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Parameters Governing Bacterial Regeneration and Genetic Recombination After Fusion of *Bacillus subtilis* Protoplasts

MAGDA H. GABOR AND ROLLIN D. HOTCHKISS*

The Rockefeller University, New York, New York 10021

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Bacterial protoplast fusion, induced by polyethylene glycol, has been made more regular and convenient by further specification and improvement of various steps in the previously used procedure. These have made it possible to obtain regularly 100% regeneration of *Bacillus subtilis* cells from protoplasts before treatment with polyethylene glycol and yields of 10 to 75% from polyethylene glycol-treated protoplasts. Genetic recombination frequencies do not increase correspondingly. Also, when regeneration is reduced by various experimental conditions, recombination does not decrease in proportion. It is concluded that regeneration of recombinant-forming cells is independently determined and not closely related to the average regeneration for the population. Kinetic studies with varying individual parental or total protoplast concentrations strongly indicate that protoplast collision and contact is not the limiting factor determining the number of genetic recombinants obtained. Recombination approximates a linear, rather than quadratic, function of the total or of the majority protoplast population present, from which it is concluded that fusion events are always adequate to produce substantially more potential recombinants than are registered. The strong effect of the majority/minority ratio upon the number of minority cells that become recombinant is independent of which parent is in excess. This shows in a direct and physiological way that both parents are equivalent partners in their genetic contributions.

Fusion of *Bacillus subtilis* protoplasts in the presence of polyethylene glycol (PEG) resulted in genetic recombination between two multiply auxotrophic cell types (10). The recombinants were recovered by selective replica plating of bacterial colonies, which had first been allowed to regenerate their cell walls on complete medium after a short exposure of mixed protoplasts to the fusogenic agent. The yields in the protoplast-to-bacterial conversion were in the range of 2 to 5% (8, 10, 11), and those of prototrophic recombinants ranged from 0.2 (8) to as many as 3 (10, 11) per 10^4 protoplasts.

In analyzing the mechanism of cellular and chromosomal interactions in the potentially diploid products of fusion, it becomes important to know whether or not the simple picture of two cells fusing to form a diploid single cell is justified. Since a large majority of the treated protoplasts were not being recovered as bacteria, it seemed possible, for example, that recombinants might arise principally from some unusual and rare product of fusion (such as a multiply fused form). We have felt obliged to ask whether the failure of many cells to recover after PEG treatment is a necessary or typical result of the fusogenic process and whether this or cell-with-cell collision may be the limiting process determining the probability of genetic recombination. Accordingly, we have made efforts, reported here, to improve and stabilize the yields of regeneration and recombination and to explore the kinetics of the process of protoplast fusion, in particular the response to protoplast concentration.

MATERIALS AND METHODS

Bacterial strains. The strains, S6-A3 and S7-C20 (Table 1), used for fusion experiments were subclones of two triple auxotrophic mutants of *B. subtilis* S6 (*rfm-486 ura-1 trpC7 thr-5*) and S7 (*rfm-486 purB34 metB5 leu-8*) (10), and were selected as single colonies showing high plating efficiency on modified synthetic medium (S) (see below). Their genetic stability is evident from Table 1.

Media and culture conditions. Bacterial stocks were maintained at −70°C in 10% glycerol. Bacteria were cultured for early experiments at 30°C in Difco nutrient broth (NB), with some adjustments (12), and for later experiments 0.5 M sucrose was added. Occasionally bacteria were also grown in "modified S," a minimal liquid medium (1), enhanced by addition of a mixture of the following amino acids (besides the biochemical requirements of the auxotrophic mutant strains): L-glutamate, 2.5 μg/ml; L-lysine, 5.0 μg/ml;

TABLE 1. *Stability toward genetic reversion of parental strains*

Strain[a]	Reversions observed	
	Marker	Frequency
S6-A3 *ura trp thr*	*thr*$^+$	$<1 \times 10^{-9}$
	trp$^+$	$<1 \times 10^{-9}$
	ura$^+$	$<1 \times 10^{-9}$
S7-C20 *pur met leu*	*leu*$^+$	8.2×10^{-8}
	met$^+$	2.4×10^{-7}
	pur$^+$	8.2×10^{-8}

[a] Subclones of S6 and S7, described in (10).

L-asparagine, 12.5 μg/ml; L-valine, 2.5 μg/ml; and a mixture of salts: $MnCl_2$, 2.5×10^{-5} M; $MgCl_2$, 2.5×10^{-4} M; $CaCl_2$, 1.5×10^{-4} M; and $FeSO_4$, 2.5×10^{-5} M, final concentrations. After the adaptive growth of precultures at 37°C in this medium (containing the required factors: amino acids at 25 μg/ml, and bases, uracil and adenine, at 40 μg/ml, final concentrations) the bacteria were grown at 30°C in S medium containing 0.5 M sucrose. After such fresh bacterial cultures had been stored in the frozen state at −70°C, for their subsequent activation and protoplastization they were incubated in a high-tonicity, supplemented Spizizen medium called PC (4). The final concentration of casein hydrolysate, when used, was decreased to 0.5 mg/ml and that of required amino acids to a usual 25 μg/ml. For regeneration of protoplasts, high-tonicity agar medium (R) was used (16), with some modifications. The concentration of sodium succinate was reduced to 2/3 amount and that of agar to 2%, and an addition was made of 0.5% gelatin (Sigma). Calf serum (Grand Island Biological Co.) in 1:200 dilution, DNase I (crude grade, Sigma) at 5 μg/ml final concentration, and the six needed growth factors were aseptically added. For testing the osmotic sensitivity of protoplasts, TBAB (14) or nutrient agar (12) was used. For selection of the recombinants, the modified S medium containing 1.5% agar (Difco) was used, to which sterile DNase (5 μg/ml) and appropriate growth factors were added. For dilution of bacteria grown in hypertonic medium and also of protoplasts, SMMD (10) was used. Addition to this of 1% bovine serum albumin (Sigma) produced SMMAD. The letter "D" stands for DNase.

Fusion procedure. (i) With freshly grown cells. The parental cultures were grown separately with shaking at 30°C or 37°C in hypertonic NB or S to an absorbance at 650 nm of 0.6 to 0.8 (reading actually 0.3 to 0.4 in sucrose). After centrifugation at 7,000 rpm for 10 min at 11 to 18°C, they were resuspended in SMMAD to an absorbance at 650 nm of 2.

(ii) With activated cells. Cultures were grown as above, but after harvesting they were resuspended in 0.1 volume of modified PC at an absorbance at 650 nm of 8, subdivided in 0.5- to 0.6-ml aliquots, and kept frozen at −70°C. When needed they were quickly thawed (at 37°C), diluted 1:10 in PC containing 5 μg of DNase per ml, and shaken in shallow layers in a 37°C waterbath for 30 min. After centrifugation, cells were suspended in SMMAD to a calculated absorbance at 650 nm of 2. Cell samples were drawn as needed and plated on R medium for colony-forming unit monitoring.

Protoplastization. When not otherwise specified, cell suspensions in shallow layers were transferred to a 42°C waterbath and left for 1 to 2 min for temperature equilibration. Lysozyme (Miles Laboratories) in SMMD was added to a 100 μg/ml final concentration. After 30 min without shaking at 42°C, samples were plated on TBAB (shock sensitivity test) and R (regeneration test) and inspected under a microscope. The main protoplast suspensions were centrifuged and usually resuspended in 1/10 or 1/5 volume of SMMAD. Samples were sometimes again plated on RD. At this point protoplast suspensions were kept in shallow layers, without shaking, at room temperature, to be used as soon as feasible, since even if regeneration frequency does not decrease, that of subsequent recombinants may.

PEG treatment. The following PEG preparations of the indicated molecular weights, at 40% concentration (wt/vol) in SMMD, were used: 600 and 1,500 from Fisher Scientific Co., and 6000 from Matheson-Coleman & Bell. Parental protoplast suspensions were mixed at 1:1 (or other specified ratio), and 0.1 ml of this mixture was added to 0.9 ml of PEG solution and vigorously shaken. After specified times, usually 2 min of standing at room temperature, PEG-treated protoplast mixtures were diluted in SMMAD and plated on RD. After 48 h of incubation at 37°C, colonies were counted and/or replicated with sterile velvets upon specified selective media containing DNase. Colonies on these plates were counted after 24 to 40 h of incubation at 37°C.

RESULTS

Optimal conditions for growth, fusion, and regeneration. A series of background experiments led to the procedures specified in Materials and Methods. Cultures grown in medium containing 0.5 M sucrose, employed also by others (5, 13, 15), proved less sensitive to resuspension of the cells in sucrose, and more uniformly convertible to protoplasts. Reduction of the osmotically active sodium succinate of R medium to 2/3 of that specified by Wyrick and Rogers (16) proved a suitable compromise for protoplasts of S6 (stable) and S7 (sensitive to high levels). Calf serum instead of horse serum, supplementation with the six growth factors for the auxotrophic parents, and mineral salts were adopted after specific tests. The enhancing mixture of additional nutrients, glutamate-lysine-asparagine-valine, was developed as useful to increase the efficiency of plating of the parent bacterial strains in supplemented minimal media.

The presence of DNase is crucial principally for regeneration and selection steps, since it is known that DNA-mediated transformation and transfection do not occur after the loss of the cell wall (13, 15). Control experiments in this laboratory confirmed others' findings (5, 11) that

protoplasts are not susceptible to transformation, and also that mixed bacteria do not give rise to recombinants if DNase is present in regeneration and selection plates (10, 11). Presence or absence of DNase in PEG or buffers did not in fact change our results with protoplast mixtures, but its presence prevented borderline genetic exchange that could occur in bacterial mixtures (data not presented). Nevertheless, DNase was included in suspending buffers and media at all times throughout the experiments as a matter of precaution.

Certain additions to the protoplast dilution medium and regeneration environment led to substantial improvements and reproducibility. The presence of 1% bovine serum albumin in the hypertonic dilution and suspension buffer, SMMD, increased the yield of regenerants from 10-fold to several hundredfold (Table 2). Use of a low concentration, 0.5%, of gelatin in the R plates could improve it an additional threefold, to reach 100% for the same sensitive parent, S6. When there were short bacterial chains present, the viable protoplasts and consequent regenerant colonies could reach 1.5 or even 2 times the bacterial colony-forming units, suggesting that lysozyme was producing more than one viable protoplast from short chains of bacteria.

Temperature specifications at different stages established further modifications. It was postulated that temperature would affect the fluidity of the protoplast membranes and hence the ability to fuse. A differential between the temperature of bacterial growth and that of lysozyme treatment tended to increase the yield of regenerating bacteria and of recombinants (Table 3), confirming some of the findings with fungal protoplasts (2). Treatment with PEG at 0°C in our hands generally reduced both yields, in some contrast with earlier recommendations (11). Our preferred procedure accordingly employs growth at 30°C, lysozyme treatment at 42°C, and PEG treatment at room temperature.

Later we adopted from DeCastro-Costa and Landman (4) procedures for freezing of cultures

TABLE 3. *Effect of temperature on regeneration and recombination*[a]

Temperature (°C) during:			Frequency after PEG treatment[b]		
Growth[c]	Lysozyme treatment	Fusion (PEG)[d]	% Regenerants	Recombinants per 10^5	
				MLTU	ALθU
30	37	0	8.8	3.4	1.04
30	37	28	10.6	8.8	3.8
30	42	0	3.9	1.22	2.5
30	42	28	9.7	10.1	3.1
37	37	0	14.7	2.3	1.3
37	37	28	14.2	3.6	2.3
37	42	0	21	5.2	1.20
37	42	28	34	12.6	5.6

[a] General note for this and Tables 5, 7, and 9: if calculated as in the accompanying papers (7, 9), the recombinant frequencies would be twice as large, since those authors use as base one-half of the potential pairs—the fraction assumed to be (randomly) heterologous. Our calculation uses as base the minority protoplast number (even from the nominal 1:1 mixtures), not assuming the randomness of pairings. The input protoplasts are based upon the (highly efficient) regeneration of the parents, before they have been exposed to PEG.

[b] Regenerants are related to total input bacteria, recombinants to individual (minority) parent input. Marker symbols: M represents independence of methionine requirement; similarly, L, T, U, A, and θ indicate wild-type alleles for the leucine, threonine, uracil, purine (adenine), and tryptophan deficiencies of the parent strains.

[c] Parent cells S6 and S7 freshly grown in hypertonic medium; input for each: 1.9×10^8 (30°C culture), 1.65×10^8 (37°C).

[d] Molecular weight of PEG used, 600.

TABLE 2. *Effect of albumin and gelatin on regeneration*

Cells[a] (medium)[b]	Colonies recovered (per ml) from			
	S6 (*ura trp thr*)		S7 (*pur met leu*)	
	SMM[c]	SMM + BSA[c]	SMM	SMM + BSA
Input bacteria	2.5×10^7		1.0×10^7	
After lysozyme (R + gelatin)	5.2×10^5	8.9×10^7	1.7×10^6	9.9×10^6
After PEG treatment				
(R − gelatin)	5.7×10^4	3.4×10^7	1.1×10^6	6.5×10^6
(R + gelatin)	1.0×10^5	1.1×10^8	8.0×10^5	9.6×10^6

[a] Cultures of S6-A3 and S7-C20, freshly grown in NB containing Ca and Fe supplements, with 0.5 M sucrose.

[b] Plating medium R (16), modified as described in the text; counts after lysozyme and after PEG are regenerating protoplasts.

[c] SMM, sucrose-Mg-maleate; BSA, bovine serum albumin.

to be protoplasted, and an activation process that reached an optimum at 30 min for our strains.

Prolonging the exposure to PEG (Table 4) had an adverse effect upon regeneration but much less upon the number of recombinants, thus leading to an apparent two- to threefold increase in recombinants per survivor. Comparing the two experiments in the Table, one sees even more strikingly the independence of regeneration and recombination, the former generally being highest just when the latter is lowest.

The validity of the procedure of replicating from mass regeneration plates onto selective media in the basic procedure (10, 11) has been demonstrated in experiments in which replicas were made upon selective media in which one or the other of the parents can grow. Crowded and less crowded plates gave growth distributed as expected, and from the latter essentially complete or reasonable recoveries of parental cell types could be obtained (Table 5).

Kinetics of protoplast fusion. Having some evidence that the process of fusion as measured by recombination was critically affected by crowding, diffusion, and other local conditions on the regeneration plate, we diluted PEG-treated protoplasts ten-fold in SMMAD and allowed them to remain at room temperature for various intervals. The purpose was to leave the protoplasts in an environment closely resembling that in which fusion is initiated, but with the PEG reduced to a concentration at which "toxicity" is not noticeable, possibly to "complete" the fusion process. We did not observe a significant increase of regularity or yield of regeneration or recombination. Subsequently we learned from Schaeffer and associates that they had observed progression of certain cytological and fusion-induced phage complementation effects occurring in post-PEG incubation of treated protoplasts in hypertonic medium at 37°C (data now available for recombinants also; 7, 9). Stimulated by their findings, we extended our experiments to the higher temperatures and richer environments.

When we incubated PEG-treated protoplasts in hypertonic minimal medium at 37°C, we found that regeneration of bacterial forms was not greatly affected; however, when the minimal medium was supplemented with all of the six factors required by the two auxotrophs, regen-

TABLE 4. *Effect of length of PEG treatment*

Expt	Duration of PEG[a] treatment (min)	Regenerants[b] (%)	Recombinants found per ml[c]		Recombinant frequency[d]	
			MLTU $\times 10^{-3}$	ALθU $\times 10^{-3}$	MLTU $\times 10^{-4}$	ALθU $\times 10^{-4}$
I	2	20	1.2	0.86	17	12.5
	4	14	1.7	1.3	35	26
	8	15	2.2	0.91	41	17
II	1	5.8	12.2	4.7	47	18
	2	5.4	17.2	2.65	71	11
	6	2.1	7.6	3.0	81	32
	17	1.3	8.6	2.0	149	35

[a] Molecular weight of PEG: experiment I, 1,500; experiment II, 600.

[b] Regenerants, based on total input protoplasts (experiment I: S6, 1.42×10^7; S7, 3.5×10^6; experiment II: S6, 4.4×10^7; S7, 8.9×10^7 per ml).

[c] Symbols as in Table 3, footnote b.

[d] Recombinant frequency referred to minority parent actually regenerated from the same treated population.

TABLE 5. *Fusion: regeneration, parent recovery, and recombination*

Expt[a]	Time (min)	Relative protoplast concn	Input protoplasts ($\times 10^6$)		Regenerants $\times 10^6$ Mixture	Recovered parents[b] ($\times 10^6$)		Recombinant frequency[c] $\times 10^{-4}$ (MLTU)[+]
			S6	S7		S6	S7	
I	2	1	42.5	10.4	49.1	16	18	0.68
		1:4	10.6	2.6	13.5	7.3	7.2	1.09
		1:9	4.2	1.5	3.2	2.2	2.2	2.8
II	2	1	42.5	10.4	7.3	5.2	3.8	4.1
		1:4	10.6	2.6	2.0	1.7	1.5	1.7
		1:9	4.2	1.5	0.9	0.7	0.5	2.8
III	2	1:3	14.2	3.5	3.5	2.1	2.7	3.3
	4		14.2	3.5	2.5	1.5	1.8	4.9
	8		14.2	3.5	2.7	1.5	1.3	6.3

[a] Experiment I, PEG 6000; experiment II, PEG 600; experiment III, PEG 1500.

[b] Replicas on parental selective media from regeneration plates bearing appropriately diluted PEG-treated bacterial mixtures. Note that the sum of the two parent values approximates that of total regenerants.

[c] Recombinant frequency expressed per input protoplast, minority type. For symbols, see Table 3, footnote *b*.

eration diminished steadily with incubation (Table 6). This loss of ability to regenerate was even more striking during incubation at 30°C in hypertonic full-nutrient broth after PEG treatment. Recombinants behaved rather differently. In the minimal medium they (two different two-crossover types) were decreased moderately and rather unevenly; incubated in supplemented minimal medium they fell steadily. In the nutrient broth, recombinants decreased steadily at 30°C, but at 37°C they fell rapidly to around 25% and then remained stable. As a result, the recombinants could number as many as 0.5% (last column and line of Table 6) of the bacteria recovered from a 90-min post-PEG incubation—but these constituted by now only 0.9% of the original viable protoplasts, whereas 21% of them could be regenerated directly after PEG treatment. Thus, in hypertonic nutrient medium at 37°C, gradually more and more of the protoplasts lost the ability to regenerate a cell wall, but a disproportionately large part of those that remained able to do so were recombining two parental genomes.

Protoplast concentration and kinetics of fusion. The rather low frequency (about 10^{-4}) of genetic recombinants, and yet high proportion of multiple recombinants among them (10), led us to ask whether the limiting factor is the rate of protoplast-with-protoplast contact and fusion, or some limiting process of chromosomal interaction or expression in diploid cells. If it were the former, then recombination rates should be approximately proportional to the second power of total parental protoplast concentration.

Assaying two or three classes of recombinants from parents S6 and S7, we uniformly found that recombination yields fall off only slowly with total protoplast concentration. Table 7 presents three different experiments, among which recombination happened to range from as low as 0.2 to as high as 16 per 10^4 input protoplasts. It can be seen that neither of the independent classes of genetic recombinants showed a systematic change with protoplast concentration when expressed in relation to minority parents present (the parental ratios were approximately equal, ranging in ratio from 1.2 to 1.6). This calculation with reference to the minority protoplasts automatically normalizes the observed yields for both the protoplast concentration and also for the maximal number of pairs that could produce recombinants (if all pairings were heterologous). The data do show that at the highest concentrations used, approximately 3×10^8/ml, recovery of recombinants is not optimal. On the basis of these and similar experiments, we subsequently adopted as standard a two- to three-fold-lower cell concentration for the PEG fusogenic treatment.

TABLE 6. *Incubation in hypertonic growth media after PEG treatment*

Incubation		Regenerants $\times 10^7$	Recombinants found		Recombinants per survivor[b] ($\times 10^{-4}$)	
Medium[a]	min		(MLTU)$^+$ $\times 10^3$	(AL$\emptyset$U)$^+$ $\times 10^3$	(MLTU)$^+$	(AL$\emptyset$U)$^+$
Minimal	0	4.9	6.8	1.64	5.6	1.4
	30	4.2	2.3	1.05	2.2	1.0
	120	5.4	2.9	1.75	2.2	1.3
Minimal + 6F	15	4.4	3.0	2.7	2.8	2.4
	75	2.5	2.5	2.9	4.0	4.7
	90	2.0	1.3	0.9	2.6	1.8
Nutrient broth, 30°C	0	6.5	10.8	4.7	4.5	2.0
	30	4.0	4.8	1.8	3.3	1.25
	60	2.1	2.9	1.2	3.8	1.6
	105	0.29	0.97	0.43	9.2	4.0
Nutrient broth, 37°C	15	5.6	2.8	1.4	1.4	0.66
	45	1.0	1.6	1.0	4.2	2.5
	75	0.38	1.2	0.78	8.8	5.5
	90	0.28	2.4	1.6	23.3	51.5

[a] Media: "Minimal," modified S; 6F, supplemented with all six auxotrophic requirements. Cells from frozen cultures, grown in S, were supplemented with their own requirements during growth. Input protoplasts: (minimal medium experiment) S6, 9.7×10^7; S7, 3.2×10^7; (nutrient broth experiment) S6, 2.6×10^8; S7, 4.4×10^7. Incubation was at 37°C except where noted. Molecular weight of PEG, 600.

[b] Recombinants are referred to minority parent actually regenerated from the same treated population. For symbols, see Table 3, footnote *b*.

TABLE 7. *Protoplast concentration and recombinant yield*

Expt	Protoplast concn[a]	% Regenerants	Recombinant frequency[b] × 10^{-4}	
			$(MLTU)^+$	$(AL\emptyset U)^+$
I	1	17	4.8	2.6
	1:2	17	15.6	5.6
	1:4	21	10.6	3.3
	1:10	22.5	4.5	2.3
II	1	33	0.53	0.17
	1:2	22	0.73	0.43
	1:4	24	0.64	0.29
	1:10	26	0.23	0.10
III	1	88	0.96	0.95
	1:3	79	0.44	0.17
	1:9	98	0.83	0.79

[a] Initial concentrations (1) of S6 and S7 parental protoplasts respectively (×10^8): I, 1.00, 1.62; II, 2.2, 1.72; III, 0.67, 0.81. Molecular weight of PEG used: I and III, 600; II, 1500.

[b] Comments and symbols from Table 3 apply (Table 3, footnotes *a* and *b*).

When the data are expressed relative to the yields at highest concentrations (Table 8), it can be seen that they fall much closer to a linear than a quadratic function of cell concentration. The essentially first-order response over a 10-fold range of protoplast concentration strongly indicates that protoplast contact and fusion per se is not likely to be the limiting process determining the frequency of genetic recombinations. Recombination must depend more directly upon some other stage in the total process.

Parental cell ratios and recombination. Since protoplasts of the two parental cell lines do not always display equal regeneration rates, and since they might also be expected to have differing avidity for fusion, we measured recombination at different parental ratios. This would also constitute another measure of the possible limiting role of effective cell collisions (see Discussion).

As can be seen from Table 9, recombination rates do not depend characteristically upon either parent: when these data are arranged in order of absolute majority/minority ratios, disregarding which parent is in excess, the recombinant frequencies fall rather smoothly in increasing order, approximately proportional to that ratio. We show recombination frequencies in relation to the minority protoplast titer, a measure of the maximal number of heterologous pairs that could have been created. It should be noted that, since the frequency and the parental ratios are approximately proportional and both are related to the minority titer, then the yield of recombinants per majority protoplast is essentially constant (Table 9) for the whole range of parental ratios and for both types of recombinants, another evidence of simple proportionality to overall protoplast concentration.

DISCUSSION

The recommended procedures have led to 100% recovery of regenerated bacteria from input protoplasts. The features of a low gelatin content in the regeneration plates, bovine serum

TABLE 8. *Linear response of recombination to protoplast concentration*

Relative concn[a]		Regenerants, relative[b]	Recombinants, relative[b]	
(S6 + S7)	(S6) × (S7)		$(MLTU)^+$	$(AL\emptyset U)^+$
1.0	1.0	1.0	1.0	1.0
0.5	0.25	0.52	1.6	1.1
0.25	0.06	0.31	0.54	0.32
0.10	0.01	0.14	0.10	0.092
0.50	0.25	0.33	0.69	1.27
0.25	0.06	0.18	0.30	0.44
0.10	0.01	0.08	0.044	0.061
0.33	0.11	0.30	0.15	0.06
0.11	0.012	0.12	0.097	0.092

[a] (S6 + S7), Linear concentration relation; (S6) × (S7), the quadratic effect expected if limited by S6 + S7 collisions.

[b] The absolute yields of regenerants or recombinants, related to those from highest concentrations of protoplasts used for the 1:1 mixtures of Table 7. For symbols, see Table 3, footnote *b*.

TABLE 9. *Response of recombination to parental ratio but not to individual parent that is in excess*

Majority/minority[a]	Ratio, S7/S6	Recombinant frequency per 10^5 input protoplasts			
		Relative to minority		Relative to majority	
		$(MLTU)^+$	$(AL\emptyset U)^+$	$(MLTU)^+$	$(AL\emptyset U)^+$
1.17	0.86	1.70	1.70	1.46	1.45
2.1	2.1	5.0	3.5	2.3	1.66
2.9	0.34	5.1	3.2	1.75	1.08
4.3	4.3	8.7	4.0	2.0	0.94
5.8	0.17	9.3	4.4	1.59	0.75
11.7	0.09	24.0	13.5	2.05	1.16
1.2	1.2	4.6	6.1	3.8	5.1
1.7	0.60	2.5	5.1	1.5	3.1
3.0	3.0	4.9	10.4	1.6	3.4
3.3	0.30	9.7	12.7	2.9	3.8
8.3	0.12	14.8	18.1	1.8	2.2

[a] Majority/minority ratio was determined from input protoplasts and varied; were total protoplasts essentially constant.

albumin in suspension media, and other modifications of the environment and temperature for growth, preservation, and fusion of the bacteria have also served to stabilize and make more reproducible the results from experiment to experiment. Serum albumin has previously been used for stabilization of bacterial protoplasts (17). In agreement with published (3, 10, 11) and unpublished findings of various workers, we find the polymer size of the PEG to be relatively unimportant, at least above molecular weight 400.

In answer to our original question, recombination frequencies seem to remain uninfluenced by or unrelated to the average recovery of regenerated bacteria; when the latter was improved 10- to 20-fold as stated, there was no corresponding or even noticeable increase in recombination rates. In fact, we continue to observe recombination rates as before (10, 11), approximately equivalent to those obtained by our French colleagues (7, 8). The recombination rates for the *Bacillus megaterium* system, in which bacterial regeneration is essentially complete, are similar in magnitude (5).

Furthermore, under various experimental conditions, we found that the number of recombinants may remain virtually constant (Tables 4 and 6), or even be moderately increased (Tables 5 to 7) just when the average regeneration is strikingly decreasing. This was seen also in various experiments not reported here, including a series with different exposures of protoplasts to different PEG preparations and other fusogenic agents. The evidence seems unmistakable that regeneration and recombination rates very often vary independently. Sanchez-Rivas et al. (9) have also referred to their impression that higher fusion rates are accompanied by lower regeneration, and show data for two different media wherein both complementation and recombination moved in the opposite direction from regeneration frequency.

We are obliged to conclude that the average regeneration frequency cannot safely be used as a base to calculate an expected recovery of recombinants (9), since it may exaggerate the deficit in yield (e.g, 20-fold for 5% regeneration), inasmuch as recombinants and nonrecombinants do not seem to survive randomly. Likewise, we do not recommend calculating recombinants per post-PEG survivor, although we chose to do so in Tables 4 and 6 to demonstrate the apparent enrichment of recombinants as the population declined.

It should be noted that recombinants are replicated from crowded (low-dilution) regeneration plates, whereas regenerants are counted directly at high dilutions. If one supposes the crowded plates to furnish a competitively disadvantageous environment, then we may ask whether regeneration on such plates is suppressed more, or less, than is the formation of recombinants (which are somewhat suppressed in the heaviest inocula) (10, 11).

Of course, a decreasing regeneration and increasing recombination might both result from aggregation to form multicellular units; however, light microscopic examination of PEG-treated protoplasts shows no sign of irreversible aggregation commensurate with the low regeneration sometimes obtained. Electron microscopic monitoring of such suspensions (7) essentially shows the small size of the complexes that do occur.

It seems clear that some 80 to 90% of the PEG-treated protoplasts are able to regenerate their cell walls under conditions which we have partly learned to control, but that they are easily lost under various borderline conditions. One of those conditions can be the post-PEG incubation in nutrient medium (Table 6). On the other hand, Fréhel et al. (7) observed a relative stability of regenerants during such incubation; apparently their initial average of 6% regeneration never included the labile major fraction of protoplasts and so did not fall lower. We can reconcile all of their results with ours, then, if we also suppose that their hypertonic nutrient broth was sufficiently dilute (10^{-1}) in nutrients to behave more like our minimal media, preserving the recombinants with no substantial loss. Although we observed a partial loss of recombinants during post-PEG incubation, our results do not conflict with the conclusion (7, 9) that some steps of cytological fusion and complementation do occur under these conditions. It is, of course, also possible to attribute these discrepancies between our results to the use of different strains or media.

We propose that early and efficient restoration of cell metabolism will tend to induce a diploid protoplast to form a cell wall and divide before its two chromosomes have been replicated. If this is true, complete or rich media and prompt setting up of vigorous metabolism would tend to encourage segregation and reduce subsequent opportunity for recombination. The same conditions may well be destructive if they lead to growth and expansion before the protoplast has covered itself adequately with a new cell wall, and so they may also lead to low bacterial regeneration. This may happen, e.g., during post-PEG incubation in nutrient liquid media (Table 6)—regeneration being difficult or impossible to attain, as many workers have attested, in liquid medium. Since it is probable that actual recombinants (and probably postfusion diploids and polycaryotes) are subject to

metabolic lag and readjustment, this lag may serve as the basis for their ability to survive relatively better and appear to be selectively enriched during post-PEG incubation in rich medium. It is also consistent with their late appearance as colonies on selection plates (5) or as transferable growth on regeneration plates (10, 11).

In 1975, before PEG was employed, one of the authors (R.D.H.) with Schaeffer obtained, from spontaneously fused protoplasts, mixed clones which continued to segregate parental or other cell types in a complex manner on minimal medium. The observations of Fodor and Alföldi with *B. megaterium* (5) also suggested that supplemented medium did not sustain the replication of the possibly diploid (segregating) fused cells as long as did minimal media.

Our kinetic studies with differing lengths of exposure to PEG and with varying individual and total protoplast concentrations indicate strongly that protoplast collision and primary contact is not the limiting factor determining in itself the number of genetic recombinants obtained. Recombination seems to be a linear, rather than quadratic, function of the total or the majority protoplast population present, from which it can be concluded that fusion events are always adequate to produce substantially more potential recombinants than are registered.

The strong effect of majority/minority ratio, irrespective of which parent is in excess, upon recombination yield, shows in a direct and physiological way that both parents are equivalent partners in their genetic contributions. This conclusion had been reached by Lévi et al. (8) using streptomycin, and by Fodor et al. (6) using mild heat, to kill one or the other of the parents in reciprocal fusion experiments.

As suggested above, we believe that the occurrence and expression of recombination will be inversely related to the metabolic activity of the potential "diploids" during their first hours, and our most recent results with more direct selection, not yet published, seem to support this view. In the meantime, Schaeffer and colleagues have obtained rather direct evidence (7, 9), kindly made available to us, showing that cytological fusion and fusion-induced phage complementation do indeed occur in as many as 5 to 50% of the potential pairs in similar *B. subtilis* systems. We conclude, then, that the great majority of postfusion pairs segregate parental cells rather quickly, perhaps even during regeneration, and that early and complete regeneration is not conducive to preservation of "diploidy" and opportunity for recombination. We predict, and will investigate the possibility, that selective adjustment of the environment of the postfusion cells may favor stabilization of diploids and thereby also increase the opportunity to produce genetic recombinants.

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