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Fusion of protoplasts of *Bacillus megaterium*

(bacterial fusion/*Bacillus* fusion/*Bacillus* protoplasts/segregating bacteria)

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ABSTRACT **Protoplasts of two doubly auxotrophic strains of *Bacillus megaterium* were fused by nascent calcium phosphate or by polyethylene glycol treatment, and then allowed to revert to bacillary form on selection media. The colonies formed were analyzed and found to be mixed populations of individual bacteria apparently containing parental, recombinant, and segregating genotypes. The evidence suggested that segregation was transitory, lasting only for a few clonings and reisolations.**

Artificially induced fusion of vertebrate cells provides a common technique in the study of somatic cell genetics (1). Protoplasts of plant cells, and fungi, can also be induced to fuse (2–6). The primary product of any of these fusions is a cell containing in a mixed protoplasm two or more nuclei, i.e., complete genomes of both parents.

Transformation, conjugation, transduction, sexduction, and transfection—the known genetic transfer mechanisms operating in bacteria—are unidirectional processes transferring DNA, and only DNA, from a donor into a recipient bacterium. The primary products in these cases are bacterial cells into which foreign DNA has been introduced.

There is no *a priori* obstacle to the fusion of bacterial protoplasts, or to their subsequent reversion to the bacillary form. Such a fusion might be a new tool in bacterial genetics, producing diploid bacteria or hybrids of bacteria.

We therefore decided to investigate the feasibility of fusion of bacterial protoplasts. *Bacillus megaterium* was selected as the test organism since it is easily converted to protoplasts (7), which, under appropriate conditions, revert to the bacillary form (8, 9). The experiments presented here show that transient diploid and stable recombinant bacteria can be produced by fusion of protoplasts of auxotrophic strains of *B. megaterium*.

In an accompanying paper in this issue of the PROCEEDINGS Schaeffer, Cami, and Hotchkiss present similar evidence, from work started independently, with *Bacillus subtilis* (10). We thank these workers for the opportunity for free exchange of information which has been beneficial as both investigations proceeded.

MATERIALS AND METHODS

Bacterial Strains. Two doubly auxotrophic derivatives of the asporogenic KM strain of *Bacillus megaterium* were used throughout these studies. Growth of one strain (Arg^-, Leu^-) requires arginine and leucine; growth of the other (His^-, Try^-) requires histidine and tryptophan. Both strains were isolated by two-step mutagenesis, bacteria being first exposed to ethylmethanesulfonate and in a second step to nitrosoguanidine. The penicillin method was used in both cases to select the mutants. The tryptophan marker of the second strain is slightly "leaky."

Abbreviations: PEG, polyethylene glycol; YT medium, minimal medium with yeast extract and tryptone.

Media. The protoplasting medium and the hypertonic growth medium have already been described (8). Solid medium contained 1% agar (Difco) in plates, and 0.4% agar (soft agar) when used in overlays. Omission of the sucrose from the hypertonic growth medium gave our minimal medium, and addition of yeast extract (Difco) and Tryptone (Difco) to minimal medium produced the YT medium. Solid medium containing 1.5% agar was made from each.

Preparation of Protoplasts. Bacteria were inoculated into amino acid-supplemented hypertonic medium and cultured overnight at 30°. One milliliter of this culture was pipetted into 100 ml of fresh medium and incubation was resumed for 6–8 hr at 30°. The viable count was around 5×10^7 per ml at the time of harvesting. The cultures were centrifuged at 4° for 20 min at $4000 \times g$, and the bacteria resuspended in 10 ml of protoplasting medium, containing 100 μg/ml of lysozyme (Calbiochem). After 30 min at 30° (unshaken), virtually all bacteria were transformed to protoplasts. The suspensions were then centrifuged at 4° for 20 min at $4000 \times g$, and the protoplasts resuspended in the same volume of protoplasting medium.

Protoplast suspensions if osmotically shocked did not form any colonies on ordinary media from 10^7 protoplasts.

Reversion of the Protoplasts to the Bacillary Form. When the protoplast suspensions were plated in a soft agar layer onto the surface of hypertonic agar media in petri dishes according to the procedure described earlier (8, 9), 1–10% reverted to bacillary form and produced bacterial colonies after 3–4 days of incubation at 30°.

Calcium Phosphate Treatment. Equal volumes of the two bacterial suspensions in protoplasting medium, in the first minutes of the lysozyme digestion (see preparation of protoplasts), were mixed, protoplasted, and centrifuged as before. The pellet was resuspended in its original volume of protoplasting medium. Then 0.05 volumes of 0.02 M KH_2PO_4 and 1.0 M $CaCl_2$ were added in that order to the protoplast suspension. Addition of $CaCl_2$ produced a slight increase in the turbidity of the suspension. Samples (0.1 ml) were then pipetted into 2.0 ml of molten hypertonic soft agar medium (maintained at 40°) and immediately poured onto the surface of plates with hypertonic medium, supplemented with amino acids when required. Colonies appeared on these selection plates after 4–5 days of incubation at 30°.

Polyethylene glycol (PEG) treatment. Equal volumes of the two bacterial suspensions in protoplasting medium in the first minutes of the lysozyme digestion (see preparation of protoplasts) were mixed, protoplasted, and centrifuged as before. The protoplasts were resuspended in a few tenths of 1 ml of protoplasting medium. This dense suspension was then diluted 10-fold with a 40% solution of PEG (molecular weight of 6000, Fluka) in distilled water. This dilution in PEG immediately produced visible aggregation of the protoplasts. Samples were then plated as described for the calcium phosphate treatment.

Table 1. Protoplast combinations tested for genetic transfer in *Bacillus megaterium*

Cross		Frequency of colonies on minimal media	Cross		Frequency of colonies on minimal media
B. megaterium Arg⁻ Leu⁻	*B. megaterium* His⁻ Try⁻		*B. megaterium* Arg⁻ Leu⁻	*B. megaterium* His⁻ Try⁻	
Protoplasts	—	$<5 \times 10^{-7}$	Bacteria	—	$<5 \times 10^{-7}$
—	Protoplasts	$<5 \times 10^{-7}$	—	Bacteria	$<5 \times 10^{-7}$
Protoplasts	DNA	$<5 \times 10^{-7}$	Bacteria	DNA	$<5 \times 10^{-7}$
DNA	Protoplasts	$<5 \times 10^{-7}$	DNA	Bacteria	$<5 \times 10^{-7}$
Protoplasts	Lysate of protoplasts	$<5 \times 10^{-7}$	Bacteria	Lysate of protoplasts	$<5 \times 10^{-7}$
Lysate of protoplasts	Protoplasts	$<5 \times 10^{-7}$	Lysate of protoplasts	Bacteria	$<5 \times 10^{-7}$
Protoplasts	Protoplasts	10^{-6}–10^{-3}	Bacteria	Bacteria	$<5 \times 10^{-7}$

For procedures, see *Materials and Methods*.

Preparation of DNA. Highly polymerized DNA was prepared according to Sümegi Udvardy, and Venetianer (in preparation). The solution contained 38–56 μg of DNA per ml. In our experiments it was diluted 10-fold into bacterial or protoplast suspensions.

Lysates of Protoplasts. Lysates were made by resuspending a pellet of protoplasts in distilled water. The osmolarity was then adjusted to that of the protoplasting medium.

DNase Treatment. Deoxyribonuclease I (Calbiochem) at a concentration of 5 μg/ml was added before lysozyme treatment, and was present in all media used subsequently, including the selection media.

RESULTS

When *bacteria* of the two parental strains (treated as described in preparation of protoplasts, but lysozyme omitted) were mixed and plated on minimal agar, no colonies appeared. The same was true when DNA of one strain was given to bacteria of the other strain (Table 1).

Protoplasts plated in a soft agar layer on nutritionally and osmotically adequate media will revert to bacillary forms, which grow into normal bacterial colonies (8, 9). It was our expectation that complemented auxotrophic protoplasts would behave in the same way on selection media.

Because protoplasts of some bacteria might take up biologically active DNA molecules, we first wanted to know if protoplasts treated by purified DNA of the other strain would, in our conditions, form recombinant bacterial colonies. It is also known that intact DNA in crude lysates is taken up by bacteria competent for transformation (11). Lysates of osmotically shocked protoplasts were therefore tested with bacteria and with protoplasts of the other strain. No recombinant colonies were ever produced by these procedures (Table 1).

However, when bacteria of the two parents were mixed, converted to protoplasts, and plated in soft agar layer on hypertonic minimal media, bacterial colonies often grew out after 3–5 days of incubation at 30°. Their frequency was at best 10^{-6}–10^{-5} of the total protoplasts present (conclusion drawn from eight separate experiments). If centrifugation of the mixed protoplasts was omitted, then the colonies did not appear; this suggested that centrifugation might have some fusion-like effect. Unfortunately, however, this effect was poorly reproducible, in spite of a careful standardization of the procedure.

In the effective method for inducing fusion of plant protoplasts, worked out by Kao and Michayluk (4), Ca^{++} ions and PEG are used as fusing agents. The simultaneous presence of both agents is important also to induce high frequency fusion with fungal protoplasts (12). However, direct application of these procedures did not lead to successful fusion and recombination of *B. megaterium*.

We have found that nascent calcium phosphate alone, as well as PEG treatment alone (see *Materials and Methods*) of the mixture of protoplasts did lead to the appearance of bacterial colonies on minimal hypertonic agar in a reproducible manner. After calcium phosphate treatment colonies appeared after 4–5 days of incubation at 30° with a frequency of 10^{-5}–10^{-4} cells per protoplasts plated (eight separate experiments); after PEG treatment frequencies of 10^{-5}–5×10^{-3} have been observed (14 separate experiments). These yields represent 10 to 1000 times the yield from untreated protoplast mixtures. The frequencies did not decrease when the experiments were made in the presence of 5 μg/ml of DNase.

Analysis of the colonies grown on the selection media was made to learn whether these primary colonies gave rise to a progeny composed of genetically stable and identical bacteria, or not. Thirty primary colonies obtained on minimal hypertonic media, from PEG treated mixtures of the parent protoplasts, were suspended in Tris buffer. From each suspension, a drop was inoculated into 2 ml of YT broth (Culture A) and into 2 ml of liquid minimal medium (Culture B), respectively. After 20 hr of incubation at 30°, the fully grown parallel cultures were diluted and plated onto the surface of YT agar plates in order to get isolated colonies, which then were replica plated onto YT and minimal agar. The rationale of the procedure was as follows: should the primary colonies be composed of stable prototrophic bacteria, their multiplication in minimal and rich liquid media would not alter the bacteria and a sample of those populations replica plated onto minimal and rich media would yield the same number of colonies on both plates. Should, however, the primary colonies be composed of unstable prototrophic bacteria, or of a mixture of nonidentical auxotrophic bacteria, while their multiplication in rich medium would be freely allowed, in minimal medium the prototrophs would be favored. Consequently, samples from a culture grown in rich liquid medium replica plated onto rich and minimal agar media would give rise to nonidentical numbers of colonies; identical or nonidentical numbers of colonies could grow from the culture of liquid minimal medium, with prototrophs probably predominating.

Data of Table 2 show that of the 30 primary colonies tested there was only one (primary colony no. 13) which produced

Table 2. Number of colonies on YT and minimal agar media obtained from parallel subcultures (A and B) of individual primary colonies

Primary colony	Culture A		Culture B	
	YT agar	Minimal agar	YT agar	Minimal agar
1	92	90	80	35
2	55	16	30	6
3	75	35	77	66
4	87	9	50	32
5	54	47	60	60
6	44	36	66	66
7	70	11	26	1*
8	100	2	27	8
9	85	24	64	64
10	98	12	13	1
11	61	41	51	50
12	52	46	35	34
13	27	27	56	56
14	57	41	160	62
15	80	55	75	75
16	51	45	84	84
17	60	0	57	57
18	75	22	63	63
19	15	0	49	0
20	77	60	80	62
21	45	6	47	46
22	46	11	60	60
23	65	2	21	20
24	51	8	25	25
25	79	5	55	52
26	60	0	70	0
27	20	11	41	36
28	31	28	30	25
29	25	5	21	5
30	15	13	103	0

Cross: *B. megaterium* (Agr$^-$Leu$^-$) × *B. megaterium* (Try$^-$His$^-$) with PEG as the fusing agent and selection in minimal medium. Procedures: individual clones, grown on the selection medium, were suspended in Tris buffer. From each suspension, a drop was inoculated into 2 ml of YT broth (Culture A) and into 2 ml of liquid minimal medium (Culture B), respectively. After 20 hr of incubation at 30°, the fully grown parallel cultures were diluted and plated onto the surface of YT agar plates in order to get isolated colonies, which then were replica plated onto YT and minimal agar. The numbers in the table represent colonies grown on the replica plates. The asterisk (*) indicates the prototrophic colony that was subcultured (see *text*).

progeny that were able to form an equal number of colonies on YT and minimal agar media, after a passage in the two kinds of liquid media. This primary colony might, therefore, have been composed of stable prototrophic bacteria. All the other seemingly prototrophic primary colonies tested contained, or else gave rise on further growth to, auxotrophic bacteria.

The prototrophic colony identified with an asterisk in Table 2 was suspended in one ml of Tris buffer, a drop of which was inoculated into 10 ml of liquid minimal medium. From the fully grown culture, a new passage was made in the same medium. From the second subculture in liquid minimal medium, dilutions were prepared and plated on YT agar to get isolated colonies, which then were replica plated on agar media with different composition (Table 3). Surprisingly enough, not one of the 33 colonies replica plated formed colonies on minimal agar. Nevertheless, the sum of the colonies grown on the amino acid supplemented media was 35 instead of 33, and suggested that among the 33 clones prototrophic ones might have been present. Individual clones from the plates were therefore subcultured in minimal and amino acid supplemented liquid media, from which were isolated one stable prototrophic clone as well as several of both parental types, in stable form.

Table 3. Progeny analysis of primary colony no. 7

YT	No. of colony formers on agar media		
	Minimal	Minimal +arg, +leu	Minimal +try, +his
33	0	19	16

Procedure: see *text*. Each amino acid was supplemented at 50 μg/ml.

A considerable number of other prototrophic colonies resulting from fusion were studied in a like manner. We were able to distinguish the following subclasses: (*a*) colonies giving rise to mixed populations of which the prototrophic forms at least remained unstable for several passages, (*b*) colonies whose progeny consisted of mixed populations of distinct stable cell types, and (*c*) colonies which appeared at an early passage to be pure and stable (prototrophs or recombinants, depending on the selection).

From primary colonies of type (*b*) and (*c*) we have eventually obtained stable clones of both parental types, and most of the possible recombinant types (Table 4). We have not as yet attempted to determine the relative frequencies of these types.

DISCUSSION

No genetic transfer mechanism is known so far for *B. megaterium*. In this work, we were unable to detect any genetic information transfer by mixing bacteria with bacteria, or with protoplasts, or with lysates of protoplasts, or by supplying bacteria with purified DNA. Crude lysates of protoplasts, or purified DNA added to protoplasts, did not produce a transfer of genetic information.

Table 4. Genotypes of clones demonstrated from primary colonies

Genotype				Clone genotype
Arginine	*Leucine*	*Histidine*	*Trypto-phan*	
		Parental		
—	—	+	+	Observed
+	+	—	—	Observed
		Recombinant		
+	+	+	+	Observed
—	+	+	+	Observed
+	—	+	+	Observed
+	+	—	+	Observed
+	+	+	—	Observed
+	—	—	+	Observed
+	—	+	—	Observed
—	+	—	+	Not observed
—	+	+	—	Observed
—	—	—	+	Not observed
—	—	+	—	Not observed
—	+	—	—	Not observed
+	—	—	—	Observed
—	—	—	—	Observed

Mixed protoplasts from the two parental strains, subjected to centrifugation, did however produce colonies on selection media. By using fusing agents, like nascent calcium phosphate or PEG, the yield was increased 100-fold and good reproducibility was reached. The likely bursting of some protoplasts in the suspension made it necessary to insure that no transformation of protoplasts was taking place, a possibility clearly ruled out by the observed insensitivity to DNase. The observed resistance to DNase of the phenomenon described strengthens the conclusion that it is distinct from transformation.

The above mentioned evidence suggests that primary colonies, grown on the selection media, were produced from fused protoplasts of parent bacteria. A microscopic examination of the calcium phosphate or PEG treated suspensions showed a great majority of protoplasts to be strongly attached to each other in smaller or larger groups.

Genetic analysis of the primary colonies grown on the selection media further supports the protoplast fusion hypothesis. Protoplast fusion was expected to produce di- or multi-nucleated products which, either during reversion to bacillary form or subsequently, were expected to segregate and produce primary colonies composed of individual bacteria with different genotypes. This is in essence what was found. It was also demonstrated that individual bacteria of the primary colonies, after cloning, may produce progeny still of the mixed type. Nevertheless, any segregating state was always transitory, and we have not thus far observed indefinitely segregating clones. The seemingly pure primary colonies probably resulted from a very early segregation.

It should be remembered here that, since reversion of the protoplasts to bacillary form requires their growth and division (9), only complementing (therefore fused) protoplasts should be able to make proteins and regenerate cell wall under our conditions of selection.

It is clear that bacteria of primary colonies, after growth in liquid minimal medium (Culture B of Table 2), often gave a preponderance of auxotrophic progeny. This would not be expected if genuine stable prototrophs were merely cross-feeding stable parentals, even in the liquid culture. Therefore, we believe the auxotrophic progeny are in general derived from previously prototrophic products of fusion. Nevertheless, the rather unexpectedly complete separation of colonies 19, 26, and 30 into auxotrophs in minimal medium argues for investigation of another possibility: occasional cross-feeding between different auxotrophs. Ordinary experience does not seem to support this.

Bacteria are normally haploid. It is clear therefore that only those fusion products formed primary colonies on a selection medium which either were able to preserve the complementing diploid state for many generations, or at the early steps of division produced stable recombinant genotypes able to grow on the selection medium. Isolation from primary colonies of clones with stable recombinant genotypes clearly demonstrated that both segregation and recombination in fact were going on in parallel with the process of reversion to bacillary form and colony formation.

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