JOURNAL OF BACTERIOLOGY, Mar. 1978, p. 1254-1262
0021-9193/78/0133-1254$02.00/0

Vol. 133, No. 3
Printed in U.S.A.

Physiological Factors Involved in the Transformation of *Mycobacterium smegmatis*

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Received for publication 8 December 1977

Transfer of streptomycin resistance and changes from methionine and leucine auxotrophy to prototrophy were achieved in *Mycobacterium smegmatis* by transformation. Recipient cells were more resistant to mitomycin C and methyl methanesulfonate treatments than were wild-type cells. A high level of calcium ions was essential for transformation, especially during DNA adsorption, whereas the presence of magnesium ions and the exposure of recipient cells to mild doses of UV light enhanced recombination frequencies. Transformants were not isolated when recipient cell-DNA mixtures were first treated with deoxyribonuclease. Recipient cells at various stages of growth showed similar transformabilities. Transformation was successful only when recipient cells were incubated on rich agar medium after mixture with DNA. Exposure of recipient cells to Pronase before treatment with donor DNA did not affect transformation, suggesting the absence of a protein competence factor. Throughout the present experiments, cotransformation frequencies were very low and unselected-marker segregation patterns were independent, indicating that the methionine, leucine, and streptomycin markers are not closely linked in *M. smegmatis*.

Despite the medical importance of mycobacteria, knowledge of genetic recombination in this genus remains limited. Experimental procedures in transduction (12, 13, 20-22, 41) and conjugation (30, 32, 40, 44) have recently been established for various species of mycobacteria, but results of transformation experiments have been inconclusive (4, 14, 43).

Transformation in mycobacteria was first reported by Katunuma and Nakasato (23), who claimed that streptomycin resistance was transferred to *Mycobacterium avium* through DNA extracted from streptomycin-resistant strains. Tsukamura et al. (48) reported the transfer of isoniazid and streptomycin resistance to *M. avium* after exposure for 5 days to high concentrations of DNA extracted from resistant strains. Gelbart and Juhasz (12) and Juhasz et al. (S. E. Juhasz, S. M. Gelbart, and L. DeSalle, Bacteriol. Proc., p. 35, 1971) described xylose utilization transferred to *Mycobacterium phlei* through transformation.

In contrast to these observations, Bloch et al. (3) failed to transfer streptomycin and isoniazid resistance to *M. phlei*, *M. smegmatis*, *M. bovis*, and *M. tuberculosis*, using purified DNA from respective resistant strains. They were also unsuccessful in transferring the ability to synthesize pigments to *M. smegmatis* from *M. phlei* or the virulence of *M. tuberculosis* H37Rv to the avirulent *M. tuberculosis* H37Ra through transformation. Similarly, Bradley (4) was unable to transfer isoniazid, streptomycin, or cycloserine resistance or mycobacteriophage susceptibility to *M. smegmatis*, *M. kansasii*, *M. bovis*, *M. tuberculosis*, and *M. intracellulare* through transformation.

On the other hand, foreign DNA molecules, including mycobacterial and mycobacteriophage DNA as well as synthetic polynucleotides, were reportedly taken up by mycobacteria (15, 33, 36, 39, 42, 45-47). Consequently, the major difficulty encountered in transformation experiments with mycobacteria may not lie in the initial stages of the process. Rather, it seems that later stages of the transformation process, including genetic recombination, may have been defective in many mycobacterial strains used in previous studies.

Our previous studies revealed that orange-red-pigmented variants of *M. smegmatis* were significantly more resistant to UV-light irradiation than were their parental wild-type strains (15), suggesting an effective DNA repair or recombination mechanism(s) in these variants. The present study showed that these variants, also resistant to DNA-alkylating agents, were transformable by homologous DNA. The influence of various factors on transformation are described.

(This work was presented at the 77th Annual Meeting of the American Society for Microbiol-

ogy in New Orleans. [M. V. Norgard and T. Imaeda, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, H79, p. 148].)

MATERIALS AND METHODS

Strains. Bacterial strains used in this study are listed in Table 1. In addition to the nutritional and antibiotic markers presented, strains 607$^+$, 607-1, and 607-2 form rough, cream-yellow colonies and are capable of growing at 43°C and sensitive to UV irradiation at a dose of 7.2×10^2 ergs/mm^2 (15). Recipient strains (OR4 and ORuv) are characterized by their smooth, orange-red-pigmented colonies, inability to grow at 43°C, and resistance to UV irradiation at a dose of 7.2×10^2 ergs/mm^2 (15). Strains OR4 and ORuv do not cross-feed each other.

Media and growth conditions. Mycobacteria were cultivated in Penassay-glycerol (PG) (15) (for strains OR4, ORuv, and OR4-1) or PG-Tween (15) (for strains 607$^+$, 607-1, and 607-2) broth. Agar plates (PGA) contained 1.5% (wt/vol) agar (Difco Laboratories, Detroit, Mich.) (15). For the selection of streptomycin-resistant organisms, 500 μg of dihydrostreptomycin sulfate (Sigma Chemical Co., St. Louis, Mo.) per ml was added to PGA. The pH of all media was 7.0 to 7.2.

Minimal agar was prepared as described by Clowes and Hayes (7), wherein 2% (vol/vol) glycerol was substituted for glucose. When various mutants and transformants were tested for their amino acid requirements, L-amino acids (Sigma) were added to a final concentration of 20 μg/ml. Prototrophic, streptomycin-resistant transformants were selected on minimal agar plates containing 500 μg of dihydrostreptomycin sulfate per ml. Media, reagents, buffers, and DNA stock solutions were examined for sterility by inoculating a 0.1 to 1.0-ml volume of each onto PGA followed by incubation at 37°C for 7 days.

Bacteria were routinely cultivated in 50-ml broth cultures incubated at 37°C while shaking at 125 rpm in a water bath shaker. Log-phase cells of mycobacteria were obtained by inoculating about 10^8 colony-forming units (CFU) into 50-ml broth cultures and incubating them at 37°C for 6 to 12 h with constant shaking. Growth reached the early stationary phase at about 18 h.

Isolation of mutants. Auxotrophic mutants of *M. smegmatis* were isolated after treatment of OR strains with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (K & K Laboratories, Inc., Plainview, N.J.). An inoculum (0.2 ml) containing about 2×10^7 CFU was evenly spread on each of several petri dishes containing PGA. After a small crystal of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was placed in the center of each plate, plates were incubated at 37°C. At the end of 7 days, tiny colonies growing at the periphery of the zone of inhibition were examined for their amino acid requirements.

TABLE 1. *Bacterial strains*

Strain	Relevant genotype or phenotype	Source
607$^+$	Wild type, Str^s	American Type Culture Collection (ATCC) 607.
607-1	Str^r [a]	This laboratory, formerly N701.
607-2	Arg$^-$ Str^s	Y. Mizuguchi, formerly N715.
OR4	Met$^-$ Str^s	This laboratory (15).
ORuv	Met$^-$ Str^s	This laboratory (15).
OR4-1	Met$^-$ Leu$^-$ Str^s	*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutant of OR4.

[a] Resistant to 500 μg of streptomycin per ml.

Characteristics of recipient strains. Spontaneous single- and double-marker reversion frequencies of strains OR4 and ORuv (Met$^-$ Str^s) to Met$^+$ Str^r were examined by inoculating 3.0×10^{11} to 1.0×10^{12} CFU on appropriate selective media followed by incubation at 37°C for 4 weeks. Spontaneous multiple-marker reversion for strain OR4-1 (Met$^-$ Leu$^-$ Str^s) to Met$^+$ Leu$^+$ Str^s, Met$^-$ Leu$^+$ Str^s, Met$^+$ Leu$^-$ Str^r, or Met$^+$ Leu$^+$ Str^r was similarly investigated. Various strains were tested for their sensitivity to mitomycin C (MMC) and methyl methanesulfonate (MMS) as follows. MMC (Sigma; M-0503) or MMS (Aldrich Chemical Co., Milwaukee, Wis.; no. 12, 992-5; 97%) was added to prewarmed, log-phase PG cultures (about 10^8 CFU/ml) of strains OR4, ORuv, and 607$^+$ at a final concentration of 1 μg/ml or 0.35% (vol/vol), respectively. Zero-time and 10-min-interval samples were diluted 100-fold immediately in cold 0.15 M NaCl–0.05% (vol/vol) Tween 80 and mixed vigorously with a Vortex device. The CFU in each sample were assayed on PGA after incubation at 37°C for 7 days.

DNA isolation. Mycobacterial DNA was isolated, using the procedure of Mizuguchi and Tokunaga (31). Cell lysis was aided with 0.2 mg of Lytic Enzyme 2 (Kyowa Hakko U.S.A. Inc., New York, N.Y.) per ml followed by treatment with sodium dodecyl sulfate (Fisher Scientific Co., Pittsburg, Pa.) at a final concentration of 0.1 to 1.0% (wt/vol). Isolated DNA was further purified according to Baess (1), dissolved in sterile SSC/10 buffer (0.015 M NaCl–0.0015 M sodium citrate, pH 7.0), and stored at −15°C. The concentration of DNA in stock solutions was estimated by optical density at 260 nm. The presence of double-stranded DNA in the preparation was confirmed by a hyperchromic shift at 260 nm after alkaline denaturation. For transformation experiments, DNA was sheared by passage 10 times through a 26-gauge needle, using maximum hand pressure with a 5-ml Plastipak (Becton, Dickinson & Co., Rutherford, N.J.) disposable syringe (5).

Molecular weight determination of DNA. The molecular weight of sheared DNA was determined by two methods. Sucrose density gradient velocity centrifugation was performed according to Jacob and Hobbs (18). DNA (15 μg in 0.15 ml of SSC/10) was layered on linear 5 to 20% (wt/vol) sucrose gradients in TES (0.03 M tris(hydroxymethyl)aminomethane–0.006 M ethylenediaminetetraacetate–0.05 M NaCl, pH 8.0) (2). Tubes were centrifuged at 40,000 rpm in an SW 50.1 (Beckman Instruments, Inc., Fullerton, Calif.) rotor at 20°C for 150 min. After centrifugation, 5-drop fractions were collected in tubes containing 0.5 ml of TES and assayed for DNA as determined by optical density measurements at 260 nm. Molecular weights were determined by cosedimentation with T7 phage DNA, having a molecular weight of 2.52×10^7

(11) (T7 DNA was generously provided by H. S. Shapiro).

The size of sheared DNA was also determined by electron microscopy according to Lang and Mitani (26). Electron micrographs were taken with the aid of a Hitachi HU-12 electron microscope at direct magnifications between ×10,000 and ×20,000 at 75 kV. Lengths of 20 randomly selected DNA fragments were statistically analyzed to calculate DNA molecular weights (9), based on the relationship of 2.07×10^6 daltons/μm (25).

Radioactive labeling of DNA. Strain 607-1 was inoculated in 250 ml of PG-Tween and incubated with shaking at 37°C. When the cell density reached about 10^7 CFU/ml, sterile uridine at a final concentration of 1 mg/ml (6) and 0.25 mCi of [*methyl*-^{3}H]thymidine (sterile, 0.5 ml; NET-027X; New England Nuclear Corp., Boston, Mass.) were added. The culture was further incubated with shaking until the cells reached about 10^9 CFU/ml. Labeled cells were then treated with glycine (31) and harvested for lysis and DNA extraction as described earlier.

DNA binding assay. Strains OR4, ORuv, and 607-2 were harvested from broth containing about 10^9 CFU/ml, washed once in transformation (TF) buffer composed of 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride, 1 mM $MgSO_4$, and 0.15 M NaCl, pH 7.5, and suspended in 20 ml of TF buffer at a concentration of about 10^9 CFU/ml. Ten milliliters of each suspension was poured into a sterile plastic petri dish (100 by 15 mm) and irradiated with UV light at doses of 7.2×10^2 ergs/mm^2 for OR cells and 2.4×10^2 ergs/mm^2 for strain 607-2. The remaining 10 ml of each suspension received no UV treatment. All suspensions were kept in the dark and further divided in half, and one set of tubes (with and without UV treatment) was treated with 50 mM $CaCl_2$ at 0°C for 15 min. After the addition of sheared [^{3}H]DNA (2,340 cpm/μg) of strain 607-1 to each cell suspension at a final concentration of 4.4 μg/ml, the mixtures were incubated at 37°C with mild shaking at 60 rpm. At 5-min intervals, 1.0-ml samples of the mixtures were passed through HAWP02500 membrane filters (Millipore Corp., Bedford, Mass.) presoaked in TF buffer. The cells collected on filters were washed three times with 1.0-ml portions of cold TF buffer. Filters were then placed in scintillation vials and dried, and the amount of radioactivity remaining on the filters was determined. Blank filter values, obtained by filtering mixtures incubated without cells, were subtracted from experimental values. To determine the amount of unbound DNA in each sample, an equal volume of cold 10% (wt/vol) trichloroacetic acid (Matheson, Coleman, and Bell) was added to each filtrate, which was then kept in an ice bath for 30 min and centrifuged at 3,300 × *g* for 10 min. Trichloroacetic acid precipitates were washed twice with 5% (wt/vol) cold trichloroacetic acid, suspended in 0.5 ml of 5% trichloroacetic acid, and transferred to scintillation vials containing Whatman GF/A glass fiber disks. Supernatant fluids and wash solutions were combined to collect trichloroacetic acid-soluble materials and added to vials with GF/A disks. After drying, 10 ml of Aquasol (New England Nuclear) was added to each vial. Scintillation counting was performed in a Beckman model LS-250 liquid scintillation counter.

Transformation procedure. Recipient strains in the early stationary phase were harvested from PG, washed once in TF buffer, and suspended in TF buffer at a concentration of about 10^9 CFU/ml. Suspensions were irradiated with 7.2×10^2 ergs of UV light per mm^2 (15). A 0.9-ml sample of each suspension was kept in the dark, treated with 0.1 ml of 0.5 M $CaCl_2$ at 0°C for 15 min, and mixed with 0.2 ml (100 μg/ml) of sheared, sterile DNA of the donor strain, 607-1, for 30 min at 37°C. Cell-DNA mixtures were then transferred to PGA wells (44) or onto a 4-cm^2 surface of PGA. Plates were incubated at 37°C for 72 h. Confluent growth was removed from agar wells or the agar surface, suspended in 0.15 M NaCl at about 10^{10} CFU/ml, and plated on selective media for the detection of transformants as well as on PGA for viable counts. A control sample of cells without DNA was included in each experiment to monitor spontaneous reversion frequencies for various markers. All plates were kept for a minimum of 2 weeks at 37°C. Transformants were scored as the number of selected colonies per 10^9 CFU.

Factors affecting transformation. The effect of deoxyribonuclease (DNase) on the transformation process was investigated as follows. DNase I (Sigma; DN-100) in 0.1 M $MgSO_4$, pH 7.3, was added to a final concentration of 10 μg/ml either simultaneously to (early DNase treatment) or 20 min after (late DNase treatment) the addition of DNA to recipient cells. Transformation procedures were then completed as described.

Several cations were examined for their ability to stimulate the transformation process. For the effect of calcium ions, transformation was performed in the presence of 0, 10, 25, 50, 75, and 100 mM $CaCl_2$ in TF buffer. Due to the finding that calcium ions were required for DNA binding to recipient cells, the following experiments were carried out in the presence of 50 mM $CaCl_2$. For the effect of magnesium ions, recipient cell-DNA mixtures in TF buffer without $MgSO_4$ were exposed to 0, 0.5, and 1.0 mM concentrations of $MgSO_4$ during incubation with DNA. Similarly, recipient cell-DNA mixtures in TF buffer without $MgSO_4$ were treated with 1.0 mM concentrations of $MnCl_2 \cdot 4H_2O$ (Fisher), $FeSO_4 \cdot 7H_2O$ (J. T. Baker Chemical Co., Phillipsburg, N.J.), $Fe(NO_3)_3 \cdot 9H_2O$ (Mallinckrodt Chemical Works, St. Louis, Mo.), or $ZnSO_4 \cdot 7H_2O$ (Merck & Co., Inc., Rahway, N.J.), and the transformation procedure was completed as described.

To examine the possibility of a protein competence factor (34) for transformation, UV-irradiated recipient cells were treated with self-digested (2 h at 37°C) Pronase (Calbiochem, La Jolla, Calif.) at a final concentration of 100 μg/ml for 15 min at 37°C in the dark and then mixed with donor DNA. The relationship between competence for transformation and the stage of growth of recipient cells was examined using early-log (6 h)-, mid-log (12 h)-, early-stationary (18 h)-, and stationary (24 h)-phase cells. To determine the effect of nutrient supply on the transformability of recipient cells, $CaCl_2$ and DNA were added directly to growing PG cultures of recipient cells. Similarly, samples of

UV-treated recipient cells were inoculated into PG and treated with $CaCl_2$ and DNA.

RESULTS

Characteristics of recipient strains. The recipient strains (OR4 and ORuv) possessed auxotrophic (Met^-) and antibiotic (Str^s) markers upon isolation (15). The spontaneous reversion frequencies of these strains from Met^- Str^s to Met^+ Str^s, Met^- Str^r, or Met^+ Str^r were found to be less than 1.0×10^{-9}, 9.0×10^{-8}, and 3.0×10^{-11}, respectively. Only one additional stable, auxotrophic mutant (strain OR4-1, Met^- Leu^- Str^s) was isolated from strain OR4 after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment (Table 1). The spontaneous multiple-marker reversions from Met^- Leu^- Str^s to Met^+ Leu^+ Str^s, Met^- Leu^+ Str^r, Met^+ Leu^- Str^r, or Met^+ Leu^+ Str^r occurred at frequencies of less than 3.0×10^{-11}.

Recipient strains (OR4 and ORuv) were significantly more resistant to the DNA-alkylating agents MMC and MMS than was the wild-type strain. Within a 10-min exposure to MMC or MMS, little change was observed in the survival of strains examined. In an additional 60-min period, however, strains OR4 and ORuv showed about a 1% survival, whereas the survival of strain 607^+ dropped to 0.001%. Thus, the survival rates of both recipient strains were about 1,000 times higher than that of the wild-type strain.

Molecular weight of transforming DNA. The DNA-shearing procedure used in this study produced fragments of 5.2×10^6 daltons, as measured by sucrose gradient velocity centrifugation, or $(5.0 \pm 0.5) \times 10^6$ daltons, as determined by electron microscopy.

Effects of calcium ions and UV irradiation on DNA binding. The binding of [^{3}H]-DNA to OR strains, as well as to strain 607-2(Arg^- Str^s), used as a control, is shown in Fig. 1. The effects of calcium ions (50 mM) and UV irradiation of recipient cells on the DNA-binding

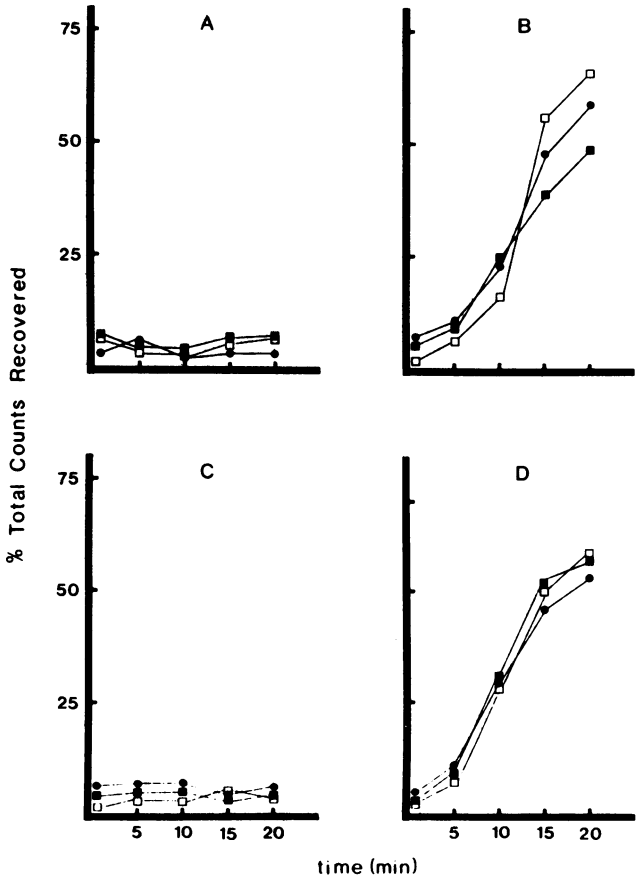

FIG. 1. *Binding of [^{3}H]DNA to strains OR4 (■), ORuv (□), and 607-2 (●). (A) −UV, −Ca; (B) −UV, +Ca; (C) +UV, −Ca; (D) +UV, +Ca.*

process were also monitored. In Fig. 1A and C, no appreciable amount of DNA was bound to recipient cells when calcium ions were omitted. In contrast, when calcium ions were present (Fig. 1B and D), a significant quantity of [^{3}H]-DNA was found in recipient cells after a 20-min exposure. UV irradiation of recipient cells treated with or without calcium ions did not affect DNA adsorption. When filtrates of these samples were assayed, the majority of ^{3}H-labeled, trichloroacetic acid-precipitable nucleotides did not bind to the recipient cells in the absence of calcium ions, whereas those of the calcium-treated samples were lost from the reaction filtrates, within 20 min (data not shown). The amount of trichloroacetic acid-soluble mono- or oligonucleotides in filtrates remained unchanged during the 20-min DNA adsorption period (data not shown). Both OR strains and strain 607-2 showed similar abilities to bind exogenous DNA, including a requirement for calcium ions during DNA adsorption.

Transfer of genetic markers. The results of a transformation experiment using the recipient strain ORuv (Met$^-$ Str^s) treated with DNA from the donor strain 607-1 (Met$^+$ Str^r) were as follows. When the recipient cells were treated with DNA for 30 min, Met$^+$ and Str^r colonies were recovered at a frequency of about 10^{-7} and 10^{-6} per CFU on PGA, respectively. Thus, the observed frequencies for Met$^+$ and Str^r were about 100- and 10-fold higher, respectively, than the spontaneous reversion frequencies described earlier. However, when DNA and DNase were simultaneously added to the recipient cells (early DNase treatment), no significant increase in the occurrence of Met$^+$ or Str^r colonies above spontaneous frequencies was observed. In contrast, when DNase was added to the cell suspension 20 min after the addition of DNA (late DNase treatment), the numbers of Met$^+$ and Str^r colonies were almost the same as those obtained without DNase treatment.

In addition to single-marker transfer, the cotransfer of markers from prototrophic and streptomycin-resistant 607-1 and ORuv transformant strains to recipient strains ORuv (Met$^-$ Str^s) and OR4-1 (Met$^-$ Leu$^-$ Str^s) was examined. As shown in Table 2, the frequency of double-marker transfer was a rare event (1×10^{-9} to 2×10^{-9}). However, this frequency was higher than the spontaneous mutation frequency of less than 3.0×10^{-11} observed for any pair of double markers. Transformants with all three markers were not isolated in the present study. Based on these findings, it is concluded that the transfer of single- or double-marker characteristics occurred as the result of transformation. In support of this conclusion, the recipient ORuv strain treated with DNA extracted from the same strain yielded no Met$^+$, Str^r, or Met$^+$ Str^r colonies (Table 2). On the other hand, when DNA extracted from a Met$^+$ Str^r transformant of ORuv was used as donor DNA, the frequencies of Met and Str marker transfer approximated those observed upon using donor DNA from strain 607-1. The ability to grow at 43°C, characteristic of the donor strain 607-1, was not evident among transformants described above.

Segregation patterns for unselected markers among transformants were also examined. Upon testing over 1,000 Met$^+$, Leu$^+$, or Str^r transformants of strain OR4-1, it was found that each of these markers segregated independently.

Effect of various factors on transformation. Throughout the transformation experiments, transformants were isolated on selective media only after recipient cells treated with donor DNA in TF buffer were subsequently incubated on PGA (agar) medium. Direct plating onto selective media immediately after DNA treatment did not result in the recovery of transformants, nor did vigorously aerated cultures of DNA-treated recipient cells in PG broth yield transformants.

An interesting characteristic of the present

TABLE 2. *Cotransformation of genetic markers in strains OR4-1 and ORuv*

Recipient	Donor DNA	Selected markers (no. of transformants/10^9 CFU)						
		Met$^+$	Leu$^+$	Str^r	Met$^+$ Leu$^+$	Met$^+$ Str^r	Leu$^+$ Str^r	Met$^+$ Leu$^+$ Str^r
OR4-1 (Met$^-$ Leu$^-$ Str^s)	None	0	9	79	<0.03	<0.03	<0.03	<0.03
OR4-1	607-1 (Met$^+$ Leu$^+$ Str^r)	127	154	841	2	2	1	0
ORuv (Met$^-$ Str^s)	None	0	NA[a]	90	NA	<0.03	NA	NA
ORuv	607-1	96	NA	1,040	NA	2	NA	NA
ORuv	ORuv	0	NA	93	NA	0	NA	NA
ORuv	ORuv transformant (Met$^+$ Str^r)	84	NA	1,000	NA	1	NA	NA

[a] NA, Not applicable.

transformation process involves the effect of UV irradiation on recipient cells. A sublethal dose of UV light increased the frequency of transformation about 10-fold, when the transfer of the Met marker from strain 607-1 to recipient cells was monitored.

As demonstrated in DNA-binding experiments, the presence of calcium ions was essential for transformation. The number of Met^+ transformants increased with the calcium ion concentration and reached a maximum at almost 100 mM (Fig. 2). However, substantial cell clumping caused by treatment with 100 mM $CaCl_2$ made viable count assays difficult. Consequently, 50 mM $CaCl_2$ was routinely used thoughout transformation experiments.

The addition of magnesium ions to TF buffer (without $MgSO_4$) containing 50 mM $CaCl_2$ resulted in a significant increase in the frequency of Met^+ transformants (Fig. 3). On the other hand, no transformants were recovered when recipient cells were treated with 50 mM $MgCl_2$ alone. One millimolar concentrations of ferrous, ferric, manganese, and zinc ions showed no ability to stimulate transformation in either the presence or the absence of calcium ions. The treatment of recipient cells with DNA and $CaCl_2$ in TF buffer seems to be essential for transformation, because UV-treated PG-broth cultures of recipient cells did not produce transformants upon incubation with $CaCl_2$ and donor DNA.

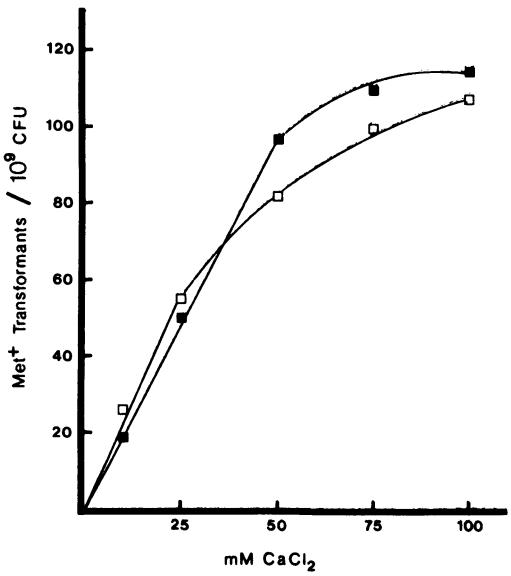

FIG. 2. *Effect of calcium ion concentration on the transformation of strains OR4 (■) and ORuv (□). Cells in TF buffer were UV irradiated and treated with DNA from strain 607-1 while exposed to various concentrations of $CaCl_2$.*

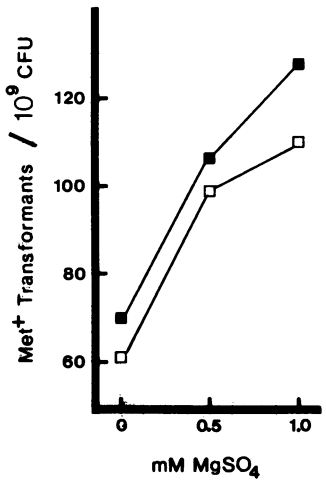

FIG. 3. *Effect of magnesium ions on number of transformants (Met^+) per 10^9 CFU from strains OR4 (■) and ORuv (□); donor strain, 607-1 (Met^+). Recipient cells in TF buffer (without $MgSO_4$) were UV irradiated and treated with DNA in the presence of 50 mM $CaCl_2$ while exposed to various concentrations of $MgSO_4$.*

Competence for transformation. The nature of competence of recipient cells was examined to determine if different growth stages influenced transformability (Table 3). When the recipient strains at various stages of growth were treated with DNA from strain 607-1, no distinct differences were observed as far as the transfer of the Met marker was concerned.

It was also found that Pronase treatment before the addition of DNA to recipient cells did not affect the number of transformants recovered. This suggests that no extracellular or surface-bound Pronase-sensitive protein competence factor is involved in this transformation system.

DISCUSSION

The present study showed that the transfer of streptomycin resistance and changes from methionine and leucine auxotrophy to prototrophy occurred in *M. smegmatis* through transformation. Several factors appear to contribute to the transformation of the recipient strains. Namely, they possess abundant mesosomes (R. J. Hawley, Ph.D. thesis, CMDNJ-Graduate School of Biomedical Sciences, Newark, N.J., 1974), which are believed to play an important role in the interaction of bacteria with exogenous DNA (19). Furthermore, they are significantly more resistant to the lethal effects of UV irradiation (15), MMC, and MMS than are donor strains. Recipient strains, therefore, seem particularly efficient in DNA repair mechanisms, which pre-

TABLE 3. *Influence of growth stages on transformation of strains OR4 and ORuv*

Strain	Culture (h)	Growth phase	Treatment[a]	No. of Met$^+$ transformants/10^9 CFU
OR4	6	Early log	−DNA	0
	6	Early log	+DNA	73
	12	Mid log	−DNA	0
	12	Mid log	+DNA	72
	18	Early stationary	−DNA	0
	18	Early stationary	+DNA	83
	24	Stationary	−DNA	0
	24	Stationary	+DNA	75
ORuv	6	Log	−DNA	0
	6	Log	+DNA	69
	12	Mid log	−DNA	0
	12	Mid log	+DNA	71
	18	Early stationary	−DNA	0
	18	Early stationary	+DNA	63
	24	Stationary	−DNA	0
	24	Stationary	+DNA	67

[a] Donor DNA was prepared from strain 607-1 (Met$^+$ Str^r).

sumably play an important role in the genetic recombination process (10). In support of this, exposure of recipient cells to mild UV irradiation before treatment with DNA significantly increased the frequency of transformation. Thus, UV irradiation before DNA uptake may induce DNA repair enzymes that can participate in recombination process, as described in the study of *Escherichia coli* (49). In this connection, it is interesting to mention that a UV-light-sensitive recipient strain of *M. smegmatis* was unable to produce recombinants after conjugation (29). The present study also revealed that strain 607-2, which is sensitive to UV light and MMC and MMS treatments, was nontransformable despite its ability to adsorb donor DNA. Thus, the failure of transformation experiments by other investigators may have been due to the use of recombination-deficient (Rec$^-$) recipient strains, although data on the recombinational abilities of these recipient strains are not available (3, 4, 42).

It has been reported that mycobacteria require calcium ions for DNA adsorption and transfection (36, 39). Consistent with these observations, the present investigation demonstrated that a high concentration of calcium ions was indispensable for the irreversible adsorption of DNA to the surface of *M. smegmatis*. Furthermore, as in other transformation systems (8, 27, 37), the presence of calcium ions was essential for the transformation of *M. smegmatis* under the present experimental conditions. Other divalent cations, such as magnesium, ferrous, manganese, and zinc ions, could not replace calcium ions in transformation.

Magnesium was the only other cation found to stimulate the transformation process in the presence of calcium ions. In pneumococcal transformation systems, a strand of the incoming double-stranded DNA is hydrolyzed by membrane DNases involved in DNA uptake (24). In contrast, our experiments showed that DNA degradation apparently does not occur at the early stages of DNA uptake by *M. smegmatis*. These data suggest that enhancement of transformation by magnesium ions may not be related to DNase activity during DNA uptake. It is possible, however, that the level of DNase activity during DNA uptake by mycobacteria may be too low to be detected with the present analyses.

The majority of DNA is presumably taken up by recipient cells within 20 min (Fig. 1). However, when these recipient cells were plated on selective media, directly or after incubation in rich liquid medium, no transformants were recovered. Incubation on rich solid medium before plating on selective media was absolutely required for the isolation of transformants. It is interesting to note that in conjugation experiments with *M. smegmatis* (30, 32, 44), *Nocardia mediterranei* (38), and *Streptomyces coelicolor* (17), recombinants can be recovered only when mating is performed on the surface of agar medium. Similarly, the genetic transformation of *Azotobacter vinelandii* (35) can only be carried out on the surface of solid media. It appears unlikely, however, that free oxygen available to DNA-treated recipient cells grown on an agar surface influences phenotypic expression, since DNA-treated recipient cells inoculated into vigorously aerated PG broth do not produce transformants.

Transformation was unsuccessful when DNA and $CaCl_2$ were incubated with UV-treated recipient cells in rich PG broth. TF buffer is, at this time, the only useful medium for the initial stage of transformation. It seems likely, therefore, that nutrient limitation is required for competence in *M. smegmatis*, similar to that required for transformation of *Haemophilus influenzae* (16). In addition, recipient cells from different growth stages were all transformed with equal efficiency (Table 3). Also, Pronase treatment before DNA treatment did not affect the number of transformants recovered, suggesting that a protein competence factor plays no role in transformation.

The frequency of transformation reported here is lower than those observed for other bacterial transformation systems (28, 34). However,

the amount of donor DNA used in our transformation experiments does not seem to be the limiting factor, because approximately 2.0×10^{12} DNA fragments per 10^9 recipient cells, or 2,000 fragments of DNA per recipient cell, have been applied in the present experimental conditions, as calculated from the following data: 1 dalton $= 1.67 \times 10^{-18}$ μg, one DNA fragment $= 5.0 \times 10^6$ daltons (present data), and 17 μg of DNA per ml present during DNA adsorption. Consequently, the low frequency of transformation for recipient cells may be attributed to an unusually small percentage of competent cells in the recipient cell population.

The occurrence of double transformants among Met, Leu, and Str markers was very low in the present system. Furthermore, the segregation of unselected markers was independent. These facts suggest that the Met, Leu, and Str markers may not be closely linked. This interpretation is in agreement with linkage data for Met, Leu, and Str markers obtained through conjugation experiments with *M. smegmatis* by Tokunaga et al. (44). However, further experiments to confirm the location of the Met, Leu, and Str markers are difficult to design at present because of the extremely low frequency of double-marker transformants.

ACKNOWLEDGMENTS

We thank S. G. Bradley for his interest and comments on this work and Nora Mann for her technical assistance.

This research was supported by a grant from the World Health Organization.

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