MUTATIONS RESULTING FROM THE TRANSFORMATION OF *BACILLUS SUBTILIS*[1]

HIROSHI YOSHIKAWA

Space Sciences Laboratory, University of California, Berkeley

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UNEQUAL crossing over was first proposed by DEMEREC (1962) to explain an increase in reversion rate in *Salmonella typhimurium* when transduced with phages carrying the same mutation. MAGNI (1963, 1964) found increases in reversion and forward mutation rates in yeast during meiosis as compared with those occurring in mitotic cells. MAGNI and associates (1964) provided evidence that the great majority of revertants occurring during meiotic processes are associated with chromosomal exchange in the region of the mutated locus. By studying spontaneous reversions of T4 *rII* mutants of various origins, STRIGINI (1965) found that recombinations of outside markers were related to reversion of proflavin-revertible spontaneous mutants. However, recombinations were not related to reversions of mutations induced by 5-bromouracil. This led to the hypothesis that reverse mutations occur as a consequence of unequal crossing over which causes base losses or insertions in DNA. Base losses and insertions have also been proposed to explain the mutagenic actions of acridine dyes (CRICK *et al.* 1961; LERMAN 1964).

Intact DNA molecules are integrated into the host chromosome in *Bacillus subtilis* transformation systems (SZYBALSKI 1961; PENE and ROMIG 1964; BODMER and GANESAN 1964). There is some direct physical evidence for single strand integration in both *B. subtilis* and pneumoccocus (BODMER and GANESAN 1964; FOX and ALLEN 1964). Transformations provide a unique opportunity for studying directly the association of mutation to recombination even though the mechanisms of integration are not known.

The present report describes increases in forward and reverse mutations during transformation of *B. subtilis* and suggests a direct association of mutation to recombination. The kinetics of transformation and mutation and the calculation of true mutation frequency are also discussed.

MATERIALS AND METHODS

Strains: Wild-type strains W23 and W168 of *Bacillus subtilis* were used throughout this investigation. Mutants requiring amino acids and adenine were obtained from the wild types by the penicillin method following ultraviolet irradiation. The characteristics of these mutants, and mapping of their markers by gene frequency analysis, have been reported (YOSHIKAWA and SUEOKA 1963). Sulfanilamide resistant mutants from W23 and W168 were isolated by growing

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cells in a minimal medium with gradually increasing concentrations of sulfanilamide. They were resistant to 200 μg/ml sulfanilamide in the absence of methionine, but to 1 mg/ml in its presence. The sulfanilamide resistance marker (*Sa*^{r}) was mapped by gene frequency analysis at 0.05 relative to *ade* in strain Mu8u5u16 [leucine$^-$(*leu*$^-$), methionine$^-$(*met*$^-$), adenine$^-$(*ade*$^-$)] derived from W168, when a whole length of chromosome was taken as unit (KIEFFER and YOSHIKAWA, unpublished data).

Escherichia coli W3110 was used as the source of *E. coli* DNA.

Media: The basal medium (medium C) contained 14 g K_2HPO_4, 6 g KH_2PO_4, 1 g sodium-citrate·$2H_2O$, 2 g $(NH_4)_2SO_4$·$7H_2O$, 5 g glucose in 1000 ml H_2O (pH 7.2). L-glutamic acid 0.01% was added to prevent lysis of the cells when they were transferred from a richer to a poorer medium. Casein hydrolyzate, 0.05% and L-tryptophan, 50 μg/ml were added to stimulate growth. Difco Antibiotic Medium 3 (Pennassay medium) was used as the enriched medium.

Other methods: Isolation of DNA and transformation were as reported by YOSHIKAWA (1965). Two layers of Whatman No. 1 filter paper were used to replicate colonies to count auxotrophic mutants.

RESULTS

A. *Forward mutation during transformation:* Competent cells were prepared from wild-type *B. subtilis* (W168) and were transformed by DNA preparations from the same strain, from W23 which was closely related to W168, and from *E. coli*. After incubation with DNA for 40 min one portion of the culture was diluted and plated on enriched (Pennassay) agar, (Condition A). The other portion was diluted tenfold in enriched medium and grown for 6 to 10 additional

TABLE 1

Forward mutation accompanying transformation using wild-type strain W168 as recipient

Experiment No.	Donor DNA 10 μg/ml	Number of cells examined†	Number of mutants found	Frequency of mutants (%)	Condition‡	Efficiency of *Sa*^{r} transformation $\times 10^{-3}$§
1	W23	5580	20	0.36	B	. .
2a*	W23Sa^r	2025	10	0.49	B	2.1
	W168Sa^r	2186	14	0.64	B	3.8
	E. coli	2351	4	0.17	B	. .
	no DNA	2450	1	0.04	B	. .
2b	W23Sa^r	3240	5	0.15	A	2.1
	W168Sa^r	3440	4	0.12	A	3.8
	E. coli	2775	1	0.04	A	. .
	no DNA	2535	2	0.08	A	. .
3a	W168Sa^r	2520	14	0.56	B	4.3
	E. coli	2470	0	0.00	B	. .
	no DNA	2150	0	0.00	B	. .
3b	W168Sa^r	2030	3	0.15	A	4.3
	E. coli	2210	1	0.05	A	. .
	no DNA	2370	2	0.08	A	. .

* a and b were in the same experiment and showed two conditions in which the frequency of mutants was examined.
† Estimated from number of colonies per plate (average of three plates) × number of plates examined (10 to 20).
‡ The two conditions are described in the text.
§ These efficiencies were measured by plating the recipient cells on plates enriched with leucine, 50 μg/ml, methionine 50 μg/ml, adenine, 20 μg/ml and sulfanilamide, 1 mg/ml after 40 min incubation with *Sa*^{r} DNA.

hours. It was then diluted and plated on enriched agar, (Condition B). Grown colonies, usually 150 to 300 per plate, were then replicated onto plates composed of medium C. Those which did not grow on medium C were counted as auxotrophic mutants.

The results of three independent experiments are shown in Table 1. Under condition B, DNAs from W168 and W23 showed marked effects on the frequency of auxotrophic mutants in the culture. *E. coli* DNA produced a level of mutant frequency similar to that of the control culture where no DNA was added. This indicated that the presence of homologous DNA was necessary to increase the frequency of mutants in the competent culture. The competence of the culture was measured by the efficiency of transformation of Sa^r markers. When the recipient cells were not in a competent state, no effects of homologous DNA were observed. Only a slight increase of mutant frequency by homologous DNA over the control was obtained in condition A.

Essentially the same results were obtained when strain Mu8u5u16 was used as recipient (Table 2). In this case colonies grown on enriched agar plates were replicated on plates containing medium C plus L-leucine, L-methionine, and adenine at 50 μg/ml each.

B. *Identification and characterization of mutants:* Sixty-two mutants obtained from strain Mu8u5u16 were tested. Fourteen required enriched medium for

TABLE 2

Forward mutation accompanying transformation using leu⁻ met⁻ ade⁻ *strain Mu8u5u16 as recipient*

Experiment No.*	Donor DNA 10 μg/ml	Number of cells examined†	Number of mutants found	Frequency of mutants (%)	Condition	Efficiency of adenine transformation $\times 10^{-3}$‡
1a	W168	4410	28	0.63	B	4.6
	E. coli	4670	5	0.11	B	..
	no DNA	4210	1	0.02	B	..
1b	W168	1680	2	0.12	A	4.6
	E. coli	1620	1	0.06	A	..
	no DNA	1845	1	0.05	A	..
2	W168	4760	32	0.67	B	4.9
	E. coli	4380	7	0.16	B	..
	no DNA	4420	3	0.07	B	..
3a	W168	3480	18	0.52	B	3.5
	E. coli	3720	4	0.11	B	..
	no DNA	3285	2	0.06	B	..
3b	W168	2530	2	0.08	A	3.5
	E. coli	2660	1	0.04	A	..
	no DNA	2490	2	0.08	A	..

* Experimental conditions were the same as in Table 1.
† Estimated as in Table 1.
‡ These efficiencies were measured by plating the recipient cells on medium C enriched with leucine and methionine, each 50 μg/ml.

growth, and a mixture of 20 amino acids and five purines and pyrimidines would not replace enriched medium. Thirty required amino acids: 1 arginine, 4 cysteine, 3 aspartic or glutamic acid, 4 lysine, 4 histidine, 2 isoleucine, 3 threonine and 4 unidentified. Eighteen were highly revertible and were not identified.

Twenty-four mutants from W168 were studied. Two required enriched medium, 16 required an amino acid, 2 required adenine, and 4 were too revertible to be identified.

All mutants examined reverted spontaneously with frequencies ranging from 1.1×10^{-7} to 1.4×10^{-10} reversions/cell/generation.

About 30% of the mutants grew very slowly on Medium C without supplementation, with growth patterns as in Figure 1. Most of these leaky mutants were temperature-sensitive auxotrophs. A typical example is shown in Figure 2.

C. *Reverse mutation correlated with transformation:* Strain Mu8u5u16 (*leu⁻ met⁻ ade⁻*) was exposed to homologous DNA isolated from the same strain in order to examine effects of transformation processes on reverse mutation. Each of

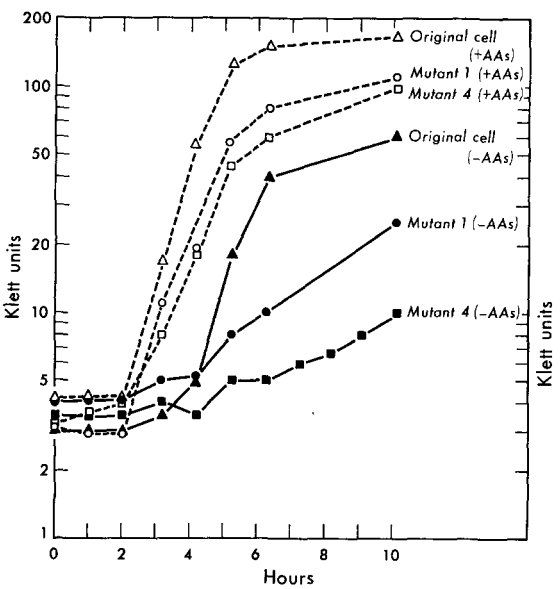

FIGURE 1.—Growth of leaky mutants. Lysine requiring mutants, 1 and 4, were obtained from strain Mu8u5u16 (*leu⁻ met⁻ ade⁻*) by transformation. Overnight cultures of the original strain and of mutants 1 and 4 in medium C plus L-leucine, L-tryptophan and adenine (50 μg/ml each) and 0.05% casein hydrolysate were diluted in the fresh medium with (open point) and without (solid point) casein hydrolysate. The cultures were shaken at 37°C and the growth was measured by a Klett-Sommerson colorimeter with a red filter.

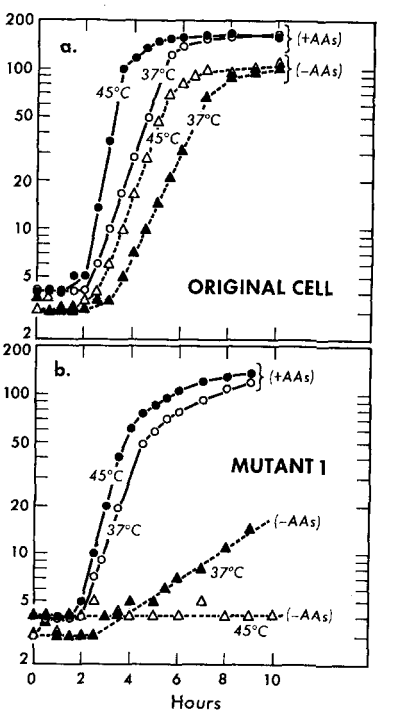

FIGURE 2.—Effect of temperature on the growth of a typical leaky mutant (No. 1, requiring lysine). a. Growth at 37°C. b. Growth at 45°C. Experimental conditions were the same as in Figure 1.

the three UV-induced markers reverted spontaneously at about 2 to 4×10^{-9} reversions/cell/generation during growth in enriched medium. DNA was isolated from Mu8u5u16 grown in enriched medium. Frequencies of revertants in the culture at the time of isolation were 2.9×10^{-8} for *ade*, 3.2×10^{-8} for *met* and 1.8×10^{-8} for *leu*. Approximately 10 to 100 times more revertants were produced during transformation than were produced spontaneously (Tables 3,4). The reversion frequency of the adenine marker during transformation was 1.2×10^{-6}, while transformation efficiency of the marker by wild-type DNA was 5×10^{-3} using competent cells of the same type (Table 3). Therefore, the frequency of reverse mutation in every transformation event could be calculated as 2.4×10^{-4} for adenine; for methionine it was 1.7×10^{-4}. The observed reversion during transformation was not due to transformation by contaminating DNA, since at the time of isolation the amount of wild-type DNA in the mutant DNA preparation, from reverted cells in the culture, was less than 1×10^{-7} for each marker.

In contrast to forward mutations, reverse mutations were obtained after 40

TABLE 3

Reverse mutation accompanying transformation of leu⁻ met⁻ ade⁻ *strain Mu8u5u16 with homologous DNA*

Experimental conditions*	adenine revertants		methionine revertants		leucine revertants	
	Number†	Frequency‡	Number	Frequency	Number	Frequency
A + DNA	238	1.2×10^{-6}	60	5.0×10^{-7}	4	3.3×10^{-8}
— DNA	1	8.0×10^{-9}	4	3.4×10^{-8}	3	2.5×10^{-8}
B + DNA	198	3.0×10^{-7}	44	8.8×10^{-8}	6	1.2×10^{-8}
— DNA	9	1.6×10^{-8}	12	2.4×10^{-8}	8	1.6×10^{-8}

* Strain Mu8u5u16 was transformed by DNA from the same strain. After 40 min incubation with 10 μg/ml DNA, 0.1 ml of treated cells was plated on agar plates which lacked one of the three requirements (condition A). Another aliquot of the cells was diluted ten times with enriched medium, grown for 10 hr, and 0.1 ml was then plated on the enriched plates (condition B). With the same competent cells, a wild-type DNA gave transformation efficiencies for *ade*, *met*, and *leu*, of 5.1×10^{-3}, 3.0×10^{-3}, and 2.8×10^{-3} with 10 μg/ml.

† Total revertants found on ten plates.

‡ Average number of cells on three plates of enriched medium with suitable dilution.

TABLE 4

Effect of varying concentration of DNA on transformation and reverse mutation using leu⁻ met⁻ ade⁻ *strain Mu8u5u16 as the recipient*

DNA concentration (μg/ml)‡	Number of transformants in 1.2×10^7 cells*			Number of revertants in 1.2×10^8 cells†		
	ade	*met*	*leu*	*ade*	*met*	*leu*
0	0	0	1	4	11	6
1	28120	14720	13320	192	106	4
2	.....	.....	.....	226	138	0
5	52400	27600	23440	256	128	4
10	61520	36880	33800	356	192	8

* Average from three plates with 200 times dilution.

† The totals from ten plates without dilution.

‡ DNA from wild-type cells (W168) was used for transformation, and homologous DNA from the same strain as the recipient was used for reverse mutation.

TABLE 5

Mutation correlated with transformation of the indole marker

Experiment number	Total number of cells examined*	Number of mutants found at		Frequency of mutants × 10^{-3}	
		37°C	45°C	37°C	45°C
1	2100	6	22	2.9	10.5
2	3680	8	21	2.2	5.7
3	4480	15	57	3.3	12.7

Experimental conditions are in the text. 10 μg/ml DNA was used in all experiments, and average efficiency of transformation of the indole marker was 1×10^{-3}.

* Estimated from number of colonies per plate (average of three plates counted) × number of plates examined (15 to 20).

minutes incubation with DNA (Condition A), and the frequency of revertants decreased during additional growth in enriched medium.

The effects of DNA concentration on transformation and reversion are shown in Table 4. Rather high concentrations of DNA were used, in order to obtain significant effect on reversion frequency. A parallel increase of both transformation and reverse mutation was observed, although DNA concentrations were not in the region where efficiency of transformation had a linear relationship to DNA concentration. The reversion frequency of leucine was not affected by transformation. The reasons for this are not clear.

D. *Mutations in transformants:* The mutation frequencies of transformed cells were measured to obtain a direct correlation between transformation and mutation events. A competent indole requiring mutant was exposed to wild-type DNA and plated on medium C enriched with casein hydrolysate. The lack of tryptophan in the medium enabled all the transformed cells to be detected including those which had acquired new mutations during transformation. Transformed cells grown on these plates were then replicated on medium C plates to find amino-acid requiring mutants. One group of replicated plates was incubated at 37°C, and one at 45°C. The results of three independent experiments are shown

TABLE 6

Linkage to the indole locus of histidine mutants arising simultaneously with transformation of indole⁻ to indole⁺

Source of DNA (mutant number)	Number of transformants in 10^7 recipient cells*	
	histidine†	indole‡
I–1	12	2240
I–2	109	3170
I–3	31	1910
I–4	63	2480
I–5	1870	3320
no DNA	2	1

* *his-2⁻*, *ind⁻* (MXIXI) as recipient and 2 μg/ml DNA for transformation from each of five his⁻ ind⁺ strains originating by transformation of *ind⁻* (M(XI)XI) to *ind⁺*.

† Averages of three plates without dilution.

‡ Averages of three plates with ten times dilution.

in Table 5. The average frequencies of mutants occurring were 0.28% at 37°C and 1% at 45°C. The frequencies of the amino-acid mutants of identical populations of cells were 0.03% at 37°C and 0.07% at 45°C before incubation with DNA. A higher frequency was expected at 45°C because most of the leaky mutants isolated during transformation were temperature-sensitive (see section B); 30 out of 36 mutants isolated at 45°C were temperature-sensitive auxotrophic mutants.

The amino-acid requirements of 52 mutants isolated from indole transformants were examined. Five of 52 mutants required histidine. DNA isolated from these five mutants was used to transform histidine$^-$(*his-2*$^-$) *indole*$^-$(*ind*$^-$) strains (MXIXI). As shown in Table 6, all DNA preparations except that from mutant No. 5 have low frequencies of transformation of *his-2*. This indicated that four of these five histidine mutants were at or near the *his-2* locus, which in turn is linked to the indole locus (NESTER, GANESAN and LEDERBEG 1963; YOSHIKAWA and SUEOKA 1963). The indole marker of the recipient cell, *ind*$^-$ *his*$^-$ (MXIXI), was identical to that of the indole-requiring mutant *ind*$^-$ (M(XI)XI) which was used for the mutation experiment. The results thus strongly suggested that mutation to *his*$^-$ occurred at a locus very close to the transformed indole locus. The same experiment was done with four *his*$^-$ mutants obtained during transformation of strain Mu8u5u16 (see Section B). In contrast to the *his*$^-$ mutants from indole transformants, they were not linked to the indole locus. Thus the efficiencies of transformation of *his-2* by DNA preparations from these mutants were more than 50% of that of wild-type DNA. No tyrosine-requiring mutants (*tyr*$^-$) were found among the 52 amino-acid mutants. Using the *his-2*$^-$ *ind*$^-$ *tyr*$^-$ strain MXIXIul the co-transfer index (NESTER and LEDERBERG 1961) of *his-2-ind* linkage was 0.58 whereas that of *tyr-ind* linkage was 0.17. The results thus suggest that integration of the DNA molecule into the recipient chromosome was the direct cause of mutation.

More than 90% of the mutants arising simultaneously with indole transformation were not linked to the indole locus. We assume that these nonlinked mutations were caused by another DNA fragment which was incorporated into the competent cell simultaneously with the DNA carrying the indole marker.

Similar mutation frequencies were obtained with transformants of the adenine, methionine and leucine markers (Table 7). Linkage between mutated loci and transformed loci has not yet been studied.

E. *Kinetics of transformation and mutation:* No increase of forward mutations over spontaneous mutations was observed if cells were plated immediately after transformation, as mentioned earlier. Mutants began to appear only a few hours after transformed cells were grown in an enriched medium. The time-course of transformation and mutation was studied in detail. In order to understand this delay in the appearance of forward mutations, a *leu*$^-$ *met*$^-$ *ade*$^-$ strain (Mu8u5u-16) was transformed by wild-type DNA. After incubation with DNA for 30 min, cells were diluted tenfold with fresh medium C enriched with casein hydrolysate, and were then shaken at 37°C. Samples were taken at various intervals and (1) total cell number, (2) total transformed cells and (3) total auxotrophic mutants

TABLE 7

Mutations originated simultaneously with the transformation of leucine, adenine, and methionine markers in strain Mu8u5u16

Experiment No.	leucine transformants		adenine transformants		methionine transformants	
	Number of cells examined	Number of mutants found	Number of cells examined	Number of mutants found	Number of cells examined	Number of mutants found
1	2805*	22	7420	25	2160	8
2	5340	28	6240	25	3620	10

leu⁻, *ade*⁻, *met*⁻ (Mu8u5u16) was transformed by wild-type DNA. After 40 min incubation with DNA cells were plated as follows: (a) Medium C plus adenine 20 μg/ml, 17 amino acids (minus leucine) each 50 μg/ml. (b) Medium C plus 18 amino acids. (c) Medium C plus adenine and 17 amino acids (minus methionine). Cells grown on plate (a) were replicated to Medium C plus adenine and methionine to count the mutants among leucine transformants. Cells grown on plate (b) were replicated to Medium C plus leucine and methionine to count the mutants among adenine transformants. Cells grown on plate (c) were replicated to Medium C plus leucine and adenine to count the mutants among methionine transformants.

* Estimated from numbers of colonies per plate (average of three plates) × number of plates examined (15 to 20).

were measured (Table 8). The over-all growth pattern of both transformed cells and total cell population agreed with the previously published data (NESTER and STOCKER 1963; KAMMEN *et al.* 1966). The ratios of frequency of mutants to the frequency of transformants were calculated (Table 8, column 5). No mutant increase over the control culture was observed in the first 3 hours. The ratio of mutants to transformants increased rapidly during a period of 3 to 4 hours and became constant after the number of transformants doubled, indicating that all the mutations induced by transformation were fixed between the 3rd and 4th hour of incubation, and thereafter mutants grew at the same rate as transformed cells.

TABLE 8

Time course of appearance of mutant and transformant cells during the growth of transformed cultures

Time (hr)	(1) Total cell number /ml × 10^8*	(2) Number of transformants in 0.1 ml × 10^4	(3) Frequency of transformants × 10^{-4}†	(4) Frequency of mutants × 10^{-4}‡		(5)
				− DNA (A)	+ DNA (B)	[(4B)-(4A)]/(3)
0	1.8	9.6	53	2.0	2.5	...
1	4.5	8.6	19	2.9	3.2	...
2	14.0	8.0	5.7	2.0	3.0	...
3	30.0	9.1	3.0	2.5	9.8	2.44
4	52.0	14.8	2.8	4.0	17.0	4.64
5	58.0	21.0	3.6	3.0	20.5	4.86
7	58.0	38.0	6.6	5.3	34.5	4.42
9	56.0	42.0	7.5	4.4	43.0	5.15
11	60.0	42.0	7.0	2.5	38.2	4.96
13	54.0	42.0	7.8	3.0	32.4	3.77

Experimental conditions were described in the text.

* Measured by plating cells on enriched medium with a suitable dilution.

†These are frequencies of adenine transformant scored by plating cells on Medium C enriched with methionine and leucine each 50 μg/ml.

‡ Number of auxotrophic mutants were scored as described in the text (section A).

B. subtilis has on the average 3.0 nuclei per cell (BODMER and GANESAN 1964). If only one nucleus mutated no mutants would be observed until the mutated cells duplicate, since original cells give rise to a mixed clone. For the same reason, at least two generations would be required to double the transformed cell. The observed difference of approximately 1.5 hours between the time when mutants began to appear and the time when transformants doubled is in agreement with this hypothesis.

F. *Estimation of competence:* (The data in this section were contributed by DR. NAT M. KIEFFER, now at Texas A and M University, College Station, Texas.) Evidence so far seen indicated that transformation was directly responsible for mutations observed in competent cultures incubated with homologous DNA. In order to analyze this phenomenon quantitatively, it was essential to estimate the actual percent of competent cells in the culture, which was a mixture of competent and noncompetent cells.

One approach to this problem is to measure the frequencies of joint transformations of nonlinked markers (BALASSA and PREVOST 1962; NESTER and STOCKER 1963). Suppose N_0 is the number of competent cells in the total N cells of a competent culture, and N_0a is the number of transformed cells of a marker A in N

TABLE 9

Efficiency of transformation and estimation of competence—I

DNA concentration (μg/ml)	Observed efficiency of transformation (number of transformants per 10^7 recipient cells)					
	adenine	methionine	*Sa*^{r}	*ade-met*	*Sa*^{r}-*ade*	*Sa*^{r}-*met*
0.2	4280	1800	2780	29	38	4
0.8	12940	5700	9420	167	248	33
3.0	34960	17620	25140	838	1092	308
12.0	63100	25980	39880	1585	2241	743
50.0	80980	32940	46960	2832	2960	1028

	Absolute efficiency					
	adenine		methionine		*Sa*^{r}	
	$\frac{ade\text{-}met}{met}$	$\frac{ade\text{-}Sa^r}{Sa^r}$	$\frac{ade\text{-}met}{ade}$	$\frac{met\text{-}Sa^r}{Sa^r}$	$\frac{ade\text{-}Sa^r}{ade}$	$\frac{met\text{-}Sa^r}{met}$
0.2	0.016	0.013	0.007	0.001	0.009	0.002
0.8	0.029	0.026	0.013	0.014	0.019	0.006
3.0	0.048	0.044	0.024	0.012	0.031	0.018
12.0	0.061	0.056	0.025	0.019	0.036	0.029
50.0	0.086	0.063	0.035	0.022	0.037	0.031

	Competence calculated from		
	ade-met	*ade-Sa*^{r}	*met-Sa*^{r}
0.2	0.027	0.033	0.180
0.8	0.045	0.050	0.143
3.0	0.072	0.079	0.146
12.0	0.105	0.122	0.136
50.0	0.094	0.129	0.149

leu$^-$ *met*$^-$ *ade*$^-$ (Mu8u5u16) was transformed by DNA from W168 *Sa*^{r}.

cells, a is defined as absolute efficiency of transformation of marker A. In the same way the numbers of transformants of marker B are N_0b, and b represents the absolute efficiency of transformation of marker B. If markers A and B are not linked, the number of cells transformed at both A and B is N_0ab. Since N_0a, N_0b and N_0ab can be measured experimentally by transformation of double mutants, one could estimate a as N_0ab/N_0b and b as N_0ab/N_0a. One could then calculate the fraction of competent cells in the total cell population, N_0/N, as $N_0a/N \times 1/a$ where N_0a/N is an observed efficiency for transformation of marker A. In practice

$$\frac{N_0}{N} = \frac{N_0a/N}{N_0ab/N_0b} = \frac{(N_0a/N) \times (N_0b/N)}{N_0ab/N} = \frac{F_a \times F_b}{F_{ab}}$$

where F_a is the observed efficiency of transformation of marker A, F_b is that of marker B, F_{ab} is the efficiency of joint transformation of markers A and B.

The absolute efficiencies of four markers were estimated by this method (Tables 9, 10). It is known that *ade* is located near the starting point of chromosome replication and that *met* is near the terminus. *Sa*^{r} is at 0.05 and *leu* is at 0.6 relative to *ade* when the unit is taken as the distance of *ade* from *met*. Therefore, *leu* is not linked to either *ade* or *met*. Likewise *Sa*^{r} is not linked to *met*. The fractions of competent cells calculated from *ade-leu*, *leu-met*, and *Sa*^{r}-*met* joint transfor-

TABLE 10

Efficiency of transformation and estimation of competence—II

DNA concentration (μg/ml)	Observed frequency of transformation (Number of transformants per 10^7 recipient cells)					
	adenine	methionine	leucine	*ade-met*	*ade-leu*	*leu-met*
0.2	12520	5480	4240	158	21	52
0.8	28120	14720	13360	708	140	256
3.0	52400	27600	23440	1930	446	982
12.0	61520	36880	33800	2556	803	1405
50.0	71800	41720	42200	3280	1057	1798

	Absolute efficiency					
	adenine		methionine		leucine	
	$\frac{ade\text{-}met}{met}$	$\frac{ade\text{-}leu}{leu}$	$\frac{ade\text{-}met}{ade}$	$\frac{leu\text{-}met}{leu}$	$\frac{ade\text{-}leu}{ade}$	$\frac{leu\text{-}met}{met}$
0.2	0.029	0.012	0.013	0.005	0.004	0.004
0.8	0.058	0.019	0.024	0.011	0.009	0.010
3.0	0.070	0.042	0.037	0.019	0.019	0.016
12.0	0.069	0.041	0.041	0.024	0.023	0.022
50.0	0.079	0.043	0.046	0.025	0.025	0.025

	Competence calculated from		
	ade-met	*ade-leu*	*leu-met*
0.2	0.043	0.104	0.110
0.8	0.058	0.148	0.129
3.0	0.074	0.125	0.145
12.0	0.089	0.150	0.153
50.0	0.091	0.167	0.167

leu$^-$ *met*$^-$ *ade*$^-$ (Mu8u5u16) was transformed by wild-type DNA

mations were similar, having an average value of 0.144. They were also independent of the DNA concentrations used. On the other hand the combinations of *ade-met* and *ade-Sa*^{r} joint transformations had considerably smaller values than 0.144, especially with lower DNA concentrations. This indicated that at low DNA concentrations joint transformations of *ade-met* and *ade-Sa*^{r} were higher than those expected from independent transformation of these markers, and suggested the presence of loose physical linkages between adenine and methionine and between adenine and *Sa*^{r}. By the same method, NESTER *et al.* obtained 1.0 to 5.0% using *his-1* and *try-2* as nonlinked markers (NESTER and STOCKER 1963).

G. *Calculation of mutation frequency caused by transformation:* The experiment shown in Table 8 demonstrated that the frequency of auxotrophic mutants in the transformed culture reached 4×10^{-3} during growth in enriched medium. If we assume that mutations take place only in those cells which incorporate DNA that recombines with the host chromosome, the observed frequencies must be corrected for the difference in the growth rate between competent and noncompetent cells and for the true proportion of competent cells in the culture. If growth of an adenine transformant represents that of the competent cells, the frequency of competent cells becomes 16% of the original frequency after growth in enriched medium. It has already been shown that competent cells comprise 14.4% of the culture. Therefore, actual mutation per competent cell immediately after transformation is $4 \times 10^{-3}/0.16 \times 0.144 = 1.7 \times 10^{-1}$. However, observed mutant frequency of indole transformants is 1×10^{-2}. If each bacterium has three nuclei, and further, if only one strand of the host chromosome recombines with an incorporated DNA molecule, the low value of mutant frequency among indole transformants would be expected.

DISCUSSION

Increased rates of forward and reverse mutations were found during transformation. It is known that some of the DNA which is incorporated by competent cells degrades within the cells. This degraded DNA might stimulate DNA replication, thus producing a higher mutation frequency. Competent cells and transformed cells are, however, metabolically less active and have a slower growth rate than noncompetent cells (NESTER and STOCKER 1963; see also section E). Furthermore, in the thymidine-requiring mutant of *B. subtilis*, both competent and transformed cells are resistant to thymineless death, indicating that DNA replication in these cells is repressed (BODMER 1965). These facts argue against the possibility that DNA incorporated during transformation acted as a growth stimulant.

An increased mutation frequency could also result from incorporated DNA acting as a mutagenic agent and increasing the mutability of genes (DEMEREC 1963). To examine this possibility, *E. coli* DNA was used as a control because it is known that this DNA inhibits transformation of *B. subtilis* by homologous DNA (SPIZIZEN 1959). Further, *E. coli* DNA incorporates into the cell but does not recombine with the *B. subtilis* chromosome (BODMER and GANESAN 1964). Our results indicated no effects on mutation frequency by *E. coli* DNA.

These results suggest a third possibility; that mutation is caused directly by transformation. To examine this hypothesis, the correlation between transformed locus and mutated loci was studied.

In *B. subtilis* chromosome at least two distinctly different histidine regions are known (EPHRATI-ELIZUR *et al.* 1961) and have been mapped (YOSHIKAWA and SUEOKA, unpublished data). One of them is closely linked to genes controlling biosynthesis of aromatic compounds (NESTER and LEDERBERG 1961). Four out of five histidine requiring mutants isolated from indole transformants were linked to *his-2*, indicating that these *his*$^-$ loci are located close to the transformed indole locus. None of the four histidine requiring mutants obtained from general transformants were linked to *his-2* locus. This evidence, though not strong statistically, is suggestive of the production of mutation by recombination of closely linked genes during transformation. Similar findings have been reported in yeast (MAGNI 1964) and phage T4 (STRIGINI 1965).

As a mechanism of mutation, it has been postulated that deletion or insertion of a base occurs owing to unequal crossing over during recombination. STRIGINI (1965) obtained direct evidence to support this hypothesis in the case of T4 *rII* mutants. Our finding that approximately 30% of mutants obtained were leaky contradicts this hypothesis because most mutants induced by insertion or deletion of a base should be nonfunctional. Further analyses of mutants are necessary to determine whether recombinations during transformation also cause base substitutions.

Two factors complicate the analysis of the kinetics of mutation and transformation: (1) unknown variations in the number of nuclei in each cell and their mode of segregation into daughter cells during cell division, and (2) the difference in the growth rates between competent cells or transformed cells and noncompetent cells. The ratio of mutants to transformants will change during the growth period following transformation if bacteria have more than two nuclei and only one of them is mutated or transformed. The ratio should increase gradually and reach a constant value after several generations if the daughter chromosomes of two original nuclei segregate into two daughter cells at random. It was found, however, that the ratio of mutants to transformants rapidly became constant even before transformants doubled. This indicates that segregation of nuclei into daughter cells during cell division does not occur in a random fashion but is controlled so that daughter chromosomes from one nucleus do not mix with daughter chromosomes from another.

The comparison between growth patterns of transformed cells and the total cell population in *B. subtilis* was extensively studied by NESTER and STOCKER (1963) and by KAMMEN, BELOFF and CANELLAKIS (1966). The results reported here are in agreement with theirs, and definitely establish a difference in growth rate between the two cell groups.

A mutation frequency of 20% in competent cells was calculated by correcting the difference in growth rate between transformants and nontransformants and determining the actual proportion of competent cells in the population. This value results from assumptions that were excessively simplified. However, the findings

in this investigation suffice to suggest strongly that recombination during transformation leads to an unusually high frequency of mutation.

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SUMMARY

The frequency of auxothrophic mutants increased during transformation. Reverse mutation also increased when competent recipient cells were transformed by homologous DNA. Several hours of growth in a rich medium following transformation were required to obtain the increase in forward mutation, whereas an increase in reverse mutation was observed immediately after transformation. A high frequency of mutation at an indole-linked histidine locus (four out of five *his* mutants tested) was found among indole transformants, indicating a direct association of mutation with recombination. Mutants induced by transformation reverted spontaneously. Approximately 30% of them were leaky, and most were temperature sensitive auxotrophic mutants. A mutation frequency of 20% in competent cells was calculated after correcting for the difference in growth rate between competent and noncompetent cells, and determining a true fraction of competent cells among the recipients.

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