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Comparison of Deoxyribonucleic Acid Uptake and Marker Integration in Bacilli and Protoplasts of *Bacillus subtilis*

IRVING L. MILLER,[1] C. DWAYNE PALMER, AND OTTO E. LANDMAN

Department of Microbiology, Northwestern State University of Louisiana, Natchitoches, Louisiana 71457, and Department of Biology, Georgetown University, Washington, D.C. 20007

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trp$^+$*his*$^-$ donor deoxyribonucleic acid (DNA) was added to highly competent *trp*$^-$*his*$^+$ recipient bacilli and to protoplasts prepared from these bacilli, and the cell-DNA complexes were incubated for 30 min. The complexes were then washed and lysed, and their DNA was analyzed on a *trp*$^-$*his*$^-$ strain for the donor marker *trp*$^+$, the resident marker *his*$^+$, and for the recombinant *trp*$^+$*his*$^+$ combination. The extracts of the bacillary complexes contained a normal percentage of donor markers (0.1–0.02%), and the number of *trp*$^+$*his*$^+$ doubles (20% of all *trp*$^+$ transformants) indicated that the donor DNA had become integrated into the resident genomes. The protoplast complexes contained 10 to 1,000 times fewer donor markers and almost no recombinants. This indicated that, in protoplasts, marker uptake was minimal and recombination was absent. Uptake was also measured with ^{3}H-labeled DNA. On the average, protoplasts took up one-fiftieth as much DNA as bacilli. It was concluded that, probably, protoplasts took up no DNA at all, that there were no DNA affinity sites on the surface of the protoplasts, and that the residual marker and radioactivity uptake was due to imperfections in the experimental system. The data and conclusions differed sharply from earlier ones of Hirokawa and Ikeda despite the fact that the techniques of these authors were followed in repeat experiments.

Miller and Landman previously reported that protoplasts of *Bacillus subtilis* were not transformable (7; Bacteriol. Proc., 1963). The inability to take up deoxyribonucleic acid (DNA) and obtain transformation was tentatively attributed to the loss of mesosomes consequent upon wall removal (7, 13). In later work of Hirokawa and Ikeda it appeared that transforming DNA was taken up by protoplasts after all and that very extensive recombination occurred between donor DNA and the DNA of the recipient protoplasts (4). The protoplast system described by Hirokawa and Ikeda had several distinctive features which set it apart from intact-cell transforming systems. (i) Two different donor DNA species recombined with each other with high frequency when protoplast recipients were present. (ii) When extracts from the donor DNA-recipient protoplast complex were assayed for donor and protoplast markers they contained about 22% as many donor markers as protoplast markers. In the corresponding control experiment with bacilli the donor markers amounted to only 1.2%. (iii) Formation of linkage was selectively inhibited by actinomycin S_3 whereas transformation was stimulated. (iv) KCN (10^{-2} M) inhibited neither the formation of DNA-protoplast complexes nor that of recombinants. In bacillary systems both uptake and recombination are inhibited by energy-uncoupling reagents (12, 17).

In sum, the results of Hirokawa and Ikeda suggested that *B. subtilis* protoplasts possessed an efficient DNA uptake and DNA recombination system perhaps different in character from the bacillary transformation system. By contrast, our earlier data (7) and subsequent experiments (13) indicated that protoplasting interrupted transformation completely and irreversibly. Several different explanations might be advanced to bring these divergent findings into partial harmony. It could be that DNA was indeed taken up by

[1] Present address: Haifa Absorption Center, Haifa, Israel.

protoplasts and recombined with resident protoplast DNA but that a late step in transformation could not occur in protoplasts. A second possibility might be that transformed protoplasts were killed selectively. A third unifying hypothesis is that, in the Hirokawa-Ikeda experiments, cell wall removal was incomplete, resulting in the formation of quasi-spheroplasts (8). These bodies are indistinguishable from protoplasts in the phase microscope, yet are highly transformable (13). Finally, it seemed possible that in the experiments of Hirokawa and Ikeda, the integrity of the protoplasts had been destroyed and that, hence, their results were attributable to cell-free components. This possibility is suggested by their finding that DNA synthesis had ceased in their suspensions (4).

The present experiments were designed to test the foregoing hypotheses by bridging the gap between our procedures and those of Hirokawa and Ikeda. By using labeled DNA we compared uptake of radioactivity in protoplasts with uptake in control bacilli. Employing *trp*$^+$*his*$^-$ donor DNA and *trp*$^-$*his*$^+$ recipient protoplasts or bacilli, we monitored the appearance of *his*$^+$*trp*$^+$ recombinant DNA. Highly competent cultures were used in both types of experiments, and bacilli and protoplasts were prepared by using both the methods of Hirokawa and Ikeda and our methods.

MATERIALS AND METHODS

Organisms and their use. *B. subtilis* strains 543M (*thy*$_{AB}$-*his*$_2$$^-$), 168 (*trp*$_2$$^-$), and SB25 (*trp*$_2$$^-$ *his*$_2$$^-$, linked) were used as follows. The *thy*$^-$*his*$^-$ strain 543M was used to prepare both labeled and unlabeled donor DNA (for transformation of *trp*$^-$ recipients to *trp*$^+$). Strain 168 (*trp*$^-$*his*$^+$) was used as the "first recipient." Donor DNA was added to bacilli or protoplasts of this strain, and then either the radioactivities of the bacillus-DNA and protoplast-DNA complexes were compared or, in separate experiments, extracts of the two complexes were assayed for their content of donor (*trp*$^+$), first recipient (*his*$^+$), and recombinant (*trp*$^+$*his*$^+$) markers. Strain SB25 (*trp*$_2$$^-$*his*$_2$$^-$) was used for these marker assays. SB25 was a gift of E. Nester.

Growth of competent cultures. Competent cultures were prepared by a modification of the procedure of Kammen, Beloff, and Cannellakis (5). Since transformation experiments nearly always yielded at least 1% transformants, a description of the modified procedure follows. Antibiotic assay broth, (AAB, Baltimore Biological Laboratories) was inoculated with a small inoculum of vegetative cells. This was grown to 40 to 50% transmission (at 660 nm) as determined with a Bausch & Lomb Spectronic 20 spectrophotometer. The cells, about 10^8 per ml, were diluted to a concentration of 4×10^4/ml in AAB containing 0.5 M sucrose, frozen immediately, and stored in a Revco freezer chest at −70 C. These cultures were used as starting inocula for the preparation of competent cultures during a 6-month period. The frozen cells were diluted 10^2 with AAB, and 0.05 ml (about 20 cells) was used to inoculate 40 ml of the same medium. This culture was incubated for 16 hr with shaking at 37 C and then centrifuged. The pellet was resuspended and used to inoculate SL1 medium (6) to 60 to 65% transmission. After 4 hr of growth with vigorous shaking at 37 C, the culture was centrifuged at 6,000 × *g* for 15 min. The cells were then suspended in a 10-fold volume of medium SL2 (6) containing 20 μg of α,α′-bipyridyl per ml (SL2-BIP) (5). This culture was incubated at 37 C with vigorous shaking for 90 min; at this time the cells were highly competent.

In two experiments, cultures were grown in MY-1 medium as described by Hirokawa and Ikeda (4). These cultures showed considerable chaining; as many as 25 protoplasts were seen to emerge from a single chain. Accordingly, we did not attempt to obtain quantitative "per cent transformation" data with these cultures.

Preparation of donor DNA and ^{3}H-labeled donor DNA. All donor DNA (*trp*$^+$) was prepared from strain 543M (*thy*$^-$*his*$^-$). Stocks were kept on nutrient agar (Oxoid) slants supplemented with 15 μg of thymidine per ml. Nonradioactive DNA was prepared from stationary-phase cultures grown in Brain Heart Infusion Broth (Difco). The method of Saito and Miura (11) was used for DNA isolation except that the ribonuclease treatments were omitted. To prepare ^{3}H-labeled DNA, an inoculum was grown on minimal agar (1) containing 20 μg of L-histidine and 10 μg of thymidine per ml. The growth medium for labeling was as follows: K_2HPO_4, 2.8 g; KH_2PO_4, 1.2 g; $(NH_4)_2SO_4$, 0.4 g; sodium citrate·$2H_2O$, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; and NaCl, 0.2 g. These were dissolved in 300 ml of deionized water and autoclaved separately. Acid-hydrolyzed casein (6.0 g) and 20 mg of L-tryptophan in 90 ml of deionized water were added aseptically after autoclaving; the medium was completed with the addition of 10 ml of 10% glucose, 0.16 ml of thymidine (5 mg/ml), and tritiated thymidine, 4 mCi (Calbiochem, 1.83 Ci/mmole). The inoculum for this medium was adjusted to 95% transmission (660 nm in Bausch & Lomb Spectronic 20 spectrophotometer), and the culture was incubated with vigorous shaking at 37 C for 3.25 hr (19% transmission). It was centrifuged at 4,800 × *g* for 20 min and washed once with saline-EDTA (0.15 M NaCl–0.1 M ethylenediaminetetraacetate, *p*H 8.0). The culture lysed spontaneously after washing; nevertheless, the lysing cells were resuspended in 10 ml of saline-EDTA containing 1 mg of lysozyme per ml. The usual procedure for the isolation of DNA was then followed (11), except for the omission of ethanol precipitation and ribonuclease treatment. The labeled crude DNA was dialized against 1.5 M NaCl plus 0.0125 M sodium citrate for several days until more than 97% of the residual radioactivity was nondializable.

Test for recombination between donor trp$^+$his$^-$ DNA and first-recipient trp$^-$his$^+$DNA: bacilli.

After 90 min of incubation in SL2-BIP medium, 10 μg of *trp*$^+$*his*$^-$ DNA per ml was added to 250 ml of competent culture, and incubation was continued for 30 min at 37 C. Transformation was then terminated by the addition of 5 μg of deoxyribonuclease per ml, and the cells were washed with SL2-BIP medium. DNA was extracted from these cells essentially by the method of Saito and Miura (11). The bacillus-DNA complexes were resuspended in 2 ml of saline-EDTA; 0.2 ml of lysozyme (20 mg/ml) was added, and the mixture was incubated for 15 to 20 min at 37 C. The lysing cells were then frozen at −70 C; 1 ml of 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 9, containing 1% sodium dodecyl sulfate (SDS) and 0.1 M NaCl, was added to the frozen cells and the preparation was heated at 60 C for 10 min. Then 3 ml of water-saturated phenol was added and the mixture was shaken gently at 0 to 1 C for 20 min. The resulting emulsion was separated by 30 min of centrifugation at 2,700 × *g* at 4 C. The water phase was drawn off, dialyzed overnight against 1.5 M NaCl–0.0125 M sodium citrate, and assayed for DNA content (2). The extract was then assayed on strain SB25 *trp*$^-$*his*$^-$ for the donor marker *trp*$^+$, the first-recipient marker *his*$^+$, and the linked recombinant markers *trp*$^+$*his*$^+$. The three classes of transformants were scored on minimal medium (1) which was supplemented with 1 mg of acid-hydrolized casein per ml, supplemented with 20 μg of L-tryptophan per ml, or unsupplemented, respectively.

Test for recombination between donor trp$^+$his$^-$ DNA and first-recipient trp$^-$his$^+$ DNA: protoplasts. Protoplast suspensions of the *trp*$^-$*his*$^+$ strain were prepared by two different methods. For experiments 6 and 7 of Table 1 the procedure of Hirokawa and Ikeda was followed exactly, from inoculation of their growth medium MY-1 to the final washing and resuspension of the protoplasts prior to DNA addition (4). In all other experiments, the protoplasts were prepared from competent cultures after 90 min of incubation in SL2-BIP medium. The cells were centrifuged at 3,800 × *g* for 20 min at 4 C, washed once in 0.01% Tris buffer (*p*H 8.0) containing 20% sucrose, and resuspended in the same medium (4). Lysozyme was added (100 μg/ml final concentration), and the culture was incubated for 30 to 35 min at 37 C. At this time more than 90% of the rods were converted to spheres. (A few rods remained even after lengthy incubation, however.) The protoplasts were centrifuged at 3,800 × *g* for 25 min (faster centrifugation causes lysis) and washed once by centrifugation in a 1:1 mixture of SL2-BIP and 20% sucrose–0.01% Tris buffer, *p*H 8.0. Then the protoplasts were resuspended in SL2-BIP medium and 25 μg of *trp*$^+$*his*$^-$ DNA per ml was added. After 30 min of incubation at 37 C with gentle shaking, transformation was terminated either by two successive washings in SL2-BIP medium or by 10 min of incubation with 5 μg of deoxyribonuclease per ml, followed by a single washing in SL2-BIP medium. Extraction of DNA from the protoplast-DNA complexes followed. The protoplasts were centrifuged and lysed by resuspension in 2 ml of saline-EDTA. Then, 1 ml of Tris-SDS-saline, *p*H 9, was added and the mixture was heated at 60 C for 10 min. Subsequently, the procedure for DNA extraction and assay was the same as the preceding one for bacillus-DNA complexes.

Measurement of ^{3}H-DNA uptake by bacilli. Suspensions (250 ml) of competent *trp*$^-$*his*$^+$ culture were centrifuged at 4,800 × *g* for 20 min at 4 C. The cells were then resuspended in 48 ml of fresh SL2-BIP medium. Test transformations before and after concentration by centrifugation showed small or negligible loss of transformability. To 48 ml of concentrated cultures, 2 ml of *trp*$^+$*his*$^-$ ^{3}H-DNA was added (60 μg; 1.16 × 10^5 counts per min per μg), and incubation was continued for 30 min at 37 C with gentle shaking. Generally, transformation was terminated by 10 min of incubation with 5 μg of deoxyribonuclease per ml. This was followed by two or three washes by centrifugation at 4 C with cold SL2-BIP medium. The supernatant fluids from each wash were monitored for radioactivity and, after two washes, the counts in the supernatant fluid were usually insignificant relative to the counts retained by the cells. After the final wash, the pellet was resuspended in 2.5 ml of a mixture of SL2-BIP (one volume) and water (two volumes). Samples (0.2 or 0.5 ml) of this suspension in scintillation vials were treated with 500 μg of lysozyme per ml for 30 min at 37 C, and then distilled water was added to bring the volume to 1.0 ml; finally 0.2 ml of 0.01 M NaOH was added. The alkaline mixture was heated in a boiling water bath for 5 min and cooled, and 20 ml of counting gel was added. The gel had the following composition: xylene, 500 ml; dioxane, 500 ml; absolute alcohol, 300 ml; 2,5 diphenyloxazole, 6.5 g; naphthalene, 104 g; Cab-o-sil (Cabot Corp; Boston, Mass.), 4 g per 100 ml of fluid.

Measurement of ^{3}H uptake by protoplasts. Suspensions (500 ml) of competent *trp*$^-$*his*$^+$ culture were centrifuged, washed once with 100 ml of 20% sucrose-0.01% Tris buffer (*p*H 8.0) and protoplasted in the same medium as described. After washing, the protoplasts were resuspended in 48 ml of SL2-BIP medium, 2 ml of *trp*$^+$*his*$^-$ ^{3}H-labeled DNA was added (60 μg; 1.16 × 10^5 counts per min per μg), and the suspension was incubated for 30 min at 37 C with gentle shaking. DNA uptake was terminated by 10 min of incubation with 5 μg of deoxyribonuclease per ml. The protoplasts were then washed two to three times with cold SL2-BIP medium by centrifugation at 3,800 × *g* for 25 min at 4 C. Samples of the protoplasts were lysed, dissolved in NaOH, and counted as described.

RESULTS

Recovery of donor markers from cell-DNA complexes and recombination of donor markers with resident markers in bacilli and protoplasts. The data in Table 1 are a compilation of experiments done to ascertain whether recombinant DNA could be recovered from recipient protoplast-donor DNA complexes or, less ambitiously, whether *any* donor markers could be found in such complexes.

Turning to control aspects first, column 1

TABLE 1. *Occurrence of recombinant DNA in extracts of bacillus-DNA and protoplast-DNA complexes*

Expt	Transformability of first recipients (*trp*⁻*his*⁺) by *trp*⁺ donor DNA before protoplasting[a] %T[c]	Transformation of *trp*⁻*his*⁻ recipients by extracts of cell-DNA complexes[b]				
		Complex extracted	Transformants/ml for			Transformability of assay strain (*trp*⁻*his*⁻) by *his*⁺ marker from complexes %T[c, d]
			trp⁺ donor marker	*trp*⁺*his*⁺ recombinant markers	*his*⁺ first-recipient marker	
	1[e]	2	3	4	5	6
1	1.9	Bacilli-DNA	1.8×10^3	4.0×10^2	8.9×10^5	0.68
2	1.7	Bacilli-DNA	1.2×10^3	2.1×10^2	1.7×10^5	0.30
3	1.2	Protoplasts-DNA	4.0×10^1	0	3.2×10^5	0.55
4	2.4	Protoplasts-DNA	1.1×10^2	5	5.1×10^5	0.51
5	1.7	Protoplasts-DNA	2	0	7.4×10^5	0.81
6	ND[f]	Protoplasts-DNA	2	0	2.7×10^5	0.35
7	ND	Protoplasts-DNA	6	0	3.5×10^5	0.45

[a] The concentration of donor DNA (*trp*⁺) was 20 μg/ml.

[b] Five to ten micrograms DNA per ml extracted from the various cell-DNA complexes was used in the assays on SB25 *trp*⁻*his*⁻.

[c] %T = (Number of transformed cells × 100)/total number of cells.

[d] The per cent *his*⁺ transformants of column 6 is based on the same data as the number of *his*⁺ transformants/ml of column 5. When competence in the *trp*⁻*his*⁻ assay cultures was assessed by using *trp*⁺ donor DNA at 20 μg/ml, the transformation frequencies ranged from 2.0 to 3.1% in all experiments except no. 3 where the frequency was 0.9%.

[e] Column number.

[f] ND = not done: Protoplasts made from filamentous cultures grown as described by Hirokawa and Ikeda (4).

shows that the first recipients were highly competent, or, in the case of protoplasts, were prepared from highly competent bacilli. Experiments 1 and 2 show that extracts of bacillus-DNA complexes assayed high for the first-recipient marker *his*⁺ and low for the donor marker *trp*⁺ (columns 5 and 3). This was obviously due to the low concentration of *trp*⁺ donor markers in the extracted complexes since 20 μg of *trp*⁺ donor DNA per ml, when added directly to the *trp*⁻*his*⁻ assay culture, gave excellent transformation efficiencies (see footnote *d*, Table 1). For the DNA from the complexes, the ratio of *his*⁺ transformations to *trp*⁺ transformations was 494 in experiment 1 and 142 in experiment 2 (columns 5 and 3). These are typical resident marker to donor marker ratios (3, 15). The *trp*⁺*his*⁺ doubles constituted 22 and 18%, respectively, of all *trp*⁺ transformants (columns 3 and 4). In view of the finding that cotransformation ("linkage") of the trp_2, his_2 loci is about 50% (9), these percentages are quite close to expectation, assuming integration had been largely completed when the bacillus-DNA complexes were extracted.

The pattern of results in the protoplast experiments was sharply different. Although assays for the first-recipient marker, *his*⁺, were the same as before (columns 5 and 6), the relative concentration of the *trp*⁺ donor marker was reduced 10-fold (experiment 4) to 1,000-fold (experiments 5 and 6). Actually, we think that even these few *trp*⁺ transformants are "false" positives due to the presence of incompletely protoplasted bacteria [i.e., quasi-spheroplasts (8)]. This conclusion is based on the observation that recombinant DNA-*trp*⁺*his*⁺ doubles are generally undetectable in the extracts of protoplast-DNA complexes (column 4, experiments 3, 5, 6, 7). Apparently, no significant integration of donor DNA into resident protoplast genomes had occurred in these experiments. This is the expected result if we assume that the donor DNA detected in the *trp*⁺ transformations (column 3, experiments 3–7) was lodged in residual wall fragments outside the cell membrane in quasi-spheroplasts (13). (The five doubles in experiment 4, 4.5% of the *trp*⁺ in this experiment, may well be due to simultaneous transformations by *trp*⁺*his*⁻ and *trp*⁻*his*⁺ DNA, the *trp*⁺*his*⁻ donor DNA having been sequestered in the wall of quasi-spheroplasts. Another possibility is that a little *trp*⁺*his*⁺ recombinant DNA was generated in a subpopulation of unprotoplasted bacilli in experiment 4.) The most plausible summary interpretation of the data of Table 1 is that true protoplasts do not take up *any* intact markers and that, consequently, no integration of donor DNA into resident DNA can occur. This interpretation applies equally to protoplasts made

from cells grown by our usual procedures (experiments 3, 4, and 5) and to protoplasts prepared and "transformed" by the methods of Hirokawa and Ikeda (experiments 6 and 7).

Uptake of radioactive DNA by bacilli and protoplasts. The uptake of labeled DNA by bacilli and protoplasts is shown in Table 2. Averaging all the data, the bacilli took up more than 50 times as much radioactivity as the protoplasts. The small amount of radioactivity taken up by the protoplast suspensions may in part be due to the presence of a small quantity (about 1%) of dialyzable tritiated material still present in our DNA preparation. More radioactive low-molecular-weight products are generated by the deoxyribonuclease treatments sometimes used to terminate transformation. Finally, a significant amount of label uptake should occur in quasi-spheroplasts or bacilli present in the protoplast suspensions. It seems probable that all the radioactivity found in the protoplasts is due to these various sources of contamination and that, in fact, clean protoplasts do not take up any intact DNA.

TABLE 2. *Uptake of labeled DNA by suspensions of protoplasts and of bacilli*

Expt	Recipient culture[a]	%T[b] (*trp*+) before protoplasting		Counts/minute[c]	
		25 μg of cold DNA/ml	1.2 μg ^{3}H-DNA/ml	Bacilli	Protoplasts
1	B	ND[d]			
	A	1.1	0.32	1.2×10^5	
2	B	3.5			
	A	1.2	0.37	3.1×10^5	
3	B	1.6			
	A	1.1	0.17		1.8×10^3
4	B	2.3			
	A	1.7	0.13	2.6×10^5	7.5×10^3[e]
5	B	2.6			
	A	ND	ND		2.8×10^3

[a] B recipients were 90-min competent cells transformed before centrifugation. A recipients were 90-min competent cells concentrated fivefold by centrifugation and then transformed.

[b] %T = (Number of transformed cells × 100)/total number of cells.

[c] The counts per minute shown are corrected, allowing for the fact that protoplast suspensions were twice as concentrated as the bacillary suspensions (see Materials and Methods). Counts were determined on lysed suspensions in a Beckman LS100 liquid scintillation counter.

[d] ND = not done.

[e] Experiment terminated by deoxyribonuclease treatment.

DISCUSSION

Our experiments showing absence of marker recombination in protoplasts and extremely low marker uptake and radioactivity uptake by protoplasts are mutually supportive and also consistent with earlier results which demonstrated that protoplasts are refractory to transformation (7, 13) and transfection (16). Our results indicate that, in *B. subtilis*, the surface of the protoplasts from competent cells does not bind DNA. It is not ruled out, of course, that binding does take place in intact cells and binding properties are lost in the course of protoplasting. Further, it is quite possible that binding properties in the membrane may appear when protoplasts are lysed and the internal membrane surfaces are exposed. The finding of membrane-associated competence proteins by other investigators (10, 14) thus would not conflict with our results, even if such proteins could be shown to bind DNA.

In several years of experimentation in the subject area covered by this publication we have not encountered any experimental trends suggestive of the phenomena described by Hirokawa and Ikeda (4). Moreover, most of the speculations offered in the introduction to explain the extensive uptake and linkage formation reported by Hirokawa and Ikeda are no longer tenable in the light of our demonstration that there is no binding of DNA to intact, naked protoplasts of *B. subtilis*. The presence of quasi-spheroplasts can be invoked to account for *some* DNA uptake but could not explain linkage formation between donor and resident DNA. Thus, we are left with the hypothesis that Hirokawa and Ikeda's observations may have been due to uptake and recombination in a partially lysing system. Since the strains used in the present study and the *B. subtilis* mutants employed by Hirokawa and Ikeda were all derived from the Marburg strain, we consider it quite improbable that the differences in results could be due to strain differences.

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