplasts.

Polyethyleneglycol mediated protoplast fusion has been recognized as a potential tool for the genetic manipulation of organisms which lack well developed methods for DNA exchange (Alfoeldi 1982, Hopwood 1981). Species of *Bacillus* and *Streptomyces* are most frequently used in the studies utilizing this method. This technique has also been applied to the introduction of DNA through liposome where the host organism has low transformation frequency (Makins and Holt 1981, Levi-Meyrueis et al 1980).

Lysozyme is the most frequently utilized enzyme to produce protoplasts of Gram positive bacteria. The susceptibility of the cells to the enzyme were increased when culture were made in the presence of penicillin (Kaneko and Sakaguchi 1979) or glycine (Okanishi et al 1974). The cell wall of Gram negative bactera can be removed by lysozyme in the presence of EDTA (Weiss 1976). *Clostridium* (Allcock *et al* 1982). *Methanobacterium* (Jarrell *et al* 1982) and *Methanosarcina* (Davies and Harris 1985) can be protoplasted by autolytic enzymes produced during the culture. The physiological states of the cell determine the efficiencies of protoplast formation and its regeneration (Hopwood 1981).

The fusion betwen protoplasts is induced by polyethyleneglycol. Di-valent cations such as Ca^{++} and Mg^{++} increase the fusion frequency (Fernczy 1981, Stahl and Pattee 1983). Recombination events after the fusion are the least understood part. Unstable diploid and stable haploid were found among the fusants. Reiterated DNA sequences were found in a stable fusant (Gabor and Hotchkiss 1983).

Fused protoplasts were regenerated using hypertonic media containing sucrose (Stahl and Pattee 1983) or sodium succinate (Kaneko and

Sakaguchi 1979). The regeneration efficiencies vary from 1% up to 50%. In addition to the physiological states of the protoplasting cells and the osmotic stabilizer, the regeneration efficiency is determined by several factors including temperature and water activity (Hopwood 1981). In this study a cellulolytic bacterium *Cellulomonas* sp CS 1-1 was used to establish a genetic manipulation process through protoplast fusion.

MATERIALS AND METHODS

Bacterial Strains and Threir Maintenance

Cellulomonas sp CS 1-1 (Choi *et al* 1978) and its auxotrophic mutants were maintained at 4°C on slops of a complex medium, potato extract medium (Kim and Wimpenny 1980) after the cultures were grown at 30°C. Auxotrophic mutants were obtained by mutagenesis by N-methyl-N'-Nitro-N-nitrosoguanidine (NTG). The phenotypes and the spotaneous reversion frequencies of the mutants are shown in Table 1.

Media and Cultural Conditions

Liquid cultures were made using potato extract broth (Kim and Wimpenny 1981) on a shaking water bath at 30°C. Potato extract medium was used in mutant selection as complete medium. The minimal medium used in the mutant selection contained (g/l): KH_2PO_4, 0.14; K_2HPO_4, 1,2; $(NH_4)_2SO_4$, 2.0; KCl, 0.5; $MgSO_4$. $7H_2O$, 0.5; $Fe_2(SO_4)_3$, 0.01; dextrose, 10, and thiamine, 1mg and biotine, 0.1mg. Mineral yeast solution (MYS) containing 3 g/l yeast extract in place of the vitamins in minimal medium was used to cultivate the cells for protoplast. Cell wall was removed from the cells using lysis fluid (LF) containing 0.2 mg/ml lysozyme, 0.4 M sucrose and 10 mM $MgSO_4$ in half strength MYS. The protoplasts were diluted in 0.02 M maleate buffer (pH 7.0) containing 0.5 M sucrose and 20 mM $MgSO_4$ (SMM). The protoplasts were regenerated in soft agar regeneration complete medium (RCM) or regeneration minimal medium (RMM) overlaied on solid medium of the same composition. Minimal medium was supplemented by 5 g/l yeast extract, 5 g/l casamino acids and 0.3 M sucrose for RCM, and 0.3 M sucrose and 1 g/l glutamic and for RMM. Solid and soft agar medium contained 15 g/l and 8 g/l agar, repectively.

Table 1. *Auxotrophic Mutants Derived from Cellulmonas sp. CS1-1.*

Strain Number	Phenotype	Reversion Frequency
CS 111 011	*ura*	6.7×10^{-8}
CS 612	*ade*	$< 10^{-10}$
CS 712	*trp*	7.5×19^{-9}

Protoplast Formation

An overnight culture in PYC broth was inoculated to 50 ml of MYS at 5% inoculum size and incubated for 6 hr at 30°C in a shaking water bath before 0.3 U/ml penicillin G was added. The culture was incubated for another 90 min without shaking. The cells were collected by centrifugation and resuspended in 50 ml LF before they were incubated at 37°C for 2 hr.

Regeneration of Protoplasts

Protoplasts were diluted in SMM immediatly after the lysozyme treatment or fusion in the presence of polyethyleneglycol (PEG). Three ml soft agar was mixed with 0.1 ml protoplast suspension before poured onto solid medium. The regenerated colonies were visible after 10 days incubation at 30°C.

Protoplast Fusion

Auxotrophic mutants were protoplasted as above. Protoplasts of two strains with different phenotypic markers were mixed approximately equal number (10 ml each) and centrifuged at 7500 x g for 15 min at 40°C. The pellet was resuspended in 2 ml SMM half of which was mixed with 9 ml of 44% (W/V) polyethyleneglycol (molecular weight 3350, Sigma Chemical Co.). The remaining 1 ml was used as control. The protoplast suspension mixed with PEG was incubated for 15 min at room temperature before dilted with SMM to regenerate on RCM or RMM. Control was regenerated without PEG treatment.

Chemicals

All chemicals used were reagent grades purchased from Sigma Chemical Co., St. Louis, MO,

USA and Wako Pure Chemical Co., Osaka, Japan.

RESULTS AND DISCUSSIONS

Effects of Penicillin G on Protoplast Formation and its Regeneration

Wild strain CS 1-1 was grown with or without 0.3 U/ml penicillin G before the cells were treated by 0.2 mg/ml lysozyme for 2 hr. Samples were taken periodically for microscopic observation and to measure the degree of lysis by osmotic shock. Cells from both of the cultures with or without penicillin G became spherical forms which lysed by osmotic shock after 30 min incubation. Table 2 compares the regeneration frequecies of protoplasts prepared from cultures with or without penicillin G. As shown in the Table 2 cells of cultures with or without penicillin treatment lysed by the lysozyme treatment. Higher regeneration frequecies were achieved in protoplast prepared from the penicillin treated culture than the control. This result indicates that penicillin treatment is not essential for protoplast formation but increases the regeneration frequency probably by stimulating cell wall synthesis.

Table 2. *Effects of Penicilln Treatment on Protoplast Formation and its Regeneration in* ***Cellulomonas*** *sp. CS1-1.*

	Trial 1		Trial 2	
	Control	Treated	Control	treated
Colony forming units before protoplast formation	1.3×10^8	1.4×10^8	3.7×10^8	3.5×10^8
Colonies regenerated on regeneration medium	3.4×10^8	5.6×10^8	1.1×10^8	1.5×10^8
Colony forming units of osmotic shocked protoplast suspension	0	0	0	0
Regeneration frequency (%)	26	40	30	46

The wild strain was grown for 3 hr at 30℃ in MYS medium followed by another 90 min with or without 0.3 unit/ml penicillin G before protoplasted by 0.2 mg/ml lysozyme for 2 hr. RCM was used for colony count and protoplast regeneration.

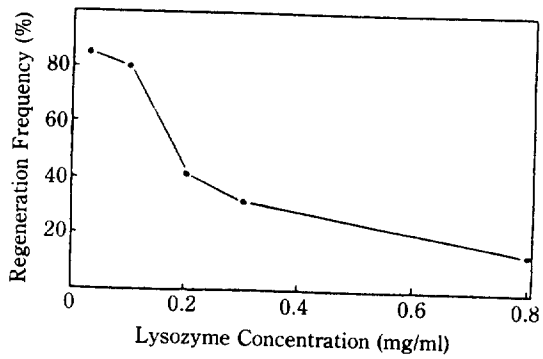

Fig. 1 *Regeneration Efficiency of Protoplasts Prepared Using Varying Concentration of Lysozyme.* Cells of wild strain grown with 0.3 U/ml penicillin G were treated for 2 hr at 37°C with given enzyme concentration.

Effects of Lysozyme Concentration

Cells grown in the presence of 0.3 U/ml penicillin were incubated for 2 hr at 37°C with varying lysozyme concentration of 0.03-0.8 mg/ml. Cells treated by any concentration of the enzyme were osmotically fragile, but the regeneration frequencies of protoplasts formed by the enzyme more than 0.2 mg/ml were 20-40%, whilst 80% of cells were regenerated when they were treated by the enzyme of less than 0.1 mg/ml (Fig 1). The higher regeneration frequencies at low enzyme concentration seem to be the results of incomplete removal of cell wall materials. For this reason 0.2 mg/ml lysozyme was used in later experiments.

Table 3. *Regeneration of Protoplast on Potato Extract Agar with Different Ostabilizer.*

Stabilizer	Regeneration frequency (%)
0.5 M sodium succinate	0
0.4 M sodium succinate	0
0.5 M sodium scetate	0
0.4 M sodium acetate	0
0.5 M sucrose	1.2
0.4 M sucrose	4.6

Protoplast suspension was mixed with soft agar of potato extract medium and poured onto solid agar of the same medium before incubated at 30° for 10 days.

Regeneration of Protoplast

Protoplasts spread on a solid agar surface using a glass rod did not show any regeneration whilst a good regeneration frequency was obtained on a plate overlaid by soft agar. Earlier attempts to regenerate the protoplasts on PYC agar were unsuccessful. Table 3 shows the regeneration frequencies of protoplasts prepared from wild strain CS 1-1 on PYC agar with different osmotic stabilizer. Less than 5% of the protoplasts were regenerated, which is much lower than 40% obtained using RCM agar. The results show that potato extract cannot be used in the regeneration medium. Sodium salts of acetate and succinate were poor osmotic stabilizer for the organism.

Protoplasts of wild strain were regenerated using RCM agar with varying concentration of sucrose (Fig 2). The best result was obtained with the initial sucrose concentration of 0.3 M.

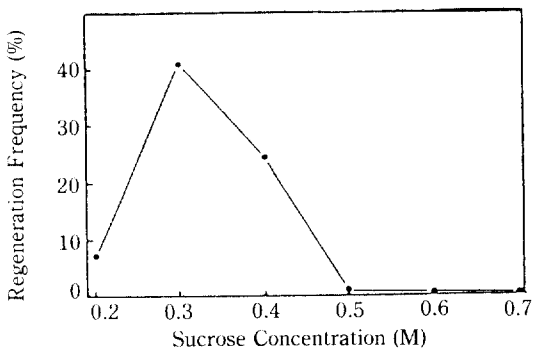

Fig. 2 *Regeneration of Protoplasts in Regeneration Medium with Varying Sucrose Concentration.* Wild strain was used.

Effects of Sucrose in Medium for Cell Propagation before Protoplast Formation

As shown in materials and methods section, MYS medium with 0.5 M sucrose was used to propagate cells for protoplast formation. Sucrose inclusion to the medium increased the regeneration frequency (Table 4). As mentioned in the introduction the regeneration frequency is heavily dependent upon the physiological states of the cells. Higher efficiency in regeneration of protoplasts prepared from culture in high molarity is interpreted as the results of the cells adaptation to the high molarity environment as in the regeneration conditions.

Table 4. *Regeneration of protoplast prepared from cultures with or without sucrose.*

	control	with 0.5 M sucrose
Viable count before protoplasting	2.1×10^{9}/ml	9.8×10^{6}/ml
Regenerated colony from protoplast	3.3×10^{7}/ml	4.7×10^{8}/ml
Regeneration frequency	1.6%	43.0%

MYS was used to propagate cells before protoplast formation.

Regeneration Efficiency vs Cell Age

Samples were taken from a batch culture of the wild strain to determine the regeneration efficiency of protoplasts prepared from cells of different age. As shown in Fig 3, protoplast from cells of 6 hr old culture was regenerated most efficiently. In batch cultures the physiological states are determined by the growth stage of the culture. This result indictes that cells from the early exponential phase are most suitable to use in protoplast fusion.

Regeneration in the Presence of Antifugal Agents

In general regeneration of protoplasts requires a long incubation, and the bacterium used in this study required about 10 days for the development of colonies from protoplast. The prolonged incubation accompanied the increased chances of contamination, especially by fungi. Sodium propionate and nystatin were tested to repress the

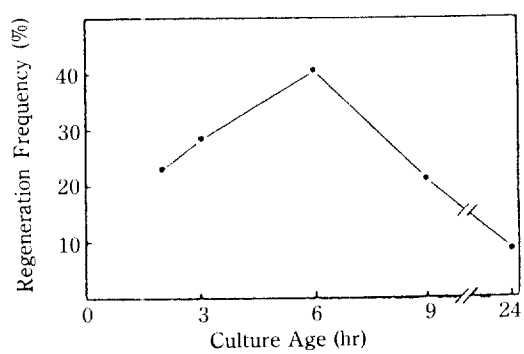

Fig. 3 *Regeneration of Protoplasts Prepared from Cultures of Different Culture age.* Wild strain was used.

Table 5. *Prototrophic Recombination between Auxotrophic Mutants by Protoplast Fusion.*

	CS 612×CS 712 (*ade*×*trp*)	CS 612×CS 011 (*ade*×*ura*)
Recombination Frequency	6.5×10^{-4}	3.6×10^{-3}
Reversion Frequency	6.2×10^{-6}	0

Recombination frequency was calculated by deviding by deviding regenerated colonies of PEG treated protoplast mixture on RMM by those on RCM and protoplast mixture without PEG treatment was used to measure the reversion frequency.

growth of the fungi isolated from a contaminated plate i.e. a *Penicillium* sp. and an *Aspergillus* sp. The protoplasts were not regenerated in the presence of 0.5% sodium propionate, which was not effective in preventing the fungal growth. On the other hand nystatin at concentration of 25 mg/l completely inhibited the fungal growth without sacrificing the regeneration efficiency. But at the concentration over 100 mg/l the antibiotic severely reduced the regeneration efficiency.

Protoplast Fusion

Conditions for fusion were studied in terms of PEG concentrtion and duration of PEG treatment. The highest recombination frequency was achieved with 40% PEG whilst the regeneration frequency decreased as the PEG concentration increased up to 75%. The viability of the protoplasts did not change during the incubation of 30 min with 40% PEG. Incubation period of 15 min was chosen for the convenience of the manipulation. The importance of Ca^{++} has been emphasized (Hopwood 1981, Frenczy 1981). Recombination frequecies over 10^{-3} obtained with 20 mM $MgCl_2$. MG^{++} seems to be as effective as Ca^{++} in protoplast fusion of *Cellulomonas* as reported in *Staphylococcus* (Stahl and Pattee 1983).

Table 5 shows the typical results of more than 3 independent experiments of protoplast fusion between auxotrophs with different phenotypic markers. Recombination frequency between *ade* and *trp* was 6.5×10^{-4} whilst that between *ade* and *ura* was 3.6×10^{-3}. The difference in the recombination frequency does not necessarily represent the relative distance of the genes on the chromosome because the numbers of cross-over between two complete genomes are not known.

Gokhale *et al* (1984) reported that *Cellulomonas* can be used in the intergeneric protoplast fusion with *Bacillus*, but did not elaborate the protocol for protoplast fusion of the cellulose utilizing bacterium. This study established the optimum conditions for protoplast formation, its fusion and regeneration to study the genetics of *Cellulomonas*. The results will be used in future studies for the development of industrially useful strains.

요 약

Cellulose를 利用하는 ***Cellulomonas*** sp. CS 1-1을 育種하는 方法으로 原形質体融合法을 応用하기 위해 영양요구변이주 간의 융합을 시도하였다. 原形質体 形成 및 이들의 融合 및 再生의 最適條件은 다음과 같다. 0.5M sucrose를 함유하는 복합배지에서 6시간 培養하고 0.2 μ/ml penicillin G로 90분 처리한다. 이들 균체를 분리하여 0.2 mg/ml lysozyme으로 2시간 동안 처리하여 原形質体를 만들고, 이들을 40% polyethyleneglycol로 15분 처리하여 10^{-3}~10^{-5}의 빈도로 遺伝子 再組合이 이루어졌다. 原形質体의 再生에서 곰팡이의 오염을 방지하기 위해 Nystatin을 이용할 수 있었다.

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