The Integration of Donor and Recipient Deoxyribonucleic Acid during Transformation of *Bacillus subtilis*

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1. DNA labelled with 5-bromo[^3H]uracil was used to transform auxotrophic strains of *Bacillus subtilis*. 2. After various times of incubation, DNA was extracted from the transformed culture and subjected to equilibrium sedimentation in caesium chloride gradients. 3. In addition to heavy donor DNA and light recipient DNA, a component with an intermediate density was found and is believed to consist of a biological hybrid of donor and recipient. 4. The component of intermediate density was isolated and found to possess activity in transformation derived from both donor and recipient strains. 5. Denaturation of the component of intermediate density followed by centrifugation gave only one component, indicating that integration had occurred in both strands of the recipient DNA. 6. No integrated band was observed after uptake by competent cells of *B. subtilis* of heavy DNA prepared from *Escherichia coli*.

The nature of the processes involved in the transfer of genetic information from donor DNA to recipient cells in bacterial transformation is not fully understood. Attempts have been made to distinguish between the two hypotheses designated as 'copy choice' and 'breakage and reunion' put forward by Lederberg (1955). In the first, donor DNA serves as a template in the replication of the host DNA but is not physically incorporated into the recipient genome. According to the alternative hypothesis, donor DNA is inserted into the chain of resident DNA by a process involving fission and repair. Several investigations (Lorkiewicz, Opara-Kubinska & Szybalski, 1961; Pène & Romig, 1964; Bodmer & Ganesan, 1964) have shown that during the transformation of cultures of *Bacillus subtilis*, insertion of donor DNA into the resident genome occurs.

It has been shown by Nester & Stocker (1963) that there is a delay in the appearance of transformants after treatment of competent cultures of *B. subtilis* with DNA. Also, Kammen, Beloff & Canellakis (1966) found that the number of transformed cells decreases with time of incubation after treatment with DNA, while at the same time non-transformed cells continue to multiply. These observations lead to the conclusion that cells that are capable of being transformed do not multiply and therefore may not synthesize new DNA. However, since growth of transformed cells is essential for the phenotypic expression of the transformation, the possibility that 'copy choice' contributes in part to the processes involved in transformation cannot be excluded.

A third alternative explanation of the transfer of genetic information during transformation has been proposed by Pène & Romig (1964), namely that a partial diploid is formed without the replacement of the resident allele. This possibility deserves further consideration in view of the results of Singh & Pitale (1968), which suggest that at least the majority of cells in competent cultures of *B. subtilis* are uninucleate.

The experiments recorded below were undertaken to find more sensitive methods of studying the nature of the physical association of donor and host DNA, and to define the process more precisely.

MATERIALS AND METHODS

Bacterial strains. The prototrophic Marburg strain of *Bacillus subtilis*, and *B. subtilis* strain 31 containing linked histidine and tryptophan markers, were given by Dr M. Saito. *B. subtilis* strains 57 (*his*⁻) and 59 (*tryp*⁻) were supplied by Dr M. S. Kelly. *Escherichia coli* strain B was a laboratory strain. All organisms were maintained by monthly transfers on to slopes of tryptose-blood-agar base (Difco Laboratories, Detroit, Mich., U.S.A.).

Preparation of [^3H]BU-DNA* from *B. subtilis*. The appropriate strain was inoculated from a slope into 100 ml. of minimal medium described by Spizizen (1958), and grown with aeration at 37° for 16 hr. A 60 ml. batch of the culture was transferred to 200 ml. of the same medium (E_{600} 0.25) and incubated with aeration at 37° for 3 hr. 5-Bromo[^3H]uracil (2 mc) (specific radioactivity 1530 c/mole) supplied by The Radiochemical Centre (Amersham,

* Abbreviations: [^3H]BU-DNA, DNA containing residues of 5-bromo[^3H]uracil; SSC, 0.15 M-NaCl-0.015 M-trisodium citrate adjusted to pH 7.0 with M-NaOH.

Bucks.) was added to the culture and incubation was continued for a further 4 hr. Cells were collected by centrifugation for 10 min. at 5000 g and washed once by centrifugation with 200 ml. of 0.15 M-NaCl containing 0.1 M-trisodium EDTA adjusted to pH 8.0 with M-NaOH. The cells were resuspended in 7.5 ml. of the same buffer and DNA was prepared from them by the method of Marmur (1961). The product was dissolved in SSC at a concentration of 0.6 mg./ml. calculated from E_{260} by the equation of Sueoka & Cheng (1962).

Radioactivity was assayed as follows: 0.1 ml. of the solution of [^3H]BU-DNA in SSC was mixed with 0.2 ml. of M-Hyamine hydroxide in methanol and heated for 1 hr. at 60°. Scintillation mixture (10 ml.) containing 0.4% (w/v) of 2,5-diphenyloxazole and 0.04% (w/v) of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in xylene were added to the cooled solution, and the sample was counted in the Packard Tri-Carb liquid-scintillation counter in one channel with a gain of 35% and window settings of 100–215. A quench window was used with a gain of 2% and a window setting of 300–1000.

Equilibrium sedimentation in caesium chloride. Solid CsCl was added to a solution of approximately 30 μg . of DNA in 0.6 ml. of SSC to give a refractive index of 1.4070. The solution was centrifuged at 35 000 rev./min. for 66 hr. in the SW39 rotor on the Spinco model L ultracentrifuge. A hypodermic needle was inserted in the bottom of the tube and 60 μl . fractions were collected. One drop (approx. 20 μl .) from each fraction was used to determine the refractive index and the rest was diluted to 0.3 ml. with 0.1 M-NaCl containing 0.02 M- K_2HPO_4 adjusted to pH 6.8 with M-NaOH. The E_{260} of the diluted solution was measured, and samples (0.1 ml.) were used for the measurement of radioactivity as described above. Buoyant densities were calculated from the measurements of refractive index by the method of Weigle, Meselson & Paigen (1959).

Heat-denaturation of fractions obtained by equilibrium sedimentation was carried out by dialysing samples for 24 hr. against SSC adjusted to pH 7 with M-NaOH, heating the dialysed solution for 10 min. at 100° and cooling quickly to 0°. The concentration of solutions heated in this way was 10 μg ./ml.

Measurement of transforming activity. Competent cultures of *B. subtilis* were prepared by inoculation of strain 31 from a slope into glucose-minimal medium (Spizizen, 1958) supplemented with 20 μg ./ml. of each of L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-cystine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine and L-valine, and 22.3 μg ./ml. of MnSO_4 (medium I). After incubation for 15 hr. at 37° with slow aeration, a sample of the culture was transferred to 300 ml. of medium I, so that E_{600} was 0.3. The culture was aerated vigorously at 37° for 4 hr. and then diluted with 1 l. of medium II, which differed from medium I in containing no MnSO_4 , and L-histidine and L-tryptophan at concentrations of 5 μg ./ml., but, in addition, 40 μg ./ml. of 2,2'-bipyridyl. The suspension of cells was incubated with slow aeration at 37° for 1 hr. Frequency of transformation of the resultant competent culture was measured as previously described (Akrigg, Ayad & Barker, 1967). Similar methods were used for preparing competent cells of strain 59 except that the concentration of L-histidine in medium II was the same as that in medium I.

RESULTS

Fate of DNA in competent cells. A 100 ml. batch of a competent culture of *B. subtilis* strain 31 was incubated at 34° with [^3H]BU-DNA, prepared from *B. subtilis* strain Marburg, at a final concentration of 5 μg ./ml. After 0, 6 and 10 min., deoxyribonuclease (5 μg ./ml.) and magnesium sulphate (0.6 mg./ml.) were added and incubation was continued for a further 5 min. at 34°. The cells were harvested as described above and were washed once with 25 ml. of 0.15 M-sodium chloride containing 0.1 M-EDTA adjusted to pH 8 with 2 M-sodium hydroxide. DNA was isolated from the washed cells and submitted to equilibrium sedimentation in caesium chloride as described in the Materials and Methods section. The results are shown in Figs. 1(a), 1(b) and 1(c). The curve for material isolated from competent cells that had been treated with [^3H]BU-DNA for 6 min. and 10 min. (Fig. 1b and 1c) exhibited a shoulder representing 260 nm.-absorbing material having a buoyant density of 1.749 g.cm.⁻³ (the I-band) which is intermediate between that of the heavy labelled DNA (the H-band) (1.786 g.cm.⁻³) and the light recipient DNA (the L-band) (1.702 g.cm.⁻³). In each case, radioactivity was found in fractions corresponding to the H-band and the I-band but was much lower in fractions corresponding to the L-band. Fractions corresponding to the I- and L-bands (Fig. 1c) were combined, dialysed against SSC and submitted to equilibrium sedimentation again under the same conditions. The results are shown in Fig. 1(d).

Heat-denaturation of fractions of DNA obtained from caesium chloride gradients. Samples of donor DNA from the H-band of Fig. 1(b), recipient DNA from the L-band of Fig. 1(d), and the I-band of Fig. 1(d) were separately denatured by heat. The denatured H- and L-bands were mixed and submitted to equilibrium sedimentation in caesium chloride; the denatured I-band was centrifuged separately under identical conditions. The results are shown in Fig. 2. The mixture of the denatured H- and L-bands gave two peaks corresponding to buoyant densities of 1.805 g.cm.⁻³ and 1.720 g.cm.⁻³. The denatured I-band gave a single peak of buoyant density 1.769 g.cm.⁻³.

Transforming activity of fractions of DNA obtained from caesium chloride gradients. Samples from the L-band and the I-band of Fig. 1(d) were separately dialysed against SSC. Frequencies of transformation by these materials were compared with those produced by unlabelled DNA prepared from *B. subtilis* Marburg strain and by [^3H]BU-DNA prepared from the same strain. The results are shown in Table 1, Expt. 1.

To determine whether transformation for the

Fig. 1. Centrifugation in CsCl gradients of DNA isolated from competent cultures of *B. subtilis* strain 31 after incubation with [^3H]BU-DNA prepared from *B. subtilis* strain Marburg for (a) 0 min. (time of manipulation approx. 3 min.), (b) 6 min., (c) 10 min., (d) re-centrifugation of the I-band from (c). ●---●, Radioactivity (c.p.m.); ■—■, E_{260} .

histidine and tryptophan markers by the I-band occurred with the same degree of linkage as with DNA from the Marburg strain, single histidine, single tryptophan and double transformants were scored and the co-transfer index calculated (Nester & Lederberg, 1961). The results are shown in Table 1, Expts. 2 and 3.

Integration of donor and recipient transforming activities. [^3H]BU-DNA was prepared from *B. subtilis* strain 57 and incubated with competent cells of *B. subtilis* strain 59. DNA was isolated from the transformed culture and submitted to equilibrium sedimentation in caesium chloride. The results are shown in Fig. 3(a). DNA was isolated

from the combined L- and I-bands and re-centrifuged as described above (Fig. 3b). Frequencies of transformation were measured with competent cells of *B. subtilis* strain 31 as acceptor and the following samples of DNA as donor: (i) [^3H]BU-DNA from *B. subtilis* strain Marburg; (ii) H-band DNA (Fig. 3a); (iii) L-band DNA (Fig. 3b); (iv) I-band DNA (Fig. 3b). As a control, frequency of transformation was also measured by using a mixture of DNA from strain 57 (2.5 $\mu\text{g./ml.}$) and strain 59 (2.5 $\mu\text{g./ml.}$) as donor. Transformants for histidine independence and tryptophan independence and double transformants were scored. The results are shown in Table 1, Expt. 3.

Fig. 2. Centrifugation in CsCl gradients of (a) a mixture of denatured H-band (Fig. 1b) and L-band (Fig. 1d); (b) material from the I-band (Fig. 1d). ●---●, Radioactivity (c.p.m.); ■—■, E_{260} .

Table 1. *Transforming activities of fractions of DNA from caesium chloride gradients*

The DNA sample was dialysed against SSC and transforming activities were measured as described in the text, with *B. subtilis* strain 31 as recipient. Results are shown for competent cultures prepared on three separate occasions, and only values obtained in the same experiment can be compared.

Expt. no.	DNA used as donor	Transforming activity (%) for markers			
		Histidine synthesis	Tryptophan synthesis	Double transformants	Co-transfer index
1	Total unlabelled DNA (Marburg)	0.61	0.52	Not measured	
	Total [³ H]BU-DNA (Marburg)	0.58	0.46		
	L-band, Fig. 1(d)	0	0		
	I-band, Fig. 1(d)	0.52	0.44		
2	Total unlabelled DNA (Marburg)	0.32	0.28	0.24	0.66
	I-band, Fig. 1(d)	0.24	0.22	0.18	0.61
3	Total [³ H]BU-DNA (Marburg)	0.55	0.48	0.41	0.64
	H-band, Fig. 3(a)	0	0.31	0	0
	L-band, Fig. 3(b)	0.44	0	0	0
	I-band, Fig. 3(b)	0.09	0.07	0.03	0.23
	Mixture of total DNA from strains 57 and 59	0.21	0.27	0	0

Centrifugation of mixtures of DNA from different sources. The following mixtures were incubated at 37° for 10 min. and then submitted to equilibrium centrifugation in caesium chloride: (a) [³H]BU-DNA from *B. subtilis* Marburg strain and unlabelled DNA from the same strain; (b) [³H]BU-DNA from strain Marburg and unlabelled DNA from *B. subtilis* strain 31; (c) [³H]BU-DNA from strain Marburg and a lysate of cells of strain 31. The results are shown in Fig. 4.

Effect of heterologous DNA on competent cells of B. subtilis. [³H]BU-DNA was prepared from *E. coli*

strain 15T⁻ derived from *E. coli* strain B by the method of Hewitt, Suit & Billen (1967). This material was incubated with competent cells of *B. subtilis* strain 31 as described above. DNA was isolated from the cells after treatment with deoxyribonuclease (Akrigg *et al.* 1967) and submitted to equilibrium sedimentation in caesium chloride. The results are shown in Fig. 5.

Competent cultures of *B. subtilis* strain 31 were transformed by using various concentrations of unlabelled DNA from *B. subtilis* strain Marburg in either the presence or the absence of an equal

Fig. 3. (a) Centrifugation in CsCl gradients of DNA isolated from a culture of *B. subtilis* strain 59 after being transformed by [³H]BU-DNA prepared from *B. subtilis* strain 57. (b) Recentrifugation of the combined L- and I-bands from (a). ●---●, Radioactivity (c.p.m.); ■—■, E₂₆₀.

Fig. 4. Centrifugation in CsCl gradients of the following mixtures after incubation at 37° for 10 min.: (a) [³H]BU-DNA from *B. subtilis* strain Marburg and unlabelled DNA from *B. subtilis* strain Marburg; (b) [³H]BU-DNA from *B. subtilis* strain Marburg and unlabelled DNA from *B. subtilis* strain 31; (c) [³H]BU-DNA from *B. subtilis* strain Marburg and a lysate of cells of *B. subtilis* strain 31. ●---●, Radioactivity (c.p.m.); ■—■, E₂₆₀.

concentration of unlabelled DNA from *E. coli* strain 15T⁻. The frequencies of transformation observed are shown in Fig. 6.

DISCUSSION

Centrifugation in a density gradient has been used extensively to study the fate of transforming

DNA. Fox & Allen (1964), using donor DNA labelled with ²H, ¹⁵N and ³²P, found that the material became associated with resident DNA of *Diplococcus pneumoniae* during the process of transformation. Ultrasonic treatment of DNA extracted from the transformed culture produced no fully heavy segments of native material, suggest-

Fig. 5. Centrifugation in CsCl gradients of (a) [³H]BU-DNA prepared from *E. coli* strain 15T⁻; (b) DNA prepared from a competent culture of *B. subtilis* strain 31 after incubation for 30 min. with [³H]BU-DNA prepared from *E. coli* strain 15T⁻. ●---●, Radioactivity (c.p.m.); ■—■, E₂₆₀.

Fig. 6. Frequencies of transformation obtained with *B. subtilis* strain 31 as recipient and DNA prepared from *B. subtilis* strain Marburg as donor. ●, Transformation for histidine synthesis with donor alone; ○, transformation for tryptophan marker with donor alone; □, transformation for histidine synthesis with donor DNA mixed with DNA from *E. coli*; ■, transformation for tryptophan synthesis with donor DNA mixed with DNA from *E. coli*.

ing that incorporation into the recipient genome had involved single donor strands, in agreement with the results of Lacks (1962). Denaturation of the

ultrasonically treated material yielded fully heavy and light subunits, confirming that incorporation did not involve double-stranded donor DNA. Some radioactive label was recovered in association with light DNA after ultrasonic treatment and denaturation of the re-extracted material, and this was attributed to the re-utilization of ³²P-containing acid-soluble degradation products of the donor DNA. In view of this, it could not be decided whether the single-stranded donor was incorporated into one or both of the strands of the recipient genome. Gurney & Fox (1968) showed by fractionation of re-extracted material of hybrid density that recipient marker activity again banded with material of hybrid density but that the ratio of recipient activity to donor marker activity was lower. It was concluded that integration of donor takes place into either strand of recipient DNA.

In contrast to the situation in *D. pneumoniae*, some transforming DNA remains in the double-stranded state after entry into competent cells of *Haemophilus influenzae* (Stuy, 1965) and no single-stranded material has been detected. Donor DNA labelled with 5-bromouracil, after extraction from a transformed culture, could not be separated from recipient DNA of normal density owing to the small extent of incorporation, and no evidence was

obtained to show whether incorporation occurred into one or both strands. Notani & Goodgal (1966) obtained results by using donor DNA containing ^2H and ^{15}N that confirm that in *H. influenzae* the donor remains double-stranded after entry. However, only one of the strands becomes integrated at any one position with the recipient DNA since the density of hybrid material extracted after further replication was unchanged. Moreover, denaturation of hybrid material resulted in an increase in density, suggesting that a light strand had been removed during the process. DNA extracted from a transformed culture gave, after denaturation, a band occupying the position expected for denatured light recipient DNA but containing isotopic label derived from both donor and recipient DNA (Notani & Goodgal, 1966, Fig. 8, p. 205). Thus no light recipient strands that were free from donor material were demonstrated in the recovered DNA and the possibility therefore exists that single-stranded donor enters both strands of the recipient genome.

Pène & Romig (1964) studied transformation of competent cells of *B. subtilis*, grown in medium containing ^{15}N , with donor DNA labelled with ^{32}P . Sedimentation in a caesium chloride gradient of DNA extracted from the transformed culture showed the presence of radioactivity in both heavy and light fractions. In a similar experiment with a competent culture that had not been grown in ^{15}N , no heavy fraction was discerned, thus excluding the possibility that the results were due to denaturation of donor DNA after entry into the cell. Moreover, non-specific association of recipient with donor material during isolation was excluded, and it was concluded that integration occurs between donor and recipient DNA.

Bodmer & Ganesan (1964) transformed competent cells of *B. subtilis* labelled with ^{32}P , by using donor DNA labelled with ^3H , ^2H and ^{15}N . Equilibrium sedimentation in caesium chloride of DNA extracted from the transformed culture showed a major band corresponding to native donor DNA with a shoulder extending across the region containing native recipient DNA, also suggesting physical association of donor and recipient DNA. It was further concluded that DNA does not become single-stranded after entry.

It is clear that physical association of donor DNA and the recipient genome occurs during transformation of *D. pneumoniae*, *H. influenzae* and *B. subtilis*. However, there appears to be no uniform way by which this association takes place. The present experiments were undertaken to attempt to define the process of integration more clearly by isolation of the complex of donor and recipient DNA in *B. subtilis*.

The results presented show the formation of a

distinct component with a buoyant density intermediate between that of donor DNA labelled with 5-bromouracil and that of unlabelled recipient DNA. We believe this to be recombinant DNA produced in the transformation process, for the following reasons. First, no material of intermediate density is formed by incubation *in vitro* of mixtures of DNA from donor and recipient cells. Secondly, no component of intermediate density is formed between resident DNA and DNA from *E. coli*, which competes with homologous DNA for entry into the cells but does not effect transformation. Further evidence that the material is produced as a result of the process of transformation rather than by incorporation of breakdown products of the exogenous DNA after entry is that it carries transforming activity for the two markers of the donor DNA and shows the same co-transfer index for them. Finally, it is clear that the material of intermediate density is a biological hybrid of donor and recipient DNA because it possesses transforming activity for markers derived from both donor and recipient cells, a property which is not exhibited by mixtures of the two types of DNA (see Table 1).

The experiments recorded do not reveal the presence of any material with the buoyant density expected of denatured donor DNA. This agrees with the results of previous work referred to above and is not incompatible with the presence of single-stranded regions as proposed by Harris & Barr (1968). Although the experiments of Bodmer & Ganesan (1964) suggested the presence of material of intermediate density, resolution was not sufficient to allow its separation. The effects of denaturation and shearing of incompletely resolved material did not allow a conclusion to be drawn whether integration of donor DNA takes place into one or both strands of the recipient genome. Moreover, Becker (1967) found that DNA extracted from *B. subtilis* cultures that had been transformed with DNA containing interstrand covalent crosslinks did not retain the crosslinks. In the present work, it has been clearly demonstrated that a biological hybrid of donor and recipient DNA is formed. Further, since denaturation of the isolated, integrated material gave a single component, the hybrid does not consist of one heavy and one light strand, but donor material is present in both strands. No conclusion can be drawn whether the biological hybrid contains donor material in corresponding segments of the duplex, or whether such material is introduced as single chains at different positions in the two strands of the recipient DNA. Consequently, it is not possible to decide whether integration occurs by replacement of one segment by another or by a process of addition of a double-stranded segment to the resident DNA.

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REFERENCES

- Akrigg, A., Ayad, S. R. & Barker, G. R. (1967). *Biochem. biophys. Res. Commun.* **28**, 1062.
- Becker, E. F. (1967). *Biochim. biophys. Acta*, **142**, 238.
- Bodmer, W. F. & Ganesan, A. T. (1964). *Genetics*, **50**, 717.
- Fox, M. S. & Allen, M. K. (1964). *Proc. nat. Acad. Sci., Wash.*, **52**, 412.
- Gurney, T. & Fox, M. S. (1968). *J. molec. Biol.* **32**, 83.
- Harris, W. J. & Barr, G. C. (1968). *Biochem. J.* **110**, 38P.
- Hewitt, R., Suit, J. C. & Billen, D. (1967). *J. Bact.* **93**, 86.
- Kammen, H. O., Beloff, R. H. & Canellakis, E. S. (1966). *Biochim. biophys. Acta*, **123**, 39.
- Lacks, S. (1962). *J. molec. Biol.* **5**, 119.
- Lederberg, J. (1955). *J. cell. comp. Physiol.* **45**, 75.
- Lorkiewicz, Z., Opara-Kubinska, Z. & Szybalski, W. (1961). *Fed. Proc.* **20**, 360.
- Marmur, J. (1961). *J. molec. Biol.* **3**, 208.
- Nester, E. W. & Lederberg, J. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 378.
- Nester, E. W. & Stocker, B. A. D. (1963). *J. Bact.* **86**, 785.
- Notani, N. & Goodgal, S. H. (1966). *J. gen. Physiol.* **49**, 197.
- Pène, J. J. & Romig, W. R. (1964). *J. molec. Biol.* **9**, 236.
- Singh, R. N. & Pitale, M. P. (1968). *J. Bact.* **95**, 864.
- Spizizen, J. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 1072.
- Stuy, J. H. (1965). *J. molec. Biol.* **13**, 554.
- Sueoka, N. & Cheng, T. Y. (1962). *J. molec. Biol.* **4**, 161.
- Weigle, J., Meselson, M. & Paigen, K. (1959). *J. molec. Biol.* **1**, 379.