FACTORS INCREASING THE FUSION EFFICIENCY OF *Bacillus subtilis*

V. N. Arbuzov, Yu. S. Kondrat'ev, and A. G. Skavronskaya*

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The obtention of hybrid forms of cells by fusion of protoplasts is widely employed in genetic research with fungi [7] and cells of animals [4] and plant [6] origin. The possibility of fusion of bacterial protoplasts, as a result of their treatment with polyethylene glycol, has also been demonstrated [2, 8]. Fusion of bacterial protoplasts is evidently a promising method for the genetic study of bacteria. This method of obtaining genetic hybrids demands special attention in work with bacteria which have none of the known systems of genetic exchange, such as conjugation, transduction, and transformation. Existing methods of fusion of bacterial protoplasts are insufficiently effective, so that there is a need for further experimental research aimed at improving them.

In this investigation an attempt was made to increase the efficiency of fusion of *Bacillus subtilis* protoplasts.

EXPERIMENTAL METHOD

Two strains of *B. subtilis* were used: SB25 trp$^-$ his$^-$ and Mi8i5i5 metB$^-$ leu$^-$ thr$^-$. Different media were used: sucrose-phosphate buffer (SPB): 0.66 M phosphate buffer, pH 6.6, 0.5 M, sucrose, 20 mM $MgCl_2$; agarized hypertonic medium (AHM): Difco agar 8 g, casaminic acid 5 g, K_2HPO_4 9.5 g, KH_2PO_4 1.5 g, succinic acid 59.5 g NaCH 40 g, gelatin 20 g, $MgCl_2$ 20 mM, glucose 5 g, tryptophan 0.1 g, normal horse serum 5 ml, H_2O to 1 liter, pH 7.3; liquid hypertonic medium (LHM): casaminic acids 4 g, tryptophan 0.1 g, K_2HPO_4, 9.5 g, KH_2PO_4 1.5 g, succinic acid 59.5 g, NaOH 40 g, $MgCl_2$ 20 mM, glucose 5 g, H_2O to 1 liter, pH 7.3; Difco nutrient agar (DNA); minimal agar (MA): M9M glucose-salt medium [3] with 1.5% Difco agar.

If necessary, amino acids were added to the above-mentioned media in a concentration of 20 µg/ml. The modifications of the experiments on protoplast fusion used in the work were made to the method described previously [8]. Bacterial cultures, in the middle of the logarithmic phase of growth, were sedimented by centrifugation, washed in physiological saline, and resuspended in SPB (concentration of bacteria 4×10^8, $OD_{570} = 2$). Lysozyme (200 µg/ml) was added to the bacterial suspensions, which were incubated for 20 min at 42°C.

The number of osmotically resistant cells, on the basis of which the percentage of protoplast formation was calculated, was determined by seeding from appropriate dilutions on DNA. The level of protoplast formation was usually 99.99%.

From suspensions of each strain treated with lysozyme 1-ml samples of suspension were taken, mixed, centrifuged, resuspended in 0.2 ml SPB, and treated with 1.8 ml of 40% polyethylene glycol 6000 (PEG). After exposure for 1 min with PEG samples were taken, diluted in SPB, and seeded on AHM in a dilution of 10^{-2} on mother dishes and in a dilution of 10^{-4}-10^{-5} to determine reversion of protoplasts. The dishes were incubated at 37°C for 48 h. Colonies growing on the mother dishes were replicated on MA and on MA with the addition of various amino acids. Colonies were counted after incubation for 78 h at 37°C.

*Corresponding Member, Academy of Medical Sciences of the USSR.

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TABLE 1. Effect of Trypsin and Incubation in LHM on Reversion of Protoplasts and Number of Colonies on MA

Treatment		% of reversions	Number of colonies on MA
incubation in LHM	trypsin		
—	—	0,1—1	1—4
+	—	10—25	17—25
+	+	60—80	67—75

TABLE 2. Frequency of Appearance of Colonies on Unenriched MA and on MA with Various Amino Acids

Enrichment of MA	Number of colonies		
	expt. 1	expt. 2	expt. 3
Unenriched MA	75	67	70
trp leu	162	148	147
trp met	173	116	121
trp thr	144	81	117
his⁻ leu	223	122	137
his⁻ met	210	178	114

To exclude transformation before lysozyme treatment, either deoxyribonuclease I (from Koch-Light Laboratories) in a dose of 5 μg/ml or (in the experiments with trypsin) thymus DNA in a dose of 100 μg/ml (from Koch-Light Laboratories) was added to the bacterial suspensions.

EXPERIMENTAL RESULTS

In agreement with data in the literature [8], treatment of *B. subtilis* protoplasts with PEG under the present experimental conditions led to their fusion (Table 1).

Evidence in support of this conclusion is given by the formation of colonies on selective MA medium containing no nutrient factors essential for the parental strains. Colonies of "primary prototrophs" on MA appeared with a frequency of 10^{-4} relative to the number of protoplasts contained in the suspension. Reversion of protoplasts in these experiments did not exceed 1%, also in agreement with data in the literature [8]. The results of experiments including our modification, leading to an increase in the frequency of reversion of protoplasts and so increasing the frequency of formation of "primary prototrophic" colonies, are also given in Table 1. This modification consisted of incubating the protoplast in LHM and treating them with trypsin. The use of this modification was based on data obtained in experiments with protoplasts of *B. subtilis*, which showed that the frequency of reversion of protoplasts to bacillary forms is increased if feeding on regeneration medium is preceded by incubation in liquid hypertonic medium with casaminic acids or by treatment of the protoplasts for 10 min with trypsin [1].

As Table 1 shows, incubation for 90 min in LHM at 37°C increases the percentage of reversion of protoplasts to 10-25, which was accompanied by a tenfold increase in the number of colonies of "primary prototrophs" of MA. After a combination of incubation in LHM and treatment for 10 min with trypsin (250 μg/ml) the percentage of reversions rose to 60-80 and the number of colonies on MA was increased by 50 times, to reach 10^{-3} relative to the number of reverting protoplasts.

It is probable that during fusion of protoplasts, not only genomes preserving their integrity are "joined" but also genomes incomplete as a result of breakages of chromosomes. Under these circumstances "primary prototrophism" can arise only in relation to certain fea-

tures. If this probability is in fact true, the number of "primary prototrophs" on selective media containing individual additives required by the parental strains ought to be increased compared with the number of MA without additives.

To test this hypothesis the effect of amino-acid enrichment of MA on the number of selective "primary prototrophs" was studied. In these experiments, besides MA, the same agar, but containing one of the amino acids required for growth of each of the partners in fusion, were used as selective media.

The results in Table 2 show that the number of colonies of "primary prototrophs" on MA with the addition of amino acids was twice to three times greater than their number on unenriched MA.

During fusion of protoplasts, not only "primary complete prototrophs" (recorded during "reprinting" on MA) may thus arise, but also "primary partial prototrophs," preserving individual auxotrophic markers of the parental strains. The latter give growth of colonies only on MA with the addition of the corresponding amino acids, but not on unenriched MA.

The number of selected "primary prototrophs" was not found to depend on the type of the added amino acids (Table 2). In other words, no difference was found in the frequency of formation of "primary prototrophs" of different phenotype (as regards essential amino acids).

The added amino acids in these experiments corresponded to genes, some of which are a considerable distance apart on the genetic map of *Bacillus subtilis*. In the case of recombinations arising during ordinary transfer of genetic material, the frequency of the recombinants produced reflects the distance apart of the genes [5]. In the case of fusion of protoplasts studied in the present experiments, the recombination process showed no regular features due to the distance between the markers.

This result may perhaps be explained, bearing in mind existing views on the recombinant nature of "primary prototrophs" and their origin, by the occurrence of multiple acts of recombination.

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