JOURNAL OF BACTERIOLOGY, August, 1966
Vol. 92, No. 2

Printed in U.S.A.

Genetic Recombination of Transforming Deoxyribonucleic Acid Molecules with the Recipient Genome and Among Themselves in Protoplasts of *Bacillus subtilis*

HIDEO HIROKAWA[1] AND YONOSUKE IKEDA

Division of Biology, Southwest Center for Advanced Studies, Dallas, Texas, and Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan

Received for publication 5 March 1966

ABSTRACT

HIROKAWA, HIDEO (Southwest Center for Advanced Studies, Dallas, Tex.), AND YONOSUKE IKEDA. Genetic recombination of transforming deoxyribonucleic acid molecules with the recipient genome and among themselves in protoplasts of *Bacillus subtilis*. J. Bacteriol. **92**:455–463. 1966.—Re-extraction of transforming deoxyribonucleic acid (DNA) from protoplasts of *Bacillus subtilis* is much more efficient than from intact competent cells. This facilitated the detection of physical recombination between donor and recipient DNA molecules, as indicated by a high cotransfer index of *ind*$^+$ and *his*$^+$ markers which were originally located in exogenous and endogenous DNA molecules, respectively. This recombinant DNA was extracted after 30 min of incubation of *ind his*$^+$ protoplasts with *ind*$^+$ *his* DNA, previously extracted from a corresponding mutant strain of *B. subtilis*. The intracellular formation of recombinant molecules (*ind*$^+$ *his*$^+$) bearing markers from two different exogenous DNA species was also detected 15 min after exposure of *ind his* recipient protoplasts to a mixture of *ind*$^+$ *his* and *ind his*$^+$ donor DNA molecules. The unity of the recombinant molecule was ascertained by dilution experiments and by its being resistant to ribonuclease and trypsin treatment (but being sensitive to deoxyribonuclease). The formation of recombinant molecules showed an inverse kinetics to that of the intracellularly induced loss of linkage between the corresponding markers in the wild-type DNA, thus suggesting a breakage and reunion process which is also favored by the absence of DNA synthesis in the protoplasts and the effect of some specific inhibitors.

The fate of transforming deoxyribonucleic acid (DNA) incorporated into recipient cells has been studied in such microorganisms as pneumococcus (4, 5, 10), *Haemophilus* (19, 22), and *Bacillus* (1, 13, 15, 21). These investigations suggest that recombination of the transforming DNA with the DNA of the recipient cells occurs shortly after incorporation.

Transforming experiments have usually been conducted on intact cells. Technical difficulties such as the low frequency of reversion to colony-forming cells have discouraged similar studies with protoplasts, although they might present potential advantages, e.g., the ease with which DNA is extracted. The reduced rate of DNA synthesis in protoplasts might also be of advantage in certain cases, as, for instance, in investigating the intermediate steps in the integration of the transforming DNA.

In this paper, it is shown that protoplasts of *B. subtilis* have an increased capacity for taking up exogenous DNA, as compred with intact cells, and that intracellular genetic recombination of donor with recipient DNA is more easily detected in protoplasts than in intact cells. Of special interest is the observation that intracellular recombination between two different exogenous (donor) molecules can also occur.

MATERIALS AND METHODS

Bacterial strains. The strains used, listed in Table 1, are prototrophs and auxotrophs of *B. subtilis* Mar-

[1] On leave from The University of Tokyo, Tokyo, Japan.

TABLE 1. *Strains of Bacillus subtilis Marburg*[a]

Strain	Genotype	Origin
Wild..	Prototroph	S. Zamenhof[b]
30.....	ind_{168}, his_{30} (unlinked)	S. Zamenhof
31.....	ind_{168}, his_{31} (linked)	S. Zamenhof
171-15.	try_{160}, arg^{15}	S. Zamenhof
301....	ind_{168}	Wild $\xrightarrow{S\text{-}1}$ x 30
311....	his_{31}	Wild $\xrightarrow{TF}$ x 31
3115...	ind_{168}, his_{31}, arg_{15}	31 $\xrightarrow{TF}$ x 171-15

[a] Abbreviation: ind_{168}, indole-requiring; his_{31} and his_{30}, histidine-requiring; try_{160}, tryptophan-requiring; arg_{15}, arginine-requiring. The symbol *wild* $\xrightarrow{S\text{-}1}$ x *30* indicates a transduction from wild type to strain 30 by the temperate phage S-1. The symbol *wild* $\xrightarrow{TF}$ x *31* indicates a transformation from wild type to strain 31.

[b] See Saito and Miura (16).

burg. Wild strain, strain 311 (*ind*$^+$ *his*), and strain 301 (*ind his*$^+$) were used as sources of exogenous DNA. Protoplasts were formed from strains 311 (*ind*$^+$*his*), 301 (*ind his*$^+$), 31 (*ind his*), and 3115 (*ind his arg*).

The *ind* (indole$_{168}$) and *his* (histidine$_{31}$) loci are linked; their cotransfer index (13) is 0.6. The *arg* (arginine$_{15}$) locus is not linked to either one.

Spores were harvested from slant cultures on potato-agar and stored at 2 to 4 C (18).

Media. Nutrient medium (NB) contained: meat extract (Difco), 10.0 g; peptone (Difco), 10.0 g; NaCl, 3.0 g.; yeast extract (Difco), 1.0 g.; and distilled water, 1,000 ml; *p*H 7.0.

Minimal medium (MM) contained: KH_2PO_4, 8.46 g; KOH, 2.26 g; Na_3-citrate·H_2O, 0.5 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; glucose, 5.0 g; and distilled water, 1,000 ml; *p*H 7.0. For specific purposes, one or more amino acids were added to this medium, in the following concentrations: L-tryptophan, 50.0 μg/ml; L-histidine, 50.0 μg/ml; L-arginine, 100.0 μg/ml.

MY-1 medium (MY-1) was MM supplemented with 0.1% yeast extract (Difco), and MY-2 medium (MY-2) was MM supplemented with 0.01% yeast extract (Difco).

Glucose, magnesium sulfate, and the amino acids were autoclaved separately and added to MY-1 or MY-2 as required. For preparing solid media, separately autoclaved agar (Difco) was mixed with the other components, to give a final concentration of 2.0%.

Chemicals. Crystalline pancreatic ribonuclease (Worthington Biochemical Corp., Freehold, N. J.) and ribonuclease T_1 (Sankyo Co., Japan) were used. The solutions were heated at 80 C for 10 min, to inactivate contaminating deoxyribonuclease.

Crystalline pancreatic deoxyribonuclease, muramidase (twice crystallized), and crystalline trypsin were products of Worthington Biochemical Corp.

5-Fluorodeoxyuridine (FUDR) was obtained from Hoffmann La Roche, Inc., Basel, Switzerland, actinomycin S_3 from Daiichi Pharmaceutical Co., Japan, and Mytomycin C from Kyowa Fermentation Industries Co., Japan.

Preparation of transforming DNA. Strains of *B. subtilis* carrying the desired genetic markers were grown overnight on a rotary shaker at 30 C in 5-liter Erlenmeyer flasks containing 1.5 liters of nutrient broth. Cells were collected by centrifugation, washed with saline-EDTA (0.15 M NaCl and 0.1 M disodium ethylenediaminetetraacetate), and exposed to muramidase (1 mg/g of wet cells). DNA was extracted by the phenol method of Saito and Miura (16). The crude DNA in the extract was precipitated by the addition of 2 volumes of ethyl alcohol and was dissolved in SSC (0.15 M NaCl and 0.015 M trisodium citrate, *p*H 7.0) (11). This solution was treated with pancreatic ribonuclease and ribonuclease T_1 (16), for 30 min at 37 C. After this treatment, the phenol extractions and ethyl alcohol precipitations were repeated two or three times. The purified DNA solution, to which 1 drop of chloroform had been added, was stored in a refrigerator. The E_{260}/E_{230} ratios of various preparations ranged from 2.1 to 2.4. DNA was assayed by the method of Burton (2), with calf thymus DNA as standard.

Preparation of protoplasts. Cells cultivated in 100 ml of MY-1 medium for 4.5 hr at 37 C were collected by centrifugation and washed with tris(hydroxymethyl)-aminomethane (Tris) buffer (120 μg/ml, *p*H 7.6). The cells were then suspended in 10.0 ml of a solution containing (per milliliter): muramidase, 100 μg; sucrose, 200 mg; Tris, 100 μg; *p*H 8.0. After 10 min, 1 ml of EDTA (50 μg/ml) was added. By 10 to 15 min after this addition, almost all rod-shaped cells had changed to protoplasts. These were carefully centrifuged, washed once with hypertonic MY-2 solution (MY-2 plus 20% sucrose), and suspended in 20 ml of the same solution. Thus, a suspension containing 5×10^8 protoplasts per milliliter was finally obtained.

Transformation. Transformation experiments were carried out by the method of Saito and Miura (16), with strain 31 (*ind his*) or 3115 (*ind his arg*) as recipients. The concentrations of recipient cells, DNA, and deoxyribonuclease were 5×10^8 cells, 0.1 μg, and 5.0 μg per ml, respectively. Platings were done on minimal agar and minimal agar supplemented with one or more amino acids, as stated in the text.

Cell countings. Each class of transformants was scored by counting the colonies on appropriate selective plates (in duplicate and at two separate dilutions).

RESULTS

Characterization of the genetic markers. When DNA from strain 301 (*ind*) was used to transform strain 31 (*ind his*), no *ind*$^+$ recombinants were formed. Analogous experiments with the same recipient strain, but strain 311 (*his*) as a donor, gave no *his*$^+$ transformants. Thus, not only by its origin (Table 1) but also by the lack of genetic

TABLE 2. *Transformation of strains 31 (ind his) and 3115 (ind his arg) by DNA from the donors 301 (ind), 311 (his) or wild type*

Recipient	Donor DNA	Concn of DNA	No. of transformants per 0.01 ml in the classes			
			arg$^+$	*ind*$^+$ *his*	*ind his*$^+$	*ind*$^+$ *his*$^+$
		μg/ml				
Strain 31-(*ind his*)	301 (*ind*)	0.1		0	345	0
	311 (*his*)	0.1		205	0	0
	Wild	0.1		454	345	1,203
	DNA-free	—		0	0	0
Strain 3115 (*ind his arg*)	301 (*ind*)	0.7	2,900	0	1,800	0
	311 (*his*)	0.7	2,900	1,300	0	0
	Wild	0.1	1,300	80	230	430
	DNA-free	-	0	0	0	0

recombination (Table 2), the *ind* site of strain 301 and the *his* sites of strains 311 and 3115 can be considered identical to those in strain 31. Similar experiments, with wild-type DNA as donor, indicated a linkage between these markers, the cotransfer index (13) being approximately 0.6. The results also indicate that the *ind* and *his* markers in strain 3115 are identical to those in strains 301 and 311, respectively (Table 2).

Transformation with re-extracted DNA. The following experiment was designed to answer the question of whether protoplasts of *B. subtilis* Marburg can take up exogenous DNA which would be able to recombine physically with the DNA of the recipient cell.

Approximately 5×10^8 protoplasts per milliliter of strain 301 or 311 were exposed to DNA from strain 311 or 301, respectively, at a concentration of 20 μg/ml, for 30 or 60 min, at 37 C. These protoplasts were then treated with deoxyribonuclease at a concentration of 5 μg/ml, for 10 min, at 37 C, rapidly cooled, carefully centrifuged at 12,000 × *g* for 10 min, and washed once with hypertonic MY-2 solution. They were then disrupted by transfer to a hypotonic solution [1% sodium dodecyl sulfate (SDS) in 0.1 M Tris buffer (*p*H 9.0)]. The DNA was extracted by methods already described. Finally, transformation experiments were carried out with this DNA (0.1 μg/ml) and competent cells of strain 31, to check cotransformation of *ind*$^+$ *his*$^+$, one marker being of exogenous and the other of endogenous origin.

As a control, intact cells were treated with the same exogenous DNA as the protoplasts. The DNA was then extracted and used in similar transformation experiments as above.

Table 3 shows that *ind*$^+$ *his*$^+$ transformants were found in every experiment. In principle, they could have been produced either in a single transformation event, by *ind*$^+$ *his*$^+$ recombinant type DNA, or in double transformation events, by *ind his*$^+$ and *ind*$^+$ *his* DNA molecules. The following experiment appears to rule out the second alternative. Mixed DNA preparations (*ind his*$^+$ and *ind*$^+$ *his* in equal proportions, in concentrations 2 to 14 times higher than in the previous experiments) were substituted for the re-extraced DNA. Here the *ind*$^+$ *his*$^+$ type transformants were found too, as shown in Table 4, and they were necessarily produced by double transformation events. The ratios of *ind*$^+$ *his*$^+$ to *ind his*$^+$ and to *ind*$^+$ *his*, however, were significant, being one or two orders of magnitude lower than those indicated in Table 3.

The results support the idea that intracellular recombination between exogenous and protoplast DNA occurred, with this recombinant DNA being responsible for the cotransformation of strain 31 to *ind*$^+$ *his*$^+$.

Intracellular recombination among two species of exogenous DNA molecules. In experiments conducted similarly, it was also shown that intracellular recombination can occur not only between recipient and donor DNA, but also between two different molecules of donor DNA taken up. Approximately 10^8 protoplasts of strain 31 (*ind his*), suspended in 5 ml of 20% sucrose-MY-2 medium, were simultaneously exposed to DNA prepared from strain 311 (*ind*$^+$ *his*) and 301 (*ind his*$^+$) at the concentrations shown in Table 5. The mixture was incubated at 37 C for 30 min, and was centrifuged at 12,000 × *g* for 5 min. After the cells were washed twice with 20% sucrose-MY-2 solution and transferred to SSC (0.15 M NaCl, 0.015 M Na_3-citrate, *p*H 7.0), lysis was induced as above, and the DNA was precipitated by the addition of 2 volumes of ethyl alcohol. The filamentous precipitate was collected with a glass rod and dissolved in SSC. These procedures were executed in a cold room.

Transformation experiments were then carried

TABLE 3. *Transformation of strain 31 (ind his) by re-extracted DNA*

Expt no[a]	Type of DNA taken up by recipients, for preincubation and re-extraction[b]	Incubation time at 37 C	Recipients, for preincubation and re-extraction of DNA[c]	No. of transformants per ml produced by re-extracted DNA in the classes[d]			Ratios		
				ind$^+$ *his*$^+$ (a)	*ind*$^+$ *his* (b)	*ind his*$^+$ (c)	Recombinant/exogenous × 100	Recombinant/residual × 100	Exogenous/residual × 100
		min							
1	311 *ind*$^+$ *his*	30 - - - - - -x	(P) 301 *ind his*$^+$	590	4,100	9,180	*14.4*	*6.5*	*44.6*
	311 *ind*$^+$ *his*	30 - - - - - -x	(R) 301 *ind his*$^+$	40	110	8,800	*36.4*	*0.5*	*1.25*
2	311 *ind*$^+$ *his*	30 - - - - - -x	(P)301 *ind his*$^+$	180	1,320	5,420	*13.6*	*3.3*	*24.3*
3	311 *ind*$^+$ *his*	60 - - - - - -x	(P) 301 *ind his*$^+$	450	1,450	10,000	*31.0*	*4.5*	*14.5*
4	301 *ind his*$^+$	30 - - - - - -x	(P) 311 *ind*$^+$ *his*	165	34,700	1,080	*15.2*	*0.5*	*3.11*

[a] In experiments 1 to 3, the transformant classes (a), (b), and (c) are of recombinant, exogenous, and residential origin, respectively; in experiment 4, they are of recombinant, residential, and exogenous origin, respectively.
[b] Concentration used: 20 μg/ml.
[c] (P) protoplasts, (R) rod-cells.
[d] Concentration of re-extracted DNA: 0.1 μg/ml.

TABLE 4. *Transformation of strain 31 by mixed DNA preparations from the donors 301 (ind his*$^+$*) and 311 (ind*$^+$ *his)*

Total concn of mixed donor DNA[a]	No. of transformants per ml, in the classes			Ratios:	
	ind$^+$ *his*$^+$ (a)	*ind*$^+$ *his* (b)	*ind his*$^+$ (c)	(a)/(b) × 100	(a)/(c) × 100
μg/ml					
1.4	330	88,000	110,000	0.37	0.30
0.7	30	48,000	58,000	0.06	0.05
0.2	0	19,200	16,000	0.00	0.00

[a] Both donor DNA preparations were mixed in equal proportions.

out with this DNA (1.0 μg/ml), with strain 3115 (*ind his arg*) as recipient, and *ind*$^+$ *his*$^+$ transformants were selected. These were produced at a high frequency, the cotransfer index being about 0.3 to 0.6, as compared with less than 0.002 in controls for which mixed donor DNA preparations had been used (Table 6). The fact that the number of *ind*$^+$ *his*$^+$ transformants decreased with the progressive dilution of the donor DNA can be explained by the decreased probability of recombination at the lower DNA concentrations. In any case, the considerable difference between the calculated values corresponding to double transformation events and the presented data makes it seem probable that the *ind*$^+$ *his*$^+$ transformants arose as a consequence of a single transformational event by an *ind*$^+$*his*$^+$-type DNA which originated by genetic recombination of exogeneous *ind*$^+$ *his* and *ind his*$^+$ donor molecules in the *ind his* protoplasts used for preincubation.

The plottings in Fig. 1 corroborate this notion, by showing that, upon dilution of the donor DNA, the number of *ind*$^+$ *his*$^+$ transformants de-decreases rather slowly when the DNA re-extracted from protoplasts is used, whereas it decreases rapidly when mixed DNA preparations are used. This is in accord with the expectation of the decrease being proportional to the dilution factor when both markers are located in one transformant molecule, as compared with an exponential decrease when separate molecules carry each marker.

Evaluation of protoplasts as recipients. Experiment 1 in Table 3 shows that larger numbers of *ind his*$^+$ and *ind*$^+$ *his*$^-$ transformants were produced by using re-extracted DNA from protoplasts as donor than when re-extracted DNA from intact cells was used. In this experimental system, the *ind*$^+$ marker was of exogenous origin and the *his*$^+$ marker originated from the recipient cell. Therefore, the ratio of total numbers of *ind*$^+$ *his* colonies to *ind his*$^+$ colonies can be considered an indicator of the re-extraction of exogenous DNA from the recipients. This ratio is 0.45 for protoplasts and 0.012 for intact cells, which indicates

TABLE 5. *Transformation of strain 3115 (ind his arg) by DNA re-extracted from protoplasts of 31 (ind his), previously incubated with mixed DNA from strains 301 (ind his$^+$) and 311 (ind$^+$ his)*

Concn of mixed DNA protoplasts were exposed to[a]	Concn of re-extracted DNA used in transformation	No. of transformants per 2×10^7 recipients of 3115 (*ind his arg*), in the classes					Cotransformation index for *ind*$^+$*his*$^+$
		arg$^+$ $\times 10^3$	*ind*$^+$ *his*	*ind his*$^+$	*ind*$^+$ *his*$^+$		
					Observed	Expected[b]	
μg/ml	*μg/ml*						
42.0	1.0	890	1,900	2,100	3,600	2×10^{-1}	0.473
0.7	1.0	670	240	590	1,200	7×10^{-3}	0.591
0.07	1.0	670	210	160	140	1.6×10^{-3}	0.274

[a] The two parental DNA preparations were mixed in equal proportions and added to protoplasts of strain 31, preincubated for 30 minutes at 37 C, and then re-extracted. Transformation experiments were carried out with this re-extracted DNA and, again, with strain 3115 as the recipient.

[b] The values expected, if transformation to *ind*$^+$ and *his*$^+$ were independent events, have been calculated from the number of transformants of each parental type (*ind*$^+$ *his* and *ind his*$^+$) as compared with the number of competent recipient cells.

TABLE 6. *Transformation of strain 3115 (ind his arg) by mixed DNA from the donor strains 301 (ind his$^+$) and 311 (ind$^+$ his)*

Total concn of mixed DNA[a]	No. of transformants per 2×10^7 recipient cells in the classes					Co-TF Index for *ind*$^+$ *his*$^+$
	arg$^+$ $\times 10^3$	*ind*$^+$ *his* $\times 10^2$	*ind his*$^+$ $\times 10^2$	*ind*$^+$ *his*$^+$		
				Observed	Expected	
μg/ml						
42.0	260	400	1,100	380	220	0.0025
1.4	310	780	810	190	314	0.0012
0.7	160	480	550	30	130	0.0003

[a] Both donor DNA preparations were mixed in equal proportions.

[b] See footnote to Table 5.

that incorporated exogenous DNA is efficiently re-extracted from protoplasts.

DNA synthesis in protoplasts. Protoplasts of strain 31 were incubated at 37 C in MY-2 medium supplemented with 20% sucrose. At intervals, samples were withdrawn and the DNA was isolated by the method of Schneider (17). The DNA content was then determined by methods previously described. The results in Table 7 show no observable increase in DNA concentration, suggesting that DNA synthesis is negligible in the protoplasts.

Effects of ribonuclease and trypsin treatment on recombinant DNA. Re-extracted DNA (approximately 10 μg/ml) was treated with ribonuclease (20 μg/ml) at 37 C for 30 min; then trypsin (10 μg/ml) was added, and the solution was incubated for 30 min more. Transformation was carried out by routine methods with strain 31 as the recipient.

Results in Table 8 show that equal numbers of transformants were obtained with treated DNA and with nontreated DNA. DNA treated with 5 μg/ml of deoxyribonuclease for 30 min gave no transformants.

Effects of metabolic inhibitors on the formation of recombinant DNA. Various types of inhibitors (Table 9) were added to mixtures of protoplasts and exogenous DNA preparations, and the mixtures were incubated for 60 min at 37 C. The DNA was re-extracted from these protoplasts, and its transformation ability was evaluated with strain 3115 as recipient. KCN, FUDR, and phenylethyl alcohol tended to enhance the formation of recombinant DNA, whereas chloramphenicol had no effect at the concentration tested. Actinomycin S_3 appeared to protect *ind*$^+$ *his* and *ind his*$^+$ DNA from degradation, simultaneously inhibiting the formation of *ind*$^+$ *his*$^+$ DNA by approximately 50%. The transforming efficiency of the *arg*$^+$ marker of the protoplasts was not affected by this drug.

As opposed to the effect of actinomycin S_3, specific inhibition of recombinant formation by mitomycin C was not observed, although mytomycin C is known to depress strongly the effi-

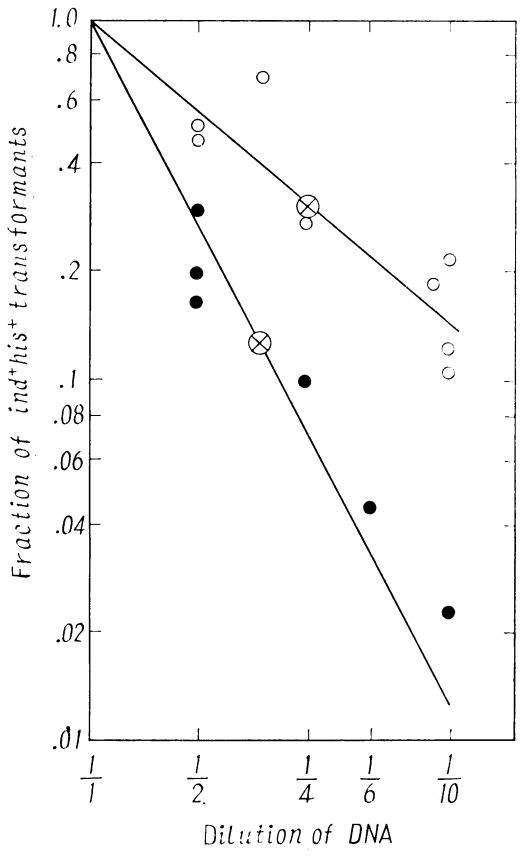

FIG. 1. *Effect of dilution on cotransforming ability of re-extracted DNA and of mixed DNA preparations. Symbols:* ○, *DNA extracted from ind his protoplasts which were treated with ind his*$^+$ *and ind*$^+$*his DNA;* ●, *ind his*$^+$ *and ind*$^+$ *his DNA mixed together. For both experiments, the DNA preparations were diluted with SSC solution. Capacity to form ind*$^+$*his*$^+$ *transformants was tested with strain 31 (ind his) as recipient. Fraction 1.0 corresponds to 120 cells per milliliter, transformed by the re-extracted DNA (1.0 μg/ml), and to 380 cells per milliliter transformed by the mixed DNA (14 μg/ml; 7.0 μg/ml each). The crossed circles show the so-called regression points.*

TABLE 7. *DNA content and numbers of protoplasts during incubation at 37 C*

Time	DNA	No. of protoplasts per ml[a]
min	*μg/ml*	
0	10.3	1.6×10^9
30	9.9	1.6×10^9
60	10.0	–
90	9.6	1.6×10^9

[a] Protoplasts were counted directly under a microscope.

TABLE 8. *Effect of ribonuclease and trypsin on the transforming ability of DNA re-extracted from protoplasts*[a]

Re-extracted DNA treated with	No. of transformants per ml in the classes			Cotransformation index for *ind*$^+$ *his*$^+$
	ind$^+$ *his*	*ind his*$^+$	*ind*$^+$ *his*$^+$	
No treatment	138	246	180	0.32
Ribonuclease and trypsin[b]	150	270	120	0.22
Deoxyribonuclease[c]	0	0	0	—

[a] Competent cells of strain 31 (*ind his*) were used as recipients.

[b] Re-extracted DNA (1.0 μg/ml) was treated with ribonuclease (20 μg/ml) for 30 min at 37 C, and, subsequently, with trypsin (10 μg/ml) for 30 min at 37 C.

[c] Re-extracted DNA (10 μg/ml) was treated with deoxyribonuclease (5 μg/ml) for 30 min at 37 C.

ciency of transforming systems by binding to DNA (8).

Loss of linkage between cotransforming markers, by preincubation with protoplasts. Ind$^+$ *his*$^+$ DNA (wild) was incubated with protoplasts of strain 31 (*ind his*). At intervals, the DNA was re-extracted from the protoplasts and its transforming activity was checked with strain 31 as recipient. The data in Table 10 indicate that within 15 min the linkage between the *ind*$^+$ and *his*$^+$ markers was reduced to approximately 50% of the original value. Thus, fragmentation of exogenous DNA by the recipient cell might have occurred during this period.

Kinetics of recombinant DNA formation. Protoplasts of strain 31 were exposed to *ind*$^+$ *his* and *ind his*$^+$ DNA under standard conditions. At timed intervals after exposure, the protoplasts were rapidly cooled to 0 C and collected by centrifugation; then the DNA was extracted. This DNA was used to transform competent cells of strain 3115.

The data in Fig. 2 show that *ind*$^+$ *his*$^+$ DNA began to be formed soon after exposure. The process was about 70% complete in 5 min, and was complete in 15 min. During this period, the transforming ability of exogenous DNA (*ind his*$^+$ and *ind*$^+$ *his*) decreased rapidly. Although the proportion of *ind*$^+$ and *his*$^+$ markers recovered in newly formed *ind*$^+$ *his*$^+$ DNA was quite low, the results strongly suggest a correlation between the disappearance of parental exogenous DNA and the appearance of recombinant exogenous DNA. The endogenous *arg*$^+$ marker was very stable,

TABLE 9. *Effects of chemicals on the formation of recombinant DNA*[a]

Chemical	Concn	No. of transformants per ml in the classes				Cotransformation index for *ind*$^+$ *his*$^+$
		arg$^+$ × 10^3	*ind*$^+$ *his*	*ind his*$^+$	*ind*$^+$ *his*$^+$	
Chloramphenicol	10 μg/ml	300	690	290	520	0.34
KCN	10^{-2} mole	120	220	250	780	0.62
FUDR	50 μg/ml	170	220	210	430	0.50
Phenylethyl alcohol	0.3%	230	200	400	630	0.51
Mitomycin C	50 μg/ml	52	50	190	100	0.29
Actinomycin S_3	10^{-3} μg/ml	300	5,450	6,550	150	0.012
No addition (control)	–	300	270	280	340	0.38

[a] The chemicals were added to the mixture of donor DNA preparations and 3115 (*ind his arg*) recipient protoplasts which were incubated for 30 min, at 37 C. The DNA was then re-extracted, and its transforming activity was checked in 3115 (*ind his arg*) recipients.

TABLE 10. *Disappearance of linkage between two markers, in wild-type DNA*[a]

Time of incubation with protoplasts	No. of transformants per ml in the classes			Cotransformation index for *ind*$^+$ *his*$^+$
	ind$^+$ *his*	*ind his*$^+$	*ind*$^+$ *his*$^+$	
min				
0	147	131	833	0.75
15	816	643	800	0.35
30	829	696	954	0.38
30 (no protoplasts)	306	206	1,620	0.75

[a] Wild-type DNA (20 μg/ml) was added to 31 (*ind his*) protoplasts. At the times indicated, the protoplasts were harvested and washed, and then their DNA was extracted. The transforming activity of this DNA was tested with competent cells of strain 31 (*ind his*) as recipients. The last line refers to a control in which wild-type DNA was used directly as transforming principle, without preincubation with *ind his* cells.

indicating that the behavior of the DNA of the protoplasts differed from that of exogenous DNA.

DISCUSSION

One of the factors which render difficult many investigations on transforming systems is the low efficiency with which transforming DNA often is taken up by intact competent cells, even under optimal conditions. It has now been shown here that protoplasts of *B. subtilis* have a much higher capacity for taking up DNA as compared with competent intact cells (see Table 3). This was demonstrated by comparing the ratios of exogenous to residential DNA in the DNA fractions re-extracted from protoplasts and from competent intact cells after their exposure to exogenous DNA molecules. These ratios ranged from 0.03 to 0.44 (average 0.22) with protoplasts, as compared to 0.0012 with intact cells.

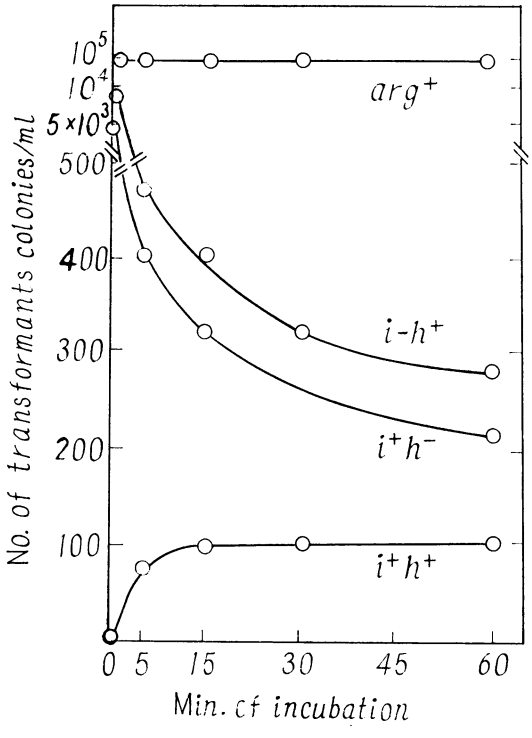

FIG. 2. *Time course of formation of recombinant DNA in protoplasts of Bacillus subtilis. Two parental DNA preparations (ind his*$^+$*, 0.8 μg/ml; ind*$^+$*his, 0.8 μg/ml) were added to protoplasts of strain 31 (ind his; 10^8/ml). At the times indicated, the protoplasts were placed in an ice bath, and their DNA was extracted. The transforming activity of this DNA was measured with competent cells of strain 3115 (ind his arg) as recipients. Ind*$^+$*his*$^+$ *is the recombinant type; ind his*$^+$ *and ind*$^+$*his are the parental types; arg*$^+$ *is an endogenous marker.*

According to Bodmer and Ganesan (1), in the re-extracted DNA from intact cells of *B. subtilis* treated the same way, this ratio was 0.0073 to 0.00075, which is in reasonable agreement with

our value. The high re-extraction of exogenous DNA from protoplasts has made it possible to observe easily such effects as genetic recombination of exogenous and endogenous markers, and especially genetic recombination between two exogenous markers originally located in separate donor DNA molecules.

Although genetic recombination of exogenous with residential DNA has already been widely reported, it appears that recombination of two exogenous markers without their integration into the recipient genome constitutes a novel finding. The following observations support this finding. The formation of double transformants (*ind*$^+$ *his*$^+$) has been observed upon treating *ind his* recipients with DNA extracted from a sample of *ind his* protoplasts to which two DNA preparations of different origins (one *ind*$^+$ *his*, the other *ind his*$^+$) had been added 15 min earlier. The appearance of double transformants under these conditions could in principle be explained by two alternative assumptions: either they arose as a consequence of two independent transformational events, or else the two markers were integrated by one simple event involving recombinant DNA. This recombinant DNA would have to be formed during the 15-min incubation of the two exogenous DNA samples with the protoplasts from which they had been re-extracted. The first alternative was ruled out (i) by reconstruction experiments and (ii) dilution experiments. In reconstruction experiments with mixtures of the two exogenous DNA preparations without preincubation in protoplasts, the frequency of double transformants was one or two orders of magnitude lower, even when much higher DNA concentrations were used. Dilution experiments showed that the preincubated DNA preparations yielded double transformants at a frequency which was proportional to the concentration of DNA, whereas the mixture of the two nonincubated exogenous DNA preparations, upon dilution, lost its ability to originate double transformants at a much higher rate. [Similar reasoning was discussed by Heriott (7) with respect to the formation of joint DNA molecules after melting and annealing *Haemophilus* DNA.]

We do not know the mechanism by which recombinant molecules arise from two exogenous DNA molecules taken up simultaneously by *B. subtilis* protoplasts. Since, in our experimental conditions, no DNA synthesis could be observed in these protoplasts, it seems likely that the parental molecules physically recombine by a breakage and reunion mechanism (9, 12). We have not investigated whether the recombinant molecules are continuous in their phosphodiester backbones or whether some gaps exist at the points of reunion as observed in the "joining form" of T4 DNA by Tomizawa and Anraku (20).

The fact that the intracellular appearance of recombinant molecules occurs parallel to the intracellular disappearance of linkage of the two markers involved when wild-type (*ind*$^+$ *his*$^+$) DNA is incubated with protoplasts also favors the idea of break and reunion.

The breakdown of linkage and the reduction of transforming ability of the parental DNA after preincubation in protoplasts suggests that protoplasts possess a nuclease activity. This effect, however, is observed on exogenous DNA only, as one endogenous marker (*arg*$^+$) was not affected at all during the incubation period. Recently, a decrease in parental (exogenous) DNA early during incubation was reported on DNA recombination in the transforming system of *H. influenzae* (22) and of *B. subtilis* (21). Therefore, recombination might be accompanied by the fragmentation of exogenous DNA in this case also. If genetic recombination by breakage and reunion is in some way related to nuclease activity, it is certainly not a straightforward relationship, because genetic recombination can be observed between exogenous and endogenous DNA (the latter not subject to nuclease breakdown) as well as between two exogenous DNA molecules subject to breakdown.

The action of inhibitors showed that the recombination process does not require protein synthesis, a supply of energy, or a normal level of DNA synthesis. Mechanisms for recombination involving single strand formation are difficult to visualize in view of our observation that phenylethyl alcohol did not interfere with recombinant formation. [According to Folsome (3) phenylethyl alcohol prevents the formation of single-stranded DNA.] Actinomycin, known to bind to DNA (6), prevented the breakage of exogenous DNA molecules as well as the formation of recombinant-type DNA. This finding also suggests that the breakage of exogenous DNA is a prerequisite for the formation of the recombinant-type DNA, which would be completed by the action of the so-called recombination or repair enzymes.

ACKNOWLEDGMENTS

We thank R. Hausmann and Jan Cooper for their help in preparing the manuscript, and H. Saito for his discussion.

LITERATURE CITED

1. BODMER, W. F., AND A. T. GANESAN. 1964. Biochemical and genetic studies of integration and recombination in *Bacillus subtilis* transformation. Genetics **50**:717–738.

2. BURTON, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**:315–323.
3. FOLSOM, C. E. 1963. Inhibition of recombination and heterozygosis in phenylethylalcohol treated phage T4-*E. coli* B complexes. Biochem. Biophys. Res. Commun. **11**:97–101.
4. FOX, M. S., AND R. D. HOTCHKISS. 1961. Fate of transforming deoxyribonucleate following fixation by transformable bacteria. Nature **187**: 1002–1006.
5. FOX, M. S., AND M. K. ALLEN. 1964. On the mechanism of deoxyribonucleate integration in *Pneumococcal* transformation. Proc. Natl. Acad. Sci. U.S. **52**:412–419.
6. GOLDBERG, I. H., AND E. REICH. 1964. Actinomycin inhibition of RNA synthesis directed by DNA. Federation Proc. **23**:958–964.
7. HERIOTT, R. M. 1961. Formation of heterozygotes by annealing a mixture of transforming DNAs. Proc. Natl. Acad. Sci. U.S. **47**:146–153.
8. IYER, V. N., AND W. SZYBALSKI. 1963. A molecular mechanism of mytomycin action: linkage of complementary DNA strand. Proc. Natl. Acad. Sci. U.S. **50**:355–362.
9. KOZINSKY, A. W., AND P. B. KOZINSKI. 1964. Replicative fragmentation in T4 bacteriophage DNA. II. Biparental molecular recombination. Proc. Natl. Acad. Sci. U.S. **52**:211–218.
10. LACKS, S. 1962. Molecular fate of DNA in genetic transformation of *Pneumococcus*. J. Mol. Biol. **5**:119–131.
11. MARMUR, J. 1961. A procedure for the isolation of deoxyribonucleic acids. J. Mol. Biol. **3**:585–594.
12. MESELSON, M. 1964. On the mechanism of genetic recombination between DNA molecules. J. Mol. Biol. **9**:734–745.
13. NESTER, E. W., AND J. LEDERBERG. 1961. Linkage of genetic units of *Bacillus subtilis* in deoxyribonucleic acid (DNA transformation). Proc. Natl. Acad. Sci. U.S. **47**:52–55.
14. NESTER, E. W., AND B. A. D. STOCKER. 1963. Biosynthetic latency in early stages of deoxyribonucleic acid transformation in *Bacillus subtilis*. J. Bacteriol. **86**:785–796.
15. PENE, J. J., AND W. R. ROMIG. 1964. On the mechanism of genetic recombination in transforming *Bacillus subtilis*. J. Mol. Biol. **9**: 236–246.
16. SAITO, H., AND K. MIURA. 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys. Acta **72**:619–629.
17. SCHNEIDER, W. C. 1946. Phosphorus compounds in animal tissues. III. A comparison of methods for the estimation of nucleic acids. J. Biol. Chem. **164**:747–751.
18. SPIZIZEN, J. 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Natl. Acad. Sci. U.S. **44**:1072–1078.
19. STUY, J. H. 1965. Fate of transforming DNA in the *Haemophilus influenzae* transformation system. J. Mol. Biol. **13**:554–570.
20. TOMIZAWA, J., AND N. ANRAKU. 1964. Molecular mechanism of genetic recombination in bacteriophage. II. Joining of parental DNA molecules of phage T4. J. Mol. Biol. **8**:516–540.
21. VENEMA, G., R. H. PRITCHARD, AND T. VENEMA-SCHRÖDER. 1965. Fate of transforming deoxyribonucleic acid in *Bacillus subtilis*. J. Bacteriol. **89**:1250–1255.
22. VOLL, M. J., AND S. H. GOODGAL. 1961. Recombination during transformation in *Haemophilus influenzae*. Proc. Natl. Acad. Sci. U.S. **47**: 505–512.