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Polyethylene Glycol-Induced Fusion of Heat-Inactivated and Living Protoplasts of *Bacillus megaterium*

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Protoplasts of *Bacillus megaterium*, incubated at 50°C for 120 min, lost the ability to revert to bacillary form. Such heat-inactivated protoplasts, however, produced recombinants when fused by polyethylene glycol treatment with normal protoplasts. Although this differential inactivation effect is not yet fully reproducible, reciprocal inactivations of the parental protoplasts in genetic crosses have clearly shown that for protoplast fusion (i) either of the parents may serve as the viable recipient for markers coming from the heated parental protoplasts, and (ii) either of the parents may be rendered nonviable and yet, when fused with a viable partner, contribute to formation of a recombinant. Heat inactivation seems to provide a way to counterselect when few markers are available and one of the parents is prototrophic.

The induction of protoplast fusion by polyethylene glycol (PEG) was first demonstrated for plant protoplasts by Kao and Michayluk (4) but later was extended to various microbial protoplasts including fungi (1, 7, 8), polyauxotrophic strains of *Bacillus megaterium* (2), *B. subtilis* (6), and *Streptomyces* (3).

Levi et al. (5) recently demonstrated that streptomycin-killed protoplasts of *B. subtilis* fused effectively with living streptomycin-resistant protoplasts to yield prototrophic recombinants, but that no recombinants could be detected when the viable parent was streptomycin sensitive. These authors explained the latter effect by concluding that streptomycin-killed protoplasts contributed with their cytoplasm enough toxic material to kill the streptomycin-sensitive fusion partner.

In recent work we have introduced heat-inactivated protoplasts as fusion partners for living protoplasts of *B. megaterium*, and here we report evidence that in genetic crosses via protoplast fusion either of the parents may be rendered nonviable and yet, when fused with a viable partner, contribute to formation of a recombinant.

MATERIALS AND METHODS

The bacteria used in these studies were wild-type, prototrophic strain *B. megaterium* KM and two of its polyauxotrophic derivatives. The tryptophan-, histidine-, and threonine-dependent, streptomycin-resistant strain *B. megaterium* THT and the arginine-, leucine-, and thymine-dependent strain *B. megaterium* ALTi are triple auxotrophs derived from the respective double auxotrophic strains used in our earlier studies (2).

Conditions for the culture of bacteria, the isolation, fusion, and culture of protoplasts, and the selection of recombinants have been described previously (2). One modification was that the parents were not mixed during the lysozyme treatment, but parental protoplasts were prepared separately. Suspensions of the protoplasts to be fused were then mixed in equal volumes before treatment with PEG (Fluka, molecular weight 6,000). In all experiments presented, the mixture was plated 30 min after the addition of the PEG.

Heat inactivation of the protoplasts. Protoplast suspensions were pipetted onto the surface of prewarmed hypertonic agar medium in Erlenmeyer flasks, then incubated for 120 min at 50°C. During this time morphological alteration of the protoplasts could not be detected by phase-contrast microscopy.

RESULTS

Selection for prototrophic recombinants was made by plating the PEG-treated mixture of the parental protoplasts directly on unsupplemented media lacking amino acid. Under these conditions, reversion to bacillary form and subsequent colony formation can be obtained only from fused and complementing protoplasts (2).

Table 1 presents the results of a cross (cross A) between the THT and ALTi strains. No colonies grew on unsupplemented media when the protoplast mixture was plated without PEG treatment, but after PEG treatment prototrophic recombinants grew even from 10^{-2} to 10^{-3} dilutions. The expression of the number of prototrophic recombinants as a function of the input number of (unheated) THT parental protoplasts represented in the fusion mixture allows comparison of separate experiments. The use of the THT parent as reference seems to be justified by its regular pattern of reversion to bacil-

TABLE 1. *Prototrophic recombinants from different crosses of the protoplasts of two polyauxotrophic strains of B. megaterium*

Types recovered	Colonies per ml[a]			
	Cross A	Cross B	Cross C	Cross D
Colonies of parental THT	2.30×10^8	2.30×10^8	3.0×10^1	3.0×10^1
Colonies of parental ALTi	1.10×10^8	9.0×10^1	1.10×10^8	9.0×10^1
Prototrophic recombinant colonies	2.64×10^5	7.60×10^3	8.10×10^3	0
Prototrophs per 10^8 colonies of original THT added	114,780	3,300	3,530	0

[a] Cross A, Protoplasts of THT × protoplasts of ALTi; cross B, protoplasts of THT × heat-treated protoplasts of ALTi; cross C, heat-treated protoplasts of THT × protoplasts of ALTi; cross D, heat-treated protoplasts of THT × heat-treated protoplasts of ALTi.

lary form. The reversion of ALTi protoplasts to bacillary form, on the contrary, is not proportional to the protoplast density. The reason for this anomaly is not known.

It can be seen from the data of Table 1 that the frequency of prototrophic recombinants from a cross of THT and ALTi protoplasts is around 0.1%, which is in the same range as that obtained with double auxotrophic *B. megaterium* strains in our earlier studies.

When protoplasts of the parents were heat treated, only 30 protoplasts of the 2.30×10^8 THT parental type and 90 protoplasts of the 1.10×10^8 ALTi parental type were able to revert to bacillary form (Table 1, cross D). Furthermore, prototrophic recombinants (cross D) were unable to grow from the PEG-treated mixture when protoplasts of both parents were heat inactivated. However, crosses B and C clearly show that when only one of the parents was heat inactivated prototrophic recombinants, at a reduced level, could be obtained from the PEG-treated mixtures. In repeated experiments the yield of this type of cross was variable, and ranged from 0 to 30% of that obtained in the standard cross (Table 2).

Further experiments demonstrated that protoplasts not only of the two auxotrophic strains but also of the wild-type, prototrophic strain *B. megaterium* KM can be heat inactivated and then used as fusion partners (Table 3).

TABLE 2. *Prototroph yields of repeated experiments*

Expt	Prototrophs[a]			
	A	B	C	D
1	100	2.9	3.1	None
2	100	0.037	0.074	None
3	100	None	None	None
4	100	25.5	30.8	None
5	100	9.9	0.029	0.023
6	100	None	0.088	None
7	100	None	0.62	None
8	100	None	None	None
9	100	14.6	16.6	None
10	100	5.0	3.8	None

[a] Crosses are as described in Table 1, footnote *a*. The yield of each A cross is taken as 100, and relative yields are given for B, C, and D crosses.

TABLE 3. *Cross of heat-treated prototrophic protoplasts with protoplasts of two polyauxotrophic B. megaterium strains*

Types recovered	Colonies per ml	
	Cross 1	Cross 2
Heated parent; prototrophic strain KM	0[a]	0[a]
Colonies of parental THT	1.06×10^8	
Colonies of parental ALTi		1.0×10^8
Prototrophic colonies	2.83×10^3	0.89×10^3
Streptomycin-resistant proportion[b]	52 of 123	

[a] *B. megaterium* KM protoplasts reverted to form 1.51×10^8 colonies per ml before heat treatment.

[b] *B. megaterium* KM is streptomycin sensitive; *B. megaterium* THT is streptomycin resistant.

DISCUSSION

Polarity of genetic information transfer has long been the general rule in bacterial genetics. Artificially induced fusion of bacterial protoplasts seemed to be the first possibility for bidirectional information transfer. Nevertheless, the first experiments had only demonstrated the feasibility of obtaining genetic recombinants by such methods (2, 6). The role of the individual partners in the formation of the recombinants could not be assessed.

In our studies, working with protoplasts of *B. megaterium*, we inactivated one of the parents by gentle heat treatment to see how it influenced the outcome of the crosses. Incubation of the protoplasts at 50°C for 120 min prevented reversion of practically every member of the population to bacillary form, yet still allowed some of them to function as fusion partners with un-

treated protoplasts. This state, however, seemed to be quite delicate, and systematic studies are needed to find out why seemingly identical experiments resulted in yields varying from 0 to 30% of the standard cross.

Nevertheless, even taking into consideration this residue of uncertainty, positive cases clearly showed that either of the parental protoplasts can be inactivated and yet function as "donor," and without heat treatment either of them may function as "recipient." These findings strongly suggest the equivalence of parents in normal fusion experiments. The equivalence of both parents is also found for the fusion of protoplasts of *B. subtilis* when one parent is killed by streptomycin and the other is resistant (5).

The state of one parental cell population can nevertheless influence fusion, as when streptomycin-killed cells fail to yield recombinants with a sensitive parent (5), or when the physiological states of the parents are varied (Fodor et al., manuscript in preparation).

The introduction of a prototrophic, wild-type partner as parent into our crosses provides further evidence in favor of the idea that any strain may function as donor. Furthermore, heat inactivation seems to be a way to select even for a prototrophic fusion product similar, or identical, to one of the parents, if few markers or few marked strains are available.

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