GENETIC STUDIES IN MYCOBACTERIA: ISOLATION OF AUXOTROPHS AND MYCOBACTERIOPHAGES FOR *MYCOBACTERIUM SMEGMATIS* AND THEIR USE IN TRANSDUCTION

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ABSTRACT

Auxotrophs of Mycobacterium smegmatis *SN2 have been isolated using ultraviolet irradiation and N-methyl-N'-nitro-N-nitrosoguanidine as mutagens. Ten mycobacteriophages have been isolated from soil. One of these phages, I3 has been shown to be a generalised transducing phage. Transduction has been found to be sensitive to antiphage serum and insensitive to deoxyribonuclease The host-range of the phages have been studied on several related mycobacterial species.*

1. INTRODUCTION

Genetic studies are to some extent responsible for understanding the action of antibiotics in *Escherichia coli*[1]. It would be very desirable to have a genetic system in mycobacteria, which would help one to understand the mechanism of action of antitubercular drugs. We have isolated several auxotrophic mutants of *Mycobacterium smegmatis* SN2, and phages which are active on *M. smegmatis* SN2 and shown that one of the phages mediates transduction in *M. smegmatis* SN2[2]. Although Karlsson,[3] Konicek,[4] and Nair,[5] obtained mutants of mycobacteria for fatty acid synthesis, pigment production and respiratory deficiency respectively, no genetic analysis has been done so far, since there was no known method of genetic exchange in mycobacteria.

2. MATERIALS AND METHODS

Bacterial strains:

The bacterial strains used in the study and their source are listed in Table 1. *M. smegmatis* SN2 (BM1) was used for isolation of auxotrophs.

TABLE I

Mycobacterial cultures used in the study

Strain No.	Bacterial species	source
BM1	*M. smegmatis* SN2	Bonicke, 1967
BM2	EB165	,,
BM3	SN46	,,
BM4	SN38	,,
BM5	$SN46AR_1$	,,
BM6	*M. smegmatis* W1123	Wayne, 1970
BM7	,, H707 (TMC 1515)	Saito, 1970
BM8	,, SD CMC 46	Kubica, 1969
BM9	*M. phlei* F89	Bonicke, 1967
BM10	*M. rabinowitsh*	Vandra, 1968
BM11	*M. butyricum*	Redmond, 1968
BM12	*M. lacticola*, 0 11	M & P Laboratory
BM13	*M. lacticola*, 0 12	,,
BM14	*M. smegmatis* ATCC607	Sellers, 1968
BM15	*M. smegmatis* 607B	Redmond, 1968
BM16	*M. smegmatis* Polanus	Vandra, 1968
BM17	SM1 ATCC 14468	Saito, 1967
BM18	SM2 *M. butyrium* ATCC 19979	,,
BM19	SM3 *M. lacticola* ATCC 19980	,,
BM20	SM4 *Mycobacterium* spp. 607	,,
BM21	SM5 402	Kubica, G.P. NCDC, Saito, 1964.
BM22	SM6 Wa-63	,,
BM23	SM7 Wa-237	,,
BM24	SM8 Wa-290	,,
BM25	SM10 R. 1103	,,
BM26	SM11 ATCC 23011	ATCC, Saito, 1967
BM27	SM12 ,, 23019	,,
BM28	SM13 ,, 23028	,,
BM29	SM14 ,, 23032	,,
BM30	SM15 ,, 23037	,,
BM31	SM16 ,, 23038	,,
BM32	*M. phlei* SN 109	Juhasz, 1970

Media :

Luria broth[6] (LB), Luria agar (LA), LB solidified with two per cent agar were used. LB with 0 05 per cent Tween 80 (polyoxy ethylene sorbitol mono-oleate) LBT, was used for dispersed growth. Bordet-Gengou Agar (BGA) base[7] contained per 750 ml. of distilled water : Potato extract 250 ml peptone 10 g; NaCl, 4g. Tryptone soft-agar contained per liter of distilled water : Tryptone, 10g ; NaCl 5g ; solidified with 7g. agar The minimal medium[8] (56) contained per liter of distilled water ; Na_2HPO_4, 12 g; NaH_2PO_4, 9.6 g ; $MgSO_4$, $7H_2O$, 0 2g ; $(NH_4)_2$ SO_4, 2g; Ca $(NO_3)_2$, 0.01g; $FeSO_4 7H_2O$, 0.0025g ; Glucose, 2g. Minimal agar was prepared by adding 2 per cent agar to minimal medium. Amino acids and purines were added separately at 50 $\mu g/ml$ concentration. One per cent citrate supplemented minimal medium was used for the mutant enrichment.

Cultural conditions :

Bacteria were routinely grown in LBT at 37°C on a rotary shaker. plating of phages and preparation of phage stocks were done on BGA plates For top layer, tryptone soft-agar was used. Minimal medium used was half-strength medium (56/2) with glucose (0.2 per cent) as carbon source and supplemented with growth factors when necessary. Minimal medium (56/2) with Tween 80 at 0 05 per cent concentration (56/2T) was used for washing the cells.

Mutagenisation with ultraviolet (UV) irradiation and N-methyl-N'-nitro-N-nitroso guanidine (NG) :

Gorini and Kaufman's[9] method of U.V. irradiation was followed using a Phillips 15-watt germicidal lamp at a distance of 30 cms. for varying lengths of time (30 to 120 seconds). BM1 cells at various stages of growth, namely 24, 48 and 72 hours in LBT were centrifuged, washed with 56/2T and suspended in 56/2T. The cells were exposed to U.V. rays in petridishes in shallow layers of about 2-3 mm. thickness. After irradiation, the cell suspension was stored in dark for 2 hours to minimise photoreactivation and and then spread on LA plates for isolated colonies. The percentages of survivors for various exposure times were calculated by plating appropriate dilution on LA plates. The viable count was reduced by a factor of 10^3 per *ml* after 2 minutes.

For nitosoguanidine (NG) treatment, the method of Adelberg *et al*[10] modified as follows : Tween 80 was incorporated in 56/2 during and after mutagenesis. Log phase cells of BM1 were suspended in 56/2T and varying concentrations of NG from 100-500 $\mu g/ml$ were incorporated. The suspensions were incubated on rotary shaker for 15-90 minutes at 37°C. At various times, cells were harvested, washed free of NG and plated for survivors.

Isolation of mutants:

After NG treatment, cells suspended in 56/2T supplemented with 1 per cent citrate and grown overnight to stationary phase at 37°C to allow phenotypic expression in the presence of 1000 units per *ml* penicillin. When penicillin was used, enrichment of mutants was not observed. Because of this, the enrichment method was modified by use of 10 $\mu g/ml$ dihydrostreptomycin as used for isolation of auxotropic mutants from *Pseudomonas aeruginosa* [11]

After single colony isolation, cells mutagenised by UV irradiation and NG were tested for auxotrophs by replica plating method on 56/2 and LA plates. Mutants isolated were screened by the auxanographic technique to characterise the mutation. Direct plating of cells after UV and NG mutagenesis was also carried out.

Isolation of bacteriophages:

BM1 was used as an indicator strain in isolating phages from soil and sewage. The enrichment technique described by Froman *et al*[7] was followed. Soil samples were collected in 100 ml. erlenmeyer flasks containing 10 ml of log phase BM1 cells. Ten ml of raw sewage samples were collected from various sewage plants in separate 100 ml erlenmeyer flasks. They were enriched by the host and incubated for three days at 37°C. After three days, the samples were further enriched by fresh log phase BM1 cells. This was repeated for three weeks, at the end of which each sample was centrifuged, and the supernatant was passed through 0.45 μ millipore filter. The filtrate was spotted on the lawn of BM1 to test for lysis. Whenever there was cell lysis, presence of phage was confirmed. Single plaque isolations were made and the phages were maintained and assayed by soft agar overlay technique [12] Plates were incubated at 37°C for 24-48 hours to observe the plaque formation.

High-titre phage lysate:

Bubbler tube method[13] with aeration was followed for making the lysates. BM1 cells grown to log phase in LBT were centrifuged and resuspended in fresh LB at a concentration of 5×10^8 cells per ml. Phage was added at a multiplicity of 0.1 and aerated for 18-24 hours. At the end, a few drops of chloroform was added and the lysate was centrifuged at $3200 \times g$. for 20 minutes. The supernatant was stored at 4-5°C with a few drops of chloroform The method gave 5×10^9 phages per ml.

Transduction:

Recipient cells were grown in LBT into log phase on the shaker. The cells were centrifuged and resuspended in prewarmed LB. They were infected at a multiplicity of 1-2 phage (J3) per cell. The phase lysates had been

freed of chloroform prior to use. After incubation for 45 minutes on the shaker at 37°C, the cells were washed thrice with 56/2T and 0 1 to 0 2 ml was spread on the surface of minimal plates, supplemented where necessary. The plates were incubated for 5-6 days. In control experiments one ml of broth replaced the phage. Rest of the treatment was same as described above. The nature of transductants was checked by streaking on 56/2 plates.

Productian of anti-phage serum :

Phage 13 was injected thrice intravenously at an interval of three days and then hyperimmunized by repeated subcutaneous injections (six times) at an interval of three days. At the end, the rabbits were bled, serum was collected and assayed. The immune serum was assayed for phage neutralisation and the constant k was calculated by the formula $k = 2.303 \times (D/t) \times \log_{10} P_0/P$ where P_0. is number of phage particles at time zero minutes, P is surviving phage particles after a time t and D is the dilution factor of serum sample used in the reaction. The control rabbits received no phage. In none of the cases, an adjuvant was used. Pre-immune serum was also tested for the phage-neutralising capacity to detect the presence of natural antibodies if any.

Nomenclature of mutants :

The proposal for uniform nomenclature in bacterial genetics by Demerec *et al*[14] was applied to classify the mutants isolated in our study. The standard symbols for various gene mutations were applied. In general, BM 1 to BM 100 are the various saprophytic mycobacterial strain in our collection. The various auxotrophs and mutants isolated in our study are numbered from BM 101.

3. Results and Discussion

Isolation of mutants :

Figure 1 shows the killing of BM1 cells by UV irradiation. Log phase cells (24 hours old culture) are more sensitive to UV irradiation than 48 and 72 hours old cells. It is known in *E. coli*[15] that log phase cells are more sensitive than stationary cells. This may be due to gross structural abnormalities such as clumping or to genetic changes resulting from the conditions of the growth.[15] It may also be due to the fact that there is heavy deposition of lipids and due to the thick cell wall of the older cells in mycobacteria. The various mutants isolated after UV irradiation are given in Table 2.

Figure 2 shows the killing of BM1 cells by NG. Routinely, 500 $\mu g/ml$ of NG for 45 minutes was used to mutagenise the cells. The percentage of survivors was fairly constant (1-5 per cent). More mutants were isolated after NG treatment than after UV treatment and thus it seems that NG is a

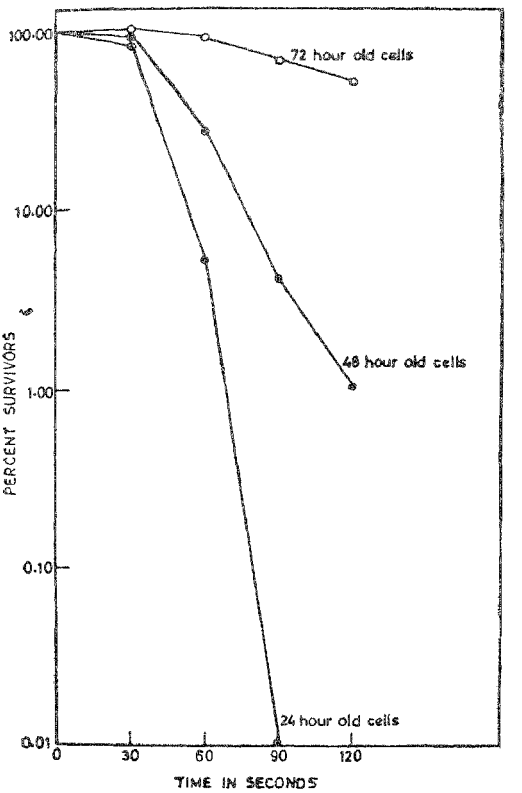

FIG. 1

Effect of UV Light on BM1

At a concentration of 1×10^8 per ml., cells were exposed to UV light at a distance of 30 cms.

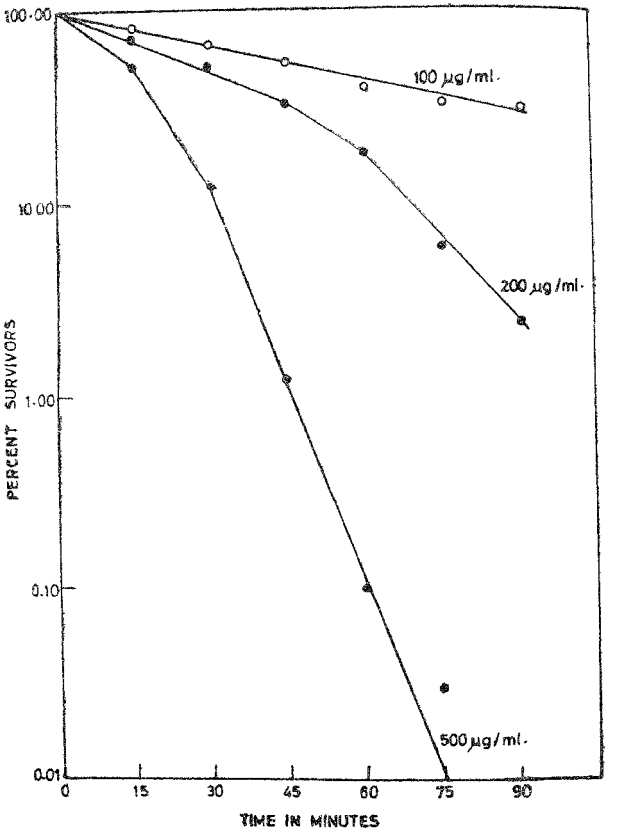

FIG. 2

Effect on NG on BMI

Log phase cells at 1×10^8 per ml. concentration were suspended in 56/2 I and subjected to various concentrations of NG.

good mutagen for *Mycobacterium smegmatis*. The various mutants isolated after NG treatment are given in Table 2. Double and triple mutants were isolated starting from a single or double mutant (see Table 2) using NG as a mutagen. All the mutants isolated in this study were derivatives of BM1. The auxotrophic mutants were very stable, the reversion frequency to prototrophy in the case of single mutants was around 1×10^{-9}. No prototrophic revertants could be seen for double and triple auxotrophs, the frequency being less than 1×10^{-10} Auxotrophs isolated in the study were by direct plating of mutagenised cells without enrichment.

Thus nearly forty mutants of BM1 cells were isolated. Various mutants were isolated requiring amino acids and purines for growth No auxotrophs for vitamin requirement were found among the mutants tested. Mycobacteria are slow growing organisms with a great affinity for clumping with each other [16] The clumping nature could be reduced to a certain extent by incorporation of Tween 80 in the medium. This hinders a proper quantitation of the cells by usual optical density measurements. Older cells have large deposits of liqids with increased clumping. This decreases the chances of mutant isolation. In order to simplify the isolation of mutants by selection, penicillin and dihydro-streptomycin were used for enrichment without success. Konicek and Malek[17] have also reported their failure in the use of penicillin for auxotrophic mutant isolation in *M. phlei*.

TABLE 2

Auxtrophic Mutants used in the study

Strain No.	Auxotrophic character	Mutagen used for isolation	Origin
BM 101	*gly*$^-$	U.V.	BM 1
BM 102	*his*$^-$	U.V.	BM 1
BM 105	*gua*$^-$	U.V.	BM 1
BM 106	*gly*$^-$ *ala*$^-$	NG	BM 101
BM 107	*gly*$^-$ *arg*$^-$	NG	BM 101
BM 127	*gly*$^-$ *ala*$^-$ *ade*$^-$	NG	BM 106
BM 140	*gly*$^-$ *leu*$^-$ *ade*$^-$	NG	BM 108

Isolation of phages :

Ten independent phages specific against BM1 were isolated. Of the different kinds of soils tested, manured garden soil was found to have phages as compared to clay and red soil. The six sewage samples tested did not show any phage. All the ten phages were purified by at least two single plaque isolations and kept sterile in *LB* with a few drops of chloroform. Three phages were lost during maintenance probably because of slow inactivation by chloroform. Bouman[18] has shown that mycobacteriophages D29, Leo, R1 and DS6A were inactivated by chloroform treatment. The remaining seven phages and two of the phages B1 and B6 obtained from Dr. Bonicke were tested against other strains of mycobacteria. Table 3 shows the host-range of phages along with the plaque morphology. The plaque size of phages varied from 2-5 mm in diameter. There were turbid and clear plaques, and the margin of the plaques were not always regular.

From Table 3, it can be see that phages I 1, I 2 and I 3 form turbid plaque on certain strains of mycobacteria and clear plaques on certain other strains. I 3 and I 5 on strains BM 4 and BM 1 respectively form plaques which are neither clear nor turbid plaques, thus forming intermediate type of plaques (weakly turbid). On strains BM3, BM24, BM25 and BM29 none of the phages form plaques. Of these strains BM3 is a natural lysogen.[19] Phage I 8 can infect and multiply on *M. smegmatis* as well as *M. phlei*. Phages I 8 and I 9 are clear plaque forming phages and may be of the virulent type.

From the above results it can be seen that mycobacteriophage are prevalent in soil samples enriched with manure. The three phage isolates which were lost during maintenance might have been probably inactivated by chloroform.

Transduction :

Of the seven phages, the phage I 3 forms turbid plaques on strain BM1 while forming clear plaques on strain BM2. Plaques on BM1 are 3-5 mm. in diameter with a distinct edge and hazy interior due to the growth of bacteria. On the strain BM2, the plaques are of 3-5 mm. in diameter. It is known from the study of *E. coli* phages λ and Plkc that these temperate phages form turbid plaques on their respective hosts and are able to mediate transfer of genetic characters from one strain to the other.[20] Since phage I 3 forms turbid plaques on strain BM1, it was thought that this could be a transducing phage. Therefore lysates of this phage were prepared on strain BM1 and tested for transduction on various auxotrophs of BM1.

TABLE 3

Phages / Bacterial strains	Host-Range of phages isolated for strain BM1								
	I1	I2	I3	I5	I6	I8	I9	B1	B6
BM1	C	C	T	C(T)	C	C	C	C	C
BM2	C	C	C	C	C	C	C	C	C
BM3	N	N	N	N	N	N	N	N	N
BM4	C	C	C(T)	C	C	C	C	C	C
BM5	C	C	C	C	C	C	C	C	C
BM6	T	C	N	T	C	C	C	C	C
BM7	N	C	N	N	C	C	C	C	C
BM8	C	C	C	C	C	C	N	C	T
BM9	N	N	N	N	N	C	N	N	N
BM10	C	C	C	T	T	C	N	N	N
BM11	N	N	N	C	N	C	N	N	N
BM12	N	T	T	T	C	C	C	C	C
BM13	N	N	N	C	N	C	N	N	N
BM14	C	C	C	C	C	C	C	C	C
BM15	C	C	C	C	C	C	C	C	C
BM16	N	N	N	N	N	N	C	C	C
BM17	N	C	N	C	C	C	C	C	C
BM18	T	T	N	N	N	N	N	C	T
BM19	N	N	T	N	N	N	N	C	C
BM20	C	T	C	C	C	C	C	C	C
BM21	N	N	N	C	C	C	C	C	C
BM22	C	C	C	C	C	C	C	C	C
BM23	C	C	C	C	C	C	N	C	N
BM24	N	N	N	N	N	N	N	N	N
BM25	N	N	N	N	N	N	N	N	N
BM26	C	C	N	C	C	C	C	C	C
BM27	C	C	T	C	C	C	C	C	C
BM28	C	C	N	C	C	C	N	N	N
BM29	N	N	N	N	N	N	N	N	N
BM30	N	N	N	N	C	C	C	C	C
BM31	N	N	N	N	C	C	C	C	C
BM32	N	N	N	N	C	N	N	N	N

On BGA plates, soft-agar with bacterial cells was overlayed and allowed to set. Over the layer, approximately 1×10^7 phages were spotted and incubated at 37°C for 48 hours. The presence or absence of bacterial lysis was indicated as T-turbid plaques; C-clear plaques; C(T)-neither turbid nor clear; and N-no plaque formation.

Transduction of histidine marker into BM102 :

Log phase recipient cells growing in LBT were centrifuged, washed and resuspended at a concentration of 5×10^8 per ml in LB. Phages grown on BM1 was added at different multiplicities of infection (0.2 to 4.0) and the mixture was incubated at 37°C with shaking for various times. It was found that during incubations beyond 45 minutes, the cells started to clump Therefore. in all transduction experiments, the phage-cell mixture was incubated only for 45 minutes. Figure 3 shows the linear relationship between the phage concentration and the number of transductants The frequency of histidine transducing particles was found by extrapolation in figure 3×10^{-6}.

Proof that the genetic exchange is mediated by phage :

To determine the nature of the transduction observed in the above section, sensitivity of transduction to DNase and anti-phage serum was tested. Table 4 shows the effect of antiserum on transduction. Control tubes without phage produced no colonies, indicating that there were no revertants in the population. Tubes containing cells, phages and LB or preimmune serum had transductants No transductants were produced when anti-phage serum was present indicating that the genetic process is phage mediated.

Table 5 shows the effect of DNase on transduction. Preincubation of the phage preparations with DNase (50 $\mu g/ml$) had no effect on transduction. These results confirm the idea that the observed genetic changes are mediated by DNA encapsulated by phage I 3 rather than free DNA that might be present in phage lysate.

It has been shown (Tables 4 and 5) that phage I 3 can transduce histidine, alanine, arginine, glycine, adenine markers and from this evidence we conclude that I 3 is a generalized transducing phage. Phage propagated on one type of auxotroph transduced the other types of auxotrophs but not the one on which it was propagated. Transduction for leucine marker could not be achieved in the same strain. Attempts are being made to isolate other auxotrophic markers for transductional analysis. Although five markers could be individually transduced, the transduced colonies did not show any cotransduction. The transduced colonies were still sensitive to the transducing phage I 3.

On BM2, phage I 3 plates with an equal efficiency and produces clear plaques. The lysates grown on BM2 were tested for the ability to transduce auxotrophs of BM1 and we could not observe any transductants in the tests.

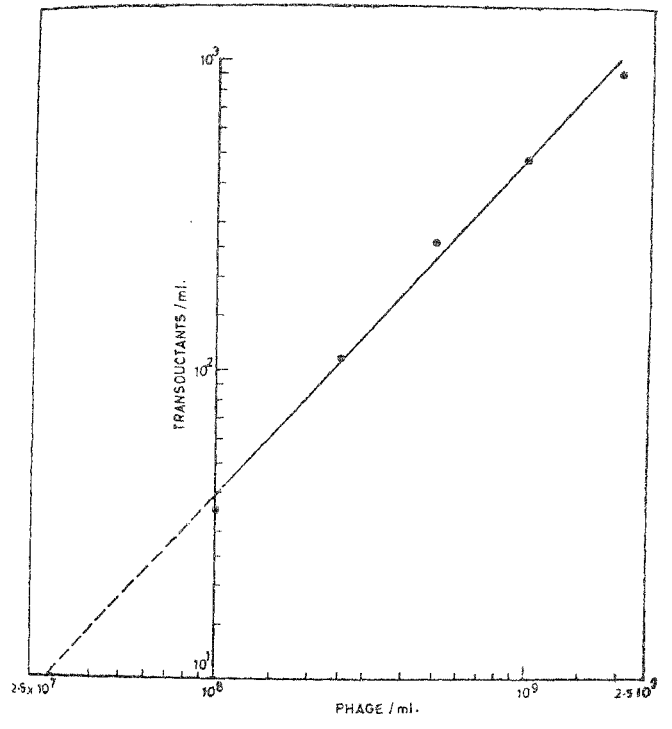

FIG. 3

Transduction of BM 102 by phage 13 grown on BM 1

Phage 13 grown on BM 1 was infected on BM 102 (*his*$^-$) at a m.o.i., 0.2 to 4 and incubated on shaker for 45 min. at 37°C. The transductants were scored on 56/2 plates.

As an approximation, only turbid plaque forming phage I 3 was tested for transduction in our study. Since I 3 could transduce, other phages were not tested. However, it is quite possible that othes phages isolated in this study could mediate transduction. It is known that *E. coli* phages P1 (vir),[21] T1[22] nnd *S. typhimurium* phage P22 c2[23] are capable of transduction. Transducing particles contain approximately 1 per cent of bacterial DNA[2] and so the mapping can be done only if the markers are closely situated. Because of this limitation, we are searching for other kinds of genetic transfer in *Mycobacterium*.

TABLE 4

Effect of Antiphage Serum on Transduction

Strain No.	Marker transduced	Multiplicity of infection	Treatment	Transductants per ml.
BM102	his^+	2.0	LB	4.0×10^2
BM102	his^+	2 0	antiphage serum	0
BM102	his^+	2.0	preimmune serum	$4\ 2 \times 10^2$
BM106	ala^+	2 0	LB	$1\ 4 \times 10^2$
BM106	ala^+	2.0	antiphage serum	0
BM106	ala^+	2.0	preimmune serum	1.4×10^2
BM107	arg^+	2.0	LB	1.4×10^2
BM107	arg^+	2.0	antiphage serum	0
BM107	arg^+	2.0	preimmune serum	$1\ 2 \times 10^2$

Cells of the three mutants of BM1, namely BM102 (his^-), BM106 (gly^-, ala^-), BM107 (gly^-, arg^-) at approximately 5×10^8 cells per ml. were infected with phage 13 and incubated and on shaker for 45 minutes. The transductants were scored for **his**$^+$ marker on 56/2 plate and for **ala**$^+$ and **arg**$^+$ markers on 56/2 plates supplemented with glycine. Antiphage serum with a K value of 10 was used in the study. LB was substituted for sera in control transduction tubes. Preimmune serum did not have phage neutralising property.

TABLE 5

Effect of Deoxyribonuclease on Transduction of BM I Mutants

Strain	Marker transduced	Multiplicity of infection	Treatment	Transductants per ml.
BM102	his$^+$	.	LB	.....
BM102	his$^+$	1 2	DNase	$4\,8 \times 10^2$
BM102	his$^+$	1 2	...	4.7×10^2
BM106	ala$^+$	.....	LB	....
BM106	ala$^+$	4 0	DNase	1.2×10^2
BM106	ala$^+$	4 0	....	$1\ 1 \times 10^2$
BM107	gly$^+$	....	LB	.....
BM107	gly$^+$	2 5	DNase	$2\ 3 \times 10^2$
BM107	gly$^+$	2 5	...	$2\ 5 \times 10^2$
BM127	ade$^+$	___	LB	.....
BM127	ade$^+$	2 4	DNase	2.4×10^2
BM127	ade$^+$	2.4	...	$2\ 4 \times 10^2$

One ml phage I3 and 0.1 ml DNase were preincubated without eells at 37°C for 30 minutes 2 ml cells were added after tnat and incubated onshaker for 45 minutes. Samples were centrifuged, washed well and plated on (1) 56/2 plates for **his**$^+$, (BM102), (2) 56/2 supplemented with glycine for **ala**$^+$, $(BM106)_+$, (3) 56/2 supplemented with arginine for **gly**$^+$, (BM107) and (4) 56/2 supplemented with glycine and alanine for **ade**$^+$, (BM127) markers Phage lysate used contained 2×10^9 phages per ml. LB (1.0 ml) was substituted for phage and DNase in control tubes for revertants.

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