
Studies on Regeneration Media for Bacillus subtilis Protoplasts*

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Bacillus subtilis protoplasts regenerate on media containing horse serum, bovine serum or gelatin. These compounds could be replaced by polyvinyl pyrrolidone or dextran, and a medium which contained 30 g polyvinyl pyrrolidone and 20 mg casamino acids per liter with chemically defined ingredients was especially useful for selection of prototrophs, e.g., by protoplast fusion. Polyvinyl pyrrolidone and other plasma expanders stimulated protoplast division in liquid media and improved protoplast survival on agar media.

Utilization of protoplasts of Bacillus subtilis was recently extended to polyethylene glycol-induced protoplast fusion1~4) and protoplast transformation5,6) which are useful for the construction of genetic exchange systems, breeding of diploid hybrids and application to genetic engineering.

On the other hand, protoplasts prepared from B. subtilis by lysozyme treatment could not regenerate to rod cells without preserving specific conditions.7) Landman and colleagues8~11) have reported that L colony growth in B. subtilis protoplasts required heat-inactivated horse serum, bovine serum albumin or gelatin (2%), and conversion of protoplasts to the bacillary cells rapidly occurred on media containing hard agar, high concentrations of gelatin, membrane filters, cell wall fractions or autoclaved microorganisms. Since gelatin, bovine serum albumin and horse serum which have often been used for regeneration media are complex proteins and horse serum contains bases, it appears to be difficult to study the mechanisms of L colony growth and regeneration, and to select prototrophic recombinants after protoplast fusion without laborious replica-platings.

This paper describes plasma expander properties, classification of the substances and the mode of growth of protoplasts in solid and liquid media.

MATERIALS AND METHODS

Bacterial strains. Bacillus subtilis 168S (trpC2, strA), B. subtilis YS11 (purB argA leuA) and B. subtilis LMHA {purA metB leuA hisA) obtained from T. Seki were used. B. subtilis YS11 and B. subtilis LMHA were used only in the experiment shown in Fig. 1.

Media. Nutrient medium contained 10 g of beef extract, 10 g of polypeptone and 2 g of NaCl per liter of deionized water. For the preparation of nutrient agar medium, 15 g of agar per liter was added. The fundamental medium, HC medium, used for a regeneration medium of B. subtilis protoplasts to the bacillary form was the modified DPA medium described by Wyrick and Rogers.12) The medium contained 5 g of glucose, 5 g of casamino acids, 3.5 g of K2HPO4, 1.5 g of KH2PO4, 0.1 g of L-tryptophan, 1.9 g of MgCl2 and 250 ml of 2 M sodium succinate (pH 7.3) per liter. HCH-10 medium was HC medium supplemented with 100 ml of heat-inactivated (56°C for 30 min) filter-sterilized horse serum per liter. For HCP-3, HCD-3 and HCV-3 media, 30 g of polyvinyl pyrrolidone, 30 g of dextran and 30 g of vinyl pyrrolidone were added per liter of HC medium, respectively. HMP-3 medium was the same as HCP-3 medium except that 2 g of (NH4)2SO4 and 1 g of sodium citrate replaced the 5 g of casamino acids and 0.1 g of L-tryptophan per liter. If necessary, appropriate amino acids and base were added to final concentrations of 20 µg/ml except L-tryptophan (100 µg/ml) and 40 µg/ml, respectively. For the osmotically stabilized
plating media (e.g., HCP agar medium) used for regeneration of both protoplasts and spheroplasts, 8 g of agar per liter was added.

Preparation and regeneration of protoplasts. B. subtilis 168S grown on a nutrient agar plate overnight at 30°C was inoculated into 20 ml of nutrient broth and incubated with shaking for 4~6 hr at 30°C. When the density of the culture (OD$_{570}$ nm) reached 0.4, cell suspension was reinoculated into 25 ml of nutrient broth to give an initial OD$_{570}$ nm = 0.025 and shaken for 2 hr at 37°C. Cells from 20 ml of the culture were harvested in the mid-log phase (OD$_{570}$ nm = 0.4) by centrifugation (7200 x g for 10 min) and suspended in 4 ml of SMM (0.5M sucrose-0.02M maleate buffer, pH 6.5-0.02M MgCl$_2$) of Wyrick and Rogers$^{12}$ containing lysozyme at a final concentration of 250 µg/ml. The total mixture was kept for 45 min, unless otherwise noted, at 42°C as a shallow layer (e.g., 4 ml of suspension in a 100-ml Erlenmeyer flask). When 0.1 ml of the suspension was added to 0.9 ml of sterilized water, colonies appeared on nutrient agar plates with a frequency of about 1 x 10$^{-8}$ (No. of osmotically resistant colonies (ORC) after protoplasting/colony-forming-unit (CFU) before protoplasting). One CFU corresponded to 1.5 ~ 2.0 protoplasts on average. After incubation in SMM containing lysozyme, 3 ml of the suspension was transferred to a centrifugation tube containing 10 ml of SMM and the tube was centrifuged (4000 x g for 10 min). The pellet was suspended in HC medium and aliquots were spread onto the osmotically stabilized plating medium and then the plates were incubated for 2 ~ 5 days at 30°C. Regeneration frequency was a ratio of regenerants (colonies on regeneration media) per CFU (colonies before protoplasting). Microscopic observation showed that individual colonies grown on HC, HCH-10, HCD-3 and HCP-3 agar media after 4-days incubation, and on HC* P-3 and HC* P-3 agar media (see Results) after 5-days incubation consisted of bacillary cells, or the mixture of bacillary and L colony cells. Thus, the regeneration frequency corresponds to the survival of protoplasts by criteria of Landman$^9$ and others.$^{4,5}$

Incubation of protoplasts in liquid media. Protoplasts suspended in the osmotically stabilized media, HC, HCP-3, HCD-3 and HCH-10, were incubated without shaking at 30°C. Samples were taken from the liquid media at different intervals. Cell density was measured turbidometrically at 570 nm. No. of protoplasts was counted using a Thoma blood counter. Morphological changes of regenerating protoplasts were studied by phase contrast photomicrography. Photographs were taken with a Nikon camera attachment and Fuji commercial film (ASA 100). The magnification of samples was × 600.

Chemicals. Lysozyme was purchased from Sigma Chemical Company (Saint Louis) and polyvinyl pyrrolidone (K-30), vinyl pyrrolidone and dextran (M.W. 200,000~300,000) were from Wako Chemical Industries, Ltd. (Osaka). Horse serum obtained from Nippon Bio-Test Laboratories Inc. (Tokyo) was heat-inactivated for 30 min at 56°C and used for regeneration experiments.

RESULTS

Effect of plasma expanders on regeneration frequency

Since gelatin, horse serum and bovine serum albumin, often used for regeneration media,$^{4,5,9}$ are all plasma expanders,$^{13}$ it seemed interesting to examine the relationship between plasma expanders and the stimulative factors for regeneration frequency. For this purpose, two chemicals having the properties of plasma expanders, dextran and polyvinyl pyrrolidone, were chosen and the effect of the chemicals on regeneration frequency was tested by using HC agar medium containing 3% of polyvinyl pyrrolidone (HCP-3 agar medium) or HC agar medium containing 3% of dextran (HCD-3 agar medium). As shown in Table I, the regeneration frequency (0.8 ~ 3.0%) obtained on HCP-3 agar medium was quite similar to that (1 ~ 2%) on HC agar medium containing 10% horse serum (HCH-10 agar medium), and these two frequencies were 200 ~ 600 times higher than that on HC agar medium. Furthermore, a regeneration frequency of 0.4 ~ 0.7% was obtained on HCD-3 agar medium and this was 100 ~ 200 times higher than on HC agar medium. horse serum (HCH-10 agar medium), and these two frequencies were 200 ~ 600 times higher than that on HC agar medium. Furthermore, a regeneration frequency of 0.4 ~ 0.7% was obtained on HCD-3 agar medium and this was 100 ~ 200 times higher than on HC agar medium.

<table>
<thead>
<tr>
<th>Table I. Effect of Plasma Expanders on Regeneration Frequency of Bacillus subtilis 168S Protoplasts</th>
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<tbody>
<tr>
<td>Agar medium</td>
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<tr>
<td>-------------</td>
</tr>
<tr>
<td>HCP-3</td>
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<tr>
<td>HCP-10</td>
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<tr>
<td>HCP-3</td>
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<tr>
<td>HCD-3</td>
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</table>

* Colony-forming-unit (CFU) before protoplasting was 3.4 x 10$^{6}$/ml and No. of osmotically resistant colonies (ORC) after protoplasting was 29/ml.

* CFU was 1.8 x 10$^{6}$/ml and No. of ORC was 15/ml.
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Fig. 1. Kinetics of Osmotic Resistant Cells and Regenerants on Different Hypertonic Media during Incubation at 42°C in the Hypertonic Lysozyme Solution. Exponentially growing cells of B. subtilis YS11 and B. subtilis LMHA were suspended into the hypertonic solution containing 250 μg/ml lysozyme and incubated for 60 min. Samples were taken every 15-min intervals, and spread onto HCP-3 and HC agar plates supplemented with 40 μg/ml adenine. After 3~4 days incubation, number of colonies were counted. Osmotic resistant cells (ORC): ▲, YS11; ▼, LMHA. Regenerants: ○, YS11 on HCP-3+ade; ●, YS11 on HC+ade; △, LMHA on HCP-3+ade; ▲, LMHA on HC+ade.

Influence of concentration of dextran of polyvinyl pyrrolidone on regeneration frequency and inhibitory effect of vinyl pyrrolidone

To determine the influence of the concentration of polyvinyl pyrrolidone or dextran on regeneration frequency of B. subtilis protoplasts and to examine the properties of the two chemicals, various plating media were prepared. A concentration of 0.5% or more of polyvinyl pyrrolidone was necessary for the high regeneration frequency and 0.1% polyvinyl pyrrolidone was fairly ineffective (Table II). Even on HC agar medium containing 1% of dextran, the regeneration frequency was not different from the control on HC agar medium. When polyvinyl pyrrolidone and dextran were replaced with the monomers (vinyl pyrrolidone and glucose, respectively), stimulation of regeneration frequency by these monomers was not observed. On the contrary, increased amount of vinyl pyrrolidone decreased the regeneration frequency (Table II).

From these results, it seems that high concentrations of polymer chemicals having
Table II. Effect of Concentration of Plasma Expanders and Their Monomers on Regeneration Frequency

<table>
<thead>
<tr>
<th>Experiment I</th>
<th>Experiment II</th>
<th>Experiment III</th>
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<tbody>
<tr>
<td>Agar medium</td>
<td>Regeneration frequency</td>
<td>Agar medium</td>
</tr>
<tr>
<td>HC</td>
<td>$1.2 \times 10^{-4}$</td>
<td>HC</td>
</tr>
<tr>
<td>HCP-0.1°</td>
<td>$1.9 \times 10^{-3}$</td>
<td>HCD-1</td>
</tr>
<tr>
<td>HCP-0.5°</td>
<td>$1.0 \times 10^{-2}$</td>
<td>HCD-3</td>
</tr>
<tr>
<td>HCP-1°</td>
<td>$1.0 \times 10^{-2}$</td>
<td></td>
</tr>
<tr>
<td>HCP-3°</td>
<td>$1.9 \times 10^{-2}$</td>
<td></td>
</tr>
<tr>
<td>HCV-0.1</td>
<td>$7.6 \times 10^{-2}$</td>
<td></td>
</tr>
<tr>
<td>HCV-0.5°</td>
<td>$2.5 \times 10^{-5}$</td>
<td></td>
</tr>
<tr>
<td>HCV-1°</td>
<td>$&lt;4.5 \times 10^{-7}$</td>
<td></td>
</tr>
<tr>
<td>HCV-3°</td>
<td>$&lt;4.5 \times 10^{-7}$</td>
<td></td>
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</tbody>
</table>

a Results from three experiments are shown. Among these experiments, CFU was in the range of $3.4 \sim 4.9 \times 10^8$/ml and frequency of ORC was less than $1.0 \times 10^{-7}$.

b The "°" of HCP-nagar medium shows the % concentration of chemicals. For example, HCP-0.1 and HCV-0.1 agar media contained 0.1% of polyvinyl pyrrolidone and vinyl pyrrolidone, respectively.

c HCGlu-3 agar medium was the same as the HC agar medium, except that 30 g of glucose per liter was added.

plasma expander properties are required for the higher survival of *B. subtilis* protoplasts.

**Difference between hard agar and plasma expanders in effect on regeneration frequency**

To examine the effect of hard agar on regeneration frequency, *B. subtilis* 168S protoplasts prepared from the log-phase culture were spread onto HC medium containing 2.5% agar and/or 3% polyvinyl pyrrolidone, and incubated for 2 ~ 4 days at 30°C. Table III shows that the ratio of the regeneration frequency obtained on HC medium containing 2.5% agar (HC* agar medium) to that on HC agar medium is 6 ~ 10. Thus, the efficiency of hard agar for the increase of regeneration frequency was slightly lower than that of the plasma expanders. On the other hand, the regeneration frequency (0.1 ~ 0.2%) obtained on HC medium containing both 2.5% agar and 3% polyvinyl pyrrolidone (HCP-3 agar medium) was significantly lower than the value (1.2 ~ 1.4%) on the HCP-3 agar medium (Table III). Furthermore, large portion of colonies appeared after 2 days on HC* and HCP*-3 agar media, and almost the individual colonies consisted of bacillary cells and the residual was the mixture of bacillary and L colony cells. These results were supported by the other work that hard agar stimulated conversion of protoplasts to bacillary cells and gave rapid colony growth. On the other hand, the effect on recovery of protoplasts by hard agar was variable on the regeneration media.

**Division of protoplasts by budding in liquid media containing plasma expanders**

Landman and colleagues have reported that *B. subtilis* protoplasts do not regenerate in liquid media, but trypsin-treated protoplasts regenerate in the liquid medium containing 10% gelatin. To study the mode
of regeneration, protoplasts of *B. subtilis* 168S were suspended in HCP-3 medium and still-cultured at 30°C for various periods. Cell density and number of protoplasts were measured as described in Materials and Methods. After 5-hr incubation, cell density and number of protoplasts were 1.5 ~ 1.6 times more than initial values. This result suggested that protoplasts were dividing in HCP-3 liquid medium. Morphological changes of protoplasts cultured in liquid media: HC, HCP-3, HCD-3 and HCH-10 media were investigated using phase contrast photomicrography (Fig. 2). Protoplasts swelled in HC, HCP-3, HCD-3 and HCH-10 media, but division by budding was only observed in HC media containing plasma expanders (Fig. 2). Furthermore, dividing protoplasts aggregated in the HCH-10 medium. After 24-hr incubation, few bacillary cells were observed in HCP-3, HCD-3 and HCH-10 media, but this amount was considerably low compared with the previous

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**Fig. 2. Phase Contrast Illuminated Photomicrography of Protoplasts and Dividing Protoplasts of *B. subtilis* in HC, HCP-3, HCD-3 and HCH-10 Liquid Media.**

Protoplasts were still-cultured in HC, HCP-3, HCD-3 and HCH-10 media for 1.5 hr, 7.5 hr and 13.5 hr. Key: a, 1.5 hr culture in HC medium; b, 7.5 hr in HC; c, 13.5 hr in HC; d, 1.5 hr in HCP-3; e, 7.5 hr in HCP-3; f, 13.5 hr in HCP-3; g, 1.5 hr in HCD-3; h, 7.5 hr in HCD-3; i, 13.5 hr in HCD-3; j, 1.5 hr in HCH-10; k, 7.5 hr in HCH-10; l, 13.5 hr in HCH-10. Bar represents 10 μm.
Table IV. Influence of Casamino Acids on Regeneration Frequency

<table>
<thead>
<tr>
<th>Agar medium</th>
<th>Regeneration frequency</th>
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<tbody>
<tr>
<td></td>
<td>Experiment I</td>
</tr>
<tr>
<td>HCP-3</td>
<td>2.2 x 10^{-2}</td>
</tr>
<tr>
<td>HC^P-3^a</td>
<td>3.0 x 10^{-2}</td>
</tr>
<tr>
<td>HC^P-3</td>
<td>1.7 x 10^{-2}</td>
</tr>
<tr>
<td>HC^P-3</td>
<td></td>
</tr>
<tr>
<td>HC^P-3^d</td>
<td>1.4 x 10^{-2}</td>
</tr>
<tr>
<td>HMP-3 + trp^e</td>
<td>2.8 x 10^{-4}</td>
</tr>
</tbody>
</table>

^a Results from three experiments are shown. CFU was in the range of 2.3 ~ 2.8 x 10^8/ml and frequency of ORC was less than 2.2 x 10^{-7}.
^b The superscript "n" of HC^P-3 agar medium shows concentration of casamino acids. "a", "b", "c", "d" and "e" denote 500, 50, 20, 10 and 5 mg/liter, respectively.
^c The rod cells emerged extremely slowly on HC^P-3 and HMP-3 + trp agar media. Therefore, the regeneration frequency of these plates was counted using the number of regenerants appearing after 7-days incubation.
^d The regeneration frequency on HC^P-3 agar medium was almost same as that on HC^*P-3 agar medium which was HC^P-3 agar medium supplemented with 2 g of (NH_4)_2SO_4 and 1 g of sodium citrate per liter.
^e 100 µg/ml L-tryptophan was added to HMP-3 agar medium.

results in the 10% gelatin medium. From these observations, it was concluded that plasma expanders influence L colony growth in which swollen protoplasts divided by budding.

Influence of casamino acids on regeneration frequency

Protoplasts were incubated without shaking in HCP-3 medium for 3 hr at 30°C. After this conditioning, protoplasts were harvested by centrifugation, washed with HMP-3 medium and suspended in HMP-3 medium. The suspension was spread onto HMP-3 agar medium supplemented with 100 µg/ml L-tryptophan and HC^P-3 agar medium which was the same as HCP-3 agar medium, except that the concentration of the casamino acids was changed (Table IV). The regeneration frequency (3.6 x 10^{-4}) obtained on HMP-3 agar medium supplemented with L-tryptophan was approximately 100-fold lower than that (3.2 x 10^{-2}) on HCP-3 agar medium. Furthermore, decreasing the amount of casamino acids from 10 mg casamino acids per liter in the regeneration medium resulted in decreasing regeneration frequency. As a control experiment, nonconditioned protoplasts regenerated on HMP-3 agar medium supplemented with L-tryptophan and they showed a very low frequency of 2 x 10^{-7}.

The role of casamino acids on regeneration frequency are not clearly understood and the several possibilities were proposed: (i) casamino acids stimulate the recovery of protoplasts injured by the preparation procedure; (ii) casamino acids support L colony growth in liquid and solid media containing plasma expanders, since omission of casamino acids or plasma expanders did not gave division of protoplasts in liquid media; (iii) casamino acids are known to accelerate the conversion of L colony cells and protoplasts to bacillary cells under gelatin tube method and increase viable cell number after 4 ~ 5 days incubation. Probably casamino acids activate general cellular metabolism and express diverse effects on regeneration.

On searching regeneration media for selection of prototrophs, HC^P-3 agar medium which was HC^*P-3 medium supplemented with 2 g of (NH_4)_2SO_4 and 1 g of sodium citrate was finally constructed, since HC^*P-3 medium contained 20 mg casamino acids per liter whose amount was not enough to support auxotrophs, and gave the same regeneration frequency as HCP-3 did. On this medium, recombinants were directly selected after polyethylene glycol-mediated protoplast fusion (T. Akamatsu et al., “Advances in Biotechnology,” Proceedings of the 6th International Fermentation Symposium/5th International Symposium on Yeasts, London, Canada, July 20 ~ 25, 1980, in press).

DISCUSSION

Plasma expanders have several properties:
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maintenance of osmotic pressure in blood, binding with various substances (iron, pigments, some soluble vitamins and drug) and transfer of these substances. Since three substances (gelatin, heat-inactivated horse serum and bovine serum albumin) often used for regeneration media in *B. subtilis*, polyvinyl pyrrolidone and dextran as the other plasma expanders were investigated for the growth of protoplasts in liquid and solid media. Horse serum, polyvinyl pyrrolidone and dextran increased survival of protoplasts in the range of 100- to 1000-fold (Table I). Colonies appeared slowly on HC agar media containing plasma expanders. Hard agar known as the stimulative factor for conversion of protoplasts to bacillary cells did not always increase the regeneration frequency (Table III), but gave the rapid growth in which almost of individual colonies consisted of only bacillary cells. In the liquid media containing plasma expanders, protoplast could swell and divide (Fig. 2), but formation of bacillary cells were very poor even after 24-hr incubation. On the other hand, protoplasts swelled in a liquid medium without plasma expanders (Fig. 2). These results strongly suggested that plasma expanders, in general, stimulate L colony growth and increase yields of regenerants.

Contrary to the rapid decrease of regeneration frequency, fusion frequency (fusants per regenerants) increased until 45-min incubation in the hypertonic lysozyme solution at 42°C (Fig. 1; unpublished results). Furthermore, another 15-min incubation of 45-min samples did not give the significant decrease of regeneration and fusion frequencies. These results suggested that decrease of regeneration frequency is not explained by the heat-damage of protoplasts at 42°C.

Chan and Cohen had reported the 10~25% viable cell counts per CFU after 2-hr incubation in SMMP containing lysozyme (2 mg/ml) at 37°C. On the other hand, the highest regeneration frequency (100% viable cell counts per input protoplasts) had been reported by Gabor and Hotchkiss after 30-min incubation in SMMD containing lysozyme (100 μg/ml) at 42°C. In both experiments, plasma expander(s) (bovine serum albumin, or gelatin and calf serum) were added to the regeneration media. When compared to the results by Gabor and Hotchkiss, approximately 10% viable cell counts per CFU in *B. subtilis* 168S were estimated on the regeneration media containing horse serum and polyvinyl pyrrolidone after 30-min incubation in the hypertonic lysozyme (250 μg/ml) solution. Still the difference of regeneration frequency may depend on the concentration of lysozyme, composition of regeneration media and/or the dilution buffer.

Furthermore, we can point out that regeneration frequency is also under the control of the genetic background of each strain. In fact, maximal of the regeneration frequency in *B. subtilis* 168S was approximately 10-fold higher than those in *B. subtilis* YS11 and *B. subtilis* LMHA (Table I and Fig. 1).

Gelatin and casamino acids were not required for regeneration media of *B. megaterium* protoplasts and, when protoplast suspension in a minimal soft agar medium was poured onto the surface of the 1% minimal agar layer, bacillary cells appeared efficiently. But, conditions used in our study were not efficient for the regeneration of *B. megaterium*, but efficient for *B. licheniformis* protoplasts (unpublished results). These informations suggested that each species has its own conditions of growth and regeneration of protoplasts.

Regeneration of protoplasts in liquid media was not often occurred in *Bacillus*. Hadlaczky et al. had reported the regeneration of *B. megaterium* protoplasts in liquid media, and indicated three phases of regeneration sequence: (i) increase in size of the individual protoplasts, (ii) non-oriented division of the protoplasts, (iii) outgrowth of the bacillary form. If the regeneration sequence is similar to that of *B. subtilis*, it is considered that plasma expanders support the conversion of protoplasts in the first phase to the second phase
In previous reports, recombinants from protoplasts fusion in *B. subtilis* were not directly selected by auxotrophic markers and the colonies grown on the hypertonic agar were replica-plated onto the diagnostic agar plates. This method has a substantially disadvantage for the detection of primary colonies which permit us to analyse heteroclines and the role of selective pressure. On the contrary, the HC*P-3* agar medium is useful for selection of prototrophs after protoplast fusion.

Acknowledgment. We thank Professor M. Shibata, Kumamoto University for providing instruments.

REFERENCES