Fractionation of Transformable Bacteria from Competent Cultures of *Bacillus subtilis* on Renografin Gradients

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A population of *Bacillus subtilis* can be fractionated into at least two components on the basis of their buoyant densities on a Renografin gradient. In competent cultures, most, and perhaps all, of the competent bacteria appear in the lighter fraction which composes 2 to 10% of the bulk population. These "lighter" bacteria are the only bacteria capable of incorporating transforming deoxyribonucleic acid.

Nester and Stocker (13) reported that transformants in a competent culture of *Bacillus subtilis* were not as sensitive to penicillin killing as the overall population, suggesting that competent bacteria form a distinct fraction of the population. Other evidence that transformable cells constitute only a fraction of a "competent population" is supported by the observation of Singh and Pitale (14) that the more slowly sedimenting bacteria of a transformed culture, in a sucrose gradient, yield a higher frequency of transformants than do the more rapidly sedimenting bacteria. We have found that this partial fractionation on sucrose may be accounted for by a difference in buoyant density between competent cells and the rest of the population. A competent culture of *B. subtilis*, submitted to isopycnic separation on a preformed linear Renografin gradient (16), separates into two major components, the lighter of which is considerably enriched in competent bacteria. This method of fractionation of a competent culture has been used to demonstrate that only bacteria fractionating as the lighter component are capable of incorporating transforming deoxyribonucleic acid (DNA).

MATERIALS AND METHODS

Strains. *B. subtilis* strain SB 25 (*his-2 trp-2*-linked markers) was used as the recipient for all transformations. DNA was isolated from SH 11 (*his⁺ trp⁺ sul^r*) obtained from L. Chasin of Massachusetts Institute of Technology and was used as a standard DNA preparation for transformation. Tritium-labeled DNA was isolated from a 168 (*thy⁻*) strain obtained from E. Kataja (Massachusetts Institute of Technology) which was derived by transformation of the 168 (*thy⁻ ind⁻*) strain obtained from F. Rothman (Brown University).

Media and competent cells. Penassay Broth (Difco

Antibiotic Medium No. 3) was used for growing overnight cultures. A modification of Spizizen's (16) minimal salts medium (referred to as LS) was used for growing competent cultures. LS medium contains 0.08% NH_4Cl , 1.4% K_2HPO_4 , 0.6% KH_2PO_4 , 0.1% sodium citrate $\cdot 2\text{H}_2\text{O}$, 0.033% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and 0.0014% Na_2SO_4 . Bacteria grown in this medium occurred in shorter chains than those grown in Spizizen's medium.

Transformants were scored for the *his⁺* marker, the *sul^r* marker, and for the double-linked marker *his⁺ trp⁺* in TG agar, a tris(hydroxymethyl)amino-methane (Tris)-chloride buffered, minimal salt medium containing 10^{-3} M KH_2PO_4 (6). Sulfanilamide was added at a concentration of 50 $\mu\text{g}/\text{ml}$ for scoring of sulfanilamide-resistant bacteria; 20 μg of L-tryptophan or L-histidine per ml was added to score for the *his⁺* or *trp⁺* transformants, respectively. Viable counts were determined on nutrient agar [1% tryptone (Difco), 0.5% yeast extract, 1% NaCl, adjusted to pH 7.0, plus 1.1% agar]. Each culture was tested for the presence of contaminants and spontaneous revertants.

Growth medium 1 (1; C. D. Laird, Ph.D. Thesis, Stanford Univ., Palo Alto, Calif., 1966) was LS medium containing 20 $\mu\text{g}/\text{ml}$ of all the common L-amino acids except arginine, lysine, methionine, and cystine (10) plus 0.5% glucose. Growth medium 2 was LS medium supplemented with 0.5% glucose, 0.02% Vitamin Free Casamino Acids, 20 $\mu\text{g}/\text{ml}$ each of L-phenylalanine, L-tyrosine, L-tryptophan, and L-histidine, and 0.02 $\mu\text{g}/\text{ml}$ each of *p*-hydroxybenzoic acid and *p*-aminobenzoic acid, as described by C. D. Laird.

A competent culture was prepared from an overnight culture of SB 25 (*his-2 trp-2*) grown in Penassay broth at 37 C. The bacteria were centrifuged at $9,000 \times g$ in a Servall SS 34 rotor for 10 min, and the packed cells were resuspended at a density of 5×10^7 bacteria/ml in growth medium 1. These bacteria were grown to stationary phase (4.5 to 6 hr at 37 C); dimethyl sulfoxide was added to 5% by volume, and portions were frozen in an ethyl alcohol-dry ice bath.

These cultures, referred to as C-1, were stored at -70°C , and could be used reproducibly for 2 to 3 weeks as an inoculum for the final growth stage. In this paper, frozen cultures are designated by a letter. If a culture was used in more than one experiment, the date of use is indicated. A sample of the frozen culture was quickly thawed at 45°C and diluted five-fold into growth medium 2. This culture was shaken at 30°C for 150 to 210 min, until it reached maximal competence, as determined from previous tests. At this time, MgCl_2 was added to a final concentration of 0.02 M, and the bacteria were used for transformation. Unless otherwise stated, transformation was carried out by exposure to $3\text{ }\mu\text{g}$ of SH 11 DNA per ml for 30 min at 30°C and terminated with $3\text{ }\mu\text{g}$ of deoxyribonuclease per ml (electrophoretically purified deoxyribonuclease I, Worthington Biochemical Corp., Freehold, N.J.) for SH 11 DNA, and with $50\text{ }\mu\text{g}/\text{ml}$ when ^3H -labeled DNA was used.

DNA isolation. SH 11 was isolated by the method described by Marmur (11). Tritium-labeled DNA was isolated from 168 (*thy*⁻) bacteria grown in growth medium 1 containing $3\text{ }\mu\text{g}$ of thymine per ml at about 0.5 c/mole of thymine for preparation A and 1.9 c/mole of thymine for preparation C (thymine ^3H -methyl from New England Nuclear Corp., Boston, Mass., was used).

Tritiated thymine-labeled bacteria were harvested in log phase, washed three times with 0.15 M NaCl-0.1 M ethylenediaminetetraacetate (EDTA) at pH 8.0, resuspended in saline-EDTA containing 0.3 M sucrose, incubated with $100\text{ }\mu\text{g}$ of lysozyme per ml at 37°C for 10 min, and lysed with 0.3% sarcosyl (Geigy Industrial Chemicals, Ardsley, N.Y.). The lysate was incubated at 37°C with $200\text{ }\mu\text{g}$ of boiled ribonuclease per ml (Worthington Biochemical Corp.) and 20 units of T_1 ribonuclease per ml (Calbiochem, Los Angeles, Calif.) and was extracted once with chloroform and isoamyl alcohol (24:1). Further purification was accomplished by isopycnic separation in a CsCl gradient. The fractions containing the DNA were pooled and dialyzed against a solution of 0.15 M NaCl plus 0.015 M sodium citrate. DNA concentrations were determined by the diphenylamine reaction (3). Preparation A had a specific activity of 5.5×10^4 counts per min per μg of DNA, and preparation C had a specific activity of 5.0×10^5 counts per min per μg of DNA.

Linear Renografin gradients. Renografin-76 (methylglucamine *N,N'*-diacetyl - 3,5 - diamino - 2,4,6 - triiodobenzoate, E. R. Squibb and Sons, New York, N.Y.) is supplied as a 76% solution in sterile 20-ml ampoules, and contains, in addition, 0.32% sodium citrate, 0.04% EDTA, 0.01% methyl *p*-hydroxybenzoate, and 0.03% propyl *p*-hydroxybenzoate. This Renografin solution was diluted with water to obtain solutions with the appropriate densities. A plot of refractive index n_D^{25} versus density (ρ) at 25°C is shown in Fig. 1.

Approximately 5×10^7 bacteria of a transformed culture were layered on a linear gradient of Renografin [4.5 ml in a 0.5×2 inch (1.3×5 cm) cellulose nitrate tube, Spinco SW 39 tube, Beckman Instruments, Palo Alto, Calif.] with a density range from $\rho = 1.19$

FIG. 1. Refractive index n_D^{25} of a Renografin solution versus the density ρ in g/cc at 25°C .

to 1.10 g/cc (Fig. 2). Centrifugation was carried out in a Spinco SW 39 rotor at 20°C for 20 min at 20,000 rev/min, and the resulting two bands can be clearly seen in Fig. 3. The tube was punctured with a no. 20 hypodermic needle, and 5- to 10-drop fractions were collected. Without removal of the Renografin, each fraction was tested for the number of colony-formers and the number of transformants present.

The lighter bacteria were found to band at $\rho = 1.136$, and the denser bacteria, at $\rho = 1.152$.

Discontinuous Renografin gradients. In a Renografin solution having a density of 1.146, only the lighter bacteria float. Therefore, all further separations were carried out at 20,000 rev/min for 20 min at 20°C in a SW 25.1 rotor in cellulose nitrate tubes [1×3 inches (2.5×7.6 cm), Spinco SW 25 tubes] containing a discontinuous gradient of Renografin solutions.

A typical discontinuous gradient had 2 ml of $\rho = 1.42$ as a cushion on the bottom, 26 ml of $\rho = 1.146$ as the separating layer, and 2 to 4 ml of cells. In the studies involving the incorporation of radioactive DNA, 21 ml of $\rho = 1.146$, plus a spacing layer of 6 ml of $\rho = 1.100$, were used. A 2-ml amount of the labeled culture was layered on top of the spacer. Bacteria, concentrated at the top and bottom interfaces of the $\rho = 1.146$ layer, were clearly visible when more than 10^7 were present. The fractions were collected with a 10-ml syringe with a no. 20 hypodermic needle by puncturing the wall of the tube in the region of the band and withdrawing the Renografin solutions at the interfaces. The upper band was always collected first to avoid mixing of the two bands. When transformation was to follow fractionation, the bacteria were washed free of the Renografin on a membrane filter (HA WP, $0.45\text{ }\mu$; Millipore Corp., Bedford, Mass.) with LS growth medium, then washed with

0.2 M $MgCl_2$, washed again with LS medium, resuspended in growth medium 2, and tested for competence. When necessary, bacteria were allowed to reach competence by further growth in growth medium 2.

Radioactive counting. To measure the amount of tritium-labeled DNA incorporated by bacteria, fractions from the Renografin gradient were washed on membrane filters (HA WP, 0.45μ) with TG medium, and then with 5% (w/v) cold trichloroacetic acid. Radioactivity was determined by scintillation counting on a Nuclear-Chicago Mark I scintillation counter with 42 ml of Liquifluor (New England Nuclear Corp.) per liter of toluene as the scintillation solution.

RESULTS

Renografin studies of competent cultures. The results of a typical fractionation on a preformed linear gradient are shown in Fig. 2. At the location of maximal cell concentration, drops 65 to 70, the transformation frequency for the *his⁺ trp⁺* markers was 0.0026%, whereas in drops 80 to 85 the frequency was 8%. Thus, transformants were enriched in the less-dense fraction. Figure 3 is a photograph of a gradient showing two distinct bands. Drop collection from the bottom of the tube resulted in trailing of the lower band during collection, causing contamination of the upper band. This trailing was, in part, due to bacteria adhering to the walls of the cellulose nitrate tubes. To avoid this difficulty, the bacteria were separated on a discontinuous gradient as described in Materials and Methods. The less-dense bacteria, designated the "T band," concentrated just below the interface between the $\rho = 1.146$ layer and the growth medium that was layered above it. When the band was difficult to see, the layer just below the interface was withdrawn. The denser band, designated the "B band," concentrated on the surface of the dense Renografin cushion. The

FIG. 2. Isopycnic separation of a transformed culture versus density. Symbols: O, viable count; ●, *his⁺trp⁺* transformants.

FIG. 3. Photograph of a linear Renografin gradient showing the two bands present in a competent culture (M9-2). White spots on the tube are reflections from dust particles.

B band stuck noticeably to the sides of the tube, but most of the contents of this band could be recovered by moving the point of the hypodermic needle around the edge of the tube and withdrawing the solution at the edge. The bands were assayed for total number of transformants and viable count. As can be seen from Table 1, transformants were indeed separated from the rest of the culture, and there was between 60 and 100% recovery from the gradient, as determined by viable count. It should be noted that more than 98% of the transformants occurred in the top band where their frequency was 1,000-fold higher than in the dense fraction.

TABLE 1. Fractionation of transformed cultures

Culture	No. of bacteria put on gradient		Band	No. of bacteria collected		Recovery (%)		Transformation frequency (%) ^a	
	Total	Transformants ^a		Total	Transformants ^a	Bacteria	Transformants ^a	After fractionation	Before fractionation
G	4.4×10^8	8.8×10^5	T	0.15×10^8	5.9×10^5	3.4	67	3.9	0.2
			B	3.1×10^8	0.081×10^6	71	0.9	0.003	
K 7-13b	2.7×10^8	2.4×10^5	T	0.051×10^8	2.3×10^5	1.9	96	4.5	0.09
			B	1.5×10^8	0.04×10^6	56	0.6	0.002	
L 8-10	5.6×10^8	32×10^5	T	0.23×10^8	32×10^5	4.1	100	13	0.57
			B	3.9×10^8	0.45×10^6	70	1.4	0.01	
M 8-13	2.6×10^8	23×10^5	T	0.17×10^8	14×10^5	6.5	61	8.1	0.92
			B	2.5×10^8	0.39×10^6	96	1.7	0.02	

^a Transformants—scored for *his*⁺*trp*⁺ markers.

To show that the fractionation of transformants was not a consequence of the exposure of the population to DNA, a competent culture was fractionated and the two isolated fractions were transformed. The isolated fractions were assayed for viable count and *his*⁺ *trp*⁺ transformants. As shown in Table 2, the cultures could be transformed after fractionation, and the T band was very substantially enriched in competent bacteria. The frequency of transformants in the T band was roughly the same whether the culture was transformed before or after separation (culture G in Tables 1 and 2). The frequency of *his*⁺ *trp*⁺ transformants was 3.9 and 5.2% for these cultures, respectively.

Study of bands formed at C-1 stage. It was also observed that a culture fractionates into two bands on a discontinuous gradient at all stages during the competence regimen. We directed our attention to the C-1 stage to determine whether bacteria fractionating in the T band at that stage were the only cells predestined to become competent. A culture at the C-1 stage was fractionated on a discontinuous gradient; the two fractions were washed, resuspended in growth medium 2, grown to competence, and transformed. As shown in Table 3 (Part A), bacteria from both the T and B bands at the C-1 stage became competent; in addition, further refractionation separated the transformants from the nontransformable bacteria in the usual manner (Table 3, Part B). Both T and B bands isolated at the C-1 stage gave rise to two bands when fractionated at the competent stage. Variability was observed among different cultures in the extent to which competent bacteria originated from those present in either the T or B band at the C-1 stage. Cultures K and M represent ex-

TABLE 2. Transformation of T band and B band fractions of a competent culture

Culture	Band	No. of bacteria/ml	No. of <i>his</i> ⁺ <i>trp</i> ⁺ transformants/ml	Frequency of <i>his</i> ⁺ <i>trp</i> ⁺ transformants (%)
AA	T	5.7×10^7	12×10^4	0.21
	B	7.5×10^7	0.02×10^4	0.0003
G	T	1.3×10^7	67×10^4	5.2
	B	31×10^7	1.3×10^4	0.004

amples of such variability at the C-1 stage. There was no obvious difference between these two kinds of cultures in terms of growth history or final turbidity reached at the C-1 stage.

To examine further the time course of the generation of "light" competent bacteria, 4 ml of culture M 8-27, which separated into two visible bands, were fractionated at the C-1 stage on a discontinuous gradient. The entire T and B bands were used to inoculate two parallel cultures of 8 ml of growth medium 2. At 0.5-hr intervals, equal portions from both cultures were removed and transformed. Figure 4 shows the number of *his*⁺ *trp*⁺ transformants observed as a function of time for the two cultures that were inoculated with the T and B band bacteria. During this 3.5-hr period, the viable count in *each* band increased by a factor of 4, while the number of competent bacteria increased by a factor of 100. These curves show that the appearance of competent bacteria followed the same time course in both cultures. The observations described in Table 3 (Part B) show that the transformants at the 3.5-hr stage were virtually all in the T band when refractionated from the two parallel cul-

TABLE 3. Transformation of fractions isolated at C-1 stage
Part A: After reaching the competent stage

Culture	Band	No. of bacteria/ml	No. of <i>his⁺trp⁺</i> transformants/ml	Frequency of transformation, <i>his⁺trp⁺</i> (%)
K 7-13b ^a	T	1.5×10^7	3.6×10^4	0.24
	B ^b	3.5×10^7	4.3×10^4	0.12
M 8-27 ^c	T ^d	3.7×10^7	110×10^4	3.0
	B ^d	6.5×10^7	21×10^4	0.32

Part B: Refractionation after becoming competent

Culture	Initial band at C-1	Refractionated band	Total <i>his⁺trp⁺</i> transformants collected	Frequency of transformation (<i>his⁺trp⁺</i>) after refractionation (%)	Recovery (%)	
					Total	Transformants
K 7-13b	B	T	1.5×10^5	10	1.4	105
		B	0.01×10^5	0.001	78	0.8
M 8-27	B	T	4.3×10^5	5.9	5.6	100
		B	0.063×10^5	0.006	75	1.5
M 8-27	T	T	20×10^5	10	27	91
		B	0.016×10^5	0.03	74	0.37

^a A 1-ml amount of C-1 culture was fractionated, and T and B bands were resuspended in 4 ml of growth medium 2.

^b A 3.2-ml amount was refractionated 3.5 hr after C-1 and is shown in Part B.

^c A 4-ml amount of C-1 culture was fractionated and both bands were resuspended in 8 ml of growth medium 2.

^d A 2-ml amount was refractionated 3.5 hr after C-1 and is shown in Part B.

tures. "Light" bacteria at C-1 yielded "heavy" bacteria during this 3.5-hr incubation and vice versa; appearance in the T band at C-1 was not a requirement for achieving competence after further incubation (Table 3, Part B). Furthermore, there appeared to be no additional time increment imposed on the members of the "heavy" population for achieving competence.

DNA incorporation. The technique of separating transformed bacteria from the bulk of the population was used to compare DNA incorporation and transformation in the fractionated population. Two separate preparations of ³H-thymine-labeled DNA were used in these experiments (see Materials and Methods). The data in Table 4 show that essentially all of the macromolecular DNA incorporated was associated with bacteria fractionating in the T band. Controls with deoxyribonuclease-treated DNA resulted in little or no incorporation of the radioactive label by the bacteria. Other experiments were carried out to determine the amount of DNA fixed by the competent bacterial population per bacterium transformed for the *his⁺* marker, as a function of concentration of DNA, as well as of the duration of exposure to DNA. The ratio of DNA fixed to

bacteria transformed was relatively constant over a wide range of DNA concentrations (Table 5) and was independent of the duration of exposure of the competent culture to DNA (Table 6). (Less than 10 transformants were counted for the 3-min sample, giving this measurement a very large uncertainty.)

Frequencies of transformation for unlinked markers. The frequency of bacteria transformed for both of a pair of unlinked markers has been used to estimate the fraction of a population participating in transformation (7, 9). It is argued that, if all the bacteria in a culture were competent, one might as a first approximation expect $f[AB]/(f[A] \times f[B]) = 1$, if fragments of DNA are distributed randomly among the recipient bacteria; $f[A]$ and $f[B]$ represent the frequencies of bacteria transformed for the unlinked markers A and B, respectively, and $f[AB]$ is the frequency of bacteria transformed with respect to both markers. In a population in which only a fraction of the bacteria participate in transformation, one might expect $f[AB]$ to exceed the product $f[A] \times f[B]$. This relationship was applied as a crude criterion for estimating the fraction of the bacteria appearing in the T band that were com-

FIG. 4. *T* and *B* bands tested for transforming ability at times after fractionation at the C-1 stage. The graph gives the number of bacteria transformed for *his*⁺ *trp*⁺ versus the time at which the culture was transformed. *T* and *B* bands of culture M 8-23 are represented by Δ and $\blacktriangle$, respectively; $\circ$ and $\bullet$ represent the *T* and *B* bands of culture M 8-27. A 4-ml amount of a M C-1 culture was fractionated. The *T* and *B* bands were washed and resuspended in 8 ml.

petent. The frequencies of transformation for the unlinked markers *his*⁺ and *sul*^r, and for the *his*⁺ *sul*^r double transformants were determined in unfractionated populations and in the light fraction of those populations. The frequency of double transformants exceeded the expected frequencies in the unfractionated populations (Table 7). After fractionation, fewer double transformants were found than expected on the basis of the naive model. In any case, the rough estimate of the frequency of bacteria in the unfractionated population participating in transformation comes to about 10%. This is consistent with the fraction of bacteria recovered in the *T* bands.

DISCUSSION

Competent cultures of *B. subtilis* could be fractionated into two components on a discontinuous Renografin gradient. More than 98% of the transformants in a transformed culture could be isolated in the "lighter" fraction. The "heavier" fraction contained 90 to 95% of the bacteria fractionated and contained less than 2% of the transformants. Those transformants that appeared in the heavier fraction might be accounted for by the existence of chains or clumps of bacteria. Overloaded gradients clearly manifest clumping, and microscopic examination of the fractionated culture revealed more bacteria occurring in chains in the "heavy" fraction than in the "light" fraction.

TABLE 4. DNA uptake: top band and bottom band

Culture	DNA concn ($\mu\text{g/ml}$)	Band	Total no. of <i>his</i> ⁺ <i>trp</i> ⁺ transformants	Frequency of transformants (%)		Total counts/min ^b	DNA per <i>his</i> ⁺ <i>trp</i> ⁺ trans- formant (μg)
				After fractionation	Before fractionation		
K 7-10	Prepn A 0.81	T	16.0×10^5	3.1	0.28	1,700	1.9×10^{-8}
		B	0.16×10^5	0.007		109	1.5×10^{-8}
	0.81 ^a	T	$<0.001 \times 10^5$			44	
		B	$<0.001 \times 10^5$			20	
L 8-15	1.62	T	9.9×10^5	3.5	0.27	1,320	2.3×10^{-8}
		B	0.15×10^5	0.005		49	0.6×10^{-8}
	0.81 ^a	T	$<0.001 \times 10^5$			0	
L 8-18	Prepn C 1.8	T	6.6×10^5	5.1	0.51	8,726	2.9×10^{-8}
		B	0.19×10^5	0.01		298	3.4×10^{-8}
	0.9 ^a	T	$<0.001 \times 10^5$			2	
L 8-20	0.9	T	3.4×10^5	2.4	0.26	3,150	2.1×10^{-8}
		B	0.096×10^5	0.009		125	2.4×10^{-8}
	0.9 ^a	T	$<0.001 \times 10^5$			24	

^a DNA was incubated with 50 μg of deoxyribonuclease prior to incubation with bacteria.

^b Background, 24 counts/min.

TABLE 5. DNA uptake as a function of concentration (T band only)^a

Culture	DNA concn (μg/ml)	Total <i>his</i> ⁺ transformants	Frequency after fractionation (%)	Transformants before fractionation (%)	Total counts/min ^b	DNA per <i>his</i> ⁺ transformant (μg)
L 8-15	<i>Prepn A</i>					
	1.6	16.0×10^5	5.9	0.34	1,320	1.5×10^{-8}
	0.81	12.0×10^5	6.3	0.34	794	1.2×10^{-8}
	0.4	8.2×10^5	3.2	0.25	580	1.3×10^{-8}
	0.16	3.9×10^5	1.4	0.15	337	1.5×10^{-8}
	0.08	1.4×10^5	0.7	0.07	217	2.8×10^{-8}
	0.8 ^c	$<0.001 \times 10^5$	—	—	0	—
L 8-18	<i>Prepn C</i>					
	4.8	17.0×10^5	12.2	1.9	11,465	1.3×10^{-8}
	1.8	12.0×10^5	9.2	1.0	8,726	1.5×10^{-8}
	0.9	5.9×10^5	4.6	0.5	7,095	2.3×10^{-8}
	0.45	5.0×10^5	4.3	0.34	5,520	2.2×10^{-8}
	0.09	1.9×10^5	1.4	0.16	2,147	2.3×10^{-8}
	0.9 ^c	$<0.001 \times 10^5$	—	—	29	—

^a A 2-ml competent culture was transformed and fractionated.^b Background, 24 counts/min.^c Incubated for 5 min at 30 C with 50 μg of deoxyribonuclease before being added to bacterial culture.TABLE 6. Uptake of DNA^a versus time (T band only)^b

Time (min)	Total transformants collected		Transformation frequency, <i>his</i> ⁺ (%)	Total counts/min ^c	DNA/transformant (μg)	
	<i>his</i> ⁺ <i>trp</i> ⁺	<i>his</i> ⁺			<i>his</i> ⁺ <i>trp</i> ⁺	<i>his</i> ⁺
0	$<0.001 \times 10^5$	$<0.001 \times 10^5$		24		
3	0.002×10^5	0.005×10^5	0.04	86	5.0×10^{-8}	2.1×10^{-8}
7	0.17×10^5	0.26×10^5	0.35	272	2.9×10^{-8}	1.8×10^{-8}
15	1.4×10^5	1.9×10^5	1.9	1,350	1.9×10^{-8}	1.4×10^{-8}
30	3.4×10^5	4.3×10^5	3.2	3,150	1.8×10^{-8}	1.4×10^{-8}

^a Prepn C, 0.9 μg/ml of DNA.^b Culture L 8-20 transformed and fractionated.^c Background, 24 counts/min.

TABLE 7. Transformation frequencies in unfractionated and fractionated cultures

Culture	Transformation frequencies (%)						$f[his + sul^r]/(f[his^+] \times f[sul^r])$	
	After fractionation			Before fractionation			After fractionation	Before fractionation
	<i>sul</i> ^r	<i>his</i> ⁺	<i>sul</i> ^r <i>his</i> ⁺	<i>sul</i> ^r	<i>his</i> ⁺	<i>sul</i> ^r <i>his</i> ⁺		
L 8-18 ^a	5.1	3.4	0.17	0.29	0.26	0.009	0.91	12
M 8-21 ^a	16	13	1.3	1.3	0.82	0.1	0.68	10
M 8-27 ^b	20	14	0.95	6.7	4.2	0.25	0.34	0.89

^a Cultures grown to competence, transformed, and fractionated.^b T band obtained at C-1 stage and refractionated after transformation (see Fig. 4).

Bacteria which fractionated in the heavier band appeared to be capable of giving rise to "light" progeny, and "light" bacteria were capable of yielding "heavy" progeny. Whatever the mech-

anism is for the establishment of competence, it appears to operate only on bacteria which have become light (Table 3). Furthermore, competence seems to occur with about the same time delay

in both "light" and "heavy" fractions of a population separated at an early stage of the competence procedure.

Tritium-labeled DNA was used to demonstrate that incorporated DNA is used genetically with equal efficiency under a variety of conditions. There is no evidence for a significant variation in the ratio of DNA incorporated to the yield of transformants when the bacteria were exposed to DNA for various lengths of time. The same ratio was also observed when the bacteria were exposed to various concentrations of DNA, including those exceeding the linear response.

Macromolecular DNA was incorporated predominantly, if not exclusively, by the "light" bacteria. The amount of DNA incorporated by the "light" population for each *his*⁺ transformant observed was 1.7×10^{-8} and 1.9×10^{-8} μ g, respectively, for DNA preparations A and C (Tables 5-7).

Since the frequency of *sul*^r transformants was about 1.5 times that of *his*⁺, about 10^{-8} μ g of DNA was incorporated for each *sul*^r transformant. If a nuclear complement of *B. subtilis* is 6.2×10^{-9} μ g (5), then approximately 2 bacterial equivalents of DNA must be incorporated by a competent population to yield a single transformant. This value agrees with that reported by Bodmer (1).

These techniques were also used to examine the proposal that the product of the frequencies of transformation for two unlinked markers divided by the frequency of double transformants, $f[A] \times f[B]/f[AB]$, could be used to assess the frequency of competent cells in a population (7, 12). The frequency of double transformants was about 10 times higher in cultures L 8-18 and M 8-21 (Table 7) than would be expected if transformation for single markers were a random event and the whole population were participating. In this culture, the number of "light" bacteria was 10% of the total population, as might be expected on the basis of the above hypothesis.

The question of whether or not all the "light" bacteria in a competent culture are competent is still a matter of speculation. It is clear that bacteria which are in the light fraction at the C-1 stage are poorly competent, while at maximum competence the frequency of random double transformants is equal to, or, in fact, most often less than, the expected product of the frequencies of single transformants. Culture M 8-27 in Table 4 fractionated at the C-1 stage gave a ratio $f[his^+ sul^r]/(f[his^+] \times f[sul^r])$ of 1, but when refractionated after transformation gave a ratio of 0.34; ratios of 0.5 were often observed. The observation that fewer double transformants

were observed than were expected could be accounted for by the presence of multiple nuclei or by single-strand integration of the DNA during transformation (2, 4, 8) or both. Thus, to a first approximation, half of all double transformants would not be counted, since these double transformants have integrated the two markers either into alternate nuclei or into the opposite strands of a single genome, and each of the transformant types would therefore separate at the first doubling. The fraction of "light" cells that are competent might be very high, but this is certainly not always the case.

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