Infection of Competent *Mycobacterium smegmatis* with Deoxyribonucleic Acid Extracted from Bacteriophage B1

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A relatively competent state of *Mycobacterium smegmatis* for infection with deoxyribonucleic acid (DNA) extracted from phage B1 was found in the late log phase of bacterial growth. This state of the culture was used in quantitative studies on the infectivity of the DNA. The buoyant density of B1 DNA was 1.728 g/cc in CsCl, and 1 μ g of the DNA produced 84 infective centers, the phage equivalent of which was 1.5×10^{-8} . The infectivity was destroyed by catalytic amounts of deoxyribonuclease but not by specific B1 antiserum. Tween 80, which prevents phage adsorption, did not prevent DNA infection. The response of plaque-forming ability to DNA concentration suggested that two or more molecules are required to initiate an infective center. The low efficiency of DNA infection in mycobacteria was considered to be caused by a limiting population of competent cells in the culture employed; in this experiment less than 10^{-5} of the cells were infected with DNA. A typical cycle of infection was observed, although the latent period was prolonged and the burst size reduced after DNA infection. The transition of B1 DNA infection to deoxyribonuclease insensitivity had a lag period of about 10 min, and increased linearly with a velocity of about 0.24 infective centers per min per μ g of DNA. Half of the infective titer was inactivated by heating at 92 C for 15 min. The melting temperature was about 96 C. Species barriers were not crossed by B1 DNA; however, the DNA was infectious for a B1-resistant mutant of the host.

Romig (16) first reported that deoxyribonucleic acid (DNA) isolated from a phage, SP3, could infect transformable *Bacillus subtilis*. Subsequently this finding was extended to *Mycobacterium* by Tokunaga and Sellers (Bacteriol. Proc., p. 146, 1963) and to *Haemophilus* by Harm and Rupert (7). Further studies with such host systems have been reported by many investigators (1, 3, 5-7, 12-15, 18, 22-25). Földes et al. (5) termed as "transfection" such an infection of cells with the nucleic acid isolated from a virus which resulted in the production of a complete virus. In all of the transfection reported, except that with mycobacteria, the host employed was competent cells, which have the ability to become transformed by uptake of donor DNA. Although the transfection of mycobacteria was demonstrated in many host systems (18, 23, 24), including tubercle bacilli (28), no successful transfection has yet been reported. In these transfection experiments, growing cultures, but not a particular state of competent cells, were employed as the host.

We previously found a state of mycobacterial culture at which the efficiency of phage DNA infection was relatively high (24). Using this relatively competent culture, we attempted quantitative analysis of transfection of mycobacteria.

MATERIALS AND METHODS

Bacteria and bacteriophages. A relatively smooth clone of *M. smegmatis* ATCC 607, isolated by S. Froman and given to us by M. I. Sellers, was employed (hereafter referred to as 607). Phage B1, isolated and supplied by K. Takeya (21), was used. In addition, *M. phlei* (G. Penso), *M. tuberculosis* H37Ra, *Micrococcus lysodeikticus*, *B. subtilis* 168 (H. Saito), and *Escherichia coli* K-12 Hfr Cavalli were employed. A mutant of 607 which is resistant to B1 infection was isolated and used. *Phagus phlei* from G. Penso and a male-specific, ribonucleic acid (RNA)-containing phage, MS2, isolated by A. J. Clark and donated by K. Takeya, were also employed.

Media. Tryptone-Soy broth (Nihon Eiyo Kagaku Co., Tokyo, Japan), hereafter referred to as broth, contained (per liter) tryptotone, 1.7 g; soypeptone, 3 g; glucose, 2.5 g; KH_2PO_4 , 2.5 g; NaCl , 5 g; and was

supplemented with CaCl_2 , glycerol, and Tween 80 at final concentrations of 0.002 M, 2%, and 0.06%, respectively. For phage propagation and phage infection, Tween 80 was removed from the broth. Agar was added to the broth at final concentrations of 1.5 and 0.6%, respectively, for plates and overlay.

Enzymes and special reagents. Ribonuclease and deoxyribonuclease, once crystallized, were purchased from Worthington Biochemical Corp., Freehold, N.J. Liquefied analytical reagent phenol was purchased from Wako-Junyaku Corp., Tokyo, Japan. Tris(hydroxymethyl)aminomethane (Tris) used for buffer was purchased from Sigma Chemical Co., St. Louis, Mo. and Tween 80 used was from Atlas Chemical Industries, Inc., Wilmington, Del.

Buffer. A 0.02 M Tris-chloride buffer at pH 7.2, supplemented with 0.15 M NaCl and 0.002 M CaCl_2 , was employed.

Preparation of bacteriophage and its DNA. High-titered phage lysate was obtained by the method of Sellers et al. (17). The crude lysate usually had a titer of 2×10^{10} to 6×10^{10} per ml. The lysate was centrifuged at $5,500 \times g$ for 20 min, and the supernatant fluid was treated at 37 C for 30 min with 0.1 μg of deoxyribonuclease per ml and 1 μg of ribonuclease per ml. Then the lysate was centrifuged in a Spinco model L ultracentrifuge at $40,000 \times g$ for 2 hr. The pellets were resuspended in Tris buffer, and bacterial debris and clumps were removed by low-speed centrifugation. This cycle of enzyme treatment and differential centrifugation was repeated twice. The final concentration of B1 phages was 3×10^{12} to 2×10^{13} per ml. In some experiments, the phages were further purified by a sucrose density gradient centrifugation. A 5 to 20% (w/v) sucrose gradient was used (Tris buffer, pH 7.2). Samples were centrifuged for 50 min at 22,500 rev/min in a Spinco SW39 rotor. The phage fraction, checked by ultraviolet (UV) absorption, was collected and dialyzed against Tris buffer.

A small amount of the concentrated material was rotated with an equal amount of phenol at 58 rev/min for 20 min. The mixture was then centrifuged to separate phenol and aqueous layers; the aqueous layer was collected with a capillary pipette. This extraction was repeated three times. Residual phenol was removed from the aqueous layer by dialysis against Tris buffer. The recovery of UV-absorbing material (260 $m\mu$) was about 70%. The DNA concentration of the extract was determined by the diphenylamine method (4).

Preparation of competent cells. Broth inoculated with strain 607, about 10^6 cells per ml, was shaken at 42 times per min at 37 C overnight. When the cell concentration reached about 10^8 colony-formers per ml (optical density = 0.7; $\lambda = 660 m\mu$), the efficiency of the DNA infection was found to be maximal. Cells from this culture were used as the host in all experiments for the DNA and for some of the phage infections.

Assay for infectious DNA. A 0.1-ml quantity of phage DNA was added to tubes containing 0.9 ml of the competent culture; the tubes were shaken gently at 37 C. After an appropriate time of incubation, deoxyribonuclease (0.1 ml of a 100 $\mu\text{g}/\text{ml}$ solution)

was added to terminate the uptake of DNA. The number of infective centers produced was assayed by the soft agar-layer technique.

In some experiments, DNA was directly plated with the competent culture and the plaques produced were assayed in the same way.

Density gradient centrifugation of DNA. Microdensitometer tracing of UV photographs of B1 DNA was carried out after centrifugation for 23 hr in CsCl , the density of which was 1.715 g/cc, at 44,770 rev/min and at 20.0 ± 0.05 C in a Spinco analytical E ultracentrifuge.

Anti-B1 phage serum and phage neutralization. Anti-phage serum was obtained by repeated injection of high-titered B1 lysate into rabbits. The serum, the K value of which was 25.8, was added to phage or DNA suspensions at a final concentration 10^{-2} of the original, and was incubated at 37 C for 10 min, unless otherwise stated.

One-step growth experiments with B1 phage and its DNA. Methods described by Sellers et al. (17) were employed for phage. For the infectious DNA, Tween 80 was added to the competent culture at a concentration of 0.1% before DNA addition, to prevent the readsorption of released phages from infected cells. A 0.9-ml sample of the competent cells was added to 0.1 ml of B1 DNA, and the mixture was gently shaken at 37 C for 10 min. Deoxyribonuclease (0.1 ml of a 100 $\mu\text{g}/\text{ml}$ solution) was added to terminate the uptake of DNA and incubation continued at 37 C with gentle shaking. At intervals, samples were taken and plated with soft agar to count numbers of infective centers.

Thermal inactivation. DNA and phages were diluted in Tris buffer at a concentration of 10 to 20 $\mu\text{g}/\text{ml}$ and about 5×10^6 plaques/ml, respectively. A 0.5-ml amount of each sample was taken in a glass-stoppered quartz cuvette, and was placed in a water bath adjusted at a desired temperature within an error of ± 0.5 C. When a temperature exceeding 100 C was desirable, a certain amount of NaCl was added to the water; in that case, fluctuation of the temperature was ± 1 C. After 15 or 60 min of exposure of the sample in the bath, the sample was cooled quickly in an ice bath, and was plated with indicator cells for counting infective centers. In the renaturation experiments, the sample was cooled slowly after heating; the sample was first dipped into a water bath at 90 C and allowed to cool with the heaters turned off. After 190 min, it dropped to 40 C. Measurement of UV absorption ($\lambda = 260 m\mu$) was carried out with a Shimadzu spectrophotometer, model QR-50.

RESULTS

Some properties of the infectious agents. The amount of DNA per B1 phage particle was estimated to be approximately 1.7×10^{-16} g. The maximal UV absorption of the phenol extract was seen at 258 $m\mu$ and the minimum was at 230 $m\mu$. Absorption ratios of 260 to 280 $m\mu$ and 260 to 230 $m\mu$ were both 1.8.

The buoyant density gradient centrifugation in CsCl showed a single peak at 1.728 g/cc. The

mole proportion of guanine plus cytosine (GC) was calculated to be 69.4% according to this value (11).

When the preparation was plated with competent cells, a maximum of 84 plaques per μg of DNA was produced. The phage equivalent of the infectious DNA was about 1.5×10^{-8} .

Antiserum against phage B1, sufficient to inactivate >99% of the mature phage present in control within 20 min of contact, as shown in Fig. 1, had essentially no effect on the infectiousness of the DNA preparation.

However, complete loss of infectivity resulted from deoxyribonuclease treatment. Preincubation with 10^{-5} μg of deoxyribonuclease destroyed 89% of the infectivity of the phenol extract. With 1 μg of deoxyribonuclease, the infectivity of 1,000 μg of the DNA, an amount sufficient to produce 84,000 plaques, was destroyed completely. A typical hyperchromic effect of UV absorption was observed after the deoxyribonuclease treatment of the extract; the value of αp was 1.35 ($\lambda = 260 \text{ m}\mu$).

Tween 80 at a concentration of 0.06% prevents adsorption of mycobacteriophages to the hosts (27). A 0.9-ml amount of the competent culture containing 0.06% Tween 80 was added to 0.1 ml of B1 phage ($5 \times 10^4/\text{ml}$) or B1 DNA (125 $\mu\text{g}/\text{ml}$). The mixtures were shaken gently at 37 C. Samples were taken at intervals, and 5 $\mu\text{g}/\text{ml}$ of deoxyribonuclease was added to them. After 10 min of the deoxyribonuclease treatment at 37 C, the samples were plated with the bac-

terial indicator. In an experiment lasting for 240 min, no growth was observed in the culture with phages, whereas the number of infective centers increased significantly in the culture with added DNA (Fig. 6).

These results suggested that DNA is an active component of the phenol extract and that mature phage particles do not account for the infectivity present.

Competence for transfection in mycobacteria. Competence, the ability of cells to bind DNA irreversibly and to become transformed, is a genetic property which was found in certain strains of *B. subtilis*, *Haemophilus*, pneumococci, and other organisms. It has been known that the state of competence develops in a cell population only during a certain phase of growth. The correlation between infection with B1 DNA and the growth phase of strain 607 is shown in Fig. 2. After a lag phase of about 60 min, bacterial growth increased logarithmically. The generation time was about 120 min. When the cell concentration reached about 8×10^7 colony-formers/ml (optical density = 0.5, $\lambda = 660 \text{ m}\mu$) or more, the increase of the optical density gradually became slower. Even if Tween 80 was present in the medium, clumping of the bacteria was unavoidable. In late log phase, large numbers of clumping bacteria were observed microscopically; therefore, the number of colonies formed does not indicate directly the number of living cells present.

At intervals, 0.5-ml samples were taken from the growing culture, and added to 0.1 ml of B1 DNA (100 $\mu\text{g}/\text{ml}$ solution). The mixture was gently shaken at 37 C, and after 60 min of incubation 0.1 ml of deoxyribonuclease (100 $\mu\text{g}/\text{ml}$ solution) was added. Relative numbers of infective centers produced in three experiments are plotted in Fig. 2. All through the growth cycle, DNA could produce plaques, but it was noticed that at a certain stage of late log phase (optical density = 0.6 to 0.7, $\lambda = 660 \text{ m}\mu$, corresponding to about 10^8 colony-formers/ml), production of infective centers was the highest, with about 100 times more produced than at the other stages. After passing through this particular stage, the number of infective centers decreased quickly, while the optical density of the culture still continued to increase. The cells at this stage also showed the highest susceptibility for transfection when plated with soft agar as well as in broth. If the cultivation started at a rather high cell concentration, such as at an optical density of 0.3, this peak of infective centers did not appear.

As the population of competent cells was still very low at this stage, endeavors to obtain a much higher competent state are necessary. However,

FIG. 1. Effects of anti-B1 serum on B1 phage and B1 DNA. The experimental procedures are given in the text.

there is no doubt that a competent state for phage DNA uptake is present in mycobacteria. This relatively highly competent culture could be maintained for long periods at -21°C with little loss of competence.

Infective centers as a function of B1 DNA and cell concentrations. Samples, each 0.1 ml, of B1 DNA diluted in Tris buffer at various concentrations were added to 0.3 ml of competent culture and assayed for infective centers (Fig. 3). A linear relationship exists between DNA concentration and infectivity in the region from 0.1 to about $7\text{ }\mu\text{g/ml}$. A saturation phenomenon was observed in the DNA infection, when the amount of DNA exceeded about $7\text{ }\mu\text{g/ml}$.

A competent culture was centrifuged and resuspended in fresh broth at various concentrations. Samples (0.5 ml) of the suspensions were mixed with a 0.1-ml amount of B1 DNA at a final concentration of $10\text{ }\mu\text{g/ml}$, and were shaken gently at 37°C for 60 min. After deoxyribonuclease treatment, the mixture was plated for the determination of plaque-forming units (Fig. 4). A linear relationship between cell concentration and infectivity was observed. These facts indicate that the saturation phenomenon shown in Fig. 3 was caused by a limiting number of competent cells present in the competent culture.

One-step growth experiments with B1 and B1 DNA. One-step growth curves of B1 phage and the phage DNA are shown in Fig. 5. The latent

FIG. 2. Growth curve of 607 and its susceptibility to B1 DNA infection. The bacteria were incubated in broth with shaking at 37°C overnight and were then diluted with fresh broth to 0.005 optical density ($\lambda = 660\text{ m}\mu$, corresponding to 1.1×10^6 colony-formers/ml). The diluted culture was shaken at 37°C . The growth curve was obtained by measurement of optical density ($\lambda = 660\text{ m}\mu$). At intervals, 0.5-ml samples of the culture were added to 0.1 ml of B1 DNA ($100\text{ }\mu\text{g/ml}$ of solution). The mixture was gently shaken at 37°C , and, after 60 min of incubation, 0.1 ml of deoxyribonuclease ($100\text{ }\mu\text{g/ml}$ solution) was added. Relative numbers of infective centers produced were plotted.

FIG. 3. Effect of DNA concentration on infective center formation. Amounts of 0.1 ml of B1 DNA diluted in Tris buffer at various concentrations were mixed with 0.3 ml of a competent culture, and were immediately plated with soft agar for the determination of infective centers.

FIG. 4. Effect of bacterial concentration on infective center formation. A competent culture containing 10^8 colony-formers/ml was centrifuged and resuspended in fresh broth at various concentrations. Amounts of 0.5 ml of the suspensions were added to 0.1 ml of B1 DNA at a final concentration of $10\text{ }\mu\text{g/ml}$, and shaken gently at 37°C for 60 min. After deoxyribonuclease treatment, the mixture was plated for the determination of infective centers.

period of B1 phage is 60 min, the rise period is 20 min, and the burst size is 80. With B1 DNA, the latent period is 90 to 95 min, the rise period is 35 min, and the burst size is 49. The characteristic extension of the latent period was observed. Such a prolongation of the time of lysis has also been observed in D29 DNA (24) and *B. subtilis* phage DNA (13, 15).

Kinetics of DNA infection. A competent culture was added to B1 DNA, and incubated at 37 C with or without shaking. At intervals, samples were transferred to tubes containing deoxyribonuclease and incubated for 10 min. As shown in Fig. 6, it took about 10 min for the transition of viral infection to deoxyribonuclease stability. An almost linear relationship between infection and contact time was observed. Increase of infective centers occurred faster in the shaking tube than in the standing one; after 30 min of incubation, the number of infective centers produced in the former was about fourfold higher than that of the latter. However, in the latter case, the increase in the number of infective centers showed almost the same slope as the former after about 30-min contact. The velocity of the infection was about 12 plaque-formers/min (0.24 per min per μg of DNA). Although not clearly illustrated on the curve in Fig. 5, the curves reached to a saturation at about 350 infective centers per ml.

Inactivation of B1 and B1 DNA by heating. Comparison of heat inactivation with an elevated temperature between B1 phage and B1 DNA is shown in Fig. 7. In B1 phage, the number of plaque formers decreased logarithmically when temperatures exceeded about 50 C. The temperature necessary to inactivate half of the original titer was about 50 C for 15 min. After 60 min at about 45 C, the titer decreased to 1% of the original. On the other hand, in B1 DNA, a much higher temperature was necessary to inactivate the infectivity. A slight inactivation was observed at about 91 C for 15 min, and half of the titer was lost at about 92 C. More than 99% was in-

FIG. 5. One-step growth curves of 607 after B1 and B1 DNA infection. The experimental procedure is given in the text.

FIG. 6. Time course of infection of the competent culture containing Tween 80 with B1 DNA and B1 phage. A competent culture of 607 (1.2×10^8 colony-formers/ml) grown in broth containing 0.06% Tween 80 was added to B1 DNA at a final concentration of 100 $\mu\text{g}/\text{ml}$. The mixture was incubated at 37 C with (■) or without (●) mild shaking. At intervals of 10 min, samples were taken, treated with 10 $\mu\text{g}/\text{ml}$ of deoxyribonuclease for 10 min, and plated for the determination of plaque-forming units. As a control, B1 phage was added instead of B1 DNA in the same way (○). No increase of infective centers was observed, which indicates that B1 phage could not be absorbed to the host in broth containing Tween 80 (27).

activated when it exceeded 94 C, suggesting that the active material was double-stranded DNA. With heating for 60 min, the inactivation occurred at about 74 C, half of the titer was lost at 75 to 76 C, and more than 99% was lost at 79 C. In the prolonged exposure for 60 min, inactivation of the DNA may be caused by mechanisms other than the breakdown of its secondary structure (11).

A hyperchromic effect of UV absorption was seen at about 92 to 93 C, with a correspondence to the beginning of the loss of biological activity. Melting temperature (T_m) was 96 to 97 C. Although suspending buffers and heating methods employed in our experiments with DNA were different from those of Murmer et al. (10), the mole per cent GC of B1 DNA estimated from the T_m -GC curve (0.15 M NaCl plus 0.015 M Na

FIG. 7. Heat inactivation of B1 phage and B1 DNA. Experimental procedure is given in the text. (a) Infectious activity of B1 phage after heating at the indicated temperatures for 60 min; (b) same as (a), heating for 15 min; (c) infectious activity of B1 DNA after heating at the indicated temperatures for 60 min; (d) same as (c), heating for 15 min. In addition, UV absorption ($\lambda = 260 \text{ m}\mu$) of B1 DNA after heating at the indicated temperatures for 15 min (O) is indicated.

citrate) (10) was about 66%. This value was a little lower than that computed from the buoyant density in CsCl described previously.

Reactivation experiments performed by annealing infectious DNA after heat inactivation failed, possibly because the infectivity of the original material was too low.

Infection of B1-resistant strain 607 with B1 DNA. Spizizen reported that a preparation of disrupted T2 could produce infectious virus in protoplasts from bacterial species which are resistant to T2 infection (19). Whether or not phage nucleic acids can infect bacteria which are resistant to phage infection was tested previously (18), with negative results. A further attempt was made in the present investigation. In addition to B1 DNA, viral DNA was extracted from *Phagus phlei*, which infects *M. phlei* but not strain 607, and viral RNA was extracted from MS2 which infects only male bacteria. Host bacteria examined, in addition to 607, were *M. phlei*, *M. tuberculosis*, *M. lysodeikticus*, *B. subtilis*, and *E. coli* (Hfr). *B. subtilis* was used in its competent state (28) and *M. phlei* was employed at the same cell concentration as the competent culture of 607. Of 18 test systems, only 3 showed positive results; B1 DNA could produce infectious virus in 607 and *M. tuberculosis*, and DNA of *Phagus phlei* could infect *M. phlei* only. Details of the infection of *M. tuberculosis* were published separately (25). These results showed the same

conclusion as the previous paper (18): species barriers were not crossed by DNA.

It was reported that transfection could be observed in phage-resistant clones of *B. subtilis* (13, 15). B1 DNA was tested for propagation in 607/B1, and it was found that only DNA could propagate in the bacteria (Table 1).

DISCUSSION

Evidence that DNA is a necessary factor of the phenol extract of B1 phage in infective center formation in 607 is based on the following observations: (i) treatment of the material with an anti-B1 serum did not affect its infectivity; (ii) infectivity was lost when the preparation was preincubated with a catalytic amount of deoxyribonuclease; (iii) presence of Tween 80 did not reduce the infectivity; (iv) a single band was obtained in CsCl; (v) infectivity was variable, depending upon the growth phase of the host cells; (vi) inactivation experiments by heating showed a typical inactivation curve of double-stranded DNA which is quite different from that of mature phages; and (vii) infectivity was observed against B1-resistant 607. The observations described by i, iii, iv, and vi above, suggest that a complex structure, such as that of the disrupted T2 particle, is not the infectious agent.

However, genetic transformation of 607, with the use of the competent state for the transfection, has not yet succeeded (T. Tokunaga and R. M. Nakamura, unpublished data). Part of the explanation may be the low population of competent cells in the culture used and the difficulty of ob-

TABLE 1. Infection of B1 phage-resistant *Mycobacterium* with B1 DNA^a

Incubation time (min)	No. of plaque-formers unabsorbed/ml	
	B1 phage	B1 DNA
0	241	0
60	229	0
120	200	1
180	223	0
240	209	101

^a A 0.1-ml amount of B1 phage or B1 DNA was added to competent culture of 607/B1 at the final concentration of about 200 plaque-forming units/ml or 50 $\mu\text{g/ml}$, respectively, and then incubated at 37°C. At 60-min intervals of the incubation, samples were taken and were treated with deoxyribonuclease. After treatment for 10 min, the samples were centrifuged, and the numbers of plaque-formers in the supernatant fluids were scored.

taining DNA of host bacteria in a native form, because the cell wall of mycobacteria is quite resistant to chemical agents, such as phenol, chloroform, lysozyme, etc. However, the possibility exists that a limited amount of protein present in the DNA preparation could enhance the DNA infection of competent cells.

Competence appears in early log phase of bacterial growth in *Streptococcus* (14), and in late log phase in *H. influenzae* (2) and *B. subtilis* (22). In *Neisseria meningitidis*, cells are capable of being transformed all through the regular growth cycle, but the highest transformation frequency is obtained in an early part of the log phase (9). In pneumococcus, it was found that the most important factor affecting the time course of the phenotypic expression process was the cell concentration (26). In mycobacteria, the number of colony-formers does not directly indicate the exact number of living cells, because of clumping of the bacteria. However, a correlation between the optical density of the culture and competence was noticed. Cells susceptible to transfection appeared all through the growth cycle; however, a special increase of transfected cells was found in late log phase.

To obtain a competent culture, various nutritional conditions have been employed: in *B. subtilis* and *H. influenzae*, a step-down from a rich medium to a poor medium is necessary (28; S. H. Goodgal, Federation Proc. 23: 318, 1965); in *Neisseria*, the highest competence is seen in media exhibiting the shortest time of generation (9); in pneumococcus, it depends only on cell concentration (26). Preliminary experiments with mycobacteria suggested that high competence appears in rich medium, as with *Neisseria* (T. Tokunaga and R. M. Nakamura, unpublished data).

When the competent culture was concentrated by centrifugation, a significant increase of infective centers was observed; for instance, about 240 infective centers per μg of DNA were produced. As the infectivity of D29 DNA was reported to be 2.4 to 3.2 plaque-forming units per μg in the previous report (18), the value reported here represents a significant increase in specific activity over those obtained before. However, Reilly et al. reported an infectivity of $>5 \times 10^6$ infective centers per μg of DNA in a *B. subtilis* phage (15) and about 10^{-7} in a *Haemophilus* phage (5), whereas it was about 1.5×10^{-8} in B1. One of the reasons that infectivity of mycobacteriophage DNA is so low compared to those reported for *B. subtilis* or *Haemophilus* phages is the low population of competent cells in the so-called competent culture obtained, as described above. Only less than 10^{-5} of the cells were susceptible

to DNA infection in the case of mycobacteria, whereas 4.5% of the cells were susceptible in *B. subtilis* (15). It is necessary to obtain cultures of 607 with much higher competence for further analysis of transfection of mycobacteria. Recently, spheroplasting of mycobacteria has succeeded (Y. Mizuguchi and T. Tokunaga, unpublished data). This fraction is being used for further analysis of mycobacterial competence.

The role of recombination in the transfection of *B. subtilis* was reported by Okubo et al. (13) and Green (6). When the DNA samples are shaken with a Vortex mixer or sheared by being blown through the needle of a hypodermic syringe prior to the addition of cells, the activity of the sample is affected only a little. This observation suggests that the cooperation of two or more molecules of DNA is required for the establishment of the infective center. Recently isolated temperature-sensitive mutants (R. M. Nakamura and M. I. Sellers, unpublished data) are being used in studies on the possible role of recombination.

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