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Electron Microscopic Study of *Bacillus subtilis* Protoplast Fusion

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When protoplasts derived from sporulating cells of *Bacillus subtilis* were fused by exposure to polyethylene glycol (PEG) and fixed immediately thereafter, protoplasts with two enclosed prespores could be seen by electron microscope. The number of fusion events was greatly increased, and multiply fused protoplasts appeared, when the PEG-treated suspension was diluted in hypertonic broth and reincubated before fixation. This post-PEG incubation effect is taken to indicate a fusion mechanism of two steps: a short, PEG-dependent step of membrane activation, followed by a slow, metabolism-requiring step completing fusion. When prespore-bearing protoplasts from two genetically different strains were mixed and fused, the extent of fusion could also be followed by counting clones of recombinant bacteria. Maximal from the start, their number (1% of each parent type protoplast present) was unaffected by post-PEG incubation. Fusion in this case is apparently completed after plating on the wall-regeneration medium. After optimal post-PEG incubation, the majority of the protoplasts were seen to participate in fusion, and the cytological fusion observed, corrected for wall-regeneration frequency, accounted quantitatively for the prototrophic bacteria eventually recovered. These results are in good agreement with those obtained independently by Sanchez-Rivas and Garro (J. Bacteriol. **137**:1340-1345, 1979).

With gram-positive species, stable prototrophic bacteria are formed in the continued presence of DNase after fusion of protoplasts derived from two complementary polyauxotrophic strains, followed by cell wall regeneration (2, 6, 20). With *Bacillus subtilis* most of these prototrophs are haploid recombinants (20), and the highest frequency recorded for a particular class of these is 0.03% (21), or approximately 10% of the regenerants. No prophage induction occurs when one of the parental strains is lysogenic (20), and the fertility of crosses made between viable and killed cells does not depend on which of the parental cells were killed (3, 9). For these reasons the observed recombinants are believed to result from cell fusion, a process in which parental cytoplasms are mixed and whole parental chromosomes are made available to multiple exchanges, rather than from a mere gene transfer. Thus, recombinant frequency ought to depend not only on fusion frequency, but also on the frequency of wall regeneration and on the genetic recombinations required to produce progeny viable on a given selective medium.

In this work fused cells have been visualized and their frequency determined directly by electron microscopic (EM) examination of *B. subtilis* protoplasts that had just been treated with polyethylene glycol (PEG). In an accompanying paper (19), fusion frequency was assayed among PEG-treated protoplasts by a complementation test.

MATERIALS AND METHODS

Bacterial strains. Protoplasts from a single strain, MO11T (*trpC2 spoIV11T*; 8), were used whenever fusion was to be followed by EM examination only. When recombinants were to be counted, mixed protoplasts were used, from MO83U (*trpC2 spoIII 83U*; 8) and MO209 (*leu-8 spoIII83U*). The latter strain was derived by transformation of MO83U recipient cells by excess DNA from S1, a Trp^+ Leu^- strain (20), and by picking a Leu^- Spo^- clone among the Trp^+ transformants obtained.

Culture conditions. Two procedures were used for growth and sporulation. In procedure I, cells were grown in a suitable nutrient broth (22) and harvested 3.5 h after growth had stopped. Procedure II was that of Sterlini and Mandelstam (23), in which cells were harvested after a 3.5-h incubation period in the resuspension medium. For both procedures incubation was at 42°C with shaking, and prespores had formed at the time of harvesting.

Formation and fusion of protoplasts. Digestion of the cell wall by lysozyme in SMM buffer and fusion of the resulting protoplasts (or their 1:1 mixture) by exposure to PEG have already been described (20, 21). Keeping the unshaken protoplasts overnight at room temperature in the presence of lysozyme before they are centrifuged and PEG treated has no deleterious consequence and has been found convenient in this study.

Postfusion incubation. Immediately after their 1-min exposure to PEG, protoplast suspensions were diluted 10-fold and incubated without shaking at 37°C (post-PEG incubation, or PPI) in PPI medium. Samples taken at various times were fixed and processed as described below. PPI medium is SMM buffer to which 0.1 volume of LB broth (12) without glucose is added.

Selection of prototrophic recombinants. Recombinants were selected as described (20, 21) by transferring from a hypertonic nutrient agar medium, on which protoplasts had regenerated their walls and grown as bacterial colonies, by replica plating onto a minimal nonhypertonic selection medium. DNAse (5 μg/ml) was present in each of these media.

Processing of the fused protoplasts for EM examination. Protoplasts were prefixed with 1% glutaraldehyde (18) in 0.2 M cacodylate buffer (pH 6) containing 0.5 M sucrose and 10 mM Mg^{2+}. After centrifugation, pellets were resuspended in nutrient broth containing 2.5% agar and 0.5 M sucrose (24). Blocks of agar, kept overnight in the presence of 2.5% glutaraldehyde in the same buffer, were washed for 1 h with Michaelis Veronal buffer (17) and postfixed for 1 h with 1% sodium tetroxide and then with 1% uranyl acetate (17). Dehydration was with acetone, and embedding was in Epon (10). Sections, cut with a diamond knife on a Sorval microtome, were stained with lead citrate (14) and observed with a Siemens Elmiskop 101 electron microscope.

Calculation of cytological fusion frequency. When fusion events were counted by EM examination, their frequency was expressed per pair of protoplasts present ($0.5n$ when n protoplasts are considered), since two protoplasts are required for one fusion. For the sake of simplicity, this has been applied even in the case of multiply fused cells, where it is at best an approximation.

Estimation of the number of pRFU from EM data. Potential recombinant-forming units (pRFU) are fused cells containing at least one of each parental chromosome. The large majority of the fused cells observed in the EM (even after PPI) derive from two or three protoplasts. One-half of the first kind of fused cells and three-fourths of the second kind are expected to be pRFU. Actual calculations are found in the Tables.

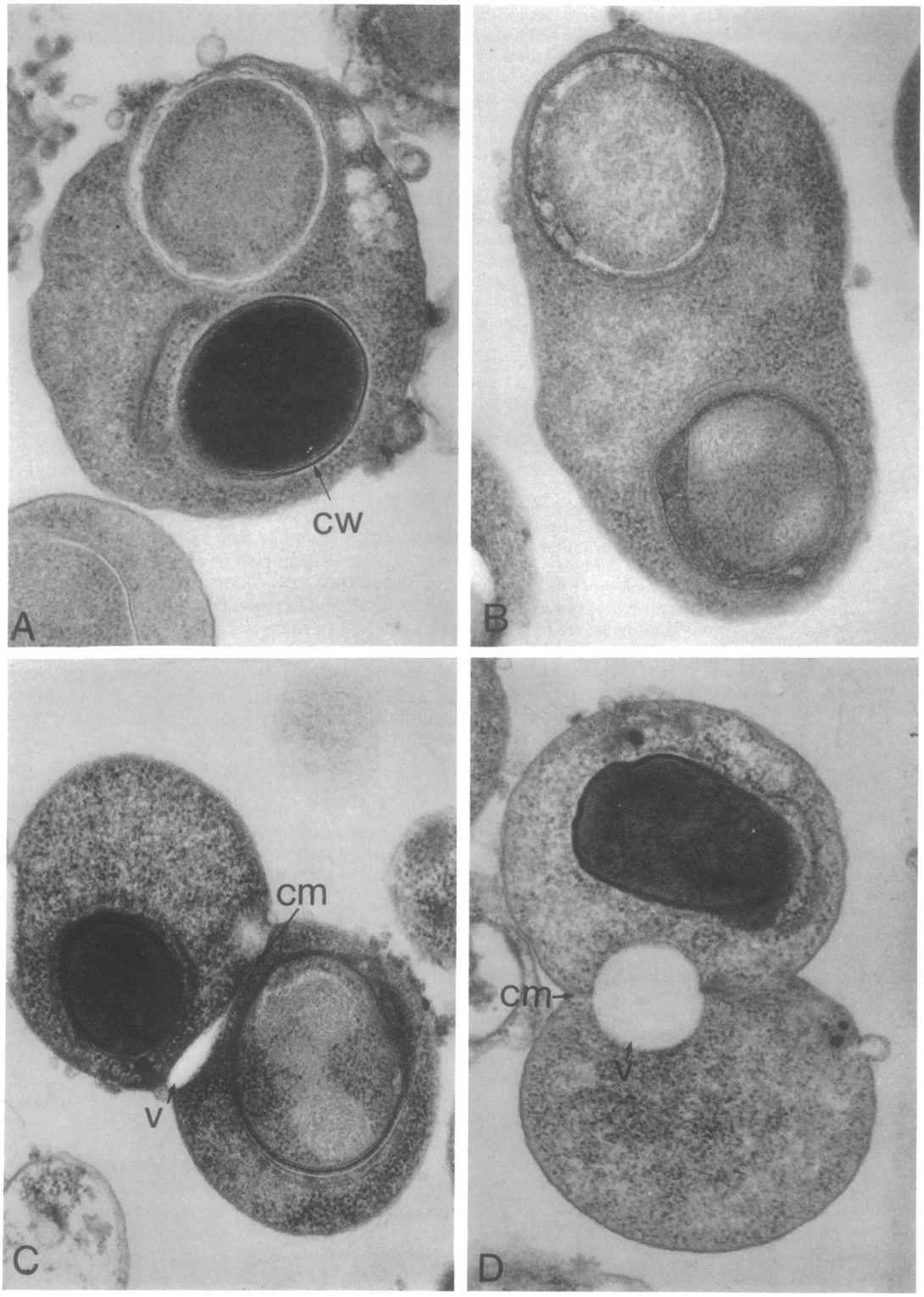
RESULTS

Preliminary technical considerations. Protoplasts from growing Spo^+ cells were first examined by EM in negative-contrast preparations, and their increased size was initially considered a possible criterion for their fusion. Due to the relative opacity of these protoplasts, however, distinction of fused protoplasts from merely aggregated ones was not easy, suggesting that previous sectioning would be an improvement. Disregarding aggregation was easier with sections, due to better visibility of cytoplasmic membranes. With the diameter of the sectioned cells depending on the tangentiality of the cut, however, apparent size is no criterion for fusion. For this reason we chose to use prespores as cell markers, and looked for protoplasts carrying at least two of them.

Thus, it became important to start with cultures with as high a fraction of prespore-bearing cells as possible. This is best achieved using cells genetically blocked at stage III or IV of sporulation and incubating them long enough to allow the more slowly sporulating cells to catch up with the faster ones, which, being blocked, are unable to proceed further, as Spo^+ cells would do (16). These considerations account for the choice of the strains used, and for the late harvesting time. Incubation at 42°C was a mere convenience, to shorten the experiments.

Visualizing cell fusion with the help of prespores. When the experiment was done with MO11T cells grown according to procedure I, fusion figures were obtained (Fig. 1 and 2). Spherical or ovoid protoplasts, showing two enclosed prespores with no trace of cytoplasmic membrane between them, can be seen in Fig. 1A and B (the latter is from a biparental cross). Some cell pairs were apparently caught at an earlier stage in the fusion, as suggested by their bispherical form (Fig. 1C and D and 2A). This early stage is also characterized by a partially dissolved membrane and by what appears to be an empty vesicle in place of the missing membranes. The vesicles were always symmetrically placed with respect to the fusion plane, and may be in some way involved in the dissolution of the membrane. The bispherical forms showed two entrapped prespores (Fig. 1C), one only (Fig. 1D), or even none at all (Fig. 2A). This is explained partly by the (variable) fraction of cells in any culture that do not initiate sporulation, and partly by the fact that not every prespore present in the cell is seen in a given section. Multiple fusions (Fig. 2C and D) also occurred (see below).

Direct EM determination of fusion frequency and effect of a post-PEG-fusion incubation period. In the experiment just described, the cells were fixed immediately after a 1-min exposure to PEG. Under these conditions only a few percent of the protoplasts are seen to take part in fusion, and hardly any multiple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fusions are observed (see Tables 1 and 2, 0-min PPI).

With somatic cells of higher organisms, fusion has been said to take place mostly after the PEG is diluted out (11, 13), and multiple fusion seems to be frequent (15). An additional period of incubation at 37°C of the PEG-treated protoplasts was therefore introduced after a 10-fold dilution into PPI medium (the resulting PEG concentration, 4%, is ineffective in promoting fusion; 21). After various times PPI, protoplasts were fixed and examined as before.

The results of such an experiment are given in Table 1. Fusion frequency was seen to increase with time up to 60 min postincubation, when it reached 65%, with less than one-half of the protoplasts seemingly unfused. This high value is partly due to the appearance, mostly during the first 30 min, of multiple fusions.

A new cell type appeared during PPI, which is illustrated in Fig. 2B. Since it was not clear whether this type results from fusion or not, an experiment was performed in which PEG treatment was omitted. No fusion figures were found in this experiment, but cells of the new type appeared as before, and with the same kinetics (data not shown). Some kind of degeneration of the protoplasts in PPI medium can thus be responsible for this unexpected aspect, and since it is clearly unrelated to PEG treatment or fusion, protoplasts showing it were considered unfused and counted as such.

Some lysis detectable by turbidimetry, which occurs after 60 min postincubation, and a process of prespore ejection observed at such late times (data not shown), are presumably responsible for the decreasing numbers of fused protoplasts counted after 60 min (Table 1). Practically the same maximal fusion frequency (57% at 60 min) was observed with cells prepared by procedure II (data not shown).

Relationship between cytological fusion and recombinant frequency and the effect of PPI. The frequencies of cytological fusion and of prototroph production were both determined in crosses between MO209 and MO83U cells. Parental bacteria were grown according to procedure I, and the protoplast suspensions (2×10^8 cells of each parent per ml) prepared from them were subdivided and worked upon separately, but as simultaneously as possible, in two laboratories.

In one of them, equal volumes from the two suspensions were mixed, and the mixture was exposed to PEG for 1 min. This exposure was immediately followed by a 10-fold dilution in PPI medium, and incubation at 37°C was resumed. At various times thereafter samples were removed, fixed, and processed as described in Materials and Methods. Cytological fusion frequency increased sharply during the first 30 min postincubation, to a maximum of 43% (Table 2).

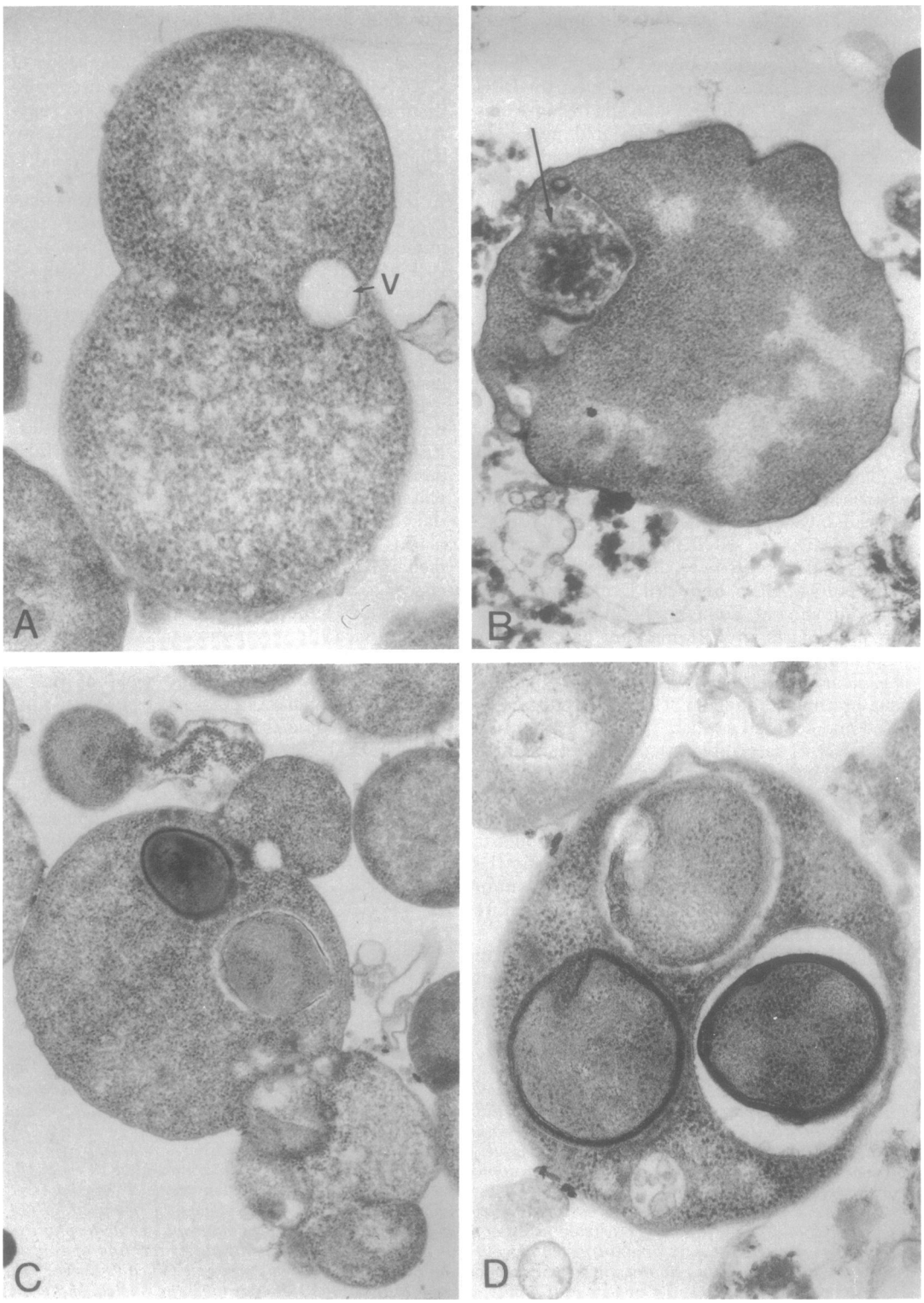
In the other laboratory a mixture of the same protoplast suspensions was made, and it was PEG treated, diluted, and incubated exactly as described above, but then the samples were plated for regeneration and prototrophic bacterial colonies were counted as usual, after replica plating from the regeneration to the selection medium. The same regeneration test, applied also to each of the unmixed parental protoplast suspensions, supplied the desired information on their capacity to regenerate cell walls.

The efficiency of wall regeneration by the parental protoplasts and the yield of prototrophic recombinants obtained from their mixture remained constant during the 60 min PPI (Table 3). The latter result is particularly striking when compared with that in Table 2, since the increase in fusion figures automatically means that the estimate of pRFU would increase during this incubation. Thus, at 30 min postincubation the pRFU would be expected to be 3.8×10^7, or nearly 10% of the total population.

DISCUSSION

Genetic evidence presented in earlier papers on *B. subtilis* (9, 20) and in recent papers on *Streptomyces* by Hopwood et al. (6, 7) strongly suggested that the recombinant-forming process

FIG. 1. *Thin sections of protoplasts fixed immediately after PEG treatment. (A) Fused protoplast from M011T cells. Two prespores are seen enclosed within this protoplast: the one in which a cell wall (CW) has formed is at stage IV, whereas the other, surrounded by a mere double membrane, is at stage III (16). No remains of cytoplasmic membranes, and no vesicle, are visible between the two prespores (×47,000). (B) Two prespores at stage III are enclosed in this protoplast, from a 1:1 mixture of MO209 and MO83U cells. No membrane remains, and no vesicle can be seen in the cytoplasm. As in (A), fusion seems to be completed (×37,000). (C) and (D) Although derived from the same sample of MO11T fused cells as the one used for (A), these protoplasts seem to have been fixed at an earlier stage of fusion. Both photographs show bispherical protoplasts, in which the individual parental cells can still be clearly recognized. At the line of contact between them, remains of cytoplasmic membranes (cm) and an apparently empty vesicle (V) can be seen. Two enclosed prespores at stage IV appear in (C), only one in (D). In all four photographs, the nuclear material, dispersed in the cytoplasm, can hardly be seen. (C) ×40,000; (D) ×45,000.*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triggered by exposure of bacterial protoplasts to PEG was initiated by cell fusion rather than genetic transfer. Deduced initially from the absence of prophage induction after fusion between lysogenic and phage-sensitive cells (20), the mixing of the parental cytoplasms involved in the fusion process has now been visualized directly by EM examination (Fig. 1 and 2). Since by this method high frequency of cytological fusion could be obtained, an attempt was made to quantitatively relate protoplast fusion with the yield of bacterial recombinants. As this discussion will show, when the frequency of cell wall regeneration is taken into account, the prototroph yield can be predicted fairly accurately from the frequency of cytological fusion, determined after PPI as prescribed. Various aspects of the work will now be discussed.

(i) When PEG-treated protoplasts were diluted 10-fold in hypertonic broth (PPI medium) and incubated with gentle shaking before they were fixed with glutaraldehyde, the frequency of cytological fusion was seen to increase by a factor of nearly 7, when after 30 to 60 min at 37°C a maximum was reached (Tables 1 and 2). Maximal values higher than 50% have repeatedly been noted (similar fusion frequencies have simultaneously been obtained by Sanchez-Rivas and Garro [19], following a quite different approach). Admittedly, our calculations do not take multiple fusions into account, and therefore provide underestimates for incubated samples. There is no easy way around this difficulty, which has been well discussed by Ringertz and Savage (15), particularly when one is dealing with bacterial cells in which "nuclei" cannot easily be counted.

TABLE 1. *Effect of PPI on cytological fusion frequency of M011T protoplasts*[a]

PPI (min)	A	B1	B2	C	D	E	F
0	513	26	0	26	52	565	9
30	498	54	73	200	327	825	48
60	342	88	78	244	410	752	65
90	353	62	70	202	334	687	59
120	365	48	49	146	243	608	48

[a] Protoplasts were derived from M011T bacteria only, grown according to procedure I. A, Unfused protoplasts; B, fusion figures between two (B1) or more (B2) protoplasts; C, fusion events; D, protoplasts involved in fusion; E, total protoplasts observed; F, fusion frequency percentage. A, B1, and B2 were counted directly by EM; C, D, E, and F were calculated as follows: C = B1 + 2B2; D = 2B1 + 3B2; E = A + D; F = 100 × (C/0.5E). The technical difficulty of detecting all fusion events in a sample must mean that the counts B1 and B2 (and C, D, and E, derived from them) are underestimated. C is underestimated further by the fact that the rare fusion figures seen as involving more than three protoplasts are counted as involving only three.

TABLE 2. *Effect of PPI on cytological fusion in a biparental and on pRFU's*[a]

PPI (min)	A	B1	B2	E	F	G	H (×10⁶)
0	632	21	0	674	6	10.5	6
30	429	80	34	691	43	65.5	38
60	435	73	32	677	41	60.5	36

[a] Protoplasts were derived from M083U and M0209 bacteria grown according to procedure I. After PEG treatment, their 1:1 mixture was incubated at 37°C in PPI medium for various times, prior to fixation with glutaraldehyde. A, B1, B2, E, and F, see Table 1, footnote *a*; G, pRFU expected among the observed protoplasts; H, pRFU expected per milliliter of PEG-treated suspension (4 × 10^8 protoplasts). Since half the number of the B1 figures and three-fourths the number of the B2 figures are pRFU (containing at least one copy of each parental chromosome), G = (B1/2) + (3B2/4).

TABLE 3. *Effect of PPI on the frequency of prototrophic bacteria*[a]

PPI (min)	% Regeneration of parental protoplasts		Prototrophic recombinants found per ml (×10⁶)
	M0209	M083U	
0	10	2.0	4
30	12	2.5	5
60	8	1.7	3.6

[a] The mixed protoplast suspension is the same as in Table 2, but at various times of PPI it was used to count prototrophic recombinants. At each time, regeneration of each of the parental protoplast suspensions was also measured.

FIG. 2. *Thin sections of PEG-treated protoplasts from MO11T cells. (A) No prespores are seen in these fusing protoplasts, but the bispherical shape and the presence of an empty vesicle (V) at the contact line are unmistakable signs of early fusion (×42,000). (B) Prior to fixation, protoplasts in this case had been incubated at 37°C for 60 min in PPI medium after PEG treatment. A peculiar type of protoplast, like the one shown here, appeared during this incubation. The irregular cell shape, the apparent dispersion of the nuclear material into distinct peripherally located patches, and a prespore-like inclusion (arrow) suggest a degenerative origin for this unusual appearance (×37,000). (C) and (D) Multiple protoplast fusions. Some of the fusing protoplasts can still be distinguished in (C). In (D) two completed fusion events have led to one protoplast containing three prespores. (C) ×16,000; (D) ×45,000.*

(ii) One major finding in this work is that the fusion-boosting effect of PPI, clearly seen when cytological fusion is followed against time (Tables 1 and 2), is not apparent when recombinants are counted (Table 3). This discrepancy would be accounted for if fusion occurred in two steps: a short prefusion step, presumably consisting of membrane "activation," dependent on high PEG concentration and undetectable by EM; and a much slower step of fusion sensu stricto, or "completion," seemingly requiring energy or specific biosyntheses, taking place either in PPI medium or (even better) after plating on the rich regeneration medium. Cell fixation would suppress completion in cells processed for EM examination, but a few activated cells would go through completion while in the presence of 40% PEG. This interpretation, at which Sanchez-Rivas and Garro also arrived (19), is an appealing alternative to a model proposed by Maggio et al. (11), which assumes that prior to their dilution "polymer molecules act as a barrier between cells." It also can explain why the observed fusion figures before PPI provide a poor estimate of the pRFU's that are actually going to be formed, whereas this estimate is correct after some incubation (see below).

(iii) As seen in Table 2, the expected pRFU's at 30 min of incubation were 38×10^6 per ml of the mixed protoplast suspension. Six percent of those (Table 3), or 2.3×10^6 per ml, will regenerate a wall, if we assume that the mean regeneration rate of the PEG-treated parents would apply. At the same time, at 30 min PPI, 5×10^6 prototrophic bacteria per ml, nearly the same number, were actually counted (Table 3). An identical calculation carried out on data collected at 0 min PPI shows that the number of prototrophic bacteria formed (4×10^6/ml, Table 3) is 10 times as great as that of the expected pRFU that had regenerated their wall ($[6 \times 10^6] \times 0.06$, or 3.6×10^5). The meaning of this is simply that early fixation of the protoplasts had prevented completion of their PEG-induced fusion, and that determination of the frequency of cytological fusion was incorrect in this case. Also supporting this interpretation is the repeated observation that the number of fusion figures counted at 0 min fluctuates widely from one experiment to another (often 6×10^5 or even less), whereas that determined 30 min later, when cytological fusion is maximal, fluctuates very little and agrees with the prototroph yield much better.

As this work was being completed we learned from now-published experiments by Gabor and Hotchkiss (5) that genetic recombinants do not reflect the prevailing general regeneration rates in many series of experiments, and that "the average regeneration rate used as a base for an assumed random loss of recombinants may give an unreliable, often exaggerated estimate of total recombination." In the present work, although use was made of the average recovery of the PEG-treated parental protoplasts to calculate the expected recombinant bacteria from the EM data (a choice to which we see no alternative), the calculation resulted in a very nearly correct estimation of the recombinant bacteria actually recovered (in fact, the calculated value was somewhat low, as cytological fusion must be when determined by looking at cell sections). Since such a good agreement, repeatedly observed, could hardly be fortuitous, it is concluded that the probability for the heterozygous diploids formed in these crosses to produce at least one prototroph in their progeny is very high, and that no mechanism other than fusion is producing these diploids.

Three remarks also belong with this discussion. At an early stage of fusion, when a bispherical cell is formed, an apparently empty vesicle was regularly seen somewhere along the border between the fusing protoplasts (Fig. 1C and D). We suggest a fusion mechanism in which this vesicle would be moving along the border, disassembling the double membrane ahead of it, and becoming loaded with micelles of phospholipids and possibly proteins, which would eventually be discharged to the medium at the end of the way. This hypothesis can be tested experimentally.

In this work, for the first time, protoplasts were made and fused from cells far advanced in the sporulation process, rather than from exponentially growing cells. Yields of prototrophic recombinants as high as 2% of the heterologous pairs of parental cells have been obtained. Higher than yields observed in the past (20, 21) or in more recent work (5) with *B. subtilis,* these yields may be due to the markers being used (their number, nature, and chromosomal location), to the proneness of membranes of sporulating cells to fuse, or to both. A special study might be rewarding, since branched fatty acids, which are known to increase membrane fluidity, accumulate in the membrane after the end of growth (1).

Sporangia at stage III of sporulation are unable to resume a cell division cycle, even at the mother-cell side, when fresh medium is added back to the sporulating culture (4). Fusion between two such sporangia (Fig. 1A and B) would therefore seem unlikely to produce a viable recombinant clone after transfer to the regeneration medium. Why is it, then, that recombinant

clones arise in high numbers? An answer is easily found in the fact that, in the experiment depicted in Table 3, only 20% of the MO209 cells, and 30% of the MO83U cells, had engaged in sporulation and contained a prespore. Even if a viable recombinant could only arise by fusion between two nonsporulating cells, pRFU would still be 70% of the number expected with cultures containing no sporulating cells, and the reduction in yield of recombinants would not be significant. There is no indication, therefore, that recombinants formed when protoplasts from sporulating cultures are fused may derive from fusion between two prespore-bearing protoplasts, or that prespore genomes participate in recombination.

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