Genetic Recombination in Mycobacteria

TOHRU TOKUNAGA, YASUO MIZUGUCHI, AND KIYOKO SUGA

Department of Tuberculosis, National Institute of Health, Kamiosaki, Shinagawa-ku, Tokyo, Japan

Received for publication 10 October 1972

Evidence for genetic recombination between *Mycobacterium smegmatis* strain Rabinowitchi (Rab) and strain Jucho or PM5 is presented. Backcrosses of recombinants by either parental strain indicated four different types of mating behavior, suggesting that the mycobacterial compatibilities are controlled by at least two different factors. No sex factor that transfers at a high frequency or that is sensitive to acridine dyes was detected. Analysis of segregation of unselected markers revealed that strain Jucho, or PM5, contributes the majority of alleles in almost all recombinants obtained from different selective media. Efforts to construct linkage maps for the markers employed failed because of ordering ambiguities. Mating medium containing streptomycin prevented genetic recombination when strain Rab was resistant to the antibiotic and Jucho, or PM5, was sensitive, but it did not prevent recombination when Rab was sensitive to streptomycin and Jucho, or PM5, was resistant. Very low frequency of recombinant formation was observed when Jucho, or PM5, had been treated with streptomycin, whereas recombinants were formed at fairly high frequencies when Rab had been treated with the antibiotic, suggesting that the roles of parental strains in zygote formation were not identical. The results suggest a polar transfer of genetic material from Rab to Jucho, or PM5, although an alternative possibility of cell fusion followed by exclusion could not be excluded.

Evidence for genetic recombination in mycobacteria was previously shown in crosses between two different strains of *Mycobacterium smegmatis*, designated as Lacticola and Jucho (19). Crosses between nutritionally complementary auxotrophs derived from one strain were infertile whereas those from different strains formed recombinants. Conjugation rather than transformation or transduction was suggested as the gene transfer mechanism (19).

A further search for a recombination system in the mycobacteria was carried out and resulted in the observation that two groups of strains belonging to the species *M. smegmatis* were compatible to each other. The present paper describes the genetic recombination between strain Rabinowitchi and strain Jucho or PM5. Features of mycobacterial conjugational behavior which appear as a transfer of genetic material unidirectionally are also described.

MATERIALS AND METHODS

Media. Tryptosoy broth (Eiken, Tokyo) was used as a complete medium. Agar plates were prepared by adding 1.5% agar (Eiken, Tokyo) to tryptosoy broth. As a minimal medium (MM), Karlsson's medium with the following composition was used: glycerol,

2%; $(\text{NH}_4)_2\text{SO}_4$, 0.1%; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002%; MgSO_4 , 0.01%; CaCl_2 , 0.001%; KH_2PO_4 , 0.15%; K_2HPO_4 , 0.4%; and agar, 1.5% (15).

Bacterial strains. *M. smegmatis* strain Jucho (Jucho or J) (20) and strain Rabinowitchi (Rab or R) received from G. Penso (Istituto Superiore di Sanità, Rome, Italy) and strain PM5 (PM5 or P) received from M. Shishido (Yokohama City University, Yokohama, Japan) were employed. These three strains can grow at 45°C on MM agar and belong to *M. smegmatis* according to *Bergey's Manual* (the 7th edition), but are distinguishable from each other by their colonial appearance and phage susceptibility (20). Auxotrophic mutants of these strains were isolated by the method described previously (19). The concentrations of amino acid supplements necessary for the growth of all auxotrophic mutants are 10 µg/ml, and in the case of thiamine, 1 µg/ml. Streptomycin (SM)-resistant mutants were isolated by inoculating bacterial cells on tryptosoy agar plates containing 1,000 µg of SM per ml. The mutant strains employed are listed in Table 1. Because revertants from auxotrophy gradually accumulated in some strains upon prolonged storage at 4°C (such a phenomenon was reported also in *Nocardia* [1]), the mutants were repurified before being employed, and the reversion incidences were checked in each experiment.

Mating procedures. Two technically different procedures were devised and employed as described

below. (i) Each parental strain grown to log phase at 37 C on tryptosoy agar plates was suspended in saline, washed twice, and then resuspended in saline to a concentration of approximately 2×10^8 colony formers/ml. Equal volumes of the two parent suspensions were mixed, and 0.1 ml of the mixture, diluted appropriately by saline, was spread with a bent glass rod over the surface of the MM plates supplemented for selection. In all crosses, each parent alone was plated as a control to measure reversion frequency. The number of colony formers contained in the cell suspensions was also measured by plating on complete agar plates. All plates were incubated at 37 C for 4 to 5 days. Frequency of recombinant formation was expressed as a ratio of the number of recombinants produced on selection plates to that of total cells. This method is referred to hereafter as the direct plating method. (ii) The method used for *Nocardia* (1) was employed with the following modifications. Cylindrical plastic tubes, the inner diameter of which was 1 cm, were prepared. The several sterile tubes were placed on the bottom of a sterile petri dish, 20 ml of melted tryptosoy agar was poured into the dish burying the lower half of the standing tubes, and then the agar was solidified at a room temperature. Thus, plastic cups, the bottoms of which were made of tryptosoy agar, were prepared. Cell suspension of each parent strain containing 2×10^8 to 5×10^8 colony formers/ml was prepared in saline or broth, and equal volumes of the parent suspensions were mixed. A 0.1-ml amount of each of the mixtures was dropped into the cups prepared and spread with a thin glass rod to cover the agar surface on the inside of the cups. Each parent suspension alone was dropped into a separate cup to serve as a control. These agar cups were then incubated for varying periods of time (usually for 48 h). After the incubation, 0.5 ml of saline containing 0.06% Tween 80 was poured into each of the cups and then stirred with a thin glass rod to make a cell suspension. These cell suspensions were shaken vigorously with a homogenizing mixer, diluted appropriately with saline, and spread over plates suitable for selection of recombinants. Expression of frequency of recombinant formation was the same as in the direct plating method. Results obtained by the method using agar cups were more reproducible than those obtained by the procedure described previously (19). This method is referred to hereafter as the indirect plating method.

Segregation analysis. With sterile toothpicks, recombinant colonies were picked from selective media and were subcultured on the same media as that used for the initial selection, except Tween 80 was added at a concentration of 0.1%. These master plates were incubated for 48 h and then replicated with sterile velveteen pads to a medium identical to the selective medium and to differential media. Addition of Tween 80 in master plates makes replications of mycobacterial colonies easier and more exact.

Killing of bacteria by SM. Cultures of strain R-4 and P-1 were incubated in broth to log phase, usually for 18 h, and then diluted 10-fold with fresh broth.

TABLE 1. *Mutant strains employed*

Strain ^a	Marker ^b
J-1	<i>arg-3</i>
J-3	<i>gly-2</i>
J-4	<i>gly-2, str-4</i>
J-5	<i>gly-2, leu-7</i>
J-7	<i>arg-3, str-3</i>
R-4	<i>arg-6</i>
R-15	<i>arg-6, met-5</i>
R-41	<i>arg-6, str-7</i>
P-1	<i>thi-5</i>
P-11	<i>thi-5, str-13</i>
P-17	<i>leu-11, his-13</i>
P-18	<i>leu-11, his-13, str-22</i>

^a Strains of Jucho are prefixed J (J = wild type Jucho); strains of Rabinowitchi are prefixed R; strains of PM5 are prefixed P.

^b Abbreviations used for nutritional requirements: *gly*, glycine; *arg*, arginine; *his*, histidine; *leu*, leucine; *met*, methionine; *thi*, thiamine; for resistance marker: *str*, resistance for streptomycin.

These cultures were shaken for further 3 h, and then SM was added at a final concentration of 1,000 µg/ml, and shaken for 60 min. Cells were then washed and resuspended in a volume of saline equal to the original one. Numbers of colony formers contained in the cell suspensions were measured before and after the SM treatment.

RESULTS

Crosses between biochemical mutants of mycobacteria. A double auxotrophic mutant of Rab, R-15, (*arg, met*), was crossed by the indirect plating method with a double auxotrophic mutant of Jucho, J-5 (*gly, leu*), or PM-5, P-18 (*leu, his, str-r*). Results obtained are shown in Table 2; frequencies of revertants for individual markers of the parental strains are also shown. The frequencies of colonies recovered on the selective media are significantly higher than those of revertants for single markers in the parental strains, indicating a genetic interaction between the strains crossed.

To determine the genotype of the putative recombinants, 520 colonies grown on different selective media from a cross between R-15 and J-5 and 534 colonies from a cross between R-15 and P-18 were analyzed for segregation of unselected markers. Results obtained are shown in Table 3 and Table 4.

The variety of phenotypes shown by the tested colonies indicates that the interactions between the strains are due to genetic recombinations. The results also suggest the possibility of a polarized cross in which the J-5 and P-18 strains appear to contribute the majority of

TABLE 2. Results of crosses R-15 (*arg, met*) $\times$ J-5 (*gly, leu*) and R-15 $\times$ P-18 (*leu, his, str*)

Cross	No. of colonies counted on complete medium	No. of recombinants on selective media supplemented with ^a					Frequency of revertants for individual markers in the parental strains			
		None	Arg Gly	Arg Leu	Met Gly	Met Leu	<i>arg-6</i>	<i>met-5</i>	<i>gly-2</i>	<i>leu-7</i>
R-15 $\times$ J-5	1.3×10^7	140	460	315	490	240	2×10^{-8}	$<1 \times 10^{-8}$	$<3 \times 10^{-8}$	$<3 \times 10^{-8}$
		None	Arg Leu	Arg His	Met Leu	Met His	<i>arg-6</i>	<i>met-5</i>	<i>leu-11</i>	<i>his-13</i>
R-15 $\times$ P-18	3.2×10^7	34	520	650	490	730	3×10^{-8}	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$	7×10^{-8}

^a Abbreviations: Arg, arginine; Gly, glycine; Leu, leucine; Met, methionine; His, histidine.

TABLE 3. Results of cross R-15 (*arg, met*) $\times$ J-5 (*gly, leu*)

Recombinant genotypes				No. of recombinants with selected markers:			
				<i>met</i> ⁺ <i>leu</i> ⁺	<i>met</i> ⁺ <i>gly</i> ⁺	<i>arg</i> ⁺ <i>leu</i> ⁺	<i>arg</i> ⁺ <i>gly</i> ⁺
+	+	+	+	14	25	12	14
<i>arg</i>	+	+	+	1	1		
+	<i>met</i>	+	+			1	4
+	+	<i>gly</i>	+	113	120		
+	+	+	<i>leu</i>		101		103
<i>arg</i>	+	<i>gly</i>	+	5			
<i>arg</i>	+	+	<i>leu</i>		6		
+	<i>met</i>	<i>gly</i>	+			0	
+	<i>met</i>	+	<i>leu</i>				0
Totals				133	133	133	120

alleles in nearly all recombinant progeny obtained from different selective media, although the number of unselected markers employed was too limited to allow a conclusion.

Crosses between nutritionally complementary auxotrophic mutants derived from either strain Rab, Jucho or PM5 were infertile.

Stability of the recombinants. To test the stability of recombinants, single colonies of prototrophs obtained from the crosses of R-15 by J-5 and P-18 were transferred serially to complete media and MM. Results indicated that the prototrophic colonies were genetically stable; they did not segregate in the serial transfers, indicating that they are not heterogenotes.

Attempts to obtain transductants or transformants. To test the possibility that the prototrophic colonies that appeared in mixed cultures of Rab with Jucho or PM5 might be produced by transduction with temperate phages released from either strain or by

transformation with deoxyribonucleic acid (DNA) released spontaneously from cells in mixed cultures, experiments were carried out by using the methods reported in the previous paper (19). Incubation of mutants of one strain mixed with a culture filtrate or DNA of another compatible strain extracted by our method (18) produced no significant increase of prototrophic colony formation as compared to controls in which broth was used instead of culture filtrate or DNA.

Pretreatment of each type of parental cells with deoxyribonuclease and addition of deoxyribonuclease in mating medium did not affect the frequency of recombination. These facts suggest that the recombinants are not produced by a typical transducing or transforming mechanism but by a conjugal principle.

Mating types of the F₁ recombinants. In the previous paper (19), it was noticed that backcross of recombinants obtained from a cross between strains Jucho and Lacticola with either of the parental strains showed that there were four different mating types in the recombinants. A similar backcross experiment was performed with the F₁ recombinants shown in Table 3; 103 leucine-requiring recombinants which were obtained from the cross of R-15 (*arg, met*) and J-5 (*gly, leu*) on MM supplemented with methionine and leucine were picked and crossed with R-41 (*arg, str-r*) and J-4 (*gly, str-r*) by the indirect plating method. Recombinant selection was made on MM plates. Out of the 103 colonies, 100 were fertile with R-41 but infertile with J-4. One among 103 was fertile with J-4 but not with R-41, one was fertile with both R-41 and J-4, and one failed to recombine with either strain. These F₁ recombinants showing the four different mating types were tentatively named types I, II, III, and IV, respectively. Three clones of type I and one of each of types II and III, all of

TABLE 4. Results of cross R-15 (*arg*, *met*) × P-18 (*leu*, *his*, *str*)

Recombinant genotypes					No. of recombinants with selected markers:			
					<i>arg</i> ⁺ <i>leu</i> ⁺	<i>arg</i> ⁺ <i>his</i> ⁺	<i>met</i> ⁺ <i>leu</i> ⁺	<i>met</i> ⁺ <i>his</i> ⁺
+	+	+	+	<i>str-r</i>	6	3	5	11
+	+	+	+	<i>str-s</i>	0	1	1	0
<i>arg</i>	+	+	+	<i>str-r</i>			0	0
<i>arg</i>	+	+	+	<i>str-s</i>			0	0
+	<i>met</i>	+	+	<i>str-r</i>	0	0		
+	<i>met</i>	+	+	<i>str-s</i>	0	0		
+	+	<i>leu</i>	+	<i>str-r</i>		118		107
+	+	<i>leu</i>	+	<i>str-s</i>		9		7
+	+	+	<i>his</i>	<i>str-r</i>	127		114	
+	+	+	<i>his</i>	<i>str-s</i>	5		5	
<i>arg</i>	+	<i>leu</i>	+	<i>str-r</i>				4
<i>arg</i>	+	<i>leu</i>	+	<i>str-s</i>				0
<i>arg</i>	+	+	<i>his</i>	<i>str-r</i>			5	
<i>arg</i>	+	+	<i>his</i>	<i>str-s</i>			1	
+	<i>met</i>	<i>leu</i>	+	<i>str-r</i>		4		
+	<i>met</i>	<i>leu</i>	+	<i>str-s</i>		0		
+	<i>met</i>	+	<i>his</i>	<i>str-r</i>	2			
+	<i>met</i>	+	<i>his</i>	<i>str-s</i>	0			
Totals					138	135	132	129

which required leucine and were sensitive to SM, were crossed with the compatible parental strains on MM, and the resulting F₂ recombinants were analyzed for segregation of *str-r*. In the F₂ recombinants obtained from crosses of R-41 (*arg*, *str-r*) with three clones of the type I, five, six, and seven out of 100 tested were SM resistant. Ninety-three percent of the F₂ recombinants from a cross of J-4 with the type II were SM resistant. The percentages of SM-resistant colonies in the F₂ recombinants from crosses of type III with R-41 and J-4 were 6 and 92, respectively. Thus, the phenotype of Jucho was expressed in most of the F₂ recombinants when the F₁ recombinants were compatible with Jucho, and the phenotype of the F₁ recombinant was expressed in most of the F₂ recombinants when the F₁ recombinants were compatible with Rab.

Attempt to find an infectious sex factor.

The possibility of an infectious sex factor was examined in our system based on the hypothesis that Rab is a donor strain and Jucho is a female. R-4 (*arg*) was mixed with J-4 (*gly*, *str-r*) at a cell ratio of 4:1, incubated on tryptose agar for 48 h, and then transferred on to tryptose agar containing SM. To see whether they had received the hypothetical infectious sex factor, about 100 single colonies (*gly*, *str-r*) produced on the SM agar plates were crossed with J-1 (*arg*). All crosses were infertile.

To test whether or not the F₁ recombinant from crosses between natural male and female

strains transfers the sex factor to the female strains at a high frequency, as reported in *Pseudomonas aeruginosa* (13, 14), two leucine-requiring recombinants, obtained from a cross of R-15 (*arg*, *met*) by J-5 (*gly*, *leu*) and fertile with Jucho, were selected, crossed with J-7 (*arg*, *str-r*) at a cell ratio of 4:1 on tryptose agar plates, and transferred onto tryptose agar containing SM. About 100 single colonies (*arg*, *str-r*) produced on the SM plates were crossed with J-3 (*gly*) on MM plates. All crosses were infertile.

Pretreatment of Rab and Jucho cells with acriflavine or acridine orange by the method described previously (19) failed to eliminate the ability to form recombinants. These facts suggest the mycobacteria, at least Rab and Jucho, possess no acridine dye-sensitive episomal sex factor that can transfer at a high frequency.

Effect of SM on frequencies of recombination. To substantiate whether a polar transfer exists in the mycobacterial conjugation system, an experiment was carried out by using the method described by Hayes (12), in which unidirectional transfer of genetic material in conjugation of *Escherichia coli* was demonstrated. SM-resistant mutants R-41 (*arg*, *str-r*), J-4 (*gly*, *str-r*), and P-11 (*thi*, *str-r*) were isolated. No growth of the original strains R-4, J-3, and P-1 was observed when the cells were inoculated at a final cell concentration of about 10⁹/ml in a tryptose broth containing 1 μg of SM/ml. To determine

whether the *str-r* loci of these mutants were allelic or not, segregation of the SM-sensitive character was looked for in recombinants from crosses of R-41 $\times$ J-4 and R-41 $\times$ P-11 selected on MM. The 1,016 recombinants obtained from the former cross and the 470 obtained from the latter were picked and inoculated onto tryptose agar plates containing 1,000 μ g of SM/ml. All of the recombinants tested were resistant to SM, indicating that the *str* loci were allelic to each other.

Cell suspensions of R-4 (*arg*), R-41 (*arg*, *str-r*), J-3 (*gly*), and J-4 (*gly*, *str-r*) were prepared separately in saline, and reciprocal crosses were performed on MM agar plates with or without SM. Each strain alone was also spread on similar plates as a control. In a similar way, four crosses between R-4 and P-1 (*thi*), R-4 and P-11 (*thi*, *str-r*), R-41 and P-11, and R-41 and P-11 were performed on MM plates with or without SM. Results obtained are shown in Table 5. In the cross between R-4 and either J-4 or P-11, roughly the same number of prototrophic recombinants was formed when the mixture of bacteria was immediately spread on a medium with or without SM. However, in the cross between R-41 and either J-3 or P-1, no recombinant was produced when the mixture of bacteria was spread on a medium with SM.

Reciprocal crosses similar to those described above were also performed by the indirect method; the crosses were carried out on tryptose agar plates with or without SM and then transferred onto MM containing SM. Results obtained are shown in Table 6. Frequency of prototrophic and SM-resistant colony forma-

TABLE 5. Effect of SM on frequencies of prototrophic colony formation in crosses between Rab and Jucho and between Rab and PM5 (direct plating method^a)

Cross ^b	Frequency of prototroph formation on	
	MM	MM plus SM
R-4 <i>arg</i> $\times$ J-3 <i>gly</i>	5.9×10^{-6}	$<1.0 \times 10^{-8}$
R-41 <i>arg</i> , <i>str</i> $\times$ J-3	1.3×10^{-6}	$<1.0 \times 10^{-8}$
R-4 $\times$ J-4 <i>gly</i> , <i>str</i>	1.9×10^{-6}	1.6×10^{-6}
R-41 $\times$ J-4	1.4×10^{-6}	1.3×10^{-6}
R-4 $\times$ P-1 <i>thi</i>	3.5×10^{-6}	$<1.0 \times 10^{-7}$
R-41 $\times$ P-1	3.3×10^{-6}	$<1.0 \times 10^{-7}$
R-4 $\times$ P11 <i>thi</i> , <i>str</i>	2.2×10^{-6}	2.7×10^{-6}
R-41 $\times$ P-11	2.3×10^{-6}	2.6×10^{-6}

^a Details of the method can be seen in the text.

^b Frequency of revertants for individual markers in parental strains: *arg*, $<1.3 \times 10^{-8}$; *gly*, 5.6×10^{-8} ; *thi*, 4.1×10^{-8} .

TABLE 6. Effect of SM on frequency of prototrophic colony formation in crosses between Rab and Jucho (indirect plating method^a)

Cross ^b	Medium used for mating	Medium used for selecting	Frequency of prototroph recovered
R-4 $\times$ J-3	Complete agar	MM containing SM	1.4×10^{-8}
R-41 $\times$ J-3			4.1×10^{-8}
R-4 $\times$ J-4			5.8×10^{-4}
R-41 $\times$ J-4			4.3×10^{-4}
R-4 $\times$ J-3	Complete agar containing SM	MM containing SM	c
R-41 $\times$ J-3			3.3×10^{-8}
R-4 $\times$ J-4			4.7×10^{-6}
R-41 $\times$ J-4			8.1×10^{-4}

^a Details of the method can be seen in the text.

^b Frequency of revertants for individual markers in parental strains: *arg*, 1.8×10^{-8} ; *gly*, 6.9×10^{-8} .

^c Impossible to test, because of no bacterial growth on the plate for mating.

tion was significantly higher in the cross between R-4 and J-4 than that between R-41 and J-3 when the crosses were performed on tryptose agar plates containing SM.

These facts indicate that the antibiotic did not prevent genetic recombination when strain Rab was SM sensitive, but prevented recombination when Jucho or PM5 was sensitive to its action, suggesting that the roles of these two types of bacteria are not identical.

Frequencies of recombination in crosses between bacteria pretreated and not pretreated with SM. To determine whether or not the phenomenon described above was independent of the presence of SM in the mating medium, bacteria of SM-sensitive strains were treated with SM and then crossed with untreated bacteria of the compatible strain. By the treatment with 1,000 μ g of SM/ml for 60 min, the number of colony-forming cells of R-4 decreased from 6.8×10^5 /ml to 3.8×10^5 /ml and that of P-1 decreased from 6.0×10^5 /ml to 1.6×10^5 /ml. After washing the cells, reciprocal crosses between the SM-treated and -non-treated bacteria were performed on MM plates by the direct plating method. At the same time, control reconstitution experiments were carried out; the untreated cultures of R-4 and P-1 were diluted with saline at appropriate times to adjust the viable cell count so that untreated cultures contained almost equal numbers of colony formers as the SM-treated cultures. The results obtained are shown in Table 7. When P-1 was treated with SM and then crossed by R-4, very few recombinants

were formed. On the contrary, when R-4 had been treated by SM and then crossed with P-1, a fairly large number of prototrophic colonies was recovered. The ratios of the number of prototrophic recombinants recovered (C) to the number of colony formers of R-4 and P-1 used for the crosses (A and B, respectively) were calculated (Table 7). An unusually high value of C/B was obtained only when Rab was pretreated with SM. These facts suggest not only that the roles of Rab and PM5 in zygote formation are not identical, but also that some of the cells of Rab treated by SM which had lost their ability of colony formation could contribute to fertilize PM5 by offering genetic material, as suggested by Hayes in his system of *E. coli* (21).

DISCUSSION

The present investigations confirm and extend our previous reports (17, 19) that genetic recombination exists in the genus *Mycobacterium*. It appears that genetic interactions between the mycobacterial strains employed result from a conjugational mechanisms(s) and not from transduction or transformation. Since recombinant formation does not occur in liquid medium, relatively prolonged and tight cell-to-cell contact may be necessary for the mating event, as suggested for *Nocardia* (1). Recombinants can be formed when cell suspensions of the parental strains requiring different growth factors are mixed and immediately plated on MM agar plates suitably supplemented for selection. This phenomenon was observed in *E. coli* (16) and *P. aeruginosa* (12), but not in *Nocardia* (1).

Segregation of genes specifying nutritional requirements and SM resistance was analyzed in recombinants from crosses of strain Rab with strain Jucho or PM5. In the conjugation system of *Nocardia*, linkage maps were proposed based on the hypothesis that the best linear array results from the fewest crossover events and

that all markers are linked (2, 3, 6, 8, 9, 11). We also attempted to constitute a similar linkage map from the data shown in Tables 3 and 4. However, we were unable to determine an arrangement for loci. For instance, when R-15 (*arg*, *met*) was crossed by J-5 (*gly*, *leu*) on MM supplemented with arginine and glycine, *gly*⁻ recombinants were the most frequently observed (Table 3). To account for the most frequently observed class types as a result of the fewest crossover events, the order *met*, *arg*, *gly*, *leu* or the order *arg*, *met*, *gly*, *leu* can be postulated. However, these orders cannot be chosen on the basis of the data obtained when Met⁺, Gly⁺ recombinants were selected; in that case, the order *met*, *arg*, *leu*, *gly*, or the order *leu*, *arg*, *met*, *gly* is more preferable. If the total numbers of crossover events postulated in all cases of the four different selections are considered, only the order *leu*, *arg*, *met*, *gly* can be selected. However, even in this order, it was impossible to determine the relative distance among four markers; for instance, the relative distance between *leu* and *arg* was calculated to be 85.8% when Met⁺, Leu⁺ recombinants were selected, and it was only 10.3% when Met⁺, Gly⁺ recombinants were selected. Such contradictions were more remarkable when five loci were analyzed from the cross between R-15 (*arg*, *met*) and P-18 (*leu*, *his*, *str-r*).

Based on the facts described, two alternative explanations are postulated for the mechanism of zygote formation among mycobacterial strains. One is that zygotes are formed as a consequence of polar gene transfer from Rab to Jucho or PM5. The difficulties encountered in attempting to localize genetic characters remind us of the idea of a pseudo-linkage reported in F⁺ × F⁻ crosses of *E. coli* (21); in the crosses, a genetic recombinant possesses primarily the genetic characters of the recipient bacterium and only a small number of characters derived from the F⁺ parent. This phe-

TABLE 7. Frequencies of recombinant formation in the reciprocal crosses of R-4 (*arg*) by P-1 (*thi*) pretreated with or without SM

Cross system ^a	No. of colony formers crossed		No. of recombinants formed on MM (C)	Ratio	
	R-4 (A)	P-1 (B)		C/A	C/B
R-4 × P-1	3.4 × 10 ⁸	3.0 × 10 ⁸	6.5 × 10 ⁴	1.9 × 10 ⁻⁴	2.1 × 10 ⁻⁴
R-4 × P-1 treated with SM	3.4 × 10 ⁸	8.1 × 10 ⁸	5	1.5 × 10 ⁻⁸	6.1 × 10 ⁻⁸
R-4 × P-1 diluted 1,000 times	3.4 × 10 ⁸	3.0 × 10 ⁸	5.0 × 10 ¹	1.5 × 10 ⁻⁷	1.7 × 10 ⁻⁴
R-4 treated with SM × P-1	1.9 × 10 ⁸	3.0 × 10 ⁸	5.2 × 10 ³	2.7 × 10 ⁻²	1.7 × 10 ⁻⁵
R-4 diluted 1,000 times × P-1	3.4 × 10 ⁸	3.0 × 10 ⁸	3.0 × 10 ²	8.8 × 10 ⁻⁴	1.0 × 10 ⁻⁶

^a Reversion frequencies for individual markers in the parental strains: *arg*, 1.3 × 10⁻⁸; *thi*, 8.1 × 10⁻⁹.

nomenon was attributed, in part, to genetic polarity; in a given selection, the corresponding alleles from the donor parental types are only exceptionally transmitted to recombinants. If this is true in the mycobacteria, three alternative possibilities can be further postulated: (i) only a fragment of chromosome transfers from a donor cell to a recipient; (ii) an almost entire chromosome of the donor strain transfers with a diverse origin at a low frequency; or (iii) an almost entire chromosome transfers with a certain origin, but the majority of the donor chromosome transferred is excluded or restricted in the recipient. In the case of (i), multiple matings between parental and recombinant organisms, suggested in the nocardial conjugations (2, 9, 11), must be postulated to explain the segregation patterns by multiple crossing-over observed in mycobacterial recombinants. Another possible explanation is that zygote formation results from cell fusion to form a complete zygote and, due to a lack of homology, exclusion or restriction occurs. The data on segregation analysis of unselected markers in crosses between R-15 (*arg*, *met*) and P-18 (*leu*, *his*, *str-r*) seem to suggest the polar transfer rather than cell fusion, because that P-18 strain contributes the majority of alleles in nearly all recombinant progeny obtained from different selective media. However, we cannot exclude completely other possible hypotheses as the mechanism responsible for zygote formation, because the number of unselected markers employed was limited.

The first evidence for sexual differentiation in *E. coli* was presented by Hayes when he employed reciprocal crosses between two strains, differentially marked by *str-s* and *str-r* on a medium with or without SM supplementation (12). He also employed reciprocal crosses between two strains, sensitive to SM, pretreated with or without SM: the antibiotic did not prevent genetic recombination when the donor strain had been treated with SM, but no recombinants were produced when the female bacteria had been treated. The similar results were obtained from the cross systems of Rab by Jucho and Rab by PM5. SM had less influence on genetic recombination when bacteria of Rab had been treated, but prevented recombination when Jucho or PM5 had been treated. If the ratio of the number of recombinants produced to the number of colony-formers of Rab (C/A in Table 7) was calculated, a remarkable increase in the ratio was observed only in the cross between treated Rab and untreated PM5. To explain this phenomenon, it is unlikely that cells

possessing some metabolic disorders caused by SM treatment can increase recombinant recovery compared with metabolically unchanged strains. It seems more likely that the roles of Rab and Jucho, or PM5, are not identical, and survival of Rab is not necessary, but survival of Jucho or PM5 is indispensable to form recombinants; some of the cells of Rab which have lost the ability to form colonies by the action of SM may retain the ability to give genetic material to the cells of Jucho, or PM5. If true, the idea of polar transfer may be preferable than that of cell fusion.

The phenomena observed in mycobacterial recombination resemble those reported in *Nocardia*, which is phylogenetically related to *Mycobacterium* (5); for instance, crossing in liquid culture was not as effective as on solid medium (1, 19), recombinational events were observed in the interstrain matings but not in the intrastrain matings (1, 4), four mating genotype combinations were observed (7, 8), and crosses of some substrains produced a number of recombinant class types which show aberrant segregation patterns by multiple crossing-over in adjacent regions (6-9). An apparent difference, however, between these bacterial systems was noticed: a single, linear linkage group can be depicted with genes selected and unselected in the nocardial crosses (2, 3, 6-10), but not in the mycobacterial systems tested. If zygote formation in these bacteria is due to the mechanism of polar transfer, the transfer in *Mycobacterium* may be similar to that reported in matings of F^+ by F^- *E. coli*, whereas that of *Nocardia*, as suggested by Adams (2), may be similar to that of Hfr by F^- . If the zygote formation is due to cell fusion, on the other hand, genophore homology among *N. erythropolis* and *N. canicruria* (11) may be higher than that among Rab and Jucho or PM5.

Recently we found that the presence of Tween 80 in a mating medium inhibits the recovery of recombinants and that incubation of a mixture of parental cells at 42 C interferes with the mating process in spite of the fact that the temperature has no influence on bacterial growth (*unpublished data*). Kinetic study on genetic recombination applying these methods to interrupt the mating process is underway and is expected to throw further light on the mechanism of zygote formation in mycobacterial conjugation.

ACKNOWLEDGMENT

We thank Y. Maruyama for his technical assistance and T. Murohashi for his reviewing of this manuscript.

LITERATURE CITED

1. Adams, J. N. 1964. Recombination between *Nocardia erythropolis* and *Nocardia canicruria*. *J. Bacteriol.* **88**:865-876.
2. Adams, J. N. 1968. Partial exclusion of the *Nocardia erythropolis* chromosome in nocardial recombinants. *J. Bacteriol.* **96**:1750-1759.
3. Adams, J. N. 1970. Recombination and segregation of resistance and auxotrophic characters in crosses of *Nocardia* species. *Pneumologie* **142**:164-172.
4. Adams, J. N., and S. G. Bradley. 1963. Recombination events in the bacterial genus *Nocardia*. *Science* **140**:1392-1394.
5. Bradley, S. G. 1970. Genetic homologies among *Mycobacterium*, *Nocardia* and *Actinomyces*, p.179-188. In S. E. Juhasz and G. Plummer (ed.), *Host-virus relationships in Mycobacterium, Nocardia and Actinomyces*. Charles C Thomas Publ., Springfield, Ill.
6. Brownell, G. H., and J. N. Adams. 1967. Linkage and segregation of unselected markers in matings of *Nocardia erythropolis* with *Nocardia canicruria*. *J. Bacteriol.* **94**:650-659.
7. Brownell, G. H., and J. N. Adams. 1968. Linkage and segregation of a mating type specific phage and resistance characters in nocardial recombinants. *Genetics* **60**:437-448.
8. Brownell, G. H., and K. L. Kelly. 1969. Inheritance of mating factors in nocardial recombinants. *J. Bacteriol.* **99**:25-36.
9. Brownell, G. H., and R. S. Walsh III. 1970. Heterogenic recombinants from compatible nocardiae. *J. Bacteriol.* **104**:79-86.
10. Brownell, G. H., and R. S. Walsh III. 1972. Colony mutants of compatible nocardiae displaying variations in recombining capacity. *Genetics* **70**:341-351.
11. Clark, J. E., and G. H. Brownell. 1972. Genophore homologies among compatible nocardiae. *J. Bacteriol.* **109**:720-729.
12. Hayes, W. 1952. Recombination in *Bact. coli* K12: unidirectional transfer of genetic material. *Nature (London)* **169**:118-119.
13. Holloway, B. W. 1955. Genetic recombination in *Pseudomonas aeruginosa*. *J. Gen. Microbiol.* **13**:572-581.
14. Holloway, B. W. 1969. Genetics of *Pseudomonas*. *Bacteriol. Rev.* **33**:419-443.
15. Karlsson, J. L. 1954. Induced metabolic mutants of *Mycobacterium tuberculosis*. *J. Bacteriol.* **68**:592-593.
16. Lederberg, J., and E. L. Tatum. 1946. Novel genotypes in mixed cultures of biochemical mutants of bacteria. *Cold Spring Harbor Symp. Quant. Biol.* **11**:113-114.
17. Mizuguchi, Y. 1972. Segregation of unselected markers in mycobacterial recombinants. *Jap. J. Microbiol.* **16**:77-81.
18. Mizuguchi, Y., and T. Tokunaga. 1970. Method for isolation of deoxyribonucleic acid from mycobacteria. *J. Bacteriol.* **104**:1020-1021.
19. Mizuguchi, Y., and T. Tokunaga. 1971. Recombination between *Mycobacterium smegmatis* strains Jucho and Lacticola. *Jap. J. Microbiol.* **15**:359-366.
20. Tokunaga, T., Y. Maruyama, and T. Murohashi. 1965. Further studies on the phage typing of rapidly growing mycobacteria. *Jap. J. Bacteriol.* **20**:554-559. (in Japanese)
21. Wollman, E. L. 1953. Sur le déterminisme génétique de la lysogénie. *Ann. Inst. Pasteur* **84**:281-294.